Thyroid-specific inactivation of KIF3A alters the TSH signaling pathway and leads to hypothyroidism

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Abstract

Kinesins, including the kinesin 2/KIF3 molecular motor, play an important role in intracellular traffic and can deliver vesicles to distal axon terminals, cilia, to nonpolarized cell surfaces or to epithelial cell basolateral membranes, thus taking part in the establishment of cellular polarity. We report here the consequences of kinesin 2 motor inactivation in the thyroid of 3-week-old Kif3a<sup>Δflox</sup> Pax8<sup>Cre</sup>+/− mutant mice. Our results indicate first that 3-week-old Pax8<sup>Cre</sup>−/− mice used in these experiments present minor thyroid functional defects resulting in a slight increase in circulating bioactive TSH and intracellular cAMP levels, sufficient to maintain blood thyroxine levels in the normal range. Second, Kif3a inactivation in thyrocytes markedly amplified the phenotype observed in Pax8<sup>Cre</sup>+/− mice, resulting in altered TSH signaling upstream of the second messenger cAMP and mild hypothyroidism. Finally, our results in mouse embryonic fibroblasts indicate that Kif3a inactivation in the absence of any Pax8 gene alteration leads to altered G protein-coupled receptor plasma membrane expression, as shown for the β2 adrenergic receptor, and we suggest that a similar mechanism may explain the altered TSH signaling and mild hypothyroidism detected in Kif3a<sup>Δflox</sup> Pax8<sup>Cre</sup>−/− mutant mice.

Key Words

- thyroid
- hypothyroidism
- genetically-modified mouse
- Kif3a
- molecular motor
- kinesin 2

Introduction

Little is known about molecular motors responsible for intracellular traffic in thyroid follicle cells. However, like in other polarized cells, thyroid proteins must specifically reach the apical or basolateral membrane to be functional. In order to assure the synthesis of tri-iodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) thyroid hormones, the TSH receptor and the sodium/iodide symporter (NIS) must reach the basolateral surface, while thyroperoxidase and the dual oxidases must be transported to the thyrocyte apical membrane, and thyroglobulin (TG) exported through the apical membrane in the colloid.
Kinesins play an important role in intracellular traffic. The superfamily of kinesins is constituted of motor proteins that use energy liberated from ATP hydrolysis to transport vesicles along microtubules. These proteins are composed of a motor domain containing an ATP-binding site and a microtubule-binding site, a domain that binds vesicles, and an oligomerization domain (Scholey 1996). Most of these motor proteins, including kinesin 2, mediate vesicle transport over long distances to the plus end of microtubules. This transport pathway is highly conserved through evolution. In view of microtubule organization, kinesins can deliver vesicles to distal axon terminals, to cilia or flagella, to nonpolarized cell surfaces or to epithelial cell basolateral membranes, taking part in the establishment of cellular polarity (Hirokawa 2000). Kinesin 2 motor (or kinesin family member 3 (KIF3)) is composed of a heterotrimeric complex KIF3A–KIF3B–KAP3. KIF3A/KIFB are the motor proteins and KAP3 regulates the binding between the cargo and the KIF3 heterodimer (Hirokawa 2000). This molecular motor is expressed in all tissues, including the thyroid. KIF3A or KIF3B inactivation in mice leads to abolition of molecular motor function and to an early embryonic lethality. These knockout embryos exhibit loss of cilia ordinarily present on cells of the embryonic node, leading to a randomized establishment of left–right asymmetry and numerous structural abnormalities (Nonaka et al. 1998, Marszalek et al. 1999).

In this study, we have explored the role of the kinesin 2 molecular motor in mouse thyroid development, structure and function 3 weeks after birth. We used mice carrying a Kif3a floxed gene as well as knock-in mice expressing the paired domain transcription factor Pax8 promoter (Marszalek et al. 1999, Bouchard et al. 2002). Indeed, in these Pax8Cre/+ mice, the expression of the recombinase mimics the expression of the paired domain transcription factor Pax8, including thyroid follicle cells, kidney, inner ear, and the mid–hindbrain boundary region, all of them from the embryonic stage E8.5 (Plachov et al. 1990, Bouchard et al. 2002).

Materials and methods

Mice

Kif3aΔ/flox mice, obtained from LS Goldstein (University of California, San Diego, USA) and Pax8Cre/+ mice, obtained from M Busslinger (Research Institute of Molecular Pathology, Vienna, Austria) were on a mixed Sv129×C56BL/6 genetic background. All procedures were approved by the Ethical Committee of the Medical and the Sciences School of the Université Libre de Bruxelles.

Genotyping and analysis of Cre/lox recombination specificity

PCR was performed on tail DNA with oligonucleotide primers 1 (5'-AGGCGAGCGAAGGTG-3'), 2 (5'-CTGTTGATTTTGTGACCAGCC-3'), and 3 (5'-TGCCAGTCAAATGGACCGG-3'). Using these three primers in the same PCR, amplicons of 200, 360, and 490 bp were obtained, corresponding to the delta, wild-type, and floxed Kif3a allele respectively.

Cell culture

Large T antigen-immortalized Kif3a+/+ and Kif3a−/− mouse embryonic fibroblasts (MEFs) were maintained in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1.1 mM sodium pyruvate, 3.97 mM L-glutamine, 100 μM nonessential amino acids and 300 μg/ml G418 (Sigma–Aldrich). Cells were kept in a humidified incubator at 37 °C and 5% CO2. The medium was changed every 3 days.

Western blotting

Thyroid and MEF proteins were extracted using RIPA buffer (Tris–HCl 50 mM, pH 7.4, EDTA 1 mM, NaCl 150 mM, Triton X-100 1%, Sodium deoxycholate 1%, SDS 0.1%). Thyroid (25 μg) and MEF (60 μg) protein extracts were separated on a 10% SDS–PAGE and transferred onto PVDF membrane (Amersham Biosciences) using a Mini Trans-Blot Transfer Cell system (Bio-Rad). Membrane was preincubated for 1 h with 5% milk and 0.1% Tween in PBS, followed by overnight incubation at 4 °C with a Kif3a antibody (Sigma–Aldrich) diluted 1:2000, or with a β-actin antibody (Sigma–Aldrich) diluted 1:5000, or with a β2 adrenergic receptor (ABRR2) antibody (Abcam, Cambridge, UK) diluted 1:1000 in the same buffer. The membrane was washed in PBS and then incubated for 1 h at room temperature with peroxidase-conjugated protein A (Sigma–Aldrich) diluted 1:10 000. Proteins were visualized with Western Lightning plus-ECL (Perkin-Elmer) and exposed to X-ray film. Protein concentration was determined with Bio-Rad protein assay reagents using BSA as standard.

Serum hormone assays

Enzyme immunoassays for mouse T3 and T4 were performed using Mouse/Rat T3 ELISA kit from Calbiotech.
(Spring Valley, SK, Canada) and T4 ELISA kit from DiaSource (Louvain-la-Neuve, Belgium) respectively. TSH was measured in a bioassay using a line of Chinese hamster ovary cells stably transfected with the human TSH receptor cDNA, as previously described (Perret et al. 1990, Moeller et al. 2003). Briefly, 50,000 cells were seeded in individual test tubes and incubated for 24 h in 100 μl Ham’s F-12 nutrient mixture supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 2.5 μg/ml Fungizone. Cells were washed with 500 μl Krebs-Ringer HEPES buffer (pH 7.4), supplemented with 8 mM glucose and 0.5 g/l BSA and then preincubated for 30 min in 200 μl of the same medium. The medium was removed and 200 μl of fresh buffer containing 20 μl of serum for TSH measurement and 25 μM Rolipram, a cAMP phosphodiesterase inhibitor, were added. The incubation was continued for 1 h, at the termination of which the medium was discarded and replaced with 0.1 M HCl. cAMP was measured in the dried cell extract by RIA according to the method of Brooker (Brooker et al. 1979). Blanks were prepared as above but contained 20 μl of human serum without TSH. Thus, this assay measures the stimulus to which the cells are exposed, which reflects both the concentration of the hormone and its specific activity, i.e. its glycosylation level.

Histology
Thyroid glands were removed from 3-week-old mice and overnight fixed at room temperature in 3.7% formaldehyde. They were dehydrated through isopropanol series, cleared in Histoclear (National Diagnostics, Hessel, UK), embedded in paraffin and cut at 7 μm. For cryosectioning, thyroids were overnight fixed at room temperature in 3.7% formaldehyde. They were dehydrated through isopropanol series, cleared in Histoclear (National Diagnostics, Hessle, UK), embedded in paraffin and cut at 7 μm. Histological analysis, slides were stained with hematoxylin/eosin (Klinipath). ImageJ Software (Thermo Scientific, Rockford, IL, USA) was used to quantify follicle area as well as cell and follicle densities from a minimum of five mice per genotype. For the follicle area, more than 20 follicles were measured in each thyroid gland. For each mouse, cell and follicle densities per mm² were estimated by counting the number of cells and follicles in a total area of 0.1 mm². Colloid and epithelial areas and volumes as well as the Thyroid Activation Index were obtained as described (Kmiec et al. 1998).

Immunohistochemistry
For TG analysis, sections were deparaffinized and treated with 0.3% hydrogen peroxide in methanol for 30 min. They were incubated with blocking solution (10% goat serum in PBS) for 1 h and then with a human TG antibody (Dako, Heverlee, Belgium) diluted 1:2500 overnight at room temperature.

For iodinated TG analysis, quenching of endogenous peroxidases was performed, and then antigen retrieval was done by boiling the slides immersed in citrate buffer at pH 6. They were incubated with blocking solution (10% BSA/2% sheep serum in PBS) for 1 h and then with an antibody directed against iodinated TG (a kind gift from Prof. C Ris-Stalpers, Laboratory of Pediatric Endocrinology, Amsterdam, The Netherlands) diluted 1:2000 overnight at 4 °C.

For NIS analysis, sections were permeabilized for 20 min in PBS-0.1% Triton X-100 and treated with 0.3% hydrogen peroxide for 30 min. They were incubated with blocking solution (1% BSA and 2% goat serum in PBS) for 30 min and then with an NIS antibody (a kind gift from Prof. Carrasco, Albert Einstein College of Medicine, NY, USA) diluted 1:500 overnight at room temperature.

Semiquantitative PCR
Total RNA was isolated from thyroid tissues using RNAeasy kit (Qiagen) including a DNase treatment and was reverse transcribed with the M-MLV Reverse Transcriptase (Invitrogen) using 300 ng total RNA. The following genes were PCR amplified using sense and antisense oligonucleotide primers as follows: V-abl Abelson murine leukemia viral oncogene homolog 1 (Abl1) sense, 5’-TCGGGACGTGGGCATTT-3’ and antisense, 5’-CGCATGAGCTCTGTAAGGCTC-3’; Kif3a sense, 5’-ATGCCGATCAAATAAGTCGGAGA-3’; and antisense, 5’-GTTCCTCTCATTTCTCCACG-3’; Nis sense, 5’-CGCTA CGGTCTCTGAAGTTC-3’ and antisense, 5’-CGCAAGTCCTAGGACTGTTAGG-3’. The reaction mixtures contained 5 μl PCR buffer 10× (containing 15 mM MgCl₂; Qiagen), 1 μl DNTP mix (10 mM each DNTP), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 2 U Taq DNA polymerase (Qiagen), 15 ng template cDNA and RNase-free water to a final volume of 50 μl.

Quantitative PCR
Total RNA from thyroid tissues was isolated using RNAeasy kit (Qiagen) including a DNase treatment and was reverse transcribed with the M-MLV Reverse Transcriptase
(Invitrogen) using 120 ng total RNA. Reactions for the quantification of Kif3a, Nis (or Slc5a5), Dia2, Tshr, Gnas, Adcy3, Adcy6 and Adcy9, Prkar1a and Prkar1b, Prkar2a and Prkar2b, and Prkaza and Prkacb mRNAs were performed in a CFX96 Real-Time System (Bio-Rad), using SYBR Green as detector dye. Porphobilinogen deaminase Hmbs and acidic ribosomal phosphoprotein P0 (36B4 (Rplp0)) were used as reference genes. The reaction mixtures contained 10 µl SYBR Green PCR kit (Bio-Rad), 200 nM of each primer, 6 ng template cDNA and RNase-free water to a final volume of 20 µl. The sequences of all oligonucleotide primers are listed in Supplementary Table 1, see section on supplementary data given at the end of this article.

Statistical analysis
All results are expressed as mean ± S.E.M. and statistical analysis was done by unpaired t-test or Mann–Whitney U test, using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Results
Kif3a inactivation in mouse thyroid cells
The Cre recombinase tissue specificity and efficiency at the Kif3a locus were investigated in Kif3aflox/+ and Kif3a<sup>flox</sup>/Pax8<sup>Cre/+</sup> mice at the DNA level (Fig. 1A and B). Three oligonucleotide primers were designed to amplify the wild-type, flox, and exon 2-deleted (or Δ) Kif3a alleles in the same PCR. As expected, in Kif3a<sup>flox</sup>/ mice, amplicons of 360 and 490 bp, corresponding to the wild-type and the flox alleles respectively, were amplified out of the DNA extracted from eight different tissues. In Kif3a<sup>flox</sup>/Pax8<sup>Cre/+</sup> mice, an additional 200 bp Δ signal was specifically detected after amplification of DNA extracted from the thyroid and the kidney (Fig. 1B). The presence of the Δ signal in these two tissues was associated with a decreased intensity of the flox allele signal. These results demonstrate that the floxed Kif3a locus is efficiently recombinated by the Cre enzyme. They also confirm previous report indicating that the Cre recombinase is expressed in thyrocytes and kidney cells of Pax8<sup>Cre/+</sup> mice.

In order to investigate the role of the molecular motor component Kif3a in mouse thyrocytes, Kif3a<sup>Δ/+</sup> Pax8<sup>Cre/+</sup> mice were crossed with Kif3a<sup>flox/+</sup> mice to produce Kif3a<sup>Δ/flox</sup> pax8<sup>Cre/+</sup> ‘mutant’ mice, Kif3a<sup>Δ/+</sup> and Kif3a<sup>flox/+</sup> ‘control’ mice as well as Kif3a<sup>Δ/+</sup> Pax8<sup>Cre/+</sup> ‘Cre’ mice from the same litter. RT-PCR, quantitative PCR (qPCR) and western blot analyses demonstrated a severely reduced Kif3a mRNA and protein in the thyroid of mutant mice, as compared with control and Cre mice (Fig. 1C, D and E).

Normal thyroid migration but hypothyroidism in mutant mice
Mice with all possible genotypes were recovered at Mendelian frequency at birth, suggesting that Kif3a
Figure 1

Strategy to inactivate Kif3a in thyroid follicle cells. (A) Mice with a wild-type, a floxed, and/or a delta (Δ) Kif3a allele were used in the study. Black boxes, exons of the Kif3a gene; triangles, loxP sites; arrowheads, primers used to identify the mouse genotype in PCR. (B) Gel electrophoresis of a PCR performed on DNA extracted from different tissues of Kif3a flox/+ and Kif3aflox/+ Pax8cre/+ mice, using the three primers shown in (A). Ampicons of 490, 360, and 200 bp are identified and correspond to the floxed, wild-type, and Δ alleles respectively. The Δ allele is solely present in the thyroid and the kidney. MW: molecular weight. Kif3a mRNA identification and quantification by (C) RT-PCR and (D) qPCR performed on thyroid cDNA from 3-week-old mice. The Abl gene was used as control in the RT-PCR experiments. In qPCR, mean ± S.E.M. of five independent experiments are represented. AU, arbitrary units. Statistics (t-test with Welch’s correction): *P<0.05 and **P<0.01. (E) Western blot analysis of thyroid protein extracts from 3-week-old normal, Cre, and mutant mice with a Kif3a antibody. β-Actin served as loading control.
deletion in Pax8-expressing cells is not embryonically lethal. Nevertheless, at weaning around 3 weeks of age, all mutant mice died. Dying mutant mice presented mild growth retardation and autopsy revealed a marked increase in kidney weight and size, compared with control and Cre mice (Supplementary Figure 1A, B, C and D, see section on supplementary data given at the end of this article). Histological analysis demonstrated the presence of numerous and large cysts in the kidneys of mutant mice, providing a probable cause for their reduced survival (Supplementary Figure 1E). Indeed, in Pax8Cre/+ mice, the Cre recombinase is also expressed in kidney cells, and this was confirmed by the presence of severely decreased Kif3a mRNA and protein levels in mutant kidneys (Supplementary Figure 1F and G). No obvious alteration was detected in other organs, including the brain (data not shown). In 3-week-old mutant mice, the thyroid was normally localized in the neck and was of normal size and weight (Fig. 2A, data not shown). Mutant mice displayed hypothyroidism with low total T4 levels and increased TSH bioactivity, as compared with control and Cre mice (Fig. 2B and C). No difference was observed in total T3 levels between groups of mice (Fig. 2B). Interestingly, Cre mice, which are knock-in mice with a heterozygous insertion of the Cre cDNA into exon 3 of the Pax8 gene, presented a mild but significant increase in TSH bioactivity, as compared with control mice. However, in these mice, TSH bioactivity was still markedly below the level observed in mutant mice (Fig. 2C). Together, our results indicate that Kif3a inactivation using Pax8Cre/+ mice results in early lethality, probably a consequence of kidney failure. Importantly, they indicate that Kif3a inactivation in thyroid follicle cells leads to mild hypothyroidism. They also suggest that, on the genetic background studied, Pax8Cre/+ mice have a mild thyroid dysfunction leading to a slight increase in bioactive TSH level.

Normal thyroid structure, but reduced TG iodination and NIS expression in the thyroid of mutant mice

Histological examination of 3-week-old thyroids revealed that the general thyroid architecture as well as the follicle and cell densities was normal in Cre and mutant mice (Figs 2D and 3A). A trend for decreased follicular area (Cre mice, 2049 ± 428 μm²; mutant mice, 1394 ± 135 μm²; n = 5 mice per group; P = 0.2186), colloid area (Cre mice, 927 ± 230 μm²; mutant mice, 607 ± 111 μm²; n = 5 mice per group; P = 0.2469), colloid volume (Cre mice, 46 ± 17 mm³; mutant mice, 25 ± 7 mm³; n = 5 mice per group; P = 0.2742), epithelial area (Cre mice, 1121 ± 202 μm²; mutant mice, 787 ± 33 μm²; n = 5 mice per group; P = 0.1770) and epithelial volume (Cre mice, 74 ± 21 mm³; mutant mice, 42 ± 3 mm³; n = 5 mice per group; P = 0.0952) was observed in mutant thyroids, but the differences did not reach statistical significance when compared with Cre thyroids. A similar Thyroid Activation Index was found in Cre and mutant mice (Cre mice, 1.891 ± 0.2952; mutant mice, 2.090 ± 0.3541; n = 5 mice per group; P = 0.6772). As compared with control and Cre thyroids, Tg mRNA and protein were normal in mutant thyroid, but iodinated TG level in the colloid was significantly decreased in mutant mice, showing a very heterogeneous expression in the different follicles (Fig. 3B and C, data not shown). In order to define a potential mechanism of this iodination defect, the expression of NIS, the NIS responsible for the basal membrane iodide uptake, was investigated at the protein and mRNA levels (Fig. 3D, E and F). NIS protein expression and mRNA level were markedly decreased in mutant mice, as compared with control and Cre mice, reaching about 20% of control level and 40% of Cre level. As expected from Pax8Cre/+ mice, which are heterozygous at the Pax8 locus, a slight but significant decrease in both NIS protein and mRNA levels was observed in these mice.

Together, our results define a marked thyroid dysfunction in mutant mice. This dysfunction is probably a consequence of the nearly complete Kif3a inactivation combined with the partial Pax8 gene inactivation, since mild thyroid alterations are already present in the Pax8Cre/+ mice used to inactivate Kif3a in thyroid follicle cells. They also suggest a potential role of the TSH/cAMP signaling pathway in the pathogenesis of the mutant phenotype since Nis (Slc5a5) mRNA expression, which is altered in mutant mice, is known to be regulated by this pathway in thyroid follicle cells.

Altered TSH signaling pathway in mutant mice

Since Nis mRNA was found to be significantly decreased in mutant thyroid, mRNA levels of other genes known to be involved in or induced by the TSH/cAMP signaling pathway were investigated by qPCR in the thyroid of mutant, control, and Cre mice (Figs 4 and 5). Out of the genes tested, only the mRNA levels of Adcy3 (the adenylate cyclase 3 gene), Prkar2b (the PKA regulatory subunit 2b gene), and Dio2 (the type 2 iodothyronine deiodinase gene) were found significantly decreased in mutant thyroid, as compared with control and Cre thyroids. Expression of the Dio1 gene was decreased both in Cre and mutant thyroids, suggesting that Dio1 mRNA may be
controlled directly or indirectly by PAX8 transcription factor. By contrast, mRNA levels for the TSH receptor, G\textsubscript{as}, Adcy6, and Adcy9, the PKA α and β catalytic subunits as well as for the Prkar1α, Prkar1β, and Prkar2α were similar in mutant, Cre, and control thyroids (Fig. 5). Interestingly, among the genes tested, only Nis, Adcy3, Prkar2β, and Dio2 mRNAs expression are known to be regulated by the second messenger cAMP, through CREB transcription factor. Thus, we next investigated basal cAMP levels in mutant, Cre, and control thyroids (Fig. 6). In Cre

Figure 2
Normal thyroid migration but hypothyroidism in mutant mice. (A) Sections through the neck of 3-week-old control (Ctrl), Cre, and mutant mice showing the left and right lobes of the thyroid (arrows) and the trachea (Tr). (B) Total T\textsubscript{4} in the blood of 3-week-old control (n = 71), Cre (n = 59), and mutant (n = 46) mice and total T\textsubscript{3}, control (n = 29), Cre (n = 20), and mutant (n = 17) mice and (C) TSH bioactivity: control (n = 75), Cre (n = 63) and mutant (n = 52) mice. Means ± S.E.M. are represented. Statistics (Mann–Whitney U test), ***P < 0.001. (D) Cell and follicle densities were determined in thyroids of 3-week-old control (n = 5–11), Cre (n = 5), and mutant (n = 5–13) mice. Means ± S.E.M. are represented. No statistical difference was observed between control, Cre, and mutant mice. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-12-0219.
thyroids, basal cAMP levels were significantly increased, as compared with control thyroids. This is most probably the consequence of the increased TSH bioactivity observed in these mice (Fig. 2C). Based on this result, basal cAMP levels should be markedly increased in mutant thyroids, since TSH bioactivity was about seven- and fourfold increased in these mice compared with control and Cre mice, respectively. However, in agreement with a defect in the proximal part of the TSH/cAMP signaling pathway, cAMP levels in mutant mice were found only slightly increased when compared with control mice, and severely decreased when compared with Cre mice (Fig. 6).

Together, our results suggest that mutant mice present a defect in the proximal part of the TSH/TSH receptor/cAMP signaling pathway. They also confirm that the Pax8Cre/+ mice used to induce recombination have a thyroid dysfunction, which is compensated by increased TSH bioactivity.

Figure 3
Normal histological structure, but decreased iodinated thyroglobulin and NIS expression in mutant thyroid. (A) Hematoxylin/eosin-stained sections of thyroid from 3-week-old control (Ctrl), Cre, and mutant mice. Bars, 100 μm. Insets show follicles at higher magnification (×40). Immunodetection of (B) TG, (C) iodinated TG, and (D) NIS proteins on thyroid sections from 3-week-old control, Cre, and mutant mice. Bars, 50 μm. Nis mRNA identification and quantification by (E) RT-PCR and (F) qPCR performed on thyroid cDNA from 3-week-old control, Cre, and mutant mice. The Ab1 gene was used as control in the RT-PCR experiments. In qPCR, means ± S.E.M. of five independent experiments are represented. AU: arbitrary units. Statistics (unpaired t-test): *P < 0.05 and ***P < 0.001. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-12-0219.
Abnormal plasma membrane G protein-coupled receptor transport in Kif3a$^{-/-}$ MEFs

The faint TSH receptor expression on mouse thyroid follicle cells and/or the absence of adequate antibody directed against this receptor did not allow us to clearly localize or quantify the TSH receptor on the follicle cell basal membrane and to validate in vivo our hypothesis concerning the defect in the proximal part of the TSH/TSH receptor/cAMP signaling pathway in mutant mice (data not shown). We thus used a less complex cellular model to probe this hypothesis: Kif3a$^{-/-}$ MEF infected with RFP-tagged TSH receptor-expressing lentiviruses. Unfortunately, infections demonstrated that both RFP-TSH receptor mRNA and protein were very significantly less expressed in Kif3a$^{-/-}$ than in Kif3a$^{+/+}$ MEF, preventing the use of lentivirus to probe our hypothesis (data not shown). As an experimental surrogate, we decided to study the subcellular localization and signaling downstream of

Figure 4
Reduced Dio1 and Dio2 mRNA expression in thyroid of mutant mice. mRNA encoding Dio1 and Dio2 were quantified by qPCR performed on thyroid cDNA from 3-week-old control, Cre, and mutant mice. Means ± S.E.M. of four or five independent experiments are represented. AU, arbitrary units. Statistics (unpaired t-test or Mann–Whitney U test): NS, nonsignificant; *P < 0.05, **P < 0.01 and ***P < 0.001. Dio1, type 1 iodothyronine deiodinase; Dio2, type 2 iodothyronine deiodinase.

Figure 5
Reduced Adcy3 and Prkar2b mRNA expression in thyroid of mutant mice. mRNA encoding (A) Tshr and Gnas1, (B) Adcy3, 6, and 9 as well as (C) the a and (D) b subunits of Prkar1 (R1a and R1b), Prkar2 (R2a and R2b), and Prkac (Ca and Cb) were quantified by qPCR performed on thyroid cDNA from 3-week-old control (Ctrl), Cre, and mutant mice. Means ± S.E.M. of four or five independent experiments are represented. AU, arbitrary units. Statistics (unpaired t-test or Mann–Whitney U test), *P < 0.05. Tshr, TSH receptor; Gnas1, protein G$\alpha$; Adcy, adenylate cyclase; Prkar, protein kinase A regulatory subunit; Prkac, protein kinase A catalytic subunit.
another G protein-coupled receptor (GPCR) naturally expressed by MEF: the ADRB2. Indeed, this receptor belongs to the same GPCR subclass as the TSH receptor and is similarly expressed in Kif3a<sup>+/+</sup> and Kif3a<sup>−/−</sup> MEF (Fig. 7A). Despite the similar protein expression when tested by western blotting on MEF protein extracts, a significantly reduced ADRB2 expression was detected by flow cytometry at the cell surface in Kif3a<sup>−/−</sup> cells (Fig. 7B). Therefore, in response to stimulation by isoproterenol, an ADRB2 agonist, significantly lower cAMP levels were observed in Kif3a<sup>−/−</sup> MEF, as compared with Kif3a<sup>+/+</sup> MEF (Fig. 7C). Altogether, these results suggest that the absence of Kif3a leads to alterations in β<sub>2</sub> adrenergic GPCR transport to the plasma membrane and to defective downstream signaling.

### Discussion

We report here for the first time that 3-week-old Pax8<sup>Cre/+</sup> mice present minor thyroid functional alterations and that inactivation of Kif3a, a kinesin 2 molecular motor subunit, with these Cre mice results in a mild hypothyroidism at 3 weeks of age, probably secondary to defects in TSH receptor signaling. This mild hypothyroidism is characterized by a decreased circulating T<sub>4</sub> and an increased TSH level, a probable consequence of a reduced production of intracellular T<sub>3</sub> from T<sub>4</sub> in pituitary cells. Our results also indicate that a lack of Kif3a in cells leads to decreased cell surface expression of the endogenous ADRB2 and a decreased signaling in response to stimulation.

In man, at least ten different mutations have been identified in the PAX8 gene, leading to thyroid dysgenesis and congenital hypothyroidism (Meeus et al. 2004, Montanelli & Tonacchera 2010). All affected people are heterozygous for the PAX8 mutation and transmission is autosomal dominant (Meeus et al. 2004, Montanelli & Tonacchera 2010). Unexpectedly, no structural or functional thyroid defect has been previously reported in Pax8<sup>Cre/+</sup> mice, suggesting that, in this species, the presence of one functional Pax8 allele is sufficient for normal organogenesis and function (Amendola et al. 2005). Unlike published data on these mice, we show here that Pax8<sup>Cre/+</sup> mice present minor thyroid functional defects, including a significant decrease in Nis mRNA expression, resulting in a slight compensatory increase in circulating TSH and intracellular cAMP levels, sufficient to maintain blood T<sub>4</sub> levels in the normal range. The discrepancy between our results and those reported by Amendola (normal TSH and T<sub>4</sub> levels) may be explained by a difference in the mouse genetic background and/or in dietary levels of iodine. These findings certainly complicate the interpretation of our results on the role of Kif3a in the thyroid.

It has been extensively demonstrated that the kinesin 2 molecular motor, including Kif3a, is essential for correct primary cilium assembly (Rosenbaum & Witman 2002). Indeed, Kif3a participates in the intraflagellar transport in this antenna-like structure and Kif3a inactivation in mice leads to the absence of primary cilium at the cell surface and developmental alterations characteristic of ciliopathies (Marszalek et al. 1999, Lin et al. 2003). Antenna-like structures related to primary cilium have been detected on thyroid cells during development and on mature follicular cells, but major differences exist between species (Sobrinho-Simões & Johannessen 1981). In adult mice, it has been reported that only 2% of thyroid cells present a cilium, and its precise role has not been addressed so far (Wetzel & Wollman 1969). We were unable to detect an acetylated α-tubulin-positive primary cilium on thyroid cells in mouse at embryonic or adult stages (data not shown). However, we show here that Kif3a inactivation from E8.5 in the thyroid bud had no obvious effect on thyroid development and migration, suggesting that Kif3a and potential primary cilium have no essential role in these processes, at least in mice.

Correct cell-surface expression of receptors, including TSH and β<sub>2</sub> adrenergic GPCR, is essential for binding of water-soluble ligands and for initiating downstream intracellular signaling. In MEF in the absence of any Pax8 gene alteration, our results clearly indicate that Kif3a
inactivation leads to significantly lower ADRB2 expression at the cell surface and, as a consequence, to markedly decreased downstream signaling in response to agonist stimulation. The kinesin 2 molecular motor is known to participate in ‘non-intraflagellar’ transport in the cell, like retrograde traffic between the Golgi and the endoplasmic reticulum (Stauber et al. 2006), endosome and lysosome transports (Banani et al. 2004, Brown et al. 2005), endocytosis and recycling of cell-surface receptors like transferrin, cubulin, and megalin receptors, or of other proteins like Clc-5, the H⁺/Cl⁻ exchange transporter (Schonteich et al. 2008, Reed et al. 2010). The KIF3 molecular motor also plays an important role in the transport of vesicles containing GluR2 and GLUT4 receptors or MT1-MMP and MMP9 metalloproteinases from the cytosol to the plasma membrane (Imamura et al. 2003, Wiesner et al. 2010, Hanania et al. 2012, Lin et al. 2012). Unfortunately, we were unable to analyze the intracellular traffic or the cell-surface expression of the TSH receptor in vivo or in vitro due to the lack of specific antibody working on tissue sections (and probably also to the very low expression of the TSH receptor at the thyrocyte surface) and the unexpected consequence of Kif3a inactivation on TSH receptor expression directed by lentivirus infection. Given that the TSH and the ADRB2s belong to the same subclass of GPCR and have similar posttranslational modifications (glycosylation, sialylation, and palmitoylation), we speculate here that they use similar mechanisms for transport from the Golgi to the plasma membrane and/or for recycling at the cell surface.

Figure 7
Reduced surface expression and signaling response of β2 adrenergic receptor in Kif3a⁻/⁻ cells. Total cellular and cell surface expression of the β2 adrenergic receptor were respectively analyzed (A) by western blotting (n=3) and (B) by flow cytometry (n=8). Red, ADRB2; blue, negative control. (C) cAMP levels in response to isoproterenol or forskolin stimulation were determined in Kif3a⁺/⁺ and Kif3a⁻/⁻ cells (n=9). Statistics (Mann–Whitney U test): NS, nonsignificant; P>0.05 and ***P<0.001. Adrb2, β2 adrenergic receptor. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-12-0219.
Thus, based on our in vitro results on the ADRB2 in Kif3a-deficient MEF, we suggest that Kif3a inactivation in thyrocytes may lead to altered transport and/or recycling of the TSH receptor, decreased expression at the basolateral membrane and altered TSH signaling. TSH signaling is clearly altered in Kif3aΔ/ΔfloxB Pax8Cre/+ mutant mice. First the expression of specific genes controlled by cAMP through CREB transcription factors, like NIS, Adcy3, Prkar2b, and Dio2, is significantly decreased in these mice. Second, as compared with thyroid of Cre mice, the cAMP levels in mutant thyroid are markedly lower. Thereby mutant mice are not able to compensate a thyroid dysfunction despite of increased TSH bioactivity, unlike Cre mice. Our mutant phenotype is indeed reminiscent of the phenotype observed in TSH receptor-deficient mice. In the latter mice, normal thyroid development is also associated with TSH resistance, decreased Nis mRNA expression and congenital hypothyroidism (Postiglione et al. 2002). However, our mutant phenotype is less pronounced than the TSH receptor-deficient phenotype. As for the ADRB2 in Kif3a−/− MEF, it is probable that a significant fraction of the TSH receptor is still expressed at the thyrocyte cell surface and is functional. Partial compensation of kinesin 2 deficiency by other kinesins, like Kinesin 1 which is expressed in thyrocytes (data not shown), to carry cargo to their final destination is abundantly described in the literature (Brown et al. 2005, Hirokawa et al. 2009, Wiesner et al. 2010). It is noteworthy here that mice were analyzed only at 3 weeks of age, given the experimental difficulties to record similar data earlier in mice. The thyroid phenotype of Kif3aΔ/ΔfloxB Pax8Cre/+ mutant mice is thus unknown at earlier developmental stages and it could eventually change with age, as reported in type 3 deiodinase knockout mice (Hernandez et al. 2006). Furthermore, the Kif3a mutation analyzed here is not strictly thyrocyte specific and the mice have a severe kidney phenotype, which probably explains the postnatal lethality. Severe chronic kidney disease in man results in decreased circulating T₄, T₃, and TSH levels, a consequence of changes in peripheral hormone metabolism, thyroid hormone-binding proteins, and central defects (van Hoek & Daminet 2009). Although the consequences of chronic kidney disease have not been clearly reported in the literature for 3-week-old mice, the human phenotype is not identical to the Kif3aΔ/ΔfloxB Pax8Cre/+ mouse phenotype where only T₄ level is decreased and where increased TSH level is associated with low thyroid cAMP level and with altered expression of specific genes controlled by cAMP, which together sign the presence of TSH signaling defects.

In conclusion, our results in 3-week-old Kif3aΔ/ΔfloxB Pax8Cre/+ mutant mice indicate first that the Pax8cre/+ mice used in these experiments present minor thyroid functional defects resulting in a slight increase in circulating bioactive TSH and intracellular cAMP levels, presumably allowing to maintain blood T₄ levels in the normal range. Second, Kif3a invalidation in thyrocytes markedly amplifies the phenotype observed in Pax8cre/+ mice, resulting in an altered TSH signaling upstream of the second messenger cAMP and a mild hypothyroidism 3 weeks after birth. Finally, our results in MEF indicate that Kif3a inactivation in the absence of any Pax8 gene alteration leads to altered β₂ adrenergic GPCR plasma membrane expression; we suggest that a similar mechanism may explain the altered TSH signaling and mild hypothyroidism observed in Kif3aΔ/ΔfloxB Pax8Cre/+ mutant mice. In future genetic studies on congenital hypothyroidism or high TSH serum level, it will be important to look for variants in the Kif3a gene.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-12-0219.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
E D is researched data, contributed to discussion and wrote manuscript. S G and C M are researched data. J V S, J E D and B R are contributed to discussion. J F R is supplied material. S S is designed project, contributed to discussion and wrote manuscript.

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