THEMATIC REVIEW

The complex regulation of NIS expression and activity in thyroid and extrathyroidal tissues

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Abstract

The sodium/iodide symporter (NIS) is an intrinsic plasma membrane protein that mediates active iodide transport into the thyroid gland and into several extrathyroidal tissues. NIS-mediated iodide uptake plays a pivotal role in the biosynthesis of thyroid hormones, of which iodide is an essential constituent. For 80 years, radioiodide has been used for the diagnosis and treatment of thyroid cancer, a successful theranostic agent that is extending its use to extrathyroidal malignancies. The purpose of this review is to focus on the most recent findings regarding the mechanisms that regulate NIS both in thyroid and extra-thyroidal tissues. Among other issues, we discuss the different transcriptional regulatory elements that govern NIS transcription in different tissues, the epigenetic modifications that regulate its expression, and the role that miRNAs play in fine-tuning NIS after being transcribed. A review on how hormones, cytokines, and iodide itself regulate NIS is provided. We also review the present stage of understanding NIS dysregulation in cancer, occupied mainly by convergent signaling pathways and by new insights in the route that NIS follows through different subcellular compartments to the plasma membrane. Furthermore, we cover NIS distribution and function in the increasing number of extrathyroidal tissues that express the symporter, as well as the role that NIS plays in tumor progression independently of its transport activity.

Key Words

- sodium/iodide symporter (NIS)
- gene regulation
- thyroid
- iodide
- extrathyroidal tissues

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Introduction

The year 2021 is the 80th anniversary of the first use of radioiodide treatment. On March 31, 1941, Saul Hertz administered radioiodide (^{131}I) to treat a woman with hyperthyroidism (Graves' disease) (Hertz *et al.* 1942, Hertz 2019). A few years later, the first clinical trial leading to a series of 29 patients successfully treated with ^{131}I was published (Hertz & Roberts 1946). Hertz further declared: 'My new research is in cancer of the thyroid which I believe holds the key to the larger problem of cancer in general' (Hertz 2019). Evidence of such prediction finally arose in the 1980s, when Mazzaferri and cols published a large retrospective series showing the effectiveness of radioiodide as an adjuvant treatment for patients with differentiated thyroid cancer (Mazzaferri & Young 1981). In a time where cancer caused devastating effects, as no effective treatments were available, the success of radioiodide treatment was extraordinary and, not surprisingly, it is still considered one of the best and longest molecularly targeted and

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imaging-based treatments ever devised for cancer (De la Vieja & Riesco-Eizaguirre 2021).

Not until 1996, the molecule responsible for iodide uptake was finally cloned and characterized (Dai *et al.* 1996). Nancy Carrasco's group demonstrated that a sodium/iodide symporter (NIS) actively transports two ions of sodium along with one of iodide into the cells using the favorable gradient of Na^+. NIS is localized in the basolateral membrane of the thyroid follicular cell and represents the first step in thyroid hormone (TH) synthesis (Portulano *et al.* 2014). Once iodide is transported from the circulation into the cytosol, iodide is then further transported through the apical membrane by pendrin and other proteins into the colloid (De la Vieja & Santisteban 2018). Iodide is then oxidized by thyroid peroxidase (TPO) using H_2O_2 provided by dual oxidase (DUOX2), both enzymes localized at the interphase between the colloid and the apical membrane. TPO also incorporates iodide to the tyrosyl residues of the thyroglobulin (TG) and then couples two iodinated tyrosyl residues to form tri-iodothyronine (T3) and thyroxine (T4), which are stored in the colloid covalently linked to TG in a process called organification. This iodide organification process, involving NIS, TPO, DUOX, and TG, enhances the iodide retention time in the thyroid gland and is, therefore, able to increase the therapeutic efficacy of radioiodide that enters thyroid follicular cells via NIS. Finally, TG, stored in large amounts in the colloid, is taken into the cell through endocytosis and then broken down by lysosomes, releasing T3 and T4, which are then transported to the circulation mainly through MTC8; the remaining iodide that was not used in the production of TH is recycled by the iodotyrosine dehalogenase (DEHAL1).

The experimentally tested secondary structure model for NIS shows a hydrophobic protein with 13 transmembrane segments, an extracellular amino terminus and an intracellular carboxy terminus (Levy *et al.* 1998, Ravera *et al.* 2017). There are three sites of glycosylation in the mature form of NIS, and the major phosphorylation region is the COOH terminus. NIS dimerizes and needs to be located at the basolateral membrane in order to be functional (Huc-Brandt *et al.* 2011). The human NIS gene is localized on chromosome 19p12–13.2 and encodes a glycoprotein of 643 amino acids (aa) with a molecular mass of approximately 90–100 kDa that can vary between tissues (De la Vieja & Santisteban 2018). The gene comprises 15 exons interrupted by 14 introns and has an open reading frame of 1929 nucleotides. NIS is a member of solute carrier family 5A and has been designed as *SLC5A5* by the Gene Nomenclature Committee, according to the Human Genome Organization.

NIS research has become an evolving field of considerable scope. It ranges over the structure/function properties of plasma membrane transporters, gene therapy, thyroid pathophysiology, theranosis of several types of cancer (thyroid, breast, ovary, etc.), and even environmental pollutants and public health, among others. Therefore, discoveries in this field have had a remarkable impact on numerous basic and translational areas, with important clinical implications. The purpose of this review is to focus on the most recent findings regarding the mechanisms that regulate NIS, both in thyroid and extra-thyroid tissues. In the past 2 decades, the mechanisms governing NIS function and expression have turned out to be increasingly complex. The more mechanistic information we have, however, the better equipped we are to extend the clinical applications for this molecule.

Promoters and enhancers regulating NIS transcription

Transcriptional regulatory elements in complex genomes are key players in the dynamic transcription of genes during development, tissue homeostasis, and disease (Schaffner 2015, Andersson & Sandelin 2020). Identifying the promoters and enhancers driving important biological processes can be challenging and has been relatively unexplored in thyroid pathophysiology on a genome-wide scale. Given the importance of NIS in iodide metabolism and given its functional expression in several tissues, it is to be expected that NIS transcription is under the control of several promoters and enhancers to regulate temporal and spatial expression in tissues. Substantial progress has been made in two tissue-specific contexts, the thyroid and mammary gland (Fig. 1).

In the thyroid

It is well established that specific gene transcription in the thyroid is orchestrated by the joint expression of three transcription factors: NKX2.1 (previously known as TTF1), a homeodomain-containing protein; FOXE1 (previously known as TTF2), a forkhead protein; PAX8, a paired domain-containing protein (De Felice & Di Lauro 2004) (Fig. 1 left panel). Initially, a proximal rat NIS promoter to which NKX2.1 binds to was identified, conferring a thyroid-specific transcription (Endo *et al.* 1997). A thyroid-stimulating hormone (TSH)-responsive element (TRE) and

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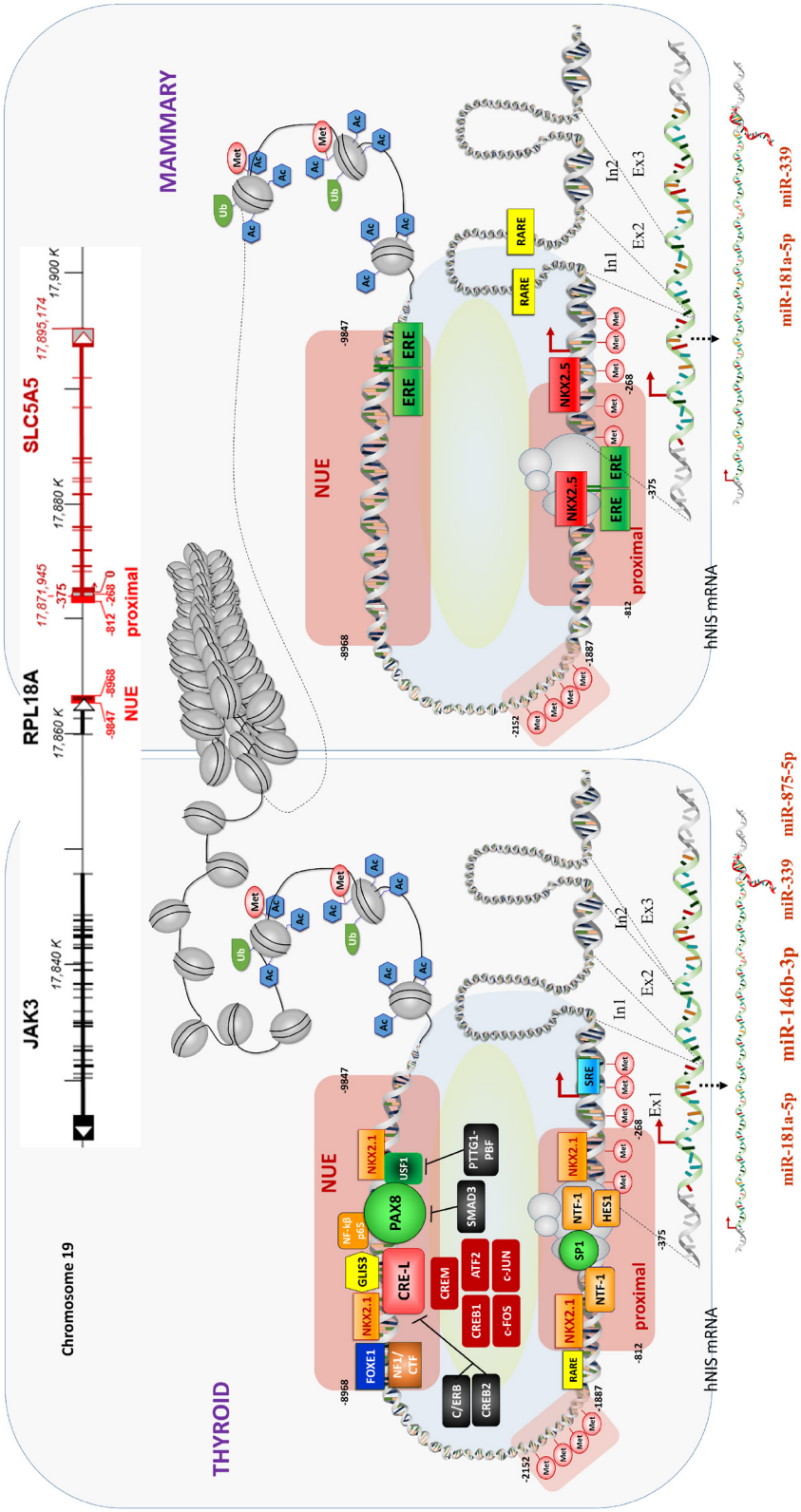

Figure 1

Transcriptional and epigenetics regulation of NIS/*SLC5A5* in thyroid and mammary cells. NIS gene (*SLC5A5*, solute carrier family 5 member 5) is localized in the human chromosome 19 from 17,871,945 to 17,895,174 bp, in a span of 23,230 nucleotides (in the latest assembly CRCh38.p13). In mice, it is localized at chr8 and in rats, at chr16. The open reading frame of 1929 nucleotides, generated by 15 exons, encodes a human protein of 643 amino acids (aa) (rat NIS contains 618 aa). *SLC5A5* gene regulation starts with epigenetics modifications (methylation and histone modification) (top), and finishes with mRNA negative regulation by miRNAs, mainly by miR-146b-3p (bottom). *SLC5A5* transcriptional regulation is tissue-specific with important differences in the thyroid (left) and mammary gland (right). There are two main regulatory elements, the proximal promoter (−268 to −812 bp) and the NIS upstream enhancer (−8968 to −9847), also called NUE. In the thyroid, the PAX8 transcription factor (negatively regulated by SMAD3) binds to the NUE region and has a major role in NIS upregulation. Many other factors have been shown to bind to NUE regions such as CRE-like binding site (positively regulated by CREM, CREB1, ATF2, c-Fos, and c-Jun, or negatively regulated by CREB2 and C/ERB), FOXE1, NKX2.1, USF1 (negatively regulated by PTTG1-PBF), GLIS3, and NF-kappaB. Transcription factors that upregulate NIS expression in the thyroid and have been found to bind to the proximal promoter are NKX2.1, NTF-1, SP1, and HES1. Regulation by methylation has been observed in two regions: around the exon 1 and the most important at positions −1887 to −2152. In the mammary gland, the NUE region does not seem to participate much in NIS regulation. In this tissue, NKX2.5 and several estrogen receptor elements (ERE) at the proximal promoter have a major role in NIS regulation. ERE elements at the NUE may also exert similar effects to PAX8, and this could occur in the mammary gland and ovary, but its specificity has not been totally demonstrated. In addition, RAR and RXR interaction in the intronic regions of NIS act like enhancers of NIS expression.

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a functional GC box (to which Sp1 protein binds) were further identified as contributing to NIS transcription at the proximal promoter (Ohmori *et al.* 1998, Xu *et al.* 2002). However, NKX2.1 exerts only a modest effect on NIS transcriptional activity. Soon after, it was demonstrated that PAX8 was a much stronger inducer of NIS transcription by binding to a distant region upstream of the proximal promoter. This region, named NIS upstream enhancer (NUE), is located between −2264 and −2495 in the rat promoter, having two PAX8 binding sites (Ohno *et al.* 1999) and between −9847 and −8968 in the human promoter, having one PAX8 binding site (Schmitt *et al.* 2002, Taki *et al.* 2002). In addition, NKX2.1 and FOXE1 have also been found to bind to the NUE in the rat promoter (Ohno *et al.* 1999, Fernández *et al.* 2013). Overall, the enhancer NUE is the essential regulatory element for NIS transcription in the thyroid, and although the three transcription factors that define the differentiated thyroid phenotype binding to this enhancer, it is well accepted that PAX8 is the main factor mediating NIS transcription.

In addition to the binding sites for the above transcription factors, a cAMP response element (CRE-like) binding site has also been shown to be crucial in the rat and human NUE (Ohno *et al.* 1999, Taki *et al.* 2002). This is not surprising as it has been well established for decades that thyroid iodide transport activity is markedly stimulated by TSH and cAMP (Weiss *et al.* 1984). The NUE responds to cAMP in both PKA-dependent and -independent manners, requiring PAX8 for the full cAMP transcriptional action (Ohno *et al.* 1999). Moreover, several basic-leucine zipper (B-ZIP) proteins have been identified in the thyroid to bind the CRE-like site and regulate NIS transcription positively (CREM, CREB1, ATF2, c-Fos, and c-Jun) and negatively (C/ERB and CREB2) (Chun & di Lauro 2001, Chun *et al.* 2004, Fenton *et al.* 2008). Overall, the enhancer NUE is considered the main regulator of NIS transcription in the thyroid, with both PAX8 and CRE-like binding sites being essential (Taki *et al.* 2002). The full activity of this enhancer seems to be cell-specific as it activates NIS transcription in thyroid cell lines but not in MCF-7 breast cancer (Taki *et al.* 2002). However, the expression of PAX8 in non-thyroid cells results in transcriptional activation of NUE, suggesting that tissues other than the thyroid which express PAX8 (i.e. ovary) may also be dependent on NUE activation (Riesco-Eizaguirre *et al.* 2014).

Other transcription factors have been involved in the transcription of NIS in the thyroid (Fig. 1 left panel). One of the most prominent is the Krüppel-like zinc finger transcription factor GLI-similar 3 (GLIS3), a transcription factor whose deficiency is associated to a syndrome characterized by neonatal diabetes and congenital hypothyroidism both in humans and mice. GLIS3 is essential for the induction of TSH-mediated NIS transcription, being able to bind the NUE region and activate it (Kang *et al.* 2017). Another transcription factor is nuclear factor-KB (NF-KB), a ubiquitous transcription factor that is involved mainly in inflammatory and immune responses, and more specifically, it has been involved in thyroid autoimmune diseases, thyroid orbitopathy, and thyroid cancer, being fundamental for the expression of the main thyroid-specific genes (Giuliani *et al.* 2018). There is a conserved NF-KB site within the NUE region, and NF-KB p65 subunit appears to physically interact with PAX8 inducing a synergistic effect on NIS transcription in response to certain stimuli such as lipopolysaccharides (LPS) (Nicola *et al.* 2010).

In addition to GLIS-3 and NF-KB, there is a binding site for upstream stimulating factor 1 (USF1) that lies within the PAX8 consensus being this overlapping PAX8/USF1 sequence highly conserved among species (Lin *et al.* 2004). USF are ubiquitously expressed proteins, which belong to the basic helix-loop-helix (HLH) leucine zipper family of transcription factors and are also involved in FOXE1 regulation (Landa *et al.* 2009). Discrete mutation of the USF1 site resulted in a significant disruption of promoter activity suggesting that USF1 binding is required for full hNUE activity (Boelaert *et al.* 2007). The PAX8/USF1 site plays a critical role in mediating the repression of PTTG and PBF on the human NIS promoter (Boelaert *et al.* 2007). Primary tumor-transforming gene 1 (PTTG1), a proto-oncogene originally identified in pituitary tumors (Pei & Melmed 1997) and the cofactor PBF (PTTG1-binding factor), essential for PTTG1 to be functional, is overexpressed in thyroid cancer and has been proven to be tumorigenic in both cell lines and transgenic mice repressing iodide uptake (Stratford *et al.* 2005, Read *et al.* 2011). Finally, retinoic acid (RA), a derivative of vitamin A known for its potent proliferation-inhibiting and differentiation-inducing properties, exerts its regulatory effect on hNIS promoter through a retinoic acid response element (RARE) located at −1375 relative to the ATG codon (Schmutzler *et al.* 2002). While RA treatment of normal non-transformed thyrocytes resulted in decreased iodide uptake and reduced NIS expression, RA treatment of human follicular thyroid carcinoma cell lines had the opposite effect, both NIS mRNA and iodide uptake were elevated (Schmutzler *et al.* 1997).

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In the mammary gland

NKX2.5, a transcription factor that belongs to the NK2 family (which includes NKX2.1), is a potent inducer of the NIS proximal promoter in the breast by binding to two specific sites (Dentice *et al.* 2004) (Fig. 1 right panel). This is surprising because, although the consensus binding sites of NKX2.5 and NKX2.1 are virtually identical, NIS is only weakly responsive to NKX2.1 in the thyroid. NKX2.5 is expressed in the heart and in the thyroid primordium during development (Lints *et al.* 1993), and mutations affecting this gene have been reported in individuals with congenital hypothyroidism, with NKX2.5 null embryos having thyroid bud hypoplasia (Dentice *et al.* 2006). However, NKX2.5 is virtually absent from the adult human thyroid and thus unlikely to be involved in maintaining NIS expression in the adult thyroid. Curiously, NKX2.5 is expressed in papillary thyroid carcinomas and its presence correlates to better prognosis (Penha *et al.* 2018). Importantly, NKX2.5 is expressed in mammary glands during lactation as well as in breast cancer, being critical in RA-induced NIS up-regulation in MCF-7 cells (Dentice *et al.* 2004).

In addition to NKX2.5, there are several estrogen receptor element (ERE) sites upstreaming NIS (Fig. 1 right panel), one in the proximal promoter and two ERE sites in the NUE. Estrogens regulate NIS expression *in vivo* in lactating mammary gland as well as in the ovary and fallopian fimbriae during the reproductive cycle, yet they seem to act through different transcriptional elements (Tazebay *et al.* 2000, Riesco-Eizaguirre *et al.* 2014). Also, they may have a regulatory role in the thyroid gland (Furlanetto *et al.* 1999). Estrogens mediate their effects in target tissues through two members of the nuclear receptor superfamily, estrogen receptor-α (ERα) and ERβ. In breast cells, experimental evidence has shown that ERα strongly stimulates NIS transcriptional activity at the proximal promoter in both ligand-dependent and -independent manner, yet the NUE was not analyzed in this study (Alotaibi *et al.* 2006). However, RA, which stimulates NIS gene transcription only in the presence of active ERα, solely acts at the proximal promoter but not at the NUE (Alotaibi *et al.* 2010). By contrast, in the ovary and fallopian fimbriae, ERα strongly stimulates NIS transcriptional activity at the NUE in both ligand-dependent and -independent manner, but very weakly at the proximal promoter (Riesco-Eizaguirre *et al.* 2014). ERβ had little effect on its own, antagonizing the positive effects of ERα on NIS transcription in the ovary (Riesco-Eizaguirre *et al.* 2014). Interestingly, in the ovary, PAX8 significantly cooperates with ERα on NIS transcription suggesting that there is a synergism between ERα and PAX8 upon NIS transcriptional activation at the NUE region (Riesco-Eizaguirre *et al.* 2014). In the thyroid, ERs seem to be operating in the opposite way, as estradiol inhibits TSH-induced NIS expression in FRTL5 cells (Furlanetto *et al.* 1999).

NIS regulation by RA in breast cancer cells has been extensively reviewed (Kogai & Brent 2012, Alotaibi *et al.* 2017). In contrast to the thyroid gland, in the mammary gland, an alternative enhancer in the first intron of NIS has been shown to mediate direct regulation by all-trans-retinoic acid (tRA)-stimulated nuclear receptors (Alotaibi *et al.* 2010). tRA is a potent ligand that enhances NIS expression in several breast cancer cell lines and in experimental breast cancer models. *In vitro* as well as *in vivo* DNA-protein interaction assays have revealed a direct association of retinoic acid receptor-alpha (RARα) and retinoid X receptor (RXR) with this intronic enhancer. Moreover, multiple introns of NIS gene (7 out of 14 introns) contain identical functional RARE sequences potentially interacting with the NIS promoter and regulating the transcriptional activation of the symporter (Alotaibi *et al.* 2010). In addition, because tRA-upregulated NIS expression is restricted to ERα-positive mammary gland cell lines (Alotaibi *et al.* 2006), these intronic elements stimulate the initiation of NIS gene transcription only in the presence of active ERα. RA induction of NIS in MCF-7 cells is mediated by rapid activation of the PI3K pathway and involves direct interaction with RAR and retinoid X receptor (Ohashi *et al.* 2009).

General view

Overall, different regulatory elements on NIS gene with varying degrees of enhancer and promoter activities operate depending on the species and tissue context to modulate NIS transcription (Fig. 1). In the thyroid, there are a low proximal promoter and a high NUE activity mediated by TSH/cAMP stimulation that acts through a synergism between PAX8 and CRE-like binding sites. In the lactating mammary gland, there is a high proximal promoter but low NUE activity ruled by estrogens and other lactogenic hormones that act through NKX2.5 and ERα; in this setting, an enhancer on intron 1 based on RARE elements strongly potentiates NIS transcription through RA stimulation. In the ovaries, there is a low proximal promoter and high NUE activity due to a synergism between ERα and PAX8, most likely under the control of gonadotrophins.

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Epigenetic modification on NIS gene

Epigenetic modification alters the regulation of gene expression mainly by three mechanisms: DNA methylation, histone modification, and nucleosome positioning (Portela & Esteller 2010). The modifications observed are usually the sum of different interactions of epigenetic factors, as well as positive and negative feedback mechanisms. miRNAs, also considered an epigenetic phenomenon, will be included in the next section.

DNA methylation

DNA methylation of CpG dinucleotides tends to cluster in CpG-islands, or in CpG-island shores in regions of lower CpG density. In normal human cells, about 60% of the promoters are usually unmethylated or methylated in a tissue-specific manner during early development or in differentiated tissues (Portela & Esteller 2010). Aberrant DNA methylation plays a crucial role in thyroid carcinogenesis (Xing 2007, Zafon *et al.* 2019). Typically, papillary thyroid carcinomas (PTC), poorly differentiated thyroid carcinomas (PDTC), and anaplastic thyroid carcinomas (ATC) exhibit more hypomethylation and less hypermethylation events than in normal thyroid tissues but the contrary occurs in follicular thyroid carcinomas (FTC). This pattern also differs with respect to the driver mutation, with *BRAF*-mutated tumors harboring more hypomethylations and RAS-mutated tumors more hypermethylation (Agrawal *et al.* 2014).

Examination of 5000 bp in the 5′ region of the NIS gene has identified two CpG-islands, the first one between nucleotides −466 to +246 and the second one between nucleotides −2152 and −1887 relative to the ATG site (Fig. 1) (Venkataraman *et al.* 1999, Galrão *et al.* 2014). NIS gene methylation status has been explored in normal thyroid tissue and benign and malign thyroid tumors, including several thyroid cancer cell lines. The methylation levels in the first CpG-island were similar in tumoral and non-tumoral surrounding thyroid samples, and no correlation was observed between NIS mRNA expression and methylation status (Neumann *et al.* 2004). Interestingly, the degree of methylation of the second CpG-island in tumoral tissues was significantly higher than in non-tumoral tissues in the benign and malignant groups, and a significant inverse correlation with NIS mRNA expression was discovered (Galrão *et al.* 2014). Functional analysis showed that this region is not a promoter region but has regulatory activity. Because the hypermethylation pattern was detected in both benign and malignant thyroid tumors, the authors suggest that the deregulation of NIS methylation is an early event in tumorigenesis. Other authors have also observed hypermethylation of NIS gene in PTC and FTC tumors when compared with normal tissues (Smith *et al.* 2007, Stephen *et al.* 2011) An interesting work by Choi *et al.* found that the association of $BRAF^{V600E}$ mutation and NIS down-regulation is mediated by high expression of DNA methyltransferase 1 (DNMT1) (Choi *et al.* 2014).

Several groups have attempted the use of demethylation agents as a strategy to restore NIS function in thyroid cell lines with contradictory results. In some cases, the results have been positive (Venkataraman *et al.* 1999, Galrão *et al.* 2014), but in others, the results have been modest or negative, even though some of the cell lines were the same (Tuncel *et al.* 2007, Massimino *et al.* 2018). Currently, there are several clinical trials underway testing the use of 5-AZA or analogs in thyroid cancer. Intravenous decitabine (5-AZA) injected in 12 patients with metastatic papillary or follicular thyroid tumors did not restore iodide accumulation in patients (NCT00085293). Azacitidine is currently being evaluated in a phase 1 trial (NCT00004062).

Histone modification

One of the most important events in gene regulation is histone acetylation and deacetylation. Histone acetylation status is regulated by the opposing activity of histone acetyltransferases (HAT) and histone deacetylases (HDAC). Histone acetylation promotes gene expression, while deacetylation promotes repression of gene expression (Verdone *et al.* 2006). HDACs play a key role in cancer induction and progression, including thyroid cancer (Russo *et al.* 2013). NIS gene transcription regulation by histone acetylation at the promoter region has been demonstrated. This opened the challenge to prove whether HDAC inhibitors could be useful to restore NIS activity, not only in aggressive thyroid cancers (Puppin *et al.* 2005, Provenzano *et al.* 2007) but also in cell lines derived from non-thyroid NIS-expressing tumors such as breast, melanoma, liver, gastric, colon, cholangiocarcinoma, and Leydig testicular carcinoma (Kogai & Brent 2012).

However, there has been a great disparity among studies. Initial results were more promising, but they have not always been reproduced by other laboratories. Regardless of the thyroid tumor subtype, it has been observed that cell lines with mutations in BRAF and HRAS respond worse to treatments with HDAC inhibitors than those with other types of gene alterations. In addition, the presence of MAPK and PI3K/Akt inhibitors sometimes

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improves results. However, results in non-thyroid NIS-expressing cell lines are more complex and harder to analyze. Perhaps the most revealing experiment in preclinical trials has been the study performed with five samples from PDTC and ATC patients implanted in mice (Wächter *et al.* 2018). When treated with the HDACi Panobinostat, they observed an increase in the expression of mRNA levels of NIS in all tumors, but they only observed a very modest increase in the accumulation of radioiodide in one of them. Similar results were obtained when using the tyrosine kinase inhibitors (TKIs) sorafenib and selumetinib. In spite of modest results of tumoral NIS re-expression/function, it could be enough for radioiodide treatment to be effective. This has prompted numerous clinical trials to emerge. However, the results of clinical trials in advanced thyroid cancer or radioiodide-refractory differentiated thyroid cancer have been a bit disappointing. Evaluation with the HDAC inhibitors SAHA (Kelly *et al.* 2005), romidepsin (Amiri-Kordestani *et al.* 2013), vorinostat (Woyach *et al.* 2009), and valproic acid (VPA) (Nilubol *et al.* 2017) and depsipeptide (Xu & Hershman 2006) have not given the expected results.

The results of clinical trials with either demethylation or HDAC inhibitors highlight three important aspects. First, that the transcriptional regulation of NIS is much more complex than we can ascertain in *in vitro* experiments; secondly, transcription is only one part of the puzzle, understanding NIS trafficking to the membrane is a key element to fully reach NIS function; thirdly, that clinical trials must be preceded by robust molecular data and preclinical trials.

Challenges in understanding miRNA function in NIS regulation

NIS as a target for miRNAs

Typically, miRNAs negatively regulate gene expression; they either inhibit translation or induce mRNA degradation usually by binding to the 3'UTR of target mRNAs. Based on *in silico* predictions and experimental evidence, a number of miRNAs have been shown to target the 3'UTR of NIS and reduce its expression. miR-146b-3p, one of the most abundant and upregulated miRNAs in PTC, binds to one single specific site of the 3'UTR of NIS mildly reducing its expression (Riesco-Eizaguirre *et al.* 2015). In accordance with these data, antagonizing miR-146b-3p in human thyroid cancer cells reinduces NIS-mediated iodide uptake (Li *et al.* 2015, Hou *et al.* 2020). More importantly, miR-146b-3p also binds specifically to three sites in the 3'UTR of *PAX8* repressing its expression (Riesco-Eizaguirre *et al.* 2015). Thus, miR-146b-3p not only represses NIS directly but also indirectly through the repression of its main transcription factor, PAX8. The importance of miR-146b-3p was highlighted in the large cohort of the cancer genome atlas (TCGA) analyzing over 500 tumor samples of PTC, as this miRNA was one of the three miRNAs that most strongly negatively correlated with the expression of iodide-metabolizing genes (Agrawal *et al.* 2014).

Other miRNAs have been shown to repress NIS. miR-339 binds to the 3'UTR of NIS and is able to repress its expression in TSH-stimulated PCCl3 rat thyroid cells and in MCF-7 human breast cancer cells treated with trans retinoic acid/hydrocortisone (Lakshmanan *et al.* 2015*a*). However, this miRNA is not among the most abundantly upregulated miRNAs in thyroid carcinomas. miR-875-5p, a miRNA highly expressed in PDTC, also targets 3'UTR and represses NIS in PDTC cell lines (Tang *et al.* 2020). miR-17-92, a cluster that comprises several miRNAs and is highly expressed in human ATC, has also been shown to repress NIS and *PAX8* among other iodide-metabolizing genes (Fuziwara *et al.* 2020). Silencing of miR-17-92 via CRISPR/Cas9n gene editing induced a partial restoration of a differentiated phenotype characterized mainly by reestablishing expression of NIS, TG, TPO, PAX8, and NKX2.1. Several members of the cluster such as miR-19, miR-92, and miR-17 repress NIS, NKX2.1, and PAX8 protein levels. However, although the 3'UTR segment of rat *Pax8* mRNA contains predicted sites for miR-17-5p and miR-20a-5p, the authors failed to demonstrate an interaction between the miRNAs and the 3'UTR of *PAX8* through luciferase reporter assays. Nevertheless, this work supports the idea that miRNAs preferentially target lineage transcription factors to repress NIS and induce loss of differentiation.

miRNA–transcription factor co-regulation

Increasing evidence suggests that a complex interplay exists between the two largest classes of transcriptional and posttranscriptional regulators – transcription factors and miRNAs – to buffer gene expression and/or potentiate signaling (Bracken *et al.* 2016). Reciprocal feedback loops in which a miRNA and transcription factor co-regulate the expression of one another constitute a recurring network motif that occurs more often than predicted by chance (Martinez *et al.* 2008). Indeed, experimental evidence shows that PAX8 induces the transcription of miR-146b having several binding sites in its regulatory region and,

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hence, PAX8 limits its own activity by inducing its own repressor, miR-146b-3p (Riesco-Eizaguirre *et al.* 2015). This constitutes a reciprocal feedback loop in which the miRNA and the transcription factor co-regulate the expression of one another. Other network motifs between miRNAs and transcription factors include coherent and incoherent feedforward loops, whereby miRNAs and transcription factors regulate common targets. In thyroid cells, miR-146b-3p represses *PAX8* and its downstream target NIS, as mentioned earlier. This miR-146b–PAX8–NIS circuit is an example of an incoherent feedforward loop whereby the transcription factor and miRNA have opposing (buffering) effects. These network motifs based on miRNA-transcription factor interactions are thought to strongly induce the propagation of the signal through the cell and explain why miRNAs can have a major impact on cell behavior, yet only modestly regulate most of their direct targets (Bracken *et al.* 2016).

The spreading of regulatory effects exerted by co-regulation of PAX8 and miR-146b sets an example of what may be operating in the thyroid cell. In fact, PAX8 and miR-146b share other common target genes such as *IYD* (DEHAL) and *DIO2* that are metabolizing-iodide genes influencing iodide uptake (Riesco-Eizaguirre *et al.* 2015). Other miRNAs-transcription factor regulation pairs may also be operating with other lineage transcription factors such as FOXE1 and NKX2.1 to regulate common iodide-metabolizing target genes (Riesco-Eizaguirre & Santisteban 2016). The extent to which the influence of miRNAs is mediated, not just directly through their primary targets but also indirectly through the action of the transcription factors they regulate, has been well established (Gosline *et al.* 2016). Most of the overall gene expression changes after miRNA perturbation through DICER RNase silencing occurred at the level of transcription, rather than posttranscriptionally. Particularly, DICER1 silencing in thyroid cells (which consequently depletes most miRNAs) decreased PAX8 and NKX2.1 expression and subsequently decreased NIS expression and activity (Ramírez-Moya & Santisteban 2021).

Some cautionary notes on miRNAs

To establish the role of miRNAs, we should consider their place in a network system and their capacity to target hubs in such networks. NIS is not a common direct target for miRNAs. The reason is that NIS 3'UTR is not conserved among species and algorithms predicting mRNA targets are largely based on how well conserved the 3'UTR is among species. Thus, NIS does not seem to be a hub in a network system. By contrast, *PAX8* and other transcription factors are targets of several upregulated miRNAs in thyroid cancer, with their 3'UTR being well conserved among species and having strong scores according to *in silico* predictions. Another important issue when considering miRNAs function is their abundancy. Abundantly expressed miRNAs (i.e. miR-21, miR-146b) are expressed at thousands of copies per cell, which is sufficient to affect many different mRNAs (Bracken *et al.* 2016). However, many miRNAs, while detectable in many tissues and tumors, are lowly expressed and their function is unlikely to have a real effect. Therefore, positioning miRNAs and their targets in a network system and considering their abundancy in a certain tissue are key aspects if we really want to know the biological impact of such molecules.

Hormones, growth factors, and iodide

I^- accumulation is an active transport process finely regulated by the endocrine system in a tissue-specific manner. NIS is regulated in the thyroid, the mammary glands, and the ovaries by different hormones in different ways (Table 1). In addition, several growth factors and cytokines may be acting in a more paracrine manner in the tissue microenvironment. Here we will focus on the role played by TSH, lactogenic hormones, cytokines (i.e. TGFβ), and iodide itself. All of them elicit not only a physiological role on NIS expression but also are at the core of important pathological situations.

TSH

TSH, a glycoprotein produced by thyrotrophs in the anterior pituitary, is the major regulator of thyroid function. It is well known that TSH stimulates I^- uptake in thyroid cells. Therefore, this hormone has been identified as the main regulator of NIS expression in the thyroid as it strongly increases NIS mRNA and protein levels (Kogai *et al.* 1997). TSH mediates its action through G protein coupling receptors (GPCR), mainly by activation of Gαs-mediated cAMP production. Although a TSH-responsive element, named Ntf-1, was identified in the rat proximal promoter, its activation in response to TSH is minimal (Ohmori *et al.* 1998), as mentioned in the previous section (Fig. 1). On the contrary, the NUE site is the regulatory region that recapitulates the action played by TSH and, as we will see later, by IGF1 and TGFβ. The NUE site activates transcription in a cAMP-dependent manner, mediated

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Table 1 NIS expression, regulation, role, and molecular size in normal thyroid and extrathyroidal tissues.

Tissue	Localization	Main role of NIS	Mature form of hNIS/rNIS (kDa)	Regulation
Thyroid	Basolateral of epithelial cells	• Thyroid hormone synthesis	95–105 90–100	• TSH • Iodide (I^-) (high I^- concentrations also modulate NIS protein biogenesis, subcellular localization, and degradation) • KCNQ1/KCNE2 K+ channel • Selenium • Thyroglobulin • Growth factors and cytokines (IGF1, TGFβ, TNFα, TNFβ, IFNγ, ILα, ILβ, and IL-6) • Reactive oxidizing species (ROS) • Iodinated contrast agents
Salivary gland	Basolateral of epithelial ductal cells	• Recycling of the body I^- pool arises from deiodination of iodo-compounds in peripheral tissues and secreted to saliva • To provide I^- as antioxidant and antimicrobial agent		• Constitutive
Stomach	Basolateral of mucin secreting and parietal cells, higher expression in foveolar epithelial cells than fundic glands	• Recycling of the body I^- secreted into the gastric juice • Provide I^- as protective antimicrobial function	115–120 110–120	• Constitutive
Small intestine	Apical at the brush border of enterocytes	• The I^- pool from the diet, plus that recycled in the saliva and the stomach, is absorbed in all three regions of the small intestine (duodenum, jejunum, and ileum)	100–115	• High I^- => NIS down-regulation
Lactating mammary gland	Basolateral of the mammary alveolar cells	• To provide I^- to the newborn during lactation • Provide I^- as protective agents as antioxidant and against a wide variety of microorganisms during late pregnancy and lactation	75–85 65–70	• β-Estradiol, oxytocin, prolactin
Ovary	Basolateral of the ovarian surface epithelium	• Contribute to I^- body-pool recycling • Implicated in maintaining sterile conditions throughout the female reproductive tract	95–105	• Estradiol and follicular maturation
Fallopian tube	Basolateral of fallopian fimbriae secretory cells	• Contribute to I^- body-pool recycling • Implicated in maintaining sterile conditions throughout the female reproductive tract	95–105	
Placenta	Apical membrane of villous syncytiotrophoblasts (maternal-facing) and high expression is detected in villous syncytiotrophoblast cells	• To provide I^- from the maternal to the fetal circulation, a process critical for normal fetal thyroid function • Provide I^- than participate as an antioxidant and as a defense against infections	65–70	• hCG • Placental vascularization
Testis	Basolateral and cytoplasmic of germinal and Leydig cells			
kidney	Basolateral in the proximal tubular cells and cytoplasmic in the distal ones	• I^- elimination		
	Apical surface of the proximal and cortical collecting tubes	• I^- reabsorption		
Lung	Basolateral in the ciliated columnar cells of the bronchial mucosa	• To provide I^- as innate antimicrobial defense		

Other tissues: adrenal, appendix, bile duct, bladder, choroid plexus, ciliary body, colon, endometrium, eye, gall bladder, lacrimal sac, nasolacrimal duct, pancreas, prostate, small bowel, spleen, and uterus.

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by both PKA-dependent and independent pathways (Ohno *et al.* 1999, Taki *et al.* 2002, Chun *et al.* 2004). The mechanism involves a synergistic action between PAX8 and several other factors binding to the CRE-like site (see previous section) (Ohno *et al.* 1999).

Although the TSH effect on NIS expression and function is mainly mediated by activation of Gαs-mediated cAMP production, TSH also induces the release of other G proteins such as Gβγ dimers (Fig. 2). These Gβγ dimers stimulate PI3K signaling which in turn inhibits NIS gene expression in a cAMP-independent fashion due to a decrease in PAX8 binding to the NIS promoter (Zaballos *et al.* 2008). This action of TSH occurs mainly at high doses of the hormone, a mechanism that is thought to counterbalance the accumulation of iodide in the gland.

Finally, the TSH effect is not only elicited at a transcriptional level. Several data suggest that mechanisms other than transcriptional operate to regulate NIS activity in response to TSH (Kogai *et al.* 1997). TSH increases NIS protein, modifies its glycosylation, and influences its location at the basolateral membrane and its redistribution inside the cell vesicles (Levy *et al.* 1998, Riedel *et al.* 2001) (see also NIS traffic regulation section).

In summary, all these data reveal that TSH is the major regulator of NIS activity and I^- accumulation into the thyroid gland. TSH regulates NIS at a transcriptional, posttranscriptional, and posttranslational level, underscoring the complexity and multi-level regulation of NIS in physiological conditions.

Estrogen and lactogenic hormones

NIS is expressed in the mammary gland and in the ovary and fallopian fimbriae only in certain physiological conditions that seem to be related with the rise of estrogens and other hormones (Table 1). NIS expression in the mammary gland progressively increases toward the end of gestation and is most prominent during lactation. *In vivo* experiments in ovariectomized mice show that the combination of estrogen, prolactin, and oxytocin (in the absence of progesterone) leads to the highest levels of NIS expression in the mammary glands (Tazebay *et al.* 2000, Dohán *et al.* 2006). This combination of hormones closely resembles the relative hormonal levels in mice and rats during lactation. Of note, of all the hormones tested individually, only 17-β-estradiol led to a clearly discernible increase in NIS expression, but not oxytocin or prolactin. The ovary and the fallopian fimbriae express NIS, accumulating a significant amount of radioiodide *in vivo* both in rats and humans, being highest under maximum estrogen exposure during the reproductive cycle. This important role of estrogens in regulating ovarian NIS is reinforced by evidence demonstrating that ERα strongly stimulates NIS transcriptional activity in both ligand-dependent and -independent manner at the NUE region (Riesco-Eizaguirre *et al.* 2014). However, in the thyroid, ERs seem to be operating in the opposite way, as estradiol inhibits TSH-induced NIS expression in FRTL5 cells (Furlanetto *et al.* 1999).

Cytokines and growth factors

TSH upregulates rat NIS gene expression *in vitro*, and this induction can be modulated by cytokines. Early studies demonstrated that cytokines including interleukin-6 (IL-6), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-1α (100 U/mL), IL-1β, and TGFβ downregulated NIS function to a greater or lesser extent (Ajjan *et al.* 1998, Pekary *et al.* 1998, Spitzweg *et al.* 1999) (Table 1). Both infiltrating inflammatory cells and thyroid follicular cells produce cytokines, which affect thyroid function and growth and cause immunological changes in the gland. As cytokines are involved in the pathogenesis of autoimmune thyroid disease such as Graves' disease and Hashimoto's thyroiditis, they may explain in part the changes in NIS expression patterns seen in such forms of autoimmune thyroid disease, as well as the mechanism underlying autoimmune hypothyroidism.

Among cytokines, the mechanism played by TGFβ has been the most studied (Fig. 2). TGFβ exerts an inhibitory effect on thyroid function, including a decrease in NIS expression and I^- uptake (Kawaguchi *et al.* 1997, Pekary *et al.* 1998). TGFβ decreases NIS expression by reducing TSH transcriptional activation. The region responsible for this effect is the enhancer NUE, and the mediator of this action is the SMAD3 protein which interacts with PAX8 inhibiting its binding to NUE and, therefore, impairing NIS transcription (Costamagna *et al.* 2004). TGFβ plays a key role in thyroid cancer as $BRAF^{V600E}$, the most prevalent oncogene in thyroid cancer, is a strong inducer of TGFβ secretion both *in vitro* and *in vivo* (Riesco-Eizaguirre *et al.* 2009, Knauf *et al.* 2011), taking part in tumor progression and dedifferentiation (see 'Hormones, growth factors, and iodide' section) (Fig. 2). In addition, the inhibitory effect of TGFβ on NIS expression is not exclusive of thyroid tissue as TGFβ inhibits NIS mRNA and protein expression in cultured mammary gland explants from lactating mice (Yu *et al.* 2012). Lately, another cytokine, TNF-α, an activator of NF-κB with a central role in thyroid autoimmunity, has been studied in more detail. TNF-α downregulates

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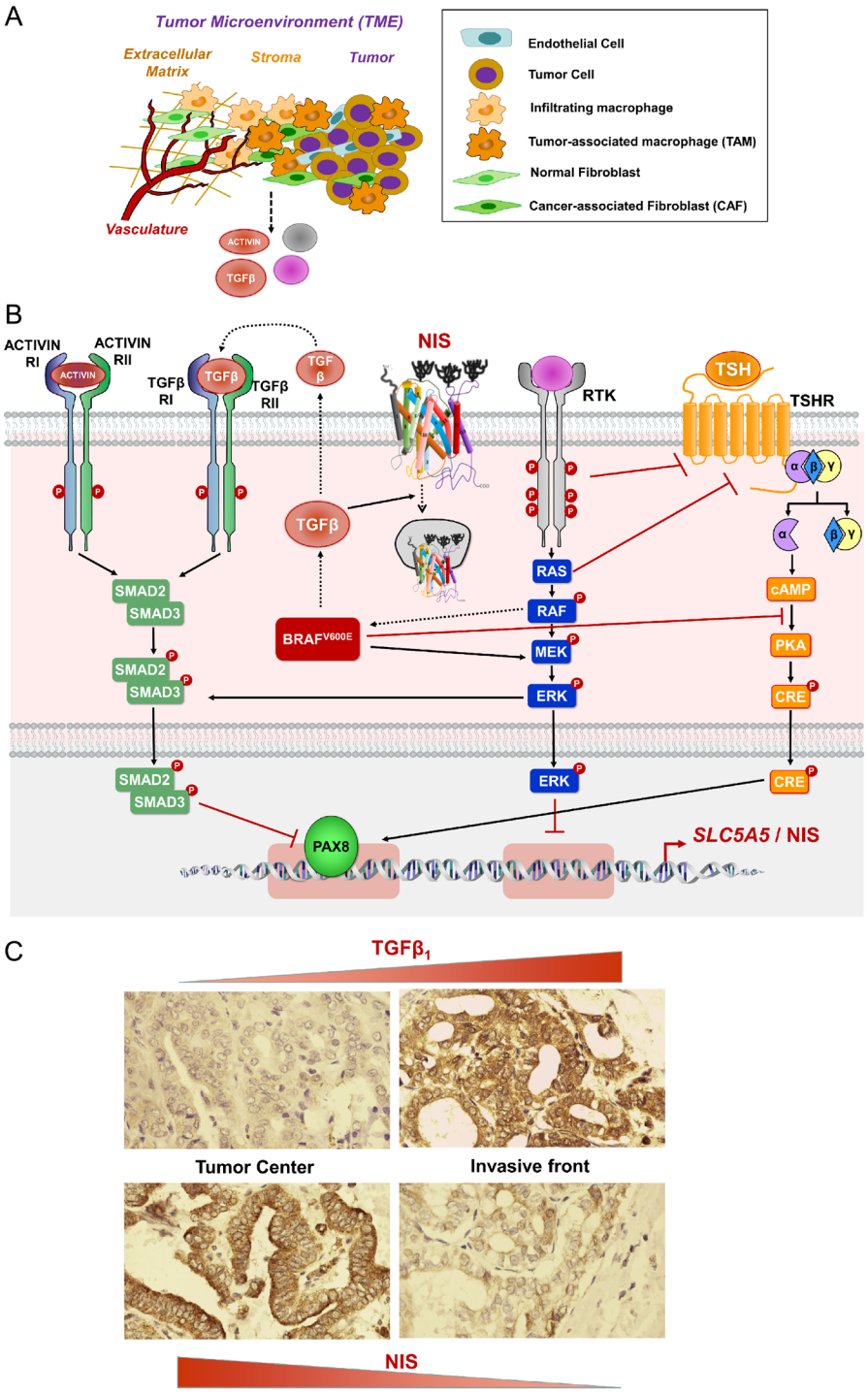

Figure 2

Crosstalk between the MAPK and TGFβ signaling pathways leading to NIS repression in thyroid cancer. (A) Tumor-associated macrophages and other cells from the stroma in the tumor microenvironment contribute to TGFβ1 secretion and other ligands such as activin. (B) In thyroid cancer cells, $BRAF^{V600E}$ induces a strong TGFβ1 and activin secretion, promoting an autocrine loop that evokes a SMAD3-dependent downregulation of PAX8 and inhibition of its binding to the NUE promoter. $BRAF^{V600E}$ also induces pERK phosphorylation at the T8 residue of SMAD, promoting its additional phosphorylation and activation by the TGFβ1 receptor. RET/PTC and $BRAF^{V600E}$ also decrease expression of the TSHR, which could also explain in part NIS repression and loss of differentiation. In addition, $BRAF^{V600E}$, but not RET/PTC, inhibits cAMP-induced levels of NIS, suggesting an alternative mechanism distal to cAMP through unknown mechanisms that are SMAD-independent. Finally, $BRAF^{V600E}$ also impairs NIS targeting to the plasma membrane, likely through TGFβ1. (C) Immunoreactive TGFβ1 is strongly increased in the invasive front of PTCs, whereas NIS expression is present mainly in the central areas of the tumors but not in the invasive front, suggesting that a negative correlation between TGFβ1 and NIS occurs also locally inside the tumor.

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TSH-induced NIS expression in the thyroid follicular cell through NF-kB activity (Pekary *et al.* 1998, Faria *et al.* 2020).

In addition, studies carried out prior to the cloning of NIS in FRTL5 thyroid cells showed that in the absence of serum, IGF1 and insulin impaired the ability of TSH/cAMP to induce iodide uptake (Saji & Kohn 1991). After the cloning of NIS, it was demonstrated that IGF1 inhibits the TSH/cAMP-induced NIS expression at the transcriptional level (García & Santisteban 2002). This transcriptional effect involved NIS promoter region closed to NUE, and unpublished results of our laboratory have shown that IGF1 inhibits the binding of PAX8 to NUE (García B, Costamagna E & Santisteban P, unpublished observations). This inhibitory effect is mainly mediated by PI3K activation, as AKT inhibitors restore iodide uptake in the presence of IGF1. Because PI3K/AKT/mTOR signaling pathway is one major pathway driving cancer growth in thyroid cancer (Xing 2010), targeting this pathway with inhibitors may be a good therapeutic strategy to reinduce NIS expression and iodide uptake.

Is NIS a major autoantigen?

In the late 1990s, the hypothesis that NIS represented a major thyroid autoantigen that elicits the formation of functionally relevant autoantibodies attracted much attention (Spitzweg & Morris 2000). NIS-directed antibodies are present in sera from patients with autoimmune thyroid disease, and these antibodies may also affect NIS functional activity. However, convincing evidence further showed that NIS-directed autoantibodies occur with low frequency among a large sample of patients with autoimmune thyroid diseases (Seissler *et al.* 2000). In addition, no evidence of specific iodide uptake inhibiting activity was obtained once sera had been subjected to dialysis and/or IgG extraction (Chin *et al.* 2000, Tonacchera *et al.* 2001). Moreover, when detected in addition to TPO and TSH receptor autoantibodies, NIS-directed autoantibodies do not appear to contribute any diagnostic power for Graves' disease and Hashimoto's thyroiditis (Heufelder *et al.* 2001). Therefore, NIS does not appear to be a major functionally relevant antigen in autoimmune thyroid diseases.

Iodide

Another regulator of NIS function is iodide itself (Table 1). It has been long established that an excess of iodide inhibits its own transport into the thyroid cell (Braverman & Ingbar 1963). Inhibition of iodide transport in FRTL5 cells by an excess of iodide seems to be mediated by an intracellular iodinated compound, as methimazole (MMI), an inhibitor of iodide organification, prevented such effect (Grollman *et al.* 1986). After the cloning of NIS in 1996, however, it became clear that NIS response to an excess of iodide was more complex than initially thought, with dissimilar results in rat thyroids *in vivo* than in thyroid cultured cells. In rats, an excess of iodide decreases NIS mRNA and protein levels at 1 and 6 days in what seems to be, at least in part, a transcriptional mechanism most likely mediated by an intracellular iodinated compound (Eng *et al.* 1999, Leoni *et al.* 2011). However, at shorter times (i.e. 5 h), the decrease of iodide uptake in rats cannot be attributed to a transcriptional mechanism because there is a much modest variation on mRNA or protein levels (Leoni *et al.* 2011). Moreover, it can neither be attributed to impaired NIS trafficking as the symporter remains at the plasma membrane (Arriagada *et al.* 2015). This suggests that, in addition to a transcriptional mechanism, an early posttranslational mechanism is taking place *in vivo*. On the other hand, in cultured thyroid cells (FRTL5 and PCCL3) this posttranslational mechanism seems to be most prominent. While a strong inhibition of iodide uptake both at short (2–5 h) and long times (24–72 h) intervals is seen, the effect on NIS mRNA and protein levels is transient and much more modest than in rats (Leoni *et al.* 2011, Arriagada *et al.* 2015). Moreover, neither NIS promoter activity nor PAX8 expression or its binding to DNA was modulated by ioidide *in vitro*.

Therefore, thyroid cultured cells are excellent models to study the posttranslational effect of iodide excess on NIS. One possible mechanism is based on alterations in the redox state. Iodide excess triggers an early and sustained increase in reactive oxidative species (ROS) production which in turn induces posttranslational modifications in NIS at the plasma membrane (Leoni *et al.* 2011). The inhibitory effect on NIS-mediated I^- transport could be recapitulated by H_2O_2 and reverted by reactive derived oxygen species scavengers (Arriagada *et al.* 2015). On the contrary, inhibition of the selenoprotein thioredoxin-reductase, which increases ROS production, increased NIS inhibition induced by iodide excess (Leoni *et al.* 2011). This attractive hypothesis suggests that the redox state of thyroid cells directly modulates NIS activity, perhaps through the effect on aa modifications or the oligomeric state of the symporter. In support of this, another modulator of the redox state such as selenium seems to increase iodide uptake through a reduction of ROS species (Leoni *et al.* 2016).

Understanding the effects of iodide excess on NIS function have important clinical implications. Excess of I^- inhibits the synthesis of TH, a process described a long

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time ago and known as the Wolff–Chaikoff effect (Wolff & Chaikoff 1948). Wolff and Chaikoff reported that high plasma iodide levels blocked iodide organification in rat thyroid *in vivo* leading to a reduction in the synthesis of thyroxine. However, there is an 'escape' or adaptation from this acute Wolff–Chaikoff effect that restores normal TH biosynthesis in approximately 2 days even in the continued presence of high plasma I^- concentrations (Wolff & Chaikoff 1949). It has been postulated that recovery of thyroid function is mediated by the early inhibition of I^- uptake through a reduction of NIS activity, which would alleviate the high intracellular iodide concentrations and restore TH biosynthesis (Braverman & Ingbar 1963, Eng *et al.* 1999, 2001). As mentioned earlier, the molecular mechanisms underlying this adaptive effect seem to be most related to the redox state, as iodide induces high levels of ROS impairing NIS activity. Eventually, the action of the selenoprotein thioredoxin reductase will reduce ROS production restoring NIS function (Leoni *et al.* 2011, De la Vieja & Riesco-Eizaguirre 2021).

Signaling pathways in NIS repression

The central role of MAPK signaling

PTC is a MAPK-driven tumor and this signaling pathway is considered to play a major role in repressing NIS and other thyroid differentiated genes (Fig. 2). Approximately more than 80% of human PTCs are associated with mutually exclusive mutations of RET/PTC or TRK, RAS (NRAS > HRAS > KRAS), or BRAF (Kimura *et al.* 2003, Soares *et al.* 2003). The oncoproteins encoded by these genes share the common property of constitutively activating MAPK signaling, which has been taken as evidence supporting a critical role of this pathway in the pathogenesis of the disease. Further evidence provided by TCGA showed that these oncoproteins activated MAPK signaling with different intensities that directly correlated to the degree of dedifferentiation (Agrawal *et al.* 2014). In other words, the higher the intensity of MAPK signaling, the higher the repression of NIS and other iodide-metabolizing genes.

This concept, that the expression of NIS and other iodide-metabolizing genes are particularly sensitive to the activity of the MAPK pathway, was first pointed in PCCL3 cells, which retain most of the differentiated properties of normal thyrocytes. Conditional activation of RET/PTC oncogene downregulates the expression of NIS, TSHR, TG, TPO, and PAX8 (De Vita *et al.* 1998, Portella *et al.* 1999). Similarly, conditional expression of oncogenic RAS, BRAF, or of constitutively active MEK recapitulates the inhibitory effect on NIS which is partially restored by treatment with MEK inhibitors (Fig. 2) (Mitsutake *et al.* 2005, 2006, Riesco-Eizaguirre *et al.* 2006, Liu *et al.* 2007*a*). Both RET/PTC and BRAF decrease expression of the TSHR (Wang *et al.* 2003, Mitsutake *et al.* 2005), which could explain in part this loss of differentiation. However, the mechanisms by which RET/PTC and BRAF interfere with TSH action distal to the receptor are different. Whereas RET/PTC markedly impairs adenylyl cyclase activity (Wang *et al.* 2003), BRAF does not affect forskolin-induced cAMP levels. BRAF inhibits cAMP-induced levels of NIS mRNA as well as NIS protein expression and membrane targeting, suggesting an alternative mechanism distal to cAMP (Mitsutake *et al.* 2005, Riesco-Eizaguirre *et al.* 2006).

In addition to PCCL3 cells, experiments in animal models have been particularly illustrative (Knauf *et al.* 2005, Chakravarty *et al.* 2011). Transgenic mice with doxycycline (dox)-inducible expression of $BRAF^{V600E}$ in thyroid follicular cells –which resembles high-grade PTCs found in humans– virtually abolished thyroid-specific gene expression including NIS and radioiodide incorporation, all of which were restored to near basal levels upon discontinuation of dox (Chakravarty *et al.* 2011). Discontinuation of dox also reestablished thyroid follicular architecture and normal thyroid histology which confirm an exquisite dependence on BRAF oncoprotein for transformation. Treatment with MEK or mutant BRAF inhibitors partially restored thyroid-specific gene expression and rendered the tumor cells susceptible to a therapeutic dose of radioiodide. However, these MAPK inhibitors had a more attenuated effect on the restoration of NIS expression levels, as well as those of TPO and TG, compared with those in dox-withdrawn mice, and therefore, the therapeutic effect of radioiodide treatment was weaker (Chakravarty *et al.* 2011). This suggests that to achieve full therapeutic benefit with MAPK inhibitors, simply dimming the signaling pathway is not enough, it needs to be almost completely suppressed, evidence of which has been shown in other cancer models (Bollag *et al.* 2010).

Therefore, potent inhibition of ERK signaling seems to be required to adequately induce iodide uptake, rendering tumor cells susceptible to a therapeutic dose of radioiodide. An allosteric MEK inhibitor (CKI) that functions as a dominant-negative inhibitor of RAF kinases (both WT and mutant) and reduces feedback reactivation of ERK signaling has been shown to achieve a potent and sustained inhibition of extracellular-regulated kinase (ERK) signaling in thyroid cells and in mice with $BRAF^{V600E}$-induced thyroid cancer (Nagarajah *et al.* 2016). The authors

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of this study found that a reduction of expression of MAPK transcriptional output markers from 70 (with selumetinib or low-dose CKI) to 85% (with high-dose CKI) had profound reciprocal effects on the expression of NIS, TG, and TSHR. Under such high-dose CKI treatment for as little as 8 days is sufficient to enable effective radioiodide therapy. However, toxicity of achieving high level ERK pathway inhibition can be a constraint on these approaches.

Subsequent clinical trials have been performed to test the effectiveness of MAPK inhibitors to restore iodide uptake and enable patients with radioactive iodide refractory thyroid cancers to be treated with high doses of I-131. The first proof-of-principle clinical trial was performed by Ho *et al.* from the Memorial Sloan Kettering Cancer Center in 24 patients with advanced thyroid cancer, confirming the ability of the MEK inhibitor selumetinib to restore radioiodide in tumors previously shown to be radioiodide resistant (Ho *et al.* 2013). This promising redifferentiation strategy has been extensively addressed in a recent review by Buffet *et al.*, summarizing all the MAPK inhibitors tested so far, including MEK inhibitors (selumetinib and trematenib) and BRAF inhibitors (dabrafenib and vemurafenib) (Buffet *et al.* 2020). Nevertheless, although MAPK inhibitors have shown to increase iodide accumulation in a significant fraction of patients with refractory metastatic thyroid cancers, such inhibitors are insufficient to induce major clinical responses to radioiodide in many of them. In line with preclinical animal models, one of the conclusions from all these clinical studies reveals that the degree of iodide avidity restoration is linked to the degree of MAPK pathway output inhibition, and toxicity derived from such inhibition is a major limitation of this approach (Nagarajah *et al.* 2016).

The crosstalk between MAPK and TGFβ signaling

The mechanism through which MAPK signaling inhibits NIS expression relies partially on TGFβ signaling (Fig. 2). As mentioned earlier, $BRAF^{V600E}$ strongly induces TGFβ secretion in thyroid cancer cell lines and animal models, having a major role in EMT and tumor progression (Riesco-Eizaguirre *et al.* 2009, Knauf *et al.* 2011). In addition, ligands other than TGFβ1 are also activating this pathway since the activin ligand subunits Inhba and Inhbb are overexpressed in $BRAF^{V600E}$-mutant thyroid cancers transgenic mice (Luckett *et al.* 2021) (Fig. 2B). In human samples, high levels of TGFβ and other components of the signaling pathway such as TβRII and p-SMAD are present in aggressive PTCs, being predominantly expressed in the invasive front of the primary tumors as well as in lymph node metastases, suggesting a widespread activation of this pathway by locally released TGFβ (Vasko *et al.* 2007, Riesco-Eizaguirre *et al.* 2009, Eloy *et al.* 2012). Interestingly, while this high TGFβ/SMAD activity is predominant in the invasive front of the tumors, NIS is preferentially expressed in the central regions, suggesting that this negative correlation between TGFβ and NIS occurs locally inside the tumor (Riesco-Eizaguirre *et al.* 2009) (Fig. 2C). BRAF induces a TGFβ autocrine loop which downregulates PAX8 and evokes a SMAD3-dependent inhibition of PAX8 binding to the Nis promoter (Costamagna *et al.* 2004, Riesco-Eizaguirre *et al.* 2009) (Fig. 2B). In addition, BRAF-induced TGFβ1 induces large amounts of ROS through NOX4, a NADPH oxidase highly expressed in thyroid cancer, and such TGFβ-induced ROS has been shown to repress NIS (Azouzi *et al.* 2017). Finally, two other considerations are worth to be noted. First, TGFβ delocalizes NIS from the plasma membrane altering its trafficking to the membrane through a yet unknown mechanism (Costamagna E, Riesco-Eizaguirre G & Santisteban P, unpublished observations) (Fig. 2B). Secondly, cells in the tumor microenvironment other than $BRAF^{V600E}$-PTC cells also contribute to the intratumoral pool of TGFβ and activin, including tumor-associated macrophages and other still unknown cells (Fig. 2A); thus, targeting TGFβ/SMAD would also block the inhibitory effects exerted by tumor microenvironment.

Several TGFβ signaling inhibitors have been shown to reinduce iodide uptake in PCCL3 cells expressing BRAF or RET/PTC, including small kinase inhibitors of TβRI and a natural TGFβ1 inhibitor, apigenin (Riesco-Eizaguirre *et al.* 2009, Lakshmanan *et al.* 2015*b*). A very recent work in $BRAF^{V600E}$-induced thyroid cancer in mice has demonstrated that vactosertib, a potent inhibitor of SMAD, was not able to restore expression of NIS or iodide uptake alone, yet the combination of either vactosertib or follistatin (a potent inhibitor of activin) and CKI (a MEK inhibitor) increased iodide uptake compared to CKI alone (Luckett *et al.* 2021). All these findings in cell lines and animal models indicate that these two pathways closely converge in the repression of NIS and iodide uptake in thyroid cancer cells (Fig. 2). The precise mechanism underlying the additive effect of SMAD and MEK inhibition on iodide uptake remains to be fully explored.

Clinical trials looking into synergistic effects of blocking SMAD and MAPK pathways in thyroid cancer look promising and are yet to be explored. Because TGFβ has also profound local immunosuppressive and immunoexclusion effects in the tumor microenvironment that are integrally involved in the failure of immune checkpoint inhibitors in some tumors, there are currently

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phase II clinical trials testing TGFβ inhibitors in refractory advanced and metastatic malignancies (NCT03834662). However, TGFβ acts as a tumor suppressor in normal tissues and early stages of cancer, making inhibition of TGFβ signaling challenging due to unwanted side effects. Therefore, blocking SMAD and MAPK pathways in thyroid cancer patients for short intervals followed by radioiodide treatment could be a reasonable strategy to limit drug toxicity (Huynh *et al.* 2019).

PI3K signaling pathway and others

PI3K/AKT signaling pathway plays an important role in tumor initiation and progression of thyroid cancer and has been shown to repress NIS and other iodide-metabolizing genes (Hou *et al.* 2010, Xing 2010). As mentioned earlier, in normal thyroid cells, IGF1 represses NIS transcriptional activity through PI3K/Akt signaling in TSH stimulated cells (García & Santisteban 2002), and high levels of TSH also stimulates AKT phosphorylation through Gβγ dimers released from G protein coupled to the TSHR, restraining cAMP-induced NIS expression (Zaballos *et al.* 2008). Thus, in normal thyroid cells, the PI3K/Akt pathway counterbalances the stimulatory effects of TSH.

Pharmacological inhibition of the PI3K pathway increases functional NIS expression in rat thyroid cells and human papillary thyroid cancer cells by several mechanisms, including posttranslational modifications (Kogai *et al.* 2008, Liu *et al.* 2012). Inhibitors for PI3K, AKT, or mTORC1 increase iodide uptake in thyroid PCCL3 cells under chronic TSH stimulation. However, although AKT inhibition increases NIS-mediated radioiodide uptake, it does not increase NIS protein levels (Liu *et al.* 2012). The authors suggest that this signaling pathway is promoting a posttranslational modification of NIS, that is, a change in its phosphorylation status, yet it is still a hypothesis that needs further confirmation. In addition, activation of PI3K/AKT signaling through MTOR leads to significant repression of iodide uptake (Plantinga *et al.* 2014, Tavares *et al.* 2018). Therefore, several inhibitors of this pathway have been proved to reinduce NIS expression and/or ^{131}I uptake (Hou *et al.* 2010). However, while the evidence targeting MAPK signaling pathway has been shown to be successful in cell lines, mice and humans, evidence targeting PI3K pathway is restricted to cell models.

Other pathways such as the NOTCH signaling pathway have been linked to the regulation of NIS and other thyroid-specific gene expression. Pharmacological Notch1 activating compounds such as resveratrol (Yu *et al.* 2013) or hesperetin (Patel *et al.* 2014) have been shown to induce NIS mRNA levels and other differentiation markers *in vitro* in ATC cell lines. Interestingly, other compounds such as the HDAC inhibitors valproic acid and SAHA or retinoic acid have also been reported to activate Notch signaling (Xiao *et al.* 2009) .

Trafficking to the membrane

NIS needs to be targeted to the basolateral plasma membrane to actively transport iodide (Fig. 3). The trafficking of NIS to the membrane seems to be a process finely regulated by TSH (Riedel *et al.* 2001). Deprivation of TSH results in the reduction of NIS protein half-life from 5 to 3 days and redistribution of NIS protein from the cell surface to intracellular compartments. In a similar way, $BRAF^{V600E}$ redistributes NIS protein from the cell surface to intracellular compartments when it is conditionally induced in thyroid cells (Riesco-Eizaguirre *et al.* 2006). $BRAF^{V600E}$ impairs TSH/cAMP signaling and also induces TGFβ secretion, a strong NIS inhibitor which not only represses NIS transcriptionally (Riesco-Eizaguirre *et al.* 2009) but also alters its trafficking (Costamagna E, Riesco-Eizaguirre G & Santisteban P, unpublished observations) (Fig. 2). Understanding how such important physiological and oncogenic events mediated by TSH and BRAF, respectively, affect NIS trafficking at a molecular level is clinically very relevant. In many human thyroid and breast cancers, NIS is still expressed or even overexpressed (Fig. 4), but unable to transport iodide because it is mainly localized in the cytoplasm (Wapnir *et al.* 2004). Therefore, targeting NIS transcriptional re-expression is undoubtedly a necessary step but not enough to render an active protein at the membrane.

The mechanisms governing NIS subcellular trafficking remain largely unknown, yet there have been some advances summarized in Fig. 3. The first attempts to elucidate the mechanisms involved in NIS trafficking focused on the posttranslational modifications of the protein, mainly glycosylation and phosphorylation. NIS is a highly glycosylated protein; there are three N-linked glycosylation sites in the mature form of NIS. Using site-directed mutagenesis in NIS-expressing COS cells, Carrasco's group demonstrated that glycosylation affects the function and stability of NIS to a certain extent, but the partial or total absence of N-linked glycosylation did not dampen much NIS activity, ranging from 90 to 50%, respectively (Levy *et al.* 1998). Further studies in thyroid and breast cancer cells using different glycosylation inhibitors have shown that alteration in the glycosylation

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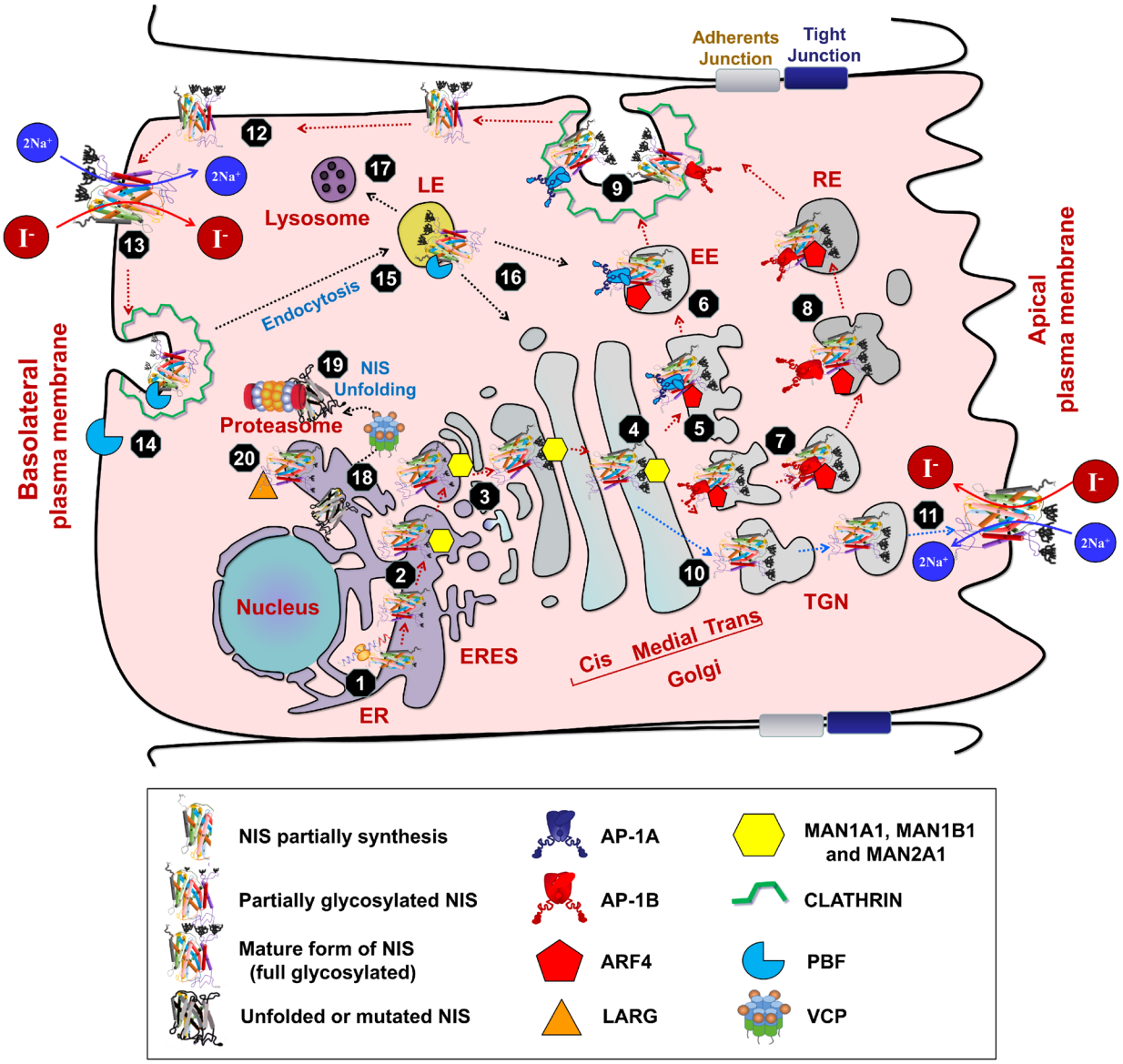

Figure 3

Mechanism of synthesis, PTM, trafficking, and degradation of NIS in thyroid and extra-thyroid. After ribosomal protein biosynthesis (1), NIS folding and N-glycosylation processes start at the ER (2). PIGU, a protein that catalyzes glycosylphosphatidylinositol in the ER, may be involved in NIS glycosylation process. This protein is regulated by MAPK signaling. Several enzymes are implicated in N-glycans protein processing. Three mannosidase enzymes (Man1a1, Ma1b1, and Man2a1) participate in NIS N-glycosylation at the GGolgi (3–4). General alteration of glycosylation in cells affects NIS trafficking. Specifically, alteration in expression levels or function of these mannosidases causes defective NIS glycosylation that affects NIS maturation and accumulation in cytoplasm organelles. Final maturation of glycosylation occurs in the trans GGolgi (4). Then, AP-1A (5,6) and AP1B (7,8) clathrin adaptor proteins are implicated in NIS trafficking from GGolgi to the basolateral plasma membrane (9). Interaction of the AP-1B subunit σ1 with an IL motif at NIS C-terminal is important for correct basolateral trafficking in epithelial cells. In the absence of AP-1 clathrin adaptors, as in kidney, small intestine, duodenum, colon, and cytotrophoblasts in placenta cells, NIS traffics to the apical plasma membrane (10–11). ARF4 binding to the VAPK motif in NIS participated in NIS trafficking from GGolgi to the plasma membrane (5–8). NIS is distributed throughout the plasma membrane (9, 12) where it exerts its iodide sodium-dependent function (13). NIS half-life at the plasma membrane is around 5 days. After this, NIS endocytosis begins. PBF is known to participate in the endocytic pathway of NIS (14). Phosphorylated PBF at residue Y174 causes NIS internalization from plasma membrane to cytoplasm in a clathrin-dependent endocytosis. Then, NIS in the LE can be recycled to the ER, LE, or RE (16) and back to the plasma membrane, or go to lysosome for degradation (17). VCP participates in NIS degradation at the ER through the proteasome (19). In addition, unfolded or cytoplasmic retained NIS (18) go to proteasome degradation (19). LARG interaction with the C-terminal PDZ motif of NIS causes NIS cytoplasm retention (20), which increases cell motility and invasion in tumor cells. This interaction is, at least in part, mediated by PTEN alteration. Nomenclature: (A) proteins: ARF4, ADP-ribosylation factor 4; LARG, leukemia-associated RhoA guanine exchange factor; PBF, pituitary tumor-transforming gene I binding factor; VCP, valosin-containing protein; PIGU, phosphatidylinositol glycan anchor biosynthesis class U; (B) subcellular organelles: ER, endoplasmic reticulum; ERES, ER export sites; EE, early endosome; RE, recycling endosome; LE, late endosome; TGN, trans-GGolgi network.

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A

	NIS Protein level				*SLC5A5* mRNA level	
Type of Cancer	Protein Expression Tumor vs. Normal tissue	% NIS positive staining	% cells with some PM staining	Are some of the metastatic tissue positive?	mRNA Expression Tumor vs. Normal tissue	fold [increase or decrease]
Bladder	↓	42			↑	3.5
Breast	↑	80	24	Yes	↑	2.3
Cervix	↑	100			↑	1.4
Colon	=	63			=	1.0
Endometrial	↑	56			↑↑	8.0
Esophagus	↑	47			↑↑	5.3
Gastric	↓	59			↓↓	-10.0
Kidney	↓	29			↑	2.0
Liver*	↓	20			=	1.1
Lung	↑	66			↑	1.5
Melanoma	↑	33			=	1.0
Ovarian	↑	95	33		↑↑	10.0
Pancreas	↓	64			↑↑	7.5
Prostate	↓	74			↑	1.4
Salivary	↓	32			↓	-7.0
Skin squamous	↑	56			=	1.0
Testicular	↑	76			↑	2.6
Thymus	↑	100			=	1.2
Thyroid	↓	73		Yes	↓↓	-10.0

B

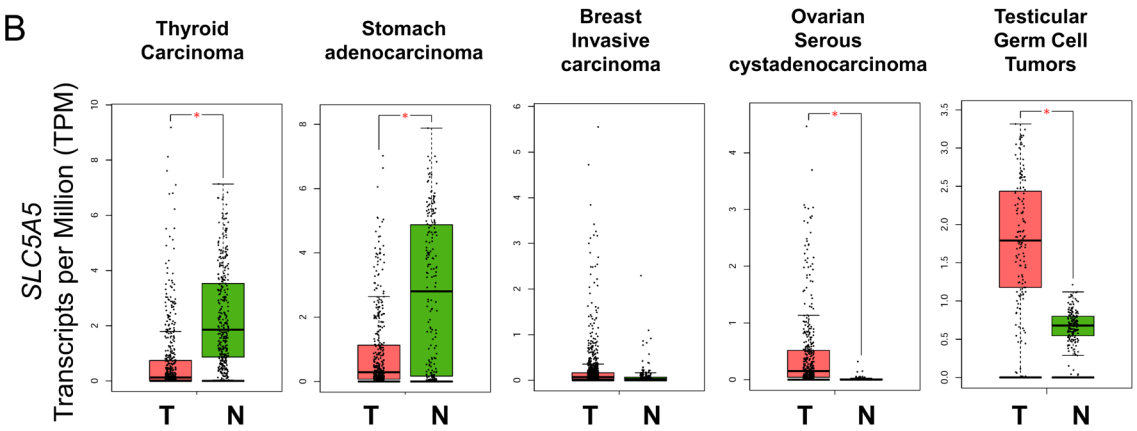

Figure 4

NIS expression regulation in tumors is tissue-dependent. (A) Levels of NIS protein and mRNA expression in tumors vs normal tissue. Relative protein levels were obtained in several studies by tissue-array. In addition, % of NIS-expressing positive cells within the tumors, % of cells expressing NIS at the plasma membrane, and metastatic cells expressing NIS are indicated. mRNA levels in tumor vs normal tissue are obtained in the GEPIA (gene expression profiling interactive analysis) portal. (B) Variation of NIS expression in five relevant tumors (T, red colors) vs normal tissue (N, green colors) is represented in box-blot. Data obtained in GEPIA portal. Normalization of RNA-seq data is represented as transcripts per million (TPM).

process affects NIS trafficking to the membrane (Beyer *et al.* 2011, Chung *et al.* 2015, Feng *et al.* 2018). However, because these inhibitors also inhibit the glycosylation of many other proteins, the specific role of NIS glycosylation is unclear. Recently, three mannosidases (MAN1A1, MAN1B1, and MAN2A1) have been proposed to be implicated specifically in NIS glycosylation (Rathod *et al.* 2019). These enzymes are likely able to condition the degree of NIS glycosylation in different tissues and tumors, making NIS have different molecular weights in the thyroid (see 'Trafficking to the membrane' section and Table 1) (De la Vieja *et al.* 2000, Riesco-Eizaguirre *et al.* 2014). NIS is also a phosphoprotein and the carboxy terminus, where most of the phosphorylation occurs under the regulation of TSH, was initially proposed to have a role in NIS trafficking and its localization at the basolateral

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plasma membrane (Riedel *et al.* 2001). Notwithstanding, using site-directed mutagenesis, the phosphorylation status of several aa residues, including those in the carboxy terminus, do not seem to affect NIS cell surface trafficking (Vadysirisack *et al.* 2007). Recent evidence has shown that a conserved monoleucine-based motif at the carboxy terminus influences the trafficking route of NIS to the basolateral or the apical membrane in polarized epithelial cells (Koumarianou *et al.* 2014, Martín *et al.* 2019). The interaction of such motif with the clathrin adaptor protein AP-1B subunit σ1 serves as a sorting signal that allows for the correct trafficking of NIS to the basolateral membrane, while the absence of AP-1 clathrin adaptors makes NIS traffic to the apical membrane (Fig. 3).

In recent years, some proteins have been identified to interact with NIS and modulate its subcellular localization (Fig. 3). The first protein shown to bind NIS and affect its trafficking was PBF (pituitary tumor-transforming gene (PTTG1)-binding factor). As mentioned in 'Promoters and enhancers regulating NIS transcription' section, this proto-oncogene acts mainly at the cell nucleus along with PTTG1 driving thyroid tumorigenesis via different mechanisms and repressing NIS transcription through USF1 (Stratford *et al.* 2005, Boelaert *et al.* 2007). However, PBF has been shown to act independently of PTTG and out of the nucleus, predominantly within intracellular vesicles and at the plasma membrane (Smith *et al.* 2009, 2011). It has a functional endocytosis motif that binds NIS and redistributes NIS away from the plasma membrane into late endosomes, particularly into clathrin-coated CD63-positive late endosomes, resulting in significantly repressed cellular iodide uptake (Fig. 3) (Smith *et al.* 2009). Evidence of PBF´s role in thyroid tumorigenesis is supported by the high levels of this protein found in human thyroid cancer, particularly in the most aggressive tumors that are refractory to radioactive iodide and by transgenic mice in which targeted overexpression of PBF induces thyroid hyperplasia and goiter (Read *et al.* 2011).

Apart from NIS regulation through an endocytosis-mediated pathway, very recently two other proteins have been shown to participate in NIS trafficking at other levels both in breast and thyroid cancer models. McCabe´s group have identified that ADP ribosylation factor 4 (ARF4) and valosin-containing protein (VCP) specifically bind NIS and regulate its trafficking or retention at the plasma membrane (Fletcher *et al.* 2020). ARF4 is a small GTPase that functions in trans-GGolgi network sorting of certain proteins into carrier vesicles. ARF4 binds to the carboxy terminus of NIS and potentiates its function by promoting its trafficking through vesicles destined to the plasma membrane. On the other hand, VCP is a chaperone that dissembles protein complexes and facilitates proteasomal degradation of proteins from the ER. VCP seems to promote dislocation of NIS from the ER and subsequent proteasome degradation. In contrast to ARF4 in which no current specific agonists exist, VCP is specifically druggable with FDA-approved inhibitors such as ebastine and clotrimazole, resulting in enhanced radioiodide uptake in thyroid cancer models. These two well-known drugs are well tolerated *in vivo* and look promising for future clinical trials in radioiodide-refractory thyroid cancer patients, most likely in combination with other redifferentiation strategies.

In another interesting work, the same group performed a high throughput screening for ~1200 FDA-approved drugs to see the most effective drug in reinducing ^{125}I uptake in a thyroid cancer cell model (Read *et al.* 2020). Of all the drugs, the most effective were ebastine, clotrimazole, disulfiram, and SAHA. The combination of ebastine (a VCP inhibitor) and SAHA (a HDAC inhibitor) was the most effective. It is interesting to note that among those drugs, MEK and BRAF inhibitors (i.e. selumetinib), alone or in combination, were not as effective. In addition, based on the drug's mechanisms of action, they figured out a hypothetical model of NIS intracellular processing in which some steps can be clearly druggable. According to their *in vitro* model, targeting trafficking (i.e. blocking ER-associated proteasomal degradation) and epigenetic mechanisms (i.e. HDAC inhibition) would be most effective.

Overall, in very recent years, some interesting mechanisms have been unveiled to understand the trafficking of NIS to the plasma membrane (Fig. 3). The fact that many tumors express or even overexpress NIS in the cytoplasm but not in the membrane showcases that reinducing radioiodide uptake is not only a matter of enhancing NIS *expression*. NIS needs to be correctly localized at the plasma membrane, and combining different drugs that target both expression and localization seems most appropriate.

NIS in extrathyroidal tissues, increasing the complexity

NIS expression does not only occur in the thyroid gland. Before NIS was identified, numerous tissues were known to be capable of accumulating I^- (Wolff 1964). After NIS was cloned in the thyroid (Dai *et al.* 1996), several

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tissues containing NIS transcript were identified and characterized at genomic and protein levels. In some cases, NIS precise cellular and subcellular localization have been determined, as well as its functional level in other cases (Table 1) (Dohán *et al.* 2003, Ravera *et al.* 2017, De la Vieja & Santisteban 2018).

NIS extra-thyroidal expression, regulation, function, subcellular localization, glycosylation level depend on each tissue (detailed summary in Table 1) and on the I^- needs/content (De la Vieja & Santisteban 2018). In many cases, it has been demonstrated that extra-thyroidal NIS participates in functions such as: (1) collect, recycle, and retain as much I^- as possible either from the diet, recycling of secondary metabolism, or the reabsorptions from urine, and thus pass I^- into the blood so that it reaches the thyroid; (2) provide I^- to the fetus and newborn so that they can synthesize their own TH; (3) provide I^- that exerts an antioxidant role, reducing ROS levels by being easily oxidized; (4) provide I^- to perform a powerful antimicrobial and antiviral protective role when oxidized to hypoiodite (IO^-) (Kussendrager & van Hooijdonk 2000) (De la Vieja & Santisteban 2018). Hence, it seems that NIS expression is more ubiquitous than initially expected. Further work will reveal the precise role of NIS in each tissue.

NIS tumoral expression in extrathyroidal tissues has also acquired much notoriety and has opened the door to propose NIS as a theranostic agent, meaning both a prognostic/diagnostic marker and a therapeutic tool through the use of radioiodide in those endogenously NIS-expressing tumors in a similar way to how it is performed in thyroid cancer. However, tumor progression and/or microenvironment affects NIS expression differently depending on the tissue. In some cases, as in the thyroid, the expression of NIS is downregulated or overexpressed (Fig. 4) (Wapnir *et al.* 2003, Altorjay *et al.* 2007, la Perle *et al.* 2013). More importantly, in some tumors, NIS is present at the plasma membrane and, therefore, target to radioiodide therapy (Tazebay *et al.* 2000, Liu *et al.* 2007*b*, Riesco-Eizaguirre *et al.* 2014, Shiozaki *et al.* 2019). On the other hand, recent work in cancer and metastases has also shown that cytoplasmic/intracellularly retained NIS triggers strong oncogenic effects via interactions with leukemia-associated RhoA guanine exchange factor (LARG) (Lacoste *et al.* 2012) that seems to be facilitated by the PI3K/AKT/mTOR signaling pathway (Feng *et al.* 2018). This metastatic potential may be another reason to redirect intracellular NIS to the plasma membrane. It would not only render tumors amenable to ^{131}I radiotherapy but could have the additional positive effect of slowing tumor progression.

Concluding remarks and perspectives

NIS increasing complexity is a challenge and at the same time an opportunity to extend its clinical applications. Significant progress has been made in understanding NIS regulation at different levels and in different tissues.

NIS transcription is under the control of different transcriptional regulatory elements in a tissue-depending manner to regulate temporal and spatial NIS expression. NIS in the thyroid gland depends largely on a distant upstream enhancer activity (i.e. NUE) mediated by TSH/cAMP stimulation that acts through a synergism between PAX8 and CRE-like binding factors, whereas NIS in the mammary gland depends primarily on the proximal promoter through the synergism of NKX2.5 and ERα enhanced by RA stimulation through intronic regulatory elements and other lactogenic hormones. Presumably, other transcriptional regulatory elements may emerge in the future through genome-wide approaches to understand the regulation of NIS in other hormonally regulated tissues.

NIS posttranscriptional regulation by miRNAs most likely depends on the co-regulation between miRNA and transcription factors, forming auto-regulatory network motifs including direct reciprocal feedback loops and feedforward loops. A prominent example is miR-146-3p and PAX8 and their shared target NIS. Other miRNA-transcription factors pairs of regulation might be operating to modulate NIS and other iodide-metabolizing genes.

Aberrant signaling pathways and impaired trafficking to the membrane are two major mechanisms underlying NIS dysregulation in cancer in which significant progress has been made. On the one hand, there is an exquisite dependence of NIS repression on the activity of MAPK signaling pathway that acts through the locally secreted cytokine TGFbeta. Indeed, MAPK inhibitors have reached clinical trials with partial success in thyroid cancer patients. The synergistic effects of blocking SMAD and MAPK pathways observed in cell lines and animal models look promising and are yet to be explored in humans. Other signaling pathways, prominent in some types of thyroid cancer (e.g. PI3K), are also to be considered. On the other hand, new insights into the route that NIS follows through different subcellular compartments have shown us druggable ways to relocalize NIS to the membrane and enhance ioidide uptake (e.g. through inhibition of ER-proteasomal degradation). Combining therapies targeting both NIS re-expression and re-localization to the plasma membrane looks like a plausible way to reinduce

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radioiodide uptake and extend the benefits of treatment to more patients with refractory thyroid cancer.

Unveiling NIS function in extrathyroidal tissues is an emerging field relatively unexplored. NIS does not only provide iodide to assure the synthesis of thyroid hormones but most likely provides iodide that has a protective role in tissues most exposed to external microorganisms. NIS takes part in the peroxidase system allowing cells to generate hypoiodite (IO^-), a strong oxidizing element that functions as an antimicrobial agent. Finally, we now know that NIS is not merely a supporting actor during tumor transformation. In fact, NIS is overexpressed and associated with worse clinical outcomes in some types of cancer. Intracellular NIS functions as an important mediator of signaling pathways promoting tumor transformation and progression, where the C-terminus plays an essential role in being independent of the symporter transport activity. This novel oncogenic function may serve as a cautionary note on re-expressing NIS artificially in cancer cells, particularly if is not expressed in the membrane.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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