Postzygotic inactivating mutations of RHOA cause a mosaic neuroectodermal syndrome

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Hypopigmentation along Blaschko’s lines is a hallmark of a poorly defined group of mosaic syndromes whose genetic causes are unknown. Here we show that postzygotic inactivating mutations of RHOA cause a neuroectodermal syndrome combining linear hypopigmentation, alopecia, apparently asymptomatic leukoencephalopathy, and facial, ocular, dental and acral anomalies. Our findings pave the way toward elucidating the etiology of pigmentary mosaicism and highlight the role of RHOA in human development and disease.

Linear hypopigmentation, which is commonly seen as a non-specific manifestation of mosaicism, is currently classified using poorly defined umbrella terms such as ‘pigmentary mosaicism’ and ‘hypomelanosis of Ito’. Because of its frequent association with various extracutaneous anomalies (especially cerebral involvement and epilepsy), hypomelanosis of Ito is often considered as a neurocutaneous syndrome, the fourth most common after neurofibromatosis, tuberous sclerosis complex and Sturge–Weber syndrome. Apart from rare reports of nonrecurrent mosaic chromosomal anomalies, the genetic causes of pigmentary mosaicism are largely unknown, which hinders diagnosis and patient care.

As part of our research program on mosaic skin disorders, we evaluated seven unrelated individuals with a remarkably similar constellation of features that did not match any known syndrome (Fig. 1, Supplementary Figs. 1 and 2 and Supplementary Table 1). Key clinical features included linear hypopigmentation and hypotrichosis following the lines of Blaschko, symmetric or asymmetric facial dysmorphism (microstomia, malar hypoplasia, downsloping palpebral fissures and broad nasal bridge), acral anomalies (brachydactyly, syndactyly and broad first toe), teeth anomalies (oligodontia, microdontia, conical teeth and abnormal enamel) and ocular anomalies (microphthalmia, strabismus and myopia). Brain magnetic resonance imaging (MRI) was available for five patients and showed diffuse cystic leukoencephalopathy with mildly enlarged lateral ventricles (Fig. 1 and Supplementary Fig. 2) in four of them. Despite this striking brain phenotype, no intellectual deficiency or neurological impairment was noted in any affected individual. Linear hypopigmentation following Blaschko’s lines, asymmetric craniofacial and brain features and sporadic occurrence were highly suggestive of mosaicism.

We hypothesized that this previously unrecognized mosaic neuroectodermal syndrome probably resulted from postzygotic mutations in the same gene. We conducted whole-exome sequencing (WES) in two parent-case trios (participants S1 and S2) using genomic DNA derived from participants’ affected skin and parental blood samples (Methods and Supplementary Table 2). We identified the same postzygotic change of RHOA (NM_001664.3:c.139G>A;
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NP_001655.1:p.(Glu47Lys)) supported by 30.6% (44 out of 144) and 2.6% (6 out of 228) of reads in participants S1 and S2, respectively (Supplementary Figs. 3 and 4 and Supplementary Table 3). We confirmed the presence and postzygotic nature of these mutations by targeted ultra-deep sequencing of the region spanning the c.139G>A substitution in all available DNA samples from the two patients and their parents (Methods and Supplementary Tables 4–6). Trio-based WES in a third patient (participant S3) led to the identification of another postzygotic RHOA change (NM_001664.3:c.211C>T; NP_001655.1:p.(Pro71Ser)) supported by 24.3% (28 out of 115) of reads (Supplementary Fig. 5), thus confirming mutations of RHOA as the cause of this previously unknown syndrome. Amplicon-based ultra-deep sequencing of RHOA coding exons in skin-derived DNA from the remaining three affected individuals, and Sanger sequencing.

Fig. 1 | Main clinical features of RHOA-related mosaic ectodermal dysplasia and RHOA mutations. a–e, Craniofacial appearance (a) and linear hypopigmentation (b) in participant S4, and dental anomalies (c), alopecia (d) and acral anomalies (e) in participant S1. f–i, Brain MRI of participant S1 at 15 years. A second MRI, conducted 6 months later, did not show any substantial change. Participants S2 and S4 had similar but milder MRI abnormalities, including enlarged temporal horns of the lateral ventricles (Supplementary Fig. 2). Sagittal T1-weighted imaging revealed preserved midline structures (f). Axial T2-weighted imaging revealed a focal hyperintense lesion in the right hemisphere of the cerebellum (g, arrowhead) and diffuse cystic leukencephalopathy with mildly enlarged lateral ventricles and cysts in the thalami and caudate nuclei (h). Leukencephalopathy and presence of multiple cysts was confirmed on fluid-attenuated inversion recovery sequences (i). j, Mutant allele fraction of RHOA mutations in the five participants studied in WES or tough decoy sequencing. k, Linear representation of RHOA and localization of the two mutations.
of RHOA in one extra individual, led to the identification of the recurrent c.139G>A change (encoding p.(Gly14Val)) in three (S4, S5 and S7) out of a total of five participants with the exact same change (Supplementary Table 6), possibly due to negative selection of mutant cells during cell culture. WES in participant S2 also revealed a previously unknown familial NC_012920.1:m.11778G>A NADH-ubiquinone oxidoreductase chain 4 in mitochondrial complex I mitochondrial DNA, causing Leber’s hereditary optic neuropathy and probably responsible for a more severe loss of visual acuity (Supplementary Fig. 6). No RHOA mutations were found in 24 additional participants with linear hypopigmentation associated with various extracutaneous features (Supplementary Table 8).

RHOA encodes a Ras-related Rho GTPase known to control a wide range of biological functions such as morphogenesis, chemotaxis, axonal guidance and cell cycle progression. Transforming protein RhoA (RhoA) has been extensively studied for its central role in signal transduction and actin cytoskeleton dynamics, through the regulation of stress fibers and focal adhesion formation.

**Fig. 2** | The inactivating effect of the two RHOA mutations. **a, b,** Cytoskeletal organization and morphology in NIH/3T3 cells transfected with wild-type, constitutively active p.(Gly14Val), dominant-negative p.(Thr19Asn), p.(Glu47Lys) and p.(Pro71Ser) forms of RhoA. **a,** Top: dual labeling for DAPI (blue) and α-tubulin (green) does not reveal significant differences in the gross organization of the microtubule cytoskeleton or nuclear morphology between different mutants. All cells (n = 20 per group) selected at random across the coverslip that were individually examined showed reduced stress fibers and limited cell spreading. Bottom: cells transfected with constructs for expression of wild-type RhoA or the p.(Gly14Val) mutant display expected increase in F-actin staining, particularly with regard to stress fibers, which are brighter, thicker and more numerous. Cells transfected with constructs for expression of p.(Thr19Asn), p.(Glu47Lys) or p.(Pro71Ser) mutants barely contain any stress fibers at all. **b,** Top: FLAG staining does not reveal any visible differences in the subcellular localization of different RHOA mutants. All mutants tended to impair cell spreading, while wild-type RHOA-overexpressing cells maintain normal morphology. Middle: dual labeling for DAPI (blue) and pMYPT1 (red) shows decrease in signal intensity of MYPT1 (pThr696) staining on transfection with p.(Thr19Asn) (control), p.(Gly14Val) or p.(Pro71Ser) mutants. Bottom: Quantification of MYPT1 (pThr696) staining shows significant decrease (n = 20, 24, 14, 30 and 13 cells, for wild-type, p.(Gly14Val), p.(Thr19Asn), p.(Glu47Lys) and p.(Pro71Ser), respectively). The following box plot elements are shown: 5th and 25th percentiles, median, mean (cross), 75th and 95th percentiles. AU, arbitrary units. **c,** Levels of phosphorylated MYPT1 (pThr696) and MLC2 (pThr19). Left: cropped images of immunoblot experiment showing the expression levels of total MYPT1, pMYPT1, total MLC2 and pMLC2. There is a visible reduction in pMYPT1 and pMLC2 when RhoA p.(Thr19Asn) or RhoA p.(Glu47Lys) are overexpressed. Middle and right: dot plot of normalized ratio (four independent experiments) for pMYPT1 and pMLC2 normalized to total MYPT1 and MLC2, respectively, indicate a reduction in MYPT1 (pThr696) and MLC2 (pThr19) on RhoA p.(Glu47Lys) or RhoA p.(Pro71Ser) overexpression. Further analyses for p.(Gly14Val) are shown in Supplementary Fig. 7. Full scans of the immunoblots are provided in Supplementary Fig. 8.
a constitutively active mutant with a p.(Gly14Val) substitution. We have delineated a clinical and molecular subset of pigmentary mosaic syndromes, which we propose to name 'RHOA-related mosaic ectodermal dysplasia'. Apart from recent reports of linear hypopigmentation in six patients with MTOR-related hemimegalencephaly, no specific genes have been implicated in pigmentary mosaic disorders. Our findings highlight the value of careful clinical phenotyping combined with massively parallel sequencing for elucidating their genetic causes. The syndrome described in this study presents both similarities and notable differences with other mosaic syndromes involving the skin, as well as disorders of the PI3K-Akt-mTOR and RAS-MAPK pathways. Transforming protein RhoA is a highly conserved protein particularly intolerant to amino acid substitutions, with only five observed missense changes in the Exome Aggregation Consortium (66.9 expected variants; z = 3.70) and no loss-of-function alleles (5.1 expected). Accordingly, RHOA is part of the 'core essentialome', a set of genes essential to cell viability, thus supporting the idea that RHOA-related mosaic ectodermal dysplasia should be added to the list of disorders resulting from lethal mutations surviving only by mosaicism, which includes Proteus, Sturge–Weber and some other mosaic syndromes.

All postzygotic mutations reported to date as causing such mosaic syndromes have been activating mutations also.

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Author contributions

P.V. and J.-B.R. designed the study. A.S., J.S.-O., P.K., J.-B.C., V.C., S.P. and V.A.K. performed the genetics experiments. J.-B.R., Y.D. and P.G. performed the bioinformatics experiments. S.S.K performed the functional experiments. P.V., A.S., B.Demeer, D.B., O.B., A.B., G.C., E.C., T.C., S.P., F.F., V.A.K., B.Devauchelle, D.G., C.J.-L., A.L., M.M.-D., J.T. and L.F. recruited and evaluated the study participants. L.G., G.B. and W.B.D. analyzed the brain MRI. P.V., L.F., M.E.R. and J.-B.R. supervised the study. P.V., S.S.K, M.E.R. and J.-B.R. wrote the manuscript. All authors revised the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Study participants. The study included seven unrelated affected individuals and their unaffected parents. Individuals were phenotyped and recruited by geneticists and dermatologists in Dijon and elsewhere in France through a collaborative nationwide effort to identify genes causing mosaic syndromes involving the skin (ClinicalTrials.gov registration no. NCT01950973; https://clinicaltrials.gov/ct2/show/NCT01950973?term=NCT01950973&rank=1). Inclusion criteria consisted of the following: sporadic condition; congenital or early-childhood onset; and cutaneous lesions with a pattern suggestive of mosaicism associated with extracutaneous anomalies. We obtained written informed consent from all participants or their legal representatives. We also obtained written consent to publish the displayed photographs of individuals. The ethics committee of Dijon University Hospital approved the study. We extracted genomic DNA from fresh skin, cultured skin fibroblasts and blood samples using the Gentra Puregene Blood and Tissue Extraction Kit (QIAGEN). We assessed genomic DNA integrity and quantity by agarose gel electrophoresis, NanoDrop spectrophotometry and Qubit fluorometry (Thermo Fisher Scientific).

WES. Exome capture and sequencing were performed at IntegraGen from 1 μg of genomic DNA per individual using the Agilent SureSelect Human All Exon V5 (trios S1 and S2) and Clinical Research Exome V1 (trio S3) kits. Libraries were sequenced on a HiSeq 2000 platform (Illumina) using paired-end 75-base pair reads. Sequences were aligned to the human genome reference sequence (GRCh37/hg19 build of the University of California Santa Cruz Genome Browser); single-nucleotide variants and small insertions/deletions were systematically detected as described previously16. Candidate de novo mutational events were identified by focusing on protein-altering and splice site changes: (1) supported by at least three reads and 10% of total reads in the proband; (2) absent in both parents, as defined by variant reads representing <5% of the total reads; (3) at base pair positions covered by at least four reads in the entire trio; and (4) present at a frequency<1% in the SNP database build 147 and 0.1% in the Exome Aggregation Consortium (http://exac.broadinstitute.org)17. Candidate low-level postzygotic changes of RHOA in participant S2 were detected as described previously16. Briefly, all coding and splice site bases of RHOA were systematically analyzed to count all sites with at least one read not matching the reference sequence, using a base quality threshold of 30.

Ultra-deep sequencing of RHOA. Coding exons of RHOA (reference accession no. NM_0016642.1) were amplified using custom intronic primers (Supplementary Table 4) and standard PCR with the PrimeSTAR GXL DNA Polymerase (Takara Bio). PCR products were purified and libraries were prepared using the transposase-based Nextera XT DNA Sample Preparation Kit (Illumina). Libraries were sequenced on a MiSeq instrument using 300-cycle MiSeq Reagent Kit v2 (Illumina) and paired-end sequencing reactions of 150-base pair reads. Ultra-deep sequencing was performed to achieve a sequencing depth of at least 1,000 reads for all targeted coding bases and splice junctions (Supplementary Table 5). As described previously7, we identified candidate single-nucleotide variants and small insertions/deletions by recording all sites of RHOA coding exons and splice junctions that do not meet at least 4 reads not matching the reference sequence, using a base quality threshold of 30 and a mapping quality threshold of 20, with a mutant allele fraction of at least 0.01. We annotated variants with SeattleSeq Annotation 138 server (http://snp.gs.washington.edu/SeattleSeqAnnotation138/) and focused on protein-altering and splice site changes present at a frequency <0.1% in the Exome Aggregation Consortium7.

In silico prediction. Nucleotide-level conservation and the impact of amino acid change of RHOA mutations were assessed with the genomic evolutionary rate profiling18 and combined annotation-dependent depletion scores19, respectively (Supplementary Table 7).

Cell culture and transfection. NIH/3T3 cells were obtained from ATCC (CRL-16581TM) and maintained in DMEM (Thermo Fisher Scientific) plus 10% FCS. NIH/3T3 cells were obtained from ATCC (CRL-16581TM) and maintained in DMEM (Thermo Fisher Scientific) plus 10% FCS.

Immunocytochemistry. NIH/3T3 cells were fixed with 0.25% glutaraldehyde and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich). Mouse anti-r-tubulin (1:5,000; catalog no. T0074; Sigma-Aldrich) and goat anti-FLAG (1:500; catalog no. A1990-101A; Bethyl Laboratories) antibodies were incubated overnight at 4°C. Appropriate secondary Alexa Fluor-conjugated antibodies (catalog no. A11055, A100042 and 141571, 1:1,000; Thermo Fisher Scientific) along with Alexa Fluor 488-conjugated phalloidin to visualize F-actin (1:100; catalog no. A12379; Thermo Fisher Scientific) were applied for 1 h at room temperature. Coverslips were mounted in ProLong Antifade Mountant (Thermo Fisher Scientific) and visualized with a 100x oil immersion objective on an inverted microscope (Zeiss) fitted with a spinning disc confocal scanner (PerkinElmer). Imaging analysis was performed using the ImageJ (National Institutes of Health) software v.1.49 as follows: confocal stacks were projected onto a single plane (maximum intensity Z-project); images were thresholded; and fluorescence intensity was measured as a mean gray value. The investigator collecting the images was blinded to the experimental groups. During analysis of the immunocytochemistry data, the investigator was blinded to the identity of the experimental groups.

Immunoblotting. NIH/3T3 cells were rinsed once with PBS and lysed in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitor cocktail (Sigma-Aldrich). A total of 15 μg of protein in lithium dodecyl sulfate electrophoresis loading buffer (Thermo Fisher Scientific) was denatured for 10 min at 70°C and separated on a 4–12% SDS-polyacrylamide gel electrophoresis gel (Thermo Fisher Scientific). Proteins were transferred onto a 0.2-μm nitrocellulose membrane (Pall) and processed for immunoblotting. Primary antibodies were used at the following dilutions: goat anti-actin (1:4,000; catalog no. sc-1616; Santa Cruz Biotechnology); mouse anti-MYPT1 (1:4,000; catalog no. 612165; BD Biosciences); rabbit anti-pMYPT1 (Thr696) (1:500; catalog no. 8505; Cell Signaling Technology); rabbit anti-RhoA (1:4,000; catalog no. 6789; Cell Signaling Technology); rabbit anti-MLC2 (1:4,000; catalog no. 8505; Cell Signaling Technology); and mouse anti-pMLC2 (Thr18/Ser19) (1:500; catalog no. A12379; Thermo Fisher Scientific) along with Alexa Fluor 488-conjugated phalloidin to visualize F-actin (1:1,000; catalog no. A12379; Thermo Fisher Scientific) and visualized with a 100x oil immersion objective on an inverted microscope (Zeiss) fitted with a spinning disc confocal scanner (PerkinElmer). Imaging analysis was performed using the ImageJ (National Institutes of Health) software v.1.49 as follows: confocal stacks were projected onto a single plane (maximum intensity Z-project); images were thresholded; and fluorescence intensity was measured as a mean gray value. The investigator collecting the immuno-blots was blinded to the identity of the experimental groups.

Statisits. For fluorescence intensity quantification, a t-test assuming unequal variance was performed; P<0.05 was deemed significant. For immunoblotting, four independent experiments for each transfection were performed; the average and s.d. reflect these replicates.

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Data analysis

For exome sequencing and targeted sequencing, reads were aligned to the human genome reference sequence (GRCh37/hg19 build of UCSC Genome Browser) with the Burrows-Wheeler Aligner (BWA, v.0.6.2), and potential duplicate paired-end reads were marked with Picard v.1.77. The Genome Analysis Toolkit (GATK) v.2.6-4 was used for base quality score recalibration, indel realignment, and variant discovery. Variants were annotated with SeattleSeq SNP Annotation v138. Variants were systematically visualized on the Integrative Genomic Viewer v2.3. Imaging analysis was performed using Imagemol software v1.49. For WB, proteins were detected using Odyssey Image Studio v4.0.21 (LI-COR).

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Sample size

Seven patients with similar phenotypical features were included. Sample size could not be predetermined as it was conditioned by the number of human research participants / patients diagnosed with RHOA mutation participating in our study (7). These patients were enrolled in the M.U.S.T.A.R.D. cohort, registered in clinicaltrials.gov under the ref NCT01950975. At the time of this study, 31 patients with hypomelanosis of Ito were included in the cohorts, as described in Supp. Table 8. Only one skin biopsy was taken for each patient, in accordance to ethical limitation. Five transfected cell lines were generated and studied using IF and Western blot. Regarding IF, 20 cells in each group were selected at random across the cover slip and were individually examined. For Western Blot, four independent experiments for each transfection was performed, and average and standard deviation reflect these replicates.

Data exclusions

No data was excluded

Replication

To verify the reproducibility of the findings, we confirmed key findings of our study using several different and independent methodological approaches. After identification of the candidate gene with exome sequencing, we used targeted sequencing of this gene to study patients with similar phenotype, as well as patient with cutaneous pigimentary disorder with a different phenotype. All patients with similar phenotype carried a RHOA mutation except for one patient whose DNA was not analyzable due to failed quality control, whereas no RHOA variant was detected in patients with a different phenotype. We also used several independent experiments to evaluate the functional consequences of the mutations (Immunocytochemistry studies of the cytoskeletal organization and morphology in NIH/3T3 cells and quantification of MYPT1(pThr696); levels of two phosphorylated downstream effectors of RhoA by Western Blot), which showed concordant results. For Western Blot, four independent experiments for each transfection was performed. All attempts of replication were successful.

Randomization

Patients with mosaic pigmentary disorders involving the skin were allocated to this study by genotype (presence of a RHOA mutation). Due to the very limited number of patients with this condition, randomization was not possible.

Blinding

The investigator collecting images was blinded to the experimental groups. During analysis of immunocytochemistry data, the investigator was blinded to the identity of the experimental groups. The investigator carrying out the Western blot experiments was not blinded to the identity of the samples. Our use of the LI-COR Odyssey, fluorescent Western blot avoids two major issues with researcher bias. First, our protocol avoids selection bias or cherry picking of data points to be included in the analysis since in this report, all data points are included. Second, the fluorescence measurement using this Odyssey system is highly standardized. Measurement is done by the software without operator manipulation, which guarantee repeatability, that is, any other operator measuring the image using the same software will get the same numbers.

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Antibodies

Antibodies used

Immunocytochemistry: Mouse anti-α-tubulin (Sigma T9026, 1:5,000), goat anti-FLAG (A190-101A, Bethyl Laboratories, 1:500), AlexaFluor-conjugated antibodies (Life Technologies, 1:1,000), AlexaFluor-conjugated phalloidin to visualize F-actin (A12379, Life Technologies, 1:100), anti-Myc antibodies (Bethyl Laboratories, 1:500). Secondary antibodies: Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 Cat # A11055, Thermo Fisher Scientific, 1:1,000; Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 Cat # A10042, Thermo Fisher Scientific, 1:1,000; Donkey anti-Mouse...
| Validation |
|-----------|
| Mouse anti-α-tubulin (Sigma T9026, 1:5,000), as per manufacturer validated for use in ICC and WB, validated for use in mouse, cited 2035 times https://www.sigmaaldrich.com/catalog/product/sigma/t9026?lang=en&region=US |
| Rabbit anti-MYPT1(pT696) (ABS45, Millipore, 1:500), as per manufacturer validated for use in WB, validated for use in mouse, cited 16 times https://www.sigmaaldrich.com/catalog/product/mm/abs45?lang=en&region=US |
| Goat anti-FLAG (A190-101A, Bethyl Laboratories, 1:500), as per manufacturer validated for use in WB and ICC, species specificity N/A, cited 15 times https://www.bethyl.com/product/A190-101A?referrer=search |
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| Mouse anti-MYPT1 (612165, Becton-Dickinson, 1:4,000), as per manufacturer validated for use in WB, validated for use in mouse, https://wwwbdbiosciences.com/us/reagents/research/antibodies-buffers/cell-biology-reagents/cell-biology-antibodies/purified-mouse-anti-myp1-20mypt1/p/612165 cited 8 times https://www.citeab.com/antibodies/2411798-612165-purified-mouse-anti-myp1?des=C6DA95FBD9320B2F |
| Rabbit anti-RhoA (2117, Cell Signaling, 1:4,000), as per manufacturer validated for use in WB, validated for use in mouse, cited 122 times https://www.cellsignal.com/products/primary-antibodies/rhoa-67b9-rabbit-mab/2117 |
| Rabbit anti-MLC2 (8505, Cell Signaling, 1:4,000), as per manufacturer validated for use in WB, validated for use in mouse, cited 28 times https://www.cellsignal.com/products/primary-antibodies/myosin-light-chain-2-d18e2-rabbit-mab/8505 |
| Mouse anti-MLC2(pT19) (3675, Cell Signaling, 1:500), as per manufacturer validated for use in WB and ICC, validated for use in mouse, cited 130 times https://www.cellsignal.com/products/primary-antibodies/phospho-myosin-light-chain-2-ser19-mouse-mab/3675 |

## Eukaryotic cell lines

**Policy information about cell lines**

**Cell line source(s)**

NIH/3T3 cells, obtained from ATCC, cat. # ATCC® CRL-1658™

**Authentication**

ATCC cell lines authentication process can be found here: https://www.atcc.org/Global/FAQs/E/A/Authenticating%20cell%20lines-249.aspx

**Mycoplasma contamination**

Mycoplasma free as per manufacturer’s specification. Cells have been cultured in antibiotic free media to monitor for sterile technique

**Commonly misidentified lines (See ICLAC register)**

NIH/3T3 line is not in the latest database of commonly misidentified cell lines https://iclac.org/databases/cross-contaminations/

## Human research participants

**Policy information about studies involving human research participants**

**Population characteristics**

Seven patients with similar phenotypical features were included.

**Recruitment**

Patients described in this study were adressed to the MAGEC reference center in Dijon to be analysed as part of the M.U.S.T.A.R.D. cohort (registered in clinicaltrials.gov under the ref NCT01950975), created in order to elucidate the genetic basis of mosaic disorders involving the skin. As it is a cohort at European scale, we postulate that it represents the diversity of patients carrying mosaic cutaneous disorder. All patients in this cohort with hypomelanosis of Ito were studied, without exclusion. Despite the apparently recurrent phenotype associated with RHOA mutation, we cannot exclude that these mutations may also cause a phenotype without cutaneous involvement (depending on the tissular distribution of the mosaic). In this case, we can expect that the phenotypic spectrum associated with RHOA mutations will broaden with further studies.
We obtained written informed consent from all subjects or their legal representatives, and the ethics committee of Dijon University Hospital approved the study.

Note that full information on the approval of the study protocol must also be provided in the manuscript.