UNIVERSITY OF NAPLES FEDERICO II

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MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXIV CYCLE

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Investigating the role of Klhl14 in thyroid differentiation

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Abstract

Early molecular events of thyroid differentiation are not fully understood even though its organogenesis is well characterized. Over the last few decades, the recently described Kelch-like protein family caught the attention for its members ability to modulate differentiation processes and tumorigenesis by targeting specific protein for degradation.

Klhl14, a member of this family, came out as one of the most enriched gene in E10.5 mouse thyroid bud, alongside with a previously uncharacterized lncRNA and Klhl14 natural antisense that was named by our group Thybe1/Klhl14-AS (as for thyroid bud enriched 1). Klhl14-AS has been recently described as a regulator of thyroid transcription factors and a modulator of Klhl14 expression, although little is known in Klhl14 involvement in thyroid differentiation.

To understand Klhl14 role, in this work we characterized its capacity to modulate thyroid factors abundance in thyroid normal cells and to elicit a negative response in survivability and proliferation of thyroid transformed cells. We found that Klhl14 silencing does not only negatively affect thyroid specific protein abundance, but also increase the presence of Klhl14-AS, thyroid transcription factors FoxE1 and Pax8, and Bcl2, all described to regulate thyroid homeostasis. Moreover, Klhl14 alteration could also direct Nis and Thyroglobulin maturation and distribution in and out of the cell. Interestingly, we also observed a negative effect on thyroid transformed cells survival that is in line with a proposed tumor suppressor role of Klhl14 described in literature.

In conclusion, we have described how the effects of tuning up or down Klhl14 expression in thyroid can induce a response that is likely to influence differentiation and homeostasis of this endocrine gland.

1. Background

1.1. Kelch-like family members are key regulators of many biological processes

The Kelch-like (KLHL) gene family is a large set of genes encoding for proteins that possess two distinctive domains by which they exert their functions. The first evidence leading to the discovery of this family of genes dates to almost four decades ago, when sequencing of the vaccinia virus genome hinted at the presence of a protein closely related to a transcription factor in *Drosophila melanogaster* named bric-à-brack 1, tramtrack, and broad-complex proteins (BTB) Koonin *et al*. 1992; Zollman *et al*., 1994). Apart from the first protein discovered in *D. melanogaster*, this group of proteins comprise a 42 members-large family in both humans and mice, all sharing sequence similarities and functional domains. Kelch-like proteins typically consist of an amino-terminal BTB fused to a zinc finger domain named Poxvirus and Zinc Finger (POZ) (BTB/POZ), which serve as a protein binding domain (Figure 1a) (Bardwell *et al.*, 1994; Albagli *et al.*, 1995; Perez-Torrado *et al*., 2006). Various functions mediated by these domains have been observed and linked to different cellular processes as cytoskeletal organization, ion channel gating, transcription suppression and targeting for ubiquitination through cullin E3 ligases (Furukawa *et al,* 2003).

On the carboxyl-terminal side, the members of this family have a highly evolutionary conserved structure consisting of a five to six repetitions of a β-sheet forming sequence called Kelch repeat or domain. These repetitions typically fold into a β-propeller structure which mediates interaction with target proteins and convey functions that range from cell-cell communication and morphology to gene expression regulation (Shi *et al.,* 2019). Between and in addition to the two domains, Kelch-like proteins contain a third domain called BTB/POZ-Associated to C-terminal Kelch repeat or BACK for short. Although no function has yet been assigned to this domain, evidence show that mutations occurring within the encoding stretch for this part of the protein could be of biological relevance given their implication in human disease (Stogios *et al.,* 2004; Liang *et al.,* 2004).

Kelch-like protein family is defined as comprising 42 genes: while genomic localization and number of coding exons vary not only among members in a single species but also between species, the number of genes itself and the overall tertiary structure of the encoded proteins are conserved between mammalian species. As for numerous other gene family, this hint to the fact that most of the functions exerted by Kelch-like proteins could be the same within different species. Kelch-like proteins play different roles, and evidence about their function are both inferred from computational analysis of protein that contains similar domains in different sequence context, and from direct observations in human and animal models disease studies (Dhanoa *et al.,* 2013). For BTB/POZ, BACK and Kelch repeat domains the binding with other proteins has been shown. A typical described interaction involves the Cullin-3 E3 ligase complex and show that Kelch-like proteins could serve as adaptors for this complex and target proteins, thus making them able to participate in the regulation of protein degradation (Figure 1b) (Canning *et al.,* 2013). To date, several downstream targets of Cul3-Kelch-like complex have been identified, many of which are involved in differentiation and survival programs, mitotic progression, protein trafficking and turnover, cytoskeletal remodeling, and secretion (Canning *et al.,* 2013).

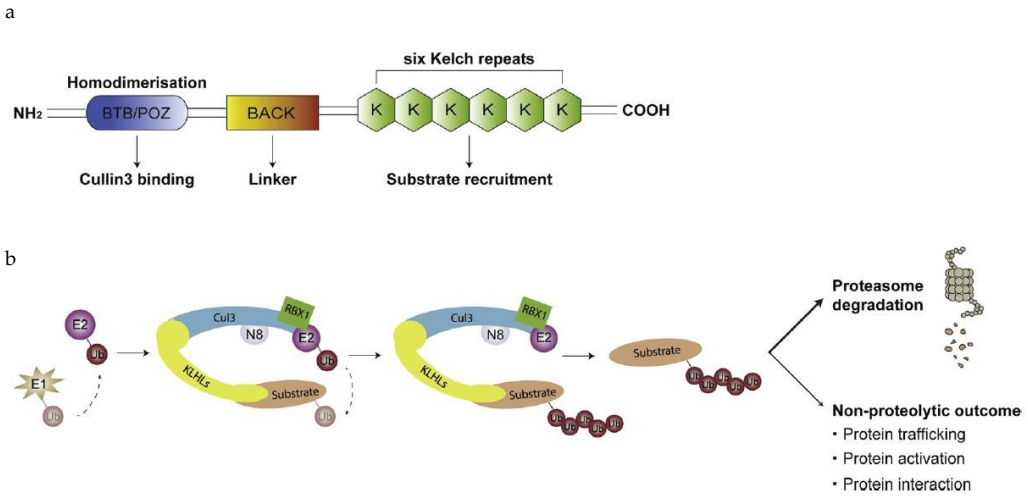

Figure 1. **Diagram of Kelch-like family typical structure and ubiquitination process mediated by them as Cul3 ligase adaptors.** (a) Kelch-like genes encode for proteins with a BTB/POZ N-terminal domain, a BTB/POZ Associated C-terminal Kelch repeat (BACK) domain, and five to six Kelch repeat domain. BTB/POZ serves to bind Cul3 ligase, while the Kelch repeats mediate substrate recruitment. (b) In the Cul3-Kelch complex model, Kelch-like proteins bring the target protein close to Cul3 ligase that attaches ubiquitin monomers. Number of added ubiquitin monomers and types of addition decide the fate of the target protein. (Adapted from Shi *et al*., 2019).

1.2. Kelch-like protein dysregulation affects tissue differentiation and homeostasis

Kelch-like proteins role can essentially be summarized in their involvement in ubiquitination process, and while their specific job in physiological conditions have not been fully understood, much of their purpose can be deduced from their substrate targets. Evidence from decades of disease studies suggest the involvement of Kelch-like proteins in key processes such as cell and tissue differentiation, cell survival and autophagy and cell trafficking. One example of Kelch-like protein operating as regulator of apoptosis and autophagy is Klhl20. Klhl20 was found to be able to target Death-Associated Protein Kinase (DAPK) for degradation, and therefore negatively regulating cell death Lee *et al.*, 2010); and target both VPS34 and Beclin1, preventing excessive triggering of autophagy (Liu *et al.*, 2016).

Perhaps, the most known example of Kelch-like protein indirectly regulating gene expression is Klhl19/Keap1. Under normal conditions, Klhl19/Keap1 targets the transcription factor Nrf2 for degradation, preventing its migration to the nucleus. A brief accumulation of reactive oxygen species (ROS) is enough to modify reactive cysteine residues within Klhl19/Keap1 BTB domain, disrupting its interaction with Nrf2, that can freely migrate into the nucleus and mount an antioxidant response Levonen *et al.*, 2014; Suzuki *et al*., 2017).

In a similar way, many differentiation processes are finely tuned by Kelch-proteins. Klhl2 transient overexpression in developing oligodendrocytes was found to modulate FYN-tyrosine kinase associated actin remodeling in lamellipodia, leading to neurite outgrowth (Williams *et al.*, 2005). Another important regulator of tissue homeostasis is Klhl6, which was found to control B-lymphocyte antigen receptor signaling and the degradation of Cyclin-dependent kinase 2 (CDK2), effectively modulating both B-lymphocyte maturation and survival (Bertocci *et al.*, 2017; Ying *et al.*, 2018). These evidence highlights the roles that Kelch-like proteins play in tissue differentiation.

Considering their engagement in the tuning of central cellular processes as well as differentiation, it was natural to hypothesize their implication in disease conditions in which these homeostatic processes are dysregulated. There is a consistent literature on Kelch-like protein related to human disease, and most of the evidence lean towards loss-of-function mutations as the main cause of the physiological imbalance at their base. These LOF mutations somehow disrupt the interaction between the Kelch-like protein and its specific target protein, and this is the major mechanism behind the downstream imbalance. Already mentioned Klhl6, Klhl19/Keap1 and Klhl20 are associated with cancer progression in diffuse lymphoma, multiple myeloma, chronic lymphocytic leukemia, and gastric cancer, as well as lung cancer, prostate cancer, and pulmonary papillary carcinoma (Walker *et al.,* 2018; Deng *et al.,* 2017; Li QK *et al.,* 2011). In the case of Klhl19/Keap1, which acts as tumor-suppressor, both epigenetic regulation and miRNA-induced silencing reduce its expression level leading to an increase Nrf2 levels that can favor tumor growth (Li QK *et al.,* 2011). Alongside these three actors, Klhl22, Klhl37 and recently Klhl14 were found associated with carcinogenesis. Klhl22 directly targets for degradation a kinase involved in spindle assembly (Metzger *et al.,* 2013), while Klhl37 normally acts promoting neural development, but when mutated it may give rise to glioblastoma and astrocytoma (Kim *et al.,* 2000) (Figure 2).

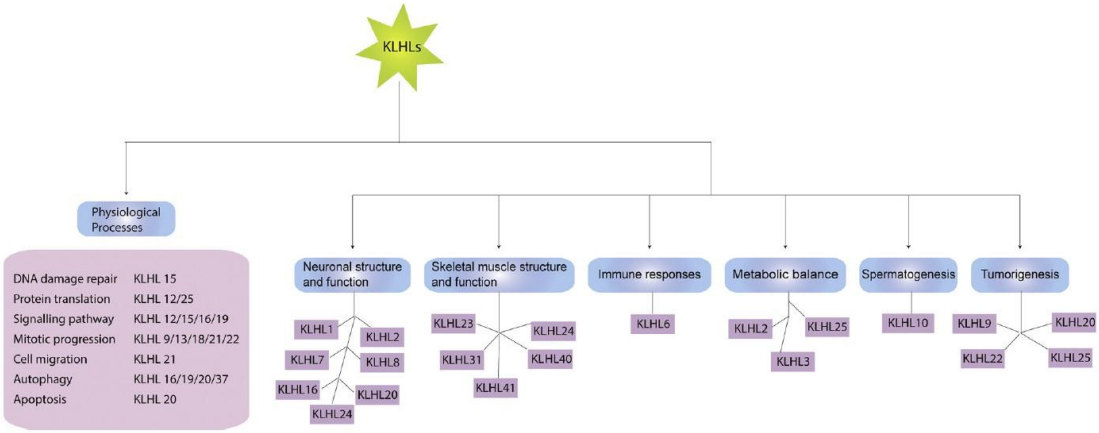

Figure 2. **Kelch-like proteins are engaged in a multitude of biological processes.** Protein from Kelch-like family are involved in many different cellular and biological processes. These proteins generally take part to these processes by either directing target degradation via proteasome or by modulating target trafficking (Adapted from Shi *et al*., 2019).

1.3. Klhl14 is a recent player in mammalian tissue differentiation and human proliferative diseases

The relatively young Klhl14 was only recently, in 2009, added to the Kelch-like gene family. This protein was found to be involved in early onset generalized dystonia, a progressive neuromuscular disorder caused by a deletion in an ATPase Associated with a variety of cellular Activity (AAA) named TorsinA. TorsinA is implicated in neuronal protein trafficking, and yeast two-hybrid screens performed by Giles and colleague found that it can be bound by a protein they named Protein interactor of TorsinA or Printor for short. They then revealed that disruption of this protein binding, which would normally occur within the endoplasmic reticulum lumen, could contribute to the physiopathology of dystonia. It was later shown via sequence homology analysis that Printor was, in fact, the human homologue of rat Kelch-like protein 14. Literature on Klhl14 is very scares and much of its functions or even partners are recent subject of discoveries. Apart from the evidence coming from large-scale transcriptome and proteome studies, namely The Genotype-Tissue Expression (GTEx) project and The Human Protein Atlas project, only a handful of studies examined its expression in specific tissue and during certain conditions. Klhl14 is prevalently expressed within B1a-lymphocytes, in thyroidal gland and spleen (Choi *et al*., 2020). Alongside these findings, Klhl14 was found to be over-expressed in ovarian and endometrial cancer (Han *et al.*, 2019) and it may promote the progression of these types of tumors by regulating signaling pathways of mTOR, WNT and TGF-β (Chen *et al*., 2020).

Nevertheless, two lines of research identified Klhl14 role in B-lymphocytes differentiation and carcinogenesis. Li and colleagues found that heterozygous mice lacking one copy of Klhl14 had a severe reduction of B1a-lymphocytes and a simultaneous increase of B1b-lymphocytes in the peritoneal cavity. Apart from these findings, mice were healthy and without apparent damage (Li *et al*., 2018). In a series of studies, Choi and colleague demonstrated that Klhl14 promotes ubiquitylation of B-Cell Receptor (BCR) subunits, decreasing its stability and increasing its turnover, and therefore, reducing the amount of exposed receptor on the cell surface. In the case of a particular subset of lymphoid malignancies, Klhl14

is found to be mutated and its function disrupted, thereby increasing the half-life of BCR on the lymphocyte surface and promoting downstream NF-kB-dependent cell survival (Choi *et al.*, 2020).

Recently, our interest in Klhl14 grew because its encoding gene is part of a gene pair, giving that it partially overlaps with a long non-coding RNA gene called Klhl14-AS (Figure 3). Klhl14-AS was found to be expressed in mouse thyroid and lung primordia at E10.5 (Fagman *et al*., 2011). Later it was shown that Klhl14-AS expression was still high in fully differentiated thyroid, as well as in spleen, kidney, brain, and gonads (Credendino *et al.*, 2017). Indeed, Klhl14-AS engages in maintaining thyroid cell homeostasis, differentiation, and survival. This lncRNA is typically down-regulated in thyroid tumor sample and cell lines, and its knock-out or silencing reduces the expression of thyroid transcription factors, thereby worsen the tumoral transformation and survival (Credendino *et al.*, 2019).

To expand what was known at the time about thyroid development, our group performed a screening for transcripts enriched in the mouse embryonic thyroid bud respect to the whole embryo by microarray analysis. Klhl14, alongside the long noncoding gene Klhl14-AS, resulted as one of the most enriched gene in E10.5 thyroid bud samples. The enriched transcripts list also includes the protein coding gene like Bcl2, together with several thyroid transcription factors already known to be expressed specifically in thyroid (Fagman et al., 2011) (Figure 4a). Klhl14-AS and Bcl2 show indeed overlapping expression profiles by in situ hybridization assays performed on thyroid bud (Fagman et al., 2011). Later, our group found a correlation between Klhl14-AS and Klhl14 expression both in thyroid normal differentiation and in neoplastic transformation. Indeed, both gene overlap in their expression pattern in different organs and at early stages of mouse development as shown in preliminary in situ hybridization data (Credendino, unpublished data; Credendino et al., 2017; Credendino et al., 2019) (Figure 4b and 4c). Although they behave similarly in various processes, with Klhl14-AS acting on Klhl14 expression, no direct evidence of involvement nor a molecular mechanism emerged for the latter (Credendino, unpublished data). Moreover, our

group found that by interfering Klhl14-AS in vitro, Klhl14 transcript expression decreases accordingly, although the molecular mechanism of this cross-regulation is still unknown.

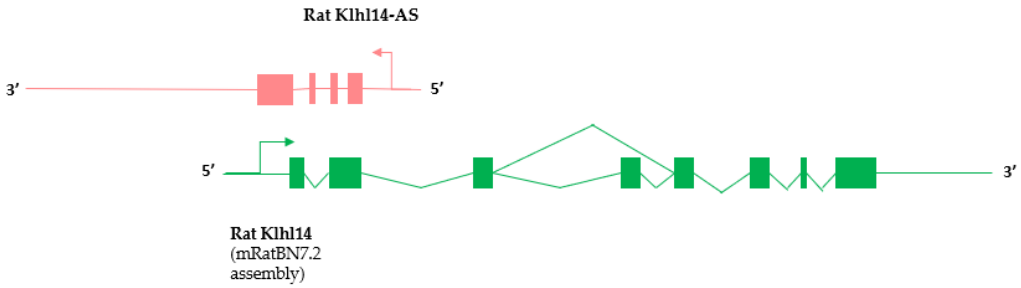

Figure 3. **Overview of Klhl14 gene locus and transcripts in rat.** Klhl14 shares its locus with a partially overlapping gene encoding for a long non-coding transcript known as Klhl14-AS.

a

Gene symbol	Fold Change	Gene title
Klhl14-AS	108.66	RIKEN cDNA unidentified at the time (Klhl14-AS today)
Iigp1	96.83	Interferon inducible GTPase 1
Hhex	87.38	Hematopoietically expressed homeobox
Prlr	79.18	Prolactin receptor
Cpne4	55.45	Copine IV
Ptpre	47.88	Protein tyrosine phosphatase, receptor type, E
Nptx1	46.05	Neuronal pentraxin 1
Bcl2	45.22	B-cell leukemia/lymphoma 2
Pax8	42.26	Paired box gene 8
Hivep3	38	HIV type I enhancer binding protein 3
Stc2	28.45	Sannocalcin 2
Pla2g7	27.81	Phospholipase A2, group VII
Klhl14	26.68	Kelch-like 14 (*Drosophila*)

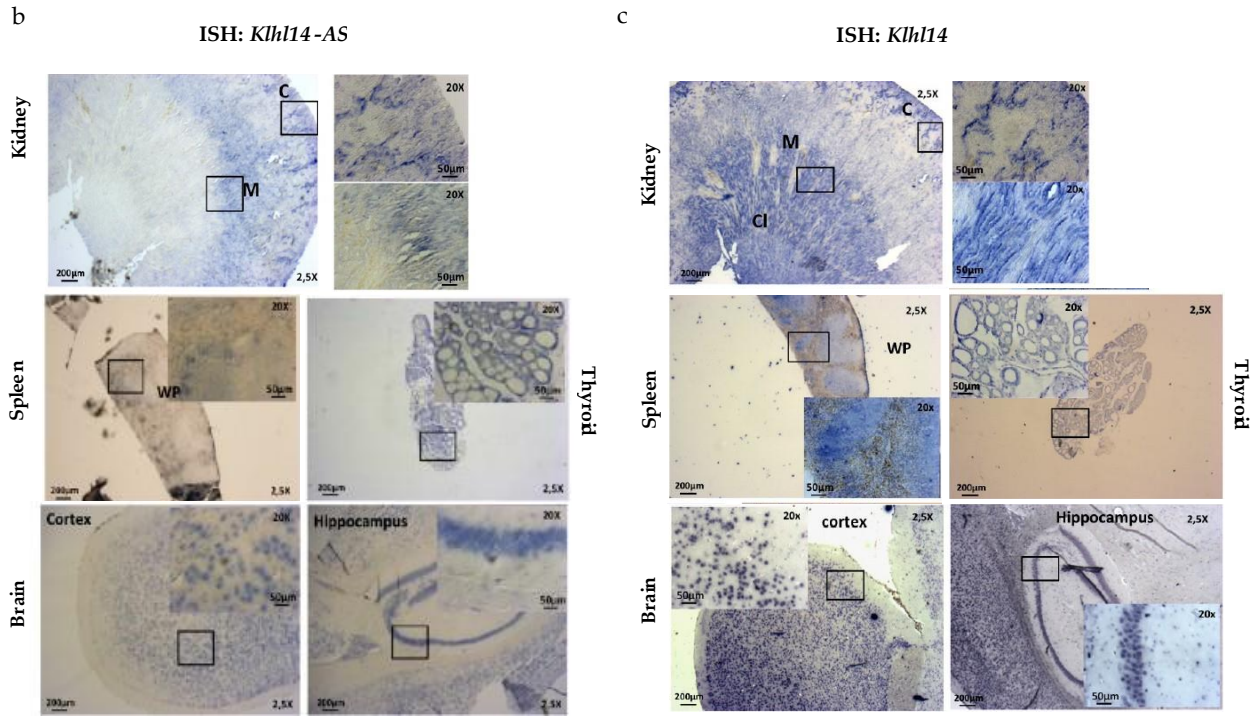

Figure 4. **Klhl14 and Klhl14-AS are remarkably similar in their expression patterns.** (a) Microarray analysis performed on developing mouse thyroid bud at E10.5. The list shows the most enriched genes (light green) among which Klhl14 (highlighted in dark green) and Klhl14-AS are the two of the most expressed. (b-c) *In situ* hybridization performed on adult mouse tissue. (b) micrographs show the expression of *Klhl14-AS* transcript in cortical kidney (upper panel), white pulp of the spleen (mid-left panel), thyroid follicles (mid-right panel) and brain cortex and hippocampus (lower panel). (c) micrograph show the expression of *Klhl14* transcript in kidney cortex and medulla (upper panel), spleen white pulp (mid-left panel), thyroid follicles (mid-right panel) and brain cortex and hippocampus (lower panel). (Adapted from Credendino *et al.,* 2017 and Credendino, unpublished data).

1.4. Thyroid gland physiology in brief

Thyroid is one of the major endocrine glands situated at the base of the neck and is responsible for producing two main metabolism regulator hormones, triiodothyronine T_3 and thyroxine T_4, and a third regulator involved in calcium homeostasis which is Calcitonin. Thyroid hormones are essential regulators of a plethora of biological processes, acting as controllers of growth and development via modulating genes expression, and upon metabolism via non-genomic actions (Armstrong M *et al.,* 2022). Thyroxine is the main secretory product of the gland, followed by triiodothyronine which is the active hormone of the pair. Both hormones are the peculiar downstream products of the unique thyroid ability to uptake and store Iodine (Crockford SJ, 2009). Thyroid follicular cells uptake circulating iodine via a basolateral membrane sodium/iodine symporter (NIS) and quickly release it in the follicle lumen via another anion exchanger called Pendrin. In the lumen, iodide anions get oxidized and incorporated, via the enzyme Thyroperoxidase (TPO), into tyrosine residues of a secreted glycoprotein, Thyroglobulin (Tg), which are then cross-linked by dual oxidases (DUOX). Iodinated thyroglobulin is reabsorbed by follicular cells and its endosomal dependent proteolysis releases of thyroid hormones T_3 and T_4. The formation of thyroid hormones greatly depends on the correct *rendez-vous* between thyroglobulin and iodide anions in the colloid, and the follicular cells spends a significant number of resources to ensure that these conditions are met. Both thyroglobulin and NIS maturation and trafficking are strictly controlled in an intricate dance that involves multiple quality checkpoints at multiple subcellular locations. Thyroglobulin processing involves rounds of quality control conducted by Endoplasmic Reticulum Associated Degradation (ERAD) proteins, in which Kelch-like proteins have a relevant role. A recent Tg interactome analysis showed that the most common flooded quality checkpoint is the ERAD, and this leads to intracellular thyroglobulin retention (Wright MT *et al.,* 2021). In a similar fashion, ion channels that should be presented on either two sides of follicular cells, like NIS or Pendrin, also get subjected to the same multiple checkpoint controls, among which the ERAD is the most relevant as it decides the fate of the examined protein

(Faria M *et al.*, 2021). The involvement of Klhl14 in these quality checks, sparked our interest to investigate such targets.

1.5. Thyroid gland development

Given the limitation of direct human studies, the best alternative mammalian model to study the anatomical feature and the embryonic specification hallmarks of this gland is the mouse (Fagman and Nilsson, 2010; Fernàndez *et al*., 2015). Fully formed thyroid gland comprise two main types of cells with similar embryonal origin: thyroid hormone-producing thyroid follicular cells (TFCs) and calcitonin-producing parafollicular or C-cells. The adult/fully formed thyroid parenchyma is mostly made of follicular structures, consisting of tightly organized TFCs around a small lumen, which contains a thyroglobulin-rich colloid they produce. Differentiated TFCs consistently express the transcription factors Ttf1/Nkx2-1, Pax8, Hhex and as other endodermal-pharyngeal derived cells, they express a Forkhead box transcription factor which is FoxE1. The ensemble of these thyroid transcription factors (TTFs) is known to specify follicular precursor cells and to preserve TFCs differentiation status and function (Fagman and Nilsson, 2010; De Felice and Di Lauro, 2004). In mouse, thyroid determination begins at E8-E8.5, when the first thyroid anlage/primordium is identifiable (De Felice and Di Lauro, 2004; Fagman and Nilsson, 2010; Fernàndez *et al*., 2015). It is only by E9 that follicular cell precursors in the thyroid anlage begin to express all the TTFs necessary to determine the thyroid bud that will sever from the pharyngeal floor at E10 to reach the trachea at E13 (De Felice and Di Lauro, 2004; Fagman and Nilsson, 2010; Fernàndez *et al.*, 2015).

Although it is still unclear how a specific subset of endoderm cells from the pharyngeal primordium assumes thyroid characteristics (De Felice and Di Lauro, 2004; Fagman and Nilsson, 2010; Fagman and Amendola *et al.*, 2011), it is known that TTFs work in an orchestrated manner to specify not only the type of thyroid cells, but also establish the spatial localization of these cells. Titf1/Nkx2-1 and Pax8 typically operate the differentiation towards TFCs and the survival of these cells (De Felice and Di Lauro, 2004). *Pax8*$^{-/-}$ mice do not form a functional thyroid and die within 2-3 weeks after birth because of a lack of thyroid hormones; *Pax8*$^{-/-}$ mice express lower levels of anti-apoptotic Bcl2 protein. *Ttf1/Nkx2-1*$^{-/-}$ mice, instead, form a smaller thyroid contrarily to wild type mice. On the other hand, FoxE1 is

necessary to commit the thyroid anlage to detach from the pharyngeal floor, as *FoxE1*$^{-/-}$ mice display a thyroid bud still attached to this pavement (De Felice and Di Lauro, 2004).

At E14.5, these TTFs control and allow the expression of thyroglobulin (Tg), and proteins required for thyroid hormone synthesis as thyroperoxidase (Tpo), dual oxidases (Duox) and thyrotropin receptor (Tshr). Finally, at E15-16 TTFs guide the expression of the ion-channels, NIS and pendrin, necessary to promote the iodine anion movement towards the colloid (De Felice and Di Lauro, 2004).

1.6. Thyroid cancer

Thyroid cancer is one of the most common endocrine malignancies and its incidence has increased over the past decades (Kitahara and Sosa, 2016). Most types of thyroid cancer are not hereditary, even though some form of it markedly present a great genetic component to the extent that rare form of it have, indeed, a hereditary component. Most of thyroid cancer derive from follicular cells and are well differentiated and usually do not pose a serious threat as they have good prognosis and survival rate Tuttle and Alzahrani, 2019). Follicular cells are usually the culprit, as their neoplastic transformation give rise to the main three type of thyroid malignancies: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and anaplastic thyroid carcinoma (ATC). PTC and FTC are the most common thyroid cancers, accounting for 85-90% of the cases, and they are generally well-differentiated and benign. ATC, on the other hand, is extremely aggressive undifferentiated tumor developing from differentiated ones. It is less frequent, but account for almost half of the death from thyroid cancer (Kitahara and Sosa, 2016).

Causative molecular alterations include point mutations and gene translocations. RAS and BRAF serine/threonine kinase are usually mutated, and chromosomal rearrangements are common in both PTC (60%) and FTC (30%). FTCs usually bear N-RAS and AKT mutations as well as PAX8/PPARγ rearrangement, while PTCs often carry BRAFV600E mutation followed by a typical series of rearrangement involving RET/PTC and a secondary MET overexpression that favor a more malignant outcome of the tumor. ATCs, the most aggressive form of thyroid cancer, usually derive from well-differentiated forms. Aside from the said alterations, they display down-regulating PTEN and up-regulating TP53 alteration, as well as CTNNB1 mutation, which drive the de-differentiation process (Katoh *et al.*, 2015).

2. Aim

Advances in thyroid research in recent years led to major breakthroughs that shed light on the development of this important gland. Even though evidence about this endocrine tissue is continuously piling up, molecular mechanisms of early thyroid developmental stages are not yet fully understood. Indeed, our group performed many analyses that advanced what is currently known about thyroid primordia, its development, and its aberrant neoplastic transformations. As of today, we were able to amass an ample collection of data showing how the interplay of the long noncoding gene Klhl14-AS and some of its miRNA targets could modulate thyroid differentiation through promoting Bcl2 and Klhl14 expression. Moreover, supportive data about the effects on Klhl14 suggest that a convoluted network exists between the two thyroid differentiation factors.

Starting from these observations, the present Ph.D. thesis is aimed at investigating the possibility that Klhl14 could be involved in maintaining thyroid differentiative state and homeostasis, and to explore the possible contribution to pathological conditions when its expression or function become altered. Furthermore, given Klhl14 involvement in E3-ligase target recruitment, we asked whether this protein could interfere with two important functional thyroid protein, NIS and Thyroglobulin.

3. Materials and Methods

3.1. Cell cultures and treatments

Normal non tumorigenic rat thyroid follicular cells FRTL-5 and tumorigenic clone ^{RASV12}FRTL-5 cells were grown in Coon's modified Ham's F12 medium (Euroclone) supplemented with 5% newborn calf serum (Hyclone Thermo Fisher Scientific), penicillin/streptomicyn, L-glutamine and a mix of six hormones containing 1mU/mL TSH (Sigma), 10mg/mL insulin (Sigma), 10µg/mL somatostatin (Sigma), 5mg/L transferrin (Sigma), 3.7µg/L hydrocortisol, and 20µg/mL Gly-His-Lys (Sigma) as described in De Vita *et al.* (2005). Locked nucleic acid oligonucleotides and plasmid DNA were transfected using Lipofectamine 2000® (Thermo Fisher Scientific) according to manufacturer's specifications.

LNA targeting Klhl14: ATCGGCTGACAAAATT

LNA scrambled control: AAGTGAGTGGAGGAGAGGA

For proteasome inhibition assay, cells were treated using 10µM MG132 (Sigma) or DMSO as vehicle only, added to the medium 12 hours upon the 48 or 72 hours timepoint was reached, depending on the experimental design.

RAS-V12 FRTL-5-V27 cells, a transformed FRTL5 clone that stably overexpress H-RAS^{V12} oncogene, was generated as a puromycin resistant clone after two-to-four weeks of selection, as described in De Vita *et al.* (2005). RAS-V12 FRTL-5 ^{Klhl14} cell line was developed from RAS-V12 FRTL-5-V27 clone by stably transfecting full-length Klhl14 ORF and selecting using 400µg/mL of G418 combined with supplemented media.

3.2. RNA isolation and quantitative Real-time PCR

Total RNA was isolated from cultured cells using Trizol® reagent (Sigma-Aldrich) according to manufaturer's specifications. Total cDNA was generated with SuperScript III Reverse Transcriptase (thermo Fisher Scientific) according to manufaturer's specifications. Real-Time PCR on total cDNA was performed with Universal SYBR Green supermix (Bio-Rad) using gene specific oligonucleotides:

3.3. Plasmids

Plasmids used for Klhl14 overexpression were 3xFlag-Klhl14-CMV14 containing the rat Klhl14 coding sequence flagged at N-terminal domain, and 3xFlag-CMV14 as empty vector control.

3.4. Protein extraction and Western Blot

Whole cell lysates were obtained using a lysis buffer made of NaCl 150mM, Tris HCl 50mM, $MgCl_2$ 5mM, sodium deoxycolate 0.5%, sodium dodecyl sulfate (SDS) 0.1%, Triton X-100 1%. To this buffer were added Dithiothreitol (DTT) 0.1mM, Phenylmethylsulfonate fluoride (PMSF) 0.5mM, proteinase inhibitor cocktail (P8340, Sigma-Aldrich) and phosphatase inhibitor cocktail (P0044, Sigma-Aldrich). Protein concentrations were measured via BCA assay and 20µg were loaded for western blot analysis. Immunoblot were incubated with the following antibodies: anti-Nis (home-made), anti-FoxE1 (home-made), anti-Pax8 (home-made), anti-Bcl2 (sc-7382, Santa Cruz Biotechnology), anti-Thyroglobulin (M0781, Dako), anti-Gapdh (sc-32233, Santa Cruz Biotechnology), anti-Klhl14 (MBS2526408, MyBioSource).

3.5. Immunohistochemistry

Embryos and organs were fixed in 4% Paraformaldehyde (PFA), dehydrated and paraffin embedded as follows:

4% PFA prepared in DEPC-treated water at 4°C o.n.;

2X saline at room temperature (RT);

Saline/Ethanol 96% at RT;

Ethanol 70% at RT;

Ethanol 70% at 4°C o.n.;

Ethanol 85% at RT;

Ethanol 96% at RT;

Ethanol 100% at RT;

Ethanol 100% at 4°C o.n.;

2X Xylene isomers mixture at RT; Xylene/paraffin (1:1) at 60°C; 3x paraffin at 60°C.

The period of room temperature and 60°C steps were determined according to the size of the processed embryo or organ. Sections with 7-10µm were obtained from dehydrated and paraffin embedded mouse embryos and organs. To perform

immunohistochemistry, the sections were deparaffinized in xylene and rehydrated with gradual decreasing concentrations of ethanol, from ethanol 100% to ethanol 50%. Sections were permeabilized with an incubation of 5 minutes with phosphate buffered saline (PBS) – 0.2% Triton X-100, then washed two times for 5 minutes with 1X PBS. Slices were treated for unmasking of epitopes in citrate buffer (10mM pH 6) 15 minutes in the microwave. Endogenous peroxidases were then saturated with methanol and 1.5% oxygen peroxide and slices were re-permeabilized using PBS – 0.2% Triton X-100 for 5 minutes, washed two times with 1X PBS for 5 minutes and then blocked 1 hour at room temperature in histology blocking solution (5% normal goat serum (Vector Laboratories S-1000), 3% BSA, 20mM $MgCl_2$, 0.3% Tween-20 in PBS). Primary antibody for Klhl14 (ab48353, Abcam) was used 1:200 dilution in blocking solution and incubated overnight at 4°C. Section were the subjected to the following protocol to reveal the signal: 5 minutes PBS – 0.2% Triton X-100; two wash in 1X PBS 5 minutes each and the incubated 1h at room temperature with a 1:200 dilution in blocking solution of the secondary antibody (biotinylated anti-rabbit IgG (H+L) BA-1000, Vector Laboratories). Sections were then treated for the last time 5 minutes with PBS – 0.2% Triton X-100, washed two times with 1X PBS, incubated 30 minutes with ABC (SK-4000, Vector Laboratories) at RT, and then with DAB substrate (SK-4100, Vector Laboratories). Slices were the de-hydrated as described for the inclusion process and covered with cover glasses using Eukitt® Quick-hardening mounting solution (03989, Sigma-Aldrich).

3.6. Immunofluorescence

Cultured FRTL-5 cells were grown on round 12mm diameter coverslips as for the required treatment time. Cells were then fixed using 4% PFA in PBS for 10 minutes at room temperature, washed 3 times with 1X PBS and stored in PBS – 0.01% sodium azide until use. Unspecific epitopes were blocked with PBS – 1% FBS – 1% BSA for 30 minutes at room temperature; cells were then incubated with 1:50 dilution in blocking solution of the anti-Nis (home-made) primary antibody for 2 hours. Three washes occurred and then cells were incubated 1h with a 1:200

dilution of an AlexaFluor 555+ conjugated anti-rabbit secondary antibody at room temperature. To prevent the wash of the primary and secondary antibody and to ensure intracellular Nis reaching, cells were re-fixed with 4% PFA in PBS for and additional 10 minutes at RT. The following permeabilization were carried out using PBS – 0.2% Tween for 5 minutes at RT, followed by extensive washing. Cells were then subjected to another cycle of primary/secondary antibodies incubation. Coverslips were then mounted on glass using a mounting medium with 1:2000 dilution of Hoechst. Coverslips were analyzed with Zeiss LSM700 confocal microscope, micrographs acquired with Zen software.

3.7. Viable cell counting, MTS assay, Clonogenic assay and BrdU assay

Cell vitality was assessed via Trypan Blue exclusion assay using 0.4% Trypan Blue solution and counting cells with Bürker counting chamber. MTS colorimetric assay was performed using CellTiter 96® AQueus One Solution Cell Proliferation Assay (Promega, G3582). Briefly, selected RAS-V12 FRTL-5 ^{Klhl14} cells and their Empty Vector control were counted and plated ate the same density on day 0 and let outgrown for 48H; after 48H they were replated in a 96-well plate, incubated for two hours with the MTS solution, and scanned using Synergy HT plate reader. Clonogenic assay was performed as previously described (De Vita *et al.*, 2005); after approximately three weeks of selection, cells passaged at two different split ratios (1:6 and 1:10) were treated with a 6% Glutaraldehyde + 0.4% Crystal Violet solution for 30 minutes at room temperature and then the excess was washed pipetting water into the dishes. BrdU proliferation assay were carried as manufacturer's specifications for 5-Bromo-2'-deoxyuridine Labeling and Detection Kit (Merck, 11296736001). Briefly, cells were plated and grown until they reached 50% confluency; then they were incubated with BrdU labeling solution at 1H at 37°C and 5%CO_2: positive cells were assayed using Zeiss LSM700 confocal microscope.

3.8. Imaging softwares

All acquisitions and micrographs, as well as immunoblot films were analyzed using FiJi software.

3.9. Statistical analysis

Statistical analyses were performed on three independent biological replicates, unless otherwise stated. Analyses were carried out using Excel statistical analyses add-on package. Homoscedastic, two-tailed *t-Student's* test were performed on qRT-PCR and immunoblot densitometries, assuming a normal distribution and similar variances *a priori*.

4. Discussion

In this work we sought to investigate the role of a poorly characterized protein of a well-known family, Klhl14, mainly in the context of thyroid differentiation and homeostasis. From its discovery, Klhl14 was the subject of some investigation that confirmed its involvement in the differentiation and neoplastic transformation of B-lymphocytes (Giles *et al.*, 2009; Li *et al.*, 2018; Choi *et al.*, 2020), and in endometrial cancer (Han *et al.*, 2019). From microarray analysis performed from our group, we found that Klhl14 is one of the most enriched gene in thyroid bud at E10.5, alongside Klhl14-AS, and these results were later confirmed by profiling the expression of both genes in mouse embryo and adult mouse tissues (Credendino *et al.*, 2017; Credendino, unpublished). Investigating Klhl14 protein expression in both in embryo and adult mouse tissue allowed us to further confirm its presence in a plethora of tissues, especially in cell types with secretory functions, as well as confirming thyroid as the one with the higher Klhl14 expression level. Furthermore, as reported in expression databases, Klhl14 strong expression in thyroid gland could hint that this protein plays a role in its homeostasis.

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