NF-κB Essential Modulator (NEMO) Is Critical for Thyroid Function*

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The IκB kinase (IKK) subunit NEMO/IKKγ (NEMO) is an adapter molecule that is critical for canonical activation of NF-κB, a pleiotropic transcription factor controlling immunity, differentiation, cell growth, tumorigenesis, and apoptosis. To explore the functional role of canonical NF-κB signaling in thyroid gland differentiation and function, we have generated a murine strain bearing a genetic deletion of the NEMO locus in thyroid. Here we show that thyrocyte-specific NEMO knock-out mice gradually develop hypothyroidism after birth, which leads to reduced body weight and shortened life span. Histological and molecular analysis indicate that absence of NEMO in thyrocytes results in a dramatic loss of the thyroid gland cellularity, associated with down-regulation of thyroid differentiation markers and ongoing apoptosis. Thus, NEMO-dependent signaling is essential for normal thyroid physiology.

The nuclear factor-κB (NF-κB) signaling pathway controls a variety of important biological functions, including immune and inflammatory responses, differentiation, cell growth, tumorigenesis, and apoptosis (1). Two distinct pathways of NF-κB activation have been reported. The classical, canonical pathway is found in virtually all mammalian cells and depends on the presence of the IKKγ/NF-κB essential modulator (NEMO)3 protein (1). In this pathway, NF-κB activation is mediated by the IKK complex, consisting of the IKK1/IKKα and IKK2/IKKβ catalytic kinase subunits and the NEMO regulatory protein (1). Basically, NF-κB transcription factors are kept inactive in the cytoplasm through binding to members of the IκB family of inhibitory proteins. Cell activation by a variety of stimuli results in the IKK-dependent phosphorylation of IκB proteins, followed by their polyubiquitination and their proteasome-dependent degradation, allowing NF-κB dimers to enter the nucleus and catalyze transcription of target genes (1).

In specific lymphoid tissues cells, an alternative, non-canonical pathway has been described that relies on IKKe-mediated phosphorylation of IκB molecules. This pathway seems to be independent of NEMO activity (1). Recent publications have also shown that diverse posttranslational modifications, including ubiquitination, sumoylation, and phosphorylation, regulate the function of NEMO in the IKK complex (2–6).

Gene-targeting experiments have shown that mice lacking p65/RELA, IKK2/IKKβ, or NEMO die during embryonic development due to liver apoptosis (7–12). The evidence that NEMO-deficient mice exhibit a phenotype similar to that of p65/RELA-deficient mice demonstrates the essential role of NEMO in the canonical NF-κB signaling. Experiments based on tissue- and/or organ-specific deletion of NEMO, obtained through Cre-mediated genetic recombination, have given diverse results regarding the physiological role of this protein. In fact, ablation of NEMO in liver parenchymal cells causes spontaneous development of steatohepatitis and hepatocellular carcinoma (13). Instead, intestinal epithelial cell-specific deletion of NEMO results in severe chronic intestinal inflammation due to apoptosis of colonic epithelial cells, impaired expression of antimicrobial peptides, and translocation of bacteria into the mucosa (14). Central nervous system-specific ablation of NEMO results in no apparent abnormalities but rather ameliorates inflammatory and autoimmune pathologies (15). NEMO inactivation in the heart did not affect embryonic cardiac development but led to spontaneous and progressive impairment of cardiac function, progression to dilated cardiomyopathy, and heart failure (16). Finally, genetic abrogation of NEMO in podocytes did not affect normal glomerular development and function under non-stressed conditions (17). Thus, it appears that canonical NF-κB signaling performs different roles and functions, depending on the type of tissue and organ.

In the present work, we have investigated the requirement for NEMO in thyroid development, differentiation, and function by generating a mouse model bearing a thyroid-specific genetic inactivation of NEMO. In these mice, development and differentiation of the thyroid gland appears normal. In contrast, after birth, thyroid-specific NEMO knock-out mice develop progressive loss of thyroid cellularity and thyroid markers, hypothyroidism, and reduced vitality due to extensive apopto-
sion of thyroid cells. Thus, our data indicate that NF-κB-dependent gene expression is essential for maintaining normal thyroid gland structure and function in the adult.

**Experimental Procedures**

_Ethics—_Procedures involving animals were conducted as indicated in the Italian National Guidelines (D.L. 100/2006 and D.L. 116/1992) and in the pertinent European Directives (EEC Council Directive 86/609, 1.12.1987), adhering to the Guide for the Care and Use of Laboratory Animals (United States National Research Council). All of the in vivo experimental activities were approved by the Animal Ethics Committee of Biogem (Italy) with ID number 4713.

_Generation of NEMO<sup>Ts</sup>-KO Mice—_To inactivate the NEMO gene in thyroid, mice expressing Cre recombinase under the control of endogenous Pax8 promoter (18) and NEMO<sup>lox/lox</sup> (12) were bred. NEMO<sup>lox/lox</sup>/Pax8<sup>Cre</sup>+ mice were named NEMO<sup>Ts-KO</sup> and used as the experimental group, whereas NEMO<sup>+/+</sup>/Pax8<sup>Cre</sup>+ littersmates were used as controls. Genotyping was performed by PCR analysis of a tail biopsy. All experiments were conducted on age- and gender-matched animals.

Metabolic Measurements—Renal parameters were evaluated in 12-month-old mice using metabolic cages as described previously (19). Mice were housed individually in metabolic cages for 5 days at 23 °C with a 12-h dark/light cycle. After 4 days of adjustment, parameters were registered on day 5. 24-h urine output was collected under mineral oil to prevent evaporation. Proteinuria was quantified by a Bradford assay, and urinary electrolytes and creatinine were evaluated using Vitrovet (Scil).

Histology and Immunohistochemistry—Mice were anesthetized by isoflurane and perfused through abdominal aorta with 4% paraformaldehyde. Blood, left kidney, and thyroid were collected before perfusion. The left kidney and one thyroid lobe were used for immunoblotting or PCR, whereas the right kidney and the other thyroid lobe were used for immunohistochemistry. After embedding in paraffin, 4-μm-thick sections were stained with hematoxylin and eosin (Sigma-Aldrich) or immunostained sections were mounted with Eukitt (Bio-Optica). Sections were then incubated with anti-NIS antibody (kindly provided by Prof. M. De Felice). Sections were then incubated with anti-NIS antibody (kindly provided by Prof. M. De Felice). Sections were then incubated with anti-NIS antibody (kindly provided by Prof. M. De Felice). Sections were then incubated with anti-NIS antibody (kindly provided by Prof. M. De Felice). Sections were then incubated with anti-NIS antibody (kindly provided by Prof. M. De Felice).

RNA Extraction and Quantitative PCR—1 μg of total RNA was isolated from a pool of three thyroid lobes using Trizol reagent (Invitrogen) and reverse-transcribed using the Quantitect reverse transcription kit (Qiagen). Quantitative PCR mixtures contained 7.5 ng of total cDNA, Power PCR Master Mix 16 (Applied Biosystems), and the following primers: Pax8, GCCATGGCTGTGAAGCAAGA (forward) and GCTTGGAGCCCCCTATCCT (reverse); Tgf1, CTACTGCAAGCCCGAACCCTG (forward) and CCATCGCCATCATATATG (reverse); and CCATCGCCATCATATATG (reverse). All quantifications were done at least three times using different biological material as sources.

**Immunoblotting—** Tissues were homogenized with a TissueLyser (Qiagen) in lysis buffer (300 mM sucrose, 25 mM imidazole, 1 mM EDTA, 1 mM PMSF) with protease and phosphatase inhibitor mixtures (Roche Applied Science). Immunoblottings were performed as described previously (20). Briefly, extracted proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Blots were developed using the ECL system (Amersham Biosciences). Anti-NEMO, anti-pIκBα/β, anti-p65, anti-p50, anti-phospho-CREB, anti-CREB, anti-caspase-3, and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. All immunoblots were done at least three times using different biological material as sources.

**Culture of Primary Mouse Thyroid—** Mice of different genotypes were anesthetized and sacrificed. Thyroid lobes were dissected aseptically and placed on a microscope slide containing a drop of Eagle’s minimum essential medium. The lobes were disrupted mechanically, using two 25-gauge needles to obtain approximately 10 fragments from each lobe. The fragments were transferred to a 1.5-ml tube containing 1 ml of digestion medium, consisting of 112 units/ml type I collagenase, 1 mM EDTA, 1 mM PMSF) with protease and phosphatase inhibitor mixtures (Roche Applied Science). Immunoblottings were performed as described previously (20). Briefly, extracted proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and incubated with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Blots were developed using the ECL system (Amersham Biosciences). Anti-NEMO, anti-pIκBα/β, anti-p65, anti-p50, anti-phospho-CREB, anti-CREB, anti-caspase-3, and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. All immunoblots were done at least three times using different biological material as sources.
and 1.2 units/ml dispase dissolved in Eagle’s minimum essential medium. The enzymatic digestion was carried out for 45 min in a 37 °C water bath. The follicles were seeded and cultured in F-12 medium (EuroClone) supplemented with 10% Nu-Serum IV (BD Biosciences), 10 ng/ml somatostatin (Sigma-Aldrich), and 2 ng/ml glycyl-L-histidyl-lysine acetate (Sigma-Aldrich) in a water-saturated incubator, under 95% air, 5% CO2 at 37 °C. Thyroid-stimulating hormone (TSH) and TNF used for thyrocyte stimulation were obtained from Sigma-Aldrich.

Free T4 and TSH Measurement—Venous blood samples were collected in microtubes without anticoagulant. After clot formation, samples were centrifuged, and the serum fraction was kept at −80 °C. Free T4 was measured using the ELISA kit from DiaMetra according to the manufacturer’s instructions. TSH levels were determined by a rat TSH radioimmunoassay kit (Institute of Isotopes, Budapest, Hungary).

Statistics—Data were analyzed by Student’s t test. A p value of <0.05 was considered significant.

Results

In order to understand the role of NEMO in thyroid development and function, we applied the Cre-LoxP strategy to specifically delete the NEMO locus in the thyroid gland. For this, females bearing a floxed Nemo allele (NemoFlox−/−), which do not show phenotype abnormalities (12), were crossed to the knock-in Pax8Cre/+ mice, which express the Cre recombinase under the control of the endogenous Pax8 gene promoter and mediate efficient Cre recombination in thyroid (18). Mutant mice were observed at the expected Mendelian frequency and were named NEMO thyroid-specific knock-out (NEMOTS-KO). As shown in Fig. 1A, site-specific recombination of the NEMO allele was observed only in NEMO floxed mice expressing the Cre recombinase. Efficient ablation of the NEMO locus was confirmed by immunoblotting analysis in the kidney cortex, inner stripe of the outer medulla (ISOM), and inner medulla (IM) of NEMOTS-KO and control mice. MW, molecular weight markers; WB, Western blotting; CTR, control.
other hand, expression of p65, IKKα/β, and p50 were not affected by genetic deletion of NEMO (Fig. 1C, right). Because Pax8Cre/+/H11001 mice express Cre recombinase also in the mesonephros and metanephros (18), excision of the Nemo floxed allele was consistently detected also in kidney tissues (Fig. 1, D and E).

NEMO<sup>Ts-KO</sup> mutant mice appeared undistinguishable from control mice at birth (data not shown). In contrast, starting from 2 months of age, mutant mice developed clearly smaller than control littermates and displayed a significantly reduced body weight (Fig. 2A). NEMO<sup>Ts-KO</sup> mice had a significantly shortened life span, and about 50% of mice die before 8 months of age (Fig. 2B). When compared with control mice, NEMO<sup>Ts-KO</sup> mice presented significantly lower levels of serum-free T4 hormone (Fig. 2C) and increased TSH levels (Fig. 2D), which is indicative of hypothyroidism. Hence, body weight reduction and early lethality observed in these mice might be due, at least in part, to thyroid dysfunction.

To exclude the possibility that the premature lethality observed in NEMO<sup>Ts-KO</sup> mice was due to renal failure, we carried out a histological and functional analysis on the kidneys of the mutant mice. The result of this analysis, shown in Table 1, indicates that partial loss of NEMO expression in specific kidney tissues was not associated with an overt kidney phenotype. In fact, monitoring of the renal function in NEMO<sup>Ts-KO</sup> mice did not reveal any physiological alteration (Table 1). Kidney histology was assessed by H&E staining and immunohistochemistry for the kidney-specific markers AQP2 and NKCC2. Again, no alteration in immunoreactivity for these markers was detected in NEMO<sup>Ts-KO</sup> kidneys with respect to control mice at 12 months of age (Fig. 3, A–C). From these results, we deduced that NEMO<sup>Ts-KO</sup> do not suffer of renal dysfunction. This conclusion is consistent with a previous observation showing that podocyte-specific NEMO-deficient mice do not show overt changes in kidney morphology and functionality (17).

To verify whether NEMO inactivation affects thyroid embryonic development, mutant embryonic day 16.5 embryos were analyzed because Cre recombinase in Pax8<sup>Cre/+/</sup> mice is active from embryonic day 8.5 (18). At embryonic day 16.5, no alteration in thyroid size and morphology could be detected (Fig. 4A), and similar results were obtained when the same analysis was performed in mice at 1 month of age (Fig. 4A). Hence, these data indicate that canonical NF-κB signaling is not required for embryonic thyroid development and differentiation.

The effect of NEMO conditional inactivation on thyroid morphology and differentiation was further analyzed. Starting

FIGURE 2. Early lethality of NEMO<sup>Ts-KO</sup> mice. A, body weight of male NEMO<sup>Ts-KO</sup> and control mice at 1 month after birth (n = 12 for each genotype). p value was calculated with Student’s t test comparing both groups. B, survival rates of NEMO<sup>Ts-KO</sup> and control mice (n = 10 for each) during 12 months after birth. C, ELISA assay of free T4 serum level in NEMO<sup>Ts-KO</sup> and control mice (n = 10 for each group) at the indicated months after birth. D, TSH serum level in NEMO<sup>Ts-KO</sup> and control mice at 12 months after birth (n = 7 for each group). CTR, control. Error bars, S.E.
**FIGURE 3.** NEMOTS-KO mice kidney phenotype analysis. Shown are H&E staining (A) and immunohistochemical analysis (B) of the kidney markers NKCC2 and AQP2 in control and NEMOTS-KO kidney sections at 12 months of age (OM, outer medulla; IM, inner medulla). C, quantification of AQP2-positive cells in the collecting ducts of the inner medulla. The count was carried out on images acquired from five NEMOTS-KO and five control mice. Data shown are representative of at least three independent experiments. CTR, control. Error bars, S.E.
from 2 months of age, thyroid glands of NEMO\textsuperscript{T5-KO} mice appeared considerably smaller compared with control mice (Fig. 4B). In the surviving 2-month-old NEMO\textsuperscript{T5-KO} mice, significant cell loss was observed, and the thyroid follicular architecture appeared highly variable in diameter with irregular outlines (Fig. 4, B–F). Immunohistochemical and immunofluorescent analysis shows that the expression level of two thyroid-specific transcription factors, namely PAX8 and TTF1, steadily declined over time (Fig. 4, C and D). A similar decline occurred for thyroglobulin and for the Na/I symporter NIS (Fig. 4, E and F). NIS, for instance, appeared barely detectable in thyrocytes isolated from 2-month-old NEMO\textsuperscript{T5-KO} mice (Fig. 4E). Masson’s trichrome staining also revealed the presence of fibrotic material in mutant thyroids (Fig. 4G). In all staining, the progressive disorganization and degeneration of the thyroid parenchyma in NEMO\textsuperscript{T5-KO} mutant mice was clearly visible. Also, reduction of cellularity and gland size in NEMO\textsuperscript{T5-KO} mice was accompanied by massive apoptosis, as indicated by the staining for active caspase 3 (Fig. 4H).

Quantitative PCR analysis confirmed the reduced expression of the thyroid markers PAX8, TTF1, TPO, and NIS in NEMO-deleted thyroids (Fig. 4I). The mRNA expression levels of TSH receptor and thyroglobulin were also both significantly reduced (Fig. 4I). Despite its reduced expression, however, TSH receptor signaling does not appear compromised in NEMO\textsuperscript{T5-KO} thyrocytes, as assessed by monitoring phosphorylation of CREB following TSH stimulation (Fig. 4J).

NF-\kappaB controls the expression of several genes, including \textit{Bfl-1/A1}, \textit{Bel-2}, \textit{Bel-xL}, \textit{c-Flip}, and \textit{lmp}, that protect cells against apoptotic cell death (23–29). Consistently, all of these anti-apoptotic genes appear significantly down-regulated in NEMO\textsuperscript{T5-KO} thyrocytes, as assessed by monitoring phosphorylation of CREB following TSH stimulation (Fig. 4J).

Taken together, these data demonstrate that in adult mice, NEMO is required for thyrocyte survival and contributes to the maintenance of thyrocyte differentiation by regulating the expression of several thyroid markers.
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Discussion

In this paper, we demonstrate that NEMO signaling is essential for normal postnatal thyroid gland structure and function. Although previous studies have clearly established a role for NF-κB in thyroid tumor progression (30–32), this is the first work that examines the role of canonical NF-κB signaling in normal thyroid development and physiology. We show that mice with thyroid-specific ablation of NEMO develop a pronounced impairment of thyroid function, eventually leading to shortened life span. On the other hand, no gross alterations of thyroid gland localization, morphology, and differentiation were observed during embryonic development and in newborn mice, indicating that the NEMO-dependent NF-κB signaling is not required for embryonic thyroid development. Our results indicate that in thyroid, NEMO is required for at least two essential aspects: (i) thyrocyte survival and (ii) maintenance of thyroid marker expression. The critical role of NF-κB in cell survival is widely supported by the available literature. Gene-targeting experiments have in fact shown that mice lacking p65/RELA, IKK2, or NEMO die during embryonic development due to liver apoptosis (7–12). Thus, the extensive apoptosis observed in NEMO<sup>TS-KO</sup> thyrocytes confirms the requirement for NF-κB in orchestrating cytoprotective pathways. Particularly interesting are the data showing the different behavior of WT and NEMO<sup>TS-KO</sup> thyrocytes when exposed to TSH. In fact, whereas both wild type and NEMO<sup>TS-KO</sup> thyrocytes proliferate following TSH stimulation, NEMO<sup>TS-KO</sup> thyrocytes also undergo extensive apoptosis (Fig. 5). This finding is consistent with previous evidence showing that TSH stimulation on thyrocytes in fact triggers both proliferative and apoptotic responses (33). Our work now shows that NF-κB signaling could play a decisive role in determining the fate of thyrocytes following TSH stimulation.

An interesting aspect of our experiments is the evidence here provided that NF-κB controls, directly or indirectly, the expression level of several thyroid markers. Although an involvement of NF-κB in the regulation of Nis expression has been already reported (34), we now show that, in addition to NIS, NF-κB controls the expression of a panel of thyroid markers, including TTF1, PAX8, TPO, and thyroglobulin. In this context, our work provides a molecular explanation for some phenotypical aspects of human disorders associated with mutations in NEMO. In humans, in fact, mutations in the X-linked NEMO gene cause two distinct genetic diseases. Mutations that completely disrupt the NEMO locus result in Incontinentia Pigmenti, a disease where male patients die in utero, whereas the phenotypic analysis of females is complicated by the fact that NEMO-deficient cells quickly disappear and are replaced by wild-type cells. A second disease, named hypohydrotic ectodermal dysplasia with immune deficiency (HED-ID), is characterized by impaired skin appendage development and severe immune deficiency. Strikingly, hypothyroidism is not uncommon in patients with HED-ID (35–38), as predicted by the data we show here.

In conclusion, our data can be easily interpreted considering the crucial role that NEMO plays in activation of NF-κB, which, in turn, regulates the expression of genes that are critical for cell survival. However, it should not be disregarded that NF-κB-independent functions have been ascribed to NEMO, and these latter are also involved in the control of cell proliferation and...
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cell death (39–41). Hence, it is very possible that NF-κB-independent functions of NEMO may contribute, at least in part, to the cellular degeneration observed in NEMO-TS-KO thyrocytes.

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