Molecular and Functional Changes in Familial Thyroid Cancer

Hugo Prazeres

Alterações Moleculares e Funcionais em Tumores Familiares da Tiróide

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VI. Hugo Prazeres, Joana Torres, Paula Soares and Manuel Sobrinho-Simões. **The Familial Counterparts of Follicular Cell-Derived Thyroid Tumors.** Int J Surg Pathol. 2010 May 5.

Em cumprimento do disposto no referido Decreto-Lei, declara que participou activamente na recolha e análise de dados e escreveu os artigos I, II, IV e V com a colaboração dos co-autores. Declara que colaborou na redacção do artigo III. Declara ainda que escreveu o artigo VI com a colaboração dos co-autores.

Nota Explicativa

A presente dissertação encontra-se escrita em Inglês na sua quase totalidade, exceptuando o Sumário, devido ao facto de alguns dos trabalhos terem sido realizados com colaboração internacional e por se prever que um dos seus arguentes principais seja estrangeiro.

Dedicado aqueles que no momento da notícia
vêm em nós a esperança
e só lhes ocorre
pedir ajuda.

Dedicated to those which when facing the news
rest their hope in us
and all that comes to their mind is
to ask for help.

Author Note

Before I started this work I felt it was in my power to help people by what I knew. I undertook this PhD to learn more so that I could help people further. Aside from fighting cancer I was seeking to put up a fight.

During the years that I spent dedicated to this work I have had the privilege to witness some major breakthroughs that set novel landmarks in the understanding the biology of the cancer cell. In fact, one of the most fascinating aspect of studying cancer relies precisely on is how much we can learn about the genetic signals, mechanisms and principles governing the workings of a cell when trying to explain the alterations underlying the abnormal behavior of the cancer cells. I was not immune to the emergence of novel concepts that are nowadays widespread and established, such as “Pharmacogenetics” and “Pharmacogenomics”; “Oncogene Addiction”; the “Histone Code” and “Bivalent Chromatin”; “miRs” (Micro-RNAs) and cancer; “Driver” and “Passenger” mutations, “Cancer Stem Cells”, the “Tumor Microenvironment” and the “Metastatic Niche” theory. As such, much of this work is set over this framework, and the discussion of the results here presented already tries to take into account this new ways of thinking about cancer.

The setting of these novel landmarks was accompanied by astonishing technological developments that have allowed the analysis of cancers in unprecedented ways. These technologies include “cDNA microarrays”; “Real-Time PCR”; “Antibody Arrays” and “Next-Generation Genome Sequencing”. I have had the advantage to employ these methods within specific rationales to bring great benefit to this work.

Overall, I am very much rewarded by what I have learned and with the experience I have gained. I have explored the effects of mutation in oncogenic signaling, witnessed how genetic and also epigenetic mechanisms govern gene expression and saw the effect of small non-coding microRNAs at the post-transcriptional level. I was unaware that I would end this work facing the observation that these genetic and epigenetic changes ultimately impacted on the composition of the extracellular micro-environment of the cancer cell, which is highly orchestrated trough pos-translational mechanisms, that affect several levels of physiology.

Facing the multitude of biological processes governing the development and progression of cancer I am now certain that my eyes are too small to see a picture that is so big.

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I once heard Professor João Lobo Antunes elaborate his thinking about the acts of Pleading and Giving in Science, as a great part of the scientist task is to get money and resources from others in order to undertake his own projects. At some point he held that one of the most honorable gestures of the human being comes from "Giving without ever making you feel like you are Pleading". During these last years I have had the chance of meeting people of such honor and humanity, who helped me because they feel more joy in giving than in getting.

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Abstract

The study of unusual families with predisposition to particular types of cancer can provide fundamental insight into the tissue-specific tumor-initiating genes. In these families, inheritance of a single mutation primes certain tissues to develop tumors. Examples of such tumor-initiating genes are represented by *RB1*, *APC* and *NF1* in tumors of the eye, colon and nervous system, respectively. In the setting of thyroid cancer, the most prominent example of a gene with such properties is provided by the REarranged during Transfection (RET) proto-oncogene. Germline activating point mutations in RET are highly penetrant for the development of hereditary Medullary Thyroid Carcinoma (MTC) arising from the parafollicular or c-Cells. RET encodes a receptor with tyrosine-kinase activity that targets several intracellular signaling cascades relevant to cell proliferation, such as RAS-RAF-MAPK, PIK3-AKT, or STAT transcription factors. Recently, several molecules have been reported to inhibit RET Tyrosine-Kinase activity, holding promise for use as therapeutic agents. Due to discriminate genotype-phenotype correlations, the classification of RET genotypes has become crucial to provide valuable information for predictive diagnosis and prophylactic treatment of hMTC and also for Kinase Inhibitor-based therapy of recurrent sporadic MTC. In project 1 we have focused on certain specificities of the spectrum of RET germline mutations. We found an increased prevalence of the Cys611Tyr mutation in our series of cases originating from the Central Region of Portugal. Haplotype studies showed that 4 out of 5 Cys611Tyr families could be attributed to the inheritance of a matching ancestral haplotype harboring the specific RET mutation. We have further assessed at the functional level 3 germline RET variants of undetermined pathogenic significance: Arg886Trp, Ser649Leu and Glu511Lys, detected in isolated cases of apparently sporadic MTC. Measurement of the capacity to transform NIH3T3 fibroblasts and of the ability to activate known RET intracellular signaling targets, namely ERK1/2, STAT-1, STAT3 and TCF4, provided a basis for the classification of these previously uncharacterized RET genotypes in their *in vitro* transforming potential, signaling properties and sensitivity to the kinase inhibitor Sorafenib. This may prove useful to define follow-up and therapeutic regimens.

In project 2 we set out to disclose genes relevant to the development of familial follicular cell-derived thyroid cancers (also termed Familial Non Medullary Thyroid Cancer – FNMTC) which, intriguingly, display the highest familial relative risk amongst all cancer sites. This epidemiological observation is strengthened by the clinical occurrence of NMTC in familial aggregation (FNMTC) and by the detection of chromosomal *loci* in linkage with the disease phenotype in such kindreds. However, no predisposing gene could be attributed to FNMTC so far. In the addressing of this issue we performed molecular studies in the tumors from familial clusters of NMTC. We obtained evidence of inactivation hits (loss of heterozygosity - LOH) in two of the regions linked to FNMTC (19p13.2 and 2q21), consistent with the Knudson model of inactivation of a tumor suppressor gene (TSG). In the follow-trough of this investigation we performed global gene expression analysis in normal thyroid and tumor samples from a FNMTC patient and analyzed expression profiling data sets from sporadic thyroid tumors. Of the 64 candidate genes located at the 2q21 region we have pinpointed the LRP1B gene (Low density lipoprotein receptor-Related Protein 1B) as the only significantly down-regulated gene in

familial and sporadic tumors. LRP1B encodes an endocytic LDL-family receptor and is amongst the 10 most significantly deleted genes across 3,312 human cancer specimens. Nevertheless, the apparently crucial role of this lipoprotein receptor in carcinogenesis in general and in thyroid cancer in particular was not clear. We have shown that in thyroid tumors and cell lines LRP1B displays frequent DNA copy number loss and CpG-island methylation resulting in mRNA under-expression. By using CpG-island reporters methylated *in vitro*, we found that DNA methylation disrupts a functional binding site for the histone-acetyltransferase p300 located at intron 1. We identified and validated a microRNA targeting LRP1B (miR-548a-5p) which is overexpressed in cancer cell lines as a result of 8q22 DNA gains. Restoration of LRP1B impaired *in vitro* and *in vivo* tumor growth, reduced angiogenesis and inhibited cell invasion. Furthermore, LRP1B expression led to overall changes in the extracellular medium, namely clearance of Growth Factors, Matrix MetalloProteinases and Cytokines. These results emphasize the role of an endocytic receptor acting as a tumor suppressor by modulating the extracellular environment composition in a way that constrains the malignant behavior of the cancer cells. Our findings open scope to use LRP1B activity as a tool for a therapeutic approach that is not centered on the cancer cell itself, but rather in its environment; aiming to control the malignant behavior of the cancer cell by normalizing the environment surrounding it.

Resumo

O estudo de famílias raras com predisposição para desenvolver certos tipos de cancro pode fornecer conhecimentos elementares acerca dos genes iniciadores de tumores no contexto específico de cada tecido. Nestas famílias, uma única mutação hereditária prepara um conjunto específico de tecidos para desenvolver tumores. Estes genes iniciadores de tumores são bem exemplificados pelo *RB*, *APC* e *NF1* em tumores da retina, cólon ou do sistema nervoso, respectivamente. No contexto do cancro da tiróide, o exemplo mais proeminente de um gene com estas propriedades consiste no proto-oncogene RET (REarranjado durante a Transfecção). Mutações germinativas activadoras do gene RET têm elevada penetrância para o desenvolvimento de Carcinoma Medular da Tiróide Hereditário (CMTH) com origem nas células parafoliculares ou Células-C. O gene RET codifica um receptor com actividade Tirosina-Cinase que induz diversas vias de sinalização intracelular relevantes para a proliferação, tais como as vias RAS-RAF-MAPK, PIK3-AKT, ou os factores de transcrição STAT. Recentemente foram descritas várias moléculas com a capacidade de inibir a actividade de Tirosina-Cinase do RET, suscitando a esperança de poderem ser utilizadas como agentes terapêuticos. Devido à existência de correlações genótipo-fenótipo bem definidas, a classificação dos genótipos do RET tornou-se crucial quer para o diagnóstico preditivo e tratamento profiláctico do CMTH, quer para o tratamento baseado em inibidores do RET das recidivas de CMT esporádico. No projecto 1 focámos certas especificidades do espectro de mutações germinativas do RET. Encontrámos uma prevalência elevada da mutação Cys611Tyr na nossa série de casos naturais da Região Centro de Portugal. Estudos de haplótipo mostraram que 4 das 5 famílias tinham em comum um haplótipo ancestral que explica a hereditariedade da mutação Cys611Tyr. Estudámos ainda, ao nível funcional, 3 variantes germinativas do RET com significado patogénico indeterminado: Arg886Trp, Ser649Leu e Glu511Lys, detectadas em casos isolados de CMT aparentemente esporádico. A medição da capacidade de transformar fibroblastos NIH3T3 e da activação de vias de sinalização intracelular alvo do RET, nomeadamente ERK1/2, STAT-1, STAT3 e TCF4, permitiu-nos classificar estes genótipos de acordo com o seu potencial transformante *in vitro*, propriedades de sinalização e sensibilidade ao inibidor de Cinases Sorafenib. Esta classificação pode ser útil para definir estratégias de acompanhamento e regimes terapêuticos.

No projecto 2 propusemos desvendar genes relevantes para o desenvolvimento de tumores derivados das células foliculares da tiróide (também designados de Carcinomas Não-Medulares da Tiróide - CNMT) que, curiosamente, apresentam o maior risco relativo familiar de entre todos os tipos de cancro. Esta observação epidemiológica é reforçada pela ocorrência clínica de CNMF em agregação familiar (CNMTF) e pela detecção de *loci* genéticos em ligação com o fenótipo da doença. Contudo, até à data, nenhum gene de predisposição foi identificado. Na abordagem a esta questão fomos o primeiro grupo a realizar estudos moleculares em tumores de agregados familiares de CNMT. Em duas das regiões ligadas ao CNMTF (19p13.2 e 2q21) observámos marcas de inactivação por Perda De Heterozigotia (PDH) consistentes com o modelo de Knudson para inactivação de genes supressores de tumores (GSTs). No seguimento desta investigação, realizámos uma análise da expressão genética global em amostras de tiróide normal e tumores de um doente com CNMTF, e analisámos dados do perfil de expressão de uma série de tumores da tiróide esporádicos. Dos 64 genes localizados na região 2q21, o gene

LRP1B (de Low density lipoprotein receptor-Related Protein 1B) surgiu como o único gene significativamente sub-expresso tanto em tumores familiares como em tumores esporádicos. O LRP1B codifica um receptor de endocitose da família dos receptores de lipoproteínas e está entre os 10 genes mais frequentemente sujeitos a delecções focais entre 3.312 espécimes de tumores humanos. No entanto, o papel aparentemente crucial deste receptor de lipoproteínas na carcinogénese em geral e no cancro da tiróide em particular ainda não é claro. Neste aspecto mostrámos que em tumores da tiróide e linhas celulares de cancro o LRP1B sofre frequentemente perdas de DNA (delecções) e apresenta metilação na ilha de CpGs resultando na sua sub-expressão ao nível do RNAm. Descobrimos que a metilação do DNA inibe um local funcional na ligação da acetil-transferase p300 localizado no intrão 1. Identificámos e validámos um microRNA que tem como alvo o LRP1B (miR-548a-5p) e que é sobre-expresso em linhas celulares de cancro em resultado da amplificação do *locus* 8q22. O restabelecimento da expressão do LRP1B abrandou o crescimento tumoral *in vitro* e *in vivo*, reduziu a angiogénese e inibiu a invasão celular. Acrescidamente, a expressão do LRP1B causou alterações globais no meio extracelular, levando nomeadamente à remoção de factores de crescimento, metalloproteinases e citocinas. Estes resultados dão ênfase ao papel de um receptor de endocitose que actua como um gene supressor de tumores modulando a composição do microambiente extracelular de tal forma que limita o comportamento invasivo das células tumorais. As nossas descobertas perspectivam a utilização da actividade do LRP1B como uma ferramenta no âmbito de uma estratégia terapêutica que não está centrada na célula tumoral em si, mas sim no seu ambiente, tendo como objectivo controlar o comportamento maligno da célula através da normalização do microambiente à sua volta.

Introduction

I1. Hereditary and/or Familial Cancers Demonstrate a Class of Tissue-Specific Tumor-Initiating Genes

Cancer is, in essence, a genetic disease. The experimental verification that cancer originates from mutated genes initially came from experiments on viral induced tumors in the early 70s, however, to this day; it is facing reinforcement by the latest analyses of individual cancer genomes [1, 2]. Work on the Rous strain of avian Sarcoma Virus (RSV) pioneered by Steve Martin and continued by Peter Duesberg, Peter Vogt and Dominique Stehelin established that some retroviruses could rapidly transform cells because they transferred an oncogene that the viruses had acquired from the genomes of the mammalian and avian cells that they infected[3, 4]. The identification of a cellular form of this oncogene product by Oppermann, Bishop, Varmus and Erikson provided the first evidence that "the seeds of cancer are within us" [4, 5]. However, the question of whether the function of this protein had been altered by the virus remained unclear. Key discoveries by the Robert Weinberg and Geoffrey Cooper groups that portions of the genome of tumor cells, when transferred into normal cells, were sufficient to cause transformation, provided the second clinching evidence [6, 7]. In 1982, the Weinberg, Barbacid and Wigler groups all cloned the first oncogene, from bladder carcinoma lines, which proved to be the cellular homologues of the *ras* genes from the Harvey and Kirsten sarcoma viruses [8-12]. Curiously, all three groups ended up discovering the same single amino-acid change: glycine to valine at position 12, highlighting that mutation was underlying the change in normal structure and activity of this gene [13-16].

These seminal discoveries led to the modern understanding of cancer as a complex interplay of genetic lesions. This concept is nowadays being strengthened by the myriad of mutations that have been found accumulated in the genomes of individual cancers [1, 2, 17-20]. Nevertheless, looking at a catalogue of hundreds to thousands of mutations provides little understanding of cancer if the functions of the products these genes encode are not fully comprehended, and if the bona fide "drivers" of the neoplastic process are not distinguished from the "passengers" [2]. Two classes of cancer genes may be recognized on the basis of the effects that mutation has on the activity of the gene product. Oncogenes are mutated in ways that render the gene product constitutively active or active under conditions in which the wild-type gene is not. Oncogene activations can result from chromosomal translocations, from gene amplifications or from subtle intragenic mutations affecting crucial residues that up-regulate the activity of the gene product in a dominant way. On the other hand, Tumor-Suppressor Genes (TSGs) are targeted in the opposite way by genetic alterations: mutations reduce the activity of the gene product and usually are required to take place in both alleles. Such inactivations arise from missense mutations at residues that are essential for its activity, from mutations that result in a truncated protein, from deletions or insertions of various sizes, or from epigenetic silencing. Although mutations in these two classes of genes seem different, they operate similarly at the physiologic level: they provide clonal growth advantage and increase tumor cell number through the stimulation of cell division or by inhibiting cell-cycle arrest or cell death. A

neoplastic cell is thus regarded as one that has acquired multiple somatic mutations resulting in selective advantage, causing it to undergo trough numerous cycles of clonal expansion [21, 22].

Nevertheless, it is evident that not all genes are equally relevant to the development of the different types of cancer. Either due to their cellular function or in virtue of taking part in a critical signaling pathway or even because of their particular relevance in certain tissue or cell type, certain genes seems to participate more prominently in cancer than others. This is sustained by the following observations:

a) The spectrum of genetic changes found in cancers is enriched in genes that are engaged in cellular functions that are crucial for the development and progression of a tumor. Examples of such functions include signal transduction, cell cycle control, apoptosis, DNA repair, hypoxia, angiogenesis, extracellular matrix remodeling and inflammation.

b) Specific genes are almost invariantly targeted by viral oncogenesis. Virtually all DNA viruses that cause tumors in experimental animals or humans encode proteins that inactivate both Rb and p53 [23, 24]. Of the hundreds of cancer genes known, these two have been singled out as targets for inactivation by all DNA tumor viruses. The explanation for this may be that p53 and Rb tumor-suppressor gene pathways inactivation is crucial for a tumor of epithelial origin to form.

c) This conjecture is further supported by studies showing that some genes are altered in a large fraction across cancer types. This is precisely the case for P53 which is found mutated in most epithelial cancers. It is also noteworthy that some genes are unusually frequent in specific cancer types, for example, a mutated c-KIT receptor tyrosine kinase can be found in more than 80% of tumors of the gastrointestinal stroma (GISTs), suggesting that tumorigenesis in this tissue is predominantly reliant on this specific gene.

d) Accordingly, a specific mutation of a widely expressed gene does not have identical effects in different mammalian cell types. RAS gene mutations provide informative examples of these complexities. KRAS2 gene mutations in normal pancreatic duct cells seem to initiate the neoplastic process, eventually leading to the development of pancreatic cancer [25, 26]. The same mutations occurring in normal colonic or ovarian epithelial cells lead to self-limiting hyperplastic or borderline lesions that do not progress to malignancy [27, 28].

e) The fact that tissue context matters in also noticeable in that many cancer genes affect different organs when mutated in mice than when mutated in humans[29].

f) A further demonstration that some genes are instrumental to the development of tumors in a tissue-specific mode comes from hereditary and/or familial cancers. The study of unusual families with predisposition to develop cancer led to the identification of genes which are sufficient to prime specific tissues to develop tumors, despite the mutation is present in every cell of the organism. For example, MMR genes seem to have identical function in every cell type by correcting specific types of mutations, such as those in homopolymer tracts [30]. Yet inherited mismatch repair defects lead to tumors in the colon and endometrium but spare most other organs, including rapidly dividing, self renewing tissues such as the small intestine and bone marrow [31]. Such tissue-specific tumor-initiating genes are well demonstrated by *RB1*,

APC and *NF1* in tumors of the eye, colon and nervous system, respectively. Mutations in these genes can occur in the germline, resulting in hereditary predispositions to cancer, or in single somatic cells, resulting in sporadic tumors. The different types of somatic cancer share many of the affected genes in different proportions, nevertheless, the models of hereditary cancer provide evidence that certain genes are of particular importance in specific tissue contexts and not in others.

Analyzing the list of cancer predisposing genes Bert Vogelstein proposes to discriminate "Caretakers", "Gatekeepers" and "Landscapers" on the basis of the cellular functions in which these genes are engaged [32].

The caretakers, or also called stability genes, include the mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes responsible for repairing subtle mistakes made during normal DNA replication or induced by exposure to mutagens as well as genes controlling chromosome breaks, segregation and mitotic recombination such as BRCA1, BLM and ATM. When stability genes are inactivated, mutations in other genes occur at a higher rate [33]. All genes are potentially affected by the resultant increased rate of mutation but eventually mutations in oncogenes and tumor-suppressor genes will affect net cell growth and thereby confer a selective growth advantage to the mutant clone.

The Gatekeepers are genes that control critical checkpoints in the cell-cycle progression that need to be surpassed for uncontrolled tumor growth to take place. Genes such as TP53 (responsible for the Li Fraumeni syndrome) or RB (causing hereditary retinoblastoma), illustrate this category.

The so called Landscaper genes, such as SMAD4 [34], have an indirect effect on the tissue that will eventually become cancerous by creating an abnormal microenvironment for the cells, probably by acting in the adjacent stroma, priming not the cancer cell, but the stromal cells [32]. The class of "Landscapers" highlights a side of cancer that concerns the physical and biochemical interactions of cancer cells with the extracellular matrix and with other cell types in the tumor microenvironment such as fibroblasts, leucocytes and endothelial cells, etc. The importance of these interactions is becoming increasingly recognized [35, 36]. Mina Bissel has been referring to them as the "tumor context" [37] and Weinberg used the term "heterotypic interactions" [38, 39] . These aspects will be discussed in depth further ahead in this thesis.

Interestingly, the most common forms of cancer predisposition, leading to breast and colon cancers, are caused by inherited mutations of stability genes. In clear contrast, oncogenes are rarely found underlying hereditary cancer, with RET, MET, KIT and PDGFRA filling this category. The lack of oncogenes in cancer predisposing syndromes can be explained by the fact that oncogenic activations are dominant and a single activated allele may be sufficient to disturb embryonic development and cause lethality. Tumor suppressor gene inactivation, on the other hand, is recessive, meaning that the wild-type allele retains function to support embryonic development and only upon a second somatic hit, acquired already in "adult" life, leads to tumor develop.

It is important to emphasize that germline mutation of cancer predisposing genes does not "cause" cancer *per se*: people with these mutations have a "head start" on the neoplastic

process, as a mutation that contributes to cancer is already present in every one of their cells. However, only a very small fraction of the total cells in an at-risk tissue become neoplastic because additional somatic mutations are required to develop a clinically detectable lesion and only when several genes are defective does an invasive cancer develop. Thus it is best to think of cancer predisposing genes as initiating, rather than "causing" cancer.

It is also becoming increasingly apparent that a better understanding of cancer comes from focusing on pathways rather than genes [40]. This emerges from the observation that similar phenotypes arise from a variety of altered genes that, nonetheless, almost invariably take part in a similar biochemical pathway. This has been solidified through the breakdown of the multiple *loci* conferring predisposition for a given type of cancer that, in the end, revealed that they most often encode genes working in the same biochemical pathway. A clear illustration is given by the colorectal cancer susceptibility genes such as hMLH1, hMSH2, hMSH6, PMS2, all of which are engaged in a mismatch repair pathway [31]. Detailed studies of somatic mutations in individual tumor types also provide evidence that genes functioning in a single pathway obey an "exclusivity principle": that is, in any single tumor, only one of the genes in the pathway is generally mutated [41]. This reasoning allows for many useful predictions for the era of molecular targeted therapies. It predicts that it will be ineffective to block a given pathway by targeting any of the genes upstream of the mutated gene actually driving increased pathway activity. On the other hand, targeting a gene in the middle of the pathway will be effective for all cases in which mutations are in the genes upstream of this target.

Overall, the contribution of studying the models of hereditary and/or familial cancer can be summed up to one sentence: Hereditary cancers highlight the key tumor initiation pathway for a given tissue-specific context. This is of fundamental importance to understand the biology and pathogenesis of particular cancers and of singular meaning to choose the targets for future diagnostic and therapeutic strategies.

I2. The impact of predisposition genes on the human side of dealing with cancer

The finding of germline mutations underlying inherited predisposition to cancer settled a novel paradigm in cancer care: for the first time knowledge about genetic origins of cancer allowed the prediction of whom, in certain families, was predisposed to develop cancer and this information could in many ways be translated into more timely patient care. Nonetheless, this new paradigm shed awareness over the human side to dealing with cancer as a little bit of genetic information is forcing patients to make very big decisions.

Before 1990, it was recognized that 5–10% of most common cancers such as breast and colon cancer occurred in familial patterns, but whether these were owing to shared environments, several interacting genes or single major genes was not known. The breakthroughs of this prolific year came with the identification of the gene underlying the childhood cancer Wilms' tumor [42, 43], Li Fraumeni syndrome [44, 45] and Neurofibromatosis [46, 47]. Familial Adenomatous Polyposis [48, 49], von Hippel-Landau disease, Retinoblastoma and Familial Medullary Thyroid Cancer and Multiple Endocrine Neoplasia 2A [50, 51] soon followed. In 1994

genes on chromosomes 2 and 17 that were associated with major fractions of colon and breast cancers, respectively were discovered. These, and related genes discovered thereafter, were MSH2 and MLH1 in hereditary non-polyposis colorectal cancer [52-56], and BRCA1 and BRCA2 in hereditary breast cancer syndromes [57-60]. These findings added to the conception established in the 1980s that cancer had a genetic basis, which now were taking place in the germline setting. The hereditary cancers were characterized by inherited changes which predisposed to cell transformation, in a tissue-specific manner, giving rise to familial aggregation of syndromic disease, according to a Mendelian transmission pattern of early onset, bilateral/multifocal tumors, often occurring in a background of benign hyperplastic conditions encountered in the remaining organ.

At the same time as researchers were finding more susceptibility genes, genetic counselors and patient-care professionals began translating these advances into advice for patients. In these families, genetic information has impact on both diagnosis and therapy: it allows for predictive diagnosis at an asymptomatic stage and thereafter the consideration of a number of cancer prevention strategies, ranging from conservative targeted surveillance of a spectrum of tumors to more radical options such as prophylactic surgery. Moreover, family members who are not at genetic risk can be obviated from the need to regularly perform uncomfortable clinical surveillance tests such as colonoscopy.

In some instances the benefits of genetic testing are mostly realized, as is well exemplified in FAP: APC mutations underlie virtually every case of FAP, they display almost complete penetrance, and prophylactic surgery is effective and accepted as the best prevention strategy. However, in others cases, the benefits of genetic testing are greatly undermined due to a number of factors. Hereditary breast cancer serves to illustrate this point. First, most of the familial breast cancers do not appear to be of monogenic hereditary origin, instead they are genetically heterogeneous and probably result from accumulated susceptibility derived from multiple *loci* still to discover. Second, the chance of developing tumors and their severity varies greatly, even when a causing mutation is found. Third, no prevention strategy is clear-cut: the surveillance methods available do not ensure timely detection of the tumor in non-invasive stage and, on the other hand, prophylactic surgery has serious implications for self-esteem.

Thus, the decision to be tested for cancer susceptibility and knowing what to do with the information is not necessarily straightforward.

Thyroid cancer in the framework of genetic predisposition

Thyroid cancer comprises a wide spectrum of tumors which can arise trough transformation of two distinct cell lineages that compose this endocrine gland (Figure 1). Oncogenesis of cells lining thyroid follicules (follicular cells) presents multiple discrete stages ranging from the common benign follicular adenoma, to the less frequent, highly aggressive, poorly differentiated and undifferentiated (anaplastic) carcinoma [61]. Between these two ends of the spectrum stays the rather common Well Differentiated Thyroid Carcinomas (WDTCs) which comprise two histological types: papillary and follicular thyroid carcinomas (PTC and FTC, respectively). The essential diagnostic criteria differ from PTC to FTC; in PTC they are cytological, based on the presence of typical nuclear features (large, pale staining, "ground glass" and irregular, "grooved" nuclei) whereas the diagnosis of FTC rests upon the histological demonstration of capsular and/or vascular invasiveness[61]. The two types of WDTC accumulate distinct genetic abnormalities during tumor progression. In PTC, somatic rearrangements of the RET proto-oncogene [62, 63], and BRAFV600E mutations [41, 64] are the most frequent events; in contrast to this, FTC have a different genetic profile: they are characterized by RAS mutations [65, 66] and PAX8/PPARγ rearrangement[67, 68]. The follicular variant of PTC (FVPTC) shares some of the molecular features of follicular tumors (FTA and FTC), namely a high frequency of RAS mutations and PAX8/PPARγ rearrangements[69], whereas a less frequent and less reported BRAFK601E form (~7%) is detected in cases of FVPTC[70]. These observations reinforce the assumption that some FVPTC cases are a sort of intermediate category between conventional PTC and FTC [71].

On the other hand, medullary thyroid carcinomas (MTCs) are derived from the calcitonin-secreting parafollicular cells, also called C-Cells, and represents only 5% of clinically evident thyroid cancers [61].

Both Medullary and Follicular-Cell derived tumors can occur in familial/hereditary settings.

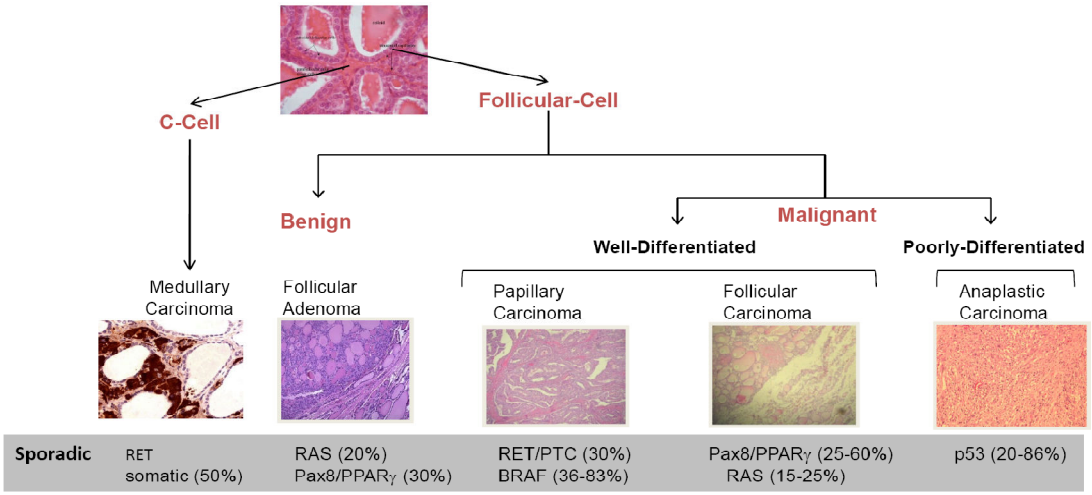


Figure 1 **Histopathologic presentations and spectrum of somatic mutations found in sporadic thyroid tumors.**

I3.1 Hereditary Medullary Thyroid Cancer

Despite being rare, Medullary Thyroid Carcinoma it has one of the highest hereditary components for any cancer site. About 25-30% of MTC cases are hereditary and constitute the first and most common clinical presentation of the autossomal dominantly inherited Multiple Endocrine Neoplasia type 2 cancer syndromes (MEN2A, MEN2B and Familial MTC) [72], [73], [74]. In 1993, germline activating mutations of the RET (Rearranged during Transfection) proto-oncogene were discovered to underlie Hereditary MTC, in the context of MEN2 [50, 51].

I3.1.1 The RET Tyrosine Kinase Receptor and its Signaling Pathways

RET encodes a membrane receptor tyrosine-kinase (RTK) composed of four extracellular cadherin-like motifs and a cysteine rich region, a transmembrane portion and an intracellular domain with tyrosine kinase activity [75], [76]. RET signals trough a ligand/co-receptor/RET complex. To date, RET ligands of the glial-derived neurotrophic factor (GDNF) family, which include GDNF, ARTN, NRTN, and PSPN and a family of GPI-linked RET co-receptors (GFR1 – 4), have been identified [77]. The formation of Ligand/co-receptor and RET complexes results in RET dimerization and triggers autophosphorylation at intracellular tyrosine residues. Phosphotyrosines 905, 981, 1015, 1062, and 1096 constitute docking sites for adaptor proteins Grb7/10, c-Src, phospholipase C and Grb2/Shc/IRS1–2/FRS2/DOK1-4-5, respectively. Phosphorylation of intracellular target proteins activates several downstream pathways (Figure 2), which include ERK1/2 mitogen-activated protein kinase, phosphatidylinositol 3-kinase, c-Jun N-terminal kinase, p38, ERK5 and cAMP-responsive element-binding protein [78]. More recently also the WNT pathway was shown to be activated by RET trough RET-mediated direct tyrosine phosphorylation of Beta-Catenin [79].

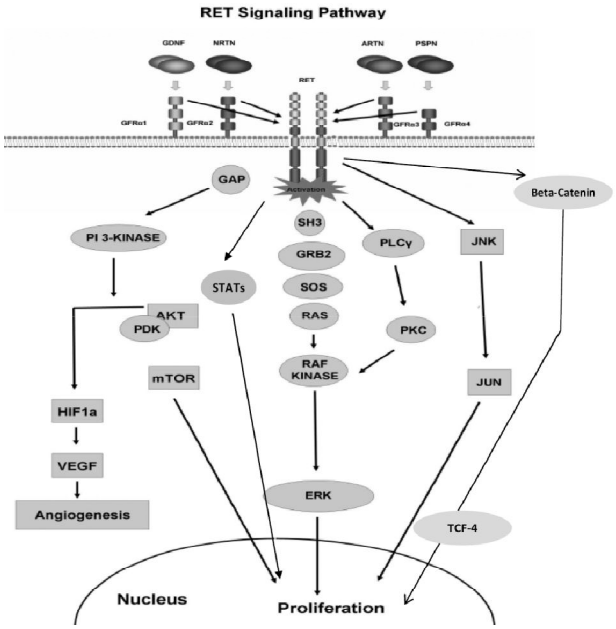


Figure 2 Squematic representation of the RET signaling pathways.
Adapted from [77-79]

I3.1.2 The Spectrum of Germline Activating RET Mutations in FMTC and MEN2

In MTC, RET mutations occur in a specific spectrum of codons and result in gain of function, increased kinase activity and receptor activation [80], [81], [82]. Mutational hotspots are located at the cysteine rich region of the extracellular domain and in the intracellular tyrosine kinase domain [72], [73], [74]. The clustering of mutations in hotspots might be explained by the fact that proto-oncogene activation requires changes at residues that specifically interact in different ways with receptor function and thus mutations cannot occur in a widespread manner. A comprehensive description of all known germline RET variations can be found at the MEN2 RET database. (http://www.arup.utah.edu/database/MEN2/MEN2_welcome), and a catalogue of somatic mutations can be found at the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/). The most common RET germline mutations are missense substitutions of extracellular cysteine residues, occurring at cysteine codon 634 in 80% of cases. Less frequently cysteine codons 609, 611, 618, 620 and 630 are affected. Other non-cysteine extracellular domain mutations, located at exons 5 and 8, have been detected [83], [84], [85] . Tyrosine Kinase domain mutations affect a more varied spectrum of aminoacids and most frequently recurring mutations replace Met918, Val804, Leu790, Tyr791 and Ala883. Less frequently residues 768, 876, 891, 886 and 912 are affected. Rare mutations found in isolated families have been reported, comprising homozygous mutations [86], duplications [85], and double mutations [87], [88].

Furthermore, a similar spectrum of somatic mutations is observed in about 50% of the cases with Sporadic MTC, which may have prognostic significance [89], [90].

I3.1.3 Mutation-specific activation mechanism of the RET proto-oncogene

Besides clinical presentation, mutation-specific activation mechanism of the RET proto-oncogene can also be distinguished. Mutations in the extracellular cysteine-rich region result in the replacement of a Cysteine residue by another aminoacid, subsequently giving rise to the loss of an intramolecular disulfide bond. As a consequence one cysteine residue becomes available for the formation of an intermolecular disulfide bond, which results in covalently bound receptors that are constitutively active because of ligand-independent receptor dimerization [80], [81], [81], [82].

In contrast, the intracellular MEN2B specific mutations and other Tyrosine kinase domain mutations affect receptor activation in a totally different way. By altering the conformation of the catalytic core of the tyrosine kinase domain they result in increased catalytic activity and alter substrate specificity [80], [82], [91].

I3.1.4 Genotype-Phenotype Correlations in MEN2 Syndromes

In MEN2 there are consistent genotype/phenotype correlations that underlie aspects such as clinical manifestation, RET activation mechanisms and disease penetrance, allowing for a mutation-specific classification of MEN2 [72], [73, 74]. In clinical terms, three disease phenotypes can be recognized: MEN2A, MEN2B, and a familial form of medullary thyroid carcinoma (FMTC). MEN2A is found to be associated with substitutions at one of six specific Cysteine residues in exon 10 (609, 611, 618, 620) and 11 (630 and 634). MEN2A cysteine mutations result in the development of MTC, along with variable expression of pheochromocytoma (50%) and hyperparathyroidism (15-30%) [72], [73, 74]. MEN2B, on the other hand, is mainly caused by a specific missense mutation located at the RET Tyrosine kinase domain (Met918Thr), which accounts for 95% of cases [92], [93], [94] .A second tyrosine kinase domain substitution (Ala883Phe) has been detected in a small proportion of MEN2B patients [95], [96]. Additionally, double mutations affecting codons 804 and 805 and 804 and 806 were described in a individual MEN2B cases [87, 88]. MEN2B kinase domain mutations give rise to a more complex clinical phenotype characterized by the early onset of a very aggressive form of MTC, concomitant with pheochromocytoma in 50% of cases, and accompanied by other non-neoplastic features, such as mucosal neuromas of the tongue, lips, and eyelids, ganglioneuromatosis of the gastrointestinal tract, thickening of corneal nerves and Marfanoid habitus [97]. In FMTC the only disease manifestation is MTC, with no additional endocrinopathies. RET mutations with low clinical expression, involving codons 321, 533, 768, 790, 791, 804 and 891 are found in these families [72], [73], [74], [84], [98, 99], [100], [101], [102]. Occasionally patients with these mutations may also develop the MEN2A phenotype, showing that FMTC and MEN2A represent a continuum of clinical expression in a common genetically related disorder [103],[100], [104], [105].

I3.1.5 Codon-specific risk of developing MTC and prophylactic therapy

Age-dependent penetrance for MTC in MEN2 is also codon-specific and classification of the risk of developing MTC can be done on the basis of genotype, reviewed by Raue F and Frank-Raue K [74]. This is of clinical relevance because the ideal timing of prophylactic thyroidectomy should take into consideration the balance between the adverse effects of thyroidectomy at early ages and the individual risk of developing MTC. Comprehensive guidelines have been issued by the American Thyroid Association concerning this aspect [106]. Very high risk, level 3 mutations are the most aggressive MEN2B-associated specific changes in codons 918 and 883. Carriers develop early onset MTC with high metastatic potential and in these cases thyroidectomy should be performed under the age of 3. High risk, level 2 mutations affecting codons 609, 611, 618, 620, 630, and 634 are found in MEN2A patients and thyroidectomy is advised before the age of 5. For low risk, level 1 mutations, comprising codons 609, 768, 790, 791, 804, and 891, thyroidectomy can be postponed until adult age and ideal timing can be decided on an individual basis [107] [108], [109], [110], [73], [111].

I3.1.6 Tyrosine Kinase Inhibitor-Based Therapy and RET Pharmacogenetics

Oncogenic forms of RET found in MTC and PTC are targets of potential interest in the therapeutic context. The finding of various compounds (
Table 1) capable of inhibiting oncogenic RET (mutated or rearranged), such as PP1 and PP2 [112], ZD6474 (Vandetanib) [113], RPI-1 [114], CEP-701,CEP-751 [115], Imatinib [116], Sunitinib (SU5416, SU11248) [117], Gefitinib [118], Sorafenib (BAY 43-9006) [119], Motesanib (AMG706) [120], Axitinib
(AG013736)[121] and XL 184, has brought further clinical relevance to the classification of the pharmacological sensitivity of RET mutants, as metastatic MTC is the most common cause of death in patients with MEN2 [122].

Table 1 Molecules used in pre-clinical and clinical trials as RET Tyrosine Kinase Inhibitors

Compound	Trade name	Structure	Targets	Clinical trials	Refs
PP1 PP2	Zaleplon	Pyrazolopyrimidine	RET	--	[112]
ZD6474	Vandetanib	Anilinoquinazoline	RET; VEGFR; EGFR	Phase II	[113]
RPI-1	--	Indolinone	RET; MET	--	[114]
SU5416 SU11248	Sunitinib	Butanedioic acid	VEGFR-2; PDGFR; c-KIT; RET; CSF-1R	Phase II	[117, 123]
ZD1839	Gefitinib	Anilinoquinazoline	EGFR	Phase II	[118, 124]
BAY43-9006	Sorafenib	Bis-aryl urea	RAF-1; BRAF; VEGFR-2/-3; PDGFR-B; Flt-3; c-KIT; RET	Phase II	[119, 125]
AMG706	Motesanib diphosphate	Diphosphate salt	VEGFR; PDGFR; KIT; RET	Phase II	[120, 126]
AG-013736	Axitinib	Benzamide	RET; VEGFR; PDGFR; c-KIT	Phase II	[121]
XL184/XL880			VEGFR2; RET and MET	Phase III	[***] page26

The small molecule TKIs mechanism of action is based on the principle that sterically blocking the ATP binding pocket results in impaired phosphorylation activity, inhibits signal transduction and prevents activation of intracellular signalling pathways relevant to tumor growth and angiogenesis.

The pyrazolopyrimidines PP1 and PP2 and the 4-anilinoquinazoline Vandetanib inhibit RET-rearrangement-derived oncoproteins with a half maximal inhibitor concentration below 100 nM. These molecules were shown to inhibit RET enzymatic activity and phosphorylation of downstream targets, such as ERK1/2. Vandetanib has been found to inhibit also RET signalling in two human PTC cell lines and to reduce tumorigenicity of RET/PTC-transformed fibroblasts

injected into nude mice [112]. Vandetanib blocks *in vivo* phosphorylation and signalling mediated by RET/PTC3 oncoprotein and of an epidermal growth factor (EGF)-activated EGF-receptor/RET chimeric receptor. Finally, it blocks anchorage-independent growth of RET/PTC3-transformed NIH3T3 fibroblasts and the formation of tumors after injection of NIH-RET/PTC3 cells into nude mice [113].

Sorafenib (BAY 43-9006) was designed originally as a RAF inhibitor [127] nonetheless, pre-clinical studies have shown that Sorafenib can inhibit the kinase activity and signalling of wild type and oncogenic RET. Sorafenib inhibited oncogenic RET kinase activity at half-maximal inhibitory concentrations (IC50) of 50 nM or less in NIH3T3 cells. It arrested the growth of NIH3T3 and RAT1 fibroblasts transformed by oncogenic RET and of thyroid carcinoma cells that harbour rearranged RET alleles. This inhibitory effects paralleled a decrease in RET phosphorylation [119]. Finally, PTC cells carrying the RET/PTC1 rearrangement were found to be more sensitive to Sorafenib than PTC cells carrying a BRAF mutation [128]. There is an ongoing phase II clinical trial using Sorafenib in patients with advanced thyroid cancer [125].

RPI-1 is a 2-indolinone derivative initially shown to inhibit RET/PTC1 activity in immunokinase assay with an IC50 of 27-42 μM. It selectively inhibited the anchorage-independent growth of NIH3T3 transformed cells expressing the RET/PTC1 gene and the transformed phenotype of NIH3T3ptc1 cells was reverted to a normal fibroblast-like morphology. In these cells, the constitutive tyrosine phosphorylation of RET/PTC1, of the transducing adaptor protein Shc and of a series of co-immunoprecipitated peptides was substantially reduced [114]. Activation of JNK2 and AKT was abolished, thus supporting the drug inhibitory efficacy on downstream pathways. In addition, cell growth inhibition was associated with a reduction in telomerase activity by nearly 85% [129].

Sunitinib was initially described as a TKI targeting VEGF and PDGFR receptors [130] and also found to inhibit c-KIT [131]. It is now approved for the treatment of GIST and renal cell carcinoma. *In vitro* kinase assays showed that Sunitinib inhibited the phosphorylation by RET/PTC3 of a synthetic tyrosine kinase substrate peptide in a dose-dependent manner. RET/PTC-mediated Y705 phosphorylation of STAT3 was inhibited by addition of Sunitinib, and the inhibitory effects of Sunitinib on the tyrosine phosphorylation and transcriptional activation of STAT3 were very closely correlated with decreased autophosphorylation of RET/PTC. Sunitinib caused a complete morphological reversion of transformed NIH-RET/PTC3 cells and inhibited the growth of TPC-1 cells that have an endogenous RET/PTC1 [117]. Treatment of two patients with progressive metastatic thyroid carcinoma (1 PTC and 1 FTC) demonstrated sustained clinical responses to Sunitinib over a period of four years [123].

Gefitinib was initially approved for NSC lung cancer since it targets oncogenic EGFR. *In vitro* data suggests that EGFR contributes to RET kinase activation, signalling and growth stimulation. Conditional activation of RET/PTC oncoproteins in thyroid PCCL3 cells markedly induced expression and phosphorylation of EGFR, which was mediated in part through mitogen-activated protein (MAP) kinase signalling. RET and EGFR were found to co-immunoprecipitate. Ligand-induced activation of EGFR resulted in phosphorylation of a kinase-dead RET, and this effect was entirely blocked by EGFR Kinase inhibitor. Gefitinib inhibited also

cell growth induced by various constitutively active mutants of RET in thyroid cancer cells as well as in NIH3T3 cells [118]. This evidence has provided a biological basis for clinical evaluation of Gefitinib in thyroid cancer. The results obtained in phase II trial showed no objective responses among the 25 patients with thyroid cancer treated with Gefitinib [124].

CEP-701 and CEP-751 are Indolocarbazole derivatives that also inhibit RET in MTC cells. Effective inhibition of RET phosphorylation in a dose-dependent manner is achieved at concentrations <100 nM. They also block the growth of MTC cells in culture. CEP-751 and its prodrug, CEP-2563, also inhibited tumor growth in MTC cell xenografts [115]. These drugs also potentiate the effects of irinotecan treatment in TT cell culture and xenografts and results in durable complete remission in 100% of the mice. CEP-751 induced a loss in the induction of the DNA repair program marked by phospho-H2AX and the checkpoint pathway marked by the activated Chk1 pathway [132]. Since preclinical models have demonstrated that both CEP-751 and CEP-2563 have antitumor activity in a variety of tumors phase I trials were undertaken [133].

Several other TKI molecules are undertaking steps in clinical evaluation for their efficacy in metastatic MTC treatment with limited published data.

Axitinib (AG-013736) [134], which in a 60 patient phase II study showed were 18 (30%) partial responses and 23 (38%) stable disease in patients with MTC [121].

Motesanib (AMG706) [135] was evaluated in differentiated thyroid cancer [120] and in a phase I study in 91 patients with either hereditary (16 cases) or sporadic MTC (75 cases), 2% showed partial response and 81% had stable disease [126].

XL184/XL880 is a compound that is rapidly evolving in the clinical evaluation process. It is a TKI that targets VEGFR2, RET and also MET and has demonstrated efficacy for several solid tumors, especially thyroid cancer ***. In patients with hereditary and sporadic MTC very interesting response rates were obtained with 9/17 patients (53%) showing partial remission. Based on this findings phase III registration trial of XL184 as a potential treatment for medullary thyroid cancer (MTC) has been initiated.

[***] - Eder JP, Appleman L, Heath E, et al. (2006). A phase I study of a novel spectrum selective kinase inhibitor (SSKI), XL880, administered orally in patients (pts) with advanced solid tumors (STs). J Clin Oncol 2006 ASCO Annual Meeting (Atlanta, GA, June 2-6) Proceedings Part I 24(18S):3041 (June 20 Suppl).// LoRusso P, Appleman L, Zhu AX, et al. (2006). Pharmacodynamics of XL880, a novel spectrum selective kinase inhibitor (SSKI) administered orally in patients with advanced solid tumors (AST). 18th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics (Prague, Czech Republic, Nov 7-10) EJC Suppl 4(12):124. (Abstract 404).// Ross RW, Stein M, Sarantopoulos J, et al. (2007). A phase II study of the c-Met RTK inhibitor XL880 in patients (pts) with papillary renal-cell carcinoma (PRC). J Clin Oncol 2007 ASCO Annual Meeting Proceedings 25(18S):15601 (June 20 Supplement).// Salgia R, Hong DS, Camacho LH, et al. (2007). A phase I dose-escalation study of the safety and pharmacokinetics (PK) of XL184, a VEGFR and MET kinase inhibitor, administered orally to patients (pts) with advanced malignancies. J Clin Oncol 2007 ASCO Annual Meeting (Chicago, IL, June 1-5) Proceedings 25(18S):14031 (June 20 Supplement).

At present, the clinical use of tyrosine kinase inhibitors in patients with thyroid cancer still do not rely in the genetic background of each tumor [121, 125, 136], nonetheless activating mutations are not functionally equivalent, and RET is paradigmatic in that respect, showing mutation-specific clinical presentation, activation mechanisms and disease penetrance. Mutant RTKs have different kinase domain conformations and, as such, are differentially sensitive to TKIs, and considerable genotype/response correlations may emerge. This is well illustrated by primary resistance of Val804 RET mutants to PP1, PP2 and Vandetanib [137] and one can envision that a codon-specific classification of RET according to drug sensitivity could be performed. This is of relevance in order to select the best class of inhibitor according to predicted primary resistance to given compounds. For instance RET mutants at Val 804 are sensitive to Sorafenib and this could provide an alternative first choice in patients harboring this mutation.

Results from clinical trial suggest that these compounds have a more cytostatic than cytolytic effect, and thus are just adding another step of selective pressure to the progressing tumor, (which buys time), but eventually secondary resistance can develop. In models such as ABL/CML (imatinib), EGFR/lung cancer (Gefitinib), or KIT/GIST (imatinib), prolonged therapy with TKIs leads to the acquisition of resistance mutations in the receptors targeted by these drugs, rendering them insensitive to therapy. Although no secondary RET mutations have been described thus far, the experience with patients undergoing clinical trials taught that some patients suddenly fail to respond while on treatment and most probably, the same underlying resistance mechanisms are at play. This implies that, in order to translate the use of these inhibitors into increased long term survival, we may need to perform molecular follow-up of the progressing lesions, in order to predict resistance.

To reduce the biology of MTC to RET activation and RAS/RAF/MAPK signaling boosting is almost certainly a simplistic view, and data accumulates concerning alternative pathways that contribute to MTC development from the precursor C-Cell Hyperplasia. This is the case of WNT pathway activation by RET-mediated tyrosine phosphorylation of Beta Catenin [79] and synergistic effects of p18 and p27, members of the RB pathway [138], [139]. This may provide additional targets for combination of RET inhibitors with other compounds targeting this pathways. The challenge for the years to come is to use the pools of knowledge generated in RET signaling pathways and MTC progression steps, to rationalize combinatory therapies, targeting different targets and different signaling pathways relevant in MTC. Also relevant to this is the recent recognition of mechanisms of cross talk between different RTKs. For instance EGFR may cooperate with RET in activating intracellular signaling pathways [118]. This provides biological basis for combining different RTK inhibitors.

I.3.2 Familial Non-Medullary Thyroid Cancer

The follicular-cell-derived thyroid cancers (termed Non-Medullary Thyroid Cancer – NMTC) occur mostly sporadically but, intriguingly, NMTC has the highest familial risk amongst all cancer sites. This epidemiological observation is strengthened by the clinical occurrence of NMTC in familial aggregation (FNMTC) and by the detection of chromosomal *loci* in linkage with the disease phenotype. FNMTC *loci* have been proposed at 14q, 1q21, 19p13.2, 2q21, 8p23, 8q24, 1q21 and 6q22 but, to date, no causative mutations were linked to FNMTC. In this chapter we focus on the clinical, morphological and molecular aspects that characterize familial tumors. Some morphological patterns may alert for a familial disease. FNMTC share several of the somatic molecular changes associated to sporadic tumors. New genes affected by somatic changes have been disclosed within regions harboring FNMTC *loci*.

I3.2.1 Familial Clustering of Follicular Cell–Derived Thyroid Carcinomas

The RET associated hereditary forms do not account for the entire fraction of thyroid cancer cases in familial clustering. In addition, cancers derived from the follicular cells (mostly PTC but also FTC) occur in familial aggregation and numerous descriptions of familial pedigrees can be found in the literature [140-156]. The subset of familial cases composed by follicular cell-derived tumors has been termed FNMTC. Most authors describe small families, comprising 2 affected members, more frequently composed of mother/daughter or sister pairs, in keeping with an apparent gender preference observed in sporadic disease. Aside from malignant disease, families show a wide variety of associated benign, relatively common conditions which fall in the group of nodular goitre [156]. Several FNMTC pedigrees show an increased incidence of multinodular goiter (MNG) [157], either as the sole presentation of thyroid pathology in some family members, or in coexistence with PTC in others [156]. One study reported that multiple benign nodules occur in about 42% of thyroid glands from patients with FNMTC compared to 29% in patients with sporadic NMTC [158]. Altogether, the association of PTC with MNG suggests a possible role for MNG in the pathogenesis of hereditary PTC.

The prevalence of FNMTC is rare, varying between little more than 1% [159, 160] up to 6.3% [161]. Most published pedigrees of large families are compatible with the transmission of an autossomal dominant gene with incomplete penetrance and variable clinical expression [156]. However, the hypothesis of polygenic inheritance cannot be excluded, at least in the smallest families. FNMTC shares the features of the hereditary cancer forms in other locations (familial aggregation, Mendelian transmission pattern, early age of onset, multifocallity, associated benign disease) but it has not been explained so far by any known genetic factors. The question which immediately emerges is whether, analogous to other cancer sites, does hereditary counterparts of NMTC actually exist? If so, how can it be differentiated at the morphologic, clinical and molecular levels from the sporadic forms? Which criteria should be weighted to select families for genetic studies and for follow-up of affected and non-affected family members? Epidemiological, clinico-pathological and molecular approaches have been used to address the aforementioned questions.

I3.2.2 Epidemiology of Thyroid Cancer in First-Degree Relatives of Thyroid Cancer Patients

Follicular-cell derived thyroid cancers have the highest familial relative risks (FRR) of all cancer sites: 8.48 in Utah and 9.51 in Sweden [162, 163] (FRR evaluates the risk that a thyroid cancer patient relative has for developing thyroid cancer). Accordingly, the standardized incidence ratio (SIR) of thyroid cancer in first degree relatives (parents or siblings) of thyroid cancer patients is actually higher than that of other cancer sites known to harbor well characterized Mendelian genetic syndromes, such as breast and ovarian cancer (in BRCA families) and colorectal cancer (HNPCC). At least 6 independent studies demonstrated that the risk of developing NMTC is about 5.2 and reaches up to 9.5 times higher in first-degree relatives of affected subjects as compared with the general population [162, 164-169]. Although highly supportive of a familial predisposition for NMTC, these studies do not constitute a definitive proof of the existence of susceptibility gene(s), because factors other than inherited genetic predisposition may explain the familial clustering. Diagnostic bias may come from the fact that family members of affected individuals are more aware of symptoms and seek to be more thoroughly screened for thyroid disease in comparison to control populations. Also, familial clustering may be due to exposure to an unidentified regional environmental factor. Finally, it may result from polygenic inheritance or synergism of low-susceptibility variants.

I3.2.3 Chromosomal *Loci* in Linkage Association with FNMTCs

Another compelling line of evidence for genetic susceptibility in FNMTC is the finding of genetic linkage of discriminate chromosomal intervals with the affected phenotype in FNMTC clusters. Several genetic genomic screens have attempted to map putative *loci* in linkage with FNMTC (Table 2). Linkage was reported at 14q in a familial MNG syndrome, in which 2 of 18 MNG subjects had lesions suggestive of PTC [170] (MNG1 *locus* – OMIM 138800), 19p13.2 in familial NMTC Tumors with Cell Oxyphilia [171] (TCO, OMIM 603386), 1q21 in a form of familial PTC associated with papillary renal neoplasia [172] (OMIM 605642), and 2q21 in familial NMTC with predominance of the follicular variant of PTC [173] (OMIM 606240). Recently, the use of high density SNP arrays, with higher genome resolution than classical panels of microsatellite markers, led to the identification of four novel *loci* at 8p23.1-p22 [174], 8q24 [175], 1q21 and 6q22 [176]. Most of these *loci* emerged from the study of a single (or few) large families and linkage was not reproduced in the entire cohort of families [170]. In several families, linkage to these *loci* has been excluded [157, 170, 177, 178] indicating that none of these *loci* is individually responsible for the majority of the families. Confirmation of linkage in independent family sets has only been reported for 14q in nontoxic familial goiter [179], for 19p13.2 in families with and without cell oxyphilia [180], and for 2q21, both in an independent sample set of 80 pedigrees [173], and in ten FNMTC families, 9 of which presented with cell oxyphilia [181]. Furthermore, *loci* at 19p13.2 and 2q21 may interact, resulting in significantly increased risk of NMTC for patients that carry both susceptibility *loci* [181].

Limited efforts have been put into screening of candidate genes within the mapped regions, because no straightforward strategy was devised to filter putative candidates and reduce the number of genes to submit to mutation screening. Targeted testing for germline mutations has

been performed on the basis of an educated selection of gene candidates, but it always resulted in negative findings. Exceptionally, a systematic screening of 14 candidate genes mapping to the region of linkage in affected TCO members led to the identification of two novel germline changes in TIMM44 [182]. However, no substantial loss of function effects were identified for the mutant alleles and no confirmation of TIMM44 mutations came from further studies in independent samples of affected individuals with TCO (Prazeres H and Máximo V, unpublished results).

In addition to the aforementioned *loci*, other putative regions candidate for FNMTC predisposition *loci*, are the ones underlying syndromes with an increased risk of NMTC, in which other cancer types represent the major component (Table 2). This is the case for familial Adenomatous Polyposis (FAP) caused by APC mutations (OMIM 175100), the Cowden syndrome - familial hamartoma syndrome (OMIM 158350) caused by mutations in PTEN, Cowden-like syndromes (OMIM 612359) resulting from mutations in Succinate Dehydrogenase subunits B and D (SDHB and SDHD), Carney complex type 1 (OMIM 160980) caused by mutations of the protein kinase A regulatory subunit type 1-alpha gene, Werner Syndrome (OMIM 277700) caused by mutations in the a homolog of the E. coli RecQ DNA helicase (RECQL2) and McCune-Albright syndrome (OMIM 174800) resulting from mutations in the alpha-subunit of the stimulatory G protein (GNAS1 gene).

In FAP, PTC or at least a PTC-like carcinoma, occurs with a frequency of about 10 times higher than that expected for sporadic PTC [183] and displays an unusual cribriform-morular growth pattern [184]. Almost every patient with the so-called cribriform-morular variant of PTC in the setting of FAP are women, whereas there is an equilibrium in gender distribution of the intestinal polyps[183]. In the Cowden syndrome, FTC occurs with an increased frequency [185]. The salient thyroid lesions in this syndrome are multicentric follicular adenomas and adenomatous (parenchymatous, hyperplastic) nodules showing a wide range of nonspecific cytoarchitectural patterns [186]. Multiple tiny cellular foci, so-called microadenomas, are also a feature of thyroid tumors in the Cowden Syndrome [186]. The occurrence of FTC is a major criterion for Cowden disease and may derive from the progression of the FTAs. In Cowden-like syndromes, distinct genetic defects underlie a spectrum of breast, PTC and renal cancers [187]. Both PTC and FTC have been found in about 11% of patients with Carney complex type 1 [188]. Patients with Werner syndrome have increased risk of PTC (the only tumor present in white patients), and the commonest in Japanese patients (84%), followed by FTC (14%) and anaplastic thyroid carcinoma (2%) [189]. Thyroid disease is the second most common endocrinopathy associated with McCune-Albright syndrome [190], consisting mostly of hyperthyroidism with and without nodular or diffuse goiter [191, 192]; PTC and clear cell carcinoma of the thyroid were also reported [192] .

However, none of the aforementioned *loci* for syndromic FNMTC seems to account for the site-specific FNMTC [193]. Altogether, these studies highlight the genetic heterogeneity of FNMTC[194].

Table 2 Summary of morphological patterns and somatic molecular genetics in syndromic and site-specific FNMTC.

Syndromic FNMTC	Chromosomal Location	Predisposing Gene	Morphological Patterns	Somatic Pathology
Familial Adenomatous Polyposis	5q21	APC	Cribriform-Morular Variant of PTC	RET/PTC1[195]
Cowden Syndrome (Multiple Hamartoma)	10q23.3	PTEN	Multiple Adenomatous Nodules; FTA, FTC	Not assessed
Cowden-like Syndromes	11q23 and 1p36	SDHB and D	PTC	Not assessed
Carney Complex Type 1	17q22-24	PRKAR1alpha	Multiple Adenomatous Nodules; FTA, PTC, FTC	Not assessed
Werner Syndrome	8p11-21	WRN	PTC, FTC, ATC	Not assessed
McCune-Albright Syndrome	20q13.2	GNAS1	Nodular and diffuse goiter FA, FC, PTC, Clear Cell Carcinoma	Not assessed
Site-specific FNMTC				
TCO - Tumors with cell oxyphilia	19p13.2	Unknown	Hurthle-cell variants	19p13.2 LOH [196] GRIMM19 [197]
Papillary Thyroid and Renal Neoplasia -fPTC/PRN1	1p13.2-1q22	Unknown	Conventional PTC; Papillary Renal Neoplasia	Not assessed
NMTC1	2q21	Unknown	Follicular Variant of PTC	LOH at 2q21 [198]
MNG1	14q32, Xp22	Unknown	PTC in background of Multinodular Goiter	Not assessed
FNMTC	8p23.1-p22	Unknown	Benign thyroid disease, PTC, FTA, FTC without distinctive features	BRAF V600E [178]
FNMTC	8q24	Unknown	Benign thyroid disease, PTC and Melanoma	Not assessed
FNMTC	1q21 and 6q22	Unknown	No distinctive features	Not assessed

Legend: FTA- Follicular Thyroid Adenoma, FTC- Follicular Thyroid Carcinoma, PTC – Papillary Thyroid Carcinoma, ATC – Anaplastic Thyroid Carcinoma.

I3.2.4 Spectrum of Clinico-Pathological Features in FNMTCs

It is still debatable whether or not FNMTC displays more aggressive clinico-pathological features than their sporadic counterparts. Clinically, familial PTCs, the commonest form of FNMTC, are characterized by increased frequency of multifocallity and greater recurrence rate, indicating that these tumors may be more aggressive and constitute a distinct clinical entity, carrying a worse prognosis [199-201]. In a large study comprising 258 FNMTC cases, the patients were more likely to have intraglandular dissemination (40.7% *vs.* 28.5%.) and multiple benign nodules (41.5 *vs.*29.8%), compared to the patients with sporadic disease[158]. There were no significant differences between the two types of patients in terms of the other pathologic features [158]. Recurrence was more frequent in the FNMTC patients compared to the control group (16.3% *vs.* 9.6%); the disease-free survival rate was significantly poorer in the FNMTC patients, but no statistically significant difference in the overall survival rate was observed [158]. Because of the high frequency of intraglandular dissemination and of lymph

node metastases, some authors recommend more aggressive therapy for FNMTC [200-202], and others recommend screening with ultrasonography for early detection in relatives of patients with FNMTC [203]. In contrast to this, an early meta-analysis study concluded that there is debatable evidence of increased clinical aggressiveness [204] and in a matched case-control study no significant difference in disease-free survival between familial and sporadic NMTC was observed [205]. Ito and colleagues [199] found that despite familial PTC showed more frequent multicentricity and recurrence than sporadic PTC, disease-free survival and cause-specific survival rates of patients with FNMTC did not differ from those with sporadic carcinomas [199]; there were no differences in other clinico-pathological parameters between the two groups [199]. One criticism that may be pointed to several of these studies is that, in most of them, a familial predisposition has been considered when only 2 family members were affected. Using this criterion alone, the FNMTC patients' population may be contaminated by a relatively large number of patients with sporadic NMTC occurring by chance. Charkes [206] found that a significant majority of two-case families are composed of clinically evident sporadic cases (probability of 62-69%) [206]. In families having 3 or more affected members, the probability of having one sporadic case by chance is lower than 6% and of two sporadic cases is lower than 0.15% [206]. When patients are stratified by the number of affected members in the family, the differences become more evident; patients with FNMTC belonging to families with 3 or more thyroid cancer-affected members carried the worst prognosis [202, 207]. Alsanea and colleagues [202] have shown that the best predictors of poor outcome in patients with FNMTC are the number of family members affected by thyroid cancer and the evidence of distant metastasis [202].

To overcome the aforementioned controversy on the clinical aggressiveness of FNMTC, strict definition of criteria for FNMTC classification is mandatory. In a meta-review, Musholt and colleagues [208] proposed the following set of criteria to define FNMTC: Primary criteria: 1. The occurrence of at least 2 first-degree relatives affected with a follicular cell-derived thyroid cancer, 2. A PTC patient with at least 3 first- or second-degree relatives presenting with multinodular goiter (MNG). Secondary criteria: 1. Cancer diagnosis in a patient younger than 35 years; 2. Multifocal or bilateral PTC; 3. Organ-exceeding tumor growth (T4); 4. Metastasis (N1, M1); 5. Familial accumulation of adolescent-onset thyroid disease. A hereditary predisposition to PTC is considered if either both primary criteria, or one primary criterion plus three secondary criteria are present [208]. Exclusion criteria include previous radiation exposure and co-existence of other cancer syndromes [208].

FNMTC displays the phenomenon of anticipation (earlier age at disease onset and increased severity in successive generations) [209]. Among 47 FNMTC with parent-child relationship, an earlier age at disease presentation and diagnosis was found in the 2nd generation compared to the 1st generation [209]. Patients in the 2nd generation were more frequently males; their tumors were more frequently multifocal and bilateral, had higher rate of lymph node metastases at surgery and worse outcome, as compared to the 1st generation [209].

I3.2.5 Spectrum of Histotypes and "Familial Variants" of FNMTCs

Detailed pathological findings of the different types of tumors included in FNMTC series were thoroughly reported by Harach in 2001 [161]. The proportion of PTC and FTA/FTC appear to be similar in familial and sporadic cases. PTC with a prominent papillary growth pattern and typical nuclear features (conventional PTC), comprises the most common histotype in most familial clusters, as well as in the sporadic setting (22). Occasionally, in FNMTC, PTC and FTC occur concomitantly in different nodules from the same patient (22). A careful pathological review of the FNMTC cases on record with published histological pictures does not seem to point to a distinct morphological variant of familial carcinomas, in contrast with the cribriform morular histotype found in association with FAP [210]. Despite the absence of a specific phenotype of FNMTC, some characteristic histology features have been observed in some families, suggesting the existence in some settings of a putative 'familial' phenotype of FNMTC [211].This is well illustrated by the finding of familial clustering of tumors with cell oxyphilia [212] that have been shown in families linked to 19p13.2 [171, 181]. Moreover, families linked to 2q21 show an apparent over-representation of the follicular variant of PTC [173]. Further stratification of families based on the presence of at least one case of follicular variant of PTC increased the LOD score for linkage to the 2q21 *locus* [173]. Other pathological features have been indicated as being over-represented in familial tumors but were not confirmed in independent series. Patients with FNMTC were reported to have a 9 times more common association with lymphocytic thyroiditis than patients with sporadic PTC [213]. These results gain relevance in the light of the finding that familial autoimmune thyroid disorders, such as Graves disease and Hashimoto thyroiditis, demonstrate linkage to the same chromosomal region as the FNMTC *locus* MNG1 at 14q [214, 215], implying the putative existence of a genetic link between chronic inflammation and benign thyroid disease.

Based on the aforementioned findings, it has been advanced a number of characteristics that might also be suggestive of a familial predisposition, when occurring in the setting of a small familial cluster of NMTC: unusual histology appearance of PTC, including PTC with cell oxyphilia; concurrence of PTC and FTC in the same patient; and the diagnosis of follicular variant PTC [198]. Familial-suggestive phenotypic observations somehow parallel the findings of different *loci* in distinct families and support the idea that additional morphologic evaluation may contribute for reducing heterogeneity in familial cohorts submitted to genetic studies[194]. Furthermore, the correct histological interpretation of the morphological patterns characteristic of hereditary/familial disease may lead to further molecular genetics evaluation of the patient and family members. For instance, if a tumor with a cribriform-morular pattern precedes any colonic manifestation, the pathologist should alert the clinician for the need to exclude FAP. In family members from patients with site-specific FNMTC, for which no predictive genetic tests are available, a personalized follow-up strategy using ultrasound may be used to detect thyroid disease at an early stage [203].

I3.2.6 Spectrum of Somatic Molecular Changes in FNMTC

The past decade was prolific in findings of well defined molecular alterations displaying high genotype/phenotype correlation with the major histotypes of sporadic thyroid tumors [216]. These include rearrangements and point mutations. The most common structural alterations in PTC are translocations involving receptor tyrosine kinases RET [217] and NTRK1 [218], comprising 20-30% of PTCs in adults [219]. Their incidence is greater in children (45-60%) [219, 220] and in populations exposed to ionizing radiation (50-80%), either accidentally or for therapeutic purposes [221, 222]. The most common rearrangement are RET/PTC1 [217], being RET/PTC3 the dominant type arising from radiation exposure. RET/PTC is typical of PTC and is absent in FTC. On the other hand, 45.5% of FTCs, 33.3% of FTAs and 37.5% of follicular variant of PTC harbor rearrangements of PAX8/PPARγ [71] which are absent in conventional PTC[68]. Activating point mutations in one of the three RAS oncogenes, H-, K-, and particularly N-RAS codon 61, occur with low frequency in PTCs (<10%) and are more relevant in FTCs (22.2%) and in the follicular variant of PTC (25.5%). A single point mutation in BRAF (V600E) is found in an high proportion of conventional PTCs (30-70%) but rarely in follicular thyroid neoplasms [41, 223]. It is conceivable to question whether the same genetic changes are found in familial forms. If holding true, it would argue for a common genetic progression pathway in sporadic and familial forms; if false, it might be an indirect indication that alternative genetic factors, of presumable germline nature, were driving tumor growth in the familial settings. There is a major gap on molecular data concerning the somatic changes in FNMTC. According to the few studies that have approached this question, the former hypothesis seems to be valid: the same genetic lesions typical of the sporadic forms are also present in familial tumors (Table 2). One study revealed that 52% of familial tumors harbored somatic mutations: BRAF-V600E mutation was observed in 41% of PTCs and H-RAS and N-RAS mutations were detected in 23% of PTCs (either conventional or follicular variant) [178], suggesting that the somatic activation of BRAF and RAS plays a role in FNMTC tumorigenesis. One could argue that these results may arise from the contamination of the population of familial cases with sporadic cases occurring by chance [206]. It has also been found that PTCs occurring in FAP patients can also harbor somatic RET/PTC1 rearrangements [195]. Out of 4 cases of the so-called cribriform-morular variant of PTC, one of which in the context of FAP, the T1799A BRAF mutation was absent [224]. We did not find in the literature any large studies of point mutations of BRAF in thyroid tumors associated with FAP, or other familial tumor syndromes, nor any data published on RAS or PAX8/PPARγ in FTCs originating from germline PTEN mutations.

To address the question of whether familial tumors constitute a molecular entity different from that of sporadic forms, in an open, genome-wide approach, Brunaud and colleagues [225] performed analysis of chromosomal aberrations in FNMTCs by Comparative Genomic Hybridization and found no common germline or somatic chromosomal aberrations that might be specifically associated to patients with FNMTC [225]. The aberrations detected in familial tumors corresponded to several locations of candidate genes already reported in sporadic tumors thus suggesting a similar tumorigenic process in both settings [225].

In the search for FNMTC susceptibility genes, a more significant insight into the tumorigenesis of the sporadic forms has been gained by identifying novel somatic lesions at regions pinpointed by linkage studies. There is evidence of somatic inactivation hits, such as LOH, within regions previously reported to harbor susceptibility *loci* for site-specific FNMTC, such as 19p13.2 and 2q2, in sporadic tumors [196]. The investigation of candidate genes within the 19p13.2 interval (a region previously associated with oxyphilic familial tumors) led to the study of GRIM-19, a dual function gene involved in mitochondrial metabolism and cell death. Somatic missense mutations in GRIM-19 were detected in 3 out of 20 sporadic Hurthle cell carcinomas [197]. A germline mutation was detected in a Hurthle cell variant of PTC arising in a thyroid with multiple Hurthle cell nodules [197]. However, no GRIM-19 mutations were detected in any of the 6 cases of a known family with Hurthle cell tumors linked to 19p13.2, thus excluding the identification of GRIM-19 as the TCO gene [197]. GRIM 19 is a member of complex 1 of the mitochondrial respiratory chain and its role as a tumor suppressor gene has been functionally emerging [226]. Interestingly, several genetic lesions affecting complex 1 component have been described in oncocytic thyroid cancers [227].

In conclusion, FNMTC seems to constitute a heterogeneous genetic entity, and probably a complex disease, with several contributing *loci* [194]. Linkage to the known *loci* has been excluded in many families and it is conceivable that novel susceptibility *loci* still remain to be identified.

It is disappointing to acknowledge the limited results obtained from screening of positional candidate genes within the linkage regions. These regions usually refer to gene-rich regions and the endeavor of selecting the genes to target for mutation screening requires therefore some sort of previous filtering strategy. One possibility is to use mapping in combination with expression profiling in order to filter genes transcriptionally deregulated within these intervals. This strategy has proven effective in the identification of a gene for pituitary adenoma predisposition [228].

Alternative genome scans may also be considered to disclose changes that might have been missed by previous approaches. The emergence of massive parallel sequencing technology will turn possible to screen systematically, in the near future, the genes in the aforementioned regions in linkage association with FNMTC.

It is important to note that the above mentioned genetic approaches should take into consideration the different histotypes of FNMTC and that it will be crucial to use strict criteria and a consistent morphological assessment for reducing the heterogeneity of cohorts of families subjected to genetic studies.

Finally, molecular pathology may help to decide whether or not familial and sporadic forms of thyroid carcinoma differ from a pathological and a clinical standpoint. The known molecular signatures of sporadic NMTC, namely RET/PTC, BRAF V600E, N-RAS, PAX8/PPARγ are poorly studied in series of familial tumors. Gene expression signatures have been produced for virtually every cancer type, including the thyroid cancer histotypes but a molecular signature of FNMTC is still lacking.

Aims

The general aims of this work were to elucidate, from genetic and functional standpoints, changes associated with familial thyroid cancer, derived either from C-Cells (project 1) or from follicular cells (project 2).

The specific aims were to:

1. Characterize the spectrum of RET mutations in sporadic and familial MTC/MEN2B;

2. Undertake haplotype studies to address possible founder effects of RET mutations in the population under study;

3. Classify novel RET variants eventually found from a functional point of view;

4. Disclose potential candidate genes involved in FNMTC susceptibility and to

5. Functionally ascertain their role in tumorigenesis of familial and sporadic NMTC.

Results

Results from Project 1

R1.1 The RET Mutation Spectrum in a Population from the Central Region of Portugal

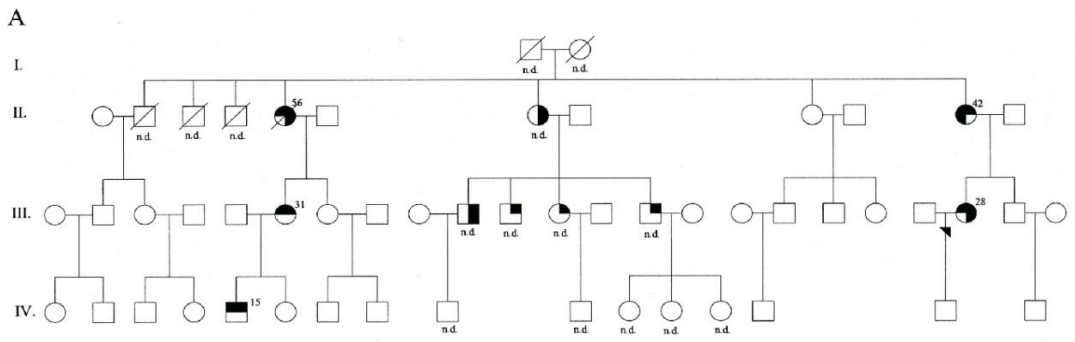
We have characterized RET proto-oncogene mutations in 6 families with MEN2 (5 MEN2A and 1 MEN2B), as well as 58 AS-MTC cases originating from the central region of Portugal (this series was updated in relation to the series published in paper I). Phenotypic and genotypic data is shown in Figure 3 and summarized in Table 3.

Table 3 RET germline mutations found in MEN2 and AS-MTC cases

Detected Mutation	Nucleotide Change	Number of families (%)	Family Annotation	Disease Phenotype
Cys634Phe	TGC-TTC	1 (9%)	A	MEN2A
Cys634Arg	TGC-CGC	2 (18%)	B, G	MEN2A
Cys611Tyr	TGC-TAC	5 (36%)	C, D*, case L, F*, J*	MEN2A/CMTF/AS-MTC
Met918Thr	ATG-ACG	1 (9%)	MEN2B case	MEN2B
Arg886Trp**	CGG-TGG	1 (9%)	E*	CMT-E
Ser649Leu**	TCG-TTG	1 (9%)	H*	CMT-E
Glu511Lys**	GAG-AAG	1 (9%)	I*	CMT-E
Total		12		

Legend: (**) indicates novel variants, (*) indicates germline mutations detected in AS-MTC.

Overall we detected four known RET mutations: Cys634Arg and Cys634Phe (3 families); Cys611Tyr (5 families); Met918Thr (1 MEN2B case). A total of 6 germline mutations were found amongst the 58 Sporadic MTC cases studied, resulting in an occult mutation rate of 10%. We additionally detected 3 novel RET variants: Arg886Trp, Ser649Leu and Glu511Lys, which were the focus of additional studies.



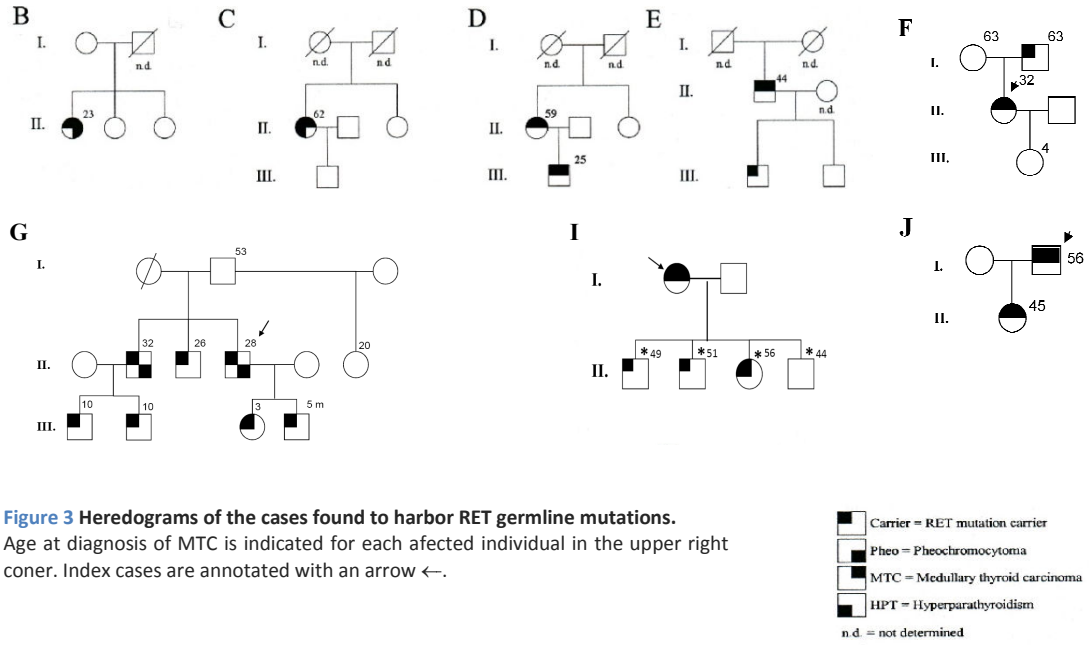


Figure 3 Heredograms of the cases found to harbor RET germline mutations.
Age at diagnosis of MTC is indicated for each afected individual in the upper right coner. Index cases are annotated with an arrow ←.

R1.2 Haplotype studies in Cys611Tyr families

Overall, 5 out of the 12 (40%) families in which RET mutations were detected harboured the Cys611Tyr mutation (Table 4). In order to investigate whether a common haplotype could underlie the inheritance of the Cys611Tyr mutation we have ascertained the haplotype cosegregating with this mutation in these families. For this purpose we genotyped microsatellite and polymorphic markers linked to the RET locus, namely D10S141 (upstream of the RET *locus*), a CA dinucleotide repeat within intron 5, and ZNF22 (3' of RET). The haplotype found to be linked with the Cys611Tyr mutation in these families is shown in Table 4. We found that four out of the five (80%) Cy611Tyr families shared a common haplotype. In families C, D, F and J, the mutant Cys611Tyr allele cosegregates with an haplotype defined by 120 bps at marker D10S141, 246 bps at intron5 and 166 bps at ZNF22. In contrast, case L did not share any allele ate these markers, indicating that an independent mutational event occurred in this case.

Table 4 Haplotype analysis of the Cys611Tyr families
The genotypes observed for each microsatellite marker are expressed in size (basepairs) of each of the alleles.

Marker	Family C	Family D	Case L	Family F	Family J
D10S141	120	120	112/122	120	120
INT5(CA)n	246	246	248/260	246	246
Cys611Tyr	A	A	A	A	A
ZNF22	166	166	148/150	166	166

With this data in hand, we investigated the geographical origin of these families we found that they circumscribed to the central region of Portugal. Further investigation of family members allowed us to trace common ancestors that link families D, F and J (Figure 4).

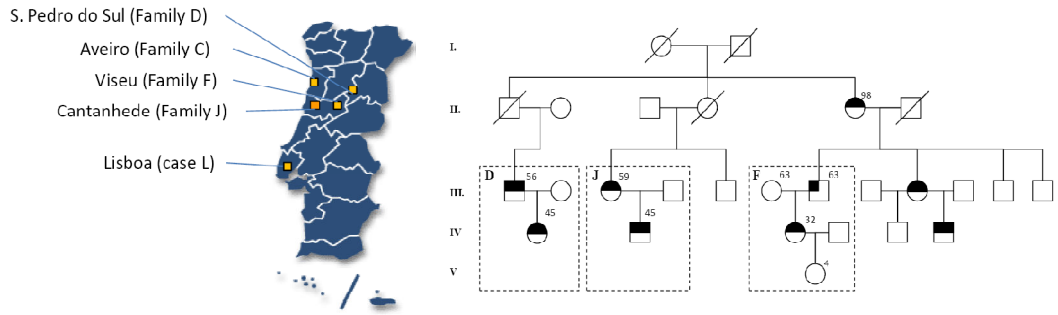


Figure 4 **Geographical origin and heredogram illustrating the common origin of three Cys611Tyr**

R1.3. Transforming Potential and Signaling Properties of Novel RET variants

To first address the pathogenic significance of RET variants Glu511Lys, Ser649Leu, and Arg886Trp we ascertained whether these variants occurred amongst individuals of the general population, so as to exclude the possibility that these constituted RET polymorphisms. For this purpose, we screened RET exons 8, 11 and 15 in a group comprising 70 regional controls (140 chromosomes) by means of Single-Strand Conformation Polymorphism (SSCP) analysis. We only found wild type alleles in the control group making it highly unlikely that these RET variants constitute polymorphisms.

We next checked whether co-segregation between carrier status and disease phenotype would be observed in these families. For this purpose we performed clinical examination, biochemical measurements of Calcitonin (CALC), Vanyl Mandelic Acid (VMA), urinary Metanephrines (MET) and Parathormone (PTH) as well as RET genetic analysis of the first degree relatives of mutation carriers in these kindreds. Family members were available for testing only in Family E (Arg886Trp) and Family I (Glu511Lys). The results of carrier status are summarized in Figure 3. In Family E, one of the two sons of the index, currently aged 27y, was shown to have inherited the Arg886Trp mutation (Figure 3). However, clinically this sibling had normal basal and pentagastrin-induced calcitonin levels and VMA, MET and PTH were also within normal ranges. In Family I, we were able to study four offspring of the index case, three of which have shown to harbour the RET Glu511Lys variant. Clinically there were no signs of MTC and all siblings showed biochemical values of CALC, VMA, MET and PTH within normal ranges (Figure 3).

Since co-segregation studies were not conclusive, we further undertook to performed functional *in vitro* analyses in order to better address the transforming potential of the uncharacterized RET variants. RET displays the classical features of an oncogene, and RET activating mutations induce transformation of NIH3T3 fibroblasts, leading to the formation of

foci. We generated vectors expressing RET mutants (chromatograms in Figure 5) and we evaluated the ability of these RET variants to transform NIH3T3 fibroblasts, relative to RET wild-type and to known RET activating mutations (Cys634Arg), by performing NIH3T3 focus formation assays.

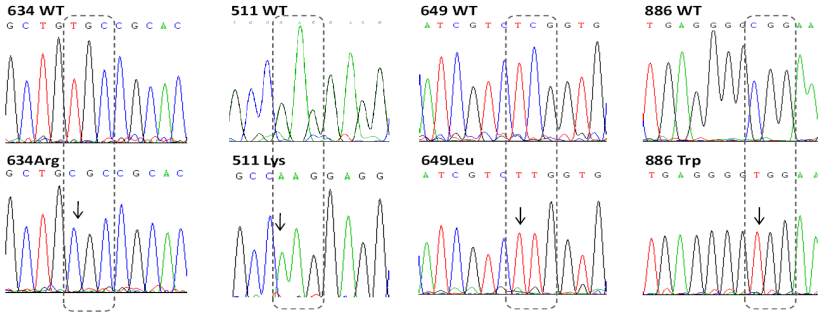
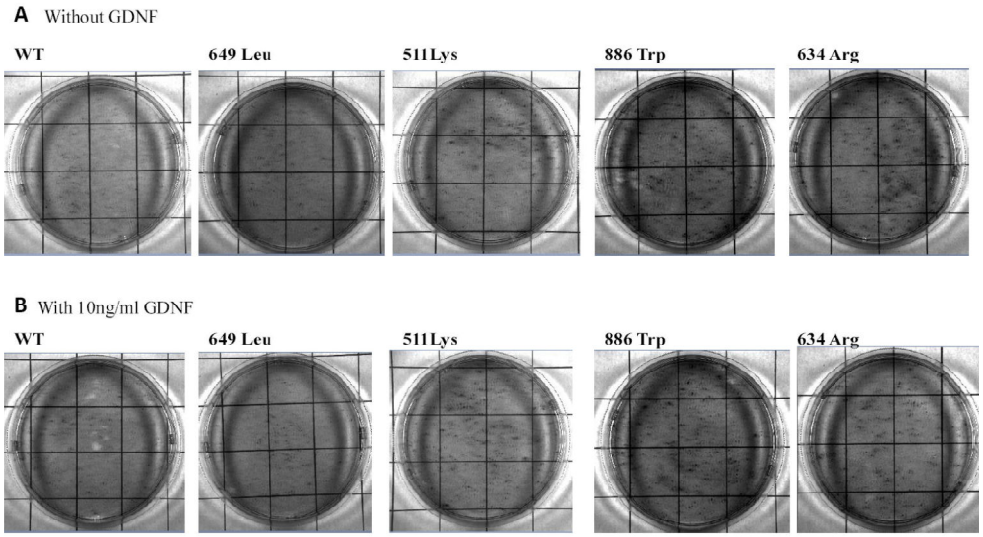


Figure 5 **Generation of RET mutants at codon 511, 649 and 886 by site-directed mutagenesis**

By counting the number of foci induced by different RET variants we found that RET Glu511Lys (in the presence of GDNF) and Arg886Trp showed increased transforming potential *in vitro.* This was demonstrated by increased number of foci relative to wild type RET, in NIH3T3 focus formation assay (Figure 6A, B and C) and by higher number of colonies in soft agar-assays (Figure 6). In contrast, the Ser649Leu variant does not induce increased number of foci or agar colonies (even in the presence of GDNF), as shown in Figure 6C and D.



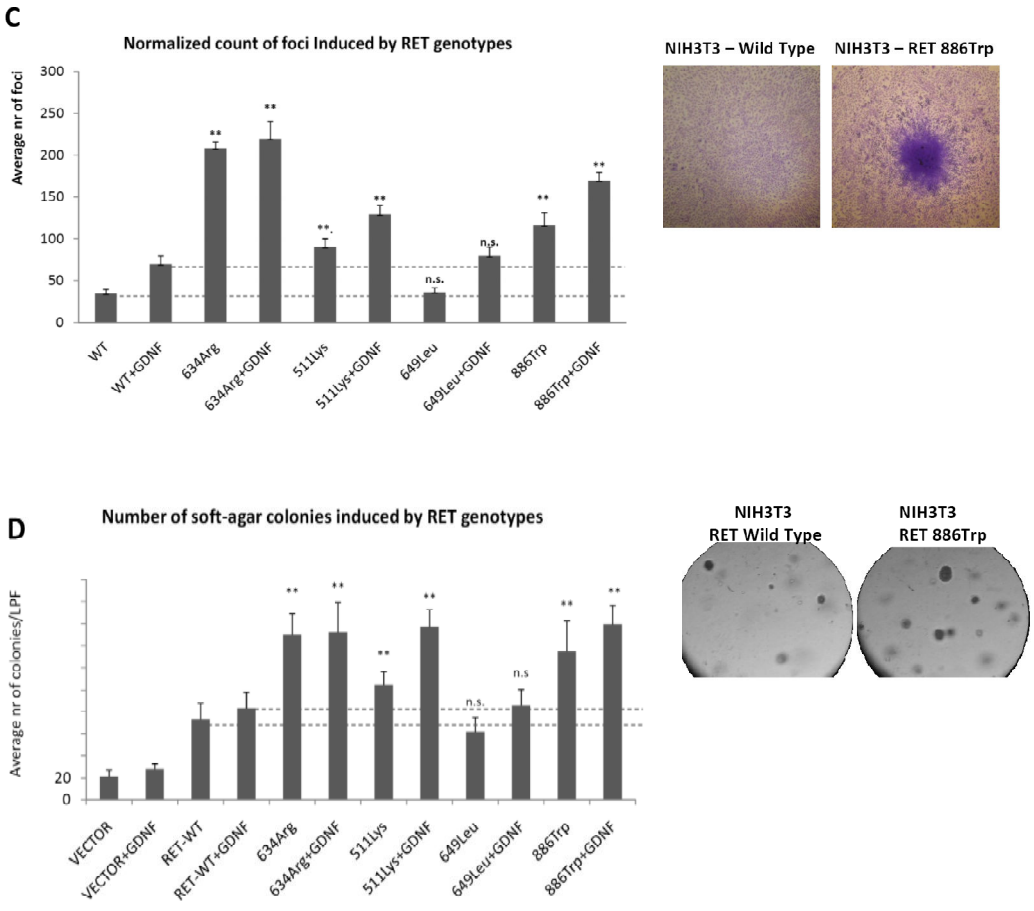


Figure 6 Transforming capacity of RET variants Glu511Lys, Ser649Leu and Arg886Trp
NIH3T3 focus formation assays and anchorage-independent soft-agar assays were performed. Representative photographs of plates obtained after focus formation experiments performed in the absence (**A**) or in the presence of 10ng/ml of GDNF (**B**). (**C**) The transformation capacity is expressed by the number of transformation foci generated by each RET mutant plasmid, normalized to the transfection efficiency estimated by counting the number of stable clones obtained under antibiotic selection conditions. Direct comparisons were made to RET wild-type under the corresponding condition (with or without GDNF) and significance of the observed differences is expressed by the P value calculated by the Student's *t*-test (on top of each bar). Significance ($p<0.05$ or $p<0,001$) is annotated by one (*) or two asterisks (**), respectively and non significant differences by "n.s.". The insert shows photographs of NIH3T3 focus formed as a result of RET-induced transformation. (**D**) Transforming activity of RET mutants was further addressed by Soft-agar assays with and without addition of GDNF. The graph presents values obtained from counting 10 low-power fields for each RET mutant. Again, direct comparisons were made to RET wild-type under the corresponding condition (with or without GDNF) and significance of the observed differences are expressed by the *p* value calculated by the Student's *t*-test (on top of each bar). Significance ($p<0.05$ or $p<0,001$) is annotated by one (*) or two asterisks (**), respectively and non significant differences by "n.s". The insert shows photographs of NIH3T3 soft-agar colonies formed as a result of RET-induced transformation.

RET is a Tyrosine Kinase receptor which induces the activation of several intracellular signaling pathways (Figure 2). We went on to ascertain whether intracellular signaling pathways known to be targeted by RET were increasingly activated as a result of transient expression of the different RET variants studied by employing Luciferase pathway reporting assays (Figure 7). RET wild-type and the activating mutation Cys634Arg were used as controls. We found that variants Glu511Lys and Arg886Trp show a 6 and 8 fold ERK activation, which is higher than wild-type RET (4 fold) (Figure 8A). Increased levels of STAT3, STA1 and TCF-4 activation were only observed for RET Arg886Trp (2.0, 2.5 fold and 3 fold versus 1.3, 1.2 and 2 fold in RET-WT, respectively) (Figure 8B, C and D).

Overall, distinct RET variants seem to display different signaling properties. We further performed a hierarchical clustering according to the signaling profile of the distinct RET genotypes Figure 8E). This classification shows that variant Cys634Arg (a mutation known to be highly activating) show stronger activation of the evaluated pathways. On the other hand, variant Ser649Leu clusters together with wild-type RET, indicating a similar signaling behavior. However, variants Glu511Lys and Arg886Trp group in a different sub-cluster since they have a higher signaling activity relative to RET wild-type, which nonetheless is lower than that of Cys634Arg (Figure 8E).

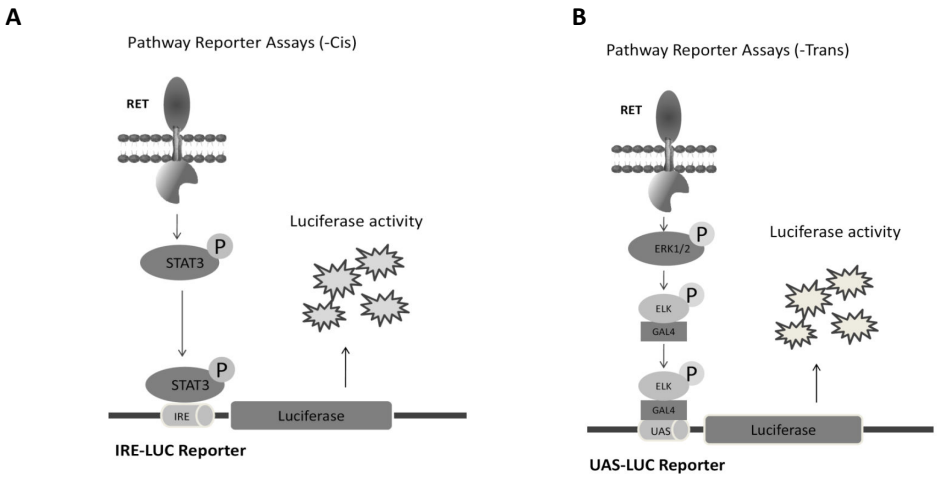


Figure 7 Luciferase pathway reporter systems.
(**A**) In the *cis* system, a vector expressing firefly luciferase upstream containing an upstream motif for the Interleukin Response Element (IRE-LUC reporter) is co-transfected with plasmid expressing RET in the different mutant configurations. RET expression and tyrosine-kinase activity phosphorylates Signal Transducers and Activators of Transcription such as STAT3. The activated STAT3 will subsequently bind to the IRE motifs and induce luciferase expression. In this sequence of events the activity of luciferase reflects the degree of RET signaling activity. (**B**) In the *trans* system, a plasmid encoding the GAL4 DNA binding domain fused to the ELK transactivation domain is transfected along with a vector containing the Upstream Activation Sequence (recognized by GAL4) and Luciferase. ERK1/2 phosphorylation will result in ELK activation luciferase transactivation through the GAL4-UAS binding.

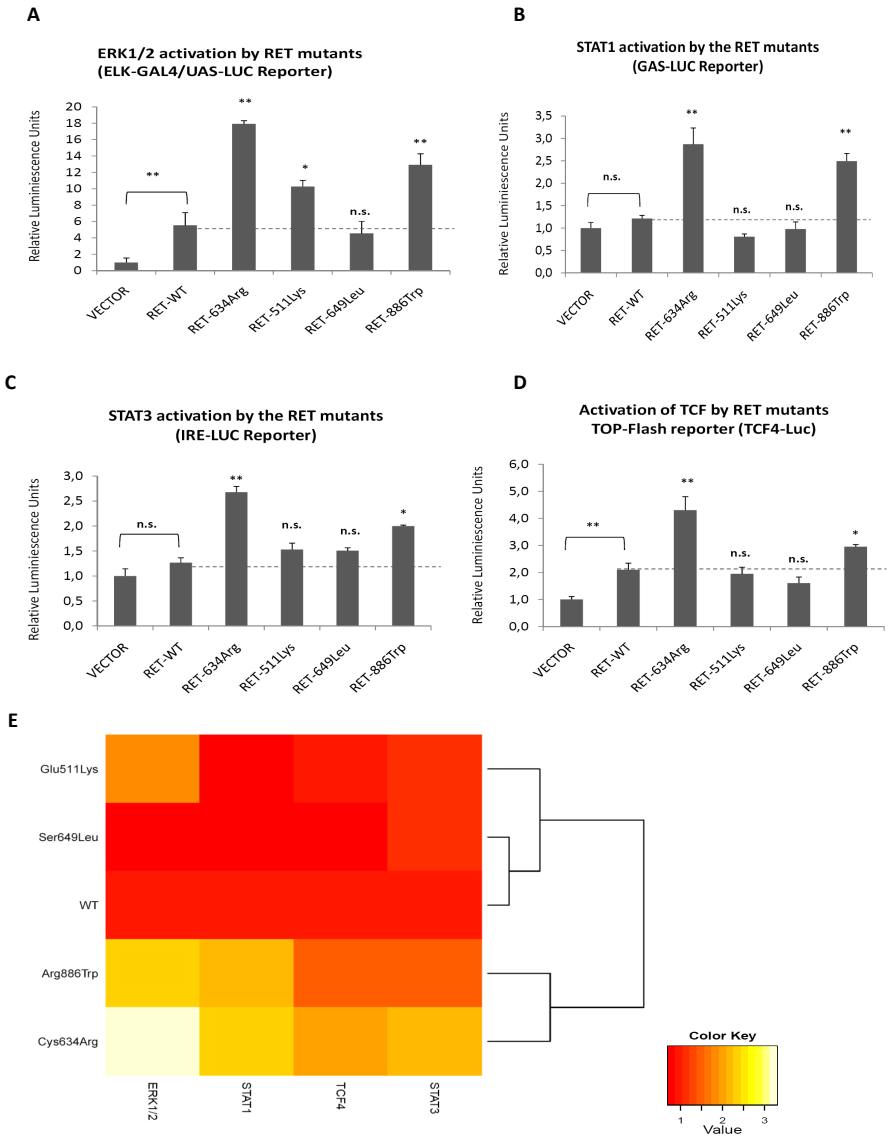


Figure 8 Signaling properties of RET variants Glu511Lys, Ser649Leu and Arg886Trp.
The degree of RET signaling activity associated with RET variants relative to wild-type RET and Cys634Arg, used as controls, was determined by employing luciferase assays reporting the activation of known RET signaling targets such as ERK1/2 (**A**), STAT3 (**B**), STAT1 (**C**), and TCF4 (**D**). Luminescence units were defined relative to the values obtained in wells in which cells were transfected with reporter vector and empty vector (pRcCMV). A comparison between empty vector (VECTOR) and wild-type RET (RET-WT) was made to check whether activation of these pathways was achieved by canonical RET. To ascertain if the RET mutants under study further induced these pathways, direct comparisons were made relative to the activation levels generated by wild-type RET. Statistically significance, determined by the Student's *t*-test, was defined at $p<0.05$ (*) or $p<0,001$ (**). Non significant differences are indicated by "n.s." Experiments were always performed in triplicates and were repeated three times for ERK1/2, STAT3 and STAT1 assays and two times for TCF4 (Topflash) reporter. Graphs present data from one representative experiment. **E** - Heat-map and clustering according to the activation levels observed for each pathway upon transfection of each of the RET genotypes under study, relative to empty vector. Graphics were produced using the R software (heatmap.2 of the gplots package).

Additionally, we performed experiments designed to evaluate the sensitivity of the RET genotypes to treatment with the kinase inhibitor Sorafenib. Although Sorafenib (BAY 43-9006) was designed originally as a RAF inhibitor [127], pre-clinical studies have shown that Sorafenib can inhibit the kinase activity and signalling of wild type and oncogenic RET [119], [128], [125]. Since ERK1/2 was the most preeminently induced pathway by the RET mutants (as we have observed in previous experiments), we used the ERK1/2 reporting system to address the degree of inhibition of RET intracellular signaling activity upon treatment with 4uM of Sorafenib for 48 hours. Treatment with Sorafenib suppressed ERK1/2 activation in slightly different extents among RET genotypes, nonetheless all three variants under study have shown some degree of response (Figure 9).

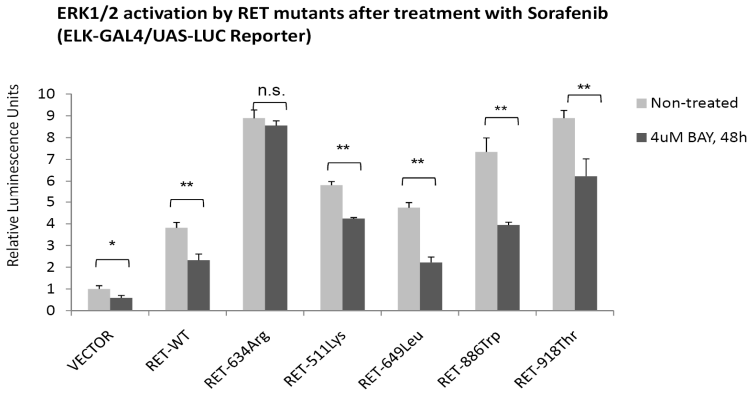


Figure 9 Sensitivity of RET genotypes to Sorafenib.
In order to check for Sorafenib-mediated suppression of ERK1/2 associated with each RET genotypes, plasmids encoding variants Glu511Lys, Ser649Leu and Arg886Trp, as well as wild-type RET, Cys634Arg and Met918Thr, (used as controls), were co-transfected along with ERK1/2 reporters after which cells were put either in medium containing either 4ug/ml Sorafenib, or in medium without Sorafenib. Luminescence units were defined relative to the values obtained in wells in which cells were transfected with reporter vectors and the empty vector (pRcCMV). Comparisons were made between treated and non-treated conditions within each genotype. Statistically significance, determined by the Student's *t*-test, was defined at $p<0.05$ or $p<0,001$ and is annotated with one (*) or two asterisks (**), respectively. Non significant differences are indicated by "n.s."

Results from Project 2

R2.1 Loss of Heterozygosity at FNMTC susceptibility *loci* 19p13.2 and 2q21 suggests that these regions harbor putative tumor-suppressor genes inactivated in the Tumors from Familial Clusters of Non-Medullary Thyroid Carcinoma

Loss of heterozygosity (LOH) is a frequent observation in tumors, occurring as a result of a deletion mutation, which can contribute to the inactivation of tumor suppressor genes. According with the Knudson's two hit theory, and in line with robust observations in a variety of hereditary cancers, the inactivation of tumor suppressor genes is often the result of a first (germline) mutational event (first "hit"), followed by deletion of the corresponding normal gene copy (second "hit"), with demonstration of LOH in tumor samples. Thus, the observation of LOH at a given chromosomal region, with selective loss of the normal allele, may provide an indication for the location of potential tumor suppressor genes conferring susceptibility for familial tumors. In accordance to this rational, we performed LOH analysis in tumors 14 tumors from 9 two-case familial clusters of NMTC (Table 5).

Table 5 Family classification and clinical-pathological features of the tumors analysed for LOH.

Family	Nr of cases	Type of Kindred	Gender	Age	Histopathological features	TNM	N/ T sample nr
A§	2	Index	F	39	PTC,	**T4N1**M0	N1/T1
		Sister	F	40	PTC, CO*	**T4**N0M0	N2TT2
B	2	Index	M	47	PTC, **MF, BL**	T2**N1**M0	N3/T3
		Sister	F	45	PTC, **MF, BL**, FV*	**T4N1**M0	N4/n.a.
C	2	Index	F	42	PTC, **MF**	T1N0M0	N5/T5
		Sister	F	**35**	PTC	**T4**N0M0	N7/n.a.
D	2	Index	F	**34**	PTC, **MF**	**T4**N0M0	N10/T10
		Mother	F	60	PTC, **MF**	T2N0M0	N9/T9
E§	2	Index	F	57	PTC, **MF, BL**, FTC*	T2N0M0	N13/T13
		Daughter	F	57	PTC, **MF**, WLV	T1bN0M0	N14/T14
F§	2	Index	M*	47	PTC, **MF**, **BL**	T3N0M0	N15/T15
		Brother	M*	**35**	PTC, FV*	T3N0M0	N16/T16
G§	2	Mother	F	44	PTC, FV*	T2N0M0	n.a./n.a.
		Index	F	**20**	PTC, **MF**, **BL**	T2N0M0	N17/T17
H	3	Index	M*	**20**	PTC, **MF, BL**, FV, FTC*	T2N0M0	N18/T18
		Mother	F	**31**	PTC, **MF, FV**, FA	T2N0M0	N19/T19
		Sister	F	**18**	**MNG**	-	n.a./n.a.
I	2	Index	F	**32**	PTC, **MF**	T1N0M0	N20/T20
		Sister	F	**30**	PTC, FV*	T2**N1**M0	n.a./n.a.

Legend: PTC: Papillary thyroid carcinoma; **MF:** Multifocal; **BL**: Bilateral; **FV:** Follicular variant; **WLV:** Warthin-like variant; **CO:** PTC with cell oxyphilia; **FTC:** Follicular thyroid carcinoma; **FA:** Follicular adenoma; **n.a.** Sample not available; **§** Cases not satisfying three secondary criteria defined by Musholt et al. [208] (see Introduction section I3.2.4); **Bold**: Features that satisfy one of the secondary criteria defined by Musholt et al. [208]; ***** Additional characteristics used to include the family in this study (see Material and Methods - section FNMTC families). **N/T: N**ormal PBL-derived DNA sample and **Tumor** DNA sample numbers.

Using paired blood (normal) and tumor DNA samples, we have allelotyped ten microsatellite markers and one SNP throughout 19p13.2 as well as fourteen microsatellite markers encompassing 2q21.The genotypes observed for each marker in normal (PBL) and corresponding tumor DNA, in the families where LOH was detected, are presented in Table 6. The chromatograms of all *loci* demonstrating LOH are shown in Figure 10. In three families (C, G and I) no LOH was found. Eight out of the fourteen familial tumors studied (57%) exhibited LOH at one or more of the markers analysed at 19p13.2 and two tumors (14%) presented losses at 2q21. In four families (B, D, E and F) LOH was found at 19p13.2 in only one of the tumors analysed (T3, T9, T13 and T16, respectively). In two families (A and H), LOH in the same markers was demonstrable in the tumors from both members of the same family (T1/T2 and T18/T19, respectively). In the two tumors from one family (T18 and T19 - family H), LOH was demonstrable at both *loci* analysed.

Table 6 Allelotyping results of 19p13.2 and 2q21 markers.

A. 19p13.2

	Family A				Family B			Family D			Family E			Family F			Family H			
Marker	N1	T1	N2	T2	N4	N3	T3	N9	T9	N10/T10	N13	T13	N14/T14	N15/T15	N16	T16	N18	T18	N19	T19
D19S1034	**235**	235	225/**235**	225/235	219/223	231/235	230/234	**231**/239	231/239	**231**/235	**227**/231	227/231	**227**/235	**227**/231	**227**/233	**227**	227/**231**	**231**	**231**	**231**
D19S901	156/**160**	156/160	152/**160**	152/160	148/**152**	**152**/154	152/154	146/**156**	146/156	152/**156**	138/**140**	138/140	**140**/148	152/**156**	**156**	156	152/**156**	**156**	146/**156**	**156**
D19S922	**238**/246	**238**	236/**238**	236/238	232/248	236/244	236/244	240/**242**	240/242	**242**	**242**/**244**	nd	**242**/**244**	234/**242**	242/250	**242**	**238**/246	**238**	**238**/240	nd
D19S884	**215**/219	**215**	**215**/227	215/227	219/221	223/225	223/225	**231**	231	**231**	**231**/237	nd	229/**231**	221/**233**	**233**/237	**233**	225/**227**	**227**	217/**227**	**227**
NDUFA7	C	C	C	C	C	C	C	CT	C	CT	C	C	C	C	C	C	CT	T	CT	C
D19S816	**102**/110	**102**	**102**/106	**102**	**94/100**	**94/100**	94/100	**102**/112	**102**	100/**102**	94/**114**	**94**	100/**114**	100/**102**	**102**	102	**102**/118	**102**	95/**102**	95
D19S391	**139**/145	139/145	**139**	139	133/165	137/153	137	139/**149**	139	**149**/157	**149**	149	147/**149**	**139**/163	**139**/163	**139**	**154**	**154**	**154**/174	**154**
D19S413	66/**74**	66/74	**74**/78	74/78	**66**/74	**66**/78	66/78	**78**	78	74/**78**	**66**	66	66/74	66/**70**	**70**/78	**70**	64/74	**64**	64/76	**64**
D19S586	236/**240**	236/240	**240**/241	240/241	236/**240**	236/**240**	236/240	**240**/**244**	240/244	**240**/**244**	**236**/241	nd	228/**236**	232/241	232/241	nd	**236**/241	**236**	**236**	**236**
D19S583	**217**/220	217/220	**217**	217	**220**/226	**220**	220	**220**/238	220/238	**220**	**220**/235	nd	**220**	**217**/220	**217**/238	**217**	220/**226**	**226**	**226**/235	nd
D19S535	**149/151**	149/151	**149/151**	149/151	143	147/155	147/155	**145**/149	145/149	**145**/153	**145**/149	145/149	**145**/155	**141**/155	**141/149**	141/149	**153**	**153**	**153**	**153**

B. 2q21

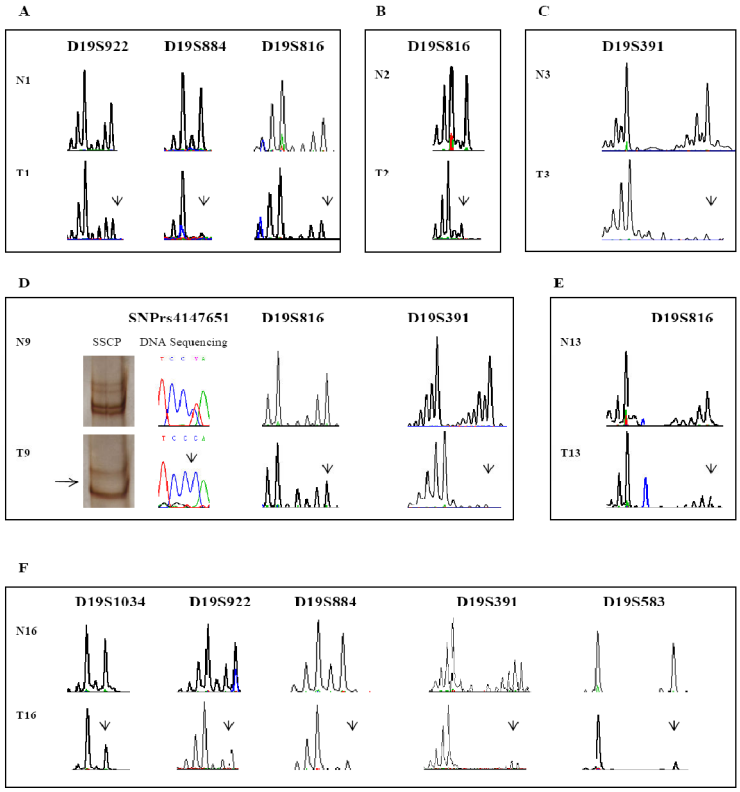
	Family H					
Marker	N18	T18-1	T18-2	N19	T19-3	T19-4
D2S2265	175/179	175/179	**175**	175/181	175/181	**175**
D2S2224	239	239	239	239/241	**241**	**239**
D2S1328	139/161	139/161	**161**	161	161	161
D2S2271	140	140	140	140/154	140/154	**140**
D2S2215	152/156	152/156	**152**	142/152	**142**	**142**
D2S1260	314/323	314/323	**314**	304/314	nf	nf
D2S112	136	136	136	136/142	136/142	**136**
D2S2219	216/218	216/218	**218**	212/218	**218**	**218**
AFMA272zg9	111/115	111/115	**115**	111/115	111/115	**115**
D2S2256	248/254	248/254	**248**	248/250	**248**	**248**
D2S114	214/230	214/230	**214**	214/216	**214**	**214**
D2S1334	282/200	282/200	**200**	286/200	nf	nf
D2S2196	255/271	255/271	**255**	255/259	**255**	**255**
D2S442	196/200	196/200	**196**	196/200	**196**	**196**

Legend: Alleles observed at each marker in blood (**N**) and tumor (**T**) DNA is given in numbers that denote allele size (base pairs) for the polymorphic microsatellite markers. Alleles shared between affected individuals are annotated in **bold**. Markers showing LOH are presented in black shading. Non-informative markers are in gray shading. **nd or nf:** not determined due to non functioning PCR amplification in the tumor DNA; the marker is considered non-informative.

Allelotyping throughout the *loci* allowed the determination of the shared haplotype in mother/daughter families (D, E and H). In the remaining families, determination of the shared haplotype was not possible, due to the small number of affected members. However, it is noteworthy that in sib-pair families A and F, there were always demonstration of a shared

allele at each marker throughout the 19p13.2 *locus*, strongly indicating a shared haplotype. In contrast, allelotyping seems to exclude linkage to 19p13.2 in sib-pairs from families B and C.

The pattern of allelic losses from tumors in which LOH was demonstrable is addressed inTable 6. Detailed haplotype analysis showed that in four tumors from two families, the pattern of LOH was consistent with the selective retention of the shared allele. This was demonstrated in family A (T1 and T2) and family H (in T18 and at some markers in T19). In families B, D and E, the pattern of LOH involved random losses (Table 6).



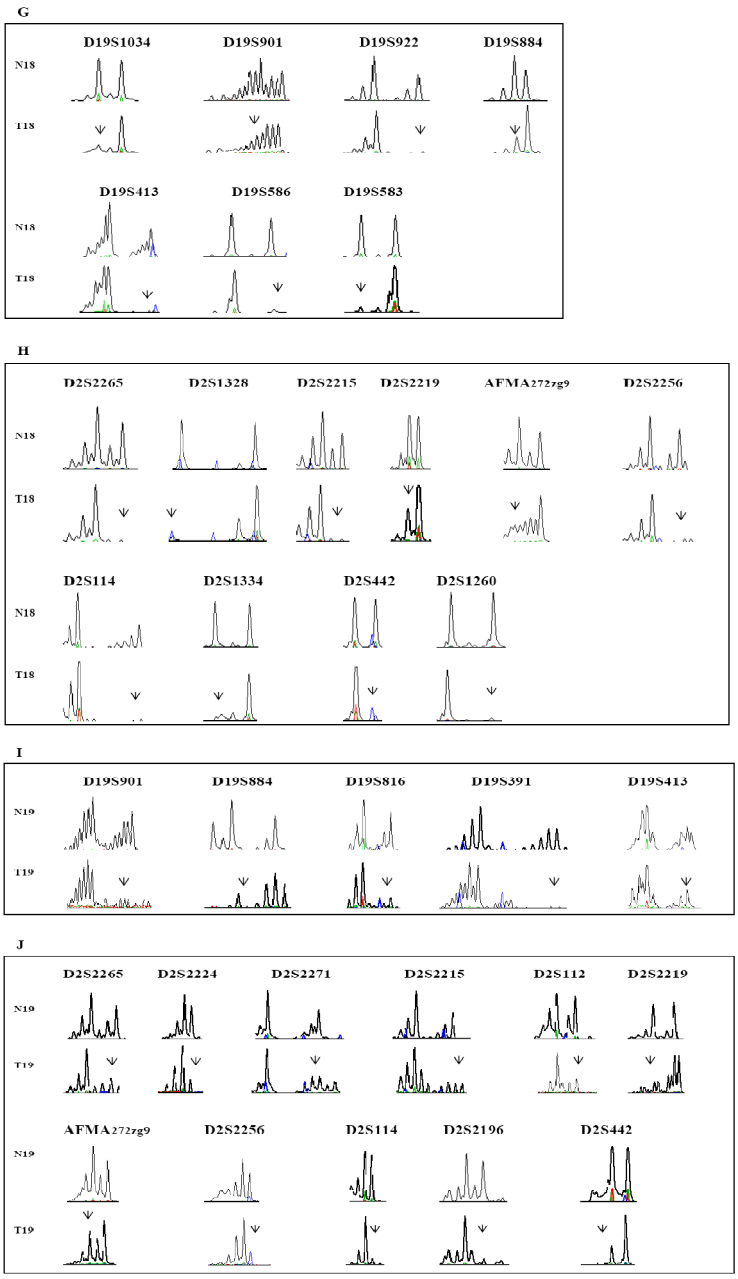


Figure 10 LOH analysis in tumors from familial clusters of NMTC.
Chromatograms corresponding to matched normal and tumor alleles are presented for markers in which LOH was detected. **Panels A** and **B**: Family A; **Panel C**: Family B; **Panel D**: Family D; **Panel E**: Family E. **Panel F**: Family F; **Panels G - J**: Family H. The X axis indicates the allele size and the Y axis, the relative allele height. The arrow ↓ indicates the allelic deletion. In Panel D, the SSCP gel and sequencing chromatograms are presented in order to illustrate LOH at the SNP4147651.

From heron we centered our analysis in tumors from family H from which fresh tumor material could be collected. We have focused in the study of the susceptibility region at 2q21 since this family comprises cases of the FVPTC a featured observed to be overrepresented in families linked to this *locus* [173]. Moreover, the pattern of LOH at 2q21 showed consistent loss of a presumable susceptibility haplotype in 3 out of 4 tumors, in accordance with the mode of inactivation of a tumor-suppressor gene (Figure 11).

R2.2 Global gene expression profile of familial and sporadic NMTC tumors filters LRP1B as the only significant de-regulated gene at 2q21

We performed gene expression profiling in the only tumor (out of four) without LOH at 2q21 by using Affymetrix U133A Plus 2.0 Genechips. Amongst the probe sets which encompass the 2q21 region (>200 probesets; 64 candidate genes, Supplementary Information Table 1) LRP1B was the only gene found to be appreciably de-regulated. In complement to this, analysis of 2q21 probesets was done in the dataset derived from expression profiling (Affymetrix Human Exon 1.0 ST Array) of a validation series comprising sporadic thyroid tumors (12 normal samples, 12 follicular thyroid adenomas and 18 follicular thyroid carcinomas). Concordantly, we found that LRP1B was the single probe-set in 2q21 region showing significant de-regulation (3 fold cutoff) in sporadic tumors (Supplementary Information Table 2).

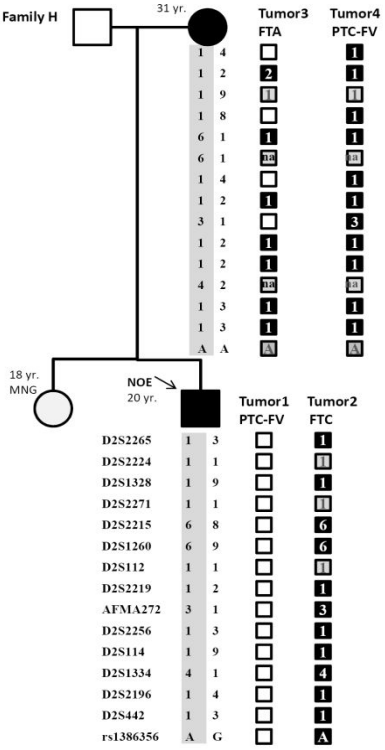


Figure 11 Pattern of LOH amongst distinct tumors of the index FNMTC family H.
The Heredogram summarizes the clinical features (age at diagnosis and histotype) and the pattern of allelic losses in distinct tumors from different members of family H. ***The herein designated Tumor 1, 2, 3 and 4 correspond to the previously termed T18-1, T18-2, T19-3 and T19-4, respectively.*** Allelotyping of the 2q21 interval was performed for 14 microsatellite markers and 1 LRP1B intragenic SNP. A code for each allele was generated on the basis of the size of the microsatellite fragment. The index case is annotated with an arrow (←). The haplotype shared by the affected individuals is overshadowed in grey. The alleles detected for each marker in the distinct tumor nodules derived from affected members are represented along side of the respective case. **FTA** – Follicular Adenoma; **PTC** – Papillary Thyroid Carcinoma; **FV** - Follicular Variant; **FTC** – Follicular Thyroid Carcinoma; □ - Retention of Heterozygosity; ■ - Loss of Heterozygosity; ▣ - Non Informative Marker; **na** - not amplified.

The Low density lipoprotein receptor Related Protein gene (LRP1B), originally isolated on the basis of homozygous deletions (HD) detected in human lung cancer cell lines [229-231], was recently reported amongst the top 10 most significantly deleted genes across 3,312 human cancer specimens [232]. LRP1B encodes for a member of the endocytic Low Density Lipoprotein Receptor (LDLR) superfamily. Because LRP1B is most highly expressed in the brain and thyroid gland [233], [234] and a susceptibility *locus* for familial non-medullary thyroid cancer (fNMTC) at 2q21 [173], encompassing LRP1B, as well as loss of heterozygosity at 2q21 have been detected in familial [198] and sporadic NMTC [196], we undertook to elucidate the mechanisms of LRP1B inactivation in cancer cells and to investigate the key roles of this lipoprotein receptor in the carcinogenesis of familial and sporadic thyroid tumors.

Quantitative Real-Time-PCR (qRT-PCR) was used to validate the results obtained with the microarrays. LRP1B was shown to be down-regulated to similar levels in the 2 fNMTC samples when assessed by cDNA microarray or qRT-PCR (-12.15 and -10.1, respectively, Figure 12). Furthermore, LRP1B was completely silenced or down-regulated to variable levels in all the thyroid cancer cell lines analyzed (Figure 12), suggesting that LRP1B might be relevant in the carcinogenesis of both familial and also sporadic thyroid cancer.

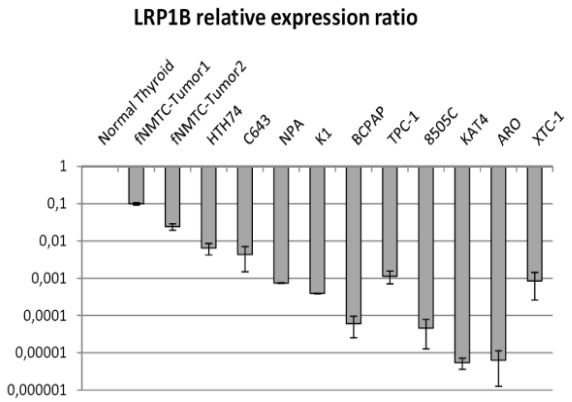


Figure 12 LRP1B is silenced in FNMTC tumors and in thyroid cancer cell lines
Mesenger RNA expression of LRP1B in thyroid cancer cell lines, normal thyroid (N) and tumors derived from the index fNMTC case (fNMTC-Tumor1 and fNMTC–Tumor2). Expression values were normalized to the endogenous control and divided by the relative expression of the normal thyroid sample.

We sequenced, in the index family member, the16Kbs of the entire coding sequence (91 exons) and flanking intron boundaries of LRP1B, as well as cDNA fragments encompassing several exons. Aside from previously reported SNPs and intronic variants of unknown significance (Figure 13A) we were unable to detect any causative germline mutation in the fNMTC case studied. We additionally screened for mutations in 5 sporadic thyroid tumor DNAs. In one sNMTC we could demonstrate a frameshift deletion: del68_146 at the transcript level (nucleotide +1 is the ATG initiation codon) which is predicted to translate a truncated LRP1B protein (Figure 13).

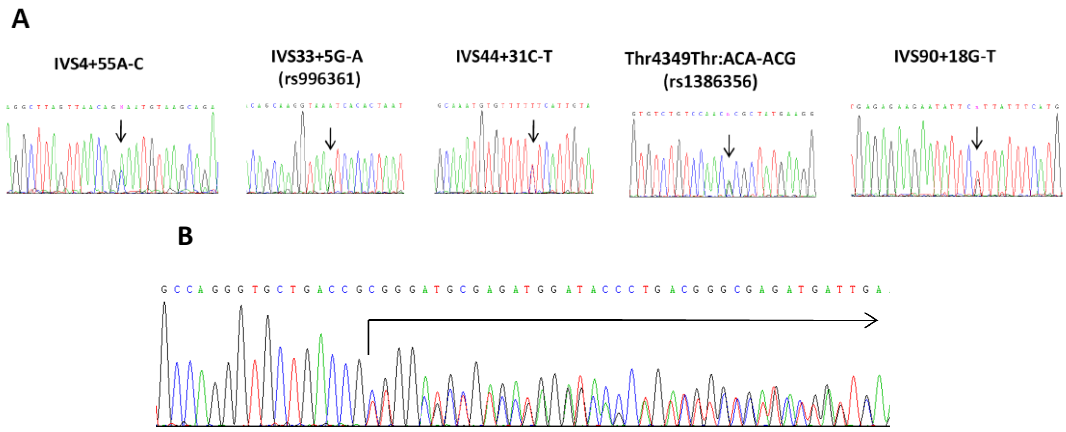


Figure 13 LRP1B genetic variants in familial NMTC and mutation is sporadic NMTC. (**A**) Genetic variants detected upon germline sequencing of the entire LRP1B coding exons and respective intron boundaries in the index fNMTC case. (**B**) Sequencing chromatogram illustrating a LRP1B deletion mutation encompassing exon1 to exon2 (del68_146 at the transcript level) detected in 1 out of 5 sporadic NMTCs scanned for LRP1B mutations. The arrow indicates the deletion site after which an overlap of the wild-type and deleted alleles is observed.

R2.3 Genomic Loss of LRP1B is frequent in sporadic thyroid tumors

Since LRP1B was initially identified by the observation of homozygous deletion in lung cancer cell lines, which led to the initial designation of LRP-DIT - a gene found to be Deleted In Tumors, we were prompted to investigate whether LRP1B is subjected to genomic loss. For this purpose, we used quantitative genomic real-time PCR assays directed at LRP1B sequences in the upstream, middle and downstream part of the gene (exons 5, 44 and 90, respectively) as well as sequences near the chromosome 2 centromere. We found that LRP1B frequently displays genomic loss both in cell lines (5/10 – Figure 14B) and also in sporadic tumors (13/20 FTAs, 13/15 FTCs, 14/19 PTCs and 6/10 UTCs –Figure 14C). In most cases, losses at LRP1B are accompanied by loss at the centromeric *locus*, indicating that probably the entire chromosomal arm was deleted (Figure 14C). We could observe cases in which copy number values dropped in internal exons of LRP1B, indicating that the deletion breakpoints were intragenic (for example PTC-14). We could also detect cases with a presumable homozygous loss, displaying a calculated Copy Number value close to zero (Figure 14C).

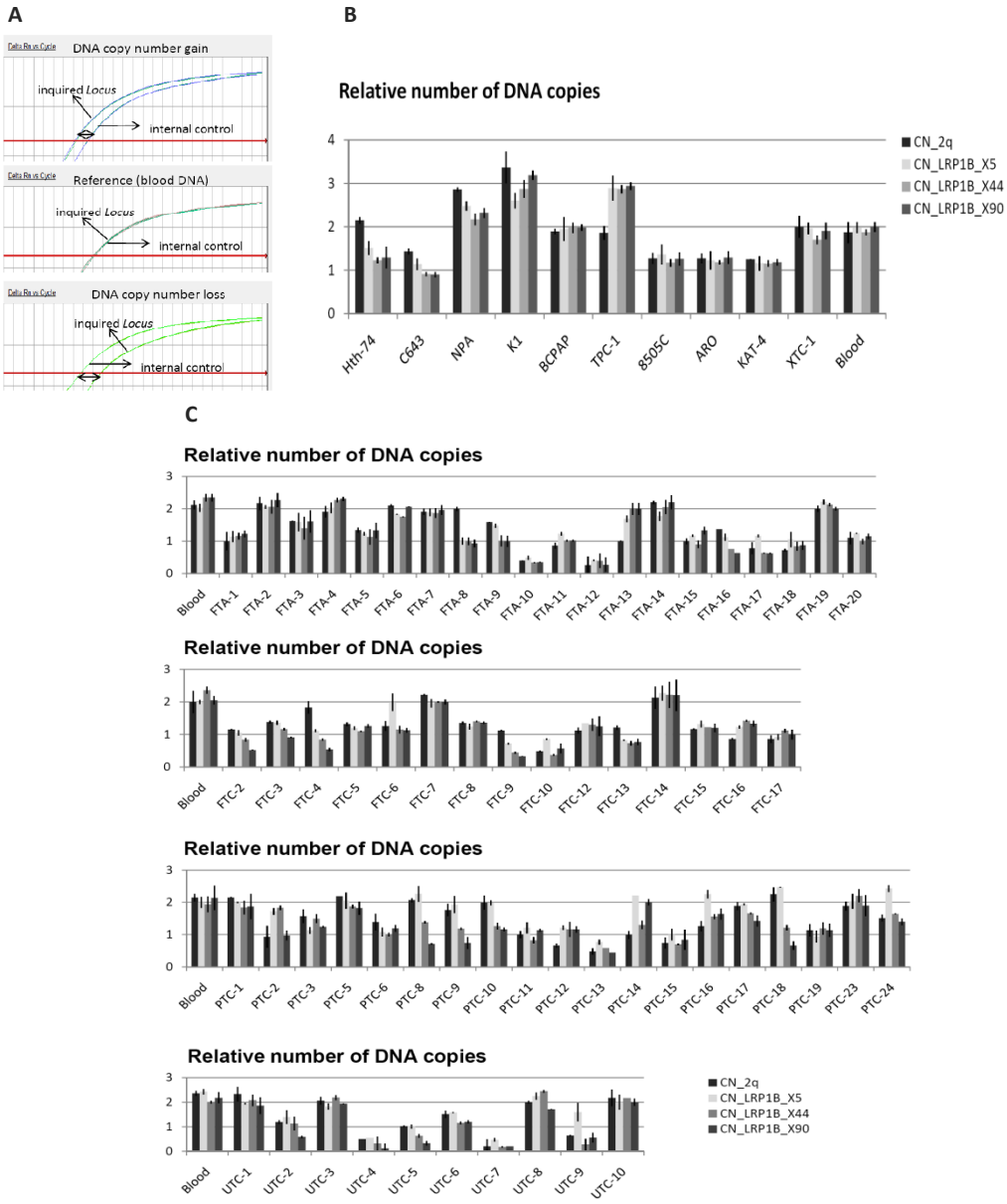
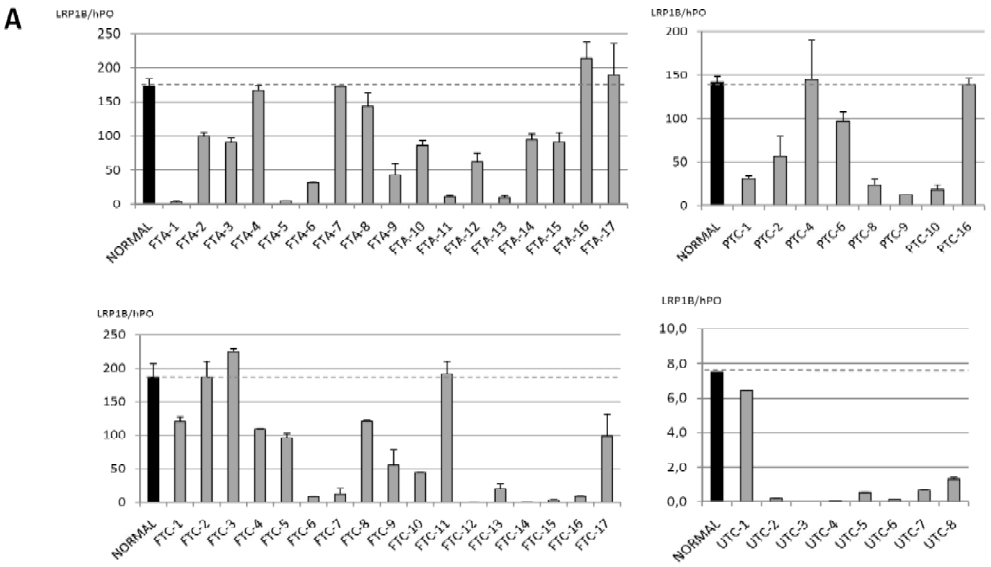


Figure 14 Genomic Loss of LRP1B is frequently found in thyroid cancer cell lines and sporadic thyroid tumors. LRP1B DNA copy number was estimated by quantitative genomic real-time-PCR assays (**A**) in 10 thyroid cancer cell lines (**B**) and thyroid lesions (20 FTAs, 15 FTCs, 19 PTCs and 10 UTCs) (**C**) was determined by genomic quantitative real-time-PCR employing primer/probe pairs targeting different genetic regions of LRP1B, namely exon 5, 44 and 90 (CN_LRP1B_X5; _X44 and_X90) and at a region near the 2q centromere (CN_2q). The abundance of DNA copies at each site was normalized to an endogenous control for RNAse P run in multiplex reactions and copy number values were calibrated by running reactions with blood-derived DNA sample in the same plate. Data was generated with the Copy Caller software.

R2.4. LRP1B under-expression follows thyroid cancer progression and correlates with vascular invasion.

We analyzed the expression levels of LRP1B at the mRNA level. In accordance with the results observed in thyroid cancer cell lines, we found under-expression at variable degrees both in pre-malign lesions, such as FTA, as well as in carcinomas such as FTC, PTC and UTC (Figure 15A).The only case of UTC displaying normal LRP1B mRNA expression was shown to harbor the abovementioned somatic frameshift mutation (Figure 13B). Noteworthy, the expression level of LRP1B was significantly lower in thyroid lesions, compared with normal thyroid tissue ($p<0.02$), and the expression in UTCs is significantly lower than in differentiated thyroid cancers ($p=0.036$, Figure 15B). The levels of LRP1B were significantly lower in cases with vascular invasion, compared with cases without vascular invasion, namely in FTC ($p=0.04$, Figure 15C). A statistically significant under-expression of LRP1B was reproduced in the validation set comprising 12 FTAs and 18 FTCs compared against 12 normal thyroid samples ($p=0.03$) (Figure 15D). This was further confirmed in cases in which paired Normal and Tumor specimens were available ($p=0.015$) (Figure 15E). However, the association with vascular invasion was not statistically significant in the validation series.



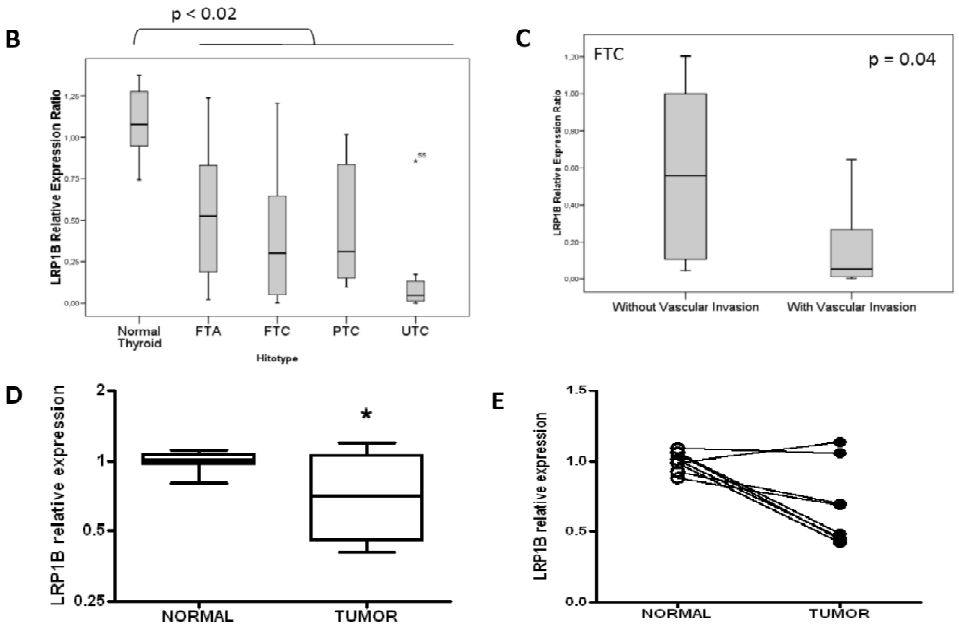


Figure 15 LRP1B under-expression follows thyroid cancer progression and correlates with vascular invasion. (**A**) Real-Time-PCR quantification of the LRP1B transcript in mRNA samples derived from microdissected thyroid lesions. Expression values were normalized to an endogenous control (hPO) and a normal reference (a pool of 9 mRNAs obtained from normal thyroid samples subjected to the same treatment as the cases) was quantified in triplicate in the same plate. (**B**) Differences in the relative expression ratios for each histotype of thyroid lesions are displayed in box-plot graphics. LRP1B is significantly under-expressed in thyroid tumors relative to normal thyroid and in UTC *versus* differentiated thyroid tumors (FTC and PTC). Statistical significance p values were obtained by using the Man-Whitney test. (**C**) LRP1B mRNA levels are significantly lower in FTCs with vascular invasion. (**D**) In a validation series gathered by an independent group and analysed by a different method (exon arrays), LRP1B is reproducibly found to be under-expressed in tumor versus normal thyroid tissue. (**E**) This observation is further confirmed when paired normal and tumor material was analysed.

R2.5. DNA methylation disrupts a transcription factor binding site for the histone acetyltransferase p300 at LRP1B intron 1

DNA methylation consists of a "maintenance" or *de novo* DNA MethylTransferase-mediated [235] chemical modification of cytosine to yield 5-methyl-Cytosine (5mC) predominantly, but not exclusively, at CpG dinucleotides enriched within "CpG islands" that encompass the promoters of structural genes [236]. DNA methylation modulates DNA-protein interactions at decisive sequences in a promoter [237] and serves as signal for the buildup of additional epigenetic systems (such as histone modification ad nucleossome positioning) that result in gene silencing [238-240].

Two general mechanisms of transcriptional repression have been described in vertebrates: a ‘‘direct’’ mechanism, whereby methylation of CpGs within a transcription factor binding site inhibits binding to its cognate recognition sequence [241], and an ‘‘indirect’’ mechanism, which involves the binding of one of the methyl-DNA binding domain (MBD) family of proteins, such as MeCP2, to methyl-CpGs [242, 243]. These MBD proteins interact with corepressor complexes, which mediate repression, at least in part by post-translational modification of histone [244, 245]. Histones pack and wrap nuclear eukaryotic DNA into nucleosomes formed by an equimolar octamer of four histones: H2A, H2B, H3 and H4.

The level of accessibility of DNA to transcription is affected by post translational covalent modifications of histones such as acetylation, methylation, phosphorylation, ubiquitination and sumoylation, which can be imposed upon distinct Lysine or Arginine residues at the N-terminal tails of H3 (mostly) and H4 [246, 247]. Different combinations of histone modifications have been suggested to form a so called “histone code”[248], which is “inherited” upon DNA replication and repair and interpreted to yield a defined transcriptional states (active, inactive or silent) [248]. These modifications are placed-on and removed-off by a number of enzymes (Histone AcetylTranferases and Histone DeACetylases or Histone MethylTransferases and Histone DeMeThylases, respectively) which are specific for a given modification and position[247].

Genome-wide changes in the patterns of DNA methylation and Histone modifications are seen in cancer, both at gene bodies and at promoters, epigenetically reprogramming entire sets of genes to states that confer selective advantages to tumor cells [249-251]. As a growing number of TSGs are found to be transcriptionally silenced trough epigenetic inactivation, we asked whether DNA methylation, or other epigenetic factors, could afford explanation for the observed transcriptional down-regulation of LRP1B. By bissulfite-sequencing of 2 fragments of the LRP1B CpG island we detected DNA methylation in 6/10 cell lines and in 10/19 FTAs, 9/16 FTCs, 11/17 PTCs and 5/7 UTCs (Figure 16). No methylation was observed in any of the normal thyroid samples, confirming that the observed methylation is tumor-specific and excluding tissue-specific methylation (Figure 16). The pattern of methylation was heterogeneous, without evidence of preferential methylation of a particular CpG (Figure 16). However our attention was caught by the observation of methylation in both CpG and in non-CpG sites (Figure 16 - insert).

Although the prevailing assumption is that DNA methylation is restricted to CpGs, evidence for non-CpG methylation in mammalian cells comes from several previous studies, [252], [253], [254], [255]. Moreover, recently in the first complete sequence of the human DNA methylome [256] non-CpG sites comprised as much as 25% of methylated Cytosines in an human embryonic stem cell line.

A fundamental question raised by these results was the functional significance of apparently stochastic methylation in CpG and non-CpG contexts. Analysis of all the sites found to be methylated in non-CpGs in cell lines and tumors indicated that 1/3 of all sites occur in the sequence *CCGG, with methylation of the external C.

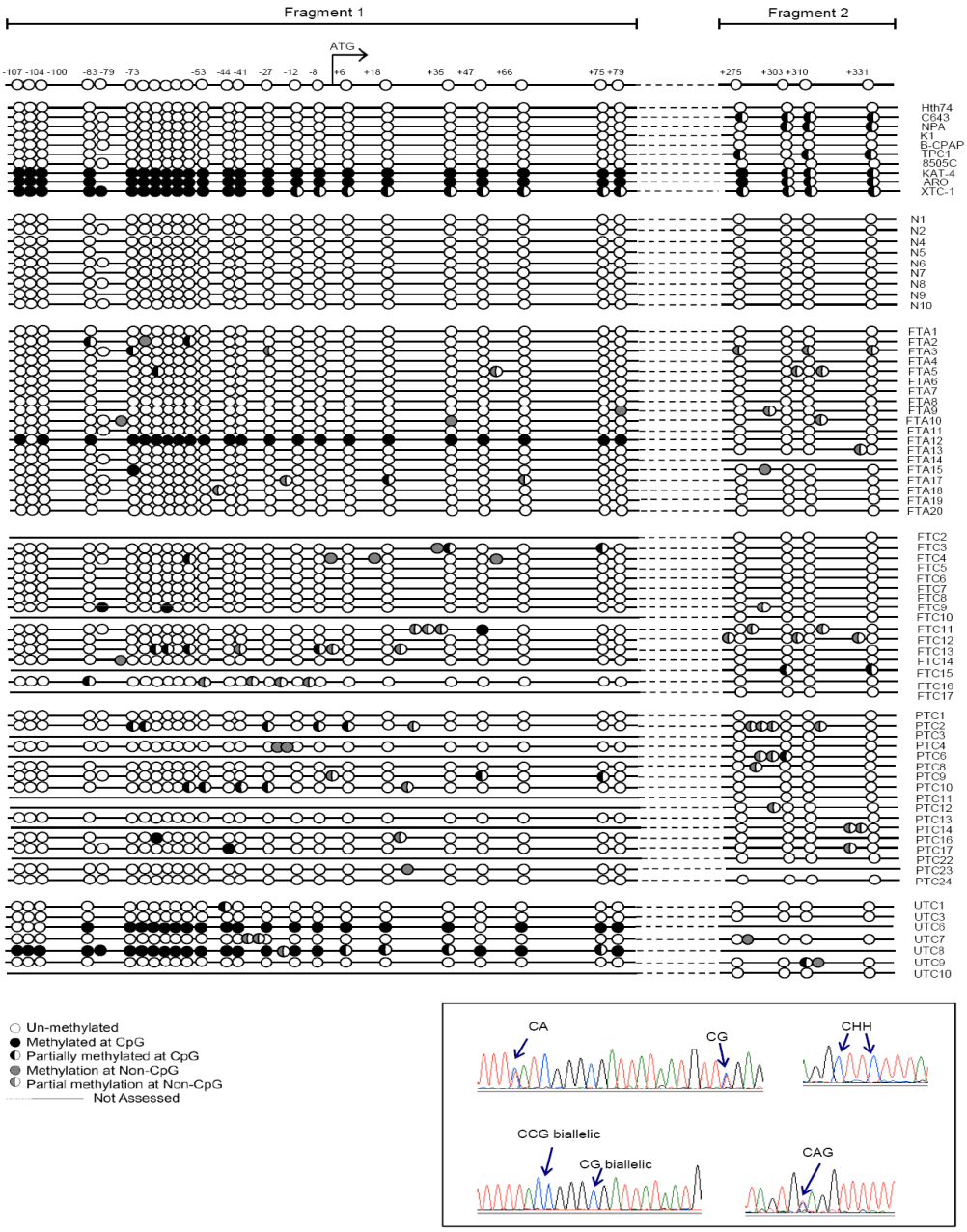


Figure 16 Patterns of DNA methylation at the LRP1B CpG island.
Profile of DNA methylation of the LRP1B CpG island in thyroid cancer cell lines, normal thyroid samples and thyroid tumors. Methylation analysis was performed by bissulfite-PCR-sequencing of two independent fragments of the CpG island (Fragment 1: -131 to +100 and fragment 2: +217 to +365, counting from ATG) in 10 thyroid cancer cell lines and in 19 FTAs, 16 FTCs, 17 PTCs and 7 UTCs. Methylation status was also assessed in 9 samples of normal thyroid tissue. Circles represent CpG and non-CpG sites in different configurations: ○ - un-methylated; - ● fully methylated; ◐ - partially methylated; ◯ - fully methylated in a non-CpG site and ◐ - partial methylation at a non-CpG. The insert contains bissulfite-sequencing chromatograms illustrating sites of non-CpG methylation; H stands for C; A or T.

To address this question, we generated CpG island luciferase reporters with two fragments of the promoter region (fragment A and B). We differentially methylated CpG island luciferase reporters by *in vitro* methylation with bacterial methyltransferases M.Sss-I (*CG), M.Msp-I (*CCGG) and M.Hpa-II (C*CGG), (Supplementary Material Figure S1). We found Luciferase activity to be increased by the unmethylated fragments of the CpG island (Figure 17A). As expected, complete M.Sss-I-mediated methylation of all *CpGs resulted in abrogation of Luciferase activity to values similar to the empty vector. Interestingly, low density methylation induced by M.Msp-I and M.Hpa-II impaired the transcriptional activity of the reporter containing fragment B of the CpG island, but not the fragment A reporter (Figure 17A).

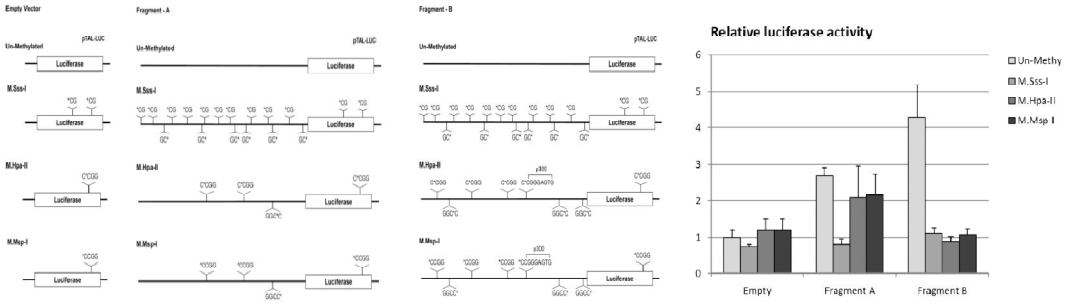
We reasoned that DNA methylation might interfere with the binding of putative transcription factors to the specific sequences flanking CCGG in fragment B. Using TFSEARCH 1.3 [257] we found that a binding motif for the p300 histone-acetyltransferase (GGGAGTG) [258] lied immediately upstream of one of the M.Msp-I/M.Hpa-II CCGG sites (CCGGGAGTG). These observations led us to investigate whether p300 activates the expression of LRP1B and if this effect is methylation sensitive. For this purpose we transfected HEK293 cells with unmethylated or methylated luciferase constructs of fragment B, either with or without the addition of a p300 expression vector. We found that addition of p300 increased Luciferase activity of the unmethylated construct. However, the p300 enhancer effect was abrogated when either M.Msp-I or M.Hpa-II methylated versions of the reporter were used (Figure 17B).

In order to confirm the direct binding of p300 to this LRP1B sequence and its possible perturbation by DNA methylation, we performed Proximity Ligation Assay (PLA) [259] using DNA probes in the unmethylated, methylated and mutated forms. We showed a 64 fold increased in interactions between p300 and a DNA probe composed of the LRP1B intron 1 unmethylated sequence as compared to probes with a mutated biding site or with methylated *CG and *CCG sites (Figure 17C). In complement to this we transfected HEK293 and C643 cells with a siRNA designed to inhibit the endogenous p300 and could demonstrated a concomitant reduction in LRP1B expression (Figure 17D).

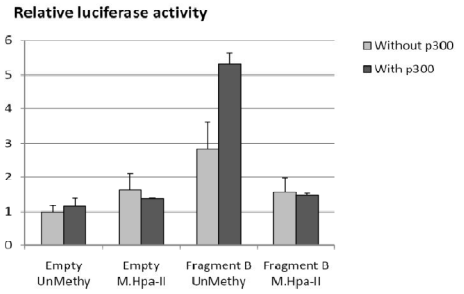
Overall these results indicate that there is a functional p300 binding site at intron 1 of LRP1B, which could be disrupted by DNA methylation of the flanking CCG sequence. Moreover, these findings highlight that methylation at unique Cytosines, even in a stochastic or non-CpG context, may have dramatic effects over gene transcription, by interfering with *cis* regulatory elements.

Since our findings imply a role for DNA methylation and histone acetylation in regulation LRP1B expression, we decided to check whether the use 5-Aza-deoxi-Cytidine (5-Aza) or HDAC inhibitor Tricostatin A (TSA) could restore expression of *LRP1B* mRNA *in vitro.* In cell lines ARO and XTC-1, in which we previously demonstrated LRP1B promoter DNA methylation (Figure 4a), we could show that LRP1B is re-expressed either by use of 5Aza or TSA alone (ARO) or in combination (XTC-1) (Figure 17E).

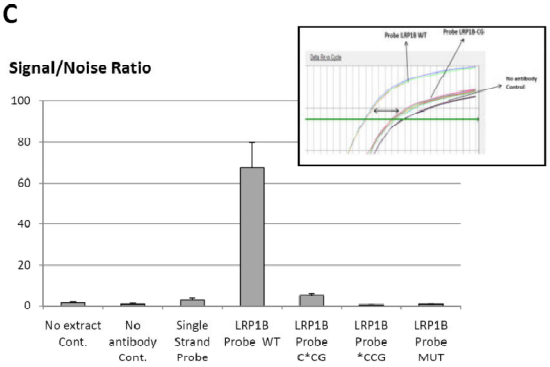
A


Empty Vector
Fragment - A
Fragment - B
Un-Methylated
pTAL-LUC
Luciferase
M.Sss-I
M.Hpa-II
M.Msp-I
p300
Relative luciferase activity
Un-Methy
M.Sss-I
M.Hpa-II
M.Msp I
Empty
Fragment A
Fragment B

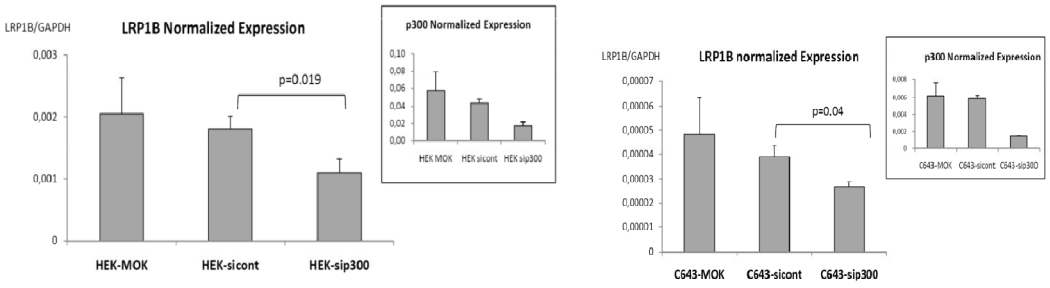
B


Relative luciferase activity
Without p300
With p300
Empty UnMethy
Empty M.Hpa-II
Fragment B UnMethy
Fragment B M.Hpa-II

C


Signal/Noise Ratio
No extract Cont.
No antibody Cont.
Single Strand Probe
LRP1B Probe WT
LRP1B Probe C*CG
LRP1B Probe *CCG
LRP1B Probe MUT

D


LRP1B/GAPDH
LRP1B Normalized Expression
p=0.019
HEK-MOK
HEK-sicont
HEK-sip300
p300 Normalized Expression
LRP1B normalized Expression
p=0.04
C643-MOK
C643-sicont
C643-sip300

E

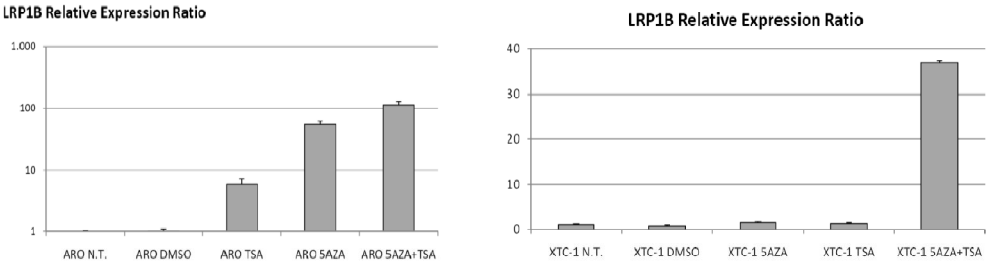

LRP1B Relative Expression Ratio
ARO N.T.
ARO DMSO
ARO TSA
ARO 5AZA
ARO 5AZA+TSA
LRP1B Relative Expression Ratio
XTC-1 N.T.
XTC-1 DMSO
XTC-1 5AZA
XTC-1 TSA
XTC-1 5AZA+TSA

Figure 17 Site-specific DNA methylation at intron 1 disrupts binding and transactivation by p300.
(**A**) Luciferase activity derived from transfection of HEK293 cells with pTAL-LUC reporter constructs containing independent fragments of the LRP1B CpG island (fragment A: -429 to -1 and fragment B: +1to +530, counting from ATG) each subjected to *in vitro* methylation using either M.Sss-I, M.Msp-I or M-Hpa-II (with *CG, *CCGG and C*CGG methylation specificities, respectively) as is represented schematically. (**B**) In the second experiment, luciferase activity of un-methylated and M-Hpa-II methylated constructs was measured in either presence or absence of a p300 expression vector co-transfected along with the reporters. (**C**) Proximity ligation-real-time-PCR quantification of the interactions between p300 and DNA probes consisting of the putative LRP1B p300 motif sequence in wild type (WT) mutated (MUT) and methylated (C*CG and C*CG) configurations. The insert displays real-time-PCR amplification curves. Results were expressed as signal/noise ratio, where the number of ligations in the sample was divided by the number of ligations in the negative control. (**D**) Expression of endogenous LRP1B upon inhibition of endogenous p300 by siRNA. HEK293 and C643 cells were transfected with a scramble siRNA control (siCont) or with a siRNA for p300 (sip300) and the effect on LRP1B expression was quantified by real-time-PCR. The inserts show concomitant under-expression of p300 in the same samples. The graphs integrate data from three experiments. (**E**) Analysis of LRP1B mRNA expression upon treatment of ARO and XTC-1 cell lines (displaying LRP1B CpG island DNA methylation) with 5-Aza-2'-deoxycytidine (5AZA), Tricostatin A (TSA) or both (5AZA+TSA). Non-treated cells (N.T.) or cells treated with DMSO solvent were used as controls.

R2.6. Overexpression of miR-548a-5p, caused by genomic gain at 8q22.3, leads to LRP1B downregulation

Despite different levels of LRP1B silencing, only three cell lines displayed widespread DNA methylation and p300 levels were generally downregulated in cell lines, which does not provide explanation for the observed differences in the degree of LRP1B down-regulation (Supplementary Information Figure S2).

MicroRNAs (miRNA) have recently emerged as alternative mediators of inactivation of renowned TSGs such as APC [260] and CDH1 [261]. MiRNAs were originally identified as non-coding developmental regulators in *C. elegans* [262-265]. Later they were found to be evolutionary conserved, endogenously encoded, small RNAs, processed to 21 to 25 nucleotides in length, capable of down-regulating the translation and/or reducing the stability of their target mRNAs in invertebreate a vertabrates [263]. Clinching evidence came from the finding that miRNAs could act as tumor-suppressor genes by targeting oncogenes [266] as notorious as RAS [267]. Since then, MiRNAs have been shown to play important roles in cancer: they are located in cancer-associated fragile sites, their expression profile is found deregulated and they take on a role of either oncogene or tumor-suppressor when targeting degradation of tumor-suppressors or oncogenes, respectively (for a comprehensive review see refs. [268, 269]). MiRNAs have shown to be engaged in circuitries targeting relevant cancer genes or their regulatory transcription factors, participating in cellular programs relevant to carcinogenesis, namely p53 induced cell cycle arrest and apoptosis[270-276], epigenetic reprogramming [277], Epithelial to Mesehnchimal Transition (EMT) [278-284], invasion [285] and metastasis [261, 286].

A first clue to the hypothesis that LRP1B might be regulated by miRNAs came from the reported observation that inhibition of Dicer, an element of the miRNA processing machinery, results in the up-regulation, at the single cell level, of several genes, among which is LRP1B [287]. Pursuing this hypothesis, we undertook to investigate whether over-expression of putative micro-RNAs (miRs) that target LRP1B could add to the mechanisms of LRP1B inactivation. To this end we started by computationally nominating potential miRs that may

target the LRP1B 3'UTR and 5'UTR using the MirBase, TargetScan, and MicroInspector prediction tools. We selected 12 miRs by this *in silico* analysis (miR-155, -200b, -429, -548a, -548b, -548d, -520d, -524, -142, -7, -497 and miR-103). We quantified the expression of the 12 selected miRs in the 10 thyroid cancer cell lines available (Figure 18) and looked for miRs with an expression level that inversely correlated with LRP1B expression.

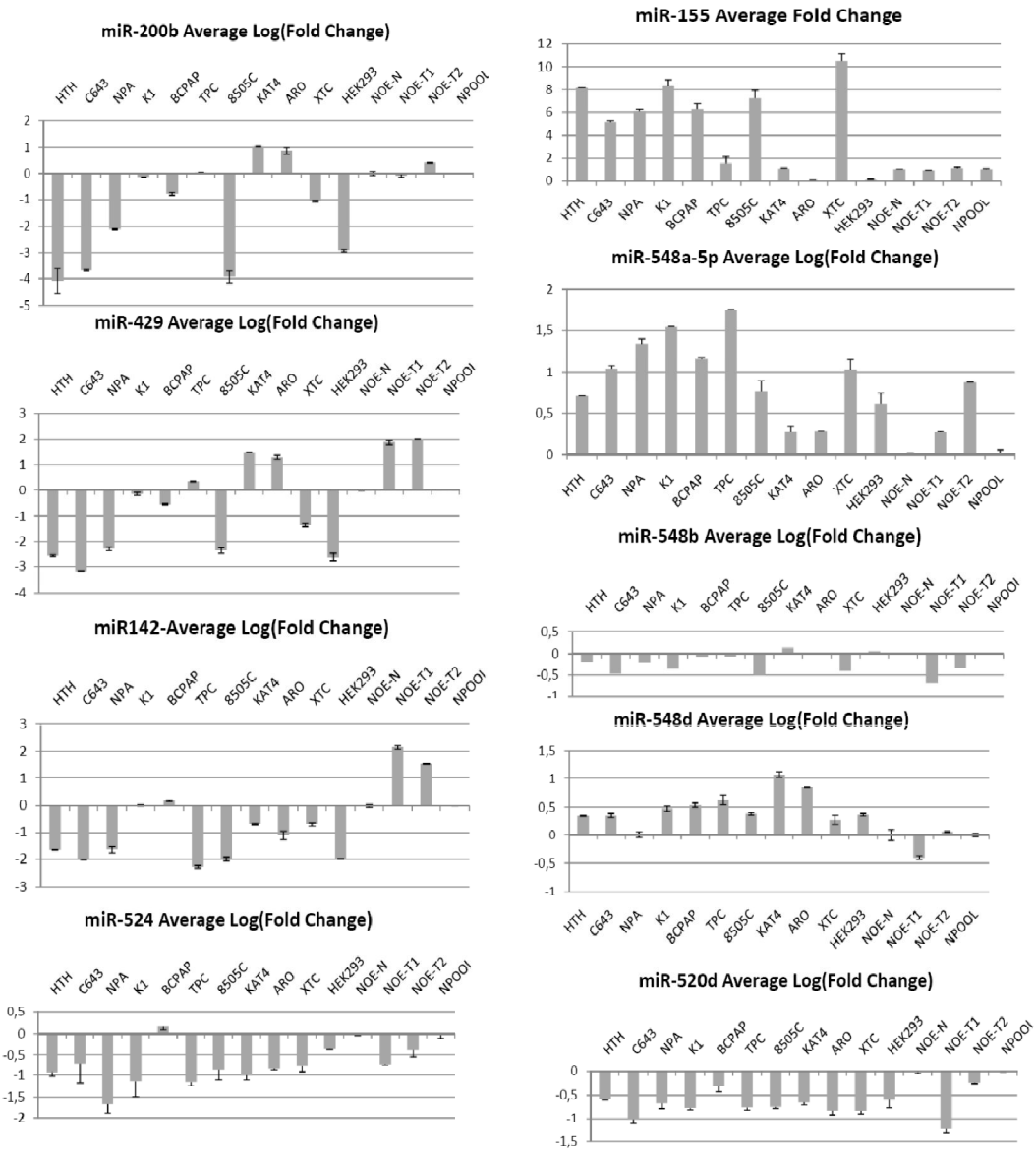


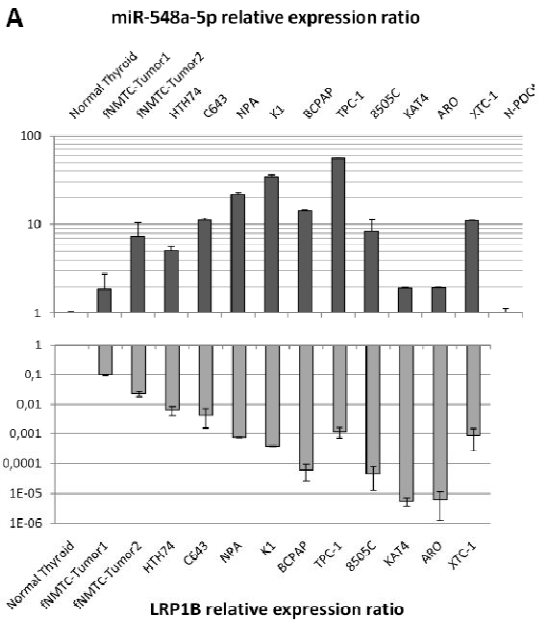
Figure 18 Quantification of miRNAs predicted to target the 3'UTR of LRP1B.
MiRNAs were quantified in 10 thyroid cancer cell lines as well as normal thyroid tissue and paired tumors of the index case. Expression values were normalized to the levels of miRNA-U6b determined in the same RNA samples. A pool of 9 RNAs derived from normal thyroid tissue (NPOOL) was used as the normal reference.

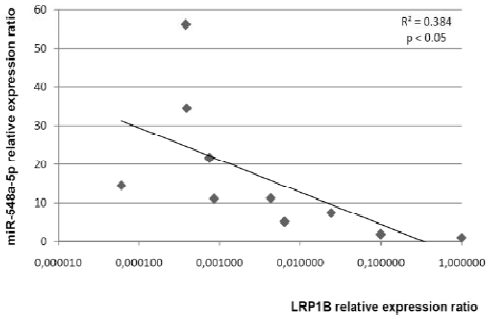
By performing this sort of analysis, we could observe that over-expression of miR-548a-5p closely paralleled the decrease in LRP1B expression, with exception of the cell lines previously demonstrating high levels of promoter methylation (ARO, KAT4 and XTC-1) (Figure 19A). This association was also observed *in vivo* in samples from the index fNMTC case. The miR548a-5p binding site at LRP1B 3'UTR is conserved across 6 species, comprising primates, mice and chicken, and shows considerable sequence complementary at the miR seed region (9-mers) (Figure 19B).

Next, we proceeded to functionally validate that LRP1B is targeted by miR-548a-5p for down-regulation. To examine whether miR-548a-5p binds to the 3'UTR of LRP1B, we generated luciferase reporters encoding the wild-type and mutated versions of the LRP1B 3'UTR. We transfected HEK293 cells with an expression plasmid encoding the precursor transcript of miR-548a-5p (Pre-miR548a-5p) and observed that overexpression of miR-548a-5p decreased the activity of the LRP1B 3'UTR luciferase reporter (Figure 6c). This effect was abrogated in the mutant LRP1B 3'UTR reporter, in which the binding site of miR-548a-5p was mutated (Figure 19Figure 19C). To further support the relationship between LRP1B and miR548a-5p, we employed anti-miR sequences to specifically inhibit miR-548a-5p expression in a cell line previously shown to have miR-548a-5p over-expression and very low levels of LRP1B (TPC-1). We verified that treatment with anti-miR-548a-5p, but not a scramble control resulted in the increase of endogenous LRP1B mRNA levels (Figure 19D).

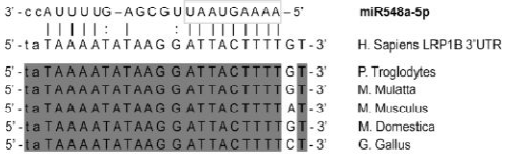
MiR-548a-5p is derived from processing of a Pri-miR intergenic transcript, encoded by a Minute Inverted repeat Transposable Element, which maps to 8q22.3. Interestingly, 8q gains were one of the most frequent cytogenetic abnormalities in thyroid cancer cell lines [288] and 8q22 amplification is a recurrent finding in several solid malignancies and may sub-classify breast cancer patients with poor prognosis [289-291]. However miRs in this location were always disregarded in surveys for genes up-regulated in this amplified region. In the interest of exploring the hypothesis that DNA gains could be the key underlying mechanism of miR-548a-5p over-expression, we employed TaqMan Copy number assays to ascertain DNA copy number at the miR-548a-5p *locus* (8q22.3). We found that 6/10 cell lines display copy number gains, whereas 2 cell lines display copy number loss (Figure 19E). Copy number gains at 8q22.3 are significantly correlated with miRNA-548a-3 over-expression (Figure 19F). These findings provide evidence for a novel mechanism of LRP1B inactivation, alternative to DNA methylation, acting trough 8q22.3 genomic gain, miR-548a-5p overexpression and direct targeting of LRP1B.

A

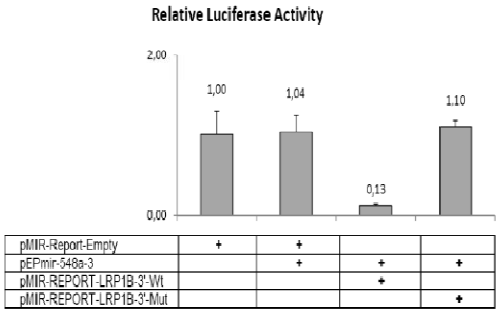

miR-548a-5p relative expression ratio
Normal Thyroid
fNMTC-Tumor1
fNMTC-Tumor2
HTH74
C643
NPA
K1
BCPAP
TPC-1
8505C
KAT4
ARO
XTC-1
N-POC
100
10
1
1
0,1
0,01
0,001
0,0001
1E-05
1E-06
LRP1B relative expression ratio


miR-548a-5p relative expression ratio
R² = 0.384
p < 0.05
60
50
40
30
20
10
0
0,000010
0,000100
0,001000
0,010000
0,100000
1,000000
LRP1B relative expression ratio

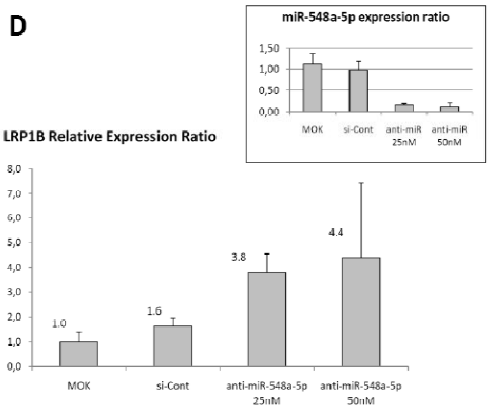
B


3' - c cA U U U UG – AG C G U UA A UG A A A A – 5'
miR548a-5p
5' - t a T A A A A T A T A A G G A T T A C T T T T G T - 3'
H. Sapiens LRP1B 3'UTR
5' - t a T A A A A T A T A A G G A T T A C T T T T G T - 3'
P. Troglodytes
5' - t a T A A A A T A T A A G G A T T A C T T T T G T - 3'
M. Mulatta
5' - t a T A A A A T A T A A G G A T T A C T T T T A T - 3'
M. Musculus
5' - t a T A A A A T A T A A G G A T T A C T T T T G T - 3'
M. Domestica
5' - t a T A A A A T A T A A G G A T T A C T T T T C T - 3'
G. Gallus

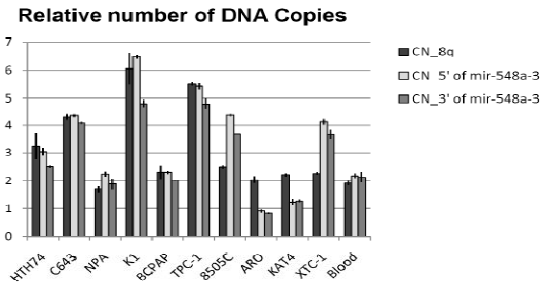
C


Relative Luciferase Activity
2,00
0,00
1,00
1,04
0,13
1,10
pMIR-Report-Empty
pEPmir-548a-3
pMIR-REPORT-LRP1B-3'-Wt
pMIR-REPORT-LRP1B-3'-Mut

D


miR-548a-5p expression ratio
1,50
1,00
0,50
0,00
MOK
si-Cont
anti-miR 25nM
anti-miR 50nM
LRP1B Relative Expression Ratio
8,0
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
1.0
1.6
3.8
4.4
MOK
si-Cont
anti-miR-548a-5p 25nM
anti-miR-548a-5p 50nM

E


Relative number of DNA Copies
7
6
5
4
3
2
1
0
CN_8q
CN_5' of mir-548a-3
CN_3' of mir-548a-3
HTH74
C643
NPA
K1
BCPAP
TPC-1
8505C
ARO
KAT4
XTC-1
Blood

F

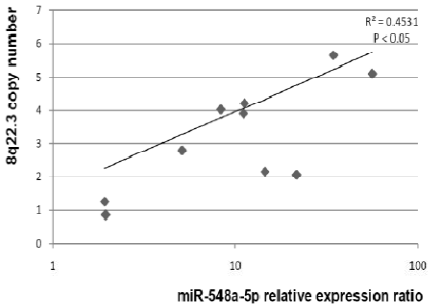

8q22.3 copy number
R² = 0.4531
P < 0.05
7
6
5
4
3
2
1
0
1
10
100
miR-548a-5p relative expression ratio

Figure 19 Overexpression of miR-548a-5p leads to LRP1B downregulation.
(**A**) Correlation between miR-548a-5p expression and LRP1B mRNA levels in thyroid cancer cell lines, normal and tumor material from the index case (Normal Thyroid, fNMTC-Tumor1 and fNMTC-Tumor2). Alignment of the two graphics illustrates the tight inverse correlation between miR-548a-5p and LRP1B which is observed both in cell lines and *in vivo* (fNMTC-Tumor1 and -Tumor2 samples). In cell lines ARO and KAT4 LRP1B is highly down-regulated without simultaneous miR-548a-5p over-expression, which is probably the result of previously demonstrated alternative inactivation events such as copy number loss and complete promoter hypermethylation (Supplementary Information Table S3). The inverse correlation between miR-548a-5p and LRP1B was demonstrated by a X/Y plot with a significant Pearson correlation (ARO and KAT4 were excluded from this analysis). (**B**) Degree of sequence complementarity between miR-548a-5p seed region and its cognate binding site at the LRP1B 3'UTR (9-mers). This site is conserved amongst primate species, mice and chicken. (**C**) Luciferase activity was assayed in HEK293 cells co-transfected with a miR-548a-5p expression vector (pEPmir-548a-3) along with either a pMIR-REPORT construct containing the wild-type 3'UTR of LRP1B (pMIR-REPORT-LRP1B-3'-Wt) or a 3'UTR sequence in which 2 nucleotides of the miR-548a-5p seed region were mutated (pMIR-REPORT-LRP1B-3'-Mut). (**D**) Endogenous expression of LRP1B upon 16 hour treatment of TPC-1 cells with anti-miR-548a-5p RNAs in 2 concentrations: 25nM and 50nM. A scramble control siRNA (siCont) and mock (MOK) conditions were used as controls. The insert shows concomitant under-expression of miR-548a-5p in the same samples. The graph integrates data from three experiments. (**E**) Analysis of miR-548a-3 DNA copy number in thyroid cancer cell lines determined by genomic quantitative real-time-PCR employing primer/probe pairs targeting genetic regions immediately upstream and downstream of the miR-548a-5p genomic *locus* (CN_5'of miR-548a-3 and CN_3'of miR-548a-3) as well as at a region near the 8q centromere (CN_8q). The abundance of DNA copies at each site was normalized to an endogenous control for RNAse P run in multiplex reactions and copy number values were calibrated by running reactions with blood-derived DNA sample in the same plate. Data was generated with the Copy Caller software. (**F**) Correlation between DNA copy number at the miR-548a-5p *locus* and miR-548a-5p expression.

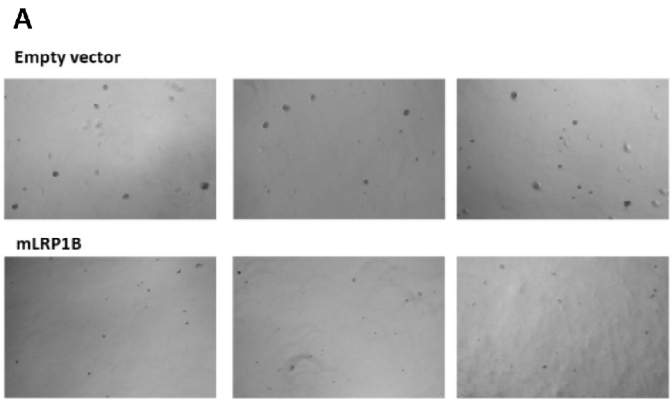
Integration of these "hits" indicates that LRP1B inactivation may arise trough diverse sequence of events during carcinogenesis (Table 7). We can infer biallelic inactivation in KAT4 and ARO cell lines to be the result of 2q21 copy number loss coupled with high density promoter methylation (>90%) of the remaining copy. In the absence of promoter methylation, an alternative combination leading to silencing comes from 2q21 copy number loss accompanied by high miR-548a-5p over-expression (8505C). The outcome is somewhat similar when there is no 2q21 copy number loss but very high miR-548a-5p over-expression/8q22.3 gain is observed (BCPAP). Cell lines K1 and TPC-1also display very high miR-548a-5p over-expression but have relatively higher levels of LRP1B expression probably because the miR-548a-5p effect is compensated by an observable low copy number gain at 2q21. NPA only displays very high miR-548a-5p over-expression. LRP1B under-expression in XTC-1 results from high miR-548a-5p over-expression combined with high DNA methylation, without 2q21 copy number loss. In Hth74 and C643 cells moderate LRP1B under-expression is attributable to copy number loss alone (Hth74) or together with miR-548a-5p over-expression/8q22.3 gain (C643).

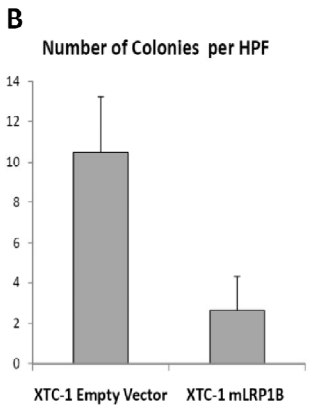
Table 7 Integration of LRP1B inactivation mechanism in cancer cell lines.
Copy number at 2q21, density of DNA methylation, miR-548a-5p overexpression and 8q22.3 copy number for each of the cell lines ascertained.

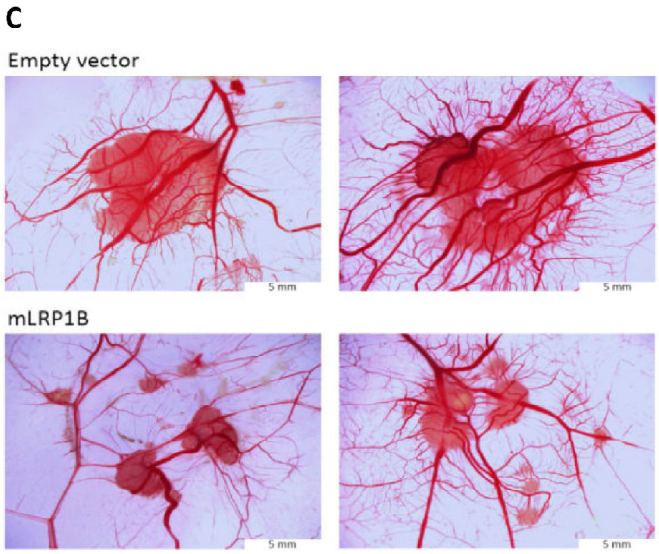
Cell Line	LRP1B Relative Expression*	2q21 Copy Number	DNA Methylation %	miR-548a-5p Expression	8q22.3 Copy Number	Summary of LRP1B Hits
KAT4	-183223	1	94%	+2	1	- Copy number loss - DNA Methylation (complete)
ARO	158357	1	94%	+2	1	Copy number loss - DNA Methylation (complete)
8505C	-21603	1	0%	+6	4	- Copy number loss - miR overexpression
BCPAP	-16433	2	0%	+15	2	-miR overexpression (high)
K1	-7551	3	0%	+35	6	- Copy number gain - miR overexpression (very high)
TPC 1	1280	3	6%	+56	5	Copy number gain - miR overexpression (very high)
NPA	-1241	2	5%	+22	2	- miR overexpression (high)
XTC-1	-1117	2	77%	+11	4	-DNA Methylation (partial) - miR overexpression (moderate)
C643	-233	1	7%	+11	4	-Copy number loss - miR overexpression (moderate)
HTH74	156	1	0%	+5	3	Copy number loss - miR overexpression (low)

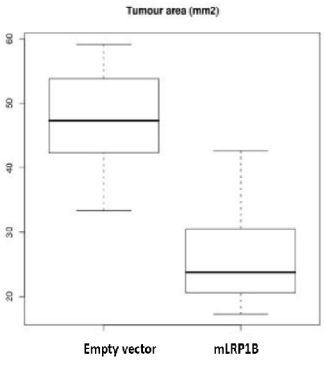
R2.7. Restoration of LRP1B inhibits *in vivo* growth and angiogenesis and impairs matrigel cell invasion.

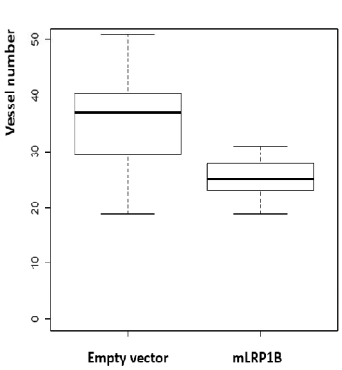
The role of LRP1B as a tumor suppressor is poorly studied. In keeping with the hypothesis that LRP1B acts as a tumor suppressor gene, we looked for a reversal of the malignant phenotype upon restoration of LRP1B. XTC-1 cells stably transfected with either a minireceptor form of LRP1B (mLRP1B), which mimics the function and trafficking of LRP1B [234] or empty vector, were used in soft agar growth assays. We found that XTC-1 cells stably expressing mLRP1B gave rise to a significantly lower number of colonies as compared with cells selected with an empty vector ($p<0.05$) (Figure 20A and B). To examine the effect of LRP1B in tumor growth in vivo, CHO cells, which are null for endogenous LRP1B, were stably transfected with either mLRP1B or empty vector and were inoculated into chicken chorioallantoic membrane (CAM). Six days after inoculation, all embryos developed tumors. However, tumors derived from mLRP1B expressing cells were significantly smaller ($p=0.002$) than tumors derived from CHO cells transfected with the empty vector (Figure 20 C and D). Furthermore, tumors from mLRP1B expressing cells had a significantly lower count of blood vessels ($p=0.022$, Figure 20C and E). In order to determine the role of LRP1B in cancer cell invasion, we performed *in vitro* matrigel invasion assays employing the otherwise highly invasive ATC cell line 8505C, in which mLRP1B was stably introduced. We observed a significant inhibition of invasive capacity of 8505C stably expressing mLRP1B relative to cells selected with the empty vector ($p<0.01$) (Figure 20F).


A
Empty vector
mLRP1B


B
Number of Colonies per HPF
14
12
10
8
6
4
2
0
XTC-1 Empty Vector
XTC-1 mLRP1B


C
Empty vector
5 mm
5 mm
mLRP1B
5 mm
5 mm


D
Tumour area (mm2)
60
50
40
30
20
Empty vector
mLRP1B


E
Vessel number
50
40
30
20
10
0
Empty vector
mLRP1B

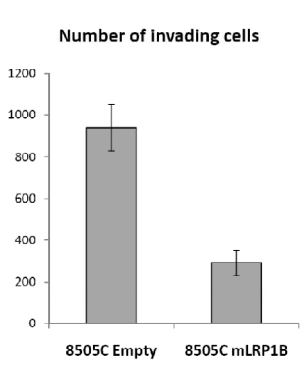

F
Number of invading cells
1200
1000
800
600
400
200
0
8505C Empty
8505C mLRP1B

Figure 20 LRP1B inhibits *in vivo* tumor growth and angiogenesis and impairs *in vitro* cell invasion.
(**A**) Soft agar growth was measured in XTC-1 cells stably selected with either an expression vector encoding a LRP1B minireceptor which mimics the function and trafficking of LRP1B (mLRP1B) or with an empty vector control (Empty vector). The XTC-1 cell line was chosen on the basis that it forms agar colonies and because endogenous LRP1B mRNA was greatly impaired. Stable clones were pooled and 5000 cells/well were mixed with soft agar in appropriate medium and plated onto 6-well plates previously coated with a bottom layer of agar. (**B**) After 2 weeks in the incubator, colonies were stained with crystal violet, photographed and counted. (**C**) *In vivo* chicken embryo growth assay were performed using CHO cells stably transfected with either mLRP1B or empty vector control. $2x10^6$ cells were inoculated into the chorioallantoic membrane and allowed to grow for 6 days. The resulting tumors were photographed. (**D**) *Ex ovo* images were used to determine the areas of the tumors (growth) and to count the blood vessels (less than 15µm diameter) developing radially towards the tumors (angiogenesis). Box-plots integrate data from 7 different inoculations of each condition. The Wilcoxon Rank Sum Test was used to calculate statistical significance. (**E**) *In vitro* cell invasion assayed by matrigel invasion chambers in 8505C cells stably transfected with mLRP1B or empty vector control. $2,5x10^4$cells were plated in 24-well plates with filter inserts containing a PET membrane of 8 micron size pores occluded by a thin layer of matrigel basement membrane matrix. After 24 hours, the invasive cells that were able to invade into the lower surface of the filter were fixed, stained with DAPI and counted under the microscope. The graph contains data from 3 experiments.

R2.8. LRP1B leads to overall changes in the "extracellular proteome"

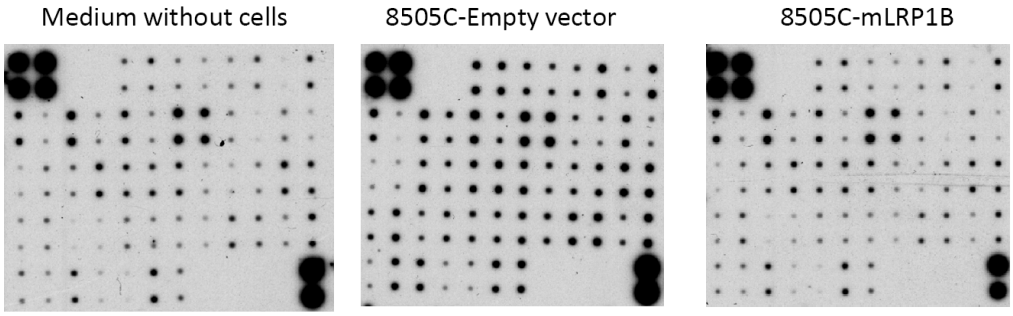
In order to investigate the impact of LRP1B in the extracellular medium, we made use of a membrane-based Cytokine antibody array as a means to compare the levels of 51 Cytokines (Figure 21A) in the conditioned medium of 8505C cells transfected with mLRP1B, relative to cells selected with empty vector. We found overall changes in the amounts of several of Cytokines quantified by this method (Figure 21B). The most prominently altered molecules were Ferritin, TACE, TRAILR2, NRG1-beta1, NrCAM, TREM1, XEDAR, all found to be reduced in media of mLRP1B expressing cells (Figure 21C and D).

Furthermore, several MMPs were found to be weakly reduced in mLRP1B 8505c cells. Nevertheless the antibody employed in the array does not discriminate between active and inactive form of MMPs. To clear the relation between LRP1B and MMPs, we performed gelatin zymography with the conditioned medium of 8505C cells. The cells were plated onto Fibronectin coated plates so as to stimulate the expression of MMPs. We found that the levels of MMP2, mainly the latent form, but not MMP9 or MMP1 (latent or active), were significantly reduced in the conditioned medium of mLRP1B expressing cells (Figure 21E). We confirmed that this difference was not attributable to different levels of MMP2 mRNA in 8505C-Empty and 8505C-mLRP1B cells, as settled by qRT-PCR quantification (Figure 21F).

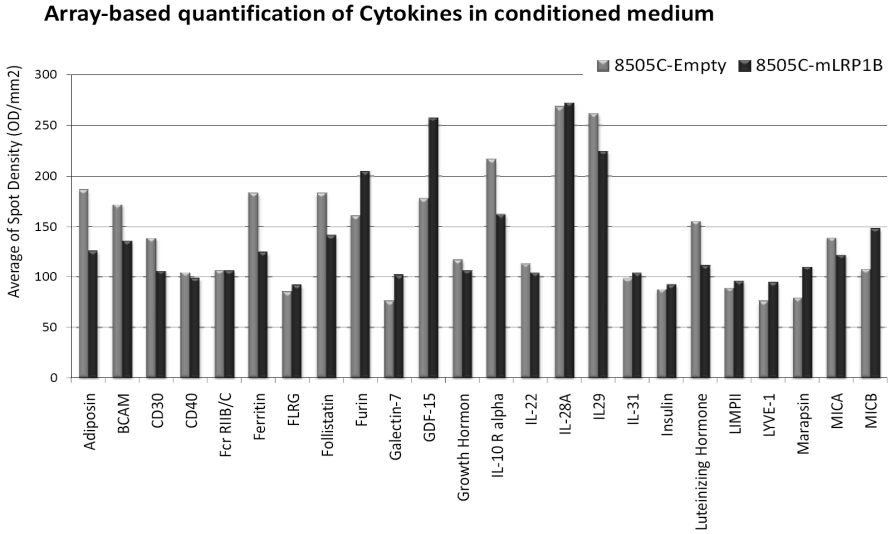
A

	a	b	c	d	e	f	g	h	i	j	k	l
1	POS	POS	NEG	NEG	Adiposin	BCAM	CD30	CD40	Fcr RIIB/C	Ferritin	FLRG	Follistatin
2	POS	POS	NEG	NEG	Adiposin	BCAM	CD30	CD40	Fcr RIIB/C	Ferritin	FLRG	Follistatin
3	Furin	Galectin-7	GDF-15	Growth Hormon	IL-10 R alpha	IL-22	IL-28A	IL29	IL-31	Insulin	Luteinizing Hormone	LIMPII
4	Furin	Galectin-7	GDF-15	Growth Hormon	IL-10 R alpha	IL-22	IL-28A	IL29	IL-31	Insulin	Luteinizing Hormone	LIMPII
5	LYVE-1	Marapsin	MICA	MICB	MMP-2	MMP-7	MMP-8	MMP-10	NCAM-1	Nidogen-1	NrCAM	NRG1-beta 1
6	LYVE-1	Marapsin	MICA	MICB	MMP-2	MMP-7	MMP-8	MMP-10	NCAM-1	Nidogen-1	NrCAM	NRG1-beta 1
7	Osteopontin	PAI-I	Platelet Factor 4	PSA-total	RAGE	RANK	Resistin	SAA	Siglec-9	TACE	TIM-1	TRAIL R2
8	Osteopontin	PAI-I	Platelet Factor 4	PSA-total	RAGE	RANK	Resistin	SAA	Siglec-9	TACE	TIM-1	TRAIL R2
9	Trappin-2	TREM-1	TSH	TSLP	VCAM-1	VEGF-C	XEDAR	Blank	Blank	Blank	Blank	POS
10	Trappin-2	TREM-1	TSH	TSLP	VCAM-1	VEGF-C	XEDAR	Blank	Blank	Blank	Blank	POS

B



C



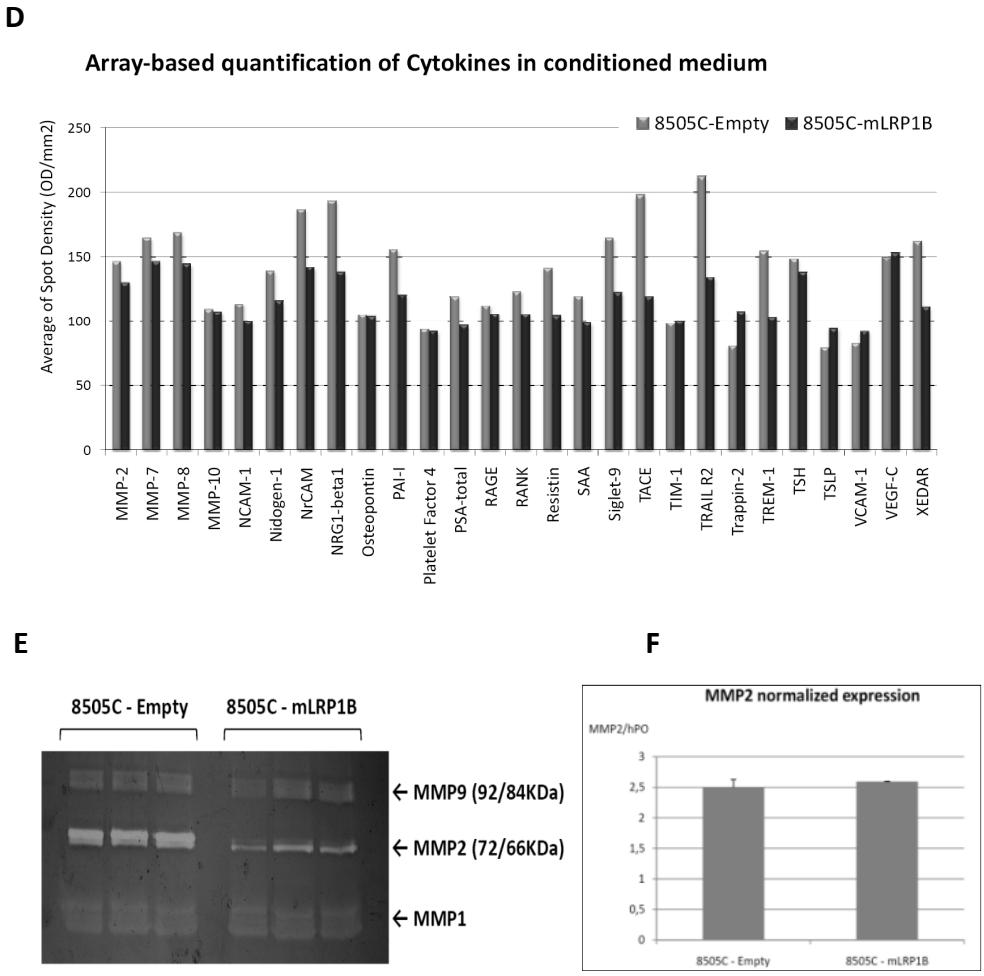


Figure 21 mLRP1B expression changes the levels of multiple Cytokines and of MMP2 in the extracellular medium.
Conditioned medium was obtained from stable 8505C mLRP1B or 8505C empty vector control cells by placing 2,5x10^5 cells in fibronectin-coated 6-well plates for 24 hours in 1ml serum free media. (**A**) Map of the antibody arrays used depicting location of antibodies for each of 51 cytokines analyzed. (**B**) For incubation of a Cytokine antibody arrays media were diluted 1:3 in sample buffer and 1ml was directly incubated with previously blocked membrane array and stained with avidin-peroxidase conjugate followed by exposure to X-ray film. (**C** and **D**) Dots were quantified using the Quantity One software and arrays were normalized trough positive controls. (**E**) Gelatin zymography performed on 20ul of the same conditioned medium which was separated in a non reducing PAGE gel containing 1mg/ml gelatin. After MMP activation gels were stained with Coomassie blue to allow the visualization of gelatinolytic bands. (**F**) Comparable levels of expression of MMP2 at the mRNA level in 8505C-empty vector and 8505C-mLRP1B stable cells.

Discussion

Discussion of Project 1

D1.1 The specificities about the spectrum of RET mutations

In project 1 we addressed the RET mutation spectrum in MEN2/FMTC as well as apparently sporadic MTC. In 5 MEN2A/FMTC families and one MEN2B case analysed for RET mutations, we have found previously described missense changes (Cys634Phe, Cys634Arg, Cys611Tyr and Met918Thr). Although these data are in accordance with the known RET mutational spectrum in MEN2 [72], 40% (five out of twelve) MEN2A/FMTC families identified in this study harboured the same specific RET mutation, Cys611Tyr (TGC-TAC), a relative frequency that is significantly different from that reported, not only by the international consortium for RET mutation [72], but also from that reported in other regions of Portugal[292]. Given the rarity of the Cys611Tyr mutation, its occurrence in five apparently unrelated families in the same geographical region suggested a possible founder effect. Our data have shown that in four out of five (80%) Cys611Tyr families the same matching chromosome 10 haplotype was found. Similar unusually high regional prevalence of specific RET mutations has been reported in other regions. This is the case of the German population, in which the frequency of codon 790 and 791 mutations is significantly higher than in other populations [100] and also in the Spanish population, where the mutation Cys634Tyr occurs in large excess[293, 294]. In Portugal, a founder chromosome, harbouring the Cys634Arg mutation, has been reported in three families originating from the same geographic circumscription in the south of the country, although settled in different places [292]. Overall, our results, in conjugation with those of others, are consistent with a cluster-like geographic distribution of families as a result of the inheritance of ancestral haplotypes. In fact, a deeper study of the Cys611Tyr families revelead that 3 of them shared commom ancestors. This may explain the incidence of hereditary MTC on a regional population basis.

We have also detected three novel occult variants located at the extracellular domain (Glu511Lys), the transmembrane region (Ser649Leu) and the Tyrosine-Kinase domain (Arg886Trp). Due to systematic RET mutation screening in all MTC cases (familial and sporadic), the finding of rare uncharacterized variants is increasingly common. This demand for functional studies to ascertain their pathogenicity, given that the number of cases (and the size of the families) is usually not sufficient to establish co-segregation, age-dependent penetrance and genotype-phenotype associations on the basis of clinical presentation. None of these variants were found in any of 70 controls from geographical. We have performed co-segregation studies and *in vitro* functional analyses to help ascertain the pathogenic potential of RET variants Glu511Lys, Ser649Leu and Arg886Trp that were found in kindreds presenting with isolated MTC. The index cases in these kindreds have been screened biochemically for pheochromocytoma and HPT and values have remained in the normal range, suggesting a MTC-only phenotype. For a number of reasons, co-segregation studies were of limited value in the clarification of the pathogenicity of these variants in the kindreds

we studied. On one hand, in one of the families siblings were not willing to be examined (as was the case for the Ser649Leu variant). On the other hand, even when siblings were found that have inherited the RET variant, they did not display clinical or biochemical symptoms of MTC (as was the case in Families E and I). In Family E one can argue that the carrier was relatively young (27 at this time) to have developed hereditary MTC and he might still develop MTC later in life, leaving the question unresolved as to MTC will not present at older age. It is known, from studies of families with MEN2A and FMTC that mutation carriers with normal calcitonin values can develop raised calcitonin levels later in life, with demonstration of C-cell hyperplasia when thyroidectomy is performed. In Family I, despite the 3 carrier siblings were older (between 49-56 years old), presumable low penetrance of the mutation may also explain lack of clinical manifestation at these ages.

In terms of their *in vitro* transformation ability, variants Glu511Lys and Arg886Trp induced significantly increased numbers of NIH3T3 transformation foci relative to wild-type RET, suggesting they somehow cause RET activation. However, the transformation efficiency of Glu511Lys and Arg886Trp is lower than that of the Cys634Arg mutation, suggesting that moderate transformation potential was achieved by these variants. Variant Ser649Leu, on the other hand, displayed very little or no gain in transforming potential, in comparison with wild-type RET (Figure 3C and D). This is consistent with other studies which have previously addressed this variant [295].

A noteworthy observation is that despite transformation induced by variant Glu511Lys is modest, it is highly augmented by GDNF treatment (Figure 3C and D). This is in clear contrast with the behavior of another extracellular mutation Cys634Arg, which nonetheless was irresponsive to GDNF (Figure 3C and D). These differences in GDNF-dependence of the transforming potential may well reflect the existence of two distinct mechanisms of receptor activation associated with two different sorts of extracellular domain mutations. In one hand, the most commonly detected extracellular mutations replacing the Cysteine residue at codons 609, 611, 618, 620, 630 and 634, cause the loss of an intramolecular disulfide bond and, as a consequence, one cysteine residue becomes available for the formation of an intermolecular disulfide bond. This generates covalently bound receptors that are constitutively active because of receptor dimerization irrespective of ligand binding [80], [81], [81], [82], leading to the experimental observation of GDNF-independent transformation potential. On the other hand, non-Cysteine mutations, such as the variant Glu511Lys herein analysed along with variants detected by others [83], [84], [85], displays a GDNF-dependent transforming capacity. This suggests that these non-Cysteine extracellular mutations mediate receptor activation trough a different mechanism, presumably by interfering with structural motifs affecting ligand-binding properties of the receptor. Indeed, Glu511Lys involves change of neutral aminoacid with a positively charged basic residue which may have strong structural implications for receptor binding properties, as explored in a recent report addressing the transforming ability of this variant, that nevertheless did not employ GDNF treatment [296].

The intracellular RET mutations such as the MEN2B specific Met918Thr and other Tyrosine-Kinase domain mutations affect receptor activation in a totally different way. By altering the

conformation of the catalytic core of the tyrosine kinase domain they result in increased catalytic activity and alter substrate specificity [80], [82], [91]. We suspect that RET Arg886Trp works in similar ways, since this change occurs in the catalytic core of the tyrosine kinase domain, in close proximity with the highly conserved region between tyrosine-kinase subdomains VI and VII, which are believed to determine substrate specificity and ATP binding at the substrate binding pocket [297]. This effect would be predicted due to the substitution of a positively charged aminoacid with tryptophan, a large, polar and hydrophobic aromatic residue, with the potential to stearically interfere with the structure of nearby regions.

Experimentally we have demonstrated that the Arg886Trp variant displays very different signalling properties, with increased activation of STAT1 and TCF4 pathways, relative to other RET variants and to RET wild-type (Figure 4). It is also of interest to emphasise the observation that wild-type RET, Cys634Arg and Arg886Trp variants were competent in activating the Beta-Catenin/TCF4 pathway, providing confirmation of the observations first made by Gujral et al [79] that this pathway contributes to RET-mediated transformation. The differences in profile and degree of activation of intracellular signalling pathways further highlight that distinct RET mutants display somehow different signaling properties (Figure 4E). This information may help design genotype-adjusted combinatorial therapies employing drugs targeting the most relevant pathways induced by each genotype. A recent, rapidly evolving field is the use of Kinase inhibitors to target specific oncogenes activated in thyroid cancer with small molecules that stearically block the ATP binding pocket and result in impaired phosphorylation activity, inhibition of signal transduction and reduction in the activation of intracellular signaling pathways relevant to tumor growth and angiogenesis. Thyroid cancer is of peculiar interest for this approach because of its apparent oncogene addiction [298]. Most oncogenic MEN2-associated RET kinase mutants are highly susceptible to inhibitors such as PP1, PP2 and ZD6474 (now known as Vandetanib/Zactima) and phosphorylation of RET mutants at codons 768, 790, 883, 918 and 634 was shown to be inhibited by these compounds [112]. In contrast, MEN2-associated swap of Valine 804 for bulky hydrophobic Leucine or Methionine in the RET kinase domain causes primary resistance to the three compounds and Valine 804 seems to be a structural determinant aminoacid mediating resistance to pyrazolopyrimidines and 4-anilinoquinazolines [137], [299]. This is also the case for the V804M/E805K tandem lesion, found in non Met918/Ala883 MEN2B, which was shown to also confer resistance to PP1, suggesting a mode of action different from the classical MEN2B mutations [87]. In contrast, Sorafenib (BAY 43-9006) is efficient in inhibiting the growth of cells carrying RET Val804Leu or RET Val804Met with an IC50 around 100-150nM, both mutants that are resistant to PP1, PP2 and Vandetanib [119], [300]. Given the accumulating evidence that response to treatment with Kinase inhibitors will be genotype-dependent, it is increasingly important to classify RET genotypes in this respect. Our results show that in vitro all three variants are sensitive to Sorafenib, which enables us to predict that patients found to harbor these genotypes will most probably respond favorably to treatment.

In terms of prophylactic therapy, age-dependent penetrance for MTC is codon-specific and classification of the risk of developing MTC can be done on the basis of the RET genotype, to define the most appropriate timing for prophylactic thyroidectomy [106]. Concerning the follow-up strategies for carriers of the herein studied variants, our functional analyses demonstrate that Ser649Leu displays a very low transforming potential and clinical management of carriers should be designed in an individual basis by monitoring calcitonin levels (basal and stimulated). On the other hand, Glu511Lys and Arg886Trp were transforming *in vitro*, but not to the same extent of Cys634Arg which, taken into account the associated clinical phenotype we would advice to classify these genotypes in the same class as Level 1 mutations usually associated with FNMTC. Indeed, other FMTC-associated mutations with lower *in vitro* transforming activity [301] often give rise to less severe forms of the disease, associated with older age at diagnosis [302].

In conclusion, this part of the work highlighted that although the RET mutational spectrum is generally conserved, there are geographical specificities in mutation frequency, which are consistent with a cluster-like distribution of specific disease-causing mutations, as a result of the inheritance of ancestral haplotypes.

We provided functional insight into rare and poorly characterized RET variants associated with isolated MTC. We confirm the indolent nature of Ser649Leu. We describe the extracellular Glu511Lys as a GDNF-dependent RET activating variant. We further present experimental data to suggest that the intracellular tyrosine-kinase Arg886Trp variant activates the receptor by changing its intracellular signaling properties. Finally we report these variants to be sensitive to Sorafenib inhibition. This information may be of value for the design of follow-up strategies and/or therapeutic regimens for patients.

Discussion of Project 2

Overall, project 2 led to the recognition of multiple levels of inactivation of LRP1B, a candidate gene for fNMTC, involving chromosomal changes (at 2q21 and 8q22.3), an epigenetic transcription factor (p300) and a specific microRNA (miR-548a-5p). LRP1B, in turn, was found to restrain cancer cell growth and invasion presumably through modulating the abundance of Growth Factors, Cytokines and MatrixMetalloProteinases in the extracellular medium (Figure 22).

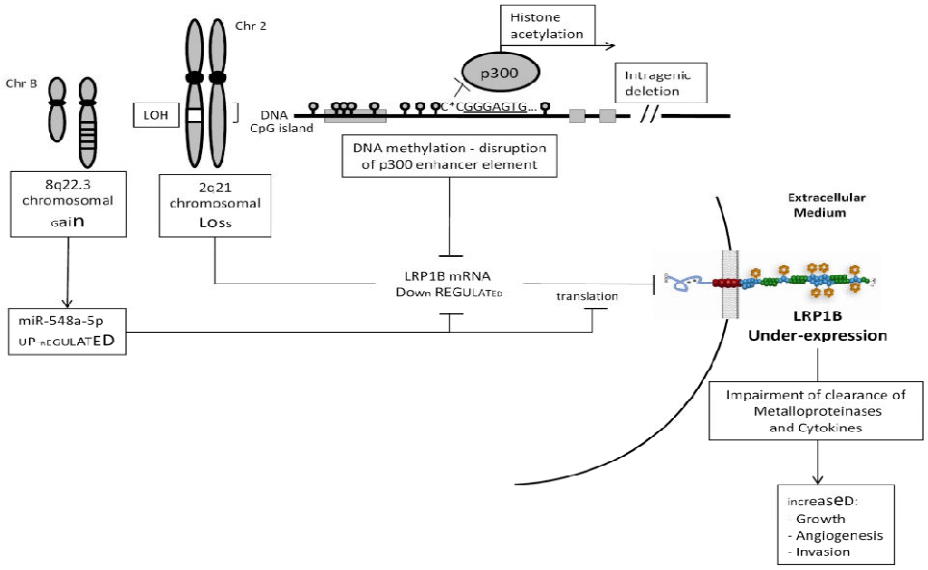


Figure 22 Multiple levels of inactivation of LRP1B, a modulator of the extracellular microenvironment
Schematic representation of chromosomal, epigenetic and microRNA-mediated changes leading to LRP1B (de)regulation which, in turn, restrains cancer cell growth and invasion by modulating the clearance of Matrix MetalloProteinases and Cytokines from the extracellular medium.

D2.1 The pattern of LOH at 2q21. The first clues implicating a tumor suppressor

As a first task of project 2 we reported loss of heterozygosity at the 19p13.2 and 2q21 regions in tumors from familial clusters of non-medullary thyroid cancer. One relevant observation in this work was the finding of LOH in the tumors from both affected members of the same family, as was the case for family A (at 19p13.2) and family H (at both 19p13.2 and 2q21). Although linkage was not tested in these families due to the small number of affected members, haplotypes were assessable in the mother/daughter families. It was noteworthy that the affected sib-pairs in families A and F consistently shared one allele at the 19p13.2 *locus*. Furthermore, the LOH pattern at 19p13.2 (in families A and H) and at 2q21 (in family H) was not random. The haplotype which was retained in tumors was the same shared by the affected members, providing evidence for the selective loss of the normal allele. These results are consistent with retention of the presumable susceptibility haplotype in keeping with the mode of inactivation of a tumor suppressor gene. In this respect, our results are in accordance with one observation reported in the paper from Stankov et al. [196], which constitutes the only previous published data of LOH analysis performed on tumoral tissue from FNMTC. In this paper Stankov *et al*. re-evaluate the haplotype at 19p13.2 in the TCO family and report deletions at chromosome 19 by FISH. The finding of loss of the normal haplotype also led Stankov *et al*. to suggest that TCO might be a tumor suppressor gene.

It is also noteworthy that in the tumors from members of family H, LOH was demonstrable at both 19p13.2 and 2q21, suggesting that, in some cases, both *loci* may cooperate in disease pathogenesis. Similar evidence has come from a linkage study by McKay et al. [181], which has demonstrated that some FNMTCs are linked to both 19p13.2 and 2q21, and that both *loci* could contribute to an increased risk of NMTC.

For the remaining cases with LOH at 19p13.2 (T3, T9, and T13), the observed LOH patterns cannot be interpreted in a similar way. This is because there was loss of the shared haplotype (in clear violation of the hypothesis of selective loss of the normal haplotype), as is observed in T13 (at marker D19S816) and in T9 (at marker D19S391), or because, despite detection of LOH, allelotyping seems to exclude linkage to the 19p13.2 region, as is the case of T3 (at marker D19S391).

These findings concur with the suspected loci heterogeneity involved in FNMTC, but can also indicate that the FNMTC series under study may be contaminated by a number of patients with patients with sporadic NMTC occurring in two members of the same family. We have used the criteria for FNMTC defined by Musholt et al. [208], (described in section I3.2.4). Families B, C, D, H and I fulfilled one primary and three secondary criteria, a pre-requisite proposed by Musholt *et al*. [208] to define FNMTC. However, we have decided to include in this study other families that, despite satisfying only one primary and one or two secondary criteria, manifested other characteristics that, in our view, may also suggest a familial predisposition when occurring in the setting of a small familial cluster of NMTC. These included unusual presentations of PTC, such as in men (family F constitutes a male sib pair), PTC with cell oxyphilia (Family A), concurrence of PTC and FTC in the same patient (Family E), and PTC of the Follicular Variant (FV) in one case (family F and G). The relevance of the

use of these criteria is justifiable by the following documented observations: PTC is unusual in men [303], some forms of FNMTC are associated with cell oxyphilia [171] and the follicular variant of PTC is more common in FNMTC kindreds [173].

In what concerns the genotype/phenotype correlation, it is interesting to notice that the two most probable true FNMTC families (A and H - in which the pattern of LOH was compatible with a non-random retention of the shared allele) were found to comprise rare morphological variants that have been previously associated with FNMTC, namely the oxyphilic and the follicular variant of PTC, respectively.

The high frequency of LOH at 19p13.2 (57%) was a surprising finding considering that in linkage studies only a minority of families were associated to this region [180], and suggests that LOH at 19p13.2 may be frequent in sporadic NMTC. Indeed, comparable frequencies of LOH at 19p13.2 and 2q21 have been previously reported in sporadic NMTCs by Stankov *et al.* [196]. Additional studies in sporadic cases could help to understand if loss of putative tumor-suppressor genes in this region can also contribute to the development of sporadic PTC (with and without oxyphilic features).

Analysis of the deleted intervals reveals a wide range of losses, spanning, in some cases, the whole length of the regions analysed, both at 19p13.2, in the case of T16 and T18, and at 2q21, in T18. In other cases, we have detected smaller deletions, limited by retentions at their boundaries, and encompassing intervals comprising one to several markers (for example in T1, T2, T3, T9 and T13 at 19p13.2). Such heterogeneous patterns of LOH, along with the heterogeneity of the families included in this study, do not allow us to restrict the previously known intervals of interest at either chromosome.

Despite several weaknesses, the findings of LOH at 19p13.2 and 2q21 constituted the first report on molecular analysis performed in tumors from familial clusters with NMTC. Furthermore the discovery of families in which losses occurring in distinct tumors of different family members consistently affected the normal/WT allele provided the first clues that the gene(s) in these *loci* was most likely tumor-suppressors in nature.

D2.2 LRP1B as an fNMTC gene? Presumed guilty until proven innocent,

LRP1B came to our attention because trough genome-wide expression profiling it was shown to be the only gene significantly targeted for downregulation within the 2q21 susceptibility *locus*, both in familial and sporadic NMTC. Despite the lack of finding a causative germline mutation in the index family, at this point we cannot exclude LRP1B as a candidate gene for fNMTC. However, we don't disregard some weaknesses that might have arisen from our strategy. In one hand, the small size of the index family (a common feature in fNMTC) did not allow ascertainment of linkage to 2q21. Despite a high probability of a susceptibility *locus* at 2q21 exists on the basis of the pattern of allelic losses, focus on 2q21 without definitive evidence of linkage may have misleaded our search from alternative fNMTC *loci* reported at 14q[170], 1q21[172], 19p13.2[171], 8p23 [174], 8q24[175], 1q21 and 6q22[176]. In the other hand, because the LRP1B coding sequence is 16Kbs long and consists of 91 exons, it was not feasible to perform exhaustive mutation screening in a larger set of samples. We are undertaking further sequencing efforts employing genome sequencing technology in a larger number of families linked to 2q21 in order to clear this issue.

D2.3 A lipoprotein receptor taking a role in cancer? An unsuspected suspect,

Despite we could not firmly assign LRP1B to familial NMTC, we were able to originally demonstrate somatic mutation and frequent genomic deletions involving LRP1B in sporadic NMTC. Moreover, besides thyroid cancer, LRP1B deletions were reproducibly observed across cancer types, namely in lung, esophagus, breast, hepatocellular, renal, neural and colorectal cancer (http://www.broadinstitute.org/tumorscape/pages/portalHome.jsf). Furthermore, the "signature" of LRP1B inactivation is archetypal of a tumor-suppressor gene. Multiple inactivation hits of structural and regulatory nature took place on both alleles trough diverse combinations of events such as genetic deletions (observable by homozygous deletion) accompanied by epigenetic silencing (DNA methylation) which, in turn, acted in alternative to microRNA overexpression. Summed up, the end results reflect selection towards biallelic inactivation and complete abrogation of the gene function.

Nonetheless, this piece of convincing evidence pointing-out LRP1B did not allow to foresee what might be the carcinogenic role of a gene best known to function as an endocytic lipoprotein receptor. Skepticism to consider LRP1B a compelling candidate also arises from the fact that endocytosis is not a cellular function renowned for its significance to tumorigenesis.

LRP1B is a member of the LDL receptor family which is composed of seven receptors structurally homologous to the LDL receptor, the prototypic gene of familial hypercholesterolemia [304]. These receptors, commonly known as Lipoprotein Receptors (LRs), include the LDL receptor, very low density lipoprotein (VLDL) receptor, apoE receptor 2, multiple epidermal growth factor-like domains 7 (MEGF7), glycoprotein 330 (gp330/megalin/LRP2), LRP1 and LRP1B (Figure 23). In addition, the family also includes additional members that are more distantly related, such as LRP5, LRP6 and SorLa/LRP11. Like other members of the LDL receptor family, the modular structures within LRP1 include

cysteine-rich complement-type repeats, EGF repeats, β-propeller domains, a transmembrane domain and a cytoplasmic domain. These domains account for three important properties of LRs. First, individual LRs have been shown to recognize multiple (more then 30) structurally unrelated ligands, that include lipoprotein complexes but, perhaps more importantly, many other categories of molecules. Second, LRs are capable of engaging endocytosis, resulting in clearance of their ligands from the extracellular milieu. Third, LRs can associate with other membrane bound receptors such as integrins and receptor tyrosine kinases, and with intracellular signaling molecules.

One hurdle that remains to be solved is how LRs are capable of recognizing such a wide variety of structurally-distinct ligands. Site-directed mutagenesis studies performed on several LR ligands, namely apolipoprotein E [305, 306], lipoprotein lipase [307], Plasminogen Activator Inhibitor-1[308, 309], α2 macroglobulin [310], and Receptor Associated Protein (RAP) [311] all point out that basic residues, Lysines or Arginines present on the ligand, are critical for recognition by LRs, as their substitution significantly reduced their affinity or resulted in loss of binding. By solving the X-ray structure of a complex between a two-module region of the CR3 and CR4 of the LDL receptor and a domain of RAP (an ER chaperone of LRs), Fisher *et al* [312] inlighted that aspartate residues in LDL receptor provide a "docking interface" for the lysine side chains of RAP. Aspartates Asp-147, Asp-149, and Asp-151(in CR4) and Asp-108, Asp-110 and Asp-112 (in CR3) surround the lysine group to form a salt bridge. Interestingly, the aspartic acid residues that form the acidic pocket responsible for docking the basic lysine residues are highly conserved amongst the CRs of LDL receptor family members, suggesting that "lysine docking" may represent a general mechanism for ligand recognition [312].

In what concerns the specific structure of LRP1B, it has four putative ligand-binding clusters (I, II, III, and IV from the amino terminus) that consist of 2, 8, 10, and 12 CRs, respectively. These domain clusters are separated from one another by three clusters of epidermal growth factor precursor repeats and (F/Y) WXD spacer repeats. LRP1B contains a putative furin endopeptidase processing site (REKR) at positions 3954–3957 [313]. This post-translational processing event results in the formation of mature LRP as a noncovalently associated heterodimer, consisting of an extra-cellular 515-kDa subunit and a transmembrane 85-kDa subunit. The transmembrane domain of LRP1B is separated from domain IV and by a cassette of six epidermal growth factor-like precursor repeats. The cytoplasmic tail of LRP1B contains two NP*X*Y motifs. Between these two is a unique insertion of 33 amino acid residues (contributed by exon 90) not present in other LRPs [313]. LRP1B has been shown to undergo regulated intra-membrane proteolysis in a gamma-secretase-dependent manner, releasing an intracellular domain (ICD) that then translocates into the nucleus [314]. However, the signaling functions displayed by the ICD in the nucleus remain unexplored.

The classical roles of LRPs are to act as scavenger in clearance of a myriad of extracellular ligands from the pericellular environment [315, 316]. The diversity of biological ligands underlies the role of LRs in multiple physiologic and pathologic processes, namely lipoprotein catabolism, blood coagulation, cell adhesion and migration, neuronal process outgrowth, and Alzheimer's disease [317]. The best studied ligands of LRs include

lipoprotein complexes and proteinase/proteinase inhibitor complexes, such α2 macroglobulin and members of the urokinase Plasminogen Activating (uPA) system (uPA, uPAR and PAI-1) [318, 319]. The close similarity in protein domain structure between LRPs strongly suggests that these receptors may share a comparable spectrum of ligands. Nevertheless this deduction may not be straightforward. For instance, LRP1B ligands of the uPA system were found to be overlapping with LRP1, its closest related member [234]. However, distinct properties are displayed by LRP1B since, in contrast to LRP1, cells expressing LRP1B display a substantially slower rate of uPA/PAI-1 complex internalization [320] which impairs the regeneration of unoccupied uPAR on the cell surface and correlates with a diminished rate of cell migration [234, 321], [322]. This emphasizes that the functions of LRs may be overlapping and yet distinct as a result of their ligand spectrum and kinetics of endocytosis [234]. Moreover, despite early evidence implicating LRP1B in cell migration (trough modulation of uPAR) it is expected that LRP1B, in analogy with other LRs, displays a myriad of ligands that impact multiple physiologic aspects, making it difficult to anticipate the multiple ways in which this receptor may contribute to tumorigenesis.

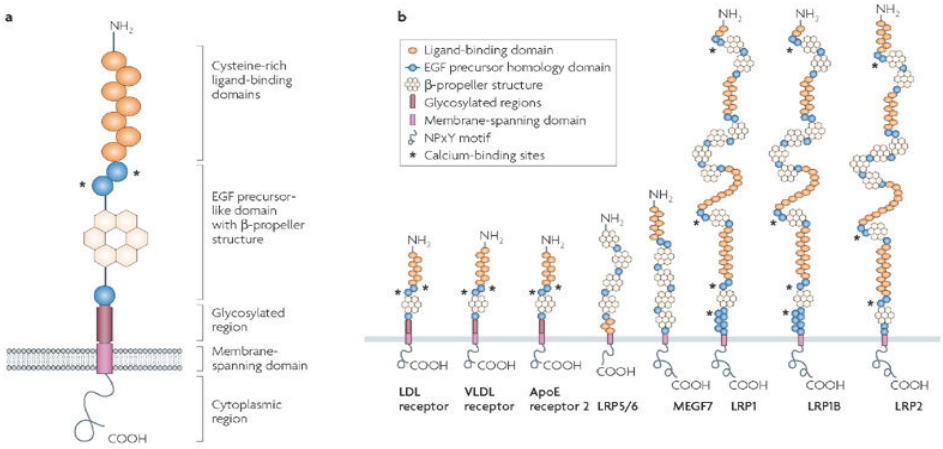


Figure 23 Structure and domains of members of the Low Density Lipoprotein Receptor Family
(**a**)The structures of the LDL Receptor Family members differ in the number and organization of nonetheless homologous modular domains, namely Cysteine ligand-binding repeats, EGF precursor-like domains with β-propeller structure, single pass transmembrane region and Cytoplasmatic domains with endocytosis NPXY motifs. (**b**)The LDL Receptor family comprises the LDL, VLDL and ApoE receptors as well as MEGF7, LRP1, LRP1B and LRP2. Adapted from [323].

D2.4 The *modus operandi*: a "landscaper" of the tumor microenvironment

Our results highlight LRP1B as an unconventional and yet key tumor-suppressor acting as a regulator of the "extracellular proteome" and constraining the abundance of matrix metalloproteinases and cytokines in the tumor microenvironment. These secreted factors are known to orchestrate a multitude of functions in shaping the tumor microenvironment (matrix, fibroblasts, infiltrating leucocytes and neo-vasculature) into a growth-promoting and invasion-prone state.

The absorbed view of cancer as a genetic disease has probably guided a reductionist focus on cancer cells and the genes within them. However, solid tumors are not simply composed of neoplastic epithelial cells and in recent years, important new insights are coming up from regarding tumors as complex tissues [324] in which cancer cells subvert normal cell types (in their surroundings and at distant sites) to serve as active collaborators in malignancy. This is leading to the appreciation that cancer cells destabilize their (local and systemic) extracellular communications much to the same extent as they disrupt their intracellular signaling pathways.

The importance of the microenvironment emerged from unforeseen observations that transformed cells, when put into a normal tissue context, reverted their malignant phenotype and were reprogrammed to behave as normal cells. This phenomenon was first clearly noted in 1975 when Beatrice Mintz and Karl Illmensee took teratocarcinoma cells from embryoid bodies *in vivo*, and injected them into developing mouse blastocysts [325]. Surprisingly, normal chimeric mice were born with no evidence of tumors[325]. Furthermore, the tumor-derived cells were present in large numbers and contributed to several unrelated tissues, all of which were functional[325]. This mysterious influence of "context" on the behavior of tumor cells became the focus of research by Mina Bissell. In 1984, she published a study, together with David Dolberg, showing that the ability of Rous sarcoma virus (RSV) to induce tumors was also context dependent [326]. At the time, the tumor-inducing behavior of RSV when injected into the wings of newly hatched chicks was already known, and the viral gene v-*src* had been identified as the sole culprit. What Bissell found, however, was that if RSV was injected into 4-day-old embryos, no tumors were produced, despite the spread of RSV infection throughout the embryo and active v-Src expression [326]. Furthermore, if the infected embryonic cells were isolated and grown in culture, they became transformed [326]. So, something about the environment of the embryos was able to block tumorigenesis, despite the presence of v-Src. The following year, her group went on to show that wounding was one important influence on the ability of a cell to succumb to tumorigenesis [327]. When RSV was injected into a chick wing to produce a local tumor, a second tumor would only be seen if a wound was simultaneously induced at a remote site[327]. The Bissell group later found that the factor responsible was transforming growth factor beta (TGF-B) [328]. Subsequent studies by other groups confirmed that the embryonic microenvironment is able to reprogramme various cancer cells, including metastatic cells, to a less aggressive phenotype [329-331]. In 1997, Bissell and colleagues showed that normal and tumor cells behaved radically different ways when in the presence of an extracellular matrix scaffold that

provides tissue architecture and that blocking integrin function was sufficient to revert the malignant phenotype of human breast cancer cells both in culture and *in vivo* [328].

Aside from the ECM, the importance of stromal fibroblasts in modulating the malignant progression of transformed epithelial cells was demonstrated by other groups. For example, in co-culture experiments, normal fibroblasts prevented the growth of initiated prostatic epithelial cells [332], and could even reverse the malignant phenotype of neoplastic epithelial cells [333]. Conversely, an abnormally activated stroma has been shown to contribute to tumorigenesis and tumor progression. Primary breast carcinoma cells formed spherical colonies when cultured in three-dimensional collagen type I, however, when co-cultivated with stromal cells, the tumor cells tend to spread and become invasive. Moreover, the degree of tumor growth increases with the density of the stromal cells [334]. Reinforcing this evidence, the groups of Luis Parada and Harold Moses, were able to show in mouse models that genetic alterations in cells of the tumor microenvironment contribute to, and can even be sufficient for initiating the development of cancer [335, 336].

A further demonstration of the potential of the stroma to initiate tumors comes from a form of predisposition to colorectal cancer. In juvenile polyposis syndrome (JPS – OMIM: 174900) patients at a young age develop multiple hamartomatous polyps of the colon in which the proliferating population of cells appears to be derived from the stroma. These polyps are markedly different from the epithelium-rich adenomatous polyps that give rise to most cases of colorectal cancer. Polyps from patients with JPS are composed largely of stromal cells, comprising a mixture of mesenchymal and inflammatory elements in which epithelium is entrapped, often forming dilated cysts. The epithelial cells within and surrounding the polyp are initially devoid of neoplastic features but nevertheless are at increased risk of becoming malignant. The gene responsible for JPS susceptibility was found to be SMAD4 [34], a central player in the signal transduction pathway activated in response to the large family of TGF-B (transforming growth factor-B)-like ligands. Interestingly, the stromal cells, and not the epithelial cells, of most hamartomas from JPS patients contain a clonal genetic alteration [337]. Similarly, clonal genetic changes have been demonstrated in the stroma, but not the epithelial cells, of endometrial polyps [338, 339]. Thus, individuals with JPS have an increased risk of colorectal cancer, in consequence of the fact that the epithelial cells associated with the polyps are more likely to undergo neoplastic transformation, as a result of an abnormal stroma microenvironment. Unlike susceptibility genes that work through a straightforward manner, through inactivation of tumor suppressor genes, in JPS patients, tumorigenesis occurs by indirectly acting cancer susceptibility genes and the increased risk of cancer therefore seems primarily the result of an altered terrain affecting growth of adjacent epithelial cell and according to Bert Vogelstein can be thought of as a "landscaper" defect [32, 340].

Overall this groundbreaking work emphasized that normal tissue environment has inherent tumor-suppressive properties which act as dominant over epithelial cells with oncogenic mutations [36, 37, 341, 342]. Moreover it advanced the idea that the protective constraints of the microenvironment are overridden by mediators of chronic inflammation that turn the stroma into an active carcinogen.

The link between chronic inflammation and cancer has been noted more than 150 years ago and provides the second body of evidence that trough altered inter-cellular communication, an activated stroma fosters tumorigenesis. In 1863, Virchow hypothesized that the origin of cancer was at sites of chronic inflammation. On the basis of observations that tumors often arose at sites of chronic inflammation, that exposure to some irritants causing tissue injury and ensuing inflammation was carcinogenic and that inflammatory cells were present in biopsied samples from virtually every tumor type, Virchow upheld that "cancers are wounds that never heal" [343]. It is now becoming clear that the tumor microenvironment is largely orchestrated by inflammatory cells and recent data has expanded the concept that inflammation is a critical hallmark of cancer [344, 345]. The link between inflammation and cancer has been substantiated trough many lines of associations:

a) Epidemiologically, many malignancies are associated with chronic inflammatory diseases or with persistent bacterial and viral infections that act at tumor initiation. An association of chronic inflammation with malignant diseases is in colon carcinogenesis arising in individuals with inflammatory bowel diseases, such as chronic ulcerative colitis and Crohn's disease [346]. Hepatitis C infection in the liver predisposes to hepatocellular carcinoma [347], whereas chronic *Helicobacter pylori* infection is the leading cause of stomach cancer [348, 349]. In the context of thyroid cancer, it has been appreciated that autoimmune thyroid diseases such as Hashimoto's thyroiditis increase the risk of well-differentiated papillary thyroid carcinomas (PTCs) (for a review see ref. [350]. Although it is still unclear whether inflammation alone is sufficient for the development of cancer, leukocytes and other phagocytic cells induce DNA damage in proliferating cells, through their generation of reactive oxygen and nitrogen species that are produced normally by these cells to fight infection [344, 351]. Repeated tissue damage and regeneration of tissue amplifies the accumulation of potential oncogenic mutations. To demonstrate this, p53 mutations are seen at frequencies similar to those in tumors in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease [352].

b) Pharmacologically, chronic suppression of inflammation through prolonged use of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) lowers the incidence of certain cancers, namely colon [353, 354] and breast cancer [355]. This antitumor activity occurs through inhibition of cyclooxygenase-2 (COX2), an enzyme that is involved in the synthesis of pro-inflammatory prostaglandins, illustrates how the several tissue types can collaborate to promote tumor progression, as fibroblasts, immune system cells and cells involved in neoangiogenesis are all part of this pathway [356, 357].

c) Signaling pathways involved in inflammation have been shown to operate as major components downstream of oncogenic mutations. A useful setting in which to explore the connection between oncogenes and an inflammatory microenvironment is RET. In freshly isolated human thyrocytes, the activation of RET induces a transcriptional program that is similar to that which occurs during inflammation [358]. RET induces the expression of various inflammatory factors [358-360] and furthermore, key protein components of the RET-activated 'inflammatory' program were found in tumor specimens taken by biopsy, and larger amounts of these inflammatory molecules were found in the primary tumors of

patients with lymph-node metastasis than in primary tumors in the absence of lymph-node metastasis (reviewed in [350]). These and other results [361, 362] [363] connect the activation of oncogenes to inflammation.
d) Inflammatory cells, mainly tumor-associated macrophages (TMAs) [364-366] and biochemical mediators of inflammation including major inflammatory cytokines [367, 368] (such as IL-1β[369], IL-6[370], IL-23[371] and TNF-α[372]) and transcription factors (such as NF-κB [373] and STAT3[374, 375]) are abundantly found in the microenvironment of all tumors in experimental animal models and humans, since the earliest stages of development.

Yet another extensive line of evidence that cancerigenesis will often only be realized when cells sculpt a favorable tissue environment comes from the observation of tumor-induced angiogenesis, initially addressed by Judah Folkman [376] .The onset of angiogenesis, also known as the "angiogenic switch" [377], is recognized as a rate-limiting discrete step that occurs at a given stage of tumor progression so as to provide access to the host vascular system and produce an adequate supply of oxygen and metabolites that support tumor growth and allow premalignant and/or dormant lesions to progress [376-378]. The induction of the angiogenic switch depends on pro-angiogenic stimuli, such as the hypoxia which results from increased tissue mass, and also by oncogene activation or tumor-suppressor mutation [376-378]. Thus, neovascularization also typifies an ability of cancer cells to induce other cell types, endothelial cells in this case, so as to liven up their environment.

Summed up, the above mentioned body of evidence sustains that an otherwise tumor-suppressive microenvironment is shifted by cancer cells trough subversion of physiological processes such as inflammation and angiogenesis to act as nurturing ground for carcinogenesis. This primed microenvironment is then composed of a functional architectural ECM (not an inert scaffold), activated (and sometimes mutated) stromal cell types such as fibroblasts, endothelial cells (comprising blood and lymphatic networks), pericytes and various tumor associated BMDCs, including macrophages, neutrophils, mast cells, myeloid cell-derived suppressor cells and mesenchymal stem cells [324, 379, 380].

The question now stands to clarify which are the secreted soluble biochemical factors, released by cancer cells or by other cell types recruited to their surroundings, which mediate these disturbed interactions. By reviewing literature spanning nearly a century Dvorak [381] proposed that tumors activate some of the mediators of the normal wound-healing responses. Wound healing and tumor-environment communications are dynamic, progressive processes that involve the interaction of several cell types [382], and according to Dvorak [381], comparison of the two reveals many mechanistic similarities. In wound-healing, immediate reaction to tissue injury leads to activation of platelets that form a haemostatic plug and also release vasoactive mediators to increase vascular permeability and to enable the influx of serum fibrinogen to generate the fibrin clot. Platelets produce chemotactic factors, including transforming growth factor-B (TGF-B) and platelet-derived growth factor (PDGF). These factors initiate the formation of granulation tissue by activating fibroblasts to produce MMPs and a number of growth factors, such as fibroblast growth factor-2 (FGF-2). These factors degrade dermal extracellular matrix, stimulate infiltration of macrophages and promote the development of new blood vessels. These interactions are

potentiated by reciprocal signaling between the epidermis and dermal fibroblasts through growth factors, MMPs, and members of the TGF-B family. The complex reaction to wounding reduces epithelial adhesiveness and increases epithelial-cell mobility to re-form an intact sheet of tissue over the wound. Production of MMPs and proteolytic enzymes such as urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) facilitates this re-epithelialization. Blood vessels can then enter the fibrin clot as epidermal cells resurface the wound.

Although developing tumors do not disrupt the vascular tissue in the same way as in wounding, many of the processes occur in parallel. Tumor cells produce many of the same growth factors that activate the adjacent stromal tissues in wounding or fibrosis. Activated fibroblasts and infiltrating immune cells (macrophage) secrete MMPs and cytokines such as TGF-β, FGF-2, and PDGF [383, 384]. These factors potentiate tumor growth, stimulate angiogenesis, and induce fibroblasts to undergo differentiation into myofibroblasts and into smooth muscle. Tumor cells, myofibroblasts and activated macrophages increase production of MMPs and uPA at the invasive front to stimulate angiogenesis and proliferation. Normally, the wound-healing response is temporary and self-limited, however, in tumors, inflammation is escalating [345] and progressively amplified immune cells can overproduce growth factors and mitogenic cytokines that promote tumor cell proliferation. Also, sustained upregulation of MMPs can disrupt the ECM and facilitate invasion.

The relevance of extracellular factors secreted by tumor cells goes beyond local and may have systemic effects in regulating the settlement of cancer at metastatic sites [380, 385]. In recent years, evidence has emerged that growth factors secreted by the primary tumor prime certain tissues for tumor cell engraftment [380, 385-388]. In response to these soluble factors, tumor associated cells such as hematopoietic progenitor cells and macrophages cluster at “premetastatic niches” at distant organs, creating, beforehand, an environment that is conducive for tumor cell adhesion and intravasion at metastatic sites [386, 387] (the so called metastatic niches). On the pillars of the theory of the “metastatic niche” [385] are experiments in which mice bearing Lewis lung carcinomas (LLC) received intraperitoneal injections of cell culture media that had been conditioned by B16 melanoma cells [386]. In this experiment, LLC metastasis was redirected to organs characteristic of melanoma but rarely seen with LLC metastasis, such as the spleen, intestine, kidney and oviduct [386].

It is within this framework of the crucial role for secreted Cytokines [367, 368] and MMPs [389] in molding the tumor microenvironment(s) that the relevance of LRP1B in tumorigenesis now becomes clear. Our results show that LRP1B modulates many of the biochemical orchestrators of the tumor microenvironment. We have shown that molecules such as growth factors (NRG1), Cytokines (TRAIL-R2, XEDAR, TREM1, NrCAM) and MMPs (TACE and MMP2) have been depleted in medium conditioned by LRP1B expressing cells. This prompts us to advance the idea that the *modus operandi* of LRP1B is trough “landscaping” the tumor microenvironment.

The role of LRP1B is further put to relevance since it modulated a reasonable number of molecules, probably due to its inherent ability to bind multiple ligands (section D2.3).

However, at this stage we cannot set apart which molecules are directly modulated by LRP1B (presumably trough its endocytic activity) or which molecules are derived from indirect actions of LRP1B (because they may result from proteolytic shedding mediated by other LRP1B-modulated targets, such as Metalloproteinases). Binding and internalization assays need to be performed in the follow trough of this investigation in order to distinguish direct from indirect targets of LRP1B.

Concomitant with changes in extracellular medium composition, we have demonstrated that several aspects of the malignant phenotype were inhibited by LRP1B, namely growth, angiogenesis and invasion. In view of the fact that multiple molecules in the extracellular medium are directly or indirectly depleted by the activity of LRP1B, it is difficult to account responsibility for these phenotypic changes to any single of these factors.

Additionally, as the extracellular domain of LRP1B may potentially bind to a myriad of still undisclosed ligands [390] it is possible that the tumor-suppressor properties of this receptor are further explained by modulation of the extracellular concentration of other molecules that we have not addressed. So far we addressed 51 cytokines in conditioned medium, but more molecules may be tested trough other antibody arrays. Our approach is also inherently limited to the set of cytokines secreted by the single cell line (8505C) that we employed. It is expectable that LRP1B may be shown to modulate additional molecules secreted by cells derived from other cancer types or by other types of cells within the tumor microenvironment. For necessity, single cells have been used in the experiments, however it is recognized that most secreted factors in the tumor microenvironment are produced by cell types other than cancer cells. A more complete scrutiny of the impact of LRP1B in the extracellular medium would benefit from performing experiments in other (heterotypic) cell contexts. Co-culture or *in vivo* experiments would not only give a more precise idea of the consequences of LRP1B-mediated changes in terms of abundance in secreted factors but also could have the potential to verify how this affects recruitment of leucocytes, fibroblast activation or angiogenesis.

A further pending question regards to what may be the normal function of LRP1B in thyroid physiology as no specific ligand related with thyroid gland has been identified. In humans, the LRP1B transcript is much more abundant certain tissues, namely brain, salivary gland and thyroid, making it conceivable that LRP1B exerts some thyroid-specific functions in the normal context. This also remains to be ascertained by analyzing the impact of LRP1B in molecules secreted by thyroid cells in the course of their normal physiology.

In conclusion, despite the biochemical mediators of disturbed extra-cellular communications between cancer cells and their environment(s) have become reasonably identified, the mutations capable of endowing these traits to cancer cells remain largely unknown. In the end, LRP1B inactivation fits the archetype of a genetic (and epigenetic) alteration in the cancer cell that drives and orchestrates a re-setting of its extracellular environment in a way that confers growth advantage and increases invasive capacity.

D2.5 The impact of LRP1B on the extracellular microenvironment (and the hallmarks of cancer)

Working under the hypothesis that LRP1B (through its endocytic activity) modulated the amounts of certain soluble factors (such as Cytokines) which could account for the observed reduction of malignant features (growth, invasion and angiogenesis), we used antibody arrays to simultaneously ascertain 51 Cytokines in conditioned media. We cannot disregard that this experiment would benefit from increased reproducibility and confirmation by independent methods so as to firmly establish these initial findings. Previous attempts to characterize LRP1B ligands used a completely different strategy, employing immobilized recombinant extracellular sub-domains of LRP1B to perform affinity chromatography on brain lysates [234]. This previous approach inherently undermines the assessment of the full impact of LRP1B activity because the proteome which has been interrogated was that derived from cell fractions rather than the extracellular proteome which shares physiological milieu with LRP1B. Another major drawback of this approach is that it neglects potential changes occurring as an indirect consequence of LRP1B action, failing to provide the overall picture of LRP1B physiology. Despite our line of attack to this question was different, we need to bear in mind that, as others, we have only employed certain sub-domains of LRP1B. For instance we have transfected a mini-receptor form of LRP1B which only included subdomain IV of the extracellular portion of LRP1B (along with full transmembrane and cytoplasmatic domain). Thus, we need to cautiously consider that the properties we are attributing to LRP1B come from this highly artificial experimental setting. Nevertheless, trough our analysis we have been able to pinpoint molecules which were previously known (by independent strategies) to be LRP1B ligands, namely PAI-I and Ferritin [234] which, accordingly, were found to be reduced in conditioned medium of LRP1B expressing cells. The validation of these ligands provides a good deal of confidence that our experiment faithfully reflects the outcomes of LRP1B activity.

We must emphasize that this experiment is unprecedented in the way it stresses out the wide-range of physiological implications that LRP1B (directly or indirectly) could account for, covering multiple “hallmarks of cancer”. In 2000 Weinberg and Anna first enumerated a discriminate set of traits which outline the development and progression of cancer (self-sufficiency in growth signals; insensitivity to anti-growth signals; evading Apoptosis; limitless replicative potential; sustained angiogenesis and tissue invasion and metastasis) [38] which were subsequently updated to include inflamation [39, 344]. In this section we propose take on the exercise of demonstrating in which way the 7 molecules most prominently depleted by LRP1B (NRG1, TRAIL, XEDAR, NrCAM, TACE, TREM1and MMP2) could endow cancer cells with multiple traits of the “hallmarks of cancer”.

NRG1 beta (Self-Sufficiency in Growth Signals): NRG1 encodes for Neuregulin, also known as Heregulin. Neuregulin 1 was originally identified in the search for the natural ligands which interact with the HER2/NEU/ERBB2 receptor tyrosine kinase so as to increase its tyrosine-kinase phosphorylation activity [391] and, for this reason, the neuregulins are also known as heregulins. The neuregulins are the natural activators of HER/ErbB receptors and represent the largest subclass of polypeptide growth factors of the epidermal growth factor (EGF) family [392, 393]. The natural mechanism of action of Neuregulin1 is trough activation of the HER3 and 4 receptors by ligand binding [394]. Upon engagement of Neuregulin1 to HER3 or HER4 these receptors expose a dimerization arm which can interact with analogous dimerization arms of other HER receptors, including HER3 or HER4, but also HER2 [395]. Extracellular dimerisation causes intracellular transphosphorylation of the kinase region and induction of intracellular signaling pathways such as RAS/RAF/ERK or PI3K /AKT [396].

A body of evidence indicates that neuregulins have a role in the development/progression of certain types of human cancer [397, 398]. In vitro studies indicate that neuregulins act as strong mitogenic factors in cells that express HER receptors [399]. *In vivo* studies performed in mice have shown that overexpression of neuregulins in the mammary tissue results in the generation of adenocarcinomas [400] and favors the metastatic spread of breast cancer cells in vivo [401]. Furthermore, constitutively active forms of the HER/ErbB receptors have been reported in several tumors [402]. In the best example, HER2/ErbB2 is over-expressed due to gene amplification in a significant subset of patients with breast cancer [403], and Neuregulin expression correlates with clinical response to antibodies blocking HER2, such as Transtuzumab [404].

The observation that NRG1 is depleted in media from LRP1B expressing cells conveys with the growth-suppressive effect observed for LRP1B. LRP1B inactivation, found frequently in cancer cells, would otherwise result in increased levels of Neuregulin which would serve to promote growth signals.

TRAILR2 and XEDAR (Evading Apoptosis): TRAILR2 is also known as Death Receptor 5 (DR5) and consists of a so called “death receptor” that interacts with TRAIL (Tumor necrosis factor-Related Apoptosis-Inducing Ligand) [405] . Aside from TRAILR2, another death receptor (TRAILR1/DR4), along with three "Decoy Receptors" TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and osteoprotegerin (OPG) have been characterized [405-407]. Their ligand, TRAIL (also termed Apo-2L) was originally identified and cloned on the basis of sequence homology to the Fas ligand and TNF [408, 409] and has been established to selectively induce apoptosis in many transformed and tumor cell types [409]. Notably, unlike cancer cells, most normal cells are protected from to TRAIL-mediated apoptosis [409] which has raised high hopes that TRAIL could be used as a selective anticancer agent. The resistance of normal cells to TRAIL-induced apoptosis has been discovered to result from the increased expression of TRAIL decoy receptors [410]. While TRAIL-R1 and TRAIL-R2 contain cytoplasmic "death domains" that signal apoptosis, TRAIL-R4 has a truncated non-functional cytoplasmic death domain, TRAIL-R3 lacks a cytosolic region and OPG acts as a soluble decoy receptor [411]. Decoy receptors

are therefore incapable of transmitting an apoptosis signal and protect cells from TRAIL-induced apoptosis by capturing the available ligand [405, 407, 410].

A soluble form of TRAILR2 (sDR5) has also been detected in extracellular media and serum [412], and the same protective mechanism is operative . In vitro it has been shown that soluble TRAILR2 (or sDR5), which consists only of the extracellular domain of the human TRAILR2 receptor, effectively blocks TRAIL-induced apoptosis of tumor cells [413]. According to our observations, LRP1B mediates a reduction of soluble TRAILR2 decoy receptor, which would consequently increase the amount of bioavailable TRAIL and render cells more susceptible to TRAIL-induced apoptosis. This mechanism is in consistency with the tumor suppressor role of LRP1B. Conversely, LRP1B inactivation would be predicted to cause increased amount of extracellular TRAILR2 decoy receptor, subsequent reduction of active TRAIL and resistance to apoptosis, another common trait of cancer cells.

XEDAR (X-linked Ectodermal Dysplasia Receptor) is also known as EDA2R or TNFRSF27 and binds ectodysplasin (also known as EDA) [414]. Like the above mentioned TRAIL receptors, XEDAR also belongs to the TNF Receptor family and possesses the ability to induce programmed cell death, although it has a unique intracellular region which lacks a death domain [415]. Unlike Fas receptors, XEDAR does not directly bind to the death adaptors FADD, TRADD, or RIP, but activates caspase 8 via the formation of a complex containing FADD, caspase 8, and caspase 10 [415]. XEDAR has shown to be transactivated by p53 and has been proposed to act as a tumor suppressor in colorectal cancer [416]. In this study, the extracellular NH_2 terminus of XEDAR protein was reported to be released into culture media [416]. XEDAR seems to display overlapping functions with TRAIL Receptors in the induction of apoptosis. Thus, we can envision that a mechanism in which soluble receptors acting as decoys render cells insensitive to ligand-induced apoptosis, similar to that formerly described for TRAILR2, is in place.

NrCAM and MMP2 (Tissue Invasion): At some point in epithelial tumor progression cancer cells overcome the basement membrane and acquire the means to migrate through the interstitial connective tissue. These properties are partly conferred by decreasing cell adhesion, activation of cell motility and induction of proteolytic enzymes which degrade the extracellular matrix, such as MMPs.

NrCAM is a transmembrane Neuronal Cell Adhesion Molecule belonging to the family of immunoglobulin-like cell adhesion molecules [417] which has been implicated in the guidance, outgrowth, and fasciculation of neuronal cells [417, 418]. Despite NrCAM was initially discovered and most extensively studied in the nervous system [417], NrCAM is expressed in of nonneuronal tissues [419] and a role in tumorigenesis is emerging. Upregulation of NrCAM expression could be implicated in the pathogenesis and behavior of papillary thyroid cancers [420]. Nr-CAM expression was further detected in melanoma, renal carcinoma, and colon carcinoma [421]. Forced expression of NrCAM in fibroblasts conferred increased cell motility and tumorigenesis in nude mice [421]. In addition, human melanoma cells from more advanced stages of melanoma express high levels of NrCAM and form tumors in mice, whereas those lacking NrCAM do not [421]. Interestingly, the same authors showed

that the NrCAM ectodomain could account for many of the tumorigenic properties; it enhanced cell motility, conferred transforming potential and increased tumorigenesis in mice [422].

Despite MMP2 is not very prominently altered in the array-based quantifications (because the antibody employed in the array does not discriminate between active and inactive forms), results from gelatin zymography show considerable differences in MMP2 amount upon LRP1B expression.

MMP2 is an enzyme of the Matrix MetalloProteinase family which is involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, menstruation and tissue remodeling, as well as in disease processes, namely arthritis and cancer metastasis. This gene encodes an enzyme which degrades type IV collagen, the major structural component of basement membranes. Elevated expression of MMP2 is usually seen in highly invasive tumors assisting invasion of metastatic cancer cells [389]. Removal of excessive extracellular proteolysis trough use of MMP inhibitors has been shown to prevent tumor spreading [423].

From our gelatin zymography results, active MMP2 amount show consistent downregulation upon LRP1B expression. Although MMP2 is not very prominently altered in the array-based quantifications, this discrepancy is probably due to the fact that the antibody employed in the array does not discriminate between active and inactive forms.

Our findings that the conditioned media of mLRP1B expressing cells contains less soluble NrCAM and MMP2, could explain the impaired invasive capacity that was observed for these cells in matrigel assays. Altogether NrCAM and MMP2 illustrate how LRP1B inactivation, trough consequent accumulation of these molecules, could result in motility and invasive phenotype and increase tissue invasion.

TACE and TREM1 (Inflammatory microenvironment): TACE stands for Tumor necrosis factor Alpha Converting Enzyme and is also referred to as ADAM17 (A Desintegrin And Metalloproteinase -17). ADAM17 is a matrix metalloproteinase-like enzyme which was first discovered to release the active form of tumor necrosis factor alpha (TNFα) from its membrane bound precursor [424, 425]. TNF-α is a pivotal cytokine in inflammation, which is now regarded as the 7th hallmark of cancer [344]. Despite the name, TNF-α is important in early events in tumors, regulating a cascade of cytokines, chemokines, adhesions, MMPs and pro-angiogenic activities [343, 426, 427]. Although high doses of TNFα are used to treat certain malignancies [428], several animal studies suggest that this cytokine is involved in cancer promotion. In a mouse model, either a deficiency of TNFα or the administration of a neutralizing antibody directed against the cytokine inhibited the development of chemically induced skin cancer [429]. TNFα was also shown to be involved in the formation/progression of liver [5], intestinal [6] and ovarian cancer [7]. Now that links between inflammation and cancer are appreciated TNFα is viewed as a target for therapeutics of malignant disease[427]. Unfortunately TNFα was not amongst the panel of cytokines interrogated in the antibody array we used, so we could not confirm that the levels of TNFα decreases as a result of

LRP1B-mediated reduction of TACE. Nonetheless, on the basis of the established mechanisms of TACE, such effect is almost certain.

In addition to TNFα, ADAM 17 is involved in the shedding a number of several other biologically important membrane-bound ligands that have been shown to promote both cancer formation and progression [430, 431]. These ligands include transforming growth factor-α[432], amphiregulin [433], heparin-binding epidermal growth factor [434], and epiregulin [435]. In a three-dimensional culture of human breast cancer progression, inhibition of ADAM17 reversed the malignant phenotype as a result of failure to release TGFα and amphiregulin [436].

TREM1 encodes for a Triggering Receptor Expressed on Myeloid cells (TREM) which is involved in the amplification of inflammation [437]. The identity of its ligand remains unknown, yet, putative TREM-1 ligands have been reported in the lysates of necrotic cells and in the serum of patients with sepsis [438, 439]. Several members of the family of TREM receptors, namely TREM1, TREM2, and TLT1, have been shown to release soluble forms. Although the function of soluble TREM-1 is unknown, it has been detected in the biological fluids of patients and animals suffering from a variety of infections and diseases and often correlates with disease severity (for a comprehensive list see reference [440]).

Whether soluble TREM isoforms will function as ligands for other receptors or as competitive inhibitors remains unknown, nevertheless TREM1 serves to further highlight a role for LRP1B in modulating yet another inflammation-prone molecule.

Taken as a whole, considerable diverse consequences may take place as a result of LRP1B activity. An additional degree of complexity arises if we take into consideration that *in vivo*, Cytokines and Metalloproteinases are not exclusively secreted by cancer cells, but are mostly produced by other cell types in the tumor microenvironment. Further physiological implications come from regarding that these soluble factors can travel to extend their influence onto other cell types, located nearby or at a distance from the cells producing them.

D2.6 An emerging pattern - TACE as the "Godfather"?

At first glance the set of molecules most prominently changed trough LRP1B activity looks diverse, apparently disperse and deprived of any internal coherence. Nonetheless some common points may be found between them and at least two general "rules" seem to standout:

First, most of these soluble factors are initially synthesized as membrane-bound isoform precursors which turn from latent to the active extracellular form by an action commonly known as "sheddase activity", a proteolytic cleavage usually performed by a Metalloproteinase on the external face of the plasma membrane that leads to the release of the soluble factor. The same type of activity can be acknowledged to explain the release of "decoy receptors".

Second, members closely or more distantly related to the TNF or TNF receptor families seem to be overrepresented in this nonetheless small group of molecules.

These principles raise the hypothesis that the mechanism trough which most of the LRP1B-mediated changes occurred involves the initial action of a sheddase that targets members of TNF or TNF receptor family. In this case, the decreased amount of these soluble factors would not be the result of their direct endocytosis, but rather of an indirect action, by LRP1B-mediated endocytosis of a sheddase, followed by a consequent reduction in the levels of its substrates. In this perspective, and taking our results into consideration, TACE is the best candidate to be implicated in this pivotal position.
This speculation is based on the fact that TACE has affinity for TNFα and on data reporting that the release of the cytokines herein focused could be attributed to TACE, or a yet undescribed Metalloproteinase activity. For example, Neuregulin is synthesized as membrane-bound precursor (termed pro-NRG1) and TACE is one of the proteases implicated in the release of soluble Neuregulin [441]. In cells deficient of TACE activity, the release of pro-NRGa2c is blocked [441]. Furthermore, re-introduction of wild-type TACE in these cells confers regulated cleavability of pro- NRGa2c [441]. In addition, XEDAR, NrCAM, TREM1 all have been shown to require metalloproteinase activity for the release of their ectodomains. The extracellular NH_2 terminus of XEDAR protein was reported to be cleaved by a metalloproteinase and released into culture media [416]. NrCAM ectodomain was found to be shed from the cell surface, trough the activity of a Metalloproteinase [422]. Soluble TREM extracellular domains are cleaved off the cell surface by Metalloproteinases. Consistent with this, soluble TREM1 levels were decreased in the presence of metalloproteinase inhibitors [442]. Correspondingly, TREM1 surface levels were increased in these same cultures, demonstrating reduced cleavage of membrane-bound TREM1 in the presence of metalloproteinase inhibitors [442].

Taken together this provides strong argument to the hypothesis that most physiological change that results from LRP1B occurs trough indirect action over one or more Metalloproteinases, namely TACE, which aside from degrading the ECM, act as a regulator of the tumor microenvironment by relasing pro-inflammatory cytokines, liberating growth factors from their pro-forms, shedding antiapoptotic decoy receptors and “discharging” other cytokines with diverse physiologic roles [389].

By regulating TACE and other metalloproteinases, LRP1B emerges at the axis of highly orchestrated proteolytic cascades that constitute the “extracellular signaling pathways” of intercellular communications.

A final exciting consideration comes from the observations that TACE has been reported to act as a sheddase of LRP1B ectodomains[443], hypothesizing the existence of a balanced interdependent regulation between the two molecules. The existence of such mechanism requires demonstration.

D2.7 Does it run in the Family?

In the context of this discussion, endocytosis is put into relevance in the tumorigenic process because it potentially modulates the coating of membrane bound receptors and the composition of soluble factors around the cell which will ultimately be responsible for modulating its local and distant microenvironments. This predicts that other endocytic molecules in general, and other lipoprotein receptors in particular, might also take a role in cancer. The closest related member to LRP1B in the lipoprotein receptor Family is LRP1, with similarity of 59 and 52% identity at the cDNA and predicted amino acid levels, respectively [234]. However, the effects on cell invasion and MMP catabolism are contradictory seem to be dependent on the cell context. When mouse embryonic fibroblasts are grown in the presence of the 39-kDa receptor-associated protein (RAP), a natural antagonist of ligand binding to LRP1, the amount of MMP-9 in conditioned cell culture medium is significantly increased [444]. RAP also increased MMP2 levels in dermal mice fibroblasts [445]. In HT1080 fibrosarcoma cells, LRP1 mediates clearance of a pro-MMP-2/TIMP-2 complex, since addition of RAP inhibited endocytosis and lysosomal degradation of ^{125}I-pro-MMP-2/TIMP-2 [446]. According with these observations, the expression of LRP1 is diminished in stage IV and anaplastic Wilms tumors and correlated with increased levels of several known extracellular ligands that LRP1 usually recycles from the extracellular environment, including PAI-1, MMP-9, and TIMP-1 [447] and decreased expression of LRP1 was observed in invasive cell clones derived from human prostate and breast tumor cells [448]. However, in contrast with abovementioned studies, Dedieu and colleagues report that despite increased pericellular proteolytic activity, LRP-1 silencing prevents malignant cell invasion [449]. Song et al report that LRP1 promotes cancer cell migration and invasion of human glioblastoma U87 cells by inducing the expression of MMP2 and 9 [450]. Although some functions of LRP1B might be inferred for LRP1 because of aminoacid sequence homology, the abovementioned paradoxal reports demand for specific studies addressing the effects of each LRP on cell invasion and proteinase catabolism.

D2.8 Other accomplices to watch out for

It has been shown that LRP1B undergoes regulated intra-membrane proteolysis in a gamma-secretase-dependent manner, releasing an intracellular domain (ICD) that then translocates into the nucleus. The ICD alone suppresses anchorage-independent growth of LRP1B-deficient neuroglioma cells (H4 cells) [314], suggesting that additional tumor-suppressor functions may be mediated trough intracellular functions of LRP1B.

Aside from structural alterations we show transcriptional silencing of LRP1B due to DNA methylation, disrupting a previously undisclosed functional p300 binding site at intron 1 of LRP1B. The EP300 gene encodes p300, a histone-acetyltransferase that regulates transcription via chromatin remodeling and is important in the processes of cell proliferation and differentiation [451]. A role for p300 in cancer has been previously implied by the fact that it is targeted by viral oncoproteins [452], it is fused to MLL in leukemia [453], and mutations in EP300 were described in epithelial malignancies with inactivation of the second

allele [451, 454]. Our results are of significance since they place LRP1B in the p300 suppressor pathway.

In this project we have noticed that genomic gain at 8q22.3 goes together with miR-548a-5p over-expression which, in turn, directly targets LRP1B for downregulation resulting in increased invasive capacity. Chromosome 8q22 amplification has been repeatedly associated with poor patient outcome in other cancer models, particularly in breast cancer [289-291]. It remains to be seen whether poor patient outcome is due to the workings of this newly identified circuitry.

Concluding remarks

Taken as a whole, this work, undertaken in the context of familial and sporadic thyroid cancer, emphasizes the message that cancer cells harbor mutations and/or epimutations that not only alter their intracellular signaling pathways - as exemplified by RET mutations in MTC - but also subvert the extra-cellular communications regulating the tumor environment - as highlighted by our studies on LRP1B in NMTC. In this reasoning, intracellular phosphorylation and extracellular proteolysis might be thought-off like analogous circuitries of transmitting physiological message in the intracellular and extracellular milieus, respectively.

Due to their high genetic instability and potential for evolution, malignant cells are “moving targets” that can become resistant to even the most sophisticated targeted drugs, as appreciated in clinical trials employing Tyrosine Kinase Inhibitors. Using a combination of therapies that attack both the malignant cells and the “other half “of the tumor (that is, the accompanying cells in the microenvironment), which is otherwise deprived of the same degree of genetic instability and potential to acquire resistance, could therefore prove to be more effective in eliciting long-lasting therapeutic effects.

Early observations provide strong evidence that “normalization” of the environment is a valid therapeutic strategy (section D2.4.). Our findings that LRP1B-mediated targets cover multiple hallmarks of cancer (see section D2.5.) hold promise to use LRP1B activity as a tool for a therapeutic approach that is not centered on the cancer cell itself, but rather in its environment; aiming to control the malignant behavior of the cancer cell by modulating the composition of the environment surrounding it. Outstanding factors have emerged as direct or indirect targets of LRP1B physiology (section D2.6.). A future challenge will be to use the newly identified LRP1B actions and (de)regulatory mechanisms to design the strategy for such therapeutic intervention.

Material and Methods

Project 1

MEN2/FMTC families and sporadic MTC subjects

We analyzed a series of 6 MEN2 families (5 with MEN2A and 1 MEN2B) as well as 58 AS-MTC patients originating from the Central Region of Portugal. This series is partially reported in original paper 1 [455], nevertheless for the writing of this thesis it was increased and updated.

Population controls

A population of 70 regional controls, consisting of a random series of patients admitted to our institution for conditions unrelated to endocrine pathology, was used for the determination of the population frequency of RET variants.

Ethical approval

This work was approved by the Ethical Committee of the Regional Centre of Oncology of Coimbra of the Portuguese Institute of Oncology. Peripheral blood samples were obtained with informed consent in the setting of genetic counselling.

RET germline Mutation Screening

RET mutation screening was performed in DNA obtained from peripheral blood leucocytes, by PCR amplification and direct Sanger sequencing of exons 8, 10, 11, and 13-16.

Site-directed mutagenesis

A pRcCMV vector expressing RET isoform 51 (i51) was mutated in order to generate the Glu511Lys, Ser649Leu and Arg886Trp variants. Site-directed mutagenesis was performed using a PCR approach employing primers with mismatches for the intended nucleotide change. All plasmids were re-sequenced to confirm that the desired mutation was introduced without changes to the vector backbone.

Cell lines and transfections

HEK293 and NIH3T3 cell lines were maintained in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS), 100ug/ml streptomycin and 100U/ml Penicillin (PENST), in a humified atmosphere with 5% CO2 at 37ºC. For transient transfections, NIH3T3 cell were transfected by using the Lipofectamin Reagent (Invitrogen) according to supplier instructions. HEK293 were transfected by using the Calcium Phosphate method.

NIH3T3 focus formation assays

For focus formation assays, NIH3T3 cells were transfected with 1ug of expression vector in 6-well plates. 3 days after transfection, 1x105 cells were plated in 100mm plates and grown in DMEM, 5% FBS, 1%PENST. In parallel, the same number of cells were put in selection medium (DMEM, 5% FBS, 1%PENST, 400ug/µl G418). Medium was renewed every 3 days and cells were allowed to grow for a total time of three weeks. After this period plates were stained with Cristal Violet and foci were counted. Transfection efficiency was assessed by counting the number of stable clones

arising in the selection plates. Results were expressed as the number of foci normalized to the corresponding transfection efficiency.

Soft-agar assays

For soft agar assays, we prepared plates containing a bottom layer of 0,7% agar in DEMEM, 10% FBS, 1%PENST. NIH3T3 cells were transfected with 1ug of expression vector and after 1 day, 5000 cells were diluted in 2X DMEM, 20% FBS, 2% PENSTP, mixed with equal volume of 1% agar at 40ºC and plated on the agar plates. After 2-3 weeks wells were stained with Cristal Violet and colonies were counted. 10 power fields per well were photographed and images were counted using the colony counting tool of the Quantity One software (BioRad).

Luciferase reporter assays

In order to measure the degree of activation of intracellular signaling pathways induced by the different RET mutants, we performed luciferase assays. These experiments employed reporter constructs containing the firefly luciferase encoding gene under the control of DNA motifs recognized by STAT1 (GAS-LUC), STAT3 (Interleukin Response Element-LUC) and TCF4 (LEF/TCF Response Element-LUC) (Figure 1A). We further employed a system which reports the activation of ERK1/2 in Trans. In this system, we cotransfected a plasmid encoding the GAL4 DNA binding domain fused to the ELK transactivation domain along with a vector containing the Upstream Activation Sequence (recognized by GAL4) and Luciferase. In this system, ERK1/2 phosphorylation will result in ELK activation and increase luciferase activity (Figure 1B). In reporter assays, DNA mixtures consisted of 500ng expression plasmid, 1 ug of the reporter plasmid, 1ug of PDM2-LacZ (expressing B-Galactosidase) and pUC18 to complete 5ug total DNA. Cells were incubated with calcium phosphate-DNA complexes for 24 hrs, and cultured in fresh medium for additional 24 hrs. Cells were harvested, lysed in 150ul of Reporter Lysis Buffer and Luciferase and B-Galactosidase assays were performed. Fifteen ul of lysates were mixed with 50ul of SteadyLite HTS substrate and luciferase activity was measured in a luminometer. For the B-galatosidase assay, 50ul of lysate were mixed with 250ul of B-galactosidase buffer (200mM NaPO4, 2mM MgCl2, 1.33mg/ml ortho-nitrophenyl –B-D-galactopyranoside, 100mM B-mecaptoethanol), and measured in a colorimetric reaction at 405 nm. The Luciferase activity was normalized with the corresponding B-Galactosidase activity (measures transfection efficiency). All experiments were performed at least two times in triplicates.

Sensitivity of RET mutants to Sorafenib

In order to assess the sensitivity of RET mutants to the Tyrosine-Kinase Inhibitor (TKI) Sorafenib, we evaluated the degree of suppression of RET intracellular signaling by performing the above mentioned ERK1/2 luciferase reporter assays in the presence of Sorafenib. Twenty four hours after transfection of the reporter vectors, cells were incubated with fresh medium containing a final concentration of 4uM of Sorafenib for additional 48 hours, after which B-Galactosidase and Luciferase activity were measured.

Project 2

FNMTC families

We have studied matched blood and tumor tissue from cases belonging to nine two-case familial clusters of NMTC. The family histories and clinical-pathological features, namely age at diagnosis and histopathology are summarized in Table 1. FNMTC was defined according to the criteria proposed in a meta-review by Musholt et al [21] (see section I3.2.4). In the herein presented series, three families fulfilled these criteria (families B, H and I). The other six families (A, C, D, E, F and G) only matched one primary and two secondary criteria (see Table 5). In two of these families (C and D) the age at presentation (35 and 34 years old, respectively) is borderline with that proposed by the age criterion (<33 years old) and if we don't consider this difference significant, they would otherwise fulfil the proposed criteria. For the remaining four families (A, E, F and G), we have decided to include these families in the study since, despite not satisfying all the criteria (only 1 primary and 2 secondary criteria), they manifested other characteristics that, in our view, might also be suggestive of a familial predisposition when occurring in the setting of a small familial cluster of NMTC. These include: 1) unusual presentations of sporadic PTCs, including occurrence in men (family F); 2) PTC with cell oxyphilia (Family A); 3) concurrence of PTC and FTC in the same patient (Family E); and 4) PTC of the Follicular Variant (FV) (families F and G). Given the suspicious presentations of the latter families, we considered them as presenting incomplete criteria. As Musholt *et al.*[208] wrote: "... especially small families with evidence of hereditary susceptibility of Familial Papillary Thyroid Cancer/Multinodular Goiter not meeting all necessary criteria should not be excluded from further clinical and molecular investigations.". Matched blood and formalin-fixed paraffin-embedded (FFPE) or fresh tumor tissue were available in fourteen cases (Table 5).

Loss Of Heterozygosity analysis

Genomic DNA from peripheral blood leukocytes (PBL) was isolated by standard SDS/Proteinase K digestion, followed by phenol/chlorophorm extraction and ethanol precipitation. FFPE tissue samples were used to extract and purify tumor DNA, except for tumors 18 and 20 (T18 and T20) for which fresh tissue was available. In order to reduce contamination with normal cells, the tumor areas of interest (ranging from 0,5 to 1 cm^2) were carefully marked and six to twelve 5μm thick sections were microdissected. DNA extraction from FFPE tissues was performed by soaking the tissue directly in 500μl of a digestion buffer containing 0.5% SDS and 0.5μg/μl Proteinase K, followed by an overnight incubation at 56ºC. After digestion, samples were briefly centrifuged, in order to separate the overlaying layer of melted paraffin from the solution, and the undigested material. The crude digest, underlying the hard paraffin layer, was then recovered and further purified by a phenol/chloroform extraction, and quantified (between 1 and 2 μg of DNA were obtained from each case). Ten microsatellite markers and one SNP marker at the 19p13.2 region and fourteen microsatellite markers at 2q21, were analysed (Table 2). For PCR amplification of the microsatellite markers, primer sequences were obtained from the human genome database (www.gdb.org) and one nucleotide of each primer pair was labelled with 6-carboxilfluorescein (6-FAM) at the 5' end. Amplifications were carried out in a final volume of 25μl, in 1X PCR Buffer with 1,5mM of $MgCl_2$, using 2 units of DNA polymerase (BioTools, Inc) and 50ng of template DNA. Thermal cycling was performed in a Perkin-Elmer 9600 and started with an initial denaturation step at 94ºC, for 3 minutes, followed by 30-45 cycles of denaturation, at 94ºC, for 20 seconds,

annealing, at 55-60ºC (primer dependent), for 30 seconds, extension at 72ºC, for 45 seconds, and completed by a final extension step, at 72ºC, for 10 minutes. The amplified product was electrophoresed in a 2% agarose gel to confirm amplification and product size range. Fragment size analysis was performed using an ABI PRISM® 3700 Genetic Analyzer. Data collected during the runs was analysed by using dedicated software (PeakScanner), which provided allele size and quantitative information about allele peaks, namely peak height and peak area. For LOH analysis, the normal and tumor alleles were aligned and directly compared. LOH was assigned when there was loss or a clear reduction of the tumor allele peaks, as compared with PBL DNA. We used the quantitative data on height and area of normal and tumor allele peaks. LOH was defined according to the following formula: LOH index = (T2 x N1)/(T1 x N2), where T was the tumor sample, N was the normal blood sample, 1 and 2 were the peak areas of smaller and larger alleles, respectively. LOH was defined with the values of LOH index < 0.6 or > 1.67. All LOH positive *loci* and some additional markers with questionable result were re-analysed, at least once, by a repeated PCR for confirmation. One SNP marker SNPrs4147651 was also used. This SNP is constituted by two alleles, C or T, and was genotyped by single strand conformation polymorphism (SSCP)-DNA sequencing. Briefly, PCR amplification was performed with primers flanking the SNP (F: 5'gtgggctccctcagatcttt3'; R: 5'ccaaggaggcaaagtagtcg3'), using PCR conditions and a thermal profile similar to that mentioned above. SSCP was performed by denaturing samples in formamide buffer (1:1) and running a vertical electrophoresis, in MDE (Mutation Detection Enhancement) gel (Cambrex Bio Science Rockland, Inc), at 8ºC. Results were visualized by silver staining. LOH was assigned when there was loss of gel bands corresponding to either allele in the tumor, as compared with the normal DNA. Results were further confirmed by automatic DNA sequencing, using the forward PCR primer mentioned above.

Testing series of Sporadic NMTCs

The testing series consisted of 71 formalin-fixed, paraffin-embedded (FFPE) tissue samples, comprising 20 Follicular Thyroid Adenomas (FTAs), 17 Follicular Thyroid Carcinomas (FTCs), 24 Papillary Thyroid Carcinomas (PTCs) and 10 Undifferentiated Thyroid Carcinomas (UTCs). We also studied 9 samples of normal thyroid tissue dissected from the contra-lateral lobe of specimens obtained from total thyroidectomies.

Validation seriesof Sporadic NMTCs

A validation set of cases, gathered by an independent group, consisted of 30 thyroid lesions (12 FTAs and 18 FTCs) and 12 specimens of normal thyroid tissue which were independently assessed for LRP1B expression by analyzing the data concerning the LRP1B probesets represented in the GeneChip® Human Exon 1.0 ST Array (Affymetrix). Thyroid samples were collected immediately after surgical resection, snapped frozen, and stored at –80°C. All samples were visually inspected on 5-µm H&E–stained frozen sections by the pathologist (J.C-T.). Cryostat sections were disrupted by using a Polytron homogeneizer and total RNA was isolated from cryostat sections using RNeasy Mini Kit (Qiagen) following the manufacterer's instructions. RNA quality was assessed on a Bioanalyser (Agilent) and samples with RIN (RNA integrity number) >7.0 were used for array experiments. One µg of total RNA was used to rRNA reduction, synthesis, fragmentation and labeling following the standard Affymetrix Whole-Transcript Sense Target–Labeling Assay protocol. Each thyroid sample was hybridized with an Affymetrix Human Exon 1.0 ST microarray.

Background correction, normalization, probe summarization and data analysis was done with Partek Genomics Suite software using RMA algorithm.

Cell lines and transfections

We used 10 cell lines HTH74, C643, NPA, K1, BCPAP, TPC-1, 8505C, KAT4, ARO, XTC-1 otherwise reported to be derived from thyroid cancer. However, during the course of this work, Schweppe *et al, performed* STR and SNP analysis of several thyroid cancer cell lines and suggested that cell lines ARO and KAT4 may be derivates of the HT-29 colon cancer cell line. Also NPA was suggested to be a derivative of the M14/MDA-MB-435S melanoma cell line [456]. The formerly mentioned 10 cell lines were maintained in RPMI medium, supplemented with 10% Fetal Bovine Serum (FBS), 100ug/ml streptomycin and 100U/ml Penicillin (PENST), in an humified atmosphere, with 5% CO2, at 37ºC. The cell line HEK293 was maintained in DMEM medium with 10% FBS and 100ug/ml PENST. HEK293 cells, used primarily for reporter assays, were transfected by the Calcium Phosphate method. Transfection of the remaining cell lines was achieved using Lipofectamin Reagent (Invitrogen).

Microarray global gene expression analysis

Tissue samples collected immediately after surgical resection were directly put into TRIZOL (Ambion) and immediately homogenized. RNA was extracted according to the TRIZOL protocol and further clean-up was achieved by passing RNA through an RNeasy collumn (Quiagen). Complementary RNA (cRNA) was synthesized by reverse transcriptase-PCR using an oligo dT primer with a T7 promoter overhang followed by *in vitro* transcription. cRNA was labelled by incorporating biotinylated nucleotides. The cRNA target was fragmented and hybridised to the HG U133 Plus 2.0 genechip (Affymetrix). After staining and image acquisition the signal intensity data was normalized by the invariant probeset method and the expression values were computed using the dChip software.

mRNA expression

RNA was obtained from macrodissected 4x20um tissue sections with the RecoverAll RNA isolation Kit (Ambion). RNA from cell lines was purified by the TRIZOL protocol (Ambion). After cDNA synthesis employing MMLV Reverse Transcriptase (Applied Biosystems) TaqMan Gene Expression assay hs-00218582_m1 was used to measure LRP1B expression, MMP2 was assayed by TaqMan hs00234422_m1 in quantitative Real Time-PCR (qRT-PCR) experiments. A normal reference was produced by pooling RNAs from 9 samples of normal thyroid tissue, treated in the same way as the tumor samples. Data was analyzed by the absolute quantification method.

LRP1B Mutation analysis

We designed 91 sets of PCR primers which result in the amplification of flanking intronic boundaries and coding sequences of the 91 exons of LRP1B. We also used a set of 11 exonic primer pairs in order to amplify cDNA fragments ranging from 1-1,4Kb encompassing several exons. PCR products were directly sequenced. Primer sequences are available upon request.

Copy number analysis

We used TaqMan Copy number assay (Applied Biosystems) in order to estimate the copy number at several genomic locations within LRP1B, namely at exons 5 (assay hs-02812600_cn), 44 (assay

hs-02217633_cn) and 90 (assay hs-05832428_cn), and also near the centromeric region of chromosome 2q (assay hs-04631472_cn). A similar strategy was used to determine copy number at the genomic location of miRNA-548a-3 (assays hs-05036789_cn and hs-06224496_cn) and at proximity of the 8q centromere (assay hs-06217400_cn). Multiplex quantitative real-time genomic PCR reactions, containing VIC-TaqMan-primer/probes for an internal reference gene (RNAse P) and FAM-labelled TaqMan-primer/probes for the interrogated location were performed in triplicates. Results were analyzed with the CopyCaller software. DNA copy number was calibrated by using a blood sample run in the same plate.

DNA Methylation analysis

Genomic DNA was converted by bissulfite treatment using the Epitect modification Kit (Qiagen) and subsequently subjected to PCR amplification of 2 regions of the promoter: Fragment 1 (-131 to +100, from ATG) and Fragment 2 (+217 to +365). The bissulfite PCR products were directly sequenced.

Constructs and expression vectors

5'CpG island reporter constructs

Two fragments of the LRP1B CpG island, obtained by PCR amplification of nucleotides –429 to –1 from the ATG (Fragment A) and of nucleotides +1 to + 530 (Fragment B), were ligated into the pTAL-LUC vector (ClonTech), encoding the firefly Luciferase gene under a basic TATA-Like promoter region of the HSV Thymidine Kinase promoter.

In vitro methylation using Bacterial Methyltransferases

Bacterial Methyl-Transferases with distinct specificities M.Sss-I, M.Hpa-II and M.Msp-I (methylating at *CG, C*CGG and *CCGG, respectively) were used to methylate the CpG island constructs *in vitro.* Methylation reactions were set-up by incubating 2ug of plasmid DNA with 10 units of Methyltransferase, in the presence of S-adenosylmethionine (SAM), at 37ºC, overnight. After *in vitro* methylation, reporters were characterized by bissulfite sequencing.

3'UTR reporter constructs

We inserted a 645-bp sequence of the 3' untranslated region (3'UTR) of LRP1B containing the miRNA-548a-3 target site downstream of the firefly luciferase reporter in the pMIR-Report vector (Ambion). By PCR, we generated both wild-type and a mutant version of the 3'UTR, in which two nucleotides from the target sequence complementary to the seed region of miRNA-548a-3 were mutated.

Expression vectors

A pCDNA3.0 vector expressing a LRP1B minireceptor (mLRP1B) comprised of the entire cytoplasmatic tail, the trans-membrane region and extracellular sub-domain IV, which mimics the function and trafficking of LRP1B, was kindly provided by Dr Guojun Bu. An expression vector encoding the pre-microRNA-548a-5p (pEP-mir-548a-3) was purchased from Cell Biolabs, Inc. A pCMV-beta vector expressing the p300 Histone-Acetyltransferase was obtained from Dr William Sellers, trough Addgene (Addgene plasmid 10718).

Reporter Assays

DNA mixtures consisted of 0,5ug expression plasmid, 0,5 ug of the reporter plasmid, 0,5ug of PDM2-LacZ (expressing B-Galactosidase) and pUC18 to complete 5ug total DNA. Cells were incubated with calcium phosphate-DNA complexes for 24 hours, and cultured in fresh medium for additional 24-48 hours. Cells were harvested and lysed in 150ul of Reporter Lysis Buffer and Luciferase and B-Galactosidase assays were performed. The Luciferase activity was normalized with the corresponding B-Galactosidase activity (measures transfection efficiency). All conditions were performed in triplicates and experiments were performed 3 times.

Proximity Ligation Assay

Analysis of the interaction between DNA and p300 was performed by a proximity ligation assay as described by Sigurn Gustafsdottir and colleagues [259]. Briefly, a 30nM of a biotinylated anti-human p300 antibody (R&D Systems) in PBS / 1% BSA was coupled in a 1:1 ratio with 5'streptavidin-oligonucleotide conjugates (5'STV), kindly provided by Gustafsdottir S., in a volume of 5ul for 1 h at room temperature. The sequence of the 5'STV (5'-P-TCGTGTCTAAAGTCCGTTACCTTGATTCCCCTAACCCTCTTGAAAAATTCGGCATCGGTGA-3') contains a spacer sequence, primer1 target site and 12 nucleotides complementary to half of the connector oligonucleotide. HPLC-purified DNA probes were purchased from Eurofins MWG Operon Inc. (Germany). Each probe comprised a common sequence (CATCGCCCTTGGACTACGACTGACGAACCGCTTTGCCTGACTGATCGCTAAATCGTG) composed of the primer2 target site, the sequence tag, the real-time PCR tag and 12 nucleotides complementary to one half of the connector oligonucleotide. The variable region of the probe was designed to have 24 nucleotides encompassing the predicted p300 binding site at LRP1B intron 1 in the wild-type (CCCCTCCGGGAGTGTGTGCACTTG) and mutated (CCCCTCCGGGACAGTGTGCACTTG) configurations, as well as with methylated sites (CCCCTC*CGGGAGTGTGTGCACTTG and CCCCT*CCGGGAGTGTGTGCACTTG, where the " *C " represents the sites where a 5 methyl Cytosine modification was introduced). The variable part of the probes was made double-stranded by hybridizing an equimolar amount of a 24-mer oligonucleotide complementary to the variable region in 0.3x standard saline citrate (0.05 M sodium chloride/0.005 M sodium citrate, pH 7). The methylation sites were placed in symmetrical positions in the respective complementary oligonucleotide. A nuclear lysate was prepared from HEK293 cells as described by Wadman, et al [457]. Proximity ligation reactions were set-up in triplicates in final volume of 3ul by combining 1 ul of a 1:500 dilution of nuclear extract with 1ul of DNA probes (0,5nM) in a p300 biding buffer described by Rikitake, et al.[258] (20mM TrisHCl, pH7.4, 10% Glycerol, 50mM KCl, 0.1mM DTT, 0.1mM Protease Inhibitors) for 30 min at room termperature. Afterwards 2ul of p300 antibody-oligonicleotide conjugate probes (0,4nM) was added and the mixture was incubated for 4 hours at 4ºC. After incubations, 45ul of a combined mixture for ligation and amplification was added in optical PCR plates [final concentrations: 50 mM KCl,20mMTris-HCl (pH 8.4), 3.15mM MgCl2, 0.4 Weiss units of T4 DNA Ligase, 400 nMconnector oligonucleotide (TACTTAGACACGACACGATTTAGTTT), 80 uM ATP, 200uM dNTP mixed with dUTP, 100 nM primers (primer 1: CATCGCCCTTGGACTACGA; primer 2: GGGAATCAAGGTAACGGACTTTAG), 100 nM *Taq*Man probe (FAM-TGACGAACCGCTTTGCCTGA-MGBNFQ; Applied Biosystems), and 1.5units of Platinum *Taq*DNA polymerase (Invitrogen)]. The reactions were run in a real-time PCR instrument (Applied Biosystems 7000 sequence detection system) in the following thermal cycling conditions: initial activation step of 2min at 95°C, 45 cycles of 15s denaturation at 95°C and

60s annealing/extension at 60°C. The results are expressed in mean values of the Signal/Noise Ratio, where the number of ligations in the sample is divided by the number of ligations in the negative control set-up in the absence of antibody.

Treatment of Cells with 5-Aza-2'-Deoxycytidine (5AZA) and Tricostatin A (TSA)

$1x10^5$ cells per well were seeded, in triplicates, in 6well-chambers. The next day, cells were cultured in medium containing either regular medium, or medium supplemented with DMSO solvent or 5uM 5AZA (Sigma). Medium corresponding to each formulation was renewed daily for 4 days, after which nucleic acids were extracted. Treatment with TSA alone was performed by adding medium with 100nM TSA for 16 hours. For the combination of 5AZA with TSA, 100nM TSA (Sigma) was added at the 4th day of 5AZA treatment and kept for 16 hours, after which the experiment was stopped.

Quantification of microRNAs

MiRNAs we quantified using TaqMan Micro-RNA Assays (Applied Biosystems). Starting from 10ng of total RNA, primer-specific reverse transcription was performed and 2ul of cDNA was used in qRT-PCR to quantify each micro-RNA. Expression values were normalized to the levels of miRNA-U6b determined in the same RNA samples. A pool of 9 RNAs derived from normal thyroid tissue was used as the normal reference. Data was analyzed using the delta-delta-Ct method.

Soft-Agar assays

For soft agar assays, we prepared plates containing a bottom layer of 0,7% agar in DMEM, 10% FBS, 1%PENST and 5000 cells were diluted in 2X DMEM, 20% FBS, 2% PENSTP, mixed with equal volume of 1% agar at 40ºC and plated on the agar plates. After 2-3 weeks, wells were stained with Cristal Violet and colonies were photographed and counted.

Chicken embryo in vivo growth assay

The chicken embryo chorioallantoic membrane (CAM) model has been used to assay tumorigenicity as previous described with some modifications [458]. Fertilized chick (*Gallus gallus*) eggs (seven per group) obtained from commercial sources were incubated horizontally at 37.8°C in a humidified atmosphere and referred to embryonic day (E). On E3 a square window was opened in the shell after removal of 1.5-2ml of albumen to allow detachment of the developing CAM. The window was sealed with a transparent adhesive tape and the eggs returned to the incubator. At E10, a nylon ring with 5mm diameter was placed on top of the growing CAM under sterile conditions for adding the cells and medium (control). Cells ($2x10^6$ per embryo) were re-suspended in 25µl of complete medium and inoculated into the ring. Tumors were allowed to grow for 6 days at which time the embryos were euthanized by hypothermia. The resulting tumors were photographed under a stereoscope (Olympus, SZX16 coupled with a DP71 camera) at 7x magnification. *Ex ovo* images were used to determine the areas of the tumors using the image software ImageJ (Image processing and analysis in Java; NIH). Angiogenesis was assessed by counting the number of blood vessels (less than 15µm diammeter) developing radially towards the tumors. For statistical analysis the Wilcoxon Rank Sum Test was used to calculate significance.

In vitro Matrigel invasion assays

Matrigel Invasion Chambers (BD Biosciences) were used to assess the in vitro metastatic potential according to reported procedures [459]. Matrigel Invasion Chambers (BD Biosciences) were used to assess the in vitro metastatic potential of 8505C ATC cell line upon restoration of LRP1B. Briefly, parental cells were transfected with either 1ug of mLRP1B expression vector or empty vector. After a 15 day selection period, stable clones were pooled and $2{,}5x10^4$cells were plated in 24-well plates with filter inserts containing an 8 micron pore size PET membrane, coated with a thin layer of Matrigel Basement Membrane Matrix which occludes the pores of the membrane, blocking non-invasive cells from migrating and serving as a reconstituted basement membrane *in vitro*. After 24 hours, noninvasive cells on the upper surface of the filter were wiped out with a cotton swab, while the invasive cells that were able to detach themselves from and invade through the Matrigel matrix into the lower surface of the filter were fixed with methanol and stained with DAPI. Invasiveness was determined by counting the number of invasive cells in the entire membrane under the microscope. The experiment was performed three times.

Gelatin zymography

The level of gelatinases was determined as described by others [460]. The level of proteinases with gelatinolytic capacity, namely Matrix Metalloprateinase (MMP) -2 and 9, was determined in conditioned media of 8505C stable cells selected with either empty vector or mLRP1B expression vector. We plated $2{,}5x10^5$ cells onto 6-well plates previously coated with 20ug/ml Fibronectin. The next day cells were put in 1ml serum free media and left for another 24 hours. Proteins in 20ul of conditioned medium were separated by electrophoresis under non reducing conditions in a 10% polyacrilamide gel containing 1mg/ml gelatin. After electrophoresis, the gels were washed twice for 30 minutes with Triton X-100 and incubated with MMP activation buffer (Tris 50mM, 10mM CaCl2) for 24 hours at 37ºC to allow proteolysis. Gels were stained with Coomassie blue.

Antibody Cytokine arrays

A membrane-based antibody array purchased from RayBiotech containing spotted antibodies specific fror 51 Cytokines was incubated with conditioned media diluted 1:3 according to manufacturer instructions.

Statistical analyses

Differences between groups were tested by the Mann-Whitney test. Correlations were assessed by the Pearson coefficient.

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Supplementary Materials

Supplementary Materials Table 1 Genes transcriptionally de-regulated at 2q21 in tumor T1 of the index FNMTC case

Probeset	Gene_assignment	Accession	Fold Change
1554303_at	histamine N-methyltransferase	AF523356	**1.59**
224121_x_at	pleckstrin homology domain containing, family B (evectins) member 2	BC001428	**-1.20**
219770_at	glycosyltransferase-like domain containing 1	NM_024659	**1.06**
202947_s_at	glycophorin C (Gerbich blood group)	NM_002101	**-1.95**
219643_at	**low density lipoprotein-related protein 1B (deleted in tumors)**	**NM_018557**	**-12.15**
218870_at	Rho GTPase activating protein 15	NM_018460	**-1.42**
220977_x_at	erythrocyte membrane protein band 4.1 like 5	NM_020909	**1.26**
220765_s_at	LIM and senescent cell antigen-like domains 2	NM_017980	**-1.28**
202428_x_at	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	NM_020548	**1.30**
220367_s_at	mSin3A-associated protein 130	NM_024545	**-1.09**
206720_at	mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase	NM_002410	**1.09**
204385_at	kynureninase (L-kynurenine hydrolase)	NM_003937	**-1.20**
202931_x_at	bridging integrator 1	NM_004305	**-1.81**
212411_at	IMP4, U3 small nucleolar ribonucleoprotein, homolog (yeast)	BE747342	**1.18**
1558041_a_at	yeast Sps1/Ste20-related kinase 4 (S. cerevisiae)	AL834156	**1.60**
208600_s_at	G protein-coupled receptor 39	NM_001508	**1.50**
205171_at	protein tyrosine phosphatase, non-receptor type 4 (megakaryocyte)	NM_002830	**-1.36**
1559355_at	neurexophilin 2	CA336449	**-1.48**
212909_at	LY6/PLAUR domain containing 1	AL567376	**1.19**
218851_s_at	WD repeat domain 33	NM_018383	**1.65**
210926_at	ANKRD26-like family C, member 1A	AY014272	**-1.18**
1568868_at	cytochrome P450, family 27, subfamily C, polypeptide	BC039307	**1.94**
1553013_at	contactin associated protein-like 5	NM_130773	**-1.35**
204640_s_at	speckle-type POZ protein	NM_003563	**1.12**
201504_s_at	translin	AI435302	**2.31**
224775_at	IWS1 homolog (S. cerevisiae)	AW451291	**1.42**
212995_x_at	family with sequence similarity 128, member A	BG255188	**-1.28**
216660_at	myosin VIIB	AK000145	**1.13**
206259_at	protein C (inactivator of coagulation factors Va and VIIIa)	NM_000312	**-1.38**
212008_at	UBX domain containing 2	N29889	**2.06**
204645_at	cyclin T2	NM_001241	**1.24**
239538_at	zinc finger, RAN-binding domain containing 3	BG548811	**-2.01**
206611_at	chromosome 2 open reading frame 27	NM_013310	**-1.55**
210406_s_at	RAB6A, member RAS oncogene family /RAB6C, member RAS oncogene family	AL136727	**-1.09**
230602_at	aminocarboxymuconate semialdehyde decarboxylase	AW025340	**-2.48**
201623_s_at	aspartyl-tRNA synthetase	BC000629	**-1.32**
203664_s_at	polymerase (RNA) II (DNA directed) polypeptide D	NM_004805	**-1.46**
212995_x_at	family with sequence similarity 128, member B	BG255188	**-1.28**
201930_at	MCM6 minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	NM_005915	**1.34**
202100_at	v-ral simian leukemia viral oncogene homolog B (ras related; GTP binding protein)	BG169673	**1.39**
202115_s_at	DKFZP564C186 protein	NM_015658	**1.37**
223297_at	AMME chromosomal region gene 1-like	BC004208	**-1.05**
221695_s_at	mitogen-activated protein kinase kinase kinase 2	AF239798	**2.42**
224946_s_at	coiled-coil domain containing 115	AL571677	**-1.16**
207856_s_at	phingomyelin phosphodiesterase 4, neutral membrane	NM_017951	**1.24**

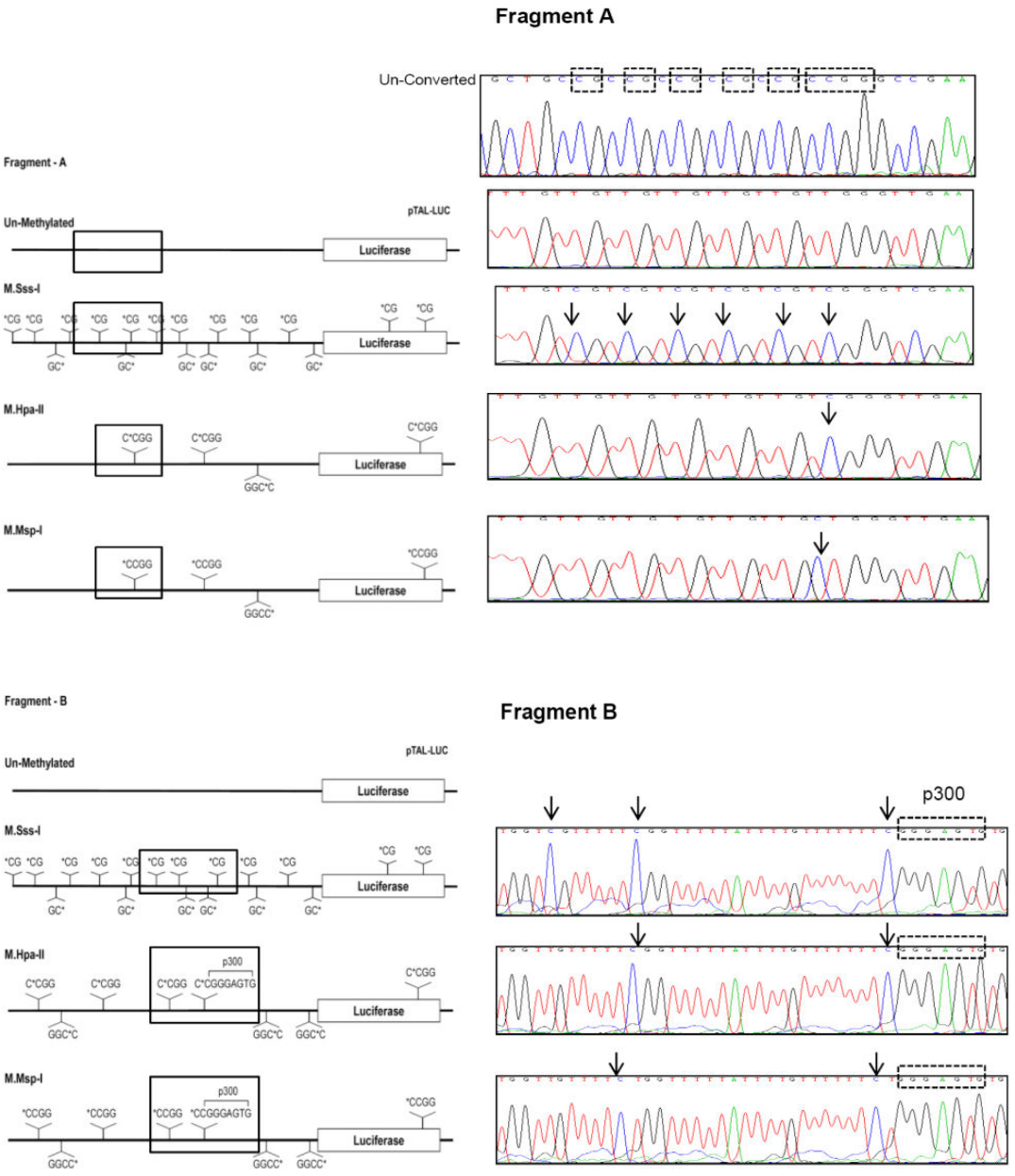
213521_at	Protein tyrosine phosphatase, non-receptor type 18 (brain-derived)	AW575379	**-1.06**
201045_s_at	RAB6A, member RAS oncogene family	BF513857	**1.41**
236638_at	amily with sequence similarity 123C	AI279217	**-1.68**
212752_at	cytoplasmic linker associated protein 1	AA176798	**1.39**
226639_at	SFT2 domain containing 3	AI304320	**-1.17**
224713_at	MKI67 (FHA domain) interacting nucleolar phosphoprotein	AL577809	**1.20**
223503_at	hypothetical protein DKFZp566N034	AF255647	**1.41**
209201_x_at	chemokine (C-X-C motif) receptor 4	L01639	**1.19**
206190_at	G protein-coupled receptor 17	NM_005291	**-1.81**
216912_at	Rho guanine nucleotide exchange factor (GEF) 4	AB029035	**1.15**
216323_x_at	alpha-tubulin isotype H2-alpha	K03460	**-1.09**
202754_at	R3H domain (binds single-stranded nucleic acids) containing	NM_015361	**-1.41**
200639_s_at	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	NM_003406	**1.21**
223753_s_at	cripto, FRL-1, cryptic family 1	AF312769	**1.69**
202176_at	excision repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing)	NM_000122	**1.82**
229946_at	similar to CDNA sequence BC043098	BF056651	**1.44**
225263_at	heparan sulfate 6-O-sulfotransferase 1	BC001196	**-1.38**
213530_at	RAB3 GTPase-activating protein	AI040009	**1.23**
222569_at	UDP-glucose ceramide glucosyltransferase-like 1	AU153746	**1.10**

Supplementary Materials Table 2 Genes transcriptionally de-regulated at 2q21 in the validation series of sporadic NMTCs.

Transcript ID	Gene_assignment	Gene Symbol	p-value	Fold-Change (N vs. T)
2507896	NM_006895 // HNMT // histamine N-methyltransferase // 2q22.1 // 3176 /// NM_0010	HNMT	5,61E-10	**-1,84**
2505957	NM_017958 // PLEKHB2 // pleckstrin homology domain containing, family B (evectin	PLEKHB2	9,65E-07	**1,48**
2579439	NM_001006636 // GTDC1 // glycosyltransferase-like domain containing 1 // 2q22.2-	GTDC1	0,00260587	**-1,38**
2504328	NM_002101 // GYPC // glycophorin C (Gerbich blood group) // 2q14-q21 // 2995 ///	GYPC	0,00353118	**-1,37**
2578790	**NM_018557 // LRP1B // low density lipoprotein-related protein 1B (deleted in tum**	**LRP1B**	**0,00361758**	**-3,21**
2508611	NM_018460 // ARHGAP15 // Rho GTPase activating protein 15 // 2q22.2 // 55843	ARHGAP15	0,00384518	**1,55**
2503109	NM_020909 // EPB41L5 // erythrocyte membrane protein band 4.1 like 5 // 2q14.2 /	EPB41L5	0,00550386	**1,58**
2574984	AF527767 // LIMS2 // LIM and senescent cell antigen-like domains 2 // 2q14.3 //	LIMS2	0,01039	**1,23**
2502821	NM_020548 // DBI // diazepam binding inhibitor (GABA receptor modulator, acyl-Co	DBI	0,0133995	**-1,47**
2575196	NM_024545 // SAP130 // Sin3A-associated protein, 130kDa // 2q14.3 // 79595 /// A	SAP130	0,0249848	**1,17**
2506903	NM_002410 // MGAT5 // mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-gluco	MGAT5	0,0276574	**1,33**
2508520	NM_003937 // KYNU // kynureninase (L-kynurenine hydrolase) // 2q22.2 // 8942 ///	KYNU	0,0616844	**1,20**
2574646	NM_139343 // BIN1 // bridging integrator 1 // 2q14 // 274 /// NM_139344 // BIN1	BIN1	0,0638095	**1,17**
2505501	AK289878 // IMP4 // IMP4, U3 small nucleolar ribonucleoprotein, homolog (yeast)	IMP4	0,0659047	**-1,14**
2577644	NM_025052 // YSK4 // yeast Sps1/Ste20-related kinase 4 (S. cerevisiae) // 2q21.3	YSK4	0,072294	**-1,05**
2506570	NM_001508 // GPR39 // G protein-coupled receptor 39 // 2q21-q22 // 2863 /// EU07	GPR39	0,0782068	**1,40**
2503021	NM_002830 // PTPN4 // protein tyrosine phosphatase, non-receptor type 4 (megakar	PTPN4	0,0868897	**-1,17**
2578610	NM_007226 // NXPH2 // neurexophilin 2 // 2q22.1 // 11249 /// ENST00000272641 //	NXPH2	0,089916	**-1,11**
2576988	NM_144586 // LYPD1 // LY6/PLAUR domain containing 1 // 2q21.2 // 116372 /// NM_0	LYPD1	0,102156	**-1,10**
2575054	NM_018383 // WDR33 // WD repeat domain 33 // 2q14.3 // 55339 /// NM_001006623 //	WDR33	0,108027	**1,13**
2505993	NM_001083538 // A26C1A // ANKRD26-like family C, member 1A // 2q21.1 // 445582 /	A26C1A	0,137276	**-1,23**
2574720	NM_001001665 // CYP27C1 // cytochrome P450, family 27, subfamily C, polypeptide	CYP27C1	0,167874	**-1,07**
2503929	NM_130773 // CNTNAP5 // contactin associated protein-like 5 // 2q14.3 //	CNTNAP5	0,194054	**-1,05**

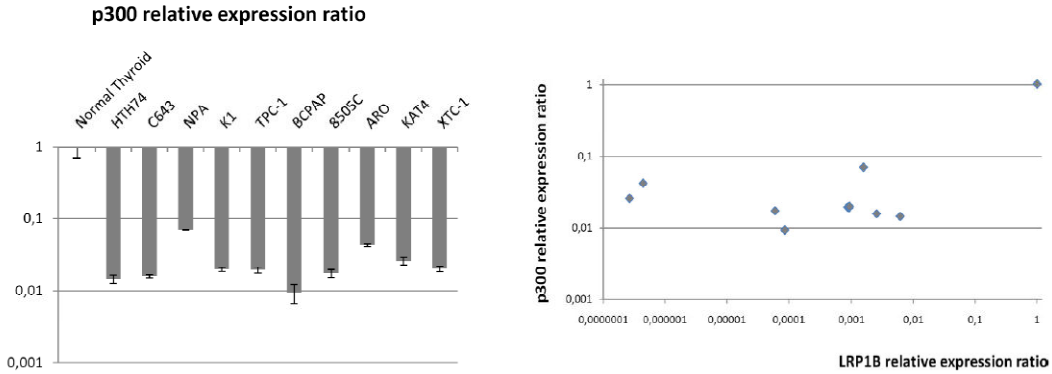
	129684			
2577106	NM_207363 // NAP5 // Nck-associated protein 5 // 2q21.2 // 344148 /// NM_207481	NAP5	0,199355	**1,07**
2508016	NM_001001664 // SPOPL // speckle-type POZ protein-like // 2q22.1 // 339745	SPOPL	0,212567	**1,13**
2503618	NM_004622 // TSN // translin // 2q21.1 // 7247 /// BC002359 // TSN // translin /	TSN	0,214684	**1,08**
2574884	NM_017969 // IWS1 // IWS1 homolog (S. cerevisiae) // 2q14.3 // 55677 /// BC01701	IWS1	0,227585	**-1,08**
2576554	NM_001085365 // FAM128A // family with sequence similarity 128, member A // 2q21	FAM128A	0,236174	**-1,22**
2504645	NM_001080527 // MYO7B // myosin VIIB // 2q21.1 // 4648 /// ENST00000389524 // MY	MYO7B	0,256876	**-1,06**
2504595	NM_000312 // PROC // protein C (inactivator of coagulation factors Va and VIIIa)	PROC	0,258901	**-1,06**
2577856	NM_002299 // LCT // lactase // 2q21 // 3938	LCT	0,258942	**-1,05**
2507495	NM_014607 // UBXD2 // UBX domain containing 2 // 2q21.3 // 23190 /// BC020806 //	UBXD2	0,272492	**1,09**
2507209	NM_058241 // CCNT2 // cyclin T2 // 2q21.3 // 905 /// NM_001241 // CCNT2 // cycli	CCNT2	0,316346	**1,14**
2577700	NM_032143 // ZRANB3 // zinc finger, RAN-binding domain containing 3 // 2q21.3 //	ZRANB3	0,328952	**1,06**
3951190	NM_013310 // C2orf27 // chromosome 2 open reading frame 27 // 2q21.1-q21.2 // 29	C2orf27	0,386347	**-1,11**
2505293	NM_032144 // RAB6C // RAB6C, member RAS oncogene family // 2q21.1 // 84084 /// N	RAB6C	0,388768	**-1,05**
2507173	NM_138326 // ACMSD // aminocarboxymuconate semialdehyde decarboxylase // 2q21.3	ACMSD	0,389214	**-1,03**
2577958	NM_001349 // DARS // aspartyl-tRNA synthetase // 2q21.3 // 1615	DARS	0,400831	**-1,07**
2575134	NM_004805 // POLR2D // polymerase (RNA) II (DNA directed) polypeptide D // 2q21	POLR2D	0,401498	**-1,05**
2505386	NM_025029 // FAM128B // family with sequence similarity 128, member B // 2q21.1	FAM128B	0,408437	**-1,06**
2577896	NM_005915 // MCM6 // minichromosome maintenance complex component 6 // 2q21 // 4	MCM6	0,432011	**1,10**
2503200	NM_002881 // RALB // v-ral simian leukemia viral oncogene homolog B (ras related	RALB	0,471963	**-1,07**
2576526	NM_015658 // NOC2L // nucleolar complex associated 2 homolog (S. cerevisiae) //	NOC2L	0,504754	**-1,04**
2575161	NM_031445 // AMMECR1L // AMME chromosomal region gene 1-like // 2q21 // 83607	AMMECR1L	0,520244	**1,04**
2505779	NM_207364 // GPR148 // G protein-coupled receptor 148 // 2q21.1 // 344561 /// BC	GPR148	0,542449	**-1,05**
2574798	NM_006609 // MAP3K2 // mitogen-activated protein kinase kinase kinase 2 // 2q14.	MAP3K2	0,573622	**-1,05**
2575980	NM_032357 // CCDC115 // coiled-coil	CCDC115	0,63409	**1,04**

	domain containing 115 // 2q21.1 // 84317 ///			
2575897	NM_017951 // SMPD4 // sphingomyelin phosphodiesterase 4, neutral membrane (neutr	SMPD4	0,662699	**1,03**
2505529	NM_014369 // PTPN18 // protein tyrosine phosphatase, non-receptor type 18 (brain	PTPN18	0,690901	**1,03**
3381607	NM_002869 // RAB6A // RAB6A, member RAS oncogene family // 11q13.3 // 5870 /// N	RAB6A	0,741363	**-1,02**
2505793	NM_152698 // FAM123C // family with sequence similarity 123C // 2q21.1 // 205147	FAM123C	0,768982	**-1,03**
2573641	NM_015282 // CLASP1 // cytoplasmic linker associated protein 1 // 2q14.2-q14.3 /	CLASP1	0,772162	**-1,02**
2504766	NM_032740 // SFT2D3 // SFT2 domain containing 3 // 2q14.3 // 84826	SFT2D3	0,790984	**-1,02**
2573786	NM_032390 // MKI67IP // MKI67 (FHA domain) interacting nucleolar phosphoprotein	MKI67IP	0,793302	**1,02**
2577482	NM_030923 // TMEM163 // transmembrane protein 163 // 2q21.3 // 81615	TMEM163	0,829125	**-1,02**
2578028	NM_001008540 // CXCR4 // chemokine (C-X-C motif) receptor 4 // 2q21 // 7852 ///	CXCR4	0,884341	**1,05**
2504743	NM_005291 // GPR17 // G protein-coupled receptor 17 // 2q21 // 2840 /// BX538082	GPR17	0,915303	**1,01**
2505833	NM_032995 // ARHGEF4 // Rho guanine nucleotide exchange factor (GEF) 4 // 2q22 /	ARHGEF4	0,947612	**1,01**
2575949	NM_207312 // TUBA3E // tubulin, alpha 3e // 2q21.1 // 112714 /// NM_080386 // TU	TUBA3E	0,96187	**-1,01**
2507380	NM_015361 // R3HDM1 // R3H domain containing 1 // 2q21.3 // 23518 /// BC001217 /	R3HDM1	0,987865	**1,00**

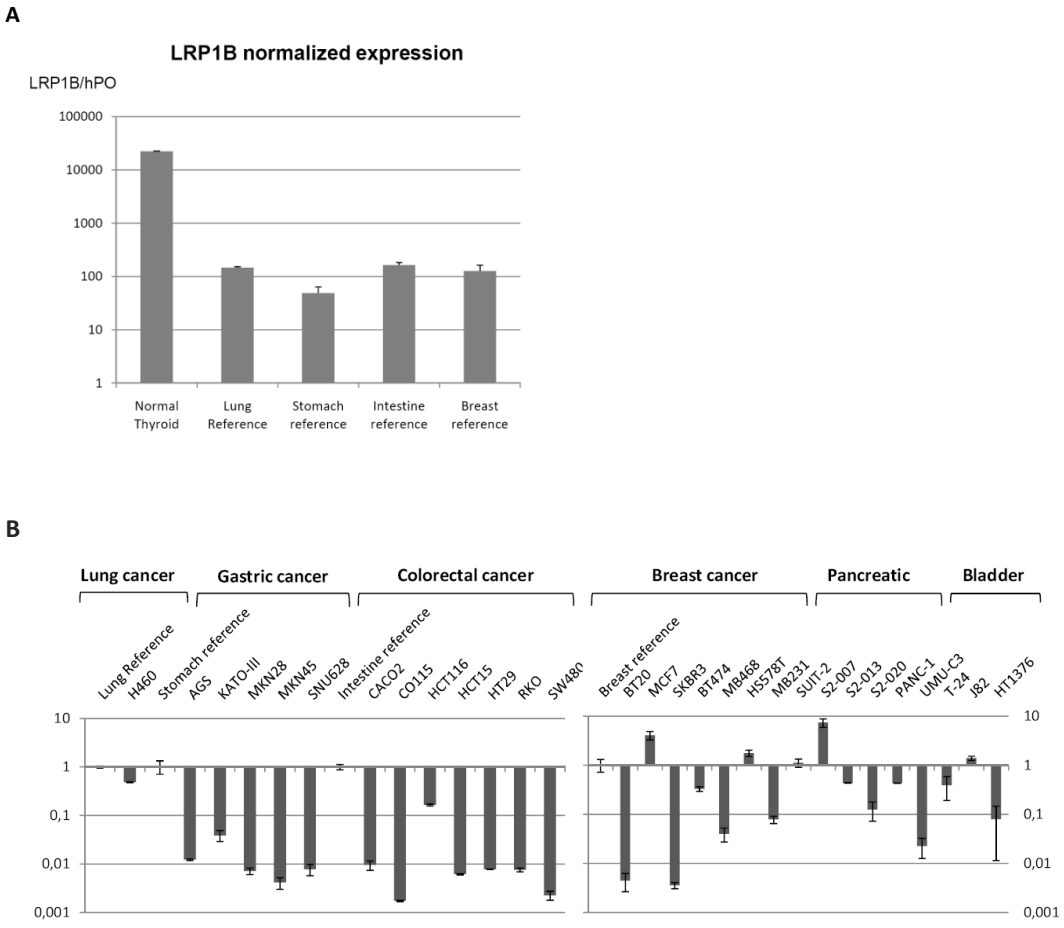


Supplementary Materials Figure 1 Methylation analysis of the CpG island reporters after in vitro methylation

In order to confirm that different patterns of DNA methylation have been generated by M.Sss-I, M.HpaII or M.Msp-I, after in vitro methylation plasmid DNA was converted by bissulfite treatment and subjected to DNA sequencing. Constructs of the CpG island fragment A (429 to -1) and fragment B (+1to +530, counting from ATG) are represented schematically on the left panel and the corresponding chromatograms are represented on the right side. Chromatograms illustrate bissulfite-sequences of the regions delimited by a solid square within the construct. Targets CG, CCGG as well as the p300 binding site are bordered by doted squares. Arrows indicate sites of methylation.



Supplementary Materials Figure 2 Real Time-PCR quantification of p300 mRNA in thyroid cancer cell lines and correlation with LRP1B mRNA expression.



Supplementary Materials Figure 3 LRP1B silencing is found across the most prevalent cancer types

(**A**) LRP1B expression is considerably higher in normal thyroid relative to control RNA (obtained commercially) derived from normal tissue of other organs such as lung, stomach, intestine and breast (obtained commercially). (**B**) Nevertheless, LRP1B silencing is also a common feature in cell lines derived from lung, gastric, colorectal, breast, pancreatic and bladder cancer, as is observable from qRT-PCR quantification of mRNA levels in cancer cell lines relative to normal reference from the corresponding organ.

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