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Interaction of Genetic Variations in *NFE2L2* and *SELENOS* Modulates the Risk of Hashimoto's Thyroiditis

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Background: Several single-nucleotide polymorphisms (SNPs) are known to increase the risk of Hashimoto's thyroiditis (HT); such SNPs reside in thyroid-specific genes or in genes related to autoimmunity, inflammation, and/or cellular defense to stress. The transcription factor Nrf2, encoded by *NFE2L2*, is a master regulator of the cellular antioxidant response. This study aimed to evaluate the impact of genetic variation in *NFE2L2* on the risk of developing HT.

Methods: In a case-control candidate gene association study, functional SNPs in the *NFE2L2* promoter (rs35652124, rs6706649, and rs6721961) were examined either as independent risk factors or in combination with a previously characterized HT risk allele (rs28665122) in the gene *SELENOS*, encoding selenoprotein S (SelS). A total of 997 individuals from the north of Portugal (Porto) were enrolled, comprising 481 HT patients and 516 unrelated healthy controls. *SELENOS* and *NFE2L2* SNPs were genotyped using TaqMan[®] assays and Sanger sequencing, respectively. Odds ratios (ORs) were calculated using logistic regression, with adjustment for sex and age. Expression of SelS was analyzed by immunohistochemistry in thyroid tissue from HT patients and control subjects. Molecular interactions between the Nrf2 and SelS pathways were investigated in thyroid tissues from mice and in rat PCCL3 thyroid follicular cells.

Results: When all three *NFE2L2* SNPs were considered together, the presence of one or more minor alleles was associated with a near-significant increased risk (OR = 1.43, $p = 0.072$). Among subjects harboring only major *NFE2L2* alleles, there was no increased HT risk associated with heterozygosity or homozygosity for the *SELENOS* minor allele. Conversely, in subjects heterozygous or homozygous for the *SELENOS* risk allele, the presence of an *NFE2L2* minor allele significantly increased HT risk by 2.8-fold ($p = 0.003$). Immunohistochemistry showed reduced expression of SelS in thyroid follicular cells of HT patients. In Nrf2 knockout mice, there was reduced expression of SelS in thyroid follicular cells; conversely, in PCCL3 cells, reducing SelS expression caused reduced activity of Nrf2 signaling.

Conclusions: The *NFE2L2* promoter genotype interacts with the *SELENOS* promoter genotype to modulate the risk of HT in a Portuguese population. This interaction may be due to a bidirectional positive feedback between the Nrf2 and SelS pathways.

Keywords: oxidative stress, selenium, *SELENOS*, selenoprotein S, *NFE2L2*, Nrf2

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Introduction

HASHIMOTO'S THYROIDITIS (HT) is a chronic autoimmune disease with an annual incidence of 0.3–1.5/1000 and a prevalence of up to 10% in the general population (1,2). It is characterized by thyroid inflammation due to expansion of thyroid antigen-specific lymphocytes that infiltrate the gland and production of thyroid autoantibodies, leading to gradual destruction of the thyroid parenchyma and often subsequent hypothyroidism (3). HT is a multifactorial disorder whose pathogenesis involves genetic and environmental predisposing factors (1,3–5). Twin studies estimate its heritability at 70% (4), and several single-nucleotide polymorphisms (SNPs) are known to increase the risk of HT. Such SNPs reside either in thyroid-specific genes (e.g., *TSHR* and *TG*) (6,7) or in genes related to autoimmunity, inflammation, and/or cellular mechanisms of defense to stress (e.g., *IL-1 β* , *IL-6*, and *TNF*) (8,9).

We have previously identified one such HT susceptibility gene, *SELENOS*, which encodes a member of the selenoprotein family, selenoprotein S (SeS) (10). *SELENOS* is expressed in multiple tissues, including thyroid follicular cells, and the protein it encodes is involved in cellular stress responses as a mediator of endoplasmic reticulum (ER)-associated protein degradation (ERAD) of misfolded proteins (11), as well as in immune and inflammatory processes (12). We previously showed that a functional SNP in the *SELENOS* promoter (rs28665122, –105G/A) is associated with increased risk of HT (10). Specifically, heterozygosity or homozygosity for the minor allele (GA or AA, respectively) was significantly more frequent in HT patients than in healthy controls, increasing the risk of HT by more than twofold (10). In luciferase reporter gene assays, the allele A shows reduced promoter transcriptional activity (12), which suggests that reduced *SELENOS* transcription may influence the proinflammatory cytokine profiles observed in thyroid autoimmunity and/or the proteostatic responses of thyroid follicular cells, thereby predisposing to HT.

We hypothesized that additional risk factors for HT might reside in cellular systems that regulate or cross talk to selenoproteins and mediate complementary and/or overlapping cellular stress responses. One such candidate is *NFE2L2*, which encodes the nuclear factor-erythroid 2-related transcription factor 2 (NFE2L2 or Nrf2), a member of the cap'n'collar leucine zipper family (13). Nrf2 is known to regulate the basal and inducible expression of a battery of cell protective genes that encode antioxidant and detoxification enzymes, proteasome subunits, and other proteostasis mediators (14). In the absence of oxidative stress, Nrf2 binds to its cytoplasmic inhibitor Kelch-like ECH-associated protein 1 (Keap1), which targets Nrf2 for polyubiquitination and proteasomal degradation (13,15). Keap1 also functions as a sensor of oxidants and electrophiles, which react with its redox-sensitive cysteines (16,17). Oxidative stressors abolish the inhibition of Nrf2 by Keap1; Nrf2 then accumulates in the nucleus where it transcriptionally activates protective genes through antioxidant response elements (AREs) in their regulatory sequences (13). Studies in different tissues have shown that Nrf2 regulates hundreds of genes, including several selenoproteins (13,14). In addition, multiple lines of evidence indicate that the selenoprotein system and the Nrf2 system can compensate for each other in antioxidant stress

responses, such that the simultaneous disruption of both systems is particularly deleterious to proteostasis (18–23).

The human *NFE2L2* gene maps to chromosome 2q31 and consists of 5 exons and 4 introns. Three functional SNPs have been identified in the *NFE2L2* promoter (24): rs35652124, –214A>G; rs6706649, –212G>A; and rs6721961, –178A>C—nomenclature according to a study (25). Each of these SNPs has been found to affect the expression level of Nrf2, with the minor alleles associated with a lower promoter activity (26,27). These polymorphisms have been associated with several diseases related to oxidative stress (25), such as acute lung injury (26), asthma (28), Parkinson's disease (29), Alzheimer's disease (30), and others, including autoimmune diseases such as systemic lupus erythematosus (31) and vitiligo (32).

Autoimmune thyroid disease has also been associated with systemic oxidative stress (33,34); however, the potential role of *NFE2L2* SNPs in susceptibility to autoimmune thyroid disease has not been addressed. Interestingly, Nrf2 knockout (KO) mice are known to develop an age-related multiorgan autoimmune syndrome (35–37), but it has not been reported whether the thyroid gland is also affected. Recently, we showed that Nrf2 positively controls thyroglobulin expression while limiting its iodination in the thyroid of mice (38). We also showed that Nrf2 positively controls the thyroidal expression of antioxidant genes, including those encoding the selenoproteins glutathione peroxidase 2 (Gpx2) and thioredoxin reductase 1 (Txnrd1) (38), which are both known to have important roles in follicular cell homeostasis (39,40). However, the involvement of Nrf2 in autoimmune thyroid disease has not been investigated. For these reasons, we investigated whether the known functional *NFE2L2* promoter SNPs, either individually or in combination with each other and/or with the known *SELENOS* promoter functional SNP, modulate the risk of HT.

Materials and Methods

Study subjects

Associations between the three *NFE2L2* promoter SNPs and the *SELENOS* promoter SNP and the risk of HT were assessed in a case–control study using cohorts that have been previously described in detail (10). Briefly, a total of 997 subjects were enrolled; these included 481 genetically unrelated HT patients (441 females and 40 males; mean age 55.3 years; range 30–62 years) enrolled at the Department of Endocrinology of Hospital of S. João (Porto, Portugal). The control group comprised 516 subjects (479 females and 37 males; mean age 46.3 years; range 18–83 years) recruited among unrelated healthy blood donors. Patients were enrolled between 2007 and 2013; diagnosis was made based on the presence of positive serum autoantibodies against thyroid peroxidase and/or thyroglobulin and negative serum autoantibodies against the thyrotropin (TSH) receptor, according to methods applied before or after March 2009 in the Department of Clinical Pathology of Hospital of S. João, in conjunction with a typical ultrasound pattern of the thyroid parenchyma (i.e., diffuse hypoechogenicity and heterogeneity). Subjects with a reported history of thyroid cancer and/or prior thyroid surgery were excluded. The biochemical characteristics of the patient cohort have been reported previously (10); all patients were treated with levothyroxine for HT-related hypothyroidism. The control group, healthy

blood donors without apparent infectious and/or chronic disorders, consisted of permanent residents in the area serviced by the Hospital of S. João, selected during the assembly of the EpiPorto cohort (41). The study was authorized by the Hospital of S. João Ethics Committee and informed consent was obtained from all participants.

Formalin-fixed paraffin-embedded thyroid tissue samples from control subjects ($n=10$) or patients with HT ($n=8$), collected during thyroidectomy performed for other indications (notably benign goiter), were obtained from the archives of Lausanne University Hospital, with authorization from the Ethics Committee of the Canton of Vaud (study no. 2017-00944).

Genotyping

Genomic DNA from patients and controls was isolated from blood using standard proteinase K digestion with phenol/chloroform extraction. For the *NFE2L2* SNPs rs35652124, rs56706649, and rs6721961, polymerase chain reaction (PCR) amplifications were performed using forward primer 5'-CTGCGCTTTGGTGGGAAG-3' and reverse primer 5'-TGGAGTTGCAACCTTGC-3'. Briefly, PCR amplifications were performed in 25 μ L volume reactions containing 100 ng genomic DNA, 100 μ M of each dNTP, 0.1 μ g of each primer, 1 \times GoTaq[®] Flexi Buffer (Promega, Madison, WI), 2.5 mM MgCl₂, and 0.15 U of GoTaq G2 Flexi DNA Polymerase (Promega). Reaction mixtures were submitted to 35 amplification cycles preceded by a Taq activation step at 95 °C for 5 minutes; each cycle comprised a denaturation step at 95 °C for 30 seconds, annealing at 59 °C for 30 seconds, and extension at 72 °C for 45 seconds followed by a final extension at 72 °C for 5 minutes. PCR products were then purified by enzymatic digestion with exonuclease I (Thermo Fisher Scientific, Waltham, MA) and shrimp alkaline phosphatase (Thermo Fisher Scientific) and sequenced using the BigDye[®] Terminator v3.1 Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3100 Genetic Analyzer (Perkin-Elmer, Waltham, MA). In a randomly selected 10% of the samples, PCR and sequencing were performed twice to verify the reproducibility of the technique. The number of subjects successfully genotyped for each *NFE2L2* SNP differed slightly according to the success of the respective sequencing reaction assay. Genotyping of *SELENOS* rs28665122 in the same subjects had been performed previously using TaqMan[®] assays (10).

Mice

C57BL/6J *Nrf2*^{+/-} mice, originally developed by Prof. M. Yamamoto (42), were obtained from RIKEN BRC (Tsukuba, Japan). Mice were housed in the animal facility of the University of Patras Medical School in temperature-, light-, and humidity-controlled rooms with a 12-hour light/dark cycle. All animal procedures were approved by the local institutional review board and were in accordance with the European Commission Directive 86/609/EEC. Generation of wild-type (WT) and KO (*Nrf2*-KO) mice and tissue collection has been previously described (38).

Cell culture

PCCL3 cells, a clonal rat thyroid cell line (43), were cultured in Coon's modified Ham's F-12 under conditions described

previously (38). Culture media and supplements were all from Sigma-Aldrich (St-Louis, MO). Generation of PCCL3 *Nrf2*-KO and PCCL3 *Keap1*-KO cells using CRISPR/Cas9 technology has been previously described (38).

Cell viability assay

The CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) was used according to the manufacturer's protocol to determine the number of viable cells in culture based on the quantification of adenosine triphosphate. Briefly, cells were lysed by adding 100 μ L CellTiter-Glo reagent to each well and the luminescence intensity was measured 10 minutes later on a NOVOstar multimode reader (BMG Labtech, Ortenberg, Germany). Results were expressed as fold change over the untreated control sample.

RNA isolation and real-time PCR

Total RNA isolation and real-time PCR (RT-PCR) from mouse tissues and from cell lines were performed as previously described (38). Melt curve analysis was used to confirm gene-specific amplification. Relative gene expression was calculated by the comparative cycle threshold method using *PPIA* as reference gene for thyroid and liver tissues and *RPL19* for PCCL3 cells. Primers for RT-PCRs are shown in Supplementary Tables S1–S3.

Protein isolation, Western immunoblotting, and immunohistochemistry

Total protein isolation and Western blots were performed as previously described (38). The following primary antibodies were used: anti-SelS (HPA010025, 1:3000; Sigma-Aldrich), produced in rabbit, a Prestige Antibody, developed and validated by the Human Protein Atlas (HPA) project; anti-vinculin (SAB4200080, 1:5000; Sigma-Aldrich); and anti- β -actin (#4967, 1:5000; Cell Signaling, Danvers, MA). Secondary antibodies were anti-mouse HRP (#7076) and anti-rabbit HRP (#7074) both from Cell Signaling (1:5000).

Immunohistochemistry was performed on slices from formalin-fixed paraffin-embedded human or mouse thyroid tissues as previously described (38,44) using the aforementioned primary antibody against SelS. Specific binding was detected with the Envision Kit (Dako), and the color reaction was visualized with 3,3'-diaminobenzidine. For negative controls, blocking solution was added instead of a primary antibody.

RNA interference

Silencing of *SelS* was performed using the DsiRNA TriFECTa Kit (cat. no. 1 rn.Ri.Vimp.13; Integrated DNA Technologies, Coralville, IA). This kit contains three different small interfering RNAs (siRNAs) targeting the rat *SelS* RNA transcript and a universal negative control siRNA that was tested in parallel. Initially, each individual siRNA was tested in PCCL3 cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) at a final siRNA concentration of 20 nM. Next, conditions for siRNA transfection were optimized by pooling together the three different individual siRNAs at a final concentration of 6.6 nM each. Compound treatments were performed 24 hours later and cells were collected 48–72 hours after the siRNA transfections.

Cell treatments

Unless otherwise stated, all chemicals were from Sigma-Aldrich. The following compounds were used for cell treatments: sulforaphane (SLF, R-1-isothiocyanato-4-methylsulfinylbutane) was used as Nrf2 activator (45). Oxidative stress conditions were induced using menadione (2-methyl-1,4-naphthoquinone, vitamin K3), a redox-cycling agent widely used in the literature to induce oxidative damage (46). In preliminary experiments, various ranges of concentrations and treatment times were tested to identify conditions where the viability of WT cells remained unaffected. Finally, an 8-hour treatment at a concentration of 20 nM was used in all experiments. To induce ER stress, cells were treated with the protein glycosylation inhibitor tunicamycin A (TN) at a concentration of 0.5 mg/mL (47).

Promoter analysis, cell transfections, and reporter assays

The promoter sequence of the human *SELENOS* gene was downloaded from the EPDnew database (promoter ID: VIMP_1, <https://epd.epfl.ch/index.php>). The JASPAR core database (<http://jaspar.genereg.net>) (48) was used to locate potential Nrf2 binding sites (ARE weight matrix, MA0150.2; relative score 0.80) in a 3 kb region upstream of the translation start site of *SELENOS*. Human genomic DNA was used as a template to amplify different lengths of the *SELENOS* promoter by PCR using Kapa HiFi™ HotStart high-fidelity DNA polymerase (Kapa Biosystems, Wilmington, MA). Primers (Supplementary Tables S1–S3) were designed to contain *KpnI* and *BgIII* restriction sites to facilitate the cloning of PCR products into the PGL3 basic luciferase vector (Promega). Three promoter reporter constructs of different lengths were created, hereafter referred to as P1 (–481 to +54 bp), P2 (–1017 to +54 bp), and P3 (–2168 to +54 bp). Promoter P1 does not harbor any potential Nrf2 binding sites, P2 harbors one potential ARE (ARE1, –863 to –849), and P3 harbors ARE1 and another potential ARE (ARE2, –2062 to –2051).

Transient transfections of cells with the different promoter reporter constructs were performed in 48-well plates cultured in complete medium using a 1:2 ratio of DNA and jetPRIME transfection reagent, respectively. In all transfections, the pEGFP-N1 plasmid (Clontech, Mountain View, CA) was included for monitoring transfection efficiency and normalizing luciferase activities, as previously described (49). Treatment of cells with SLF (5 μM) or TN (0.5 μg/mL) or vehicle (DMSO or dimethylsulfoxide) was performed 24 hours after transfection. Cells were lysed 48 hours after transfection using the passive lysis buffer (Promega), and green fluorescent protein (GFP) fluorescence was measured using a NOVostar multimode reader with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The GFP fluorescence background of untransfected cells was used for blank measurements. After measuring GFP fluorescence, luciferase activities were measured in the same plates using the luciferase assay system (Promega) according to the manufacturer's protocol. Luciferase activities were normalized to the respective GFP fluorescence measurements, and relative luciferase activities were expressed as fold change over control.

Statistics

For the genetic studies, statistical analyses were performed using SPSS v.25.0 (IBM, Armonk, NY). The Hardy–Weinberg equilibrium was evaluated using a chi-square test. Comparison of genotype frequencies between HT patients and controls was performed using unconditional logistic regression. Odds ratios (ORs) with the respective 95% confidence intervals (CIs) for association of the *SELENOS* and *NFE2L2* polymorphisms with HT were calculated for the genotypic and dominant models of inheritance with adjustment for sex and age. Controlling for the effects of multiple testing was performed by the false discovery rate (FDR) method (50).

For ordinal data (staining intensity in control vs. Hashimoto's samples), the Kruskal–Wallis test was used in GraphPad Prism 8 (GraphPad Software, La Jolla, CA), which was also used to prepare all graphs. For data calculated as normalized ratios [messenger RNA (mRNA), protein, and luciferase quantifications], the Bootstratio application was used; this tool is based on bootstrapping and resampling methods without any assumption on the underlying probability distribution for the data analyzed (51). Whenever the same data were used to test multiple hypotheses, a Bonferroni correction was applied as appropriate.

Results

Distribution of NFE2L2 promoter SNP genotypes and haplotypes and HT risk

In the control group, the genotype frequencies for all three *NFE2L2* SNPs (as well as for *SELENOS* rs28665122) did not deviate significantly from those expected under Hardy–Weinberg equilibrium (all $p > 0.05$). The impact of each *NFE2L2* SNP was first examined individually and independently of the *SELENOS* SNP. These analyses included subjects successfully genotyped for each *NFE2L2* SNP: rs35652124 (403 patients and 514 controls); rs6706649 (403 patients and 543 controls); and rs6721961 (403 patients and 555 controls). On an individual basis, none of the *NFE2L2* SNP genotypes showed a significant association with the risk of HT (all $p > 0.05$; Table 1). A *per allele* analysis confirmed the absence of statistically significant associations for each of the SNPs: For rs35652124, allele A (reference allele) was present in 75.88% of controls and 73.20% of patients, and allele G was present in 24.12% of controls and 26.80% of patients (OR = 1.15 [CI 0.93–1.42], $p = 0.191$). For rs6706649, allele G (reference allele) was present in 87.57% of controls and 89.97% of patients, and allele A was present in 12.43% of controls and 13.03% of patients (OR = 1.06 [CI 0.80–1.39], $p = 0.700$). Finally, for rs6721961, allele C (reference allele) was present in 90.54% of controls and 88.71% of patients, and allele A was present in 9.46% of controls and 11.29% of patients (OR = 1.22 [CI 0.91–1.64], $p = 0.192$). Finally, when *NFE2L2* haplotypes were constructed for patients and controls, four haplotypes with frequencies $> 1\%$ were identified (Table 2). There was no difference in the frequency of each of these haplotypes between patients and controls (all $p > 0.05$; Table 2). However, each of these haplotypes was marked by only one *NFE2L2* promoter SNP minor allele (Table 2); hence, these results are not more informative than the aforementioned individual analyses (Table 1). In summary,

TABLE 1. FREQUENCIES OF INDIVIDUAL *NFE2L2* PROMOTER SINGLE-NUCLEOTIDE POLYMORPHISM GENOTYPES IN HASHIMOTO'S THYROIDITIS (HT) PATIENTS AND CONTROLS, AND CALCULATED RISK OF HT FOR EACH GENOTYPE

<i>NFE2L2</i> SNP	Genotype	Controls, n (%)	Patients, n (%)	OR [CI]	p*
rs35652124	AA	299 (58.2)	218 (54.1)	1 ^a	N/A
	AG	182 (35.4)	154 (38.2)	1.11 [0.84–1.48]	0.469
	GG	33 (6.4)	31 (7.7)	1.24 [0.73–2.12]	0.436
	AG or GG	215 (41.8)	185 (45.9)	1.13 [0.86–1.49]	0.365
rs6706649	GG	413 (76.1)	299 (74.2)	1 ^a	N/A
	GA	125 (23.0)	103 (25.6)	1.13 [0.83–1.54]	0.444
	AA	5 (0.9)	1 (0.2)	0.29 [0.03–2.57]	0.267
	GA or AA	130 (23.9)	104 (25.8)	1.10 [0.81–1.50]	0.528
rs6721961	CC	453 (81.6)	319 (79.2)	1 ^a	N/A
	CA	99 (17.8)	77 (19.1)	1.16 [0.83–1.63]	0.392
	AA	3 (0.5)	7 (1.7)	2.34 [0.58–9.42]	0.233
	CA or AA	102 (18.4)	84 (20.8)	1.21 [0.87–1.68]	0.269

Model adjusted for sex and age.

*Versus respective reference group.

^aReference groups.

CI, confidence interval; N/A, not applicable; OR, odds ratio; SNP, single-nucleotide polymorphism.

these results indicate that neither the individual *NFE2L2* promoter SNPs nor the main respective *NFE2L2* promoter haplotypes are independent risk factors for HT.

Combined *NFE2L2* promoter genotypes and HT risk

The three functional *NFE2L2* SNPs lie close together within 36 bp of the promoter, and reporter gene assays have shown that promoter activity decreases with a higher number of minor alleles (27). Therefore, we next considered the joint impact of all three *NFE2L2* SNPs on HT risk. Heterozygosity or homozygosity for any 1, 2, or 3 of the *NFE2L2* SNP minor alleles was not significantly associated with increased HT risk ($p=0.072$; Table 3). Nevertheless, the fact that the p -value was close to significance prompted us to investigate the possible interaction of the *NFE2L2* SNPs with the *SELENOS* functional SNP known to impact the risk of HT in the same population (10).

Interactions between individual *NFE2L2* and *SELENOS* promoter SNP genotypes and HT risk

Next, for each of the *NFE2L2* SNPs, its effect was assessed in combination with the previously documented *SELENOS* SNP risk allele (10). These analyses included 586 subjects

successfully genotyped for all three *NFE2L2* SNPs and for the *SELENOS* SNP (322 patients and 264 controls). First, each *NFE2L2* SNP was considered separately. In subjects not carrying the known *SELENOS* risk allele (GG homozygotes), heterozygosity or homozygosity for an *NFE2L2* SNP minor allele did not modify the risk of HT (all $p>0.05$; Tables 4–6, group 2 vs. group 1). Similarly, in subjects heterozygous or homozygous for the *SELENOS* risk allele (GA or AA, respectively), heterozygosity or homozygosity for an *NFE2L2* SNP minor allele did not modify the risk of HT (all $p>0.05$; Tables 4–6, group 4 vs. group 3). Finally, homozygosity for an *NFE2L2* SNP major allele did not abolish the increased HT risk associated with heterozygosity or homozygosity for the *SELENOS* risk allele (all $p>0.05$; Tables 4–6, group 3 vs. group 1). In summary, these results indicate that when each of the three functional *NFE2L2* promoter SNPs is considered individually, none of them either increases or abolishes the risk of HT associated with the *SELENOS* promoter functional SNP.

Interactions between combined *NFE2L2* and *SELENOS* promoter SNP genotypes and HT risk

Finally, we considered the combined impact of all three *NFE2L2* SNPs on the risk of HT associated with the *SELENOS* promoter functional SNP genotype. In subjects not carrying the known *SELENOS* risk allele, heterozygosity or homozygosity for any one of the *NFE2L2* SNP minor alleles did not modify the risk of HT ($p=0.979$; Table 7, group 2 vs. group 1). Moreover, the presence of additional *NFE2L2* SNP minor alleles (from 2 to 6 total) also did not modify the risk of HT compared with the presence of a single minor allele ($p=0.879$; Table 7, group 3 vs. group 2). These results are consistent with the notion that the *NFE2L2* promoter genotype is not an independent risk factor for HT (Tables 1–3). However, among subjects harboring only major *NFE2L2* alleles, heterozygosity or homozygosity for the *SELENOS* SNP minor allele was not associated with increased risk of HT ($p=0.704$; Table 7, group 3 vs. group 1), suggesting a protective effect of the *NFE2L2* major alleles. In further support of an interaction effect, in

TABLE 2. *NFE2L2* PROMOTER HAPLOTYPES AND RESPECTIVE CALCULATED RISK OF HASHIMOTO'S THYROIDITIS

<i>NFE2L2</i> haplotype	SNP genotypes ^a	Frequency	OR [CI]	p
1 ^b	A <u>G</u> C	0.516	1	N/A
2	A <u>A</u> C	0.128	1.13 [0.83–1.54]	0.425
3	A <u>G</u> <u>A</u>	0.102	1.31 [0.95–1.81]	0.098
4	<u>G</u> <u>G</u> <u>C</u>	0.254	1.17 [0.93–1.47]	0.182

Minor alleles are underlined. Model adjusted for sex and age.

^aBy order of position on chromosome 2: rs35652124, rs6706649, and rs6721961.

^bReference haplotype.

TABLE 3. NUMBER OF COMBINED *NFE2L2* PROMOTER SINGLE-NUCLEOTIDE POLYMORPHISM MINOR ALLELES AND RESPECTIVE CALCULATED RISK OF HASHIMOTO'S THYROIDITIS

Group	<i>NFE2L2</i> minor alleles, n	Controls, n (%)	Patients, n (%)	OR [CI]	Vs. group	p
1	0	78 (29.6)	72 (22.4)	1	N/A	N/A
2	1–6 ^a	186 (70.4)	250 (77.6)	1.43 [0.97–2.10]	1	0.072

Model adjusted for sex and age.

^aMinor allele heterozygous or homozygous for any *NFE2L2* promoter SNPs.

TABLE 4. FREQUENCIES OF THE *SELENOS* rs28665122 AND *NFE2L2* rs35652124 SINGLE-NUCLEOTIDE POLYMORPHISM COMBINED GENOTYPES IN HT PATIENTS AND CONTROLS, AND CALCULATED RISK OF HT FOR EACH GENOTYPE

Group	<i>SELENOS</i> rs28665122 genotype	<i>NFE2L2</i> rs35652124 genotype	<i>SELENOS</i> minor alleles, n	<i>NFE2L2</i> minor alleles, n	Controls, n (%)	Patients, n (%)	OR [CI]	Vs. group	p
1	GG	AA	0	0	115 (43.6)	100 (31.1)	1.00	N/A	N/A
2	GG	AG or GG	0	1–2	71 (26.9)	65 (20.2)	0.97 [0.62–1.52]	1	0.893
3	GA or AA	AA	1–2	0	42 (15.9)	80 (24.8)	2.09 [1.30–3.37]	1	0.002
4	GA or AA	AG or GG	1–2	1–2	36 (13.6)	77 (23.9)	1.15 [0.66–2.02]	3	0.621

Bold indicates statistical significance.

Model adjusted for sex and age.

TABLE 5. FREQUENCIES OF THE *SELENOS* rs28665122 AND *NFE2L2* rs6706649 SINGLE-NUCLEOTIDE POLYMORPHISM COMBINED GENOTYPES IN HT PATIENTS AND CONTROLS, AND CALCULATED RISK OF HT FOR EACH GENOTYPE

Group	<i>SELENOS</i> rs28665122 genotype	<i>NFE2L2</i> rs6706649 genotype	<i>SELENOS</i> minor alleles, n	<i>NFE2L2</i> minor alleles, n	Controls, n (%)	Patients, n (%)	OR [CI]	Vs. group	p
1	GG	GG	0	0	131 (49.6)	125 (38.8)	1.00	N/A	N/A
2	GG	GA or AA	0	1–2	55 (20.8)	40 (12.4)	0.82 [0.50–1.34]	1	0.425
3	GA or AA	GG	1–2	0	64 (24.2)	114 (35.4)	1.93 [1.28–2.91]	1	0.002
4	GA or AA	GA or AA	1–2	1–2	14 (5.3)	43 (13.3)	1.62 [0.81–3.24]	3	0.174

Bold indicates statistical significance.

Model adjusted for sex and age.

TABLE 6. FREQUENCIES OF THE *SELENOS* rs28665122 AND *NFE2L2* rs6721961 SINGLE-NUCLEOTIDE POLYMORPHISM COMBINED GENOTYPES IN HT PATIENTS AND CONTROLS, AND CALCULATED RISK OF HT FOR EACH GENOTYPE

Group	<i>SELENOS</i> rs28665122 genotype	<i>NFE2L2</i> rs6721961 genotype	<i>SELENOS</i> minor alleles, n	<i>NFE2L2</i> minor alleles, n	Controls, n (%)	Patients, n (%)	OR [CI]	Vs. group	p
1	GG	CC	0	0	152 (57.6)	133 (41.3)	1.00	N/A	N/A
2	GG	CA or AA	0	1–2	34 (12.9)	32 (9.9)	1.07 [0.61–1.88]	1	0.806
3	GA or AA	CC	1–2	0	63 (23.8)	122 (37.9)	2.20 [1.48–3.28]	1	<0.001
4	GA or AA	CA or AA	1–2	1–2	15 (5.7)	35 (10.9)	1.24 [0.62–2.50]	3	0.546

Bold indicates statistical significance.

Model adjusted for sex and age.

TABLE 7. INTERACTION BETWEEN NUMBER OF COMBINED *NFE2L2* PROMOTER SINGLE-NUCLEOTIDE POLYMORPHISM MINOR ALLELES AND *SELENOS* MINOR ALLELES, AND RESPECTIVE CALCULATED RISK OF HT

Group	<i>SELENOS</i> minor alleles, n	<i>NFE2L2</i> minor alleles, n	Controls, n (%)	Patients, n (%)	OR [CI]	Vs. group	p
1	0	0	49 (18.6)	42 (13.0)	1	N/A	N/A
2	0	1	99 (37.5)	90 (30.0)	1.01 [0.60–1.69]	1	0.979
3	0	2–6 ^a	38 (14.4)	33 (10.2)	0.95 [0.53–1.71]	2	0.879
4	1–2	0	29 (11.0)	30 (9.3)	1.14 [0.58–2.24]	1	0.704
5	1–2	1	31 (11.7)	86 (26.7)	2.84 [1.44–5.58]	4	0.003
6	1–2	2–6 ^a	18 (6.8)	41 (12.7)	0.75 [0.37–1.53]	5	0.428

Bold indicates statistical significance.

Model adjusted for sex and age.

^aMinor allele heterozygous or homozygous for any *NFE2L2* promoter SNPs.

subjects heterozygous or homozygous for the *SELENOS* risk allele, heterozygosity or homozygosity for any one of the *NFE2L2* SNP minor alleles significantly increased the risk of HT by 2.8-fold ($p=0.003$; Table 7, group 5 vs. group 4), suggesting a predisposing effect of the *NFE2L2* minor alleles. Of note, statistical significance was retained after controlling for the effects of multiple testing using the FDR method. Subjects with additional *NFE2L2* SNP minor alleles (from 2 to 6 total) had the same level of risk of HT as those with a single minor allele ($p=0.428$; Table 7, group 6 vs. group 5). In summary, these results indicate that when the three *NFE2L2* promoter functional SNPs are considered together and in combination with the *SELENOS* promoter functional SNP, *NFE2L2* major alleles abolish the HT risk associated with the *SELENOS* minor allele, whereas *NFE2L2* minor alleles increase the risk.

Reduced expression of *SelS* in thyroid follicular cells of Hashimoto's patients

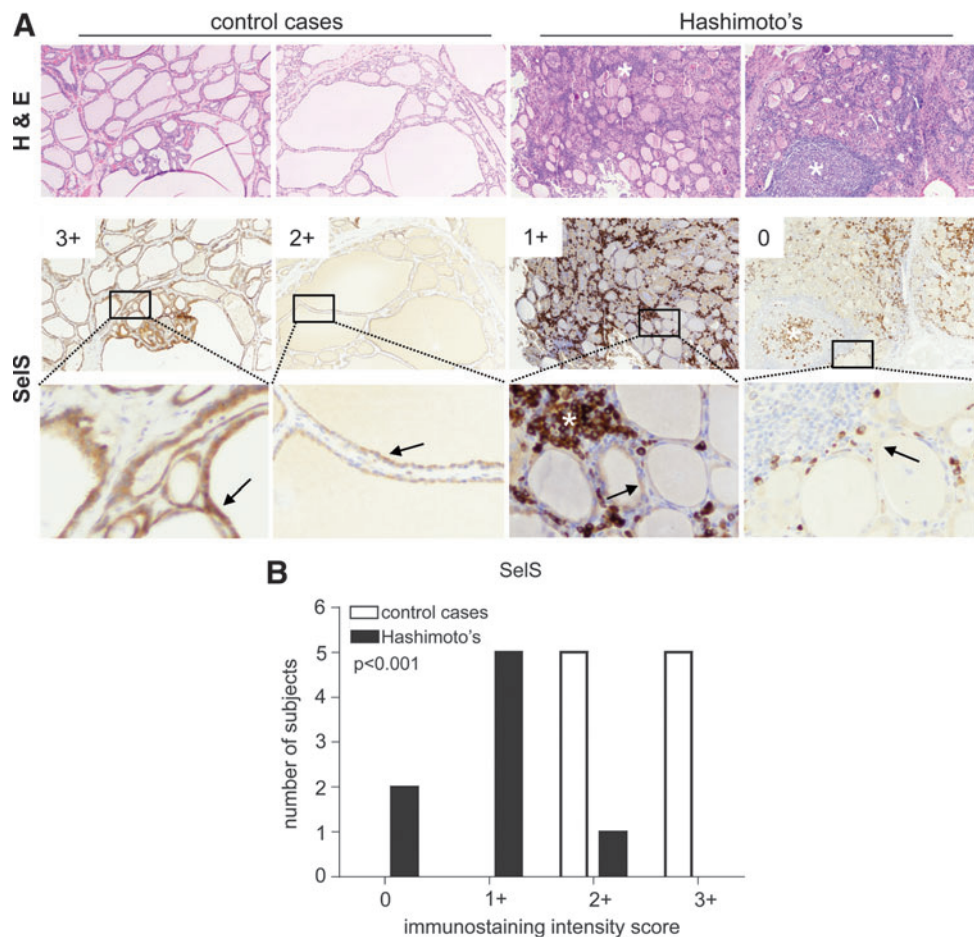
To better understand how the genetic interaction between *SELENOS* and *NFE2L2* may impact the susceptibility to Hashimoto's disease, we first analyzed the expression of *SelS* protein by immunohistochemistry in thyroid samples from control subjects ($n=10$) or patients with HT ($n=8$), collected during thyroidectomy performed for other indications (notably benign goiter). Representative immunohistochemi-

cal stainings and respective staining intensity scores are shown in Figure 1A, and the distribution of staining intensities among samples is depicted in the histograms shown in Figure 1B. There was a statistically significant lower expression of *SelS* ($p\leq 0.001$) in the thyroid follicular cells of HT compared with those in control samples. Interestingly, despite the reduced expression of *SelS* in thyroid follicular cells in Hashimoto's samples, a subset of lymphocytes in germinal centers and other lymphocytic infiltrates stained strongly for *SelS*.

Reduced expression of *SelS* in thyroid follicular cells of *Nrf2*-KO mice

We next analyzed by RT-PCR, Western immunoblotting, and immunohistochemistry the expression of *SelS* mRNA and *SelS* protein, respectively, in thyroid samples from WT and *Nrf2*-KO mice. No difference was found at the *SelS* mRNA level between the genotypes (Fig. 2A) in thyroid tissue. However, thyroidal *SelS* protein levels were significantly reduced in *Nrf2*-KO mice (Fig. 1C, E). Immunohistochemistry showed that this reduction was associated with reduced expression in the thyroid follicular cells (Fig. 1G). When liver tissue samples were analyzed, no differences were observed in either *SelS* mRNA (Fig. 1B) or *SelS* protein levels (Fig. 1D, F), suggesting that the observed differences in thyroid are a tissue-specific effect.

FIG. 1. Expression of *SelS* in samples from control subjects and Hashimoto's patients. (A) Representative immunohistochemical stainings and respective staining intensity scores. The H&E panels and the upper *SelS* panels are taken at $\times 100$ magnification; the magnified inserts are taken at $\times 300$ magnification. Arrows indicate stained areas representative of the respective overall scores. Stars indicate germinal centers and other lymphocytic infiltrates, in which a subset of lymphocytes stains strongly for *SelS*. (B) Quantification of *SelS* staining intensity in thyroid follicular cells in samples from control subjects and Hashimoto's patients. H&E, hematoxylin and eosin; *SelS*, selenoprotein S.



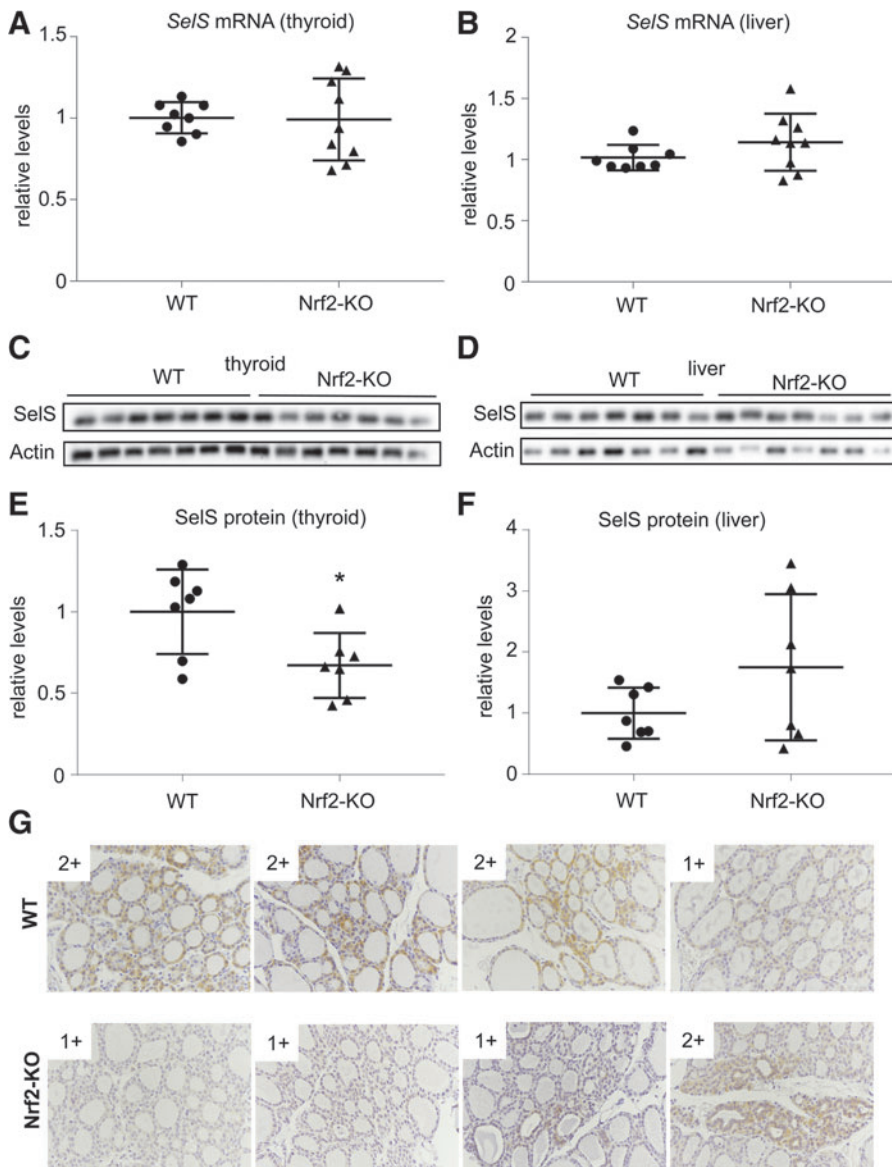


FIG. 2. Expression of *SelS* mRNA and SelS protein in WT and Nrf2-KO mice. (A, B) Relative *SelS* mRNA expression levels, quantified by RT-PCR and normalized to *PPIA* as the reference gene, in the thyroid (A) or liver (B) of WT and Nrf2-KO male mice under basal conditions. Error bars indicate mean \pm standard deviation. (C, D) SelS protein levels assayed by Western immunoblotting in protein extracts from thyroid (C) or liver (D) of WT and Nrf2-KO male mice (7 mice per group). (E, F) Densitometric quantification of relative protein levels in the mice shown in (C, D), respectively, was performed using ImageJ, with normalization to the respective actin levels and to the mean value of the WT group. Error bars indicate mean \pm standard deviation. * $p < 0.05$. (G) Representative immunohistochemical stainings and respective staining intensity scores of SelS expression in samples from WT and Nrf2-KO mice (all taken at $\times 400$ magnification). KO, knockout; mRNA, messenger RNA; RT-PCR, real-time polymerase chain reaction; WT, wild type.

SELENOS is not a transcriptional target gene of Nrf2

To further investigate how Nrf2 impacts SelS levels, we examined whether it may positively regulate the activity of the human *SELENOS* promoter. To that end, we cloned three fragments of the *SELENOS* promoter into luciferase reporter constructs to perform activity assays in transiently transfected PCCL3 rat thyroid follicular cells. All fragments contain a known ER stress-responsive element (52), and the two longer fragments also contain, respectively, one or two predicted ARE sites (Fig. 3A). In both PCCL3 WT and Nrf2-KO cells, all reporter constructs showed significant inducibility by the ER stress-inducing compound TN, but no inducibility in response to treatment with the Nrf2 inducer SLF (Fig. 3B, C). In the same cells, TN, but not SLF, significantly induced the *SelS* mRNA and SelS protein levels (Fig. 1D, E). Genetic activation of Nrf2 (as observed in Keap1-KO PCCL3 cells (38)) also had no effect on basal or inducible *SelS* mRNA and SelS protein levels (Fig. 1D, E). Taken together, these data indicate that Nrf2 does not have a direct effect on the *SELENOS* promoter but

that it positively regulates SelS protein levels in thyroid follicular cells in a posttranscriptional manner.

Reduced activity of Nrf2 signaling in thyroid follicular cells with reduced *SelS* expression

We also aimed to investigate the effects of SelS on Nrf2 signaling. To that end, we first established conditions for an efficient knockdown of SelS in PCCL3 cells, by combining three siRNA constructs yielding a strong additive effect (Fig. 3A, B). SelS knockdown decreased the mRNA expression levels of *Nqo1*, the prototypical Nrf2 target gene, both under basal conditions and in response to pharmacological induction of Nrf2 by SLF or genetic activation of Nrf2 (Keap1-KO) (Fig. 4C). The thyroidal mRNA levels of *Gpx2* and *Txnrd1* have also been previously shown to be regulated, in part, by Nrf2 (38); SelS knockdown decreased their mRNA expression levels under basal conditions and in response to genetic activation of Nrf2 (Keap1-KO), but not in response to pharmacological induction of Nrf2 by SLF (Fig. 4D–E), which may be because the latter compound can also modulate other

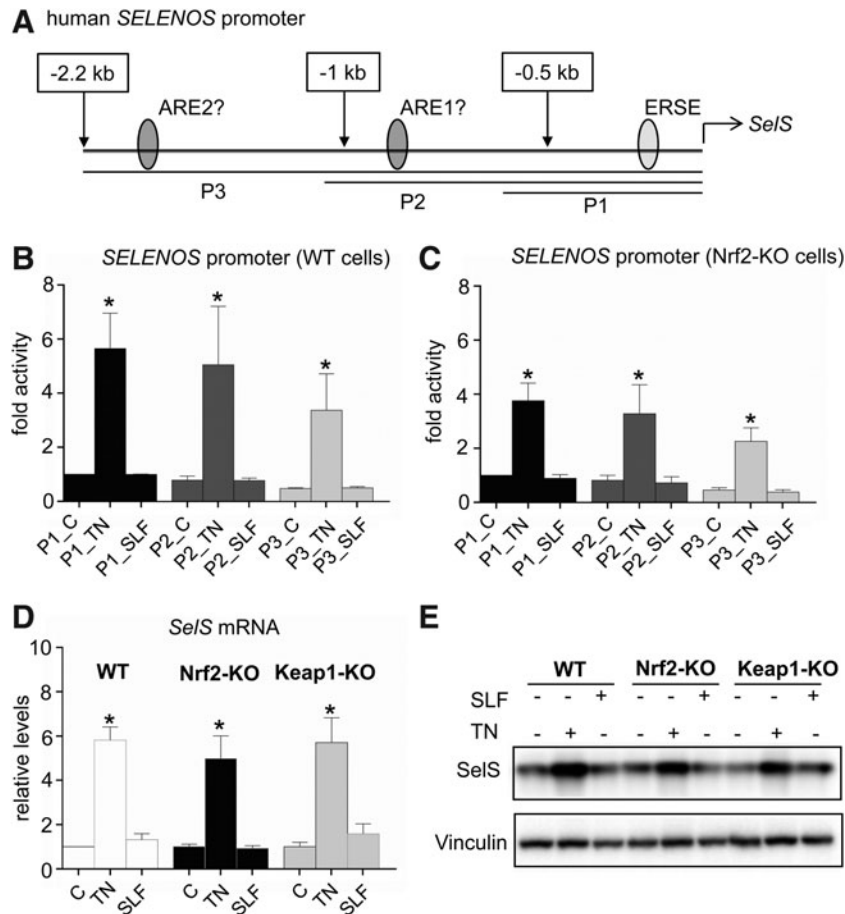


FIG. 3. Functional assessment of potential Nrf2-binding sites in the human *SELENOS* promoter. (A) Schematic representation of the human *SELENOS* promoter constructs, indicating the location of the two putative ARE sequences and of a previously characterized ESRE, as well as the length of each promoter sequence cloned in a respective luciferase reporter plasmid (P1, P2, P3). (B, C) Activity of the each *SELENOS* promoter reporter construct in WT (B) or Nrf2-KO (C) PCCL3 cells treated with vehicle (<0.1% DMSO), TN, or SLF and lysed 24 hours later to measure luciferase activity. Results are presented as fold change over the normalized activity of the P1 construct in WT (B) and Nrf2-KO cells (C), respectively. Error bars indicate mean \pm standard deviation of at least three independent experiments. * $p < 0.05$ versus respective vehicle-treated control. (D, E) Impact of Nrf2 activation status on the inducibility of *SeIS* mRNA and SelS protein expression by ER stress. PCCL3 WT or Nrf2-KO or Keap1-KO cells cultured in complete medium and treated with 0.5 μ g/mL of TN or 5 μ M of SLF or vehicle (<0.1% DMSO) were lysed 24 hours later. (D) Relative mRNA levels of *SeIS* normalized to *Rpl19* as the reference gene were quantified by RT-PCR. Error bars indicate mean \pm standard deviation of at least three independent experiments. * $p < 0.05$ versus the vehicle-treated control in WT cells. (E) Representative Western blot of one of the experiments illustrated in (D), showing SelS protein levels with vinculin as a loading control. DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; ESRE, ER stress-responsive element; SLF, sulforaphane; TN, tunicamycin.

signaling pathways and biological processes (53). Finally, we examined the effects of SelS and Nrf2 on cell viability under conditions of ER stress or oxidative stress. Cell viability was not reduced by treatment with either TN or SLF (Fig. 4F). However, knockdown of SelS reduced cell viability specifically in response to TN treatment, and pretreatment of the cells with SLF was unable to rescue this effect. Conversely, when cells were exposed to oxidative stress generated by treatment with menadione, cells were almost completely unviable, yet SLF pretreatment was able to fully rescue this toxicity. However, when SelS was knocked down, the ability of SLF pretreatment to rescue the viability of cells was significantly reduced. Taken together, these data indicate that SelS positively impacts the activity of Nrf2 signaling and its downstream protective effects against oxidative stress-induced cell toxicity.

Discussion

The present study expands the known roles of Nrf2 in the thyroid to include protection from autoimmune thyroid disease. The data indicate that *NFE2L2* SNPs do not act in isolation from other genes, but that they interact as a group specifically with the SNP in *SELENOS* that has been previously associated with HT (10). This is indicated by the results shown in Table 7, which demonstrate not only that *NFE2L2* minor alleles confer a risk when present together with the *SELENOS* minor allele but also that the presence of only *NFE2L2* major alleles abolishes the HT risk associated with the *SELENOS* minor allele. These data are consistent with the signaling interactions between the Nrf2 and SelS systems in thyroid cells described in Figures 2–4, which suggest that there is a certain level of physiological

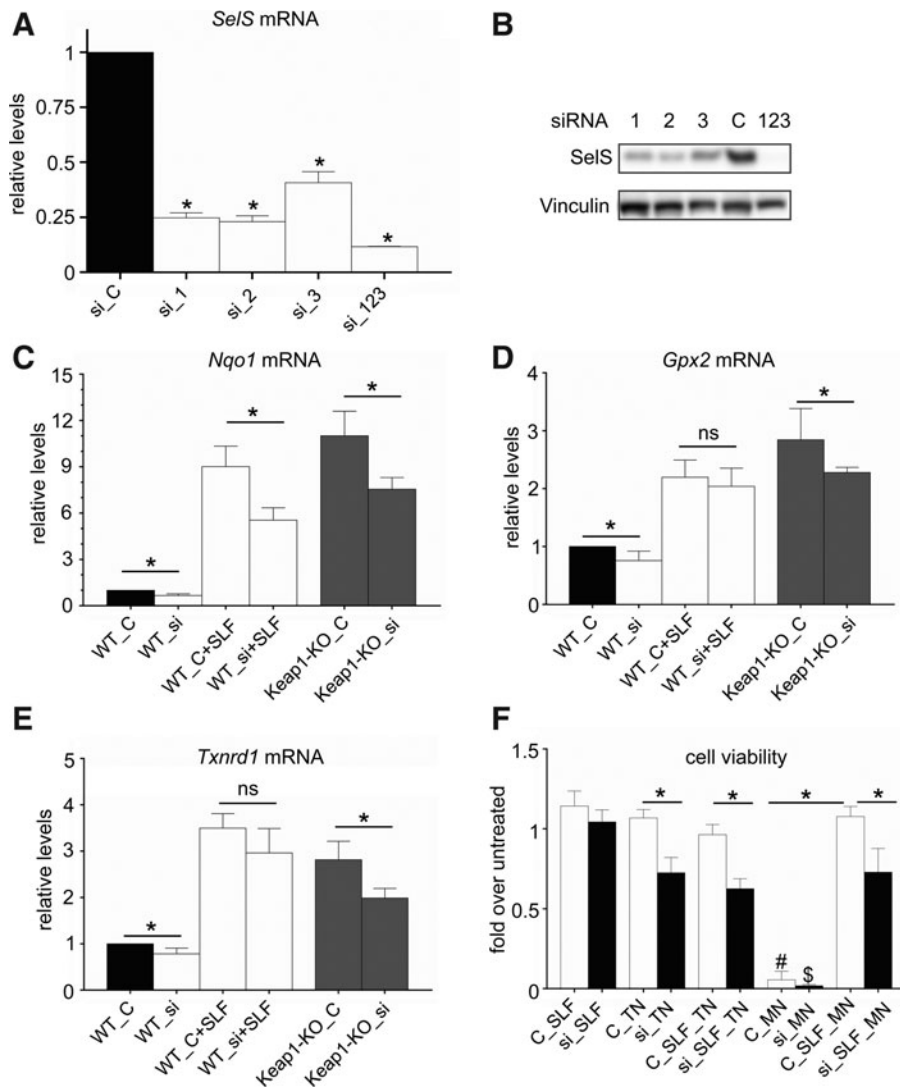


FIG. 4. Impact of *SelS* gene silencing on Nrf2 target gene expression and viability after ER or oxidative stress challenge. (A, B) Validation of siRNA-mediated silencing of *SelS*. PCCL3 cells were transfected with individual siRNAs (si_1, si_2, or si_3, all at a final concentration of 20 nM) or all three siRNAs combined (si_123, each at a final concentration of 6.6 nM); a nontargeting siRNA (si_C) was used in parallel as negative control. Cells were lysed 48 hours after transfection. (A) Relative *SelS* mRNA expression levels were determined by RT-PCR and normalized to *Rpl19*. Error bars indicate mean \pm standard deviation of at least three independent experiments. * $p < 0.05$ versus si_C. (B) Representative Western immunoblotting showing *SelS* protein knockdown with vinculin as a loading control. (C–E) Effect of *SelS* silencing on Nrf2 target genes. PCCL3 WT or Keap1-KO cells transfected with a pool of three siRNAs (_si) or with a negative control siRNA (_C), as described above, were treated 24 hours later as indicated with vehicle (<0.1% DMSO) or 5 μ M SLF for 24 hours. Relative mRNA expression levels of *Nqo1* (C), *Gpx2* (D), and *Txnrd1* (E) were determined by RT-PCR and normalized to *Rpl19*. Error bars indicate mean \pm standard deviation of at least three independent experiments. * $p < 0.05$ versus the levels in respective cells treated with negative control siRNA (_C). (F) Effects of *SelS* silencing on cell viability under ER stress or oxidative stress conditions. WT PCCL3 cells were transfected in 6-well plates with a pool of three siRNAs (si) or with a negative control siRNA (C), as described above; 12 hours later, cells were trypsinized and equal numbers of cells were plated in 96-well plates. After 24 hours, cells were treated with vehicle (<0.1% DMSO), 5 μ M SLF, or 0.5 μ g/mL TN, as indicated, for another 24 hours; 20 nM MN were added during the last 8 hours of this period. The viability of the cells was then assessed (60 hours after transfection) by an adenosine triphosphate-based luminescence assay as detailed in the Materials and Methods section, and normalized to the viability of untreated cells. Error bars indicate mean \pm standard deviation of at least three independent experiments. * $p < 0.05$ versus the levels in respective cells as indicated in each case; # $p < 0.05$ versus the levels in control untreated cells (set at “1”); \$ $p < 0.05$ versus the levels in si_SLF_MN cells. MN, menadione; ns, nonsignificant; siRNA, small interfering RNA.

compensation and bidirectional positive feedback between the two pathways.

Previous studies have implicated Nrf2 in papillary thyroid carcinoma (44), goiter (54,55), antioxidant defense in response to excess iodide (38,56), and Tg economy (38). While the mechanism through which the *NFE2L2* minor alleles contribute to the risk of HT remains to be characterized, some plausible hypotheses can already be proposed. Using Nrf2 KO mice and thyrocyte cell lines, we have recently shown that Nrf2 mediates antioxidant transcriptional responses in thyroid follicular cells and protects the thyroid from oxidation induced by iodide overload (38). In that work, we also found that Nrf2 has a dramatic impact on both the basal abundance and the TSH-inducible intrathyroidal abundance of Tg, an effect mediated by cell autonomous regulation of *TG* gene expression by Nrf2 via its direct binding to two evolutionarily conserved AREs in an upstream enhancer (38). Yet, despite upregulating Tg levels, Nrf2 was found to limit Tg iodination both under basal conditions and in response to excess iodide (38). Thus, it is possible that when Nrf2 levels are reduced due to the presence of *NFE2L2* minor alleles, Tg iodination is increased. Because increased and aberrant Tg iodination can uncover cryptic antigenic epitopes (57), an increase in Tg iodination associated with *NFE2L2* minor alleles might, over the long term, contribute to the pathogenesis of HT. Another possibility concerns the general antiautoimmunity role of Nrf2; this has been demonstrated in studies in Nrf2 KO mice. When these mice are bred into autoimmunity-permissive genetic backgrounds, a subset of them develops a multiorgan autoimmune syndrome (35–37). These studies indicate that Nrf2 has a protective role against autoimmunity, either via actions in each individual tissue (as Nrf2 is ubiquitously expressed) or via actions in the immune system that impact self-tolerance toward various autoantigens. Of course, the two aforementioned hypotheses (increased Tg iodination and deregulated autoimmunity) are not mutually exclusive.

The present study also expands the known interactions between Nrf2 and the selenium system. Selenium and Nrf2 regulate similar proteostatic processes, including antioxidant defense and the ER stress response; for example, both SelS and Nrf2 function as mediators of ERAD (11,58). Moreover, selenium and Nrf2 regulate proteostasis not only independently but also via converging on common genes and proteins. For example, Nrf2 directly upregulates the transcription of genes encoding selenoproteins, including *Gpx2* and *Txnrd1* (38), which are both known to participate in redox signaling and antioxidant defense, as well as to have important homeostatic roles in thyroid follicular cells (39,40). In this context, it is not surprising that multiple lines of evidence indicate that the selenoprotein system and the Nrf2 system can compensate for each other in antioxidant stress responses (18,19). For example, moderate selenium deficiency activates Nrf2 signaling in the liver and intestine of mice (20,21). Tissue-specific deletion of the selenocysteine tRNA gene, which inserts selenocysteine into selenoprotein enzymes, results in compensatory induction by Nrf2 of genes encoding cytoprotective enzymes (22). Thus, in these settings, the simultaneous disruption of the two systems is particularly deleterious to proteostasis, leading to enhanced disease phenotypes (22,23). These types of interactions have been documented in animal studies focusing on different tissues, including erythrocyte precursors, macrophages, and liver (22,23). Our present findings using human genetics and com-

plementary human-, animal-, and cell-based studies indicate that such an interaction is also at play in the thyroid gland, which makes sense given that both selenium and oxidation are central elements in thyroid physiology.

The genetic and signaling interactions between the Nrf2 and SelS pathways and their impact on susceptibility or resistance to HT are summarized in the model proposed in Figure 5. Nrf2 and SelS are known to favor resistance to oxidative stress and ER stress, respectively; moreover, both Nrf2 and SelS are also both known to suppress the expression of proinflammatory cytokines, including IL-6 and IL-1 β , which are two cytokines known to be involved in the pathogenesis of HT (12,59). In addition to general interactions between the Nrf2 and SelS protective systems, thyroid-specific mechanisms may also exist. For example, one explanation for the lower levels of SelS in the thyroid of Nrf2-KO mice may be the fact that these mice have lower levels of Tg (38). Since Tg comprises the bulk of the thyroid's protein content and represents a high-molecular-weight protein that depends critically on the ER for its proper conformation, the lower Tg levels in Nrf2-KO mice may impose a lower burden on the ER. Previous studies have shown that SelS is positively regulated by ER stress (52), suggesting that lower ER stress levels in Nrf2-KO mice (and, potentially, in carriers of *NFE2L2* minor SNP alleles) may lead to decreased SelS expression. Conversely,

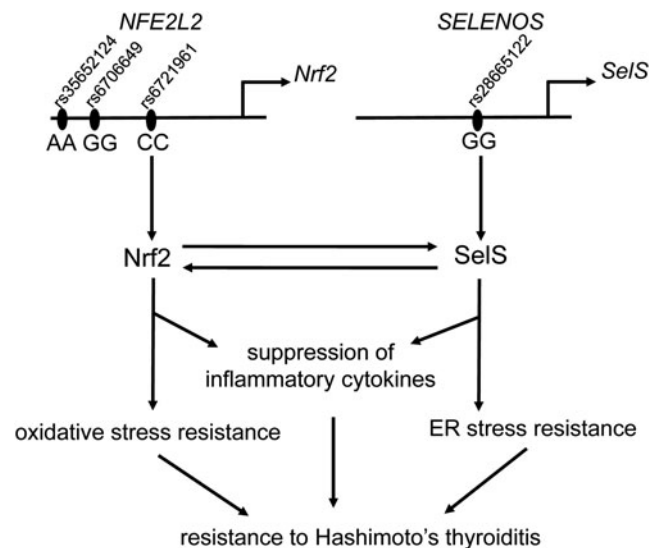


FIG. 5. Proposed model of genetic and signaling interactions between the Nrf2 and SelS pathways and their impact on susceptibility or resistance to HT. The model illustrates the expected situation in an individual who is homozygous for the major alleles of the three functional SNPs in *NFE2L2* (rs35652124, –214A>G; rs6706649, –212G>A; and rs6721961, –178A>C) and also homozygous for the major allele of the functional SNP in *SELENOS* (rs28665122, –105G/A). In this setting, the Nrf2 and SelS pathways protect against the development of HT, possibly by suppressing the expression of inflammatory cytokines and promoting resistance to oxidative and ER stress. The present study also provides evidence of a bidirectional positive feedback between the Nrf2 and SelS pathways. Specifically, Nrf2 positively regulates SelS protein levels in thyroid follicular cells in a posttranscriptional manner. Conversely, SelS positively impacts the activity of Nrf2 signaling and its downstream protective effects against oxidative stress-induced cell toxicity. HT, Hashimoto's thyroiditis.

our findings show that SelS has a positive effect on the activity of Nrf2 signaling, because reduction of SelS levels causes a reduction in the mRNA expression levels of Nrf2 target genes. Interestingly, this effect is also observed in Keap1-KO cells, suggesting that the underlying mechanism is Keap1 independent and thus likely involves a downstream step in Nrf2 signaling. In summary, the presence of a bidirectional positive feedback between the SelS and Nrf2 systems may explain why even small variations in gene expression (as a result of the SNPs) and/or activity of either pathway may be amplified, leading to phenotypic effects. Clearly, the roles of Nrf2 in the thyroid and its cross talk with other proteostatic systems in this gland warrant further elucidation.

This study has some limitations. First, it is acknowledged that a biological interaction between the two pathways is not an independent proof of a genetic interaction (epistasis). Second, it is interesting that subjects with additional *NFE2L2* SNP minor alleles (from 2 to 6 in total) had the same level of risk for developing HT as those with a single minor allele. Even though a simple gene dosage model is easier to conceptualize, for a multifactorial disease of essentially unknown pathogenesis such as HT, other models, such as a threshold model, are equally plausible. In fact, we speculate that a signaling interaction between the two genetically interacting pathways might actually better fit a threshold model. Finally, it is acknowledged that the observed interaction may not apply to all ethnicities. A recent genome-wide association study (GWAS) performed on a Croatian population suggested novel loci for HT, but did not find associations with *SELENOS* or *NFE2L2* variants (60). Of note, that study did not test specifically for an interaction between *SELENOS* and *NFE2L2* variants. Also, there were several factors that differentiate it from the present study, including the very specific and distinct ethnic origins of the participants in the two studies (Portuguese vs. Croatian); the different clinical profiles of HT patients in the two studies (e.g., overt hypothyroidism in all HT patients in the Portuguese cohort vs. inclusion of HT patients with subclinical hypothyroidism or even spontaneous euthyroidism in the Croatian study); and the relatively small population size (for a GWAS) of the Croatian cohort (838 participants with usable data in the discovery cohort) (60). The presence of striking ethnic patterns in HT susceptibility indicating the implication of ethnically restricted effects has been well acknowledged (3). It will thus be interesting to perform replication studies for the genetic interaction between *SELENOS* and *NFE2L2* variants in various independent ethnic cohorts of HT patients.

The importance of selenium for the thyroid has long been known. Selenium supplementation is recommended for the treatment of orbitopathy associated with autoimmune hyperthyroidism (Graves' disease) (61), and it is also currently being tested in a randomized controlled clinical trial as a therapeutic measure for chronic autoimmune thyroiditis (HT) (62). In view of the present findings, it might be interesting to test combinations of selenium with Nrf2 activating compounds, such as SLF-containing natural extracts, whose safety for the thyroid has actually been shown in a recent clinical trial (63).

Author Disclosure Statement

The authors have nothing to disclose.

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Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3

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