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(54) Title: RHOA (ROCK) INHIBITORS FOR THE TREATMENT OF ENFLAMMATORY BOWEL DISEASE

(57) Abstract: The inventors now identified that TTC7A deficiency is a novel cause of IBD. It results from inappropriate activation of the RhoA signaling pathway, and thus inhibition of said pathway represents a novel therapeutic strategy for the treatment of IBD. Accordingly, the present invention relates to a RhoA kinase (ROCK) inhibitor for use in the treatment of an inflammatory bowel disease in a subject in need thereof.



RHOA (ROCK) INHIBITORS FOR THE TREATMENT OF INFLAMMATORY BOWEL DISEASE

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of inflammatory bowel diseases.

BACKGROUND OF THE INVENTION:

Inflammatory bowel disease (IBD) is one of the most common chronic gastrointestinal diseases in the developed world^{1,2}. It comprises a heterogeneous group of disorders that differ in terms of the gastrointestinal sites involved and the characteristics of the inflammation. Ulcerative colitis (UC) and Crohn disease (CD) are the predominant subtype of IBD³. Whereas UC is confined to the colon, CD may occur anywhere along the gastrointestinal tract. Experimental studies and genetic evidence suggest that chronic intestinal inflammation can be triggered by various environmental factors in genetically susceptible individuals⁴⁻⁶. Maintaining a normal balance between competence to respond to intestinal pathogens and the suppression of inflammatory responses to commensal microbes depend on (i) the integrity of the mucosal barriers^{6,7}, (ii) the activity of proinflammatory signaling pathways⁸ and (iii) the regulation of innate and adaptative immune responses in the intestine and draining lymphoid organs⁹. Defects in these components have been implicated in IBD, although our fundamental knowledge of the underlying disease mechanism remains patchy. Over the last decade, genetic studies have emphasized the role of host susceptibility in the onset of IBD. About 200 risk loci, have been identified - most of which encode proteins involved in immunity, host defense against microbes, and/or gut epithelium renewal¹⁰⁻¹³. However, when considered individually, these loci only procure a minor relative risk¹³. NOD2, a pattern-recognition receptor involved in autophagy induction, is the best-established susceptibility gene for CD¹⁴.

Highly penetrant monogenic causes of IBD also exist, but are rare. It has been shown that homozygous null alleles of IL-10 and the IL-10 receptor alpha and beta, required to prevent an excessive immune response, cause very-early-onset IBD^{15,16}. Furthermore, a proportion of individuals with XIAP deficiency develop a severe Crohn's-like disease¹⁷. XIAP is an apoptosis inhibitor that is involved in NOD2's downstream activation pathway¹⁸. Genetic mutations in components of the NADPH oxidase complex that impair the respiratory

burst in phagocytic leucocytes and cause chronic granulomatous disease are associated in a high proportion of patients with a Crohn's-like IBD¹⁹.

SUMMARY OF THE INVENTION:

5 The inventors now identify that TTC7A deficiency is a novel cause of IBD. It results from inappropriate activation of the RhoA signaling pathway, and thus inhibition of said pathway represents a novel therapeutic strategy for the treatment of IBD. Accordingly, the present invention relates to a RhoA kinase (ROCK) inhibitor for use in the treatment of an inflammatory bowel disease in a subject in need thereof.

DETAILED DESCRIPTION OF THE INVENTION:

10 Molecular mechanisms underlying inflammatory bowel disease (IBD) remain largely unknown. Studies of monogenic diseases can provide insight into the pathogenesis of IBD. The inventors performed genetic-linkage analysis and candidate-gene sequencing on samples from a large consanguineous kindred in which 13 patients were affected by an early-onset IBD, progressive immune deficiency and, in some cases, alopecia. They performed a histologic analysis of patients' intestinal biopsies and carried out functional assays on peripheral-blood mononuclear cells. Gut-organoids were derived from a patient's intestinal biopsy and were analyzed for cell growth. The inventors identified a homozygous hypomorphic missense mutation (c.211G>A leading to E71K) in the *TTC7A* gene in all affected family members. 20 The mutation was associated with impaired protein expression. Partial TTC7A depletion modified proliferation, adhesion and migratory capacities in lymphocytes via inappropriate activation of the RhoA signalling pathway. Growth and polarization of TTC7-deficient gut-epithelial-organoids was also found to be dependent on the RhoA signaling pathway. In conclusion, TTC7A deficiency is a novel cause of IBD and is associated with combined immune deficiency in humans. It results from inappropriate activation of the RhoA signaling pathway, and thus inhibition of said pathway represents a novel therapeutic strategy for the treatment of IBD.

30 Accordingly; the present relates to a RhoA kinase (ROCK) inhibitor for use in the treatment of an inflammatory bowel disease in a subject in need thereof.

As used herein the term "inflammatory bowel disease" has its general meaning in the art and refers to any inflammatory disease that affects the bowel. The term includes but is not

limited to ulcerative colitis, and Crohn's disease. "Crohn's disease (CD)" or "ulcerative colitis (UC)" are chronic inflammatory bowel diseases of unknown etiology. Crohn's disease, unlike ulcerative colitis, can affect any part of the bowel. The most prominent feature Crohn's disease is the granular, reddish-purple edematous thickening of the bowel wall. With the development of inflammation, these granulomas often lose their circumscribed borders and integrate with the surrounding tissue. Diarrhea and obstruction of the bowel are the predominant clinical features. As with ulcerative colitis, the course of Crohn's disease may be continuous or relapsing, mild or severe, but unlike ulcerative colitis, Crohn's disease is not curable by resection of the involved segment of bowel. Most patients with Crohn's disease require surgery at some point, but subsequent relapse is common and continuous medical treatment is usual. IBD are characterized by abdominal pain, diarrhea (often bloody), a variable group of "extra-intestinal" manifestations (such as arthritis, uveitis, skin changes, etc.) and the accumulation of inflammatory cells within the small intestine and colon. Additional symptoms, aspects, manifestations, or signs of IBD include malabsorption of food, altered bowel motility, infection, fever, rectal bleeding, weight loss, signs of malnutrition, perianal disease, abdominal mass, and growth failure, as well as intestinal complications such as stricture, fistulas, toxic megacolon, perforation, and cancer, and including endoscopic findings, such as friability, aphthous and linear ulcers, cobblestone appearance, pseudopolyps, and rectal involvement and, in addition, anti-yeast antibodies. See, e.g., Podolsky (2002) New Engl J. Med. 347:417-429; Hanauer (1996) New Engl. J. Med. 334:841-848; Horwitz and Fisher (2001) New Engl, J. Med. 344: i 846- 1850. IBD affects both children and adults, and has a bimodal age distribution (one peak around 20, and a second around 40). IBD is a chronic, lifelong disease, and is often grouped with "autoimmune" disorders (e.g. rheumatoid arthritis, type I diabetes mellitus, multiple sclerosis, etc.). IBD is found almost exclusively in the industrialized world.

In one embodiment, the subject has a TTC7A deficiency.

As used herein, the term "TTC7A" has its general meaning in the art and refers to the tetratricopeptide repeat domain 7A. A human exemplary nucleic sequence is SEQ ID NO:1. A human exemplary amino acid sequence is SEQ ID NO:2.

SEQ ID NO:1 (NM_020458.2)

1 ggccaggggc gggctccgcag ctggcggcgc cggggcccg ggcggaggct gtggcagcag

61 ctgcagcggc gccggcggcg gcagcgccag gagctgctac agcagaggcg
 gaggttgctc
 121 ctgtacgcgt acgggccgct cggccggagc cgcagcccgg aggcgccggg
 cgggtgcgctg
 5 181 ggagctgctg gtgctgctgc tgctgctgct gccaccctc cgccgccggg
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 241 ccgcccgggc cccggctgcc gtctgcgcc ccgtcgacct cgcccgcgag
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 301 ccaggacgcc gccccgggc gggctctccac ttcttggccg caccttccat
 10 gacagcgccc
 361 gcgagaagat ggctgcgaag ggcgcgcacg gctcctacct gaagggtggag
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 421 agcgctgccg cgccgagggc cactgggacc gcatgccgga gctgggtccg
 cagctgcaga
 15 481 cgctgagcat gcccgccggc ggaggtaca ggcgaggcag cccgagcgca
 gcgttcacct
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 601 tgaaggagaa ccatgccaaa ataaaagact ccatgccttt gctggagaag
 20 aatgagccga
 661 agatgagcga agccaaaaat tatctaagca gtatccttaa ccatgggagg
 ctctcgccac
 721 agtacatgtg tgaggccatg ctgatcctgg gcaaactgca ttacgtggag
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 25 781 gagatgccat cagcatgtac gcacgggccg ggattgatga catgtccatg
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 841 ccctgtatca gatgcggctg ctgtcggagg cttttgtcat caaaggcctc
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 901 gcctacccaa ctccatcgcc tcccgttcc gcctgacaga gagggaggag
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 gagaagacca
 1021 caaataacag cacgtcgagg catctgaaag gctgtcaccg gcttgactat
 gagctcacct
 35 1081 acttcttgga agctgccctc cagagcgcct atgtgaaaaa cctgaagaag
 gggaacatcg
 1141 tgaaggcat gagagagctc cgggaggtgc tgcggactgt ggagaccaa
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 1321 ctttcgccac tcaggccctg cgaaacctc acctctatga aggagacaac
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 45 1381 ccaaggacaa catcgaggaa gccctcctgc tcctcctcat cagcgaatcc
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1501 atgccgcagc catctatgac ctctgagca tcacgttggg cagaagggga
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 15 1921 tgcagacgct ggagagggct cagcagctgg cgcccagtga cccccaggtc
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 cccagagagc

2941 tctgacgacg ctgcagccgc agggagggag gggctggcca gagggagagg
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 3001 cgtgggtcag ggtggggcaa cagtggcatc aggtgcgggg cctcagggaa
 atacatcttt
 5 3061 agtgaacgcc tctgcagctg cagccctcgt tctcttggtt gggccaagag
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 3121 gatttctttg ttggtgcctt gggaaacagt ctgacttgaa ccctaagtgc
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 3181 ttttgtggtg accagacttg ctccccaaga gctgggcagc ggggagcctc
 10 acagctgtcc
 3241 ttcaccctca cccatgcctc tggcttgag tctgggtggg gggttctcac
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 3301 cagcacagta cagacttctg gatctctctc aggtcttgcc cagggcggtc
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 3601 ccgtaggctt tagatcctgt ggttgctaga tccagtcctt tctaataccc
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 3841 cagagcccag cccctctcct gtggctgagc aggcctctgt gtccatgaca
 cctgtcttcc
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 10 agggaggcca
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 aaggaaggat
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 gctaaggctg
 15 4801 ggctaaagcc tggagagggt aggaggaggc aagaggggtc caggcagggc
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 4861 ctctgacctg tccagggcga ccctgaagc ccctgctgcc tctgggcatt
 gctgggagag
 4921 gccaaaggcag gactcacgtc tgaacagaga tcccctcggg cattgctgat
 20 gggccacctt
 4981 cagctgcagg gaagaagcct aggagaggag gcatgggagg gacctgggcc
 ttgttcagat
 5041 tggccacctc tgctgagaag tccataccag tacacccta ataagttatg
 ccacatacca
 25 5101 acgtactgtg gatattataa cctgcattaa aacaactcta aagaacgctg
 ctcatthaaa
 5161 aaaaaaa

SEQ ID NO:2 (NP_065191.2):

30 1 maakgahgsy lkveselerc raeghwdrmp elvrqlqtls mpggggnrrg spsaafthpd
 61 tddfgklilla ealleqclke nhakikdsmp lleknepkms eaknylssil
 nhgrlspqym
 121 ceamlilgkl hyvegsyrda ismyaragid dmsmenkply qmrlseafv
 ikglslrlp
 35 181 nsiasrfrlt ereeevitcf eraswiaqv lqelekttnn stsrhlkgch
 pldyeltyfl
 241 eaalqsayvk nlkknivkg mrelrevlvt vetkatqnfk vmaakhlagv
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 301 wslshplpe fmgkeessfa tqalrkphly egdnlycpkd nieealllll
 40 isesmatrdv
 361 vlsvrpeqee drtvslqnaa aiydllsitl grrgqyvmls ecleramkfa
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 421 alsmvacgks ayavllrec vklrpsdptv plmaakvcig slrwleeaeh
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 dpqvilyvsl
 541 qlalvrqiss ameqlealk vrkddahalh llallfsaqk hhqhaldvvn
 maitehpenf

601 nlmftkvkle qvlkgpeeal vtcraqvlrlw qtllysfsqlg glekdgsfge
 gltmkkqsgm
 661 hltlpdahda dsgsrrassi aasrleeams eltmpssvlk qgpmqlwtll
 eqiwlqaael
 5 721 fmeqqhlkea gfciqeaagl fptshsvlym rgrlaevkgn leeakqlyke
 altvnpdgvr
 781 imhslglmls rlghkslaqk vlrdaverqs tcheawqglg evlqaqggne
 aavdcfltal
 841 eleasspvlp fsiiprel

10

In the context of the invention, the term “TTC7A deficiency” denotes that the cells of the subject or a part thereof have a TTC7A dysfunction, a low or a null expression of tetratricopeptide repeat domain 7A protein. Said deficiency may typically result from a mutation in so that the pre-ARNm is degraded through the NMD (non sense mediated decay) system. Said deficiency may also typically result from a mutation (e.g. the mutation is 211G>A) so that the protein is misfolded and degraded through the proteasome. Said deficiency may also result from a loss of function mutation leading to a dysfunction of the protein. Said deficiency may also result from an epigenetic control of gene expression (e.g. methylation) so that the gene is less expressed in the cells of the subject. Said deficiency may also result from a repression of the TTC7A gene induce by a particular signalling pathway.

Accordingly, in one embodiment, the method of treatment of the present invention comprises a first step for determining whether the subject has a TTC7A deficiency.

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In one embodiment, the first step consists in detecting the mutation that is responsible for the TTC7A deficiency. For example the presence of the mutation is 211G>A mutation may be looked for. One skilled in the art can easily identify a mutation in TTC7A gene. Typically the mutation may be detected by analyzing a TTC7A nucleic acid molecule. In the context of the invention, TTC7A 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 nucleic acid sample may be obtained from any cell source or tissue biopsy. Non-limiting examples of cell sources available include without limitation blood cells, buccal cells, epithelial cells, fibroblasts, or any cells present in a tissue obtained by biopsy. Cells may also be obtained from body fluids, such as blood, plasma, serum, lymph, etc. DNA may be extracted using any methods known in the art, such as described in Sambrook et al., 1989.

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RNA may also be isolated, for instance from tissue biopsy, using standard methods well known to the one skilled in the art such as guanidium thiocyanate-phenol-chloroform extraction. TTC7A 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 mutated site or that enable amplification of a 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 a particular TTC7A mutation. Otherwise, RNA may be reverse-transcribed and amplified, or DNA may be amplified, after which a 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 a mutation in TTC7A sequence. Actually numerous strategies for genotype analysis are available (Antonarakis et al., 1989 ; Cooper et al., 1991 ; Grompe, 1993). Briefly, the nucleic acid molecule may be tested for the presence or absence of a restriction site. When a base substitution mutation creates or abolishes the recognition site of a restriction enzyme, this allows a simple direct PCR test for the mutation. Further strategies include, but are not limited to, direct sequencing, restriction fragment length polymorphism (RFLP) analysis; hybridization with allele-specific oligonucleotides (ASO) that are short synthetic probes which hybridize only to a perfectly matched sequence under suitably stringent hybridization conditions; allele-specific PCR; PCR using mutagenic primers; ligase-PCR, HOT cleavage; denaturing gradient gel electrophoresis (DGGE), temperature denaturing gradient gel electrophoresis (TGGE), single-stranded conformational polymorphism (SSCP) and denaturing high performance liquid chromatography (Kuklin et al., 1997). Direct sequencing may be accomplished by any method, including without limitation chemical sequencing, using the Maxam-Gilbert method ; by enzymatic sequencing, using the Sanger method ; mass spectrometry sequencing ; sequencing using a chip-based technology; and real-time quantitative PCR. Preferably, DNA from a subject is first subjected to amplification by polymerase chain reaction (PCR) using specific amplification primers. However several other methods are available, allowing DNA to be studied independently of PCR, such as the rolling circle amplification (RCA), the Invader™ assay, or oligonucleotide ligation assay (OLA). OLA may be used for revealing base substitution mutations. According to this method, two oligonucleotides are constructed

that hybridize to adjacent sequences in the target nucleic acid, with the join sited at the position of the mutation. DNA ligase will covalently join the two oligonucleotides only if they are perfectly hybridized. Therefore, useful nucleic acid molecules, in particular oligonucleotide probes or primers, according to the present invention include those which specifically hybridize the regions where the mutations are located. Oligonucleotide probes or primers may contain at least 10, 15, 20 or 30 nucleotides. Their length may be shorter than 400, 300, 200 or 100 nucleotides.

The mutation may be also detected at a protein level (e.g. for loss of function mutation) according to any appropriate method known in the art. In particular a biological sample, such as a tissue biopsy, obtained from a subject may be contacted with antibodies specific of a mutated form of TTC7A protein, i.e. antibodies that are capable of distinguishing between a mutated form of TTC7A and the wild-type protein, to determine the presence or absence of a TTC7A 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 for they are more reproducible in the long run. Procedures for raising "polyclonal antibodies" are also well known. Alternatively, binding agents other than antibodies may be used for the purpose of the invention. These may be for instance aptamers, which are a class of molecule that represents an alternative to antibodies in term of molecular recognition. 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.

In one embodiment, the first step consists in determining the expression level of TTC7A gene in the biological sample obtained from the subject. Typically, said biological sample is a blood sample or a PBMC sample or is a tissue sample resulting from a biopsy (e.g. an endoscopical biopsy performed in the colon of the subject). In one embodiment, the first step consist in i) determining the expression level of TTC7A gene, ii) comparing the level determined at i) with a predetermined reference value and iii) concluding that the subject has a TTC7A deficiency when the expression level determined at i) is lower than the

predetermined reference value. Typically the predetermined reference value is the expression level determined in a healthy population of subject (e.g. the mean expression).

One skilled in the art may easily select the appropriate method for determining the expression level of the gene.

5 Typically, the expression level of a gene may be determined by determining the quantity of mRNA. Methods for determining the quantity of mRNA are well known in the art. For example the nucleic acid contained in the samples (e.g., cell or tissue prepared from the patient) is first extracted according to standard methods, for example using lytic enzymes or chemical solutions or extracted by nucleic-acid-binding resins following the manufacturer's
10 instructions. The extracted mRNA is then detected by hybridization (e. g., Northern blot analysis, in situ hybridization) and/or amplification (e.g., RT-PCR).

Other methods of Amplification include ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA) and nucleic acid sequence based amplification (NASBA).

15 Nucleic acids having at least 10 nucleotides and exhibiting sequence complementarity or homology to the mRNA of interest herein find utility as hybridization probes or amplification primers. It is understood that such nucleic acids need not be identical, but are typically at least about 80% identical to the homologous region of comparable size, more preferably 85% identical and even more preferably 90-95% identical. In certain embodiments,
20 it will be advantageous to use nucleic acids in combination with appropriate means, such as a detectable label, for detecting hybridization.

Typically, the nucleic acid probes include one or more labels, for example to permit detection of a target nucleic acid molecule using the disclosed probes. In various applications, such as in situ hybridization procedures, a nucleic acid probe includes a label (e.g., a
25 detectable label). A “detectable label” is a molecule or material that can be used to produce a detectable signal that indicates the presence or concentration of the probe (particularly the bound or hybridized probe) in a sample. Thus, a labeled nucleic acid molecule provides an indicator of the presence or concentration of a target nucleic acid sequence (e.g., genomic target nucleic acid sequence) (to which the labeled uniquely specific nucleic acid molecule is
30 bound or hybridized) in a sample. A label associated with one or more nucleic acid molecules (such as a probe generated by the disclosed methods) can be detected either directly or indirectly. A label can be detected by any known or yet to be discovered mechanism including absorption, emission and/ or scattering of a photon (including radio frequency, microwave frequency, infrared frequency, visible frequency and ultra-violet frequency photons).

Detectable labels include colored, fluorescent, phosphorescent and luminescent molecules and materials, catalysts (such as enzymes) that convert one substance into another substance to provide a detectable difference (such as by converting a colorless substance into a colored substance or vice versa, or by producing a precipitate or increasing sample turbidity), haptens that can be detected by antibody binding interactions, and paramagnetic and magnetic molecules or materials.

Particular examples of detectable labels include fluorescent molecules (or fluorochromes). Numerous fluorochromes are known to those of skill in the art, and can be selected, for example from Life Technologies (formerly Invitrogen), e.g., see, The Handbook—A Guide to Fluorescent Probes and Labeling Technologies). Examples of particular fluorophores that can be attached (for example, chemically conjugated) to a nucleic acid molecule (such as a uniquely specific binding region) are provided in U.S. Pat. No. 5,866, 366 to Nazarenko et al., such as 4-acetamido-4'-isothiocyanatostilbene-2,2' disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS), 4-amino -N- [3 vinylsulfonyl]phenyl]naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, antl1ranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcouluarin (Coumarin 151); cyanosine; 4',6-diarninidino-2-phenylindole (DAPI); 5',5"dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7 -diethylamino -3 - (4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulforlic acid; 5-[dimethylamino] naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6dic11lorotriazin-2-yDarninofluorescein (DTAF), 2'7'dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC Q(RITC); 2',7'-difluorofluorescein (OREGON GREEN®); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives such as pyrene, pyrene butyrate and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine

(R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, rhodamine green, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives. Other suitable fluorophores include thiol-reactive europium chelates which emit at approximately 617 nm (Heyduk and Heyduk, *Analyt. Biochem.* 248:216-27, 1997; *J. Biol. Chem.* 274:3315-22, 1999), as well as GFP, LissamineTM, diethylaminocoumarin, fluorescein chlorotriazinyl, naphthofluorescein, 4,7-dichlororhodamine and xanthene (as described in U.S. Pat. No. 5,800,996 to Lee et al.) and derivatives thereof. Other fluorophores known to those skilled in the art can also be used, for example those available from Life Technologies (Invitrogen; Molecular Probes (Eugene, Oreg.)) and including the ALEXA FLUOR® series of dyes (for example, as described in U.S. Pat. Nos. 5,696,157, 6,130,101 and 6,716,979), the BODIPY series of dyes (dipyrrometheneboron difluoride dyes, for example as described in U.S. Pat. Nos. 4,774,339, 5,187,288, 5,248,782, 5,274,113, 5,338,854, 5,451,663 and 5,433,896), Cascade Blue (an amine reactive derivative of the sulfonated pyrene described in U.S. Pat. No. 5,132,432) and Marina Blue (U.S. Pat. No. 5,830,912).

In addition to the fluorochromes described above, a fluorescent label can be a fluorescent nanoparticle, such as a semiconductor nanocrystal, e.g., a QUANTUM DOTTM (obtained, for example, from Life Technologies (QuantumDot Corp, Invitrogen Nanocrystal Technologies, Eugene, Oreg.); see also, U.S. Pat. Nos. 6,815,064; 6,682,596; and 6,649,138). Semiconductor nanocrystals are microscopic particles having size-dependent optical and/or electrical properties. When semiconductor nanocrystals are illuminated with a primary energy source, a secondary emission of energy occurs of a frequency that corresponds to the bandgap of the semiconductor material used in the semiconductor nanocrystal. This emission can be detected as colored light of a specific wavelength or fluorescence. Semiconductor nanocrystals with different spectral characteristics are described in e.g., U.S. Pat. No. 6,602,671. Semiconductor nanocrystals that can be coupled to a variety of biological molecules (including dNTPs and/or nucleic acids) or substrates by techniques described in, for example, Bruchez et al., *Science* 281 :20132016, 1998; Chan et al., *Science* 281:2016-2018, 1998; and U.S. Pat. No. 6,274,323. Formation of semiconductor nanocrystals of various compositions are disclosed in, e.g., U.S. Pat. Nos. 6,927,069; 6,914,256; 6,855,202; 6,709,929; 6,689,338; 6,500,622; 6,306,736; 6,225,198; 6,207,392; 6,114,038; 6,048,616; 5,990,479; 5,690,807; 5,571,018; 5,505,928; 5,262,357 and in U.S. Patent Publication No.

2003/0165951 as well as PCT Publication No. 99/26299 (published May 27, 1999). Separate populations of semiconductor nanocrystals can be produced that are identifiable based on their different spectral characteristics. For example, semiconductor nanocrystals can be produced that emit light of different colors based on their composition, size or size and composition. For example, quantum dots that emit light at different wavelengths based on size (565 nm, 655 nm, 705 nm, or 800 nm emission wavelengths), which are suitable as fluorescent labels in the probes disclosed herein are available from Life Technologies (Carlsbad, Calif.).

Additional labels include, for example, radioisotopes (such as ^3H), metal chelates such as DOTA and DPTA chelates of radioactive or paramagnetic metal ions like Gd^{3+} , and liposomes.

Detectable labels that can be used with nucleic acid molecules also include enzymes, for example horseradish peroxidase, alkaline phosphatase, acid phosphatase, glucose oxidase, beta-galactosidase, beta-glucuronidase, or beta-lactamase.

Alternatively, an enzyme can be used in a metallographic detection scheme. For example, silver in situ hybridization (SISH) procedures involve metallographic detection schemes for identification and localization of a hybridized genomic target nucleic acid sequence. Metallographic detection methods include using an enzyme, such as alkaline phosphatase, in combination with a water-soluble metal ion and a redox-inactive substrate of the enzyme. The substrate is converted to a redox-active agent by the enzyme, and the redoxactive agent reduces the metal ion, causing it to form a detectable precipitate. (See, for example, U.S. Patent Application Publication No. 2005/0100976, PCT Publication No. 2005/003777 and U.S. Patent Application Publication No. 2004/0265922). Metallographic detection methods also include using an oxido-reductase enzyme (such as horseradish peroxidase) along with a water soluble metal ion, an oxidizing agent and a reducing agent, again to form a detectable precipitate. (See, for example, U.S. Pat. No. 6,670,113).

Probes made using the disclosed methods can be used for nucleic acid detection, such as ISH procedures (for example, fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH) and silver in situ hybridization (SISH)) or comparative genomic hybridization (CGH).

In situ hybridization (ISH) involves contacting a sample containing target nucleic acid sequence (e.g., genomic target nucleic acid sequence) in the context of a metaphase or interphase chromosome preparation (such as a cell or tissue sample mounted on a slide) with a labeled probe specifically hybridizable or specific for the target nucleic acid sequence (e.g.,

genomic target nucleic acid sequence). The slides are optionally pretreated, e.g., to remove paraffin or other materials that can interfere with uniform hybridization. The sample and the probe are both treated, for example by heating to denature the double stranded nucleic acids. The probe (formulated in a suitable hybridization buffer) and the sample are combined, under
5 conditions and for sufficient time to permit hybridization to occur (typically to reach equilibrium). The chromosome preparation is washed to remove excess probe, and detection of specific labeling of the chromosome target is performed using standard techniques.

For example, a biotinylated probe can be detected using fluorescein-labeled avidin or avidin-alkaline phosphatase. For fluorochrome detection, the fluorochrome can be detected
10 directly, or the samples can be incubated, for example, with fluorescein isothiocyanate (FITC)-conjugated avidin. Amplification of the FITC signal can be effected, if necessary, by incubation with biotin-conjugated goat antiavidin antibodies, washing and a second incubation with FITC-conjugated avidin. For detection by enzyme activity, samples can be incubated, for example, with streptavidin, washed, incubated with biotin-conjugated alkaline
15 phosphatase, washed again and pre-equilibrated (e.g., in alkaline phosphatase (AP) buffer). For a general description of in situ hybridization procedures, see, e.g., U.S. Pat. No. 4,888,278.

Numerous procedures for FISH, CISH, and SISH are known in the art. For example, procedures for performing FISH are described in U.S. Pat. Nos. 5,447,841; 5,472,842; and
20 5,427,932; and for example, in Pir1kel et al., Proc. Natl. Acad. Sci. 83:2934-2938, 1986; Pinkel et al., Proc. Natl. Acad. Sci. 85:9138-9142, 1988; and Lichter et al., Proc. Natl. Acad. Sci. 85:9664-9668, 1988. CISH is described in, e.g., Tanner et al., Am. J. Pathol. 157:1467-1472, 2000 and U.S. Pat. No. 6,942,970. Additional detection methods are provided in U.S. Pat. No. 6,280,929.

Numerous reagents and detection schemes can be employed in conjunction with FISH, CISH, and SISH procedures to improve sensitivity, resolution, or other desirable properties. As discussed above probes labeled with fluorophores (including fluorescent dyes and QUANTUM DOTS®) can be directly optically detected when performing FISH. Alternatively, the probe can be labeled with a nonfluorescent molecule, such as a hapten (such
30 as the following non-limiting examples: biotin, digoxigenin, DNP, and various oxazoles, pyrrazoles, thiazoles, nitroaryls, benzofurazans, triterpenes, ureas, thioureas, rotenones, coumarin, coumarin-based compounds, Podophyllotoxin, Podophyllotoxin-based compounds, and combinations thereof), ligand or other indirectly detectable moiety. Probes labeled with such non-fluorescent molecules (and the target nucleic acid sequences to which

they bind) can then be detected by contacting the sample (e.g., the cell or tissue sample to which the probe is bound) with a labeled detection reagent, such as an antibody (or receptor, or other specific binding partner) specific for the chosen hapten or ligand. The detection reagent can be labeled with a fluorophore (e.g., QUANTUM DOT®) or with another indirectly detectable moiety, or can be contacted with one or more additional specific binding agents (e.g., secondary or specific antibodies), which can be labeled with a fluorophore.

In other examples, the probe, or specific binding agent (such as an antibody, e.g., a primary antibody, receptor or other binding agent) is labeled with an enzyme that is capable of converting a fluorogenic or chromogenic composition into a detectable fluorescent, colored or otherwise detectable signal (e.g., as in deposition of detectable metal particles in SISH). As indicated above, the enzyme can be attached directly or indirectly via a linker to the relevant probe or detection reagent. Examples of suitable reagents (e.g., binding reagents) and chemistries (e.g., linker and attachment chemistries) are described in U.S. Patent Application Publication Nos. 2006/0246524; 2006/0246523, and 2007/ 01 17153.

It will be appreciated by those of skill in the art that by appropriately selecting labelled probe-specific binding agent pairs, multiplex detection schemes can be produced to facilitate detection of multiple target nucleic acid sequences (e.g., genomic target nucleic acid sequences) in a single assay (e.g., on a single cell or tissue sample or on more than one cell or tissue sample). For example, a first probe that corresponds to a first target sequence can be labelled with a first hapten, such as biotin, while a second probe that corresponds to a second target sequence can be labelled with a second hapten, such as DNP. Following exposure of the sample to the probes, the bound probes can be detected by contacting the sample with a first specific binding agent (in this case avidin labelled with a first fluorophore, for example, a first spectrally distinct QUANTUM DOT®, e.g., that emits at 585 nm) and a second specific binding agent (in this case an anti-DNP antibody, or antibody fragment, labelled with a second fluorophore (for example, a second spectrally distinct QUANTUM DOT®, e.g., that emits at 705 nm). Additional probes/binding agent pairs can be added to the multiplex detection scheme using other spectrally distinct fluorophores. Numerous variations of direct, and indirect (one step, two step or more) can be envisioned, all of which are suitable in the context of the disclosed probes and assays.

Probes typically comprise single-stranded nucleic acids of between 10 to 1000 nucleotides in length, for instance of between 10 and 800, more preferably of between 15 and 700, typically of between 20 and 500. Primers typically are shorter single-stranded nucleic acids, of between 10 to 25 nucleotides in length, designed to perfectly or almost perfectly

match a nucleic acid of interest, to be amplified. The probes and primers are “specific” to the nucleic acids they hybridize to, i.e. they preferably hybridize under high stringency hybridization conditions (corresponding to the highest melting temperature T_m , e.g., 50 % formamide, 5x or 6x SCC. SCC is a 0.15 M NaCl, 0.015 M Na-citrate).

5 The nucleic acid primers or probes used in the above amplification and detection method may be assembled as a kit. Such a kit includes consensus primers and molecular probes. A preferred kit also includes the components necessary to determine if amplification has occurred. The kit may also include, for example, PCR buffers and enzymes; positive control sequences, reaction control primers; and instructions for amplifying and detecting the
10 specific sequences.

 In a particular embodiment, the methods of the invention comprise the steps of providing total RNAs extracted from cumulus cells and subjecting the RNAs to amplification and hybridization to specific probes, more particularly by means of a quantitative or semi-quantitative RT-PCR.

15 In another preferred embodiment, the expression level is determined by DNA chip analysis. Such DNA chip or nucleic acid microarray consists of different nucleic acid probes that are chemically attached to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. A microchip may be constituted of polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, or
20 nitrocellulose. Probes comprise nucleic acids such as cDNAs or oligonucleotides that may be about 10 to about 60 base pairs. To determine the expression level, a sample from a test subject, optionally first subjected to a reverse transcription, is labelled and contacted with the microarray in hybridization conditions, leading to the formation of complexes between target nucleic acids that are complementary to probe sequences attached to the microarray surface.
25 The labelled hybridized complexes are then detected and can be quantified or semi-quantified. Labelling may be achieved by various methods, e.g. by using radioactive or fluorescent labelling. Many variants of the microarray hybridization technology are available to the man skilled in the art (see e.g. the review by Hoheisel, Nature Reviews, Genetics, 2006, 7:200-210).

30 Expression level of a gene may be expressed as absolute expression level or normalized expression level. Typically, expression levels are normalized by correcting the absolute expression level of a gene by comparing its expression to the expression of a gene that is not a relevant, e.g., a housekeeping gene that is constitutively expressed. Suitable genes for normalization include housekeeping genes such as the actin gene ACTB, ribosomal 18S

gene, GUSB, PGK1 and TFRC. This normalization allows the comparison of the expression level in one sample, e.g., a patient sample, to another sample, or between samples from different sources.

5 Other methods for determining the expression level of a gene include the determination of the quantity of proteins encoded by said genes.

Such methods comprise contacting the sample with a binding partner capable of selectively interacting with a marker protein present in the sample. The binding partner is generally an antibody that may be polyclonal or monoclonal, preferably monoclonal. The binding partner may also be an aptamer.

10 The presence of the protein can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation,
15 etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound protein in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid
20 supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

25 More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with an antibody against the protein to be tested. A biological sample containing or suspected of containing the marker protein is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate (s) can be washed to remove unbound moieties and a detectably labeled secondary
30 binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

Alternatively an immunohistochemistry (IHC) method may be preferred. IHC specifically provides a method of detecting targets in a sample or tissue specimen in situ. The

overall cellular integrity of the sample is maintained in IHC, thus allowing detection of both the presence and location of the targets of interest. Typically a sample is fixed with formalin, embedded in paraffin and cut into sections for staining and subsequent inspection by light microscopy. Current methods of IHC use either direct labeling or secondary antibody-based or hapten-based labeling. Examples of known IHC systems include, for example, EnVision(TM) (DakoCytomation), Powervision(R) (Immunovision, Springdale, AZ), the NBA(TM) kit (Zymed Laboratories Inc., South San Francisco, CA), HistoFine(R) (Nichirei Corp, Tokyo, Japan).

In particular embodiment, a tissue section (e.g. a sample comprising cumulus cells) may be mounted on a slide or other support after incubation with antibodies directed against the proteins encoded by the genes of interest. Then, microscopic inspections in the sample mounted on a suitable solid support may be performed. For the production of photomicrographs, sections comprising samples may be mounted on a glass slide or other planar support, to highlight by selective staining the presence of the proteins of interest.

Therefore IHC samples may include, for instance: (a) preparations comprising cumulus cells (b) fixed and embedded said cells and (c) detecting the proteins of interest in said cells samples. In some embodiments, an IHC staining procedure may comprise steps such as: cutting and trimming tissue, fixation, dehydration, paraffin infiltration, cutting in thin sections, mounting onto glass slides, baking, deparaffination, rehydration, antigen retrieval, blocking steps, applying primary antibodies, washing, applying secondary antibodies (optionally coupled to a suitable detectable label), washing, counter staining, and microscopic examination.

In one embodiment the TTC7A deficiency is detected in the subject by looking for a molecular or functional consequence of said deficiency.

Typically, said molecular consequence is an increased ROCK activity in the cells of the patients (e.g. the peripheral blood mononuclear cells (PBMC) or biopsy sample obtained from the subject). For example, the expression of the phosphorylated RhoA effectors molecule such as p-ERM and p-MLC may be determined such as described in the EXAMPLE, and compared to a predetermined reference value, wherein when the determined level is higher than the predetermined reference value it is concluded that the subject has an increased ROCK activity and thus has a TTC7A deficiency.

Typically said functional consequences include but are not limited to a cytoskeletal polarization defect of the lymphocytes or of the intestinal epithelial cells, an increased

proliferation and cell cycle progression of the lymphocytes or a growth impairment of gut organoids prepared from a biopsy sample obtained from the subject. Said functional consequences may be detected as described in the EXAMPLE. For example, a biopsy sample (e.g. an endoscopic biopsy from the colon of the subject) may be analysed for analysing the structure of the gut, in particular the polarization of the epithelial cells. For example, the polarization of the cells may be analysed by using antibodies or aptamers directed against proteins allowing the visualisation of basal, apical, or lateral membranes. In particular said proteins include but are not limited to actin, integrins, laminin, ZO-1 (zonula occludens) protein, villin, or alkaline phosphatase. Additionally, the formation of vacuoles in the cellular tissue may be detected by using the same antibodies or aptamers directed against actin, integrins, laminin, ZO-1 (zonula occludens) protein, villin, or alkaline phosphatase. Multilayered regions in the tissue may also be detected.

As used herein the term “RhoA kinase” or “ROCK” has its general meaning in the art. ROCK is a member of the serine-threonine protein kinase family. ROCK exists in two isoforms, ROCK1 and ROCK2 (T. Ishizaki et al, EMBO J., 1996, 15, 1885-1893). ROCK has been identified as an effector molecule of RhoA, a small GTP-binding protein (G protein) that plays a key role in multiple cellular signaling pathways.

As used herein, the term “ROCK inhibitor” refers to a natural or synthetic compound which inhibits ROCK1, and/or ROCK2 activity. In a particular embodiment the inhibitor is selective. The selective ROCK inhibiting compounds are not limited to a particular manner of selective ROCK inhibition. For example, in some embodiments, one or more of the selective ROCK inhibiting compounds selectively inhibit ROCK1 activity over ROCK2 activity. For example, in some embodiments, one or more of the selective ROCK inhibiting compounds selectively inhibit ROCK2 activity over ROCK1 activity. Moreover, in some embodiments, one or more of the selective ROCK inhibiting compounds selectively inhibit both ROCK1 activity and ROCK2 activity with similar capability.

ROCK inhibitors are well known in the art. For example, isoquinoline derivatives, especially fasudil, are typical ROCK inhibitors. Fasudil (hexahydro-1-(5-isoquinolylsulfonyl)-1H-1,4-diazepine), also named as HA-1077, is an isoquinoline sulfonamide derivative and the only clinically available ROCK inhibitor codeveloped by Asahi Kasei of Japan and Department of Pharmacology of Nagoya University.

Hydroxyfasudil is an active metabolite of fasudil in vivo, which has higher affinity to ROCK than Fasudil. Another isoquinoline derivative, H-1152P, is optimized on the basis of fasudil. Through competitively binding to the ATP binding pocket, Y-27632, another type of ROCK inhibitor, inhibits both ROCK1 and ROCK2. Optimization of these compounds leads to a more potent ROCK inhibitor, Y-39983, which is benefit for the treatment of the glaucoma (Kubo T, Yamaguchi A, Iwata N, The therapeutic effects of Rho-ROCK inhibitors on CNS disorders. *Ther Clin Risk Manag* 2008;4(3):605-15). SLx-2119, a ROCK2-specific inhibitor, has recently been developed (Boerma M, Fu Q, Wang J, Comparative gene expression profiling in three primary human cell lines after treatment with a novel inhibitor of Rho kinase or atorvastatin. *Blood Coagul Fibrinolysis* 2008;19(7):709-18). A series of fasudil analogs were synthesized and their selectivity and inhibitory activity against ROCK were evaluated (Sato N, Toyohira Y, Itoh H, Stimulation of norepinephrine transporter function by fasudil, a Rho kinase inhibitor, in cultured bovine adrenal medullary cells. *Naunyn Schmiedebergs Arch Pharmacol* 2012;385(9):921-31; Nakabayashi S, Nagaoka T, Tani T, Retinal arteriolar responses to acute severe elevation in systemic blood pressure in cats: role of endothelium-derived factors. *Exp Eye Res* 2012;103:63-70; Sun X, Minohara M, Kikuchi H, The selective Rho-kinase inhibitor Fasudil is protective and therapeutic in experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2006;180(1-2):126-34; Yu JZ, Ding J, Ma CG, Therapeutic potential of experimental autoimmune encephalomyelitis by Fasudil, a Rho kinase inhibitor. *J Neurosci Res* 2010;88(8):1664-72; Hou SW, Liu CY, Li YH, Fasudil ameliorates disease progression in experimental autoimmune encephalomyelitis, acting possibly through antiinflammatory effect. *CNS Neurosci Ther* 2012;18(11):909-17; LoGrasso PV, Feng Y. Rho kinase (ROCK) inhibitors and their application to inflammatory disorders. *Curr Top Med Chem* 2009;9(8):704-23; Engel J Jr. A proposed diagnostic scheme for people with epileptic seizures and with epilepsy: report of the ILAE Task Force on Classification and Terminology. *Epilepsia* 2001;42(6):796-803; Fisher RS, van Emde Boas W, Blume W, Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia* 2005;46(4):470-2. Inan S, Buyukafsar K. Antiepileptic effects of two Rho-kinase inhibitors, Y-27632 and fasudil, in mice. *Br J Pharmacol* 2008;155(1):44-51; Meihui Chen, Anmin Liu, Ying Ouyang, Yingjuan Huang, Xiaojuan Chao, Rongbiao Pi Fasudil and its analogs: a new powerful weapon in the long war against central nervous system disorders? *Expert Opinion on Investigational Drugs* Apr 2013, Vol. 22, No. 4, Pages 537-550.). Other examples of ROCK inhibitors include those described in the international patent publications WO98/06433,

WO00/09162, WO00/78351, WO01/17562, WO02/076976, EP1256574, WO02/100833, WO03/082808, WO2004/009555, WO2004/024717, WO2004/108724, WO2005/003101, WO2005/035501, WO2005/035503, WO2005/035506, WO2005/058891, WO2005/074642, WO2005/074643, WO2005/Q80934, WO2005/082367, WO2005/082890, WO2005/097790, WO2005/100342, WO2005/103050, WO2005/105780, WO2005/108397, WO2006/044753, WO2006/051311, WO2006/057270, WO2006/058120, WO2006/072792WO2011107608A1, and WO2007026920A2.

In one embodiment the ROCK inhibitor is an inhibitor of ROCK expression. An “inhibitor of expression” refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene.

In a preferred embodiment of the invention, said inhibitor of gene expression is a siRNA, an antisense oligonucleotide or a ribozyme.

Inhibitors of gene expression for use in the present invention may be based on antisense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of the targeted mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of the targeted protein (e.g. ROCK), and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding the target protein can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

Small inhibitory RNAs (siRNAs) can also function as inhibitors of gene expression for use in the present invention. Gene expression can be reduced by contacting the tumor, subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that gene expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

Ribozymes can also function as inhibitors of gene expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of the targeted mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as inhibitors of gene expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramidite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides siRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to the cells. Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the the antisense oligonucleotide siRNA or

ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma
5 viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include
10 retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered
15 retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells
20 with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman C.O., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana Press, Inc., Clifton, N.J., 1991).

Preferred viruses for certain applications are the adeno-viruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human
25 use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hematopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into
30 human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that

the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g., SANBROOK et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

Typically the ROCK inhibitor of the invention is administered to the patient in a therapeutically effective amount.

By a "therapeutically effective amount" of the ROCK inhibitor of the invention as above described is meant a sufficient amount of the compound. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide

employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

The ROCK inhibitor of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms. Galenic adaptations may be done for specific delivery in the small intestine or colon.

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry,

especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol ;
5 and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

10 Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

15 The ROCK inhibitor of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from
20 inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and
25 the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifusoluble agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In
30 many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The ROCK inhibitor of the invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

In addition to the compounds of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations ; time release capsules ; and any other form currently used.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:**Figure 1: ROCK inhibition normalizes apicobasal polarity in patient organoids.**

5 A-E control- (A, D) and patient-derived ileum organoids (B, C, E) cultured for five days in the presence or absence of Y-27632. Immunochemical staining of $\alpha 6$ -integrin (green) and F-actin (red). Nuclei were stained with DAPI. Aberrant polarity is characterized by a multilayered epithelial structure and F-actin deposition around vacuoles (red arrows). Inverted polarity with F-actin deposition on the outer side and intra-epithelial $\alpha 6$ -integrin staining (green arrows). All scale bars are 50 μ m. F: Quantification of apicobasal polarity after five days of growth in the presence or absence of Y-27632. We scored the percentages of organoids with normal polarity (an epithelial monolayer with $\alpha 6$ -integrin outside and F-actin on the luminal side (light grey bars), aberrant polarity (multilayered epithelium with disturbed $\alpha 6$ -integrin and/or F-actin deposition, dark grey) and inverted polarity (F-actin facing outwards, $\alpha 6$ -integrin clustered inside, black bars). In all, 50 organoids were counted for each condition.

EXAMPLE 1:**20 SNP linkage analysis and sequence analysis.**

Genomic DNA was isolated by phenol/chloroform extraction. RNA was isolated using an RNeasy Mini Kit (Qiagen). A genome-wide linkage study was performed using AffymetrixGeneChip Mapping 250 K NspI, as described elsewhere¹. Homozygous regions were detected using a parametric, SNP-based linkage analysis (MERLIN software, version 25 1.1.1). Genomic DNA and cDNA were amplified, sequenced and analyzed on an ABI Prism 3700 system (using a BigDye Terminator sequencing kit from Applied Biosystems). Exome sequencing analysis of a DNA sample from an ELA patient was performed as described elsewhere².

30 Cell cultures.

Lymphoblasts were obtained by incubating PBMCs for 72 hours with phytohemagglutinin (PHA, 1:400 dilution; Sigma-Aldrich) and IL-2 (50 IU/ml; PeproTech) in Panserin medium (Biotech GmbH) supplemented with 10% human AB serum (Etablissement Français du Sang). We then added more IL-2 (100 IU/ml) and cultured the cells for 6 to 7

days. The method for establishing organoid cultures from gut biopsies has been reported previously³.

Detection of apoptotic cells with annexin-V.

5 The proportion of viable cells was determined by using the Annexin-V PE Apoptosis Detection Kit I (BD Biosciences). Cell fluorescence was measured on a FACSCanto (BD Biosciences).

Gene expression analysis

10 Total RNA was isolated from the different cell types (using an RNeasy Mini Kit), depleted of genomic DNA and then reverse-transcribed into cDNA using Quantitect (Qiagen). Quantitative PCR was performed on cDNA using TaqMan PCR Master Mix (Applied Biosystems) and TTC7A- and actin-specific primers. Fluorescence during PCR and subsequent dissociation was measured in triplicate on an ABI 7900 cycler and analyzed using
15 Sequence Detection Systems software (version 2.2.2, Applied Biosystems).

Protein blotting

 Lymphoblasts, primary fibroblasts and LBLs were lysed in radio-immunoprecipitation/glycerol buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton
20 X-100, 2 mM EDTA, 1% sodium deoxycholate) supplemented with protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma-Aldrich). Cell extracts were separated by SDS-PAGE, blotted and then stained with the appropriate specific antibodies: anti-ROCK, anti-p-ERM, anti-Ezrin and anti-p-MLC (from Cell Signaling), anti- α -tubulin (from Sigma) and polyclonal antibody against human TTC7A prepared by immunizing
25 rabbits with a TTC7A-GST fusion protein and then using affinity purification (from AgroBio). After staining with a horseradish- peroxidase-conjugated secondary antibody, the immunoblot was developed with an Enhanced Chemiluminescence Detection Kit (Amersham).

Flow cytometry.

30 Antibodies were purchased from eBioscience or BD Biosciences; standard flow cytometry methods were used for staining cell surface markers. Blood lymphocytes were stained with PeCy7-conjugated anti-CCR7, PE-conjugated anti-CXCR4, APC-conjugated anti-CD11a, FITC-conjugated anti-CD18 (all from BD Biosciences) and AL-57 antibody⁴.

Data were collected on a FACSCanto system and analyzed with FlowJo8.8.4 software (TreeStar).

Proliferation assays.

5 PBMCs from patients and controls were purified by density gradient centrifugation and cultured for three days with PHA (5 µg/mL) and for five days with tetanus toxoid antigen (0.125 lf/ml; Statens Serum Institute). [³H] thymidine was added for the last 18 hours. Cell proliferation was determined as the cpm of [³H] thymidine incorporated. Proliferation was monitored by labeling T cells with 10 µM CFSE (Invitrogen) prior to stimulation with anti-
10 CD3/CD28 beads (Invitrogen), in accordance with the manufacturer's instructions.

Cell cycle analysis.

Cell cycle analyses were performed by measuring the incorporation of the nucleoside analogue EdU into newly synthesized DNA two or five days after stimulation with anti-
15 CD3/CD28 beads, PHA or PMA/ionomycin, in accordance with the manufacturer's instructions (Click-itEdU, Invitrogen). EdU incorporation was measured according to the abundance of fluorescent product and analyzed on a FACSCanto system with FlowJo 8.8.4 software.

Lymphocyte-polarization assay.

20 Day 7 lymphoblasts in suspension were stimulated with 100 nM CCL21 and immediately plated on poly-L-lysine-coated glass coverslips (Sigma-Aldrich) for incubation at 37°C. After 30 min, T cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then permeabilized with 0.05% saponin in PBS. Immunostaining was
25 performed with Alexa Fluor 488-conjugated anti-rabbit phalloidin (Invitrogen). Confocal fluorescence images were acquired with a LSM 700 microscope (Carl Zeiss). The intensity of phalloidin contact zones was quantified using ImageJ 1.4.3.67 Launcher Symmetry Software (NIH).

Chemotaxis assay.

30 Chemotaxis assays were performed using Transwell chambers (5 µm pore size) with 200 µl of cell suspension (1×10^6 cells/ml) in the top chamber and 600 ng/ml CCL21 or 1 µg/ml CXCL12 in the bottom chamber. After 3h at 37°C in 5% CO₂, the proportion of migrated cells was calculated by flow cytometry.

Cell adhesion and proliferation assays using xCELLigence technology (Roche). For the adhesion assay, the E-plate wells were coated with poly-L-lysine in the presence or absence of ICAM-1 or VCAM-1. 50 µl of cell culture medium was added to the E-plate wells. The background signal was then measured. Meanwhile, PBMCs were resuspended in the appropriate cell culture medium, adjusted to 200,000 cells per 100 µl and then incubated for 1 hour with a ROCK inhibitor (10 µM Y27632). The cells were added to wells containing 50 µl of medium. Adhesion of PBMCs was automatically monitored every 10 s over 4 hours. For the proliferation assay, 1500 fibroblasts from an ELA patient were incubated in the presence or absence of 10 µM Y27632 for 1 hour and then added directly to E-plate wells containing 50 µl of medium. After a 20 min incubation at room temperature, the E-plate was placed in the cell incubator. Lastly, cell proliferation was monitored every 10 min for up to 147 h. Electrical impedance was measured by the xCELLigence system's integrated software and expressed as a cell index.

Rescue experiments.

For rescue experiments, full-length TTC7A cDNA was subcloned into the EcoR1 and BamH1 restriction sites of the pEGFP-C1 plasmid (Clontech) using the following primers: TTC7A forward 5'-AGG AAT TCC ATG GCT GCG AAG GGC GCG CAC GGC-3' (SEQ ID NO:3) ; reverse 5'-GGG GAT CCC TCA GAG CTC TCT GGG GAT GAT GG -3' (SEQ ID NO: 4). Empty vector was used as a control. Primary fibroblasts (70% confluent) were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and Western blotting was performed 24h post-transfection.

Statistical analysis.

Analyses were performed with Prism software (version 4 for Macintosh, GraphPad Inc.). Statistical hypotheses were analyzed using T-tests.

Accession codes.

The sequences referenced in the present study are available from GenBank under the following accession codes: TTC7A: NM_020458.2

EXAMPLE 2:

Methods:

Study Design

Clinical information and blood samples were collected from the patients, their relatives and controls, all of whom had given then prior, informed consent to participation in the study. Genetic studies and data collection procedures were approved by the local investigational review board and the French Advisory Committee on Data Processing in Medical Research. Genotyping, sequence analysis and gene expression analysis were performed as described elsewhere²⁰ and are detailed in EXAMPLE 1.

Protein studies and functional analysis

Peripheral blood mononuclear cells were isolated from blood samples taken from enteropathy lymphocytopenia and alopecia (ELA) patients and controls. B lymphoblast cell lines transformed with Epstein-Barr virus and fibroblast cell lines were established as described elsewhere²⁰. Cell proliferation, adhesion and migration and cell-cycle progression assays, Western blotting and the antibodies used to detect TTC7A, ROCK, phosphorylated ezrin/radixin/moesin (p-ERM), phosphorylated myosin light chain (p-MLC) and actin are described in EXAMPLE 1. Additional information on other assays, reagents and statistical techniques is also provided in EXAMPLE 1.

Results

Manifestations of the ELA syndrome

Thirteen members of a large, consanguineous French kindred displayed enteropathy associated with a progressive T, B and NK cell combined immunodeficiency. The family members' pedigree and clinical features are shown in the Table 1 and the Supplementary Table. All patients had developed IBD within the first days to months of life and displayed recurrent, severe diarrhea, sometimes associated with rectal bleeding, and weight loss. Enteral or total parenteral nutrition were repeatedly required in nine patients (Table 1). Enteropathy was fatal in one patient (E3). The severity of gastrointestinal manifestations gradually decreased with age. Five patients developed alopecia and four developed onychopathy. With age, four patients also developed autoimmune manifestations, autoimmune hepatitis in B4, autoimmune hemolytic anemia in A3, psoriasis and type I diabetes in B4 and autoimmune thyroiditis in C1. Two patients (N3 and F5) underwent hematopoietic stem cell transplantation (HSCT). N3 died soon after, whereas F5 succumbed 9 months later from

infection. It is noteworthy that the gastrointestinal disease in F5 was not resolved by HSCT, despite full donor chimerism and the absence of skin or liver graft-versus-host manifestations. This fact suggested the presence of an intrinsic gut disease.

Immunohistopathologic analysis of the digestive tract revealed major lesions in the antrum and colon in all patients. In contrast, the small intestine was relatively unaffected. The antrum was characterized by changes in the surface and foveolar epithelium that were exactly the same in biopsies from an 8 month-old patient (O3) and a 50 year-old patient (C1). The disorganized architecture of the epithelium consisted in tufting, loss of apical mucin, abnormal, pseudostratified cell organization and enlarged, hyperchromatic nuclei - all of which are characteristics of both degenerative and regenerative processes. The lamina propria contained an inflammatory infiltrate with mononuclear cells and a high eosinophil count. Antrum sections were *Helicobacter pylori*-negative. Colon lesions were severe and widespread. The epithelium was dedifferentiated and little mucus-secreting tissue remained. A combination of glandular necrosis, cell apoptosis and crypt abscesses in various sites was also noticed. The lamina propria was infiltrated by polymorphous inflammatory cells including mononuclear cells (CD4, CD8 T cells, B cells and macrophages) and polymorphonuclear (PMN) cells notably eosinophils.

The patients showed increased susceptibility to infections. From the first year of life onwards, six of the eight patients not receiving pre-emptive IgG replacement therapy displayed lower and/or upper respiratory tract infections, which eventually resulted in bronchiectasis in patient C1. IBD was combined with evidence of protracted CMV infection on the basis of PCR analyses of blood and gut samples in six out of thirteen cases. Diarrhea was not improved by anti-CMV treatment.

Immunological studies (Supplementary Table 1) revealed normal PMN cell and monocyte counts and progressive T, B and NK cell lymphocytopenia. TCR α/β and γ/δ cells were equally affected. CD4 lymphocytopenia, and notably naïve T cell lymphocytopenia predominated. The CD8 T cell counts were close to those of age-matched controls. However, in older patients, naïve CD8 T cell counts decreased while effector-memory phenotype CD8 T cells were overrepresented (Supplementary Table 1). Counts of regulatory T cells, as identified by Foxp3⁺CD25^{hi}CD4⁺ co-expression, were low (Supplementary Table 1). NK cell counts were profoundly low in most patients (Supplementary Table 1). The patients' B-cell counts were close to normal in early-life but then declined. Memory (CD27⁺) B cell counts were always very low (Supplementary Table 1). Hypogammaglobulinemia (IgM, IgG and IgA) was noted, requiring Ig replacement therapy (Supplementary Table 1). Antibody

responses could not be evaluated. Most of the patients had normal T cell proliferation in response to mitogens but low or no proliferation in response to antigens (including to CMV in chronically infected patients, Supplementary Table 1). It is noteworthy that a proportion of circulating T cells expressed HLA-DR, independently of CMV infection status, suggesting *in vivo* T cell activation.

Identification of a mutation in *TTC7A*

With a view to identifying the gene underlying this ELA syndrome and assuming an autosomal recessive inheritance, we performed genome-wide homozygosity mapping with a single nucleotide polymorphism (SNP) array. This mapping revealed a common 4 Mb region on chromosome 2p21. Of the region's 23 genes, *TTC7A* appeared to be the most plausible candidate because (i) *TTC7A* is known to be expressed in lymphoid tissues and (ii) *TTC7A* deficiency induces forestomach papillomas in the mouse. Evidence of linkage to 2p21 was supported by a logarithm of the odds ratio (LOD score) of 7. Sequencing of the *TTC7A* gene's 20 coding exons revealed a homozygous missense mutation in exon 2 in all 13 patients. The mutation prompts a 211G>A nucleotide change and an E71K amino acid substitution. The latter glutamate acid residue is highly conserved in all species, and is predicted to be "probably damaging" (score 0.997) by PolyPhen2, "deleterious" by SIFT (score:0.00) and "disease causing" (p-value:1.0) by MutationTaster. Exome sequencing in one of the patients also identified this substitution and showed that *TTC7A* was the only mutated gene within the defined genetic linkage region on chromosome 2p21. All tested parents were heterozygous for this mutation. The 211G>A mutation was not found in control subjects, in-house exome sequencing data and any of the public databases (including the dbSNP129 and 1000 Genomes datasets). Heterozygous individuals were asymptomatic.

We next analyzed *TTC7A* transcript expression by using quantitative real-time PCR. The *TTC7A* missense mutation did appear to have any influence on the steady-state level of mRNA *TTC7A*. *TTC7A* was found to be ubiquitously present in a panel of cDNAs from a wide range of normal haematopoietic and non-haematopoietic tissues, with the highest levels in pancreas and thymus. Expression in the intestine was low but detectable. No significant difference in *TTC7A* expression levels was observed between T cell subsets.

Defective expression of *TTC7A* protein

We assessed the expression of the *TTC7A* protein in cells obtained from O3, F5 and N3 patients by using a rabbit polyclonal antibody generated against the human *TTC7A*

protein. TTC7A protein was not found in lymphoblastoid B cell lines (LBLs) nor in T lymphoblasts and was barely detectable in fibroblasts from ELA patients relative to control cells. These data indicate that the ELA syndrome was associated with the partial loss of TTC7A protein expression caused by a loss-of-function mutation in the *TTC7A* gene.

5 TTC7A belongs to a large family of tetratricopeptide repeat (TPR)-containing proteins. The TPR domain is a 34 amino-acid consensus motif that is found in varying numbers of tandem repeats in different proteins^{21,22}. Sequence analysis of TTC7A indicates that the E71 is highly conserved among TCC7 proteins and might be included in a degenerated TPR repeat. The impaired cellular expression observed for the E71K mutant
10 suggests that E71 is required for correct folding of TTC7A or an essential, stabilizing interaction with a partner.

Molecular consequences of TTC7A deficiency

Given the perturbation of the RhoA signalling pathway observed in the gut epithelial
15 cells of patients with null mutations in *TTC7A*, we sought to determine whether a similar perturbation could also be detected in TTC7A-deficient lymphocytes from ELA patients. We first analyzed the protein expression of RhoA kinase (ROCK) and the phosphorylation status of RhoA effector molecules. Whereas the ROCK protein expression was not modified, the expression of p-ERM and p-MLC was much higher in the patients' peripheral blood
20 mononuclear cells (PBMCs) or LBLs than in control cells. ROCK is known to enhance myosin II activity by phosphorylating the regulatory MLC and inhibiting MLC phosphatase²³. Our findings thus reflect increased ROCK activity in the patients' cells.

Functional consequences of TTC7A deficiency

Increased cytoskeletal polarization profile of TTC7A-deficient lymphocytes

We then evaluated the consequences of RhoA signalling dysregulation on lymphocyte functionality, given that p-ERM is known to regulate the cytoskeletal rearrangements that underlie lymphocyte polarization and migration²³. Following CCL21 stimulation, a switch
30 from a spherical shape to a polarized shape was observed in both control and TTC7A-deficient T cells. However, the intensity of F-actin accumulation at the cell-cell interface was significantly greater for TTC7A-deficient T cells than for controls, albeit with no change in the contact size. Similar observations were made with activated TTC7A-deficient T cells in the absence of CCL21 stimulation, suggesting constitutive changes in the cytoskeletal anchor.

Since polarization is required for cell migration, we also analyzed the migratory profile of TTC7A-deficient T cells. Impaired migration of TTC7A-deficient lymphocytes was observed in response to CCL21 and CXCL12, and was associated with a concomitant decrease in expression of the corresponding chemokine receptors CCR7 and CXCR4.

We also assessed the T cells' adhesion capacity of T cells. Adhesion to ICAM-1, and to a lesser extent VCAM-1, was consistently higher for the ELA patients' T cells than for controls' T cells. This difference in the patients' cells was associated with an increase of LFA-1 integrin expression. Expression of VLA-4 integrin (VCAM-1 ligand) was similar in cells from patients and controls. Expression of the high-affinity, open conformation of LFA-1 by activated or non activated patients' cells was not increased relative to controls (data not shown). Overall, these data suggest that the increased adhesion of TTC7A-deficient cells results from greater expression of B2-integrin and potentially higher affinity of VLA4 for VCAM1, related to a modified cytoskeletal anchor.

Increased proliferation and cell cycle progression of TTC7A-deficient cells

TTC7A-deficient patients had CD4 T cell lymphocytopenia associated with preserved T cell proliferation to mitogens but markedly impaired T cell proliferation to antigens. Further assessment of the proliferation potential of TTC7A deficient T cells was investigated in response to anti-CD3/CD28 stimulation, by using a sequential CFSE dilution assay. The TTC7A-deficient T cells exhibited a significant increase in T cell proliferation between days 1 and 3. Accordingly, a high fraction of patient's T cells were in the S phase at day 2 of stimulation (as measured by EdU incorporation) and a corresponding lower proportion of cells in G1 phase, when compared with controls. Similar results were obtained when other stimuli were used (PHA or PMA/Ionomycin). It is noteworthy that no difference in proliferation, cell cycle progression nor changes in cell death, (evaluated by annexin-V-positive cells) were observed, when comparing control T cells and the patients' T cells at later time points (days 5 to 8). Taken as a whole, these data suggest that the TTC7A protein is involved in the control of cell cycle progression and T cell proliferation.

Inhibition of ROCK rescues the TTC7A-deficient phenotype in T cells

To test whether inhibition of ROCK could rescue the TTC7A-deficient phenotype, PBMCs from ELA patient T cells were incubated in the presence of the synthetic ROCK inhibitor Y27632. The patients' PBMCs indeed showed expression of p-ERM and p-MLC at much the same level as control PBMCs. Incubation of control PBMCs with Y27632 in the

same setting did not have a significant effect on p-ERM or p-MLC expression. Addition of Y27632 inhibited the hyperproliferation of TTC7A-deficient T cells observed at day 3 after anti-CD3/CD28 stimulation. Similarly, a one-hour pre-treatment of TTC7A-deficient fibroblasts with Y27632 reduced cell over-proliferation, as evidenced by the rapid loss of electrical impedance observed over the following six days. As expected, Y27632 had no effect on control fibroblast proliferation. Lastly, to determine whether *TTC7A* deficiency was enough to account for the patients' functional defects, wild-type *TTC7A* transfection of fibroblasts from ELA patients restored p-ERM expression to control levels. Expression of an empty vector had no effect.

Growth impairment of gut organoids from ELA patient

A three-dimensional epithelial "organoid" culture^{24,25} was established from a rectum biopsy from an ELA patient (O3). The gut organoids displayed a disturbed epithelial architecture with condensed cell aggregates. Interestingly, continuous addition of ROCK inhibitor (Y27632) resulted in lumen expansion and restored normal expansion of patient organoid. The inhibitor was dispensable for the growth of control organoid²⁵. These data further suggest that *TTC7A* has a primary role in the enteropathy that characterizes the ELA phenotype.

Discussion:

Here, we described a previously unrecognized autosomal recessive ELA syndrome, characterized by familial enteropathy and progressive, chronic inflammatory disease, and defined its genetic cause. The underlying gene mutation results in the loss of *TTC7A* expression, which in turn modifies activation of the Rho kinase pathway. This abnormality could be corrected *in vitro* by the expression of wild-type *TTC7A*. The symptoms displayed by the kindred members combined features of a T/B cell immune deficiency with those of severe IBD, notably in early childhood. All the patients display progressive T, B and NK lymphocytopenia and defective antigen-specific T cell responses *in vitro*. Inflammatory bowel disease is another key manifestation in patients with ELA syndrome and is life threatening. It is noteworthy that despite full hematopoietic chimerism, IBD was not improved by HSCT in the two treated individuals; this finding suggests that *TTC7A* deficiency is a major inducer of local inflammation in enterocytes.

Spontaneous mutation of *ttc7a* has been reported in the flaky skin mutant mice (*f_{sn}*) as well as in two other models, i.e. the *hea* and the *int* mutants^{22,26-28}. The flaky skin mice have a longer life spans and were thus analyzed in more details. The flaky skin mouse phenotype overlaps (at least in part) with that of ELA patients. The mice display pleiotropic skin abnormalities, with the development of thick white scales and patchy alopecia that turns into a papulosquamous disease resembling human psoriasis^{28,29}. Although only one adult ELA patients developed psoriasis, five displayed alopecia and four displayed onychopathy. Furthermore, flaky skin mice progressively develop (i) massive papillomatosis of the stratified squamous epithelium of the forestomach, (ii) hyperplasia and dysplasia of the glandular stomach, (iii) increased apoptosis of cecal enterocytes and (iv) a moderate, mixed inflammatory cell infiltrate in the lamina propria^{30,31}. These gastrointestinal changes are reminiscent to those observed in ELA patients and in combined immune deficiency with multiple intestinal atresia (CID-MIA) patients carrying a null mutation in *TTC7A*. These epithelial anomalies further suggest that *TTC7A* has a key role in gut intestinal epithelial cells. In addition, flaky skin mice exhibit an hyperproliferative immune disorder combining splenomegaly, lymphadenopathy (accumulated T and B lymphocytes), increased proliferation in response to mitogens and features of systemic autoimmunity that persist over time. Although the immunological phenotype is not strictly the same in murine and human *TTC7A* natural mutants, there are common features. Both murine and human phenotypes display excessive T lymphocyte proliferation and an impaired cellular response to antigens. Mouse vs. human disparities in phenotypic expression may be related to the time at which the assessment is carried out, the nature of the mutation, the disease's environment and, of course, species-specific differences. The *TTC7A* protein does not have a known function. It contains 9 cognate TPR domains, which may be involved in multiple protein-protein interactions. Indeed, TPR domains are involved in a number of cellular processes, including cell-cycle control³², protein transport³³ and the assembly of multiprotein complexes²¹. Disorders resulting from mutations in genes that encode TPR-containing proteins include Fanconi anemia³⁴, Bardet-Biedl syndrome³⁵, Charcot-Marie-Tooth disease type C³⁶ and tricohepatoenteric syndrome³⁷. The latter is also characterized by the presence of a protracted gut disease. Nevertheless, relatively little is known about the function of proteins that contain TPR domains or the pathogenesis of conditions in which these proteins are defective. Here we show that *TTC7A* deficiency leads to inappropriate activation of the RhoA signaling pathway – a pathway that is known to orchestrate the interplay between the plasma membrane and the cortical cytoskeleton³⁸. In particular, *TTC7A* deficiency induces the phosphorylation of

several ROCK effectors (such as ERM and the MLC) known to regulate cell adhesion and migration^{23,39} notably in hematopoietic cells⁴⁰. Accordingly, T lymphocytes from ELA patients adhered more strongly to integrin ligands and hence showed a lesser capacity to migrate in response to a range of chemoattractants. Defective control of the RhoA signaling pathway activation also modulates the proliferation capacity of both lymphocytes and epithelial cells. However, the mechanism by which TTC7A regulates the ROCK pathway remains to be established.

In conclusion, we have identified a new cause of IBD that results from a loss of function mutation in TTC7A. The mutation affects both lymphocyte and epithelial cell functions. Our investigation of the *in vivo* and *in vitro* consequences of TTC7A deficiency show that this protein is critical for fine-tuning the cells' proliferation, adhesion and migration capacities and avoiding premature cell death. TTC7A deficiency is associated with an increase in ROCK activity. The fact this increase can be reverted *in vitro* by treatment with a ROCK inhibitor suggests that this ELA syndrome is amenable to pharmacological manipulation.

Patient ID	R3	N3	F5	E3	K4	N4	O3	K3	D8	B3	A3	B4	C1
Gender	f	f	m	m	m	m	f	f	m	m	m	f	f
Age at last follow up (months or years)	5m	6m	6m	8m	10m	10m	2y	4y	4y	14y	14y	28y	50y
Inflammatory bowel disease	+	+	+	+	+	+	+	+	+	+	+	+	+
Enteral nutrition	-	-	-	-	+	+	-	+	-	-	-	-	-
Total parenteral nutrition	+	+	+	+	-	-	+	-	+	+	-	-	-
Recurrent upper (U) and lower (+) respiratory tract infections	U	NE	+	-	NE	-	-	NE	NE	+	+	+	+
CMV infection	+	-	+	+	+	-	+	+	-	-	-	-	-
Alopecia	-	-	-	-	-	-	-	+	-	+	+	+	+
Onychopathy	-	-	-	-	-	-	-	+	-	+	-	+	+
Hepatomegaly	+	-	+	-	-	-	+	-	+	+	-	-	-
Autoimmunity	-	-	-	-	-	-	-	-	-	AH	AIHA	IDDM, psoriasis	thyroiditis
Outcome	alive	died soon after HSCT	died 9 months after HSCT	died (enteropathy)	alive	alive	alive	alive	died (septic shock)	died (gastric adenocarcinoma)	alive	alive	alive

Table 1: Summary of the ELA patients' clinical characteristics

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Patients ID ^A	R3	Control Values	N3	F5	K4	K3	N4	Control Values (6-12)/12	O3	D8	Control Values	A3	B4	C1	Control Values
Age (years)	4/12		6/12	6/12	7/12	9/12	10/12		2	4	2-6	14	28	50	12-adult
Lymphocytes (per $\mu\text{l} \times 10^{-3}$)	2.9	3.9-9.0	1.9	2.2	2.8	1.9	1.4	3.4-9.0	2.8		2.3-5.4	0.7	0.5	0.80	1.4-3.3
<i>T cells</i>															
CD3+ (per $\mu\text{l} \times 10^{-3}$)	2.3	2.5-5.6	1.1	2.0	1.7	1.4	0.7	1.9-5.9	1.7	0.1	1.4-3.7	0.6	0.3	0.67	1.0-2.2
CD4+ (%)	51	35-56	23	40	26	0.5	23	31-56	21	6	28-47	36	25	49	31-52
CD4+CD45RA+CD31+ naïve	33	60-72	35		63	71	47	60-72	47		50-85	11	11	2	43-55
CD4+CD45RO+	67	6-23	40		27		45	7-36	35		14-47	88	85	94	34-67
CD8+ (%)	25	12-23	33	35	30	0.8	19	12-24	33	3	16-30	57	35	25	18-35
CD8+CD45RA+CCR7+ naïve	49	52-68	93				81	52-68	28		52-68	6	6	0.25	30-50
CD8+CD45RA+CCR7-	16	16-28	4				5	16-28	65		16-28	92	27	9	8-20
CD8+CD45RA-CCR7+	1	3-4	1		3-4		4	3-4	0.6		3-4	0	2	6	6-16
CD8+CD45RA-CCR7-	33	11-20	2				10	11-20	8		11-20	2	65	85	20-40
Treg CD4+CD25 ^{hi} FoxP3+ (%)	0.7	2-6			2.6	1.4		3.5-8	1.1		2.5-6	0.4	1.1		2.8-4.2
<i>T cell proliferation (cpm $\times 10^{-3}$)</i>															
PHA	48	>50	30	116	205	86	104	>50	74	38	>50	31	36	4	>50
CD3	6	>30		78				>30	42	nd	>30		44	10	>30
tetanus toxoid	ND		ND		1	0	1.5	>10	8	44	>10	1	8	1	>10
CMV	0.3	>10	ND	2		0	0	>10	0.5	1	>10	16.5	0.9	5	>10
Candida antigen	1	>10	15	1	0	0	1	>10	1	73	>10		7.5		>10
<i>Natural killer cells</i>															
CD56+CD16+ (per $\mu\text{l} \times 10^{-3}$)	0.09	0.2-0.8	0.06		0.05	0.02	0.04	0.2-0.9	0.06	0.01	0.2-0.9	0.01	0.01	0.11	0.07-0.58
<i>B cells</i>															
CD19 ⁺ (per $\mu\text{l} \times 10^{-3}$)	0.46	0.43-3.0	0.89	0.29	1.1	0.5	0.7	0.6-2.6	1.0	0.05	0.7-2.6	0.04	0.09	0	0.09-0.57
CD27 ⁺ /CD19 ⁺ memory (%)	3	7-27	2		1.5	3		7-27	2		10-27	5	4	0	24-45
<i>Immunoglobulin levels (g/l)</i>															
IgM	0.14	0.34-0.95	0.31	0.06	<0.04	0.32		0.4-1.0	<0.05	0.26	0.5-1.4	0.1	0.17	0.19	0.5-2.1
IgG	2.49	2.35-4.37	1.7	1.3	2.6	1.5		2.3-6.23	6.08	2	4.0-7.5	2	8.97	3.7	6.6-12.3
IgA	0.08	0.20-0.62	0.07	<0.07	0.08	0.22		0.2-0.8	0.25	0.12	0.3-1.1	0.48	2.3	0.15	1.16-2.6

Supplemental Table 1: Immunological features of patients with ELA

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by
5 reference into the present disclosure.

CLAIMS:

- 5 1. A method for the treatment of an inflammatory bowel disease in a subject in need thereof comprising administering the subject with a therapeutically effective amount of at least one RhoA kinase (ROCK) inhibitor.
2. The method of claim 1 wherein the subject has a TTC7A deficiency.
3. The method of claim 2 wherein the TTC7A deficiency results from a mutation in so that the pre-ARNm is degraded through the NMD (non sense mediated decay) system.
- 10 4. The method of claim 2 wherein the TTC7A deficiency results from a mutation which is 211G>A so that the protein is misfolded and degraded through the proteasome.
5. The method of claim 2 wherein the TTC7A deficiency results from a loss of function mutation leading to a dysfunction of the protein.
- 15 6. The method of claim 2 wherein the TTC7A deficiency results from an epigenetic control of gene expression such as methylation so that the gene is less expressed in the cells of the subject.
7. The method of claim 2 wherein the TTC7A deficiency results from a repression of the TTC7A gene induce by a particular signalling pathway.
- 20 8. The method of claim 2 which comprises a first step for determining whether the subject has a TTC7A deficiency.
9. The method of claim 8 wherein the first step consists in detecting the mutation that is responsible for the TTC7A deficiency.
10. The method of claim 9 wherein the the mutation is 211G>A mutation
- 25 11. The method of claim 9 wherein the mutation is detected by analyzing a TTC7A nucleic acid molecule.

12. The method of claim 9 wherein the mutation is detected at the protein wherein a biological sample, such as a tissue biopsy, obtained from a subject is contacted with antibodies specific of a mutated form of TTC7A protein.
13. The method of claim 8 wherein the first step consists in determining the expression level of TTC7A gene in the biological sample obtained from the subject.
14. The method of claim 8 wherein the first step consists in i) determining the expression level of TTC7A gene, ii) comparing the level determined at i) with a predetermined reference value and iii) concluding that the subject has a TTC7A deficiency when the expression level determined at i) is lower than the predetermined reference value.
15. The method of claim 14 wherein the expression level of TTC7A gene is determined by determining the quantity of mRNA.
16. The method of claim 2 wherein the TTC7A deficiency is detected in the subject by looking for a molecular or functional consequence of said deficiency.
17. The method of claim 16 wherein the molecular consequence is an increased ROCK activity in the cells of the patients such as the peripheral blood mononuclear cells (PBMC) or biopsy sample obtained from the subject.
18. The method of claim 16 wherein the expression of the phosphorylated RhoA effectors molecule such as p-ERM and p-MLC is determined and compared to a predetermined reference value, wherein when the determined level is higher than the predetermined reference value it is concluded that the subject has an increased ROCK activity and thus has a TTC7A deficiency.
19. The method of claim 1 wherein the ROCK inhibitor is an inhibitor of ROCK expression.

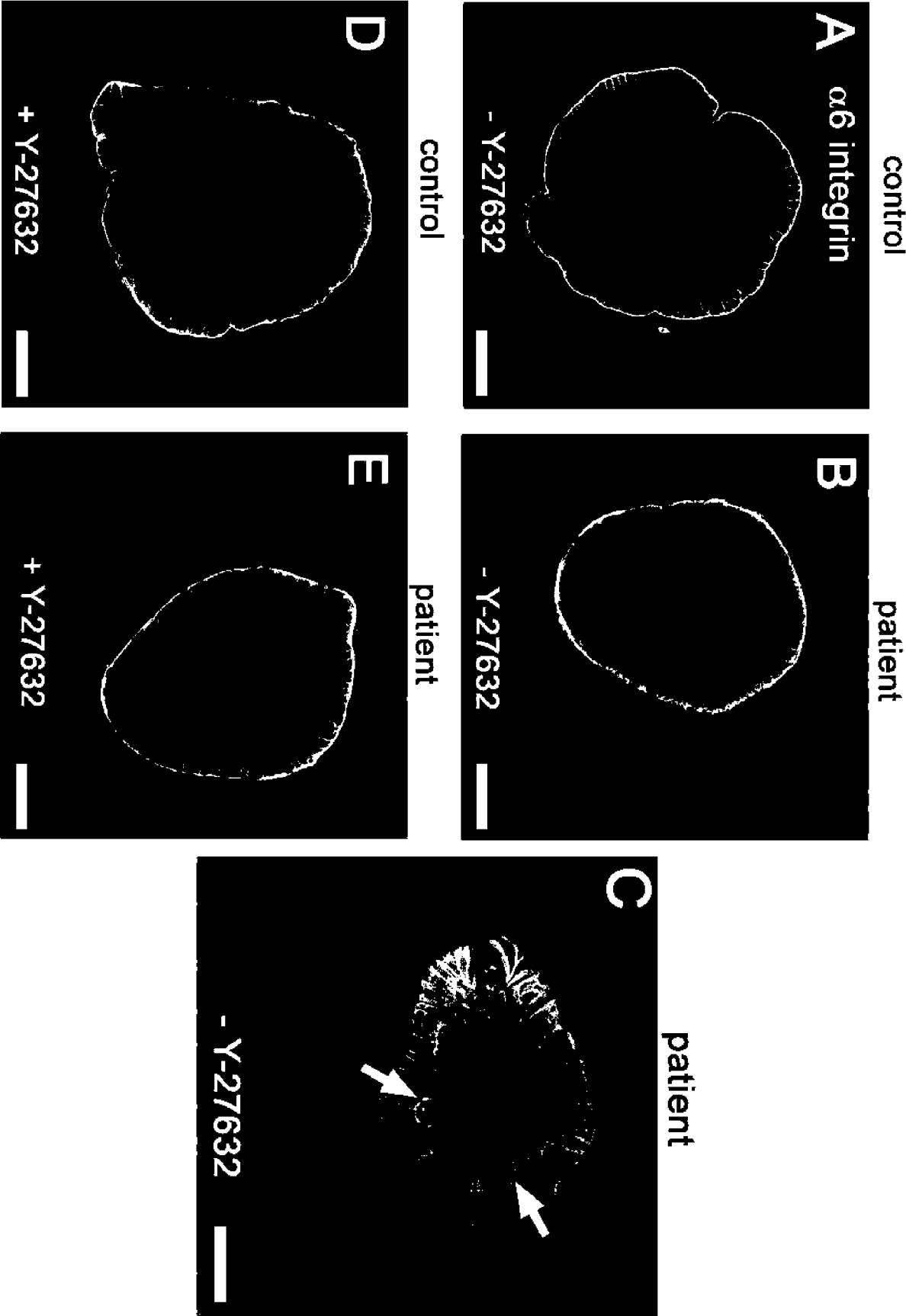


Figure 1A, 1B, 1C, 1D, and 1E

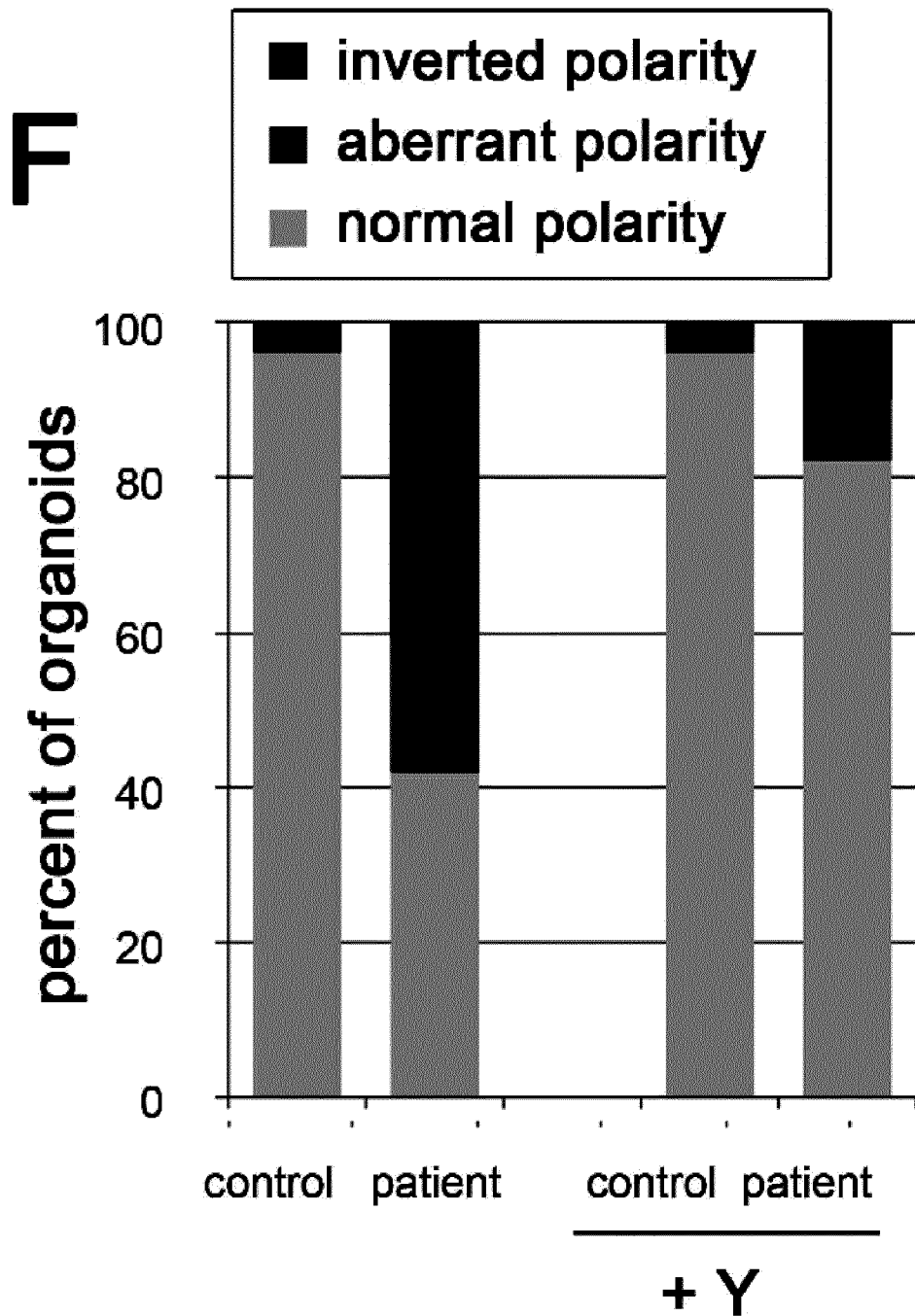


Figure 1F

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2014/058997

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)

☐

 on paper

☒

 in electronic form
 - b. (time)

☒

 in the international application as filed

☐

 together with the international application in electronic form

☐

 subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/058997

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/437 A61K31/4409 A61K31/551 A61P1/04
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)

A61K A61P

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, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/086047 A1 (BOEHRINGER INGELHEIM INT [DE]; BOEHRINGER INGELHEIM PHARMA [DE]; DAHMA) 17 July 2008 (2008-07-17) page 2, paragraph 5 - page 3, paragraph 1 page 166, line 22 claim 12	1-19
X	----- US 2003/134775 A1 (UEHATA MASAYOSHI [JP] ET AL) 17 July 2003 (2003-07-17) paragraph [0108]	1-19
X	----- WO 2005/074642 A2 (SMITHKLINE BEECHAM CORP [US]; DREWRY DAVID HAROLD [US]; HUNTER ROBERT) 18 August 2005 (2005-08-18) cited in the application page 22, line 4 ----- -/--	1-19



Further documents are listed in the continuation of Box C.



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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

2 July 2014

Date of mailing of the international search report

10/07/2014

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Albayrak, Timur

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/058997

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/082890 A1 (SMITHKLINE BEECHAM CORP [US]; LEE DENNIS [US]; GOODMAN KRISTA B [US]) 9 September 2005 (2005-09-09) cited in the application claim 5 -----	1-19
X	EP 1 632 492 A1 (ASAHI KASEI PHARMA CORP [JP]) 8 March 2006 (2006-03-08) paragraph [0183] -----	1-19
X	US 2009/325960 A1 (FULCHER EMILEE H [US] ET AL) 31 December 2009 (2009-12-31) paragraph [0229] - paragraph [0230] -----	1-19
X	WO 2005/103050 A2 (VERTEX PHARMA [US]; GREEN JEREMY [US]; MILLER ANDREW [GB]; JIMENEZ JUA) 3 November 2005 (2005-11-03) cited in the application claim 52 -----	1-19
X	SEGAIN JEAN-PIERRE ET AL: "Rho kinase blockade prevents inflammation via nuclear factor kappaB inhibition: Evidence in Crohn's disease and experimental colitis.", GASTROENTEROLOGY, vol. 124, no. 5, May 2003 (2003-05), pages 1180-1187, XP002699002, ISSN: 0016-5085 the whole document -----	1-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/058997

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008086047	A1	17-07-2008	AR 064726 A1 22-04-2009
			CA 2674389 A1 17-07-2008
			CL 38742007 A1 16-05-2008
			EP 2114869 A1 11-11-2009
			JP 5258790 B2 07-08-2013
			JP 2010514842 A 06-05-2010
			TW 200843770 A 16-11-2008
			US 2010041645 A1 18-02-2010
			WO 2008086047 A1 17-07-2008
US 2003134775	A1	17-07-2003	AT 359822 T 15-05-2007
			AU 738620 B2 20-09-2001
			AU 3785197 A 06-03-1998
			BG 63991 B1 30-09-2003
			BG 63992 B1 30-09-2003
			BG 103246 A 31-05-2000
			BG 107645 A 31-05-2004
			BR 9711154 A 17-08-1999
			CA 2263425 A1 19-02-1998
			CN 1233188 A 27-10-1999
			CZ 9900460 A3 14-07-1999
			DE 69737631 T2 27-12-2007
			DK 0956865 T3 03-09-2007
			EE 9900050 A 16-08-1999
			EP 0956865 A1 17-11-1999
			ES 2286834 T3 01-12-2007
			HK 1022436 A1 14-12-2007
			HU 9903694 A2 28-03-2000
			IS 4973 A 11-02-1999
			JP 3669711 B2 13-07-2005
			KR 20000029918 A 25-05-2000
			KR 20050055022 A 10-06-2005
			NO 990622 A 12-04-1999
			NZ 334613 A 01-02-2002
			NZ 513800 A 28-09-2001
			PL 331561 A1 19-07-1999
			PT 956865 E 30-07-2007
			RU 2206321 C2 20-06-2003
			US 6218410 B1 17-04-2001
			US 2002032148 A1 14-03-2002
			US 2003134775 A1 17-07-2003
			WO 9806433 A1 19-02-1998
WO 2005074642	A2	18-08-2005	EP 1708697 A2 11-10-2006
			JP 2007519753 A 19-07-2007
			US 2008293716 A1 27-11-2008
			WO 2005074642 A2 18-08-2005
WO 2005082890	A1	09-09-2005	EP 1718638 A1 08-11-2006
			JP 4857128 B2 18-01-2012
			JP 2007523186 A 16-08-2007
			JP 2011173915 A 08-09-2011
			US 2008058384 A1 06-03-2008
			WO 2005082890 A1 09-09-2005
EP 1632492	A1	08-03-2006	CA 2528497 A1 16-12-2004
			EP 1632492 A1 08-03-2006
			KR 20060027802 A 28-03-2006

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/058997

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		US 2005096310 A1	05-05-2005
		WO 2004108724 A1	16-12-2004

US 2009325960	A1	31-12-2009	NONE

WO 2005103050	A2	03-11-2005	AR 049333 A1 19-07-2006
		AU 2005236002 A1	03-11-2005
		CA 2561724 A1	03-11-2005
		CN 101014597 A	08-08-2007
		EP 1756108 A2	28-02-2007
		JP 5019612 B2	05-09-2012
		JP 2007531740 A	08-11-2007
		KR 20070002081 A	04-01-2007
		US 2006003968 A1	05-01-2006
		WO 2005103050 A2	03-11-2005
