(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
04 January 2018 (04.01.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/002147 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/EP2017/066005

(22) International Filing Date:
28 June 2017 (28.06.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
16305807.6 30 June 2016 (30.06.2016) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: DETECTION OF BOREALIN MUTATIONS FOR DIAGNOSING THYROID DYSGENESIS

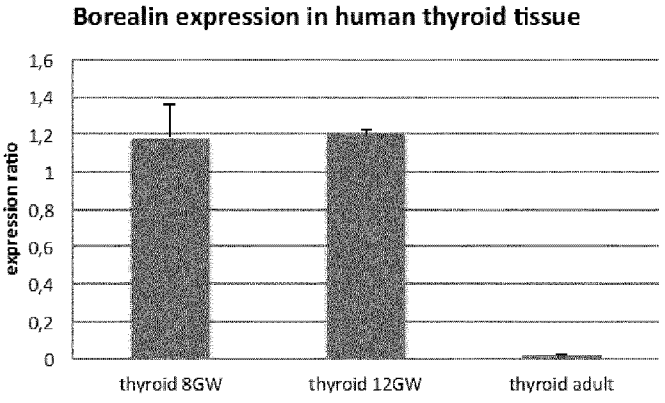

Figure 2: Borealin expression during thyroid development

(57) Abstract: The present inventors have identified Borealin, which is a major component of the Chromosomal Passenger Complex (CPC), as being involved in thyroid dysgenesis. They have demonstrated that patients suffering from thyroid dysgenesis displayed mutations in the borealin gene. Thus, the present invention relates to a method for diagnosing or predicting thyroid dysgenesis in a subject, wherein a mutation in the CDCA8 gene is detected in a sample comprising DNA and/or RNA from said subject and wherein the presence of a mutation in the CDCA8 gene is indicative of thyroid dysgenesis. Mutations in the CDCA8 gene can be detected in any appropriate sample, particularly in a blood sample or a tissue sample.

WO 2018/002147 A1

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Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

DETECTION OF BOREALIN MUTATIONS FOR DIAGNOSING THYROID DYSGENESIS

Field of the invention

The present invention relates to a method for diagnosing or predicting thyroid dysgenesis by detecting a mutation in borealin.

Background of the invention

The thyroid is one of the earliest endocrine organs to differentiate (from embryonic day 22) and has an important hormonal role in development of the embryo. Any defect in the specification, proliferation, migration, growth or organization of thyroid cells during embryogenesis may thus result in thyroid development abnormalities.

Thyroid dysgenesis is a type of primary congenital hypothyroidism (CH) which is the most common neonatal endocrine disorder and causes a permanent thyroid hormone deficiency from birth. Thyroid dysgenesis results from an abnormality in the development of the thyroid. It represents 80 to 85 percent of permanent congenital hypothyroidisms and is estimated to affect 1-5 in 10 000 people. It occurs in various forms such as an ectopic location of the thyroid gland (thyroid ectopy), a complete absence of thyroid tissue (called athyreosis) or an incomplete thyroid development (thyroid hypoplasia).

The genetic component of thyroid dysgenesis remains unclear. Around 2% of cases have been shown to be family-inherited: this supports a Mendelian inheritance but does not exclude other mode of inheritance such as multigenetic, multifactorial as well as epigenetics (Léger et al, J Clin Endocrinol Metab., 575–580 (2002); Castanet et al, N Engl J Med., 441–2 (2000); and Castanet et al, J Clin Endocrinol Metab., 2009–14 (2001)). Mutations in the FOXE1, NKX2-1, NKX2-5, PAX8 or TSHR genes have been reported, but they only account less than 5% of the thyroid dysgenesis cases (Carré et al, Thyroid, 649–54 (2014); Carré et al, Hum Mol Genet., 2266–76 (2009); Ramos et al, Eur J Endocrinol., 499–507 (2014); Sura-Trueba et al, Endocrinology, 1043–50 (2009); Dentice et al, J Clin Endocrinol Metab., 1428–33 (2006)).

Thus, there is a real need to identify the pathways, molecules and/or genes involved in thyroid dysgenesis so as to provide methods allowing the early and accurate detection of this disease.

Detailed description

The present inventors have identified Borealin, which is a major component of the Chromosomal Passenger Complex (CPC), as being involved in thyroid dysgenesis. They have indeed demonstrated that patients suffering from thyroid dysgenesis displayed mutations in the borealin gene.

Borealin, also known as CDCA8 (cell division cycle associated 8), is a major component of the Chromosomal Passenger Complex (CPC) along with Survivin, AuroraB kinase (AURKB) and INCENP (inner centromere protein). The CPC guides chromosomes during all the steps of the mitosis. The CPC allows the stability of the bipolar mitotic spindle, the stabilization of microtubules and cytokinesis. Borealin is encoded by the CDCA8 gene (available under the reference ENSG00000134690 (gene) and ENST00000373055 (transcript) in the Ensembl Gene Database).

The present inventors have demonstrated that further to its role during mitosis, borealin is also involved in the adhesion and migration of the thyrocytes, and, accordingly, that it plays a major role during thyroid genesis.

Thus, in a first aspect, the present invention relates to a method for diagnosing or predicting thyroid dysgenesis in a subject, said method comprising detecting a mutation in the CDCA8 gene in a sample comprising DNA and/or RNA from said subject.

Particularly, in the context of the present invention, the presence of a mutation in the CDCA8 gene is considered to be indicative of thyroid dysgenesis.

In the context of the present invention, “thyroid dysgenesis” refers to a permanent thyroid hormone deficiency present from birth and resulting from an abnormality in the development of thyroid. Thyroid dysgenesis comprises e.g. thyroid ectopy, athyreosis and thyroid hypoplasia

A "mutation" refers to any detectable change in genetic material, e.g. DNA, RNA, cDNA, or in an amino acid sequence encoded by such a genetic material. This includes gene mutations, in which the structure (e.g. DNA sequence) of a gene is altered any gene as well as protein mutations, in which the amino-acid structure of the protein is altered. Generally a mutation is identified in a subject by comparing the sequence of a nucleic acid or of a polypeptide expressed by said subject with the corresponding nucleic acid or polypeptide expressed in a control population.

The term "allele" used herein is one of two or more forms of a gene or a genetic locus (generally a group of genes). Diploid organisms, (such as humans) have two sets of chromosomes (i.e. two sets of homologous chromosomes) and have one copy of each gene (and therefore one allele) on each chromosome. If both alleles are the same, they are homozygotes. If the alleles are different, they are heterozygotes.

In the context of the present invention, the mutation of CDCA8 can consist in the mutation of one or more nucleotide in the CDCA8 gene/mRNA sequence. In a particular embodiment, the mutation according to the present invention consists in the mutation of one single nucleotide in the nucleotide sequence of CDCA8, particularly in the substitution of one particular nucleotide by another.

The presence of a mutation in the CDCA8 gene according to the present invention may be detected by analysing a CDCA8 nucleic acid molecule. In the context of the invention, CDCA8 nucleic acid molecules include mRNA, genomic DNA and cDNA derived from mRNA. DNA or RNA can be single stranded or double stranded. These may be utilized for detection by amplification and/or hybridization with a probe, for instance. The nucleotide sequence can be obtained from a genomic DNA sample isolated from the biological sample. In this case, any biological sample containing genomic DNA (e.g. not pure red blood cells) can be used.

CDCA8 mutations may be detected in a RNA or DNA sample, preferably after amplification. For instance, the isolated RNA may be subjected to coupled reverse transcription and amplification, such as reverse transcription and amplification by polymerase chain reaction (RT-PCR), using specific oligonucleotide primers that are specific for a the mutated site or that enable amplification of a the region containing the mutated site. According to a first alternative, conditions for primer annealing may be chosen to ensure specific reverse transcription (where appropriate) and amplification; so that the appearance of an amplification product be a diagnostic of the presence of the particular mutation according to the invention. Otherwise, RNA may be reverse-transcribed and amplified, or DNA may be amplified, after which the mutated site may be detected in the amplified sequence by hybridization with a suitable probe or by direct sequencing, or any other appropriate method known in the art. For instance, a cDNA obtained from RNA may be cloned and sequenced to identify the mutated sequence of CDC8A

The mutation of the CDCA8 gene results in a mutated mature CDCA8 protein (borealin). Thus, according to a further embodiment, the mutation of CDC8 may be detected at the protein level by detecting the mutated form of the borealin. In this case, any biological sample wherein the mature borealin protein is present may be used for detecting mutated forms of this protein.

Suitable samples for detecting the mutated borealin according to the present invention are e.g. blood samples.

This mutated form of the borealin may be detected according to any appropriate method known in the art. In particular a sample, such as a tissue biopsy, obtained from a subject may be contacted with antibodies specific of the mutated form, i.e. antibodies that are capable of distinguishing between the mutated form and the wild-type protein (or any other protein), to determine the presence or absence of the mutation specified by the antibody.

The antibodies may be monoclonal or polyclonal antibodies, single chain or double chain, chimeric antibodies, humanized antibodies, or portions of an immunoglobulin molecule, including those portions known in the art as antigen binding fragments Fab, Fab', F(ab')2 and F(v). They can also be immunoconjugated, e.g. with a toxin, or labelled antibodies.

Whereas polyclonal antibodies may be used, monoclonal antibodies are preferred because they are more reproducible in the long run.

Procedures for raising “polyclonal antibodies” are also well known. Polyclonal antibodies can be obtained from serum of an animal immunized against the appropriate antigen, which may be produced by genetic engineering for example according to standard methods well-known by one skilled in the art (see e.g. Harlow et al. (1988)).

A "monoclonal antibody" in its various grammatical forms refers to a population of antibody molecules that contains only one species of antibody combining site capable of immunoreacting with a particular epitope. A monoclonal antibody thus typically displays a single binding affinity for any epitope with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different epitope, e.g. a bispecific monoclonal antibody. Laboratory methods for preparing monoclonal antibodies are well known in the art (see, for example, Harlow et al., 1988).

Aptamers, which are a class of molecule that represents an alternative to antibodies in term of molecular recognition, can also be used for detecting the KIT^{M541L} form in the context of the present invention. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible

modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996).

All the probes, primers, aptamers or antibodies used in the context of the present invention may be labelled with a detectable molecule or substance, such as a fluorescent molecule, a radioactive molecule or any others labels known in the art. Labels are known in the art that generally provide (either directly or indirectly) a signal.

In the context of the present invention, the "biological sample" can be any sample allowing the detection of a mutation in CDCA8. Examples of such samples include fluids, tissues, cell samples, organs, biopsies, etc. Preferred biological samples are a cell or tissue sample. Preferred biological samples are whole blood, serum, plasma or urine. In a particular embodiment, the sample obtained from the subject himself.

As disclosed above, thyroid dysgenesis results from an abnormality of the development of thyroid during embryo development. The present invention is particularly interesting for detecting thyroid dysgenesis just after birth, i.e. wherein the subject is a new-born. In this case, the subject is less than 2 weeks old, preferably less than one week old, more preferably less than three days old.

In one particular embodiment, the method according to the present invention is performed just after birth and after having suspected thyroid dysgenesis during embryo development, e.g. by observing an abnormality in the development of the embryo's thyroid during an ultrasonography, particularly obstetric ultrasonography.

In another particular embodiment, the method according to the present invention is performed in a child, preferably a new-born, whose parents (1 or both of them) present thyroid dysgenesis. Particularly, the method according to the present invention is performed in a child, preferably a new-born, whose parents (1 or both of them) are identified has displaying a mutation in the CDCA8 gene.

In one further embodiment, the method according to the present invention is performed after having suspected thyroid dysgenesis during embryo development and treated the foetus thereby identified as presenting an abnormality in the thyroid development.

An example of treatment which can be administered to a foetus identified as presenting an abnormality in the development of thyroid is intra-amniotic injection of Levothyroxine. Such treatment is e.g. disclosed in Léger et al (ESPE-PES-SLEP-JSPE-APEG-APPES-ISPAE; Congenital Hypothyroidism Consensus Conference Group. European Society for Paediatric Endocrinology consensus guidelines on screening, diagnosis, and management of congenital hypothyroidism. J Clin Endocrinol Metab. 2014 Feb;99(2):363-84) and in Ribault et al (French Fetal Goiter Study Group. Experience with intraamniotic thyroxine treatment in nonimmune fetal goitrous hypothyroidism in 12 cases. J Clin Endocrinol Metab. 2009 Oct;94(10):3731-9).

The method according to the present invention is interesting for detecting an abnormality in the thyroid development at the embryo stage.

In one embodiment, the present invention relates to a prenatal method for diagnosing or predicting thyroid dysgenesis in a foetus by detecting a mutation in the CDCA8 gene in a sample obtained from said foetus or from said foetus's mother.

In this case, the sample may be an amniosynthesis sample. The sample may further be any biological sample wherein foetal DNA may be detected. For instance, in this case, the biological sample may be a sample obtained from the mother but wherein foetal DNA can be found. As disclosed e.g. in Hixson et al (J Lab Autom; 20(5):562-73, 2015) or in the reference EP patent N° 0994963, foetal DNA is detectable in maternal serum or plasma samples. Abnormalities in the foetus genetic material can thus be detected by directly analysing the foetal DNA present in the mother's blood. Thus, in a particular embodiment, the biological sample according to the present invention is a maternal serum or plasma sample.

In the context of the present invention, the "subject" is preferably a human. More particularly, the subject is a new-born or a foetus. In the context of the present invention, a "new-born" is a child who is less than 2 weeks old, particularly less than 1 week old, more particularly less than 3 days old. Thus, when the method is method is performed "just after birth", it means that the method is performed within 2 weeks, particularly 1 week, more particularly 3 days after birth.

The present inventors have further identified different mutations of the CDCA8 gene involved in thyroid dysgenesis.

One of these mutations is a homozygous mutation of the CDC8A gene which is a substitution of a cytosine residue into a thymine residue at position 443 of the CDCA8 gene (C443T). This mutation is accessible under the reference rs546751848 in the Single Nucleotide Polymorphism Database (dbSNP), which is a free public archive for genetic variation within and across

different species developed and hosted by the National Center for Biotechnology Information (NCBI) in collaboration with the National Human Genome Research Institute (NHGRI). This C443T mutation encodes for the substitution of a serine in position 148 by a phenylalanine in the mature borealin protein (c.443C>T, p.S148F, rs546751848).

Another mutation identified by the present inventors is a heterozygous mutation which is a substitution of a guanine residue into an adenine residue at position 341 of the CDCA8 gene. This mutation is accessible under the reference rs35565540 in the Single Nucleotide Polymorphism Database (dbSNP) and encodes for the substitution of an arginine in position 114 by a glutamine in the mature borealin protein (c.341G>A, p.R114Q, rs35565540).

One further mutation identified by the inventors is a heterozygous mutation which is a substitution of a thymine residue into a guanine residue at position 530 of the CDCA8 gene. This mutation is accessible under the reference rs140856315 in the Single Nucleotide Polymorphism Database (dbSNP) and encodes for the substitution of a leucine in position 177 by a tryptophan in the mature borealin protein (c.530T>G, p.L177W, rs140856315).

Thus, in one particular embodiment, the mutation of CDCA8 according to the present invention is selected from the group consisting of rs546751848, rs35565540 and rs140856315.

These three particular mutations are missense mutations, i.e. point mutations that result in a codon that codes for a different amino acid. Thus, in one particular embodiment, the mutation of CDCA8 according to the present invention is a missense mutation.

None of these three particular mutations have an effect on cell mitosis. However, they all induce a decrease in cell migration and spreading. They also induce a decrease in the expression of several genes such as TLN1, ACTN1, ITGA3, CAV1, which are all involved in adhesion of human primary thyrocytes.

Thus, in a particular embodiment, the mutation according to the present invention induces a decrease in thyrocytes migration and spreading. In this context, thyroid dysgenesis is diagnosed/predicted by detecting a mutation in CDCA8, wherein said mutation induces a decrease in the migration and spreading of thyrocytes.

The Examples of the present application disclose methods allowing the skilled person to evaluate whether a mutation induces a decrease cell migration and spreading.

Brief Description of the figures

Figure 1: Molecular genetics.

a. Pedigrees of families with Borealin mutations:

Family F1: Consanguineous family/Homozygous missense mutation, c.443C>T, p.S148F. Familial pedigree with four children including two siblings with TD (in black) and the homozygous mutation. Parents are heterozygous for the mutation. The mother has an asymmetric thyroid lobes (in gray).

Family F2: Heterozygous missense mutation, c.341G>A, p.R114Q, in the mother and her daughter. The daughter has CH with ectopy (in black) and the mother had an asymmetric thyroid and developed later a papillary thyroid cancer (in gray).

Family F3. Heterozygous missense mutation, c.530T>G, p.L177W, in a girl with CH and an athyreosis (in black);

b. Schematic representation of the Borealin protein with domain structure;

c. Multiple sequence alignment of Borealin proteins: for the 3 missense mutations (in gray) conservation across evolution of altered amino acid residues is shown.

Figure 2: Gene expression of Borealin in thyroid tissue at 8GW, 12GW and in adult thyroid, reported to one thyroid tissue at 8GW.

Figure 3: a. G2/M phases in Nthy transfected with Borealin-wt, Borealin-114, Borealin-148 and Borealin-177. Transfection of cells with Borealin increased the mitosis. The percentage of cells was determined by flow cytometry. Values are represented as mean ± SE from three independent measurements; **b.**Graph representing the effect of the different Borealin mutants on the length of mitosis

Figure 4: a. The length of migration in um was measured by quantifying the total distance that the positively transfected cells (GFP) moved from the edge of the wound toward the center of the wound in 8h. Mean of the length of the migration from four independent experiments. *P 0,04, 0,0036, 0,202 respectively for Nthy with Borealin-114, Borealin-148 and Borealin-177 in comparison with Nthy with Borealin-wt. **b.** The percentage of spread and unspread Nthy was estimated by counting Nthy for each cell line (light gray, unspread; medium gray, weakly spread; dark gray, strongly spread). The graph represents the mean of 5 independent experiments. *P< 0,05, P<0,01 calculated by t test.

Figure 5: a. Genes with decreased expression in transcriptome analysis of the thyroid tissue with Borealin-114 compared to thyroid controls. On the left, KEGG Pathway analysis of expressed mRNAs. Top fifteen enriched down-regulated pathways. On the right, GO enrichment analysis

of expressed mRNAs. Top fifteen enriched down-regulated pathways. *Pathways involved in adhesion and/or migration; **b**. Gene expression of Borealin-114 tissue compared to control in trancriptome analysis and validated by quantitative PCR (black, thyroid controls; medium gray, Borealin-114 tissue; light gray, validation by quantitative PCR in Borealin-114 tissue); **c**. Genes involved in adhesion and/or migration in transfected HPT (black, Borealin-wt HPT, dark gray, Borealin-114 HPT; medium gray, Borealin-148 HPT; light gray, Borealin-177 HPT). The graph represents the mean of 4 or 5 independent experiments. *P < 0,05, **P<0,01 calculated by t-test.

Figure 6: a. Gene expression in transcriptome analysis of the thyroid tissue with Borealin-114 (in gray) compared control thyroid tissues control (in dark); **b.** Gene expression in transfected HPT with Borealin-wt (black) or Borealin-114 (dark gray), Borealin-148 (medium gray) and Borealin-177 (light gray). The graph represents the mean of 4 independent experiments. *P < 0,05, **P<0,01 calculated by t-test.

EXAMPLES

Example 1: Borealin mutations are involved in thyroid dysgenesis

Thyroid Dysgenesis (TD) occurs in 80-85% of congenital hypothyroidism (CH), the most common neonatal endocrine disorder, while the remaining 10-15% are owing to inherited defects of thyroid hormone synthesis called dyshormonogenesis (Thania Endocrine clinic of North America). TD includes a large spectrum of developmental anomalies with or without hypothyroidism varying from the absence of thyroid tissue (athyreosis), the presence of ectopic tissue to hypoplasia of an orthotopic gland or hemiagenesis[1]. Ectopic thyroid is by far the most common cause of CH (60%), followed by athyreosis (20%). In contrast, thyroid hypoplasia is rare as well as hemithyroid (5%). In human development, the median anlage invaginates from the floor of the foregut starting around embryonic day 22 (E22) and expresses *NKX2-1*, *PAX8*, and *FOXE1* (E32-33)[2,3]. From E26 onwards, the ultimobranchial bodies develop from the fourth pharyngeal pouch on each side[4]. The median and lateral anlagen actively migrate and then fuse, at E44 at the definitive pretracheal position[5]. The cells differentiate into thyrocytes expressing thyroglobulin (TG, from 8GW[Gestational Weeks]) and producing T4 (from 11GW) or into C-cells expressing calcitonin[6]. The functional unit of the thyroid is represented by the follicles where thyroid hormone synthesis takes place. Defects in any step of thyroid development (such as specification, proliferation, migration, growth, organization, differentiation, and survival) may result in a congenital anomaly and/or impaired hormonogenesis, leading to variable degrees of hypothyroidism. However, DT are not always associated with CH. Mutations in five of these genes *PAX8*, *NKX2-1*, *FOXE1*, *NKX2-5*, *TSHR* have been reported in individuals with CH and TD, demonstrating the genetic heterogeneity of this pathology, accounting for a small number of

patients with TD, less than 5%[7–11]. TD is usually considered as a sporadic disease, although a prevalence of 2% of familial cases support the Mendelian inheritance but do not exclude other mode of inheritance such as multigenetic, multifactorial as well as epigenetics[1,12,13].

To overcome the limitations posed by the rarity of new mutated genes associated with CH and TD, we performed WES for siblings with childhood-onset TD and thereby identified mutations of *Borealin/CDCA8* as a single-gene responsible for TD [14,15]. By Sanger sequencing of the *Borealin* gene, we analyzed 134 TD cases. We identified two more mutations in patients with CH and TD.

Borealin is a major component of the Chromosomal Passenger Complex (CPC) along with Survivin, AuroraB kinase (AURKB) and INCENP (inner centromere protein)[16,17]. The CPC guides chromosomes during all the steps of the mitosis. The CPC allows the stability of the bipolar mitotic spindle, the stabilization of microtubules and cytokinesis. The role of Borealin in the thyroid development and in the TD was unknown. We described for the first time the mutations of Borealin in TD and a new role for this protein in migration and adhesion of the thyrocytes cells.

Results

Mutations in Borealin and clinical data

Family F1 was a consanguineous French family originating from Sri Lanka and of Tamil ethnicity. Parents were first cousins (I-1, I-2) and had four children sharing three different pathologies (Figure 1A). One daughter died at four months of leprechaunism (II-2), while two children, one daughter (II-3) and one son (II-4), had hemolytic and uremic syndrome (HUS). Finally, two siblings had TD, one with ectopic thyroid and CH (II-3) and the other one with hemiagenesis and no CH (II-1). The parents and the son (II-4) were euthyroid. Using the recessive model of transmission, the WES revealed mutations potentially responsible for the three pathologies. Firstly, a homozygous frameshift mutation in the sibling with leprechaunism (II-2) was found in *INSR* (p.G5AfsX60). Secondly, homozygous codon-stop mutation was identified in *ADAMSTS13* (p.R1119X) in two children with HUS (II-3, II-4). These two mutated genes (*INSR*, *ADAMTS13*) correlated well with the clinical phenotype[18,19]. Finally, a missense homozygous mutation found in *CDCA8/Borealin* (c.443C>T, p.S148F, rs546751848) in two daughters with TD (II-1, II-3). Parents carried the three mutations in the three different genes at the heterozygous state. The euthyroid son (II-4) was carrier of the heterozygous Borealin mutation. Patients II-1 and II-3 were born at term and II-3 had CH detected by the systematic CH

neonatal screening performed in France. TD as hemiagenesis for II-1 and thyroid ectopy for II-3 was diagnosed on ultrasound. Thyroid ultrasound revealed nodules in the father (I-2) and an asymmetric thyroid for the mother (I-1).

In family F2, we identified a heterozygous mutation of Borealin, c.341G>A, p.R114Q (rs35565540), in a daughter with CH due to ectopic thyroid gland. Her mother carried the same heterozygous mutation. She had an asymmetric thyroid (right lobe 5,89cm3, left lobe 3,87cm3) and developed papillary thyroid cancer with nodule in the left thyroid lobe. The father was euthyroid and did not carry this mutation. No autosomal monoallelic expression of the mutation p.R114Q of Borealin was identified in the cDNA of the thyroid tissue of the F2-I by Sanger sequencing (citer Johnny, Thyroid, 2016) (*data not shown*).

In family F3, we identified an heterozygous mutation of Borealin, c.530T>G, p.L177W (rs140856315), in a proband with CH with thyroid agenesis. DNA of parents was not available.

For the Borealin-114 and 177 mutations, we sequenced the cDNA of the white blood cells and we found the mutation at the heterozygous state.

Mutations Borealin-114 and 148 were not in a domain known to be involved in the mitosis function (Figure 1B). Borealin-177 is localized in the dimerization domain of the Borealin. Moreover, the three mutations altered an amino acid residue were mildly conserved from chicken to human (Figure 1C).

Mutation Borealin-114 (p.R114Q) is predicted to be benign according to Polyphen and Sift prediction tools, while mutations Borealin-148 (p.S148F) and Borealin-177 (p.L177W) are predicted to be possibly damaging by Polyphen and deleterious by Sift. On ExAC (Exome Aggregation Consortium) database, the mutation Borealin-114 has a reported frequency estimated at 0,001368, Borealin-148 a frequency at 0,0001411 and Borealin-177 a frequency at 0,0001153.

Borealin localizes in thyroid during development

Borealin is mainly expressed in development of organs during mitosis. Few adult tissues expressed Borealin except for within high proliferation rate, such as the testis and intestine. By quantitative PCR, we observed *mRNA* encoding *Borealin* expression in human thyroid at 8 and 12GW (Figure 2). No expression was found in adult thyroid samples. We confirmed this Borealin expression in thyroid tissues by immunofluorescence. At 8GW, Borealin was colocalized with E-cadherin-expressing cells (a marker of epithelial cells) undergoing mitosis. At

12GW, while the thyroid was thoroughly differentiated, we observed a clear colocalization of Borealin in the nucleus of some TG-expressing cells surrounding colloid in thyroid follicles. No expression was found in adult thyroid tissue (*data not shown*). We therefore confirmed the Borealin expression in human thyroid tissue during development.

Mutations in Borealin do not disturb the mitosis

To understand the role of Borealin mutations identified in patients with TD, we studied the known role of Borealin as a regulator during mitosis. We transfected an immortalized thyroid epithelial cell line, Nthy, with pFlagBorealin wild-type (wt) or mutated (114, 148 and 177 mutants) and pSuperBorealin (sh) to inhibit endogenous Borealin to study the effects of only Borealin transfected wt or mutated[21]. After propidium iodide (PI) staining, we quantified cell cycle distributions by flow cytometry (Fig. 3A). Expression of Borealin mutants had no effect on the percent of cells in G2/M compared to Borealin-wt (Fig. 3A). Borealin mutants localized normally to centromeres, midzone and midbody, structures where CPC coordinates important mitotic events (*data not shown*). In addition, overexpression of GFP-tagged Borealin mutants in combination with Borealin shRNA had no effect on the duration of mitosis in HeLa cells (Fig. 3B). Therefore, the three Borealin mutations found in TD patients do not appear to affect the mitotic functions of the protein.

Borealin mutations decrease cell migration and cell spreading

Two patients with the Borealin mutations 114 and 148 had an ectopic thyroid gland and two parents had an asymmetric thyroid gland. Accordingly, we decided to analyze the role of Borealin mutants on Nthy cell migration. We tracked Nthy transfected with sh and Borealin-GFP vector (containing Borealin-wt or Borealin-114, Borealin-148, Borealin-177) by microscopy during a time lapse of 8 hours following a wound-healing assay. We observed a significant decrease in the length of migration path of Nthy cells with three Borealin mutants compared to Borealin-wt (decrease of 20% for three mutants compared to wt of the length of the tracking, $P<0,05$) (Figure 4A). We can therefore conclude that Borealin mutants could alter the migration of Nthy cells.

To understand this altered migration ability, we analyzed the adhesion of Nthy cells transfected with Borealin-Flag. Cell spreading was observed by immunofluorescence after staining of the actin cytoskeleton with phalloidin (*data not shown*). We found a significantly increased number of unspread Nthy cells transfected with Borealin mutants compared to Borealin-wt Nthy (respectively with Borealin-114, Borealin-148 and Borealin-177: increase of 74%, 168%, 213% $P<0,05$) (Figure 4B). Furthermore, we observed a weaker spreading of Nthy cells with mutants

compared to Borealin-wt Nthy (respectively with Borealin-114, Borealin-148 and Borealin-177: decrease of 36%, 45%, 42%, *P<0,05*).

The three Borealin mutants on Nthy cells have led to defect in spreading of cells that could explain the decrease in cell migration. We next asked the question of the involvement of Borealin mutations on the cell adhesion.

Impact of Borealin mutations on the expression of genes involved in adhesion

Transcriptome analysis of human thyroid bearing the Borealin-114 mutation

We performed the transcriptome analysis of the thyroid tissue of the mother with asymmetric thyroid lobes (I-1) of the pedigree F2, carrying the mutation 114 was performed. By an unbiased analysis with GSEA software (Gene Set Enrichment Analysis, Broad Institute), the whole gene expression pattern revealed a decrease in different pathways as "Focal Adhesion", "Regulation of actin cytoskeleton", "ECM receptor interaction" (Extracellular Matrix) in the KEGG classification and "Proteinaceous extracellular matrix", "Cell substrate adhesion", "Protein complex binding", and "Extracellular Matrix structural constituent" in the GO (Gene Ontology) classification in the mutated Borealin-114 thyroid tissue in comparison with thyroid controls (Figure 5A). In these pathways, we interestingly observed a decrease expression of *VCL*, *TLN1*, *PXN*, *CAV1*, *ITGA3* and *ITGB1,* directly implicated in the focal adhesion compared to thyroid control tissues (Figure 5B). Expression of the *LAMB1*, *LAMA4*, *LAMC1*, which are laminins, extracellular matrix glycoproteins, implicated in part in cell adhesion, migration and differentiation were also decreased. Furthermore, we observed a diminution of *ACTN1*, a cytoskeletal protein involved in adherens junctions and *FBLN1*, a glycoprotein protein of extracellular matrix, playing a role in cell migration and adhesion (Figure 5B). *VCL*, *TLN1*, *PXN*, *ITGA3*, *ITGB1*, *ACTN1*, *LAMA4*, *LAMB1*, *CAV1* and *FBLN1* genes with decreased expression found by transcriptome analysis were validated by quantitative PCR on same tissues (Figure 5B).

Adhesion genes expression in transfected human primary thyrocytes

By quantitative PCR, we analyzed gene expression of human primary thyrocytes (HPT) transfected with Borealin-wt or Borealin mutant (114 or 148 or 177) (Figure 5C). We found a significantly lower expression of *VCL* in HPT transfected with mutants Borealin-114 and Borealin-148 and a trend in Borealin-177 compared to Borealin-wt. We also observed a significant decrease in *TLN1*, *ITGA3*, *ITGB1*, *ACTN1*, *LAMB1*, *CAV1* and *FBLN1* expression with three mutants (Borealin-114 or Borealin-148 or Borealin-177) compared to HPT transfected with Borealin-wt. No difference was shown with the *PXN* gene expression. We observed also a

decrease of a this same set of genes involved in the adhesion in Nthy transfected with Borealin mutants compared to Borealin-wt (*data not shown*). Our results in HPT confirmed results of the transcriptome analysis of the thyroid with Borealin-114. All these genes are involved in cell adhesion and/or cell migration. As a consequence our results obtained in very unique human thyroid tissue of patient with Borealin mutation strengthen our hypothesis of the involvement of Borealin mutants in cell adhesion and migration.

As the expression of thyroid transcription factors is known to modulate thyroid development, we then studied the expression of these genes according to the Borealin mutation[7].

Study of genes involve in thyroid development and thyroid function

Thyroid transcription factors and the timing of their expression are critical for normal thyroid development; therefore, we determined whether the expression of these genes was altered by the mutant forms of Borealin[24]. *FOXE1* expression was significantly decreased in HPT expressing mutant Borealin-148 and Borealin-177, but increased in cells expressing mutant Borealin-114 (Fig. 6B). *FOXE1* expression in the thyroid tissue-114 was also increased compared to thyroid control tissues (Fig. 6A). No significant change in *PAX8* and *NKX2-1* expression was observed for Borealin mutants except for Borealin-177, leading to a *NKX2-1* decrease. Surprisingly, we found a significant decrease in TG in HPT transfected with Borealin-114 or 148 or 177 and a significant increase in thyroid peroxidase (TPO) with Borealin-114 and Borealin-148 as in thyroid tissue Borealin-114 (Fig. 6B). The altered level of expression of *FOXE1* induced by Borealin mutation is in line with data linking thyroid development and *FOXE1* gene expression level[24DDI]. Importantly, these experiments provide evidence that Borealin modulates developmental gene expression and appears to contribute to a program associated with thyroid development.

Discussion

We describe here for the first time the impact of mutations in Borealin as a cause of TD in humans. We have shown that Borealin is expressed in thyroid tissue during development and localizes in thyrocytes. Through functional studies in human thyrocytes, we have demonstrated that Borealin is involved in the adhesion and in the migration of these cells.

Mutations in Borealin are linked to TD

To date, no Borealin mutation was linked to human pathology. We identified three Borealin mutations in patients with different types of TD: ectopic thyroid gland, hemithyroid, athyreosis

and asymmetric thyroid lobes. Mutations Borealin-114 and 148 were localized in a domain, which was unrelated to the known function with CPC during mitosis. Mutation p.S148F leads to a change in the protein from serine to phenylalanine. The phosphorylation of the Borealin conditioned this activity and some Borealin serine were studied but not this serine may be because of its localization. Mutation Borealin-177 is located in the dimerization domain of the Borealin[17]. In all cases, no significant disturbing of mitosis was shown between Nthy or HeLa transfected with Borealin-wt or mutants. Borealin knock out mice died very early during development because of its crucial function during mitosis[23]. We can hypothesize that the mutations described here were not in a known crucial domain for the function of Borealin. However, if they were located in two important domains for mitosis (Figure 1), we should expect that they would be lethal or associated with more syndromic features.

Mutations in Borealin altered cell migration: a new function of this protein

The three Borealin mutations identified in patients with TD lead to defect in migration of transfected Nthy. The thyroid gland migration is a very important step during thyroid development. Any perturbation during the migration process can lead to TD. The mechanism is not very well known but different concomitant actions take place as active migration of progenitor cells, the effects of surrounding tissues and vessels organization, and the neck elongation[3]. Collective cell migration during morphogenesis was discussed for thyroid tissue. Molecular mechanisms of collective migration implicate cell motility, cell-cell adhesion, signaling and ECM (cell-extracellular matrix) remodeling allowing the integrity of cell-cell junctions, cell polarization, organization of the actin cytoskeleton generating traction and protrusion, and the guidance by chemical and physical signals of surrounding environment[24,25]. The decrease in migration shown by tracking of transfected Nthy reports the impact of Borealin mutations on this mechanism. Moreover, the defect in spreading of Nthy transfected with Borealin mutants and the decrease of molecules involved in adhesion as *TLN1*, *ITGA3*, *ITGB1*, *CAV1* lead to defective adhesion in case of Borealin mutants. We also observed a decrease in *FBLN1*, *LAMCB1*, proteins of ECM whose remodeling determines the migration. These decreases in molecules involved in the adhesion and in the ECM were obtained by experiments in transfected HPT and in the trancriptome of thyroid tissue of the patient with Borealin-114. The thyroid tissue trancriptome strengthened our data of *in vitro* studies. Indeed, all our experiments to unravel the Borealin function in adhesion/migration were performed in human thyroid context.

Mutations in Borealin led to *FOXE1* expression deregulation

We observed in transfected HPT a significant decrease in *FOXE1* expression with Borealin-148 and Borealin-177 and a clear significant increase in *FOXE1* expression with Borealin-114 compared to HPT transfected with Borealin-wt. This striking *FOXE1* increase was also shown by the transcriptome of the thyroid tissue of patients with the mutation Borealin-114. *FOXE1* was early expressed at E33 in the thyroid primordium and then persisted in the thyroid gland throughout development[27]. Moreover, Foxe1 is a thyroid transcription factor essential for the thyroid migration during development as shown in invalidated mice[21]. Human *FOXE1* mutations were identified in patients with TD and functional studies showed that mutations lead to decrease or increase of TG or TPO activity[7,22,28]. Therefore, a proper gene dosage of *Foxe1* is required for the normal development and function of the thyroid. Accordingly, FOXE1 dysregulation can contribute to the TD phenotype of patients with Borealin mutations.

On the other hand, we found a significant decrease in *TG* with HPT transfected with the three Borealin mutants compared to wt. However, *TPO* expression was increased with Borealin-114 and Borealin-148. This *TPO* raise with Borealin-114 was also observed in the transcriptome of the thyroid tissue of the patient with Borealin-114. The increase in *TPO* may be due to the increase in FOXE1 with Borealin-114. However, the *TPO* increase with Borealin-148 may be explained by an altered activation of TPO in case of lower *FOXE1* gene dosage.

Phenotypic variability of Borealin mutations

Borealin mutated patients had a range of TD, from asymmetric thyroid to ectopic thyroid gland and athyreosis while we found for three mutant decrease in migration and spreading. These are already shown for other transcription factors involved in TD such as PAX8 and NKX2-1 and patients presented also a large phenotypic variability, as parents[8,9]. These data confirmed that TD, despite different types, represents the same disease and is modulated by epigenetic and environment factors[12].

To conclude, the WES, performed in a consanguineous family with TD, allowed us to identify a new gene involved in TD, Borealin. Two other mutations of Borealin in two distinct families with TD were also found. Our experiments showed an impact of Borealin mutants on cell adhesion and on cell migration. These results were well correlated with the decrease in expression of genes known to be involved in the cell adhesion, and found in the transcriptome analysis of the thyroid tissue of a patient with a Borealin mutation. Finally, these findings broaden the understanding of TD and more particularly highlight the integrity of cell adhesion required for the proper thyroid development. Moreover, we demonstrated a new function of the Borealin in the cell adhesion allowing a proper migration in thyrocytes.

Material and methods

Subjects

A consanguineous family and 134 patients with CH and TD were included in the study (69 with ectopic thyroid gland, 31 with athyreosis, 3 with thyroid *in situ* of normal size, 6 with hypoplastic thyroid gland and 25 with hemiagenesis). The study was approved by the institutional review board.

WES and Sanger sequencing

Genomic DNA was isolated from whole blood. Exome capture and sequencing were performed at the Genomic platform of the Imagine Institute from 3 ug of genomic DNA per individual using the SureSelect Human All Exon V6 kit (Agilent Technologies, Santa Clara, USA). The resulting libraries were sequenced on a HiSeq 2500 HT (Illumina, San Diego, USA) according to the manufacturer's recommendations for paired-end (2x130) 76 bp reads. Variant prioritization followed the detailed strategy: (i) variants with a frequency below 1% in public databases (dbSNP, 1000genomes, Evs, ExAc), (release date April, 2016)), (ii) and previously identified in less than five individuals from the 7670 in-house exomes.

The entire coding exon of *CDCA8/Borealin* (NM_001256875.1) was amplified by PCR, and DNA sequencing was performed (3500xL Genetic Analyzer, Thermo Fisher Scientific, Waltham, USA). Primers sequences and PCR conditions are available on request.

Plasmids, cells culture and transfection

The vectors pSuper, pSuperBorealin (sh), pFlag-tagged-humanBorealin, phumanBorealin-tagged GFP were already described by William Taylor[20]. The mutant clones of *Borealin* were generated by a PCR-based site-directed mutagenesis method as described previously, using the Stratagene Quikchange® kit (Agilent Technologies, Santa Clara, USA)[29]. Nthy (Nthy-ori 3.1) cells, which are immortalized human thyroid cell lines, were culture as previously described[30]. Normal human thyroid tissue specimens were collected at the Cochin University Hospital, Paris, France, in accordance with local and national ethical requirements. Informed consent was obtained from all donors. Human Primary thyrocytes (HPT) were prepared as previously described and cultured with DMEM/F-12, glutaMAX supplement, MEM Non-Essential Amino Acids solution,

Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, USA), supplemented with six nutritional factors 1U/L bovine TSH (Sigma–Aldrich, Saint-Louis, USA), 10mg/L human insulin (Roche Applied Science, Penzberg, Germany), 10 mg/l somatostatin (Sigma–Aldrich, Saint-Louis, USA), 6 mg/l human transferrine (Roche Applied Science, Penzberg, Germany), 10^{-8}M hydrocortisone (Roche Applied Science, Penzberg, Germany), and 10 mg/L glycyl-histidyl-lysine acetate (Sigma–Aldrich, Saint-Louis, USA)[31].

Nthy were plated at $1{,}25 \cdot 10^5$/well in a 6-well plate 24h before transfection and 1.10^5/well in a 12-well plate for HPT. Nthy and HPT were transfected using XtremeGENE HP DNA, as recommended by the manufacturer (Roche Applied Science, Penzberg, Germany) to co-transfect cells transiently with 700ng in Nthy, 280ng in HPT of pSuperBorealin and with 300ng in Nthy, 120ng in HPT of wild type or mutated *Borealin* (vectors containing Borealin tagged with Flag or with GFP). After 24h, cells were used for the spreading and migration assays and after 48h for quantitative RT-PCR and flow cytometer test.

Tissue sample

After approval by the Institutional Ethical Committee of the experimental design and protocols, embryonic and adult thyroid tissues were obtained from either elective termination of pregnancy or thyroid surgery. Tissue samples were snap-frozen and stored at -80°C before RNA analysis. For immunohistochemical studies, tissues were fixed by immersion in 3.7% buffered formalin and then embedded in paraffin. Subsequently, 4um-thick sections were mounted on StarFrost adhesive slides (Knittel Glaser, Braunschweig, Germany) and processed for immunohistochemistry.

RNA extraction and Quantitative RT-PCR

Total RNA of cells or thyroid tissue was isolated using the Qiagen RNeasy Microkit or Minikit (Qiagen, Valencia, USA). Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA) was used for reverse transcription of 250 ng of each RNA sample. The synthesized cDNA was diluted to 1/20, and 5ul was used per PCR reaction. Each reaction consisted of TaqMan Universal PCR Master Mix or SybrGreen PCR Master Mix (Thermo Fisher Scientific, Waltham, USA) and primers. Peptidylpropyl isomerase A was used as an endogenous control. Real-time PCR was performed using the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA). The data were analyzed using the comparative cycle threshold method and presented as the fold change in gene expression, normalized for a

calibrator whose value equaled 1. Primers sequences and PCR conditions are available on request.

Immunofluoresence Staining

Immunohistochemistry was already described[32]. The primary antibodies were used at the following dilutions: rabbit anti-Borealin (1/1000, given by William Taylor), rabbit anti-Ecadherin (1/100, Becton Dickinson, Franklin Lakes, USA), and mouse anti-TG (1/100, Dako-Cytomation, Glostrup, Denmark). The used fluorescent secondary antibodies were Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse antibodies (1/400, Thermo Fisher Scientific, Waltham, USA). Photographs were taken using a fluorescence microscope (Leitz DMRB; Leica, Wetzlar, Germany) and digitalized using a chilled 3CCD camera (C5810; Hamamatsu Photonics, Japan).

Flow cytometer

Two days after transfection, Nthy were fixed with PFA4% (paraformaldehyde solution) 10 minutes at -20°C and permeabilised using TBS 1X with 0,1% triton then incubated with 10µg/ml of Brilliant Violet 605™ anti-mouse Ki-67 antibody (1/20, Biolegends, San Diego, USA) or anti-FLAG M2-FITC antibody (1/100, Sigma–Aldrich, Saint-Louis, USA) during 30 minutes at RT. After washing with PBS 1X, cells were incubated with PI/RNase staining buffer (propidium iodide, Becton Dickinson, Franklin Lakes, USA) during 15 minutes at RT before flow cytometric analysis (FACS Aria II, Becton Dickinson, Franklin Lakes) and FlowJo software (FlowJo LLC, Ashland, USA).

Time lapse

To analyze progression through mitosis, HeLa cells were transfected with wild-type or mutant Borealin fused to GFP. Live cells were tracked at 37°C using an environmental chamber attached to an Olympus inverted IX81 microscope. Images were captured with a Photometrics Coolsnap HQ2 camera every 12 minutes for 16 – 18 hours depending on the experiment.

Cell migration assay

Nthy were plated in Culture-Inserts on micro-slides (IBIDI, Martinsried, Germany) and transfected under the same conditions than above but adapting the quantity of vectors. One day after transfection, the insert was taken off and slides were incubated at 37°C in growth medium for 8h. Phase contrast and fluorescence images were taken every 8 minutes until the wound was closed (approximately 12h). The length of migration was calculated measuring the distance

moved toward the center of the wound in 8h using Imaris Software allowing imaging-based computerized motility analysis method.

Cell adhesion assay

Nthy were transfected as described above. 24h after transfection, cells were trypsinated and plated on glass slides previously coated with polylysine. After 1h30 incubation at 37°C, cells were fixed with PFA4%. We performed staining of Borealin-Flag (mouse anti-Flag M2, 1/1000, Sigma–Aldrich, Saint-Louis, USA) following with Alexa Fluor 488 goat anti-mouse antibodies (1/400, Thermo Fisher Scientific, Waltham, USA) and filamentous actin with rhodamine coupled phalloidin (1/400, Sigma–Aldrich, Saint-Louis, USA).

Microarray and analysis

For gene expression analysis, 200ng of RNA was used for the thyroid tissue with Borealin-114 and three thyroid tissue controls. The Human Transcriptome Array 2.0 (HTA 2.0; manufactured by Affymetrix Inc., Santa Clara, USA) was employed in this study. HTA 2.0 covers global profiling of full-length transcripts, containing more than 40,000 non-coding and 245,000 coding transcripts in human genome; each transcript is accurately identified by specific exon or exon-exon splice junction probes. RMA method was used to normalize arrays using Bioconductor *affy* package in R^{33}. Normalized expression values were then Log2-transformed before statistical analysis were performed. All results of the thyroid tissue with Borealin-114 were compared with the mean of the three thyroid tissue controls.

Statistics

Results are presented as mean ± SEM for the number of experiments indicated in the figure legends. Statistical analysis of continuous data was performed with 2-tailed Student's t test. $P < 0.05$ was considered statistically significant.

CLAIMS

1. A method for diagnosing or predicting thyroid dysgenesis in a subject, said method comprising detecting a mutation in the CDCA8 gene in a sample comprising DNA and/or RNA from said subject, wherein the presence of a mutation in the CDCA8 gene is indicative of thyroid dysgenesis.

2. The method according to claim 1, wherein said mutation is a missense mutation.

3. The method according to claim 1, wherein said mutation is a single nucleotide polymorphism consisting of rs546751848.

4. The method according to claim 1, wherein said mutation is a single nucleotide polymorphism consisting of rs35565540.

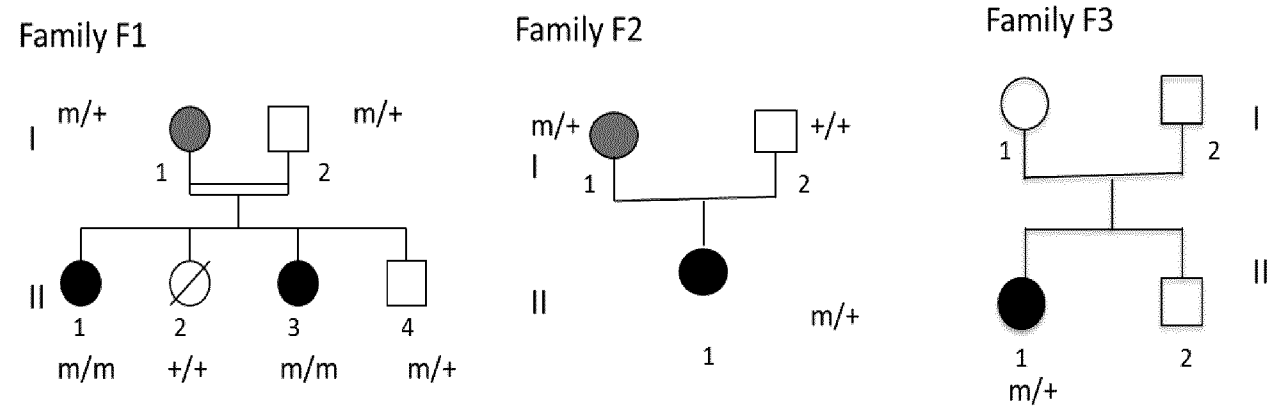
5. The method according to claim 1, wherein said mutation is a single nucleotide polymorphism consisting of rs140856315.

6. The method according to any one of claims 1 to 5, wherein said sample is a blood sample.

7. The method according to any one of claims 1 to 6, wherein said subject is less than two weeks old.

Figures

a



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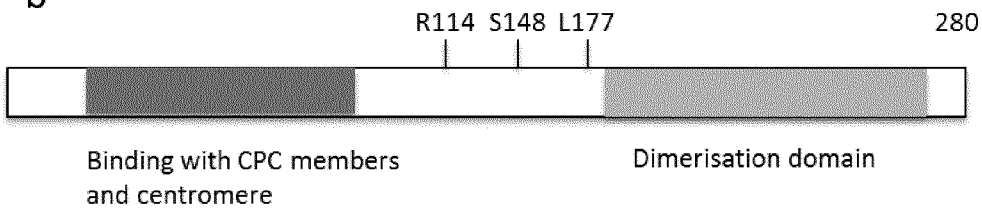

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SP|P86346|BORE1_CHICK EKSKQGIEATEEAAEVPLLPPAKKAKHDSQLLLEQAAASTNPRNGKVRTSTKKAPVSRSR 169
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SP|P86346|BORE1_CHICK RAPSA-RVKRVSKRSSKSSFITPATGRNLDLCARGGASIVTPRFDSRVFKTPGLRTPAVN 228
                      *:    .     :* ::    **: .     .    :   ***:*  : ***.**:
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Figure 1 : Molecular genetics

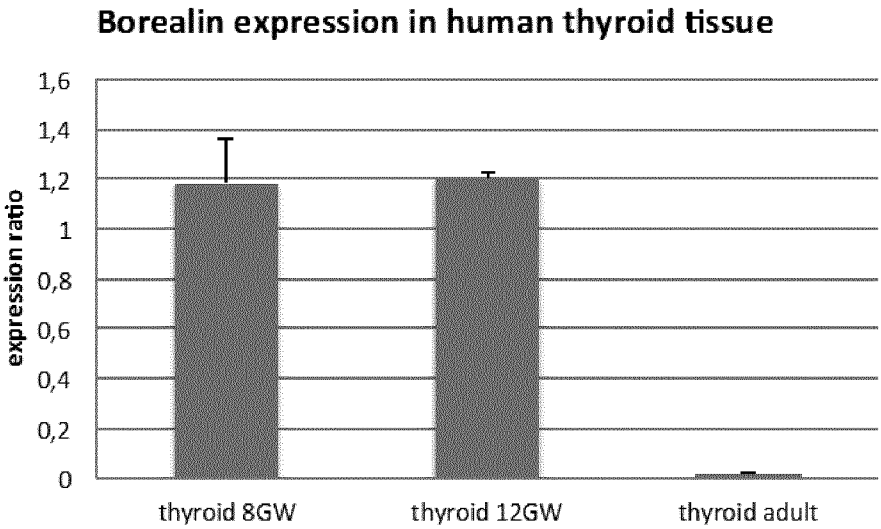


Figure 2: Borealin expression during thyroid development

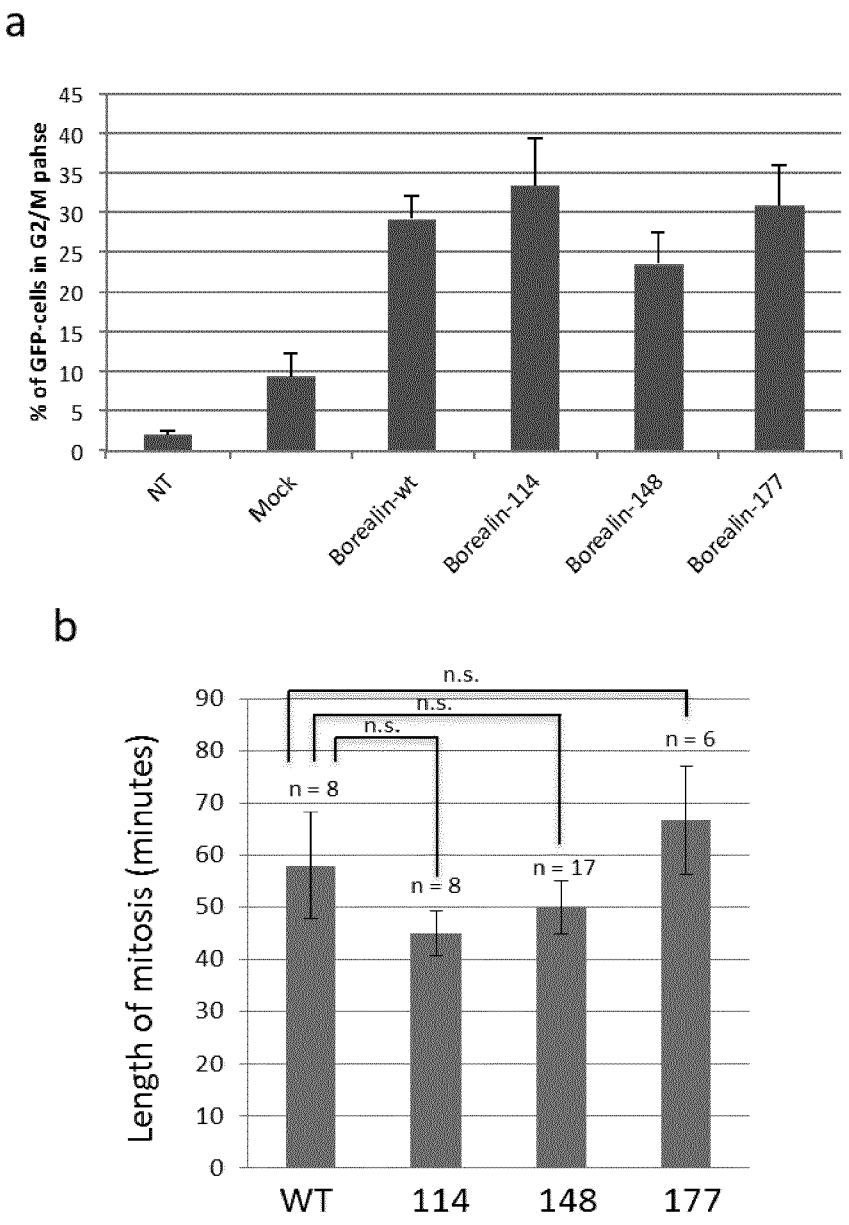


Figure 3: Effects on mitosis of Borealin mutants

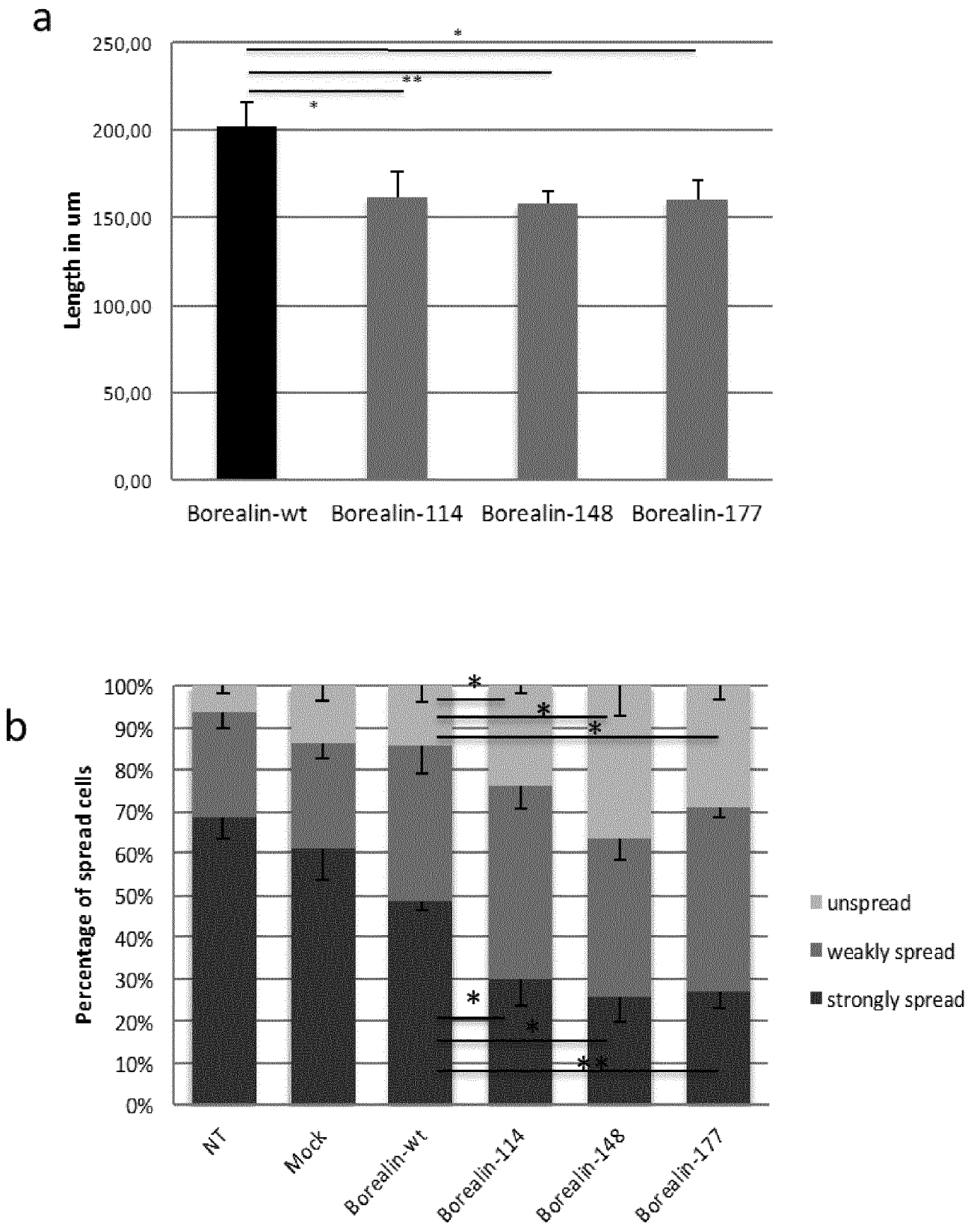
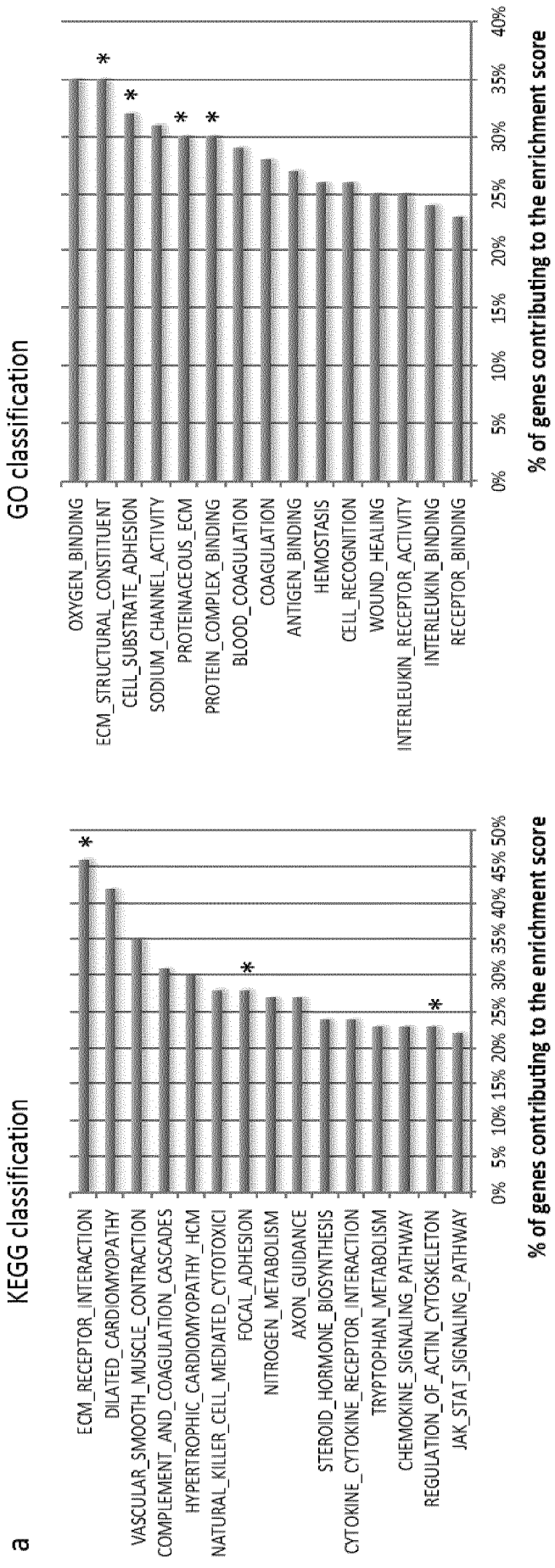


Figure 4: Modifications in migration and spreading of cells by Borealin mutants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KEGG classification
ECM_RECEPTOR_INTERACTION
DILATED_CARDIOMYOPATHY
VASCULAR_SMOOTH_MUSCLE_CONTRACTION
COMPLEMENT_AND_COAGULATION_CASCADES
HYPERTROPHIC_CARDIOMYOPATHY_HCM
NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICI
FOCAL_ADHESION
NITROGEN_METABOLISM
AXON_GUIDANCE
STEROID_HORMONE_BIOSYNTHESIS
CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION
TRYPTOPHAN_METABOLISM
CHEMOKINE_SIGNALING_PATHWAY
REGULATION_OF_ACTIN_CYTOSKELETON
JAK_STAT_SIGNALING_PATHWAY
0% 5% 10% 15% 20% 25% 30% 35% 40% 45% 50%
% of genes contributing to the enrichment score
GO classification
OXYGEN_BINDING
ECM_STRUCTURAL_CONSTITUENT
CELL_SUBSTRATE_ADHESION
SODIUM_CHANNEL_ACTIVITY
PROTEINACEOUS_ECM
PROTEIN_COMPLEX_BINDING
BLOOD_COAGULATION
COAGULATION
ANTIGEN_BINDING
HEMOSTASIS
CELL_RECOGNITION
WOUND_HEALING
INTERLEUKIN_RECEPTOR_ACTIVITY
INTERLEUKIN_BINDING
RECEPTOR_BINDING
0% 5% 10% 15% 20% 25% 30% 35% 40%
% of genes contributing to the enrichment score

Figure 5a

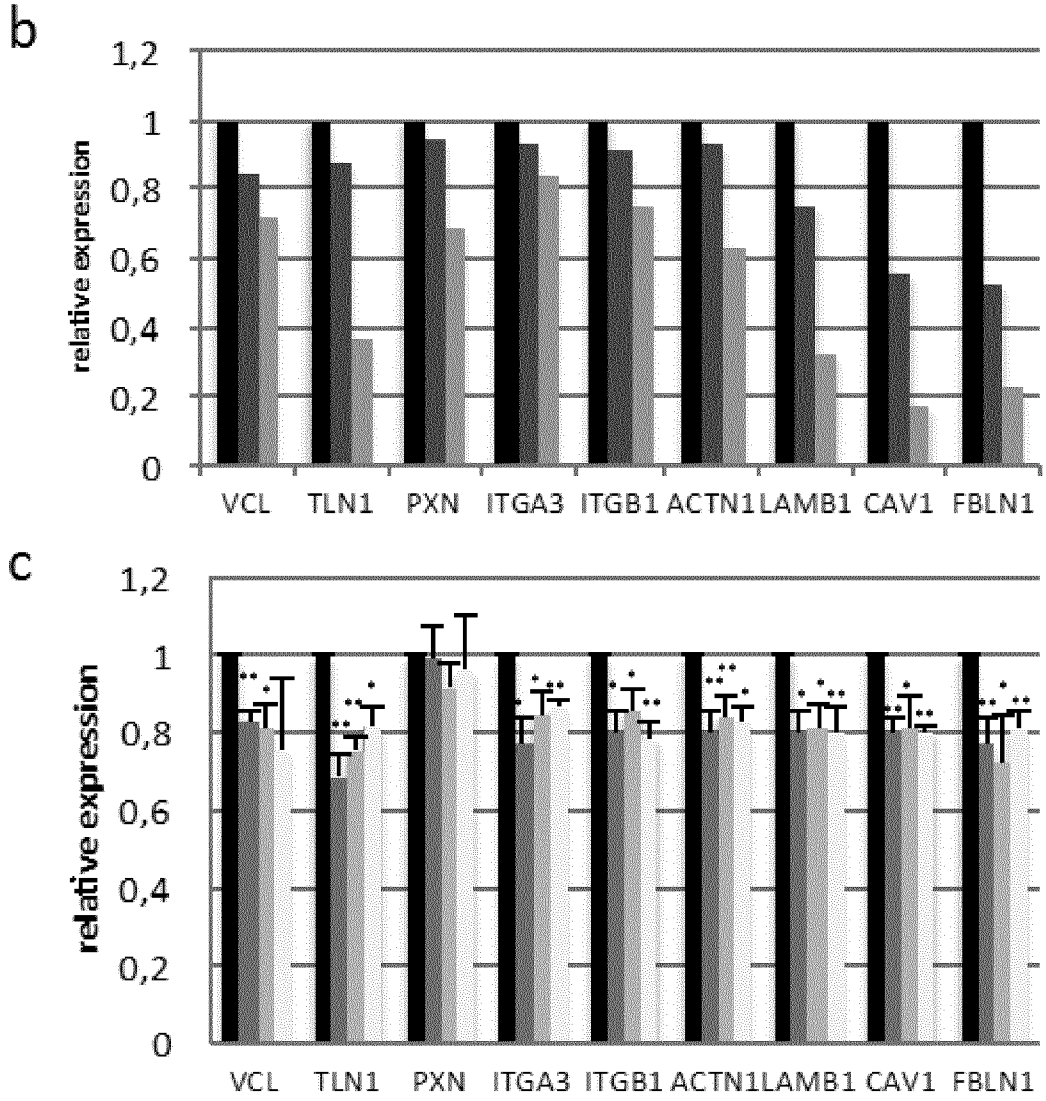
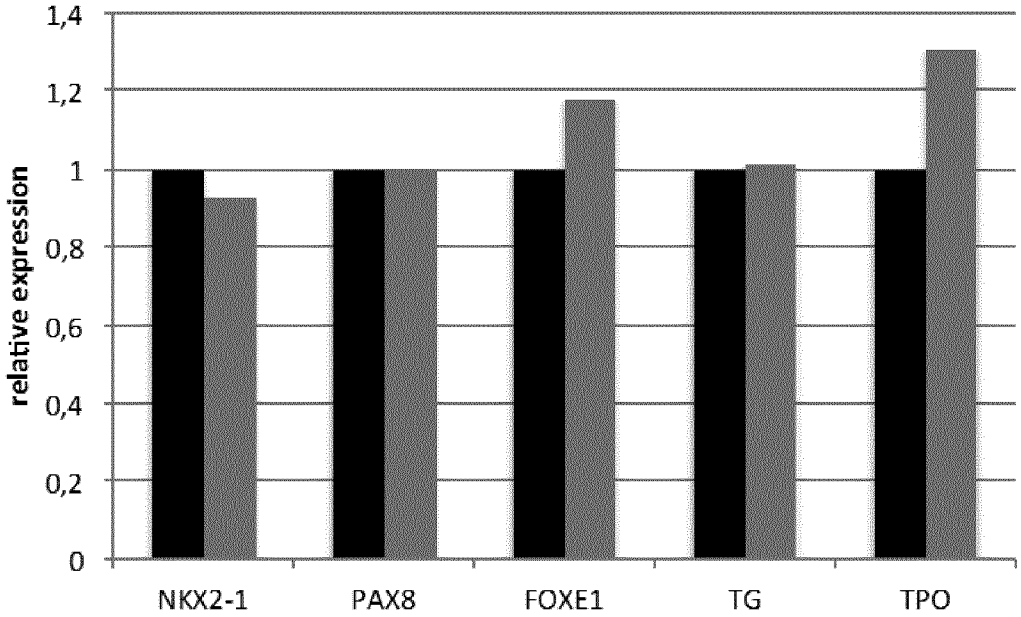


Figure 5b and 5c

Figure 5: Transcriptome analysis of thyroid tissue with Borealin-114 and quantitative PCR in transfected HPT

a



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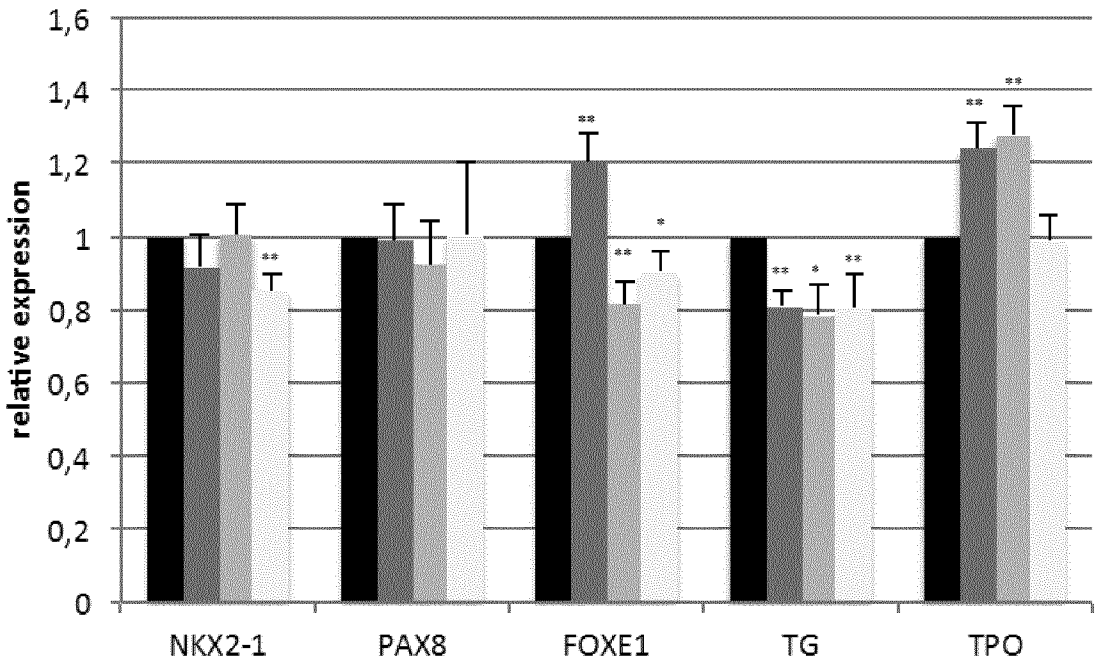

Figure 6: Expression of genes involved in thyroid function

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/066005

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PAOLO EMIDIO MACCHIA ET AL: "PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis", NATURE GENETICS., vol. 19, no. 1, 1 May 1998 (1998-05-01), pages 83-86, XP055325007, NEW YORK, US ISSN: 1061-4036, DOI: 10.1038/ng0598-83 the whole document page 85, column 1, paragraph 2 -----	1-7
A	WO 2011/035239 A1 (UNIV CORNELL [US]; ABBOTT GEOFFREY [US]; ROEPKE TORSTEN [DE]) 24 March 2011 (2011-03-24) claims 3-6 ----- -/--	1-7

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
5 September 2017	13/09/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schmitt, Anja

3

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3

INTERNATIONAL SEARCH REPORT

Information on patent family members

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