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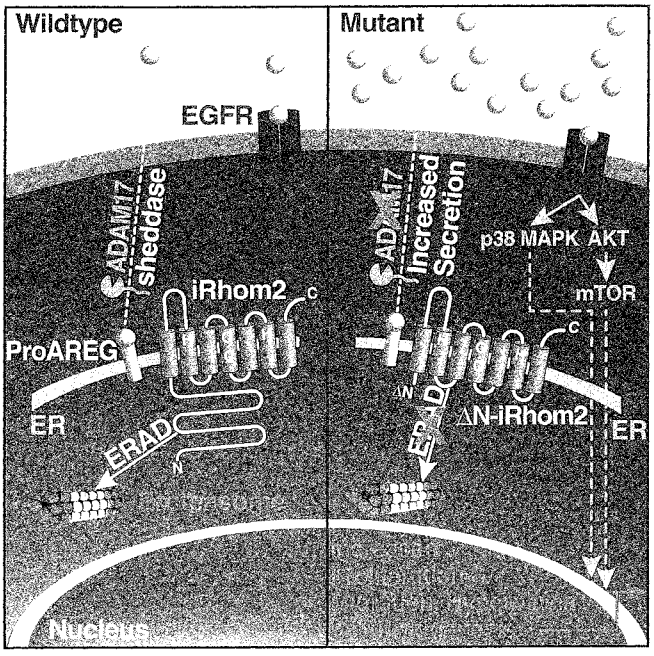


FIG. 8

(57) Abstract: Methods for identifying a compound that modulates activity of an iRhom polypeptide according to aspects of the present invention are described herein which include contacting a cell expressing an iRhom polypeptide with a test compound; and determining the effect of the compound on activity of the iRhom polypeptide. Detection of a decrease in proteolytic activity of the iRhom polypeptide indicates that the compound is capable of one or more of: reducing tumor growth, reducing tumor progression, treatment of cancer and promoting hair growth in a subject, and detecting an increase in proteolytic activity of the iRhom polypeptide indicates that the compound is capable of accelerating wound healing in a subject.

WO 2015/172143 A1

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METHODS FOR IDENTIFYING COMPOUNDS THAT ALTER THE ACTIVITY OF iRHOM POLYPEPTIDES AND USE THEREOF

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Applications Serial Nos. 61/991,423, filed May 9, 2014 and 61/992,152, filed May 12, 2014, the entire content of both of which is incorporated herein by reference.

GRANT REFERENCE

[0002] This invention was made with government support under Grant Nos. CA034196, HLO77642, and DK057199, awarded by the National Institutes of Health. The Government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] Methods for identifying compounds that alter the activity of iRhom polypeptides and uses thereof are generally described herein. According to specific aspects, methods for identifying compounds that modulate the proteolytic activity of iRhom polypeptides, such as increasing, stimulating, reducing, inhibiting or abolishing the proteolytic activity of iRhom polypeptides are disclosed herein along with uses thereof.

BACKGROUND OF THE INVENTION

[0004] The rhomboid proteases are a family of enzymes that exist in almost all species. Rhomboids are intramembrane serine proteases and proteolytic cleavage by Rhomboid proteases is important for cellular regulation. The active site of intramembrane proteases is buried in the lipid bilayer of cell membranes, and they cleave other transmembrane proteins within their transmembrane domains.

[0005] Inactive rhomboids (iRhoms) are highly conserved intramembrane proteins. Prior to the discovery of the present invention, it was thought that iRhoms were proteolytically inactive due to the lack of a serine residue in the putative active site.

SUMMARY OF THE INVENTION

[0006] In one aspect, the present invention is based on the discovery that iRhoms are short-lived proteins, but dominant mutations increase their protein stability and stimulate secretion of specific EGF family ligand amphiregulin independently of metalloprotease

activity. In another aspect, the invention is based on the discovery that mammalian iRhoms function in regulating an EGFR signaling event that promotes accelerated wound healing and triggers tumorigenesis. In yet another aspect, the present invention is based, at least in part, on the discovery that iRhoms have the ability to regulate EGFR signaling in parallel with metalloproteases, and may function as therapeutic targets in impaired wound healing and cancer.

[0007] In one aspect the invention provides methods for identifying a compound that activates the proteolytic activity of an iRhom polypeptide on a substrate comprising a) contacting a cell expressing an iRhom polypeptide with a test compound; and b) determining an increase in stability of the iRhom polypeptide relative to an appropriate control, wherein an increase in stability of the iRhom polypeptide indicates that the compound activates the proteolytic activity of the iRhom polypeptide on a substrate.

[0008] In another aspect, the invention provides a method for identifying a compound capable of accelerating wound healing or tissue repair in a subject comprising a) contacting a cell expressing an iRhom polypeptide and an EGFR ligand with a test compound; b) determining an increase in stability of the iRhom polypeptide relative to an appropriate control; and c) determining an increase in secretion of an EGFR ligand by the cell relative to an appropriate control, wherein an increase in stability of the iRhom polypeptide and an increase in secretion of an EGFR ligand by the cell indicates that the compound is capable of accelerating wound healing.

[0009] In another aspect, the invention provides a method for identifying a compound that inhibits the cytoplasmic domain of an iRhom polypeptide comprising a) contacting a cell expressing an iRhom polypeptide with a test compound; b) determining an increase in stability of the iRhom polypeptide relative to an appropriate control, wherein an increase in stability of the iRhom polypeptide indicates that the compound inhibited the cytoplasmic domain of the iRhom polypeptide.

[0010] In one embodiment, the method further provides that an increase in stability of the iRhom polypeptide is determined by analyzing the half-life of the iRhom polypeptide following exposure to the compound.

[0011] In some embodiments, the substrate is an EGFR ligand or an EGF-like substrate.

[0012] In another embodiment, an increase in stability of the iRhom polypeptide is determined by detecting an increase in secretion of an EGFR ligand. In another embodiment, an increase in stability of an iRhom polypeptide is determined by detecting an increase in the

level of soluble EGFR ligands. In some embodiments, the EGFR ligand is selected from the group consisting of AREG, HB-EGF, TGFα and EPGN.

[0013] In another embodiment, an increase in stability of an iRhom polypeptide is determined by detecting an increase in EGFR signaling activity.

[0014] In yet another embodiment, the compound of the invention inhibits an interaction between the iRhom polypeptide and a proteasome.

[0015] In yet another embodiment, the compound inactivates the cytoplasmic domain of an iRhom polypeptide. In some embodiments, the inactivation of the cytoplasmic domain is transient.

[0016] In one embodiment, the compound of the invention cleaves and/or deletes the cytoplasmic domain of an iRhom polypeptide such that the polypeptide has proteolytic activity or altered biological activity. In some embodiments, mouse iRhom2 is cleaved between amino acid residues 1 and 268 or human iRhom2 is cleaved between amino acid residues 1 and 298. In other embodiments, mouse iRhom1 is cleaved between amino acid residues 1 and 272 or human iRhom1 is cleaved between amino acid residues 1 and 316. In another embodiment, the compound activates the peptidase domain of an iRhom polypeptide.

[0017] In one embodiment, the iRhom polypeptide is iRhom1 or iRhom2. In another embodiment, the iRhom polypeptide is a human or mouse iRhom polypeptide.

[0018] In one embodiment, the compound of the invention is selected from the group consisting of a small molecule, a peptide or a polypeptide decoy. In another embodiment, the compound is attached to a cell penetrating peptide. In another embodiment, the compound is cell-permeable. In yet another embodiment, the compound is an ubiquitin protease inhibitor.

[0019] In one embodiment, the compound of the invention accelerates migration of keratinocytes. In yet another embodiment, the compound accelerates proliferation of fibroblasts.

[0020] In one aspect, the invention provides a method for identifying a compound that inhibits the proteolytic activity of an iRhom polypeptide on a substrate comprising a) contacting a cell expressing an iRhom polypeptide with a test compound; and b) determining a decrease in secretion of a physiological target of the iRhom polypeptide relative to an appropriate control or a decrease in EGFR activity relative to an appropriate control, wherein a decrease in secretion of a physiological target of the iRhom polypeptide or a decrease in EGFR activity indicates that the compound inhibits the proteolytic activity of the iRhom polypeptide on a substrate.

[0021] In another aspect, the invention provides a method for identifying a compound capable of reducing tumor growth and/or progression or treating cancer in a subject comprising a) contacting a cell expressing an iRhom polypeptide with a test compound; and b) determining a decrease in secretion of a physiological target of the iRhom polypeptide relative to an appropriate control or a decrease in EGFR activity relative to an appropriate control, wherein a decrease in secretion of a physiological target of the iRhom polypeptide or a decrease in EGFR activity indicates that the compound is capable of reducing tumor growth and/or progression or treating cancer in the subject.

[0022] In another aspect, the invention provides a method for identifying a compound capable of promoting hair growth in a subject comprising a) contacting a cell expressing an iRhom polypeptide with a test compound; and b) determining a decrease in secretion of a physiological target of the iRhom polypeptide relative to an appropriate control or a decrease in EGFR activity relative to an appropriate control, wherein a decrease in secretion of a physiological target of the iRhom polypeptide or a decrease in EGFR activity indicates that the compound is capable of promoting hair growth in the subject.

[0023] In one embodiment, the tumor is a solid tumor. In some embodiments, the cancer is an epithelial cancer. In other embodiments, the cancer is cancer of the esophagus, lung, brain, colon, kidney, prostate, skin, liver, pancreas, stomach, uterus, ovary, breast, lymph glands or bladder.

[0024] In one embodiment, the substrate is an EGFR ligand or an EGF-like substrate.

[0025] In another embodiment, the inhibition of proteolytic activity of the iRhom polypeptide is determined by detecting a decrease in secretion of a physiological target of the iRhom polypeptide. In some embodiments, the physiological target of the iRhom polypeptide is an EGFR ligand. In one embodiment, the inhibition of proteolytic activity of the iRhom polypeptide is determined by detecting a decrease in the level of soluble EGFR ligands. In yet other embodiments, the EGFR ligand is selected from the group consisting of AREG, HB-EGF, TGFα and EPGN.

[0026] In one embodiment, the inhibition of proteolytic activity of an iRhom polypeptide is determined by detecting a decrease in EGFR activity.

[0027] In another embodiment, the compound inhibits the peptidase domain of an iRhom polypeptide.

[0028] In yet another embodiment, the compound affects the activity of an iRhom polypeptide by inactivating one or more amino acid residues in the proteolyic site of the

iRhom polypeptide peptidase domain. In one embodiment, the iRhom polypeptide is mouse iRhom2 and the compound inactivates one or more amino acid residues in the proteolytic site of mouse iRhom2 selected from the group consisting of Histidine 635 on TM helix 2, Glutamine 695 on TM helix 4, Cysteine 701 on TM helix 4, and Histidine 744 on TM helix 6. In other embodiments, the iRhom polypeptide is human iRhom2 and the compound inactivates one or more amino acid residues in the proteolytic site of human iRhom2 selected from the group consisting of Histidine 664 on TM helix 2, Glutamine 724 on TM helix 4, Cysteine 730 on TM helix 4, and Histidine 773 on TM helix 6.

[0029] In one embodiment, the iRhom family member is iRhom1 or iRhom2. In other embodiments, the iRhom family member is a human or mouse iRhom family member.

[0030] In one embodiment, the compound of the invention is selected from the group consisting of a small molecule, a polypeptide decoy, an miRNA molecule, an siRNA molecule, an shRNA molecule, a dsRNA molecule, an antisense molecule, a ribozyme specific for *Rhbdf2*; or a polynucleotide encoding the miRNA, siRNA, shRNA, dsRNA; or a biological equivalent of each thereof. In other embodiments, the compound is attached to a cell penetrating peptide. In some embodiments, the compound is cell-permeable.

[0031] In one aspect, the invention provides an isolated polypeptide comprising a variant iRhom polypeptide.

[0032] In another aspect, the invention provides an isolated polypeptide comprising human iRhom2 with a deletion of the cytosolic N-terminal domain.

[0033] In another aspect, the invention provides an isolated polypeptide comprising mouse iRhom2 with a deletion of the cytosolic N-terminal domain.

[0034] In yet another aspect, the invention provides an isolated mouse iRhom2 polypeptide comprising a mutation at one or more amino acid residues in the proteolytic site of mouse iRhom2 selected from the group consisting of Histidine 635 on TM helix 2, Glutamine 695 on TM helix 4, Cysteine 701 on TM helix 4 and Histidine 744 on TM helix 6.

[0035] In another aspect, the invention provides an isolated human iRhom2 polypeptide comprising a mutation at one or more amino acid residues in the proteolytic site of human iRhom2 selected from the group consisting of Histidine 664 on TM helix 2, Glutamine 724 on TM helix 4, Cysteine 730 on TM helix 4 and Histidine 773 on TM helix 6.

[0036] In one embodiment, the invention provides an isolated nucleic acid molecule encoding any one of the foregoing polypeptides. In another embodiment, the invention

provides a vector comprising the foregoing nucleic acid molecule. In another embodiment, the invention provides a host cell expressing the foregoing vector.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] Figure 1A is a graph showing quantitation of MEFs using the proliferation assay described herein; cells were seeded at the numbers indicated on the X-axis and incubated for 24h, with the extent of fluorescence being proportional to the amount of total cellular DNA; data is shown as mean ± s.d.;

[0038] Figure 1B is a graph showing a quantitation of migration of *cub/cub mcub/mcub* and +/+ *mcub/mcub* MEFs using a scratch-wound assay described herein; the width of the scratch wound was measured at time zero (100% open), and the increase in wound closure at each time point was calculated as a percent of that original width; data is shown as mean ± s.d.;

[0039] Figure 1C is an image showing an immunoblot analysis of *cub/cub mcub/mcub* or +/+ *mcub/mcub* MEFs for various markers of EGFR signaling in which cell lysates were run in duplicate with actin serving as a loading control;

[0040] Figure 1D shows representative images of regenerating ear tissue in 6–40-week-old female *cub/cub mcub/mcub* and +/+ *mcub/mcub* mice (n=3 per group) at 0,7,14, 21 and 28 days post-wounding;

[0041] Figure 1E is a graph showing a quantification of ear hole closures shown in Figure 1D; data is shown as mean ± s.d.;

[0042] Figure 1F shows images of cross-sections of ears from *cub/cub mcub/mcub* and +/+ *mcub/mcub* mice at 0, 7 and 14 days post-wounding stained with Hematoxylin/Eosin (original magnification, x10); note the undifferentiated and thickened epidermis (E) (10–12 nucleated layers) and the extensive degree of proliferation (M) in the ears of *cub/cub mcub/mcub* mice; the dotted line indicates the site of excision;

[0043] Figure 2A is a diagram showing that the *cub* mutation is a 12,681bp deletion in the mouse *Rhbdf2* gene in which the deletion starts midway between exons 1 and 2 and encompasses exons 2-6, ending shortly after exon 6;

[0044] Figure 2B shows images of results from a reverse-transcriptase PCR on MEFs from +/+ *mcub/mcub*, +/*cub mcub/mcub* and *cub/cub mcub/mcub* mice using primers against exons 2, 5, 12 and 19; ntc represents the no template control;

[0045] Figure 2C is a graph showing results from a qPCR on cDNA extracted from skin tissues using TaqMan gene expression assays against the indicated exon boundaries with actin serving as an endogenous control; data is normalized to +/+ *mcub/mcub* actin levels, and samples were run in triplicate with four biological replicates; data is shown as mean ± s.d.;

[0046] Figure 2D is a graph showing results from a qPCR using a custom TaqMan gene expression assay to detect transcripts with an exon 1-exon 7 boundary; samples were run in triplicate with three biological replicates (each bar represents an individual mouse); data is shown as mean ± s.d.;

[0047] Figure 2E is an image of an anti-Flag immunoblot of HEK293 cells transiently expressing Flag-tagged Hu*Wt* (human full-length *RHBDF2* cDNA) and Hu*Cub* (human version of *cub* cDNA) with actin serving as a loading control;

[0048] Figure 2F shows representative images of Flag-tagged Hu*Wt* and Hu*Cub*–expressing B6 primary MEFs stained using a Flag-specific antibody, and DAPI was used to counterstain the nucleus; arrows represent cytoplasmic expression of both full-length and mutant proteins;

[0049] Figure 3A is an image showing the results from a PCR for wild-type product in KOMP *Rhbdf2* knockout mice with an expected product length of 2181bp; L represents the 1kb DNA ladder obtained from New England Biolabs, and ntc represents the no template control;

[0050] Figure 3B shows images for reporter gene analysis of *Rhbdf2* expression; the whole-mount X-gal stained E18.5 $Rhbdf2^{-/-}$ embryo shows strong expression of β-gal in the epidermis (arrowhead) (original magnification, x2.5), and the X-gal stained two-week-old female $Rhbdf2^{-/-}$ skin shows β-gal positivity in the inner and outer sheath layers of hair follicles (arrow) (original magnification, x5); no staining was observed in the hair shaft; scale bar,1 mm;

[0051] Figure 3C is a graph showing that mice with a null mutation of *Rhbdf2* lack a regenerative phenotype as demonstrated by significantly delayed wound closure in the ears of six to eight-week-old female $Rhbdf2^{-/-}$ mice compared with those of $Rhbdf2^{cub/cub}$ mice;

[0052] Figure 3D shows images demonstrating that mice with a null mutation of *Rhbdf2* have normal skin and hair morphology; H&E stained sections of adult skin from $Rhbdf2^{cub/cub}$ and $Rhbdf2^{-/-}$ mice taken at indicated times after wounding in which the adult $Rhbdf2^{cub/cub}$ skin displays a thin hypodermal fat layer (F), abnormal hair follicles (H), thick epidermis (E),

enlarged sebaceous glands (SG), dense interlacing bundles of collagen fibers (C), and no full differentiation of hair follicles and hair bulb (HF); the adult *Rhbdf2*$^{-/-}$ skin shows normal epidermis and hair follicles; scale bar, 100 μm; original magnification, x10 and x40;

[0053] Figure 3E is an image showing that *Rhbdf2*$^{-/-}$ mice develop a normal hair coat (arrowhead), whereas compound mutant *Rhbdf2*$^{-/cub}$ mice exhibit a sparse hair coat (arrow);

[0054] Figure 4A is a diagram showing that *Mcub* is a T-to-G point mutation that disrupts the normal donor splice site (exon 1) in the *Areg* gene, causing the use of an alternative downstream splice site;

[0055] Figure 4B is a graph showing the healing of ear holes in 6–8-week-old female *Rhbdf2*$^{+/+}$ *Areg*$^{+/+}$, *Rhbdf2*$^{cub/cub}$ *Areg*$^{+/+}$, and *Rhbdf2*$^{cub/cub}$ *Areg*$^{Mcub/Mcub}$ mice (n=3 mice per group) over a period of 28 days; data is shown as mean ± s.d.;

[0056] Figure 4C shows images of hematoxylin/eosin-stained sections showing post-excision healing of ear holes of *Rhbdf2*$^{cub/cub}$ *Areg*$^{Mcub/Mcub}$ mice in which the dotted line indicates the site of excision; original magnification, x10; E represents epidermis and M represents proliferation; compare with Figure 1F;

[0057] Figure 4D is a graph showing serum AREG levels in age-matched *Rhbdf2*$^{+/+}$ *Areg*$^{+/+}$, *Rhbdf2*$^{cub/cub}$ *Areg*$^{+/+}$, and *Rhbdf2*$^{cub/cub}$ *Areg*$^{Mcub/Mcub}$ female mice, in which AREG was not detected (n.d.) in the serum of *Rhbdf2*$^{cub/cub}$ *Areg*$^{Mcub/Mcub}$ mice; data is shown as mean ± s.d. of three independent experiments; *** $p<0.001$;

[0058] Figure 4E is a graph of an ELISA quantitation of AREG levels in the supernatants of cultured mouse epidermal keratinocytes (MEKs) isolated from *Rhbdf2*$^{+/+}$ *Areg*$^{+/+}$, *Rhbdf2*$^{cub/cub}$ *Areg*$^{+/+}$, and *Rhbdf2*$^{cub/cub}$ *Areg*$^{Mcub/Mcub}$ mice, in which AREG was not detected (n.d.) in MEKs from *Rhbdf2*$^{cub/cub}$ *Areg*$^{Mcub/Mcub}$ mice; data is shown as mean ± s.d. of three independent experiments ***$p<0.001$;

[0059] Figure 4F is a graph showing results obtained from a qPCR of EGFR ligands: cDNA was extracted from skin tissues of *Rhbdf2*$^{cub/cub}$ *Areg*$^{+/+}$ and *Rhbdf2*$^{+/+}$ *Areg*$^{+/+}$ using TaqMan gene-expression assays where actin served as an endogenous control; data is normalized to *Rhbdf2*$^{+/+}$ *Areg*$^{+/+}$ actin levels, data is shown as mean ± s.d. of three independent experiments; *$p<0.05$;

[0060] Figure 5A is a diagram showing the schematic representation of the full-length human *RHBDF2* (Hu*Wt*) gene and a human construct mimicking the mouse *Rhbdf2*^{cub} gene (Hu*Cub*);

[0061] Figure 5B is a graph showing an ELISA quantitation of cleaved AREG after co-expression of Hu*Wt* or Hu*Cub* with the *AREG* gene in 293T cells; at 24 h post-transfection, cells were either incubated with DMSO or 10 μM marimastat (MM) for 24 h and AREG levels were analyzed in the conditioned medium with the transfections performed in duplicate, and the conditioned medium diluted five-fold;

[0062] Figure 5C is a graph showing the quantitation of serum TNFα levels by ELISA 3h after LPS injection of 8–12-week-old female mice of the indicated genotypes, in which TNFα was not detected (n.d.) in the serum of *Rhbdf2*$^{+/+}$ mice with no LPS injection; *$p<0.05$;

[0063] Figure 5D is a graph showing the ELISA quantitation of cleaved AREG/HB-EGF/EGF after co-expression of the human *RHBDL2* (Hu*RHBDL2*) gene with *AREG* or *HB-EGF* or *EGF* genes; at 24 h post-transfection, cells were incubated with 10 μM marimastat (MM) for 24 h and AREG/HB-EGF/EGF levels were analyzed in the conditioned medium, where HB-EGF and EGF conditioned medium were undiluted, and AREG medium was diluted five-fold; data represent mean ± s.d. of three independent experiments; ***$p<0.001$;

[0064] Figure 5E is a graph showing an ELISA quantitation of cleaved HB-EGF after co-expression of Hu*Wt* or Hu*Cub* with the *HB-EGF* gene in 293T cells; at 24 h post-transfection, cells were incubated with 10 μM marimastat (MM) for 24 h and HB-EGF levels were analyzed in the conditioned medium; ***$p<0.001$;

[0065] Figure 5F is a graph showing an ELISA quantitation of cleaved EGF after co-expression of Hu*Wt* or Hu*Cub* with the *EGF* gene in 293T cells; at 24 h post-transfection, cells were either incubated with DMSO or 10 μM marimastat (MM) for 24 h and EGF levels were analyzed in the conditioned medium; **$p<0.01$;

[0066] Figure 5G is a graph showing an ELISA quantitation of cleaved AREG after co-expression of Hu*Cub or* Hu*Cub* without the peptidase domain (Hu*CubΔPD*) with the *AREG* gene in 293T cells; at 24 h post-transfection, cells were incubated with 10 μM marimastat (MM) for 24 h and AREG levels were analyzed in the conditioned medium; ***$p<0.001$;

[0067] Figure 5H is a graph showing an ELISA quantitation of cleaved AREG after co-expression of Hu*Cub or* Hu*Cub* with individual alanine mutations with the *AREG* gene in 293T cells; at 24 h post-transfection, cells were incubated with 10 μM marimastat (MM) for 24 h and AREG levels were analyzed in the conditioned medium: *** $p<0.001$;

[0068] Figure 5I is a graph showing an ELISA quantitation of cleaved EGF after co-expression of Hu*Wt* or Hu*Cub or* Hu*Cub* with individual alanine mutations with the *EGF*

gene in 293T cells; at 24 h post-transfection, cells were incubated with 10 μM marimastat (MM) for 24 h and EGF levels were analyzed in the conditioned medium: **p<0.001;

[0069] Figure 5J is a diagram showing membrane topology of *Rhbdf2*^{cub}, in which the amino acids shown are critical for regulation of EGFR ligand production by *Rhbdf2*^{cub};

[0070] Figure 6A is a graph showing the quantitation of cleaved AREG by ELISA from conditioned medium of HEK293 cells transfected with *AREG* and either the human *RHBDF2* p.I186T mutant, Hu*Wt* or Hu*Cub*, in the presence of 10 μM marimastat (MM); **p<0.01;

[0071] Figure 6B is an image showing a Western blot of HEK293 cells co-transfected with *AREG* and either Hu*Cub,* Hu*Wt* or p.I186T, incubated with DMSO or MM, and immunoblotted for HA-AREG showing that co-expression of *AREG* and either Hu*Cub* or p.I186T significantly reduces the intracellular levels of pro-AREG compared with that of *AREG*/Hu*Wt* or *AREG* alone, even in the presence of 10 μM MM;

[0072] Figure 6C is a graph showing AREG levels in cells expressing various *RHBDF2 P.I186T* point mutants and co-transfected with the *AREG* gene or empty vector; each residue was mutated to alanine; the cells were incubated with 10 μM MM for 24 h and assayed for AREG levels in conditioned medium;

[0073] Figure 6D is an image of an X-gal-stained 2-week-old female *Rhbdf2*$^{-/-}$ intestine that reveals reporter gene β-gal expression in the mid and upper villous regions; original magnification, x2.5;

[0074] Figure 6E is a graph showing Kaplan-Meier survival curves of *Apc*$^{Min/+}$ *Rhbdf2*$^{+/+}$ (n=28) and *Apc*$^{Min/+}$ *Rhbdf2*$^{+/cub}$ (n=23) mice in which the median survival of *Apc*$^{Min/+}$ *Rhbdf2*$^{+/+}$ mice was 172 days, compared with 135 days for *Apc*$^{Min/+}$ *Rhbdf2*$^{+/cub}$ mice;

[0075] Figure 6F shows images of hematoxylin-eosin-stained sections of intestinal tissue from mice of the indicated genotypes at 3 months of age; original magnification, x2.5;

[0076] Figure 6G is a graph showing the size of polyps per mouse of the indicated genotypes of mice at 3 months of age; the mean tumor size was 1.8 mm^2 and 3.7 mm^2 for *Apc*$^{Min/+}$ *Rhbdf2*$^{+/+}$ and *Apc*$^{Min/+}$ *Rhbdf2*$^{+/cub}$ mice, respectively;

[0077] Figure 6H is a graph showing the number of polyps per mouse of the indicated genotypes of mice at 3 months of age; the mean number of polyps was 12 and 21 for *Apcs*$^{Min/+}$ *Rhbdf2*$^{+/+}$ and *ApcMin*$^{/+}$ *Rhbdf2*$^{+/cub}$ mice, respectively;

[0078] Figure 7A is an image of co-immunoprecipitation of human iRhom2-AREG complex in which lysates from COS7 cells were co-transfected with Flag-tagged Hu*Wt* or

Flag-tagged Hu*Cub* or Flag-tagged p.I186T and HA-tagged *AREG* were immunoprecipitated with anti-Flag magnetic beads and probed with anti-Flag and anti-HA antibodies;

[0079] Figure 7B shows flow cytometry results from 293T or COS7 (Figure 14) cells transfected with the indicated Flag-tagged genes and immunolabeled using a Flag-specific PE-labeled antibody wherein the cells were immunolabeled 48h post transfection;

[0080] Figure 7C is a graph showing the quantification of the data obtained in Figure 7B;

[0081] Figure 7D shows results of tests in which transiently transfected COS7 cells were subjected to a chase with 150 μg/ml of the protein-synthesis inhibitor cyclohexamide for the indicated times and evaluated for protein expression using Flag-specific PE labeled antibody; data is representative of one of a total of three experiments;

[0082] Figure 7E is a graph showing the quantification of the data obtained in Figure 7D;

[0083] Figure 7F is a graph showing results of tests in which transiently transfected COS7 cells were pre-incubated for 4h with 10 μM MG-132, a cell permeable protease inhibitor, followed by a chase with 150 μg/ml cyclohexamide in presence of MG-132 for the indicated times; protein expression was determined as described in Figure 7D; ***$p<0.001$;

[0084] Figure 8 is a diagram showing that wild-type iRhom2 is a short-lived protein and loss of its N terminus or mutations in its N-terminal domain, including those that underlie epithelial cancers, increase its protein stability which in turn augments the secretion of selective EGF family ligands, including AREG; inhibition of ADAM17 has no effect on AREG secretion, whereas loss of amino acids H366, Q426, C432, and H475 in the peptidase domain of iRhom2 abrogates AREG secretion and conversely, enhanced secretion of AREG leads to hyperactivation of EGFR signaling and thereby increased cell proliferation and migration;

[0085] Figure 9A is an image showing *cub/cub mcub/mcub* (arrow) and *cub/cub Mcub/mcub* (arrow head) mice;

[0086] Figure 9B is a graph showing that wound healing in *+/cub mcub/mcub* appears intermediate between *cub/cub mcub/mcub* and +/+ mice; quantification of ear hole closure rates in 6 to 40-week-old female +/+ *mcub/mcub* , *+/cub mcub/mcub* and *cub/cub mcub/mcub* mice (n=3 per group) at 0,7,14, and 21 days post-wounding (2-mm through-and-through holes); data is shown as mean ± s.d;

[0087] Figure 10A shows images demonstrating in vitro expression of Flag-tagged full-length (Hu*Wt*) and *cub* (Hu*Cub*) *Rhbdf2* clones in B6 primary MEFs followed by immunochemical assessment of protein expression with Flag-specific antibody and DAPI counterstaining for nucleus and showing that both full-length and mutant proteins co-localize with the ER marker calnexin;

[0088] Figure 10B shows images demonstrating in vitro expression of Flag-tagged full-length (Hu*Wt*) and *cub* (Hu*Cub*) *Rhbdf2* clones in B6 primary MEFs followed by immunochemical assessment of protein expression with Flag-specific antibody and DAPI counterstaining for nucleus and showing that both full-length and mutant proteins do not co-localize with the Golgi marker giantin;

[0089] Figure 11 is a diagram showing that a T to G point mutation disrupts the original splice site (exon 1) leading to an alternative splice site that adds 22 additional nucleotides to the *Areg* transcript and 16 incorrect amino acids to the protein, introducing a premature stop codon;

[0090] Figure 12A is an image of immunoblot analysis of *cub/cub Mcub/Mcub* or *cub/cub mcub/mcub* MEFs for the markers indicated in which cell lysates were run in duplicate and actin served as a loading control;

[0091] Figure 12B is a graph showing a quantitative RT-PCR determination of *Areg* expression in scramble shRNA or mouse *Areg* shRNA lentiviral infected $Rhbdf2^{+/+}$ or $Rhbdf2^{cub/cub}$ MEFs;

[0092] Figure 12C is a graph showing a quantitation of $Rhbdf2^{+/+}$ MEFs using a proliferation assay, described herein, after an infection with scramble shRNA or mouse *Areg* shRNA in which the cells were seeded at the numbers indicated on the X-axis and incubated for 24h; the extent of fluorescence is proportional to the amount of total cellular DNA;

[0093] Figure 12D is a graph showing a quantitation of $Rhbdf2^{cub/cub}$ MEFs using a proliferation assay, described herein, after an infection with scramble shRNA or mouse *Areg* shRNA in which the cells were seeded at the numbers indicated on the X-axis and incubated for 24h; the extent of fluorescence is proportional to the amount of total cellular DNA;

[0094] Figure 13A is a graph showing an ELISA quantitation of secreted AREG from a conditioned medium of HEK 293T cells transfected either with the mouse *Rhbdf1* or N-terminus truncated mouse *Rhbdf1 (ΔN-iRhom1)* or *ΔN-iRhom1* mutants together with the *AREG* gene in the presence of 10 μM marimastat (MM); ***$p<0.001$;

[0095] Figure 13B is a graph showing an ELISA quantitation of cleaved EGF from a conditioned medium of HEK 293T cells transfected either with the mouse *Rhbdf1* or N-terminus truncated mouse *Rhbdf1 (ΔN-iRhom1)* together with the *EGF* gene in the presence of 10 μM marimastat (MM); ***p<0.001;

[0096] Figure 13C is an image of the alignment of the amino acids of the peptidase domain sequences of human iRhom2 and RHBDL2, and human and mouse iRhoms;

[0097] Figure 13D is a graph showing an ELISA quantitation of secreted AREG after co-expression of Hu*Cub or* Hu*Cub* with individual alanine mutations with the *AREG* gene in 293T cells; at 24 h post-transfection, the cells were incubated with 10 μM marimastat (MM) for 24 h and AREG levels were analyzed in the conditioned medium; data represent mean ± s.d. of three independent experiments;

[0098] Figure 13E is a diagram showing the sequence alignment for the amino acids of the peptidase domains of iRHOM1 and iRHOM*2,* the active rhomboid RHBDL2, the *C. elegans* inactive rhomboids ROM3 and ROM4, and *Drosophila* iRhom wherein the asterisks represent conserved amino acids critical for regulation of EGFR ligands by iRHOM2; and

[0099] Figure 14 shows images of flow cytometry results from COS7 cells transfected with the indicated Flag-tagged genes and immunolabeled using a Flag-specific PE-labeled antibody in which the cells were immunolabeled 48h post transfection.

DETAILED DESCRIPTION OF THE INVENTION

[00100] Scientific and technical terms used herein are intended to have the meanings commonly understood by those of ordinary skill in the art. Such terms are found defined and used in context in various standard references illustratively including J. Sambrook and D.W. Russell, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press; 3rd Ed., 2001; F.M. Ausubel, Ed., Short Protocols in Molecular Biology, Current Protocols; 5th Ed., 2002; B. Alberts et al., Molecular Biology of the Cell, 4th Ed., Garland, 2002; D.L. Nelson and M.M. Cox, Lehninger Principles of Biochemistry, 4th Ed., W.H. Freeman & Company, 2004; Engelke, D.R., RNA Interference (RNAi): Nuts and Bolts of RNAi Technology, DNA Press LLC, Eagleville, PA, 2003; Herdewijn, P. (Ed.), Oligonucleotide Synthesis: Methods and Applications, Methods in Molecular Biology, Humana Press, 2004; A. Nagy, M. Gertsenstein, K. Vintersten, R. Behringer, Manipulating the Mouse Embryo: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press; December 15, 2002, ISBN-10: 0879695919; Kursad Turksen (Ed.), Embryonic stem cells: methods and protocols

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[00101] The singular terms "a," "an," and "the" are not intended to be limiting and include plural referents unless explicitly stated otherwise or the context clearly indicates otherwise.

[00102] Inactive rhomboids (iRhoms) are highly conserved intramembrane proteins that were previously thought to be protelytically inactive. iRhoms are characterized by a long cytosolic N-terminal domain, a conserved cysteine-rich inactive rhomboid homology domain (IRHD), a dormant proteolytic site lacking an active-site serine residue within the peptidase domain.

[00103] iRhoms participate in a diverse range of functions in a variety of species, including regulation of epidermal growth factor receptor (EGFR) signaling in *Drosophila melanogaster*, survival of human squamous epithelial cancer cells, misfolded protein clearance from endoplasmic reticulum membranes in mammalian cell lines, induction of migration in primary mouse keratinocytes, secretion of soluble TNFα in mice, and regulation of substrate selectivity of stimulated ADAM17-mediated metalloprotease shedding in mouse embryonic fibroblasts. EGF-like ligands may act as substrates for iRhom family members.

[00104] The *Rhbdf2* gene encodes an inactive rhomboid protease iRhom2, one of a family of enzymes containing a long cytosolic N-terminus and a dormant peptidase domain of previously unknown function. The present invention provides that iRhom2 may function in epithelial regeneration and cancer growth through constitutive activation of EGFR signaling.

[00105] Aspects of the present invention are based, at least in part, on the discovery that *Rhbdf2* mutations increase iRhom2 protein stability and drive EGFR hyperactivation through enhanced secretion of amphiregulin.

[00106] Aspects of the present invention are based, at least in part, on the discovery that iRhom2 is a short-lived protein whose stability can be increased by select mutations in the N-terminal domain. In turn, these stable variants function to augment the secretion of EGF family ligands, including amphiregulin, independently of metalloprotease ADAM17 activity.

In vivo, N-terminal iRhom2 mutations induce accelerated wound healing as well as accelerated tumorigenesis, but do not drive spontaneous tumor development. Therefore, the present invention is also based, at least in part, on the physiological prominence of iRhom2 in controlling EGFR signaling events involved in wound healing and neoplastic growth, and the function of key iRhom2 domains.

[00107] Epidermal growth factor receptor (EGFR) signal transduction plays a major role in growth, proliferation and differentiation of mammalian cells. Canonical EGFR ligands, including EGF, AREG, and HB-EGF, exist as pro-proteins expressed at the cell surface that, in order to bind EGFRs, must be shed into the extracellular compartment. Different classes of proteases cleave membrane-tethered EGFR pro-ligands to regulate a broad range of biological activities during various stages of development.

[00108] In one aspect, the present invention is based, at least in part, on the discovery that a spontaneous deletion within the *Rhbdf2* gene in mice underlies the curly bare (*cub*) mutation, in which loss of the cytosolic N-terminal domain of iRhom2 causes subsequent effects on hair-follicle development, wound healing and tumorigenesis. Furthermore, the modifier of *cub* (*Mcub*) cures or corrects balding and/or promotes hair growth (Example 1). In another aspect, the present invention is based, at least in part, on the discovery that iRhom2 is a short-lived protein but that gain-of-function mutations in the N-terminus (tylosis) or loss of the N-terminus (*cub* mutation) increase mutant protein stability leading to metalloprotease-independent secretion of the EGFR ligand amphiregulin (AREG). A genetic modifier of the *cub* phenotype (*Mcub*), was used to demonstrate that AREG is a physiological target of iRhom2. In another aspect, the present invention is based, at least in part, on the identification of key amino acids in the peptidase domain of iRhom2 that are necessary for AREG secretion, indicating that the peptidase domain of this pseudoenzyme might be functional despite lacking a serine residue in the putative active site. This invention therefore provides the function of key iRhom domains and establishes a framework for understanding the relationship between iRhoms, EGFR signaling and the biological processes involved in wound healing and tumorigenesis.

[00109] **iRhom Family Members**

[00110] **iRhom1 and iRhom2**

[00111] The rhomboid proteases are a family of enzymes that exist in almost all species. Rhomboids are intramembrane serine proteases and proteolytic cleavage by Rhomboid proteases is important for cellular regulation. The active site of intramembrane proteases is

buried in the lipid bilayer of cell membranes, and they cleave other transmembrane proteins within their transmembrane domains.

[00112] iRhoms or "inactive Rhomboids" are a subfamily of the rhomboid proteases that prior to the present invention were thought to lack protelytic activity. iRhoms are highly conserved intramembrane proteins that are characterized by a long cytosolic N-terminal domain, a conserved cysteine-rich inactive rhomboid homology domain (IRHD), a dormant proteolytic site lacking an active-site serine residue within the peptidase domain. iRhoms family members that are suitable for use in the present invention include iRhom1 (RHBDF1) and iRhom2 (RHBDF2). iRhom1 and/or iRhom2 for use in the present invention can be eukaryotic, prokaryotic, or mammalian. Additionally, iRhom1 and/or iRhom2 for use in the present invention can be from human, primate, rodent (e.g., mouse, rat, guinea pig), bovine, equine, donkey, rabbit, goat, sheep, dog, chicken, pig, drosophila, *C.elegans*, or *E.coli*.

[00113] Wild-type iRhoms are exemplified herein as mouse iRhom2 (SEQ ID No:1); mouse iRhom1 (SEQ ID No:3); human iRhom2 (SEQ ID No:5); and human iRhom1 (SEQ ID No:7). Isolated mutated sequences of iRhoms are exemplified herein as SEQ ID NO:2; SEQ ID NO:4; SEQ ID NO:6; SEQ ID NO:8.

[00114] One of skill in the art will readily recognize a nucleic acid sequence encoding the iRhom polypeptides shown herein as SEQ ID NOs: 1-8. It is appreciated that due to the degenerate nature of the genetic code, more than one nucleic acid sequence encodes a particular iRhom polypeptide or variant, and that such nucleic acid sequences may be expressed to produce the desired iRhom.

[00115] iRhom1 and iRhom2 variants generally have a sequence identity to the sequence of wild-type iRhom1 and iRhom2 as set forth in the present application of at least 50%, such as at least 60%, at least 70%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91 %, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%.

[00116] In one aspect, the number of alterations, e.g., substitutions, insertions, or deletions, in the iRhom variants of the present invention is 1 -20, e.g., 1-10 and 1-5, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 alterations compared to the corresponding wild-type iRhom1 or iRhom2.

[00117] **Mechanism of Action**

[00118] iRhoms participate in a diverse range of functions in a variety of species, including regulation of epidermal growth factor receptor (EGFR) signaling in *Drosophila*

melanogaster, survival of human squamous epithelial cancer cells, misfolded protein clearance from endoplasmic reticulum membranes in mammalian cell lines, induction of migration in primary mouse keratinocytes, secretion of soluble TNFα in mice, and regulation of substrate selectivity of stimulated ADAM17-mediated metalloprotease shedding in mouse embryonic fibroblasts.

[00119] In one embodiment, the present invention demonstrates that a spontaneous deletion within the *Rhbdf2* gene in mice underlies the curly bare (*cub*) mutation, in which loss of the cytosolic N-terminal domain of iRhom2 causes subsequent effects on hair-follicle development, wound healing and tumorigenesis. In another embodiment, the present invention demonstrates that iRhom2 is a short-lived protein but that gain-of-function mutations in the N-terminus or loss of the N-terminus increase mutant protein stability leading to metalloprotease-independent secretion of the EGFR ligand amphiregulin (AREG). Using a genetic modifier of the *cub* phenotype (*Mcub*), the present invention demonstrates that AREG is a physiological target of iRhom2. In yet another embodiment, key amino acids (H, C, Q, H) in the peptidase domain of iRhom2 that are necessary for AREG secretion were identified, indicating that the peptidase domain of this pseudoenzyme might be functional despite lacking a serine residue in the putative active site.

[00120] **Mutant forms of iRhom family members**

[00121] The present invention also relates to mutant forms of iRhom family members. In one embodiment the cytosolic N-terminus of iRhom1 or iRhom2 is deleted. The deletion of the cytosolic N-terminus activates the dormant proteolytic site within the peptidase domain of the iRhom polypeptide. In one embodiment, the cytosolic terminus of iRhom1 or iRhom2 is cleaved such that the transmembrane domain is proteolytically active. In one embodiment, mouse iRhom1 is cleaved between residues 1 and 272 and/or human iRhom1 is cleaved between residues 1 and 316 such that the transmembrane domain is proteolytically active. In another embodiment, mouse iRhom2 is cleaved between residues 1 and 268 and/or human iRhom2 is cleaved between residues 1 and 298 such that the transmembrane domain is proteolytically active.

[00122] The iRhom polypeptides may also comprise mutations in the proteolytic site of the transmembrane domain. In one embodiment, a mouse iRhom2 polypeptide comprises one or more mutations of key amino acids in the proteolytic site (peptidase domain) of the transmembrane domain selected from the group consisting of Histidine 635 on TM helix 2, Glutamine 695 on TM helix 4, Cysteine 701 on transmembrane helix 4 and Histidine 744

such that the transmembrane domain is proteolytically active. In another embodiment, a human iRhom2 polypeptide comprises one or more mutations in the proteolytic site of the transmembrane domain selected from the group consisting of Histidine 664 on TM helix 2, Glutamine 724 on TM helix 4, Cysteine 730 on transmembrane helix 4 and Histidine 773 on TM helix 6 such that the transmembrane domain is proteolytically active.

[00123] Screening Assays

[00124] The present invention provides methods for identifying a compound that alters the activity of an iRhom polypeptide, e.g., iRhom1 or iRhom2. The iRhom polypeptide may be a mutant iRhom polypeptide. For example, the iRhom polypeptide may comprise a deletion in its N-terminal cytoplasmic domain. The iRhom polypeptide may comprise a mutation within the transmembrane peptidase domain. In addition, the iRhom polypeptide may comprise a deletion in its N-terminal cytoplasmic domain and a mutation within the transmembrane peptidase domain.

[00125] In one aspect, methods of the invention generally include identifying a compound that activates the proteolytic activity of an iRhom polypeptide on a substrate. In one embodiment, the method includes contacting a cell expressing an iRhom polypeptide with a test compound and determining an increase in stability of the iRhom polypeptide relative to an appropriate control, wherein an increase in stability of the iRhom polypeptide indicates that the compound activates the proteolytic activity of the iRhom polypeptide on a substrate. In one embodiment, the substrate may be an EGFR ligand or an EGF-like substrate.

[00126] In another aspect, methods of the invention generally include identifying a compound that inhibits the proteolytic activity of an iRhom polypeptide on a substrate. In one embodiment, the method includes contacting a cell expressing an iRhom polypeptide with a test compound and determining a decrease in secretion of a physiological target of the iRhom polypeptide relative to an appropriate control or a decrease in EGFR activity relative to an appropriate control, wherein a decrease in secretion of a physiological target of the iRhom polypeptide or a decrease in EGFR activity indicates that the compound inhibits the proteolytic activity of the iRhom polypeptide on a substrate. In one embodiment, the substrate may be an EGFR ligand or an EGF-like substrate.

[00127] It is believed that Epidermal growth factor receptor (EGFR) signal transduction plays a major role in growth, proliferation and differentiation of mammalian cells. The present invention provides that iRhoms are short-lived proteins, but dominant mutations

increase their protein stability and stimulate secretion of specific EGF family ligand amphiregulin independently of metalloprotease activity. This invention demonstrates the significance of mammalian iRhoms in regulating an EGFR signaling event that promotes accelerated wound healing and triggers tumorigenesis. The invention also provides that iRhoms may regulate EGFR signaling in parallel with metalloproteases. Therefore, the invention provides a new strategy for using iRhoms as a novel therapeutic target in impaired wound healing and cancer.

[00128] Various aspects of the present invention relate to screening and assay methods and means, and substances identified thereby, for example, assays for compounds that activate or inhibit the proteolytic activity of an iRhom polypeptide on a substrate. The substrate may be an EGFR ligand or an EGF-like substrate.

[00129] Further assays are for a compound or substance that interacts with or binds an iRhom polypeptide and modulates i.e. increases, stimulates, reduces, inhibits or abolishes, its proteolytic activity.

[00130] Controls are well-known in the art and one of skill in the art would readily recognize an appropriate control and be able to determine an appropriate control for a method of the present invention with no more than routine experimentation.

[00131] According to aspects of a method for identifying a compound that modulates activity of an iRhom polypeptide according to aspects of the present invention which includes contacting a cell expressing an iRhom polypeptide with a test compound and determining the effect of the test compound on activity of the iRhom polypeptide, an appropriate control is determining the activity of the iRhom polypeptide when a cell expressing the iRhom polypeptide is not contacted with the test compound under the same or similar conditions.

[00132] An appropriate control may be a reference level of a variable such as activity of an iRhom polypeptide, level of stability of the iRhom polypeptide, level of secretion of a physiological target of the iRhom polypeptide, level of EGFR activity relative to an appropriate control and/or level of a soluble EGFR ligand, previously determined and stored in a print or electronic medium for recall and comparison to a determined effect of a test compound on activity of an iRhom polypeptide according to aspects of a method for identifying a compound that modulates activity of an iRhom polypeptide of the present invention.

[00133] An assay method for identifying a modulator of an iRhom polypeptide may include bringing into contact an iRhom polypeptide as described herein and a test compound,

determining binding of the test compound to the iRhom polypeptide and determining the proteolytic activity of the iRhom polypeptide in the presence and absence of a test compound which binds the iRhom polypeptide. Proteolytic activity may be determined by determining the cleavage of a substrate as described below. The iRhom polypeptide may be isolated or comprised in a liposome or cell.

[00134] A method of screening for and/or obtaining a substance/compound which modulates activity of an iRhom polypeptide may include contacting one or more test substances or compounds with the iRhom polypeptide in a suitable reaction medium, determining the activity of the treated polypeptide and comparing that activity with the activity of the polypeptide in comparable reaction medium untreated with the test substance or substances. The iRhom polypeptide may be in the reaction medium in an isolated form or may be comprised in a liposome or cell.

[00135] A difference in activity between the treated and untreated iRhom polypeptides is indicative of a modulating effect of the relevant test substance or substances, for example, an inhibiting or enhancing effect.

[00136] Activity of an iRhom polypeptide may be determined by determining the production of proteolytically cleaved substrate. The iRhom polypeptide may, for example, act on a membrane-bound substrate to generate a soluble product which is detected.

[00137] According to another aspect of the present invention there is provided an assay method for identifying and/or obtaining a modulator of an iRhom polypeptide, which method comprises: (a) bringing into contact an iRhom polypeptide and a test compound in the presence of a polypeptide substrate; and (b) determining proteolytic cleavage of the polypeptide substrate.

[00138] Cleavage of the substrate may be determined in the presence and absence of test compound. A difference in cleavage in the presence of the test compound relative to the absence of test compound may be indicative of the test compound being a modulator of iRhom protease activity.

[00139] Any polypeptide substrate which is proteolytically cleaved by an iRhom polypeptide may be used in an assay method as described herein. Such substrates are readily identified using standard techniques. The polypeptide substrate may be an EGFR ligand, such as an AREG or HB-EGF.

[00140] A suitable substrate may comprise a detectable label such as green fluorescent protein (GFP), luciferase or alkaline phosphatase. This allows convenient detection of the soluble cleaved product and is particularly useful in automated assays.

[00141] EGFR ligands suitable for use in the present assays are well characterized in the art and may have a structure comprising one or more Epidermal Growth Factor (EGF) domains and a single trans-membrane domain.

[00142] Preferably, suitable EGFR ligands have greater than 50% homology, greater than 60% homology, greater than 70% homology, greater than 80% homology greater than 90% homology or greater than 95% homology to a vertebrate EGFR ligand.

[00143] A chimeric ligand may have improved properties in methods described herein, for example it may be cleaved more efficiently by an iRhom polypeptide, have improved secretion properties or be more readily detected.

[00144] Another aspect of the present invention provides a chimeric EGFR ligand comprising sequence from two or more EGFR ligands, for example a chimeric ligand may comprise the transmembrane domain of a first EGFR ligand and the intracellular and extracellular domains of a second EGFR ligand. A chimeric substrate may further comprise a detectable label, such as luciferase, GFP or alkaline phosphatase.

[00145] Assay methods or other methods for obtaining or identifying modulators of iRhom activity according to the present invention may be in vivo cell-based assays, or in vitro non-cell-based assays.

[00146] Methods may be performed in the presence of Batimastat to inhibit the non-Rhomboid or non-iRhom dependent shedding of substrate and thereby decrease background.

[00147] In in vitro assays, the iRhom polypeptide may be isolated or contained in a liposome. Liposome based assays may be carried out using methods well-known in the art.

[00148] Suitable cell types for in vitro assays include mammalian cells such as mouse embryonic fibroblasts, keratinocytes, HEK293, CHO, HeLa and COS cells.

[00149] It is not necessary to use the entire full length proteins for in vitro or in vivo assays of the invention. Polypeptide fragments as described herein which retain the activity of the full length protein may be generated and used in any suitable way known to those of skill in the art. Suitable ways of generating fragments include, but are not limited to, recombinant expression of a fragment from encoding DNA. Such fragments may be generated by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be

operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Small fragments (e.g. up to about 20 or 30 amino acids) may also be generated using peptide synthesis methods which are well known in the art.

[00150] The precise format of the assay of the invention may be varied by those of skill in the art using routine skill and knowledge. For example, interaction between the polypeptides may be studied in vitro by labeling one with a detectable label and bringing it into contact with the other which has been immobilized on a solid support. Suitable detectable labels include 35S-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as a fusion protein containing an epitope that can be labeled with an antibody.

[00151] Fusion proteins may be generated that incorporate six histidine residues at either the N-terminus or C-terminus of the recombinant protein. Such a histidine tag may be used for purification of the protein by using commercially available columns which contain a metal ion, either nickel or cobalt. These tags also serve for detecting the protein using commercially available monoclonal antibodies directed against the six histidine residues.

[00152] Assays according to the present invention may take the form of in vitro assays. In vitro assays may be performed in a cell line such as a yeast strain, insect cell line or mammalian cell line in which the relevant polypeptides or peptides are expressed from one or more vectors introduced into the cell.

[00153] In assay and other methods according to such embodiments, an iRhom polypeptide may be contacted with the test compound in the presence of a substrate, such as an EGFR ligand. In such methods, the iRhom polypeptide and substrate may be present in a cell. This may be achieved, for example, by expressing the polypeptides from one or more expression vectors which have been introduced into the cell by transformation.

[00154] An assay method for identifying and/or obtaining a modulator of iRhom proteolytic activity may therefore include: (a) bringing into contact an iRhom polypeptide and a test compound in the presence of an EGFR ligand polypeptide; and (b) determining cleavage of the EGFR ligand.

[00155] Cleavage may be determined in the presence and absence of test compound. A difference in cleavage in the presence, relative to the absence of test compound is indicative of the compound being a modulator i.e. an enhancer or inhibitor of iRhom activity.

[00156] A nucleic acid encoding an iRhom polypeptide and/or polypeptide substrate as described above may be provided as part of a replicable vector, particularly any expression vector from which the encoded polypeptide can be expressed under appropriate conditions, and a host cell containing any such vector or nucleic acid. An expression vector in this context is a nucleic acid molecule including nucleic acid encoding a polypeptide of interest and appropriate regulatory sequences for expression of the polypeptide, in an in vitro expression system, e.g. reticulocyte lysate, or in vivo, e.g. in eukaryotic cells such as HEK, COS or CHO cells or in prokaryotic cells such as E. coli. This is discussed further below.

[00157] Combinatorial library technology provides an efficient way of testing a potentially vast number of different substances for ability to modulate activity of a polypeptide. Prior to or as well as being screened for modulation of activity, test substances may be screened for ability to interact with the polypeptide, e.g. in a yeast two-hybrid system (which requires that both the polypeptide and the test substance can be expressed in yeast from encoding nucleic acid). This may be used as a coarse screen prior to testing a substance for actual ability to modulate activity of the polypeptide.

[00158] The amount of test substance or compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.01 to 100 nM concentrations of putative inhibitor or activator compound may be used, for example from 0.1 to 10 nM. When cell-based assays are employed, the test substance or compound is desirably membrane permeable in order to access the iRhom polypeptide.

[00159] Test compounds may be natural or synthetic chemical compounds used in drug screening programs. Extracts of plants which contain several characterized or uncharacterized components may also be used. A further class of putative inhibitor or activator compounds can be derived from the iRhom polypeptide and/or a ligand which binds. Membrane permeable peptide fragments of from 5 to 40 amino acids, for example, from 6 to 10 amino acids may be tested for their ability to disrupt such interaction or activity.

[00160] In one embodiment, compounds for modulating (e.g., increase or decrease) the proteolytic activity of an iRhom polypeptide may be small molecule compounds. In another embodiment, the compound is cell-permeable. For example, the compound may be an ubiquitin protease inhibitor. In another embodiment, the compound is attached to a cell-penetrating peptide. The cell penetrating peptide may comprise a lysine or arginine rich sequence. For example, the cell penetrating peptide may be tat.

[00161] Other test compounds may be based on modeling the 3-dimensional structure of a polypeptide or peptide fragment and using rational drug design to provide potential inhibitor compounds with particular molecular shape, size and charge characteristics.

[00162] Following identification of a substance which modulates or affects polypeptide activity, the substance may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

[00163] Another aspect of the present invention provides the use of an iRhom polypeptide as described herein in a method for obtaining or identifying a modulator, for example an inhibitor, of iRhom proteolytic activity. Also provided are methods and uses of an iRhom polypeptide in the proteolytic cleavage of the transmembrane domain of a polypeptide substrate.

[00164] Modulators, in particular inhibitors of iRhom activity may be useful in the treatment of cancer, for example, esophageal, lung, brain, colon, kidney, prostate, skin, liver, pancreatic, stomach, uterine, ovarian, lymph glands or bladder cancer.

[00165] Modulators, in particular activators of iRhom activity may be useful in the acceleration of impaired wound healing in tissues such as skin, liver, heart, muscle or kidney.

[00166] Modulators of iRhom activity may also be useful in the treatment of inflammatory disorders or diseases, such as rheumatoid arthritis and autoimmune disease.

[00167] Thus, the present invention extends in various aspects not only to a substance identified as a modulator of iRhom activity, in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. for treatment (which may include preventative treatment) of a pathogenic infection or a condition associated with aberrant ErbB or EGF receptor activity, such as cancer, coronary atherosclerosis, psoriasis, wound healing, hair growth, curing and/or correcting balding, use of such a substance in manufacture of a composition for administration, e.g. for treatment of a pathogenic infection or a condition associated with aberrant ErbB or EGF receptor activity, such as cancer, coronary atherosclerosis, psoriasis, wound healing, hair growth, curing and/or correcting balding, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

[00168] A substance identified as a modulator of polypeptide or promoter function using an assay described herein may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many in vivo pharmaceutical uses. Accordingly, a mimetic or mimic of the substance (particularly if a peptide) may be designed for pharmaceutical use.

[00169] The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesize or where it is unsuitable for a particular method of administration.

[00170] Mimetic design, synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

[00171] The essential catalytic residues of polypeptides of the iRhom family correspond to Histidine 635 on TM helix 2, Glutamine 695 on TM helix 4, Cysteine 701 on TM helix 4, and Histidine 744 on TM helix 6 of the mouse iRhom2 polypeptide. The essential catalytic residues of polypeptides in the iRhom family also correspond to Histidine 664 on TM helix 2, Glutamine 724 on TM helix 4, Cysteine 730 on TM helix 4, and Histidine 773 on TM helix 6 of the human iRhom2 polypeptide.

[00172] A compound, polypeptide, peptide or substance able to modulate activity of a polypeptide according to the present invention may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

[00173] Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

[00174] A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

[00175] **Compounds of the Invention**

[00176] **Compounds that alter the proteolytic activity of iRhom polypeptides**

[00177] Compounds or test compounds of the invention may alter the proteolytic activity of iRhom polypeptides. In one embodiment, the compounds include activators or inhibitors of the proteolytic activity of iRhom polypeptides.

[00178] In one embodiment, the compound is a small molecule which binds an iRhom polypeptide. In another embodiment, the compound is a small molecule that inhibits the activity of an iRhom polypeptide. In yet another embodiment, the compound is a small molecule that activates the activity of an iRhom polypeptide. These small molecules may include, for example, peptides, peptidomimetics, organic compounds and the like. In one embodiment, the small molecule compound can translocate through plasma membranes and interact with the iRhom polypeptide. In some embodiment, the small molecule compound interacts with the cytoplasmic tail of the iRhom polypeptide. In other embodiments, the small molecule compound interacts with the proteolytic domain of the iRhom polypeptide.

[00179] In one embodiment, the compound is a ubiquitinase inhibitor.

[00180] In another embodiment, compounds that alter the proteolytic activity of an iRhom polypeptide may include, for example, a miRNA, a siRNA, a shRNA, a dsRNA or an antisense RNA directed to an iRhom DNA or mRNA, or a polynucleotide encoding the miRNA, siRNA, shRNA, dsRNA or antisense RNA, a vector comprising the polynucleotide. In yet another emboiment, compounds that alter the proteolytic activity of an iRhom polypeptide include, for example, antibodies, antibody fragments, a peptide or a polypeptide decoy. Additional compounds may be identified from combinatorial chemistry inhibitor libraries by screens, and then futher optimized through chemical alterations.

[00181] In another embodiment, the compound further comprises a cell penetrating peptide. The cell penetrating peptide, in one aspect, comprises a HIV-TAT peptide.

[00182] **Pharmaceutical Compositions**

[00183] The compounds on the invention that alter the proteolytic activity of an iRhom polypeptide may be administered using a pharmaceutical composition. Suitable pharmaceutical compositions comprise any one of the compounds described herein (or a pharmaceutically acceptable salt or ester thereof), and optionally comprise a pharmaceutically acceptable carrier. In certain embodiments, these compositions optionally further comprise one or more additional therapeutic agents.

[00184] Acceptable "pharmaceutical carriers" are well known to those of skill in the art and can include, but not be limited to any of the standard pharmaceutical carriers, such as

phosphate buffered saline, water and emulsions, such as oil/water emulsions and various types of wetting agents.

[00185] As used herein, the term "pharmaceutically acceptable salt" refers to those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts of amines, carboxylic acids, and other types of compounds, are well known in the art. For example, S.M. Berge, et al. describe pharmaceutically acceptable salts in detail in J. Pharmaceutical Sciences, 66: 1-19 (1977), incorporated herein by reference. The salts can be prepared in situ during the final isolation and purification of the compounds of the invention, or separately by reacting a free base or free acid function with a suitable reagent, as described generally below.

[00186] For example, a free base function can be reacted with a suitable acid. Furthermore, where the compounds of the invention carry an acidic moiety, suitable pharmaceutically acceptable salts thereof may, include metal salts such as alkali metal salts, e.g. sodium or potassium salts; and alkaline earth metal salts, e.g. calcium or magnesium salts. Examples of pharmaceutically acceptable, nontoxic acid addition salts are salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid or with organic acids such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid or malonic acid or by using other methods used in the art such as ion exchange. Other pharmaceutically acceptable salts include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, gluconate, hernisulfate, heptanoate, hexanoate, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like. Further pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations formed using

counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, lower alkyl sulfonate and aryl sulfonate.

[00187] The term "pharmaceutically acceptable ester", as used herein, refers to esters that hydrolyze in vivo and include those that break down readily in the human body to leave the parent compound or a salt thereof. Suitable ester groups include, for example, those derived from pharmaceutically acceptable aliphatic carboxylic acids, particularly alkanoic, alkenoic, cycloalkanoic and alkanedioic acids, in which each alkyl or alkenyl moiety advantageously has not more than 6 carbon atoms. Examples of particular esters include formates, acetates, propionates, butyrates, acrylates and ethylsuccinates.

[00188] As described above, the pharmaceutical compositions may additionally comprise a pharmaceutically acceptable carrier. Such a carrier includes any and all solvents, diluents, or other liquid vehicle, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's Pharmaceutical Sciences, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980) discloses various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional carrier medium is incompatible with the compound, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention. Some examples of materials which can serve as pharmaceutically acceptable carriers include, but are not limited to, sugars such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatine; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil, sesame oil; olive oil; corn oil and soybean oil; glycols; such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogenfree water; isotonic saline; Ringer's solution; ethyl alcohol, and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator.

[00189] Compositions for use in the present invention may be formulated to have any concentration of the compound desired. In some embodiments, the composition is formulated such that it comprises at least a therapeutically effective amount of the compound that alters proteolytic activity of the iRhom polypeptide. A therapeutically effective amount is an amount sufficient to achieve the desired therapeutic effect, under the conditions of administration, such as an amount sufficient to treat a cancer, accelerate wound healing or treat an inflammatory condition. In some embodiments, the composition is formulated such that it comprises an amount that would not cause one or more unwanted side effects. In certain embodiments, compositions are formulated so that the compound is present at a concentration of between about 1 mg/mL and about 20 mg/mL; between about 1 mg/mL and about 15 mg/mL; between about 1 mg/mL and about 10 mg/mL; between about 2 mg/mL and about 9 mg/mL; between about 3 mg/mL and about 8 mg/mL; between about 4 mg/mL and about 7 mg/mL; between about 4 mg/mL and about 6 mg/mL. In certain embodiments, compositions are formulated such that the compound is present at a concentration of about 5 mg/mL.

[00190] **Kits**

[00191] The invention also provides compositions and kits for prognosing the ability of a compound that alters the activity of the iRhom polypeptide to accelerate wound healing, treat cancer, or treat an inflammatory disease in a subject or for determining whether a cancer in a subject is sensitive to treatment with the compound. These kits include one or more of the following: reagents for obtaining and/or preparing samples, e.g., skin biopsy, tumor biopsy or blood samples; reagents for determining whether a sample exhibits iRhom proteolytic activity; probes and reagents for determining whether a sample exhibits a mutation in a gene, e.g., the *Rhbdf1* or *Rhbdf2* gene; probes and reagents for determining whether a sample exhibits a wild-type sequence of a gene, e.g., the *Rhbdf1* or *Rhbdf2* gene; reagents for determining the half-life of an iRhom polypeptide in a sample; reagents for determining the secretion of EGFR ligands by a sample; and instructions for use.

[00192] The kits of the invention may optionally comprise additional components useful for performing the methods of the invention. By way of example, the kits may comprise fluids (e.g., SSC buffer) suitable for annealing complementary nucleic acids or for binding an antibody with a protein with which it specifically binds, one or more sample compartments, an instructional material which describes performance of a method of the

invention, a sample of normal cells, a sample of cancer cells, a sample of wounded cells, a sample of inflamed cells and the like.

[00193] Indications

[00194] Wound healing

[00195] Wound healing is a process whereby the skin or another organ-tissue repairs itself after injury. In one embodiment, the present invention may be used to identify a compound that activates the proteolytic site of the iRhom polypeptide and thereby accelerate wound healing in a subject.

[00196] The methods of the invention may be used to identify a compound that accelerates wound healing in a subject. In one embodiment, the compound accelerates wound healing such that wound closure is achieved. In one embodiment, the wound may be an open cutaneous wound, such as a burn wound, a wound resulting from chemical (e.g., alkali) burn, a wound from physical trauma, neuropathic ulcers, pressure sores, venous stasis ulcers, and diabetic ulcers.

[00197] In another embodiment, the present invention provides compounds or pharmaceutical compositions that activate the proteolytic site of an iRhom polypeptide to accelerate, promote or enhance wound healing in a subject.

[00198] In some embodiments, the present invention provides for the use of any of the compounds described above or elsewhere herein to activate the proteolytic site of an iRhom polypeptide to accelerate healing of a wound or modulate one or more properties of cells in the wound environment or in the immediate vicinity of the wound. In some embodiments, the present invention provides for the use of the compositions described above to enhance healing of a wound or modulate one or more properties of cells in the wound environment or in the immediate vicinity of the wound.

[00199] Cancer

[00200] Compounds of the invention may be useful for treating cancer in a subject. The compounds of the invention may also be useful in reducing tumor growth and/or progression in a subject.

[00201] In one embodiment, the present invention may be used to identify a compound capable of reducing tumor growth and/or progression or treating cancer in a subject. In one embodiment, the compound inhibits the proteolytic site of the iRhom polypeptide and thereby decreases secretion of a physiological target of the iRhom polypeptide and/or decreases EGFR activity, and thereby reduces tumor growth and/or progression. In another

embodiment, the compound inhibits the proteolytic site of the iRhom polypeptide and thereby decreases secretion of a physiological target of the iRhom polypeptide and/or decreases EGFR activity, and is thereby useful in treating cancer.

[00202] In some embodiments, missense mutations in *RHBDF2* (p.I186T; p.P189L; p.D188N), the gene encoding iRhom2, may result in tylosis with human esophageal cancer, which is characterized by palmoplantar and oral hyperkeratosis.

[00203] The compounds of the invention may be useful in treating various cancers. In one embodiment, the cancer is an epithelial cancer. In another embodiment the cancer is cancer of the esophagus, lung, brain, colon, kidney, prostate, skin, liver, pancreas, stomach, uterus, ovary, breast, lymph glands or bladder.

[00204] The compounds of the invention may also be useful in treating various tumors. In one embodiment the tumor is a solid tumor.

[00205] **Inflammation**

[00206] Compounds of the invention may be useful for reducing inflammation in a subject. The compounds of the invention may be useful in treating inflammatory diseases and/or disorders. Inflammatory diseases and/or disorders, include, for example, systemic lupus erythematosus (SLE), lupus nephritis, cryoglobulinemia, vasculitis, rheumatoid arthritis, Sjoegren's syndrome, uveitis, Spondyloarthritis, miscarriage, preeclampsia, acne vulgaris, asthma, autoimmune diseases, celiac disease, chronic prostatitis, glomerulonephritis, hypersensitivities, inflammatory bowel diseases (including ulcerative colitis and Crohn's disease), Pelvic inflammatory disease, Reperfusion injury, Rheumatoid arthritis, Sarcoidosis, Transplant rejection, Vasculitis, Interstitial cystitis, Atherosclerosis, Allergies, Myopathies, leukocyte defects, psoriasis, multiple sclerosis and cancer

[00207] In one embodiment, the present invention may be used to identify a compound that is capable of reducing inflammation or treating an inflammatory disorder in a subject. In one embodiment the compound reduces the activity of proteolytic domain of the iRhom polypeptide such that there is a decrease in the secretion of a physiological target of the iRhom polypeptide and/or a decrease in TNFα secretion and thereby a decrease in inflammation. In another embodiment the compound reduces the activity of proteolytic domain of the iRhom polypeptide such that there is a decrease in the secretion of a physiological target of the iRhom polypeptide and/or a decrease in TNFα secretion and is thereby useful in treating an inflammatory disorder or disease. In yet anther embodiment, the compound of the invention reduces TNFα secretion by targeting the cytosolic domain or

transmembrane peptidase domain, and is thereby useful to reduce inflammation in the subject.

[00208] Various tests can be employed to assay the efficacy of a compound at treating an inflammatory disorder.

[00209] **Hair Growth**

[00210] Compounds of the invention may be useful for treating baldness and/or promoting hair growth in the subject. Hair loss or baldness (e.g., alopecia) is a loss of hair from the head or body. Baldness can refer to general hair loss, male pattern baldness, or hair loss in women. Some types of baldness can be caused by alopecia areata, an autoimmune disorder. The extreme forms of alopecia areata are alopecia totalis, which involves the loss of all head hair, and alopecia universalis, which involves the loss of all hair from the head and the body.

[00211] In one embodiment, the present invention is based on the discovery that the cub mutation causes balding. In another embodiment, the present invention is based on the discovery that the modifier of *cub* (*Mcub*) promotes hair growth, cures balding, corrects balding, or reduces balding (Example 1).

[00212] In one embodiment, the present invention may be used to identify a compound capable of promoting hair growth in a subject comprising: contacting a cell expressing an iRhom polypeptide with a test compound; and determining a decrease in secretion of a physiological target of the iRhom polypeptide relative to an appropriate control or a decrease in EGFR activity relative to an appropriate control, wherein a decrease in secretion of a physiological target of the iRhom polypeptide or a decrease in EGFR activity indicates that the compound is capable of promoting hair growth in the subject.

[00213] In another embodiment, the present invention may be used to identify a compounds capable of curing and/or correcting baldness in a subject comprising: contacting a cell expressing an iRhom polypeptide with a test compound; and determining a decrease in secretion of a physiological target of the iRhom polypeptide relative to an appropriate control or a decrease in EGFR activity relative to an appropriate control, wherein a decrease in secretion of a physiological target of the iRhom polypeptide or a decrease in EGFR activity indicates that the compound is capable of curing and/or correcting baldness in the subject.

[00214] This invention is further illustrated by the following examples which should not be construed as limiting. The entire contents of all references, patents and published patent applications cited throughout this application are hereby incorporated herein by reference

[00215] EXAMPLES

[00216] Example 1: The *cub* mutation leads to hyperactivation of the EGFR signaling pathway

[00217] The recessive mouse mutation named curly-bare (*cub*) is characterized by a hairless phenotype. A single copy of the dominant *Mcub* allele in combination with the *cub/cub* genotype results in a full, wavy coat rather than the hairless coat of *cub/cub mcub/mcub* mice. We investigated whether the *cub/cub mcub/mcub* genotype might be associated with aspects of altered EGFR signaling such as cell proliferation and cell migration.

[00218] We performed proliferation, cell migration (scratch-wound healing), and immunoblot assays on mouse embryonic fibroblasts (MEFs) isolated from *cub/cub mcub/mcub* and control (+/+ *mcub/mcub*) mice. Figures 1A-1F show that the *cub* mutation accelerates EGFR-related cell proliferation and migration along with cutaneous healing.

[00219] The *cub/cub mcub/mcub* MEFs had significantly higher rates of proliferation and migration relative to control MEFs (Figure 1A and 1B). These changes were associated with significant increases in the phosphorylation of canonical signal transduction proteins of the EGFR pathway, including, Akt, S6, mTOR, and p38 (Figure 1C). Further, we observed a significant reduction in cell-surface EGFR levels in *cub/cub mcub/mcub* MEFs (Figure 1C), indicating internalization and constitutive activation of EGFR signaling.

[00220] To test whether an increase in EGFR signaling corresponds to changes in epithelial proliferation, we conducted wound-healing assays in which we punched 2-mm through-and-through holes into the ears of 6–40-week-old *cub* mice and monitored the rate of closure over subsequent days (Figures 1D–1F). Within 14 days, *cub/cub mcub/mcub* mice showed accelerated closure compared with control +/+ *mcub/mcub* littermates. The +/*cub mcub/mcub* mice (which have a normal coat) also showed faster healing 14 days post-injury relative to control mice, see Figures 9A and 9B, indicating that a single mutant *cub* allele can trigger increased EGFR signaling, although not at levels high enough to block hair follicle induction. These data indicate that the *cub/cub mcub/mcub* genotype results in a hyperactive EGFR phenotype.

[00221] Example 2: *cub* is a mutation of the *Rhbdf2* gene, encoding iRhom2

[00222] Next, we examined the iRhom2 gene *Rhbdf2* as a candidate for the *cub* mutation. DNA sequencing of *cub/cub* mice identified a 12,681 base pair (bp) deletion in the *Rhbdf2* gene, which results in loss of exons 2–6 (Figure 2A). To test whether the deletion produces

an aberrant *Rhbdf2* transcript, we performed reverse-transcriptase PCR (RT-PCR) on RNA derived from wildtype and *cub/cub mcub/mcub* MEFs using primers designed to amplify exons 2, 5, 12 and 19. The *cub* transcript contained exons 12 and 19, but lacked exons 2 and 5 (Figure 2B). Further, quantitative RT-PCR (qPCR) on RNA from *cub/cub mcub/mcub* and +/+ *mcub/mcub* skin amplified *cub* transcripts in which exons 2–6 were deleted but the remaining exons were expressed, indicating that the rest of the *cub* gene is transcribed in *cub* mice (Figure 2C). To test whether the *cub* transcripts would result in splicing of exons 1 to 7, we performed qPCR with a probe specifically designed to amplify only transcripts containing exon 1 spliced to exon 7. Using this probe, we detected a PCR product in *cub/cub mcub/mcub* mice but not in +/+ *mcub/mcub* mice (Figure 2D). These findings confirm that *cub* is a mutation of the *Rhbdf2* gene, and will henceforth be referred to as $Rhbdf2^{cub}$.

[00223] Given that the normal translation initiation site in exon 3 is missing in $Rhbdf2^{cub}$ transcripts, we next tested whether these mutant transcripts could produce a mutant iRhom2 protein. Sequence analysis of $Rhbdf2^{cub}$ DNA revealed that the next in-frame translation initiation site (ATG) was in exon 8, which would result in a ~63.5 kDa protein. Since we lacked an antibody to iRhom2, we tested whether $Rhbdf2^{cub}$ transcripts could produce a protein product *in vitro* by cloning both full-length wildtype human *RHBDF2* cDNA (Hu*Wt*) as well as a version that mimicked the mutant $Rhbdf2^{cub}$ transcript (Hu*Cub*) into a C-terminal Flag-tagged expression vector. First, using immunoblotting, we determined that the Hu*Wt* clone generated a ~100 kDa protein product, whereas the Hu*Cub* mutant construct generated a ~67 kDa product, consistent with the expected molecular weight of the tagged proteins (Figure 2E). Furthermore, there was no evidence of any shortened protein products in the Hu*Wt* clone. Second, using immunostaining, we examined the localization patterns of both forms of protein after transfection of B6 MEFs (Figure 2F and Figures 10A and 10B) with either the Hu*Wt* or Hu*Cub* clones. Both forms were expressed in the endoplasmic reticulum, and no staining was observed in either the Golgi or nucleus (Figures 10A and 10B), indicating that the mutation does not lead to altered protein localization.

[00224] Example 3: Genetic non-complementation confirms that *cub* is a mutant allele of the *Rhbdf2* gene and a gain-of-function mutation

[00225] To test whether $Rhbdf2^{cub}$ is a gain-of-function rather than a null mutation, we generated *Rhbdf2* knockout ($Rhbdf2^{-/-}$) mice using ES cells from the Knockout Mouse Project (KOMP) repository, in which *lacZ* expression is under control of the endogenous *Rhbdf2* promoter (Figure 3A). *Rhbdf2* promoter–driven *lacZ* expression was predominantly observed

in the epidermis and the inner and outer sheath layers of hair follicles in the skin (Figure 3B). However, the wound healing (Figure 3C) and loss-of-hair (Figure 3D) phenotypes observed in the *Rhbdf2*$^{cub/cub}$ mice were not seen in *Rhbdf2*$^{-/-}$ mice, which appeared otherwise normal. An allele-test mating between *Rhbdf2*$^{cub/cub}$ and *Rhbdf2*$^{-/-}$ mice yielded compound mutant (*Rhbdf2*$^{-/cub}$) mice with a sparse hair coat (Figure 3E), indicating genetic non-complementation, and confirming that *cub* is a mutant allele of the *Rhbdf2* gene. Additionally, the level of hair growth in the *Rhbdf2*$^{-/cub}$ compound mutant mice was intermediate between *Rhbdf2*$^{cub/cub}$ and *Rhbdf2*$^{+/+}$ or *Rhbdf2*$^{-/-}$ mice, confirming that expression of the mutant *Rhbdf2*^{cub} protein product, rather than *Rhbdf2* deficiency, causes the *Rhbdf2*$^{cub/cub}$ phenotype.

[00226] Example 4: Genetic modifier of the *Rhbdf2*^{cub} phenotype (*Mcub*) is a loss-of-function mutation of the *Areg* gene

[00227] We next sought to map the mutation underlying the *Rhbdf2*^{cub} modifier gene (*Mcub*). We examined four EGFR ligand–encoding genes (*Epng, Ereg, Areg*, and *Btc)* as candidate loci for the *Mcub* mutation. By sequencing the exons and flanking regions of each gene in *Mcub/Mcub Rhbdf2*$^{cub/cub}$ mice, see Table II, we found that *Mcub* is a loss-of-function mutation in *Areg*, Figure 4A, a gene that encodes the autocrine keratinocyte growth factor amphiregulin (herein called AREG).

[00228] *Mcub* is a T-to-G point mutation that destroys the canonical donor splice site of exon 1 and leads to the exclusive use of an alternative downstream splice site that adds 22 extra nucleotides to the *Areg* transcript; this disrupts the coding frame and introduces a premature stop codon (Figure 11). This mutation will henceforth be referred to as *Areg*^{Mcub}. Notably, the hyperactive EGFR signaling (Figure 12A) and the rapid wound closure capability of *Rhbdf2*$^{cub/cub}$ mice is significantly reduced (Figures 4B and 4C), and the loss-of-hair phenotype prevented when a single copy of the dominant *Areg*^{Mcub} allele is present. The dominant *Areg*^{Mcub} mutation does not confer a normal hair coat to the *Rhbdf2*$^{cub/cub}$ mice, but rather a wavy hair phenotype, indicating remaining abnormalities in the EGFR pathway.

[00229] We next measured serum levels of AREG in *Rhbdf2*$^{cub/cub}$ *Areg*$^{+/+}$, *Rhbdf2*$^{cub/cub}$ *Areg*$^{Mcub/Mcub}$, and *Rhbdf2*$^{+/+}$ *Areg*$^{+/+}$ mice. We found no detectable AREG in the serum of *Rhbdf2*$^{cub/cub}$ *Areg*$^{Mcub/Mcub}$ mice, but did observe a dramatic increase in serum AREG levels in *Rhbdf2*$^{cub/cub}$ *Areg*$^{+/+}$ mice compared with *Rhbdf2*$^{+/+}$ *Areg*$^{+/+}$ mice (Figure 4D).

[00230] Measurements of supernatant AREG levels from cultured mouse epidermal keratinocytes (MEKs) of *Rhbdf2*$^{cub/cub}$ *Areg*$^{+/+}$, *Rhbdf2*$^{cub/cub}$ *Areg*$^{Mcub/Mcub}$, and *Rhbdf2*$^{+/+}$

Areg$^{+/+}$ mice yielded similar results (Figure 4E). AREG is abundantly expressed in normal skin; therefore, we next performed qPCR on skin samples from *Rhbdf2*$^{cub/cub}$ *Areg*$^{+/+}$ and *Rhbdf2*$^{+/+}$ *Areg*$^{+/+}$ mice to measure transcript levels of *Areg*, as well as six other genes known to encode EGFR ligands (*Egf*, *Tgfα*, *Btc*, *Epgn*, *Ereg* and *Hbegf*). Compared to controls, we observed a four-fold increase in *Areg*, and subtle but statistically significant increases in *Epgn* and *Hbegf* mRNAs, in *Rhbdf2*$^{cub/cub}$ *Areg*$^{+/+}$ mice. Also, there was a statistically significant decrease in *Btc* and *Egf* transcript levels (Figure 4F).

[00231] Lastly, to examine whether AREG mediates hyperactive EGFR phenotype, we silenced *Areg* expression in *Rhbdf2*$^{+/+}$ and *Rhbdf2*$^{cub/cub}$ MEFs (Figure 12B) using lentiviral shRNA and performed proliferation assays. While silencing of *Areg* had a subtle effect on proliferation of *Rhbdf2*$^{+/+}$ MEFs (Figure 12C), proliferation rates of *Rhbdf2*$^{cub/cub}$ MEFs were significantly reduced (Figure 12D). Taken together, these data suggest that enhanced AREG levels mediate the mutant phenotype of the *Rhbdf2*$^{cub/cub}$ mice via a gain of EGFR signaling. Furthermore, our data reveal that the *Rhbdf2*^{cub} phenotype is modified by *Areg*^{Mcub} expression.

[00232] Example 5: The peptidase domain of the N-terminal-truncated iRhom2 (*Rhbdf2*^{cub}) induces substrate-specific secretion of EGFR ligands independent of ADAM17

[00233] To test whether *Rhbdf2*^{cub}, with its short N-terminal domain, could induce secretion of AREG independently of metalloprotease activity, we performed *in vitro* cleavage assays in the presence or absence of marimastat (MM), a potent broad-spectrum metalloprotease inhibitor that can block both ADAM17- and ADAM10-dependent shedding of substrates. Because of the significant homology between the mouse and human *RHBDF2* genes we transfected 293T cells with human *AREG* either alone or with the human wildtype (Hu*Wt*) or human *Cub* (Hu*Cub*) *RHBDF2* genes (Figure 5A), and measured AREG levels in conditioned medium. In the absence of MM, we observed no difference in AREG levels between *AREG*-expressing and *AREG*/Hu*Cub* co-expressing cells, whereas co-expression of Hu*Wt* and *AREG* reduced AREG levels by ~60% (Figure 5B). In the presence of MM, AREG levels were approximately twofold higher in Hu*Cub*/*AREG* co-transfected cells compared with cells transfected with Hu*Wt* and *AREG* or *AREG* alone (Figure 5B). These data suggest that N-terminal-truncated iRhom2 can enhance AREG secretion, which only becomes apparent when metalloprotease activity is diminished.

[00234] ADAM17 activity can be measured in mice by examining tumor necrosis factor alpha (TNFα) secretion after stimulation with bacterial endotoxin lipopolysaccharide (LPS). We used this approach to test changes in ADAM17 activity in *Rhbdf2*^{cub} mice, by injecting LPS into *Rhbdf2*$^{cub/cub}$, *Rhbdf2*$^{-/-}$ and *Rhbdf2*$^{+/+}$ mice and measuring serum TNFα levels. We found that both LPS-injected *Rhbdf2*$^{cub/cub}$ and *Rhbdf2*$^{-/-}$ mice had a markedly lower induction of TNFα relative to LPS-injected *Rhbdf2*$^{+/+}$ mice (Figure 5C). These results suggest that ADAM17 activity is significantly attenuated in *Rhbdf2*$^{cub/cub}$ and *Rhbdf2*$^{-/-}$, and also implicate a role for the N-terminal domain of iRhom2 in regulation of ADAM17-dependent TNFα release. Notably, serum TNFα levels in LPS-stimulated *Rhbdf2*$^{cub/cub}$ mice were not completely abrogated, indicating that ADAM17 activity is attenuated but not eliminated. The observation that ADAM17 activity is attenuated in *Rhbdf2*$^{cub/cub}$ mice explains the non-lethal or non-inflammatory cutaneous phenotype in *Rhbdf2*$^{-/-}$ and *Rhbdf2*$^{cub/cub}$ mice compared with *Adam17*$^{-/-}$ mice or with transgenic mice overexpressing amphiregulin.

[00235] Next, the protease selectivity of the active rhomboid proteases for other EGF-like substrates, and whether these differed from Hu*Cub,* was assessed. We found that RHBDL2 selectively increased secretion of EGF but not AREG or HB-EGF compared with empty vector transfected controls (Figure 5D). In contrast, Hu*Cub* selectively increased secretion of AREG and HB-EGF but not EGF compared with empty vector transfected cells (Figures 5E–5G). These results suggest that the rhomboid peptidase domain confers substrate selectivity. The ability of N-terminal-truncated iRhom1 to induce AREG but not EGF secretion is consistent with this assertion (Figures 13A and 13B).

[00236] To determine how the iRhom2 peptidase domain helps to regulate the secretion of EGF-like substrates, we initially aligned sequences for amino acids of the peptidase domains of human and mouse iRhoms. We found a significant difference in protein sequence homology between the peptidase domains of human iRhom2 and RHBDL2 (Figure 13C). By contrast, the peptidase domains of iRhom1 and 2 show a 96% homology. Together, this suggests that the residues that form the active site or that play a role in substrate recognition differ significantly between active and inactive rhomboids. In addition, we found that deletion of the Hu*Cub* peptidase domain significantly diminished AREG secretion compared with native Hu*Cub* (Figure 5G), indicating that the peptidase domain is essential for secretion of EGF-like substrates. To identify the critical residues, we performed site-directed mutagenesis such that key residues in the Hu*Cub* peptidase domain were mutated to alanines. We found that glutamine-426 and cysteine-C432 in transmembrane domain four, and

histidines 366 and 475 in transmembrane domains two and six, respectively, were critical not only for mediating enhanced secretion of AREG/HB-EGF but also for suppression of EGF (Figure 5H, I). However, mutations in serines S362A, 402A and 425A, glutamic acid E436A, and glutamine Q439A did not alter AREG secretion (Figure 13D). Together, these results suggest that key residues (Figure 5J & Figure 13E) in the peptidase domain of N-terminal-truncated iRhom regulate the secretion of EGF-like substrates independent of metalloprotease activity.

[00237] Example 6: The N-terminal-truncated iRhom2 (*Rhbdf2*^{cub}) increases susceptibility to epithelial cancers

[00238] Missense mutations in *RHBDF2* (p.I186T, p.P189L, and p.D188N) may underlie a familial tylosis with esophageal cancer syndrome (TOC). To test whether an increased AREG secretion due to dominant N-terminal mutations in *RHBDF2* might drive these human pathologic changes *in vitro* by co-expressing *AREG* with a Hu*Wt* clone containing the human missense mutation (*RHBDF2* p.I186T), Hu*Cub*, or Hu*Wt* in 293T cells. Expression of the *RHBDF2* p.I186T or Hu*Cub* resulted in greater levels of AREG in conditioned medium and lower intracellular levels compared with Hu*Wt* (Figures 6A and 6B). Further, *RHBDF2* p.I186T produced AREG levels comparable to those produced by Hu*Cub*, indicating that loss of, or dominant mutations in, the iRhom2 N-terminus lead to increased AREG secretion. Additionally, we found that loss of at least one of four critical residues (H, C, Q, and H) in the peptidase domain of the *RHBDF2* p.I186T mutant resulted in significantly decreased AREG secretion (Figure 6C).

[00239] To determine whether the *Rhbdf2*^{cub} allele increases tumor susceptibility, we investigated how its expression would affect adenoma formation in *Apc*$^{Min/+}$ mice, a mouse model of human familial adenomatous polyposis. In *Apc*$^{Min/+}$ mice, spontaneous loss of one wildtype *Apc* allele induces intestinal epithelial adenoma formation and premature death at a median age of 169 days. Notably, *Rhbdf2* (Figure 6D) and *Areg* expression is observed in the small intestine, suggesting a potential functional relationship. We generated and observed *Apc*$^{Min/+}$ *Rhbdf2*$^{+/cub}$ and *Apc*$^{Min/+}$ *Rhbdf2*$^{+/+}$ mice, but because of increased lethality we could not generate enough *Apc*$^{Min/+}$ *Rhbdf2*$^{cub/cub}$ mice for meaningful comparison. Nonetheless, we did observe a significant difference in the median survival age of *Apc*$^{Min/+}$ *Rhbdf2*$^{+/+}$ (172 days) and *Apc*$^{Min/+}$ *Rhbdf2*$^{+/cub}$ (135 days) mice (Figure 6E). Necropsy of *Apc*$^{Min/+}$ *Rhbdf2*$^{+/+}$ and *Apc*$^{Min/+}$ *Rhbdf2*$^{+/cub}$ mice at 3 months of age revealed that the presence of a single *Rhbdf2*^{cub} allele significantly increased the number of polyps (Figures 6F and 6G) and

adenoma size (Figures 6F and 6H) in *Apc*$^{Min/+}$ mice, indicating that the *cub* mutation increases the growth of epithelial tumors. However, there was no spontaneous incidence of cancer in *Rhbdf2*$^{cub/cub}$ mice aged up to two years, indicating that *Rhbdf2*^{cub} mutation creates a conducive environment for, but alone does not drive, tumor development.

[00240] Example 7: Loss of the cytosolic N-terminus or dominant mutations in the N-terminus of the *RHBDF2* gene increase its protein stability

[00241] iRhom2 negatively regulates EGFR signaling by promoting degradation of EGF-like ligands through the proteasomal pathway. We determined that iRhoms induce secretion of AREG/HB-EGF when the cytosolic N-terminus is lacking (Figure 5). To test whether gain-of-function mutations in the amino terminus of iRhom2 interfere with proteasomal processing and thereby increase its stability, we examined whether the tylotic *RHBDF2* mutant p.I186T has an ability to interact with AREG. We found that, similar to Hu*Wt* and Hu*Cub,* p.I186T forms physical complexes with AREG (Figure 7A). We then compared the protein expression levels of Hu*Wt*, Hu*Cub* and p.I186T in 293T and COS7 cells by immunocytochemistry and flow cytometry. We observed that Hu*Wt* protein expression was significantly lower compared to both Hu*Cub* and p.I186T (Figure 7B,C). Further, when we subjected COS7 cells to a cyclohexamide (a protein synthesis inhibitor) chase for the indicated times, within one hour we observed an approximately 50% reduction in immunoreactivity for Hu*Wt* compared with either Hu*Cub* or the p.I186T (Figure 7D,E).

[00242] To test whether iRhom2 is a target for proteasomal degradation, we determined the protein half-life of Hu*Wt* and Hu*Cub* in the presence of a potent proteasomal inhibitor MG-132. Expectedly, the protein half-life of Hu*Wt,* but not Hu*Cub,* was significantly increased (Figure 7F), indicating that the proteasomal degradation of iRhom2 might be affecting its protein stability. We conclude that missense mutations in the amino terminus of iRhom2, similar to the *Rhbdf2*^{cub} mutation, increase its stability and contribute to enhanced AREG secretion independent of metalloprotease activity.

[00243] Example 8

[00244] Here we report that iRhom2, a member of a family of rhomboid proteases well known as regulators of EGFR signaling in *Drosophila*, has an ability to regulate EGFR signaling during cutaneous healing and tumor development. We show that iRhom2 is a short-lived protein whose stability can be increased by select mutations in the N-terminal domain. In turn, these stable variants function to enhance AREG secretion independent of metalloprotease activity. We identify an important role for iRhoms in EGFR-dependent cell

proliferation and wound healing and show how iRhom2 mutations that increase EGFR signaling, under the right circumstances, can promote cancer development.

[00245] The N-terminal and the peptidase domains have separate functions in regulating EGFR signaling

[00246] iRhoms are complex multi-domain enzymes that contain a long cytosolic N-terminus, a dormant peptidase domain and a conserved iRhom homology domain (IRHD); the function of these domains remains unknown. Under normal circumstances, iRhoms negatively regulate EGFR signaling by promoting the degradation of EGF-like substrates. However, the *Rhbdf2*^{cub} mutation is unlikely to be simply a loss-of-function mutation. *Rhbdf2*$^{-/-}$ mice failed to recapitulate the *Rhbdf2*^{cub} phenotype. In addition, we demonstrate that, similar to the *Rhbdf2*^{cub} mutation, dominant missense mutations in the N-terminus of iRhom2 induce secretion of AREG and HB-EGF, in a manner mediated by key amino acids in transmembrane helices two, four, and six of the peptidase domain. Thus, our results suggest that the cytosolic N-terminus of iRhom2 negatively regulates EGFR signaling by suppressing the peptidase domain and, consequently, secretion of AREG/HB-EGF (Figure 8). Our findings also reveal more subtle regulatory functions for iRhom2 that are unmasked in N-terminal mutations such as *Rhbdf2*^{cub}.

[00247] The *Rhbdf2*^{cub} may be considered a gain-of-function mutation. This conclusion is supported by several pieces of evidence. First, the negative regulatory role of iRhom2 seems to be minimal because *Rhbdf2* $^{-/-}$ mice do not present an overt 'EGFR hyperactive' phenotype except when combined with the *Rhbdf2*^{cub} mutation. Second, co-transfection of Hu*Cub* and *AREG* results in approximately 2–3-fold greater levels of AREG compared with transfections of either Hu*Wt* and *AREG* or *AREG* alone. Third, expression of Hu*Cub* induced secretion of membrane-anchored AREG and HB-EGF independent of metalloprotease activity. Fourth, mutant iRhom2 alleles fail to induce secretion of AREG/HB-EGF in the absence of the peptidase domain. Consistent with these observations, transgenic expression of the N-terminal-truncated but not full-length *RHBDF1* induces a strong EGFR signaling–related wing phenotype in *Drosophila*. Additionally, co-expression of truncated *RHBDF1* with *HB-EGF* intensifies the altered wing phenotype in *Drosophila*, indicating that the truncated iRhom1 might induce secretion of HB-EGF and thereby activate EGFR signaling. These results validate the concept that the cytosolic N-terminus contributes to iRhom-elicited ubiquitin processing of EGF family ligands, while the iRhom peptidase domain stimulates EGFR signaling when not suppressed by the N-terminus.

[00248] We found that mutant iRhom2 alleles induce secretion of AREG and HB-EGF in the presence of saturating concentrations of marimastat, a potent, broad-spectrum metalloprotease inhibitor. Maturation of other members of the ADAM family is unaffected by the deficiency of both iRhoms. Moreover, we demonstrate that ADAM17 activity is attenuated in *Rhbdf2*^{cub} mice. Thus, we conclude that *Rhbdf2*^{cub} and tylotic mutations selectively induce secretion of AREG and HB-EGF independent of metalloprotease activity, and the transmembrane peptidase domain is necessary for this function.

[00249] **A hyperactive EGFR pathway underlies accelerated cutaneous healing in *Rhbdf2*^{cub} mice**

[00250] The biology of wound healing is complex. In humans, adult wounds are vulnerable to non-functional fibrotic tissue formation, while prenatal wounds accomplish complete regeneration, resembling scar-free healing in vertebrates such as axolotls and planarians.

[00251] The tissue regeneration and remodeling field is still in its infancy. *In vitro* wound healing assays performed in a human keratinocyte cell line indicate that the expression of *RHBDL2* is significantly upregulated after wounding compared with unwounded controls.

[00252] The ability of *Rhbdf2*^{cub} mice to rapidly heal wounds without significant scar formation might be due to a combination of decreased TNFα secretion due to attenuated ADAM17 activity and rapid re-epithelialization induced by augmented AREG production/EGFR hyperactivation. We propose that while decreased TNFα contributes to a lesser degree of inflammation, increased AREG production facilitates accelerated proliferation and migration of keratinocytes to the wound site in *Rhbdf2*^{cub} mice. Moreover, iRhom2 is predominantly expressed in the skin, making it a potential new therapeutic target in impaired cutaneous wound healing.

[00253] **The iRhom2-AREG-EGFR pathway is constitutively active in some epithelial cancers**

[00254] In the present study, we find that N-terminal-truncated iRhom2 promotes increased AREG production independent of ADAM17 activity and thereby induces EGFR activation. In particular, the phenotype of *Apc*$^{Min/+}$ *Rhbdf2*$^{+/cub}$ mice recapitulates the increased susceptibility to epithelial cancers seen in patients with dominant *RHBDF2* mutations. However, *Rhbdf2*$^{cub/cub}$ mice did not spontaneously develop tumors, indicating that the *Rhbdf2*^{cub} mutation might not drive cancer development but instead might promote tumor growth and progression by creating a conducive environment.

[00255] MATERIALS AND METHODS

[00256] Mice were obtained, bred and maintained under modified barrier conditions at The Jackson Laboratory (Bar Harbor, ME). All genotypes, including *cub* and *Mcub,* were maintained on the C57BL/6J (B6) genetic background. To generate *Rhbdf2*$^{-/-}$ mice, ES cell clones (EPD0208_1_A09) obtained from the Knockout Mouse Program (KOMP) repository were injected into B6-*Tyr*^{c} (B6 albino) blastocysts. Males displaying >50% chimerism were mated to B6 albino females; black offspring were genotyped by PCR. Heterozygotes were mated with each other to produce homozygotes, or with *Rhbdf2*$^{cub/cub}$ mice to produce *Rhbdf2*$^{-/cub}$ mice. The Animal Care and Use Committee at The Jackson Laboratory approved all the experimental procedures.

[00257] Cell culture and Reagents- We isolated MEFs as previously described. Mouse epidermal keratinocytes (MEKs) were isolated according the manufacturer's instructions (CELLnTEC/Zenbio, Research Triangle Park, NC). Cells were grown in a humidified chamber at 370C with 5% CO2. MEF reagents [Phosphate buffered saline (PBS), HyClone Dulbeccos Modified Eagles Medium, High Glucose (DMEM), penicillin-streptomycin and HyClone fetal bovine serum) and MEK reagents (progenitor cell targeted medium CnT-07, antibiotic/antimycotic solution ABM, and protease Dispase) were purchased from Fisher Scientific (Boston, MA) and Zen-Bio (Research Triangle Park, NC), respectively. TrypLETM Select was obtained from Life technologies (Chicago, IL). 293T and COS7 cells were grown in DMEM and RPMI medium, respectively.

[00258] Histology- Preparation of the tissues for histological examination was performed as previously described.

[00259] Ear hole closure: Mice were wounded using a surgical ear punch device Napox KN-292B to make 2 mm through-and-through holes in the center of the left ear, and the closure rates were analyzed over a period of 28 days. At the indicated times, mice were euthanized and images of the ear holes and surrounding tissue were captured using the 4x objective (Olympus BX41 microscope). ImageJ software analyzed the wound area. Ears were fixed in 10% neutral buffered formalin (NBF) for 24 h at room temperature and processed for paraffin embedding and sectioning. The physical wound area was measured at day 0 and measurements at all subsequent time points were calculated as a percent of the original area. Proliferation and scratch-wound assays: Both sets of assays were performed using MEFs. For the proliferation assays, the indicated numbers of cells were grown in collagen-coated 96-well plates at 37^{0}C. After 24h of incubation, the growth medium was removed and the plates

were frozen at -80^{0}C until use. CyQUANT® Cell Proliferation kit was used to determine the relative cell numbers (Life technologies). Fluorescence intensity was measured using Victor^3 multilabel plate reader (PerkinElmer, Boston, MA). For the scratch-wound assays, MEFs were grown to confluence in collagen-coated 6-well plates and a strip of cells was removed from the confluent monolayer by drawing a sterile p200 pipette tip across the well. Scratches were imaged at 0, 3, 6, 9 and 12 h using the 5x objective on a Zeiss Observer phase contrast microscope and Axio Vision 4.6.3 software. Using the GRID plugin we overlaid a grid on each image. The grid size was such that there were at least 10 lines bisecting the scratch image. The widths for each scratch were compiled and averaged. ImageJ software analyzed the captured images from the 0, 3, 6, 9 and 12 h time series.

[00260] Human AREG, HB-EGF and EGF ELISA: Transient transfections were performed in 293T cells seeded in 24-well plates. Using lipofectamine LTX with Plus reagent 16.5 fmol of HuWt or HuCub was co-transfected with 25 ng of AREG or 50 ng of HB-EGF or 200 ng of EGF. The total amount of transfected DNA was made up to equal amount per well with pCMV6-AC-HA vector. After 24h, DMSO or 10 μM MM in 1ml of fresh medium replaced the old medium. After 24 h, 100 μl of supernatant was either undiluted (HB-EGF and EGF) or diluted five-fold (AREG) and subjected to ELISA as per the manufacturer's instructions (DY262, DY259, and DY236).

[00261] Expression plasmids containing cDNA encoding human *AREG* (RC203150), human *HB-EGF* (SC108485), human *EGF* (SC127840), human *RHBDF2* (RC203923), mouse *Rhbdf1* (MC205414), and human *RHBDL2* (RC219882) were obtained from OriGene (Rockville, MD). Mutations (deletions or insertions) in the full-length *RHBDF2* or mouse *Rhbdf1* were introduced using the QuikChange II XL mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions and verified by DNA sequencing. Primers for site-directed mutagenesis were generated using the Primer Design Program (QuikChange® Primer Design, Agilent Technologies).

[00262] Immunochemistry: MEFs plated in BD BioCoat™ Collagen I 2-well culture slides were grown to ~60% confluence and transiently transfected with Lipofectamine™ LTX (Life technologies) according to the manufacturer's instructions. After 48 h, MEFs were fixed in acetone and subjected to immunocytochemistry. Briefly, cells washed in 1X ice-cold PBS were fixed in acetone at -20^{0}C for 10 min followed by three additional washes in PBS. Endogenous peroxidase activity was blocked with 3% H2O2, followed by incubations in blocking buffer (PBST, 10% goat serum and 1% BSA) for 1h and DDK-specific antibody

(Origene) at 4^0C overnight. The following day, cells were washed in PBST for 25 min and incubated in HRP-conjugated secondary antibody for 30 min at room temperature. After three washes in PBST, cells were incubated in DAPI solution (IHC World, Woodstock, MD) for 10 min before cover slipping and sealing the slides. Calnexin and giatin antibodies were obtained from Abcam (Cambridge, MA). Secondary antibodies were obtained from Life technologies.

[00263] Immunoprecipitation: 293T and COS7 cell transfections were carried according to the manufacturer's instructions (Life technologies). Cells plated in 6 well plates were treated with 400 µls of Cell Lysis Buffer (Cell Signaling, Danvers, MA) containing complete mini protease inhibitors (Roche Applied Science, Indianapolis, IN), incubated on ice for 10 min and centrifuged at 14,000g for 10 min in a cold room. Cell supernatant was incubated overnight with 20 µls of magnetic beads conjugated with Anti-DDK antibody (Origene) in a cold room on a rocking platform. Following overnight incubation, beads/slurry was washed with three times with TBS-T, and protein was collected using 2X SDS-PAGE loading buffer.

[00264] SDS-PAGE and Immunobloting: Cells were washed once with ice cold PBS and incubated with RIPA buffer (Cell Signaling, Danvers, MA) containing complete mini protease inhibitors (Roche Applied Science, Indianapolis, IN) on ice for 10 min. Cell lysate was centrifuged at 14,000g for 10 min at 4^0 C. Supernatant was collected and stored at -80^0 C until further use. Total amount of protein was quantified using a Qubit Fluorometer (Life technologies), and 10 µg of protein was loaded onto 4-12% pre-cast gels (Lonza, Allendale, NJ) and run at 140V until the low molecular weight Precision plus protein standard (Bio-Rad) reached the bottom of the gel. Resolved proteins were electrophoretically transferred onto PVDF membranes using an iBLOT (Life technologies) before blocking in 5% BSA (Cell Signaling Technology) in TBST (20 mM Tris, 137 mM sodium chloride, and 0.1% Tween-20) for 1h at room temperature. Membranes were then incubated overnight at 4^0C in primary antibodies (1:1000) followed by three 15 min washes in TBST and incubation in secondary antibodies (1:2000) for 1h at room temperature. After another wash in TBST for 90 min, membranes were exposed to SuperSignal West Femto Substrate (Fisher Scientific) for 5 min for detection of horseradish peroxidase generated signal. Membranes were re-probed with actin-specific antibody. All the antibodies were purchased from Cell Signaling Technology (Danvers, MA). DDK-antibody was obtained from Origene (Rockville, MD).

[00265] Amphiregulin silencing in primary MEFs: Murine *Areg* shRNA clone TRCN0000089050 (in lentiviral vector pLKO.1) and scramble control shRNA in pLKO.1

(plasmid #1864) were obtained from Sigma Aldrich (St. Louis, MO) and Addgene (Cambridge, MA), respectively. Lentiviral particles were produced in 293T/17 cells (ATCC) using plasmids pMDLg/pRRE, pRSV-rev, and pMD2.g (Addgene). Lentiviral supernatants were collected at 48h and 60h, combined, and filtered through 0.45 μm PVDF filters. MEFs were infected twice on successive days with lentiviral supernatants in the presence of 5 μg/ml polybrene, followed by selection with puromycin (2 μg/ml) for 72 hours prior to being used in downstream experiments.

[00266] Polyp counts and tumor area- Intestinal tracts removed from 12-week-old euthanized mice were flushed and fixed in 10% neutral buffered formalin for at least 24 h and subjected to polyp counting or histological examination. H&E stained slides were scanned using a digital scanner NanoZoomer 2.0HT (Hamamatsu, Japan). Polyps were either counted directly under a dissecting microscope or from the scanned digital images. Notably, there were no significant differences in polyp counts between the two methods. Tumor area was also measured from the scanned digital images using the NanoZoomer 2.0HT software.

[00267] Quantitative real-time PCR (qPCR)- Purified RNA from skin was isolated as per the manufacturer's instructions using the TRIzol® Plus RNA purification kit (Life Technologies, Chicago, IL). The Agilent 2100 Bioanalyzer determined the quality of purified RNA. qPCR cycling conditions have been published previously. We obtained the following list of predesigned TaqMan gene expression assays from Life Technologies (Chicago, IL), Table I.

Table I

Gene	Assay	Spanning Exons
Rhbdf2	*Mm00553469_m1*	*2 – 3*
Rhbdf2	*Mm00553471_m1*	*4 – 5*
Rhbdf2	*Mm01248820_g1*	*7 – 8*
Rhbdf2	*Mm00553476_m1*	*9 – 10*
Rhbdf2	*Mm00553479_g1*	*12 – 13*
Rhbdf2	*Mm01248816_g1*	*14 – 15*
Rhbdf2	*Mm01248818_g1*	*17 – 18*
Areg	*Mm00437583_m1*	-
Hbegf	*Mm00439307_m1*	-
Ereg	*Mm00514794_m1*	-

Epgn	*Mm00504344_m1*	-
Tgfa	*Mm00446232_m1*	-
Egf	*Mm00438696_m1*	-
Btc	*Mm00432137_m1*	-
Actb	*4352933E (Part #)*	-

[00268] Mouse TNFα and AREG ELISA– For mouse TNFα ELISA, 8-12 week-old female mice of the indicated genotype were injected intravenously with 70 μg of Lipopolysaccharide from *E.coli* O111:B4 (Sigma-Aldrich, St. Louis, MO). Serum was collected after 3h and frozen at -80^{0}C. For AREG ELISA, serum collected from all genotypes was used immediately or stored not more than 24h at -20^{0}C. Samples were assayed using DuoSet ELISA Developmental kits (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions (DY989 and DY410).

[00269] Flow cytometry- 293T and COS7 cells plated in 6 well plates were transiently transfected with 600 ng of Hu*Wt* or Hu*Cub or* Hu*Wt* p.I186T cDNAs using Lipofectamine™ LTX reagent. After 40h, cells were incubated in 150 μg/ml of cyclohexamide for 0, 0.5, 1, 2 and 4h. Following that, cells were fixed in 150 μls of freshly prepared cold 4% PFA at RT for 40 min. Cells were washed once with 2ml PBST and resuspended in 150 μls of PBST for 15 min at RT in the dark. After another wash, cells were resuspended in 125 μls of PBST containing phycoerythrin-labeled anti-DDK primary antibody (1:50) on ice for 30 min. After a final wash, cells were resuspended in FACS buffer and subjected to flow cytometry analysis (BD FACSCalibur).

[00270] Reverse transcriptase PCR (RT-PCR)- RNA was extracted from MEFs using RNAqueousTM-4PCR (Ambion) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA using reverse transcriptase (Promega reverse transcription systems, Promega). cDNA was amplified using specific primers with HotMaster TAQ (5-Prime Inc.).

[00271] Statistical analysis- GraphPad Prism v4 (GraphPad Software, San Diego, CA) was used to generate Kaplan-Meier survival curves and calculate protein half-life (One phase exponential decay equation). One-way ANOVA and t-tests determined statistical differences. P value less than 0.05 was considered statistically significant.

[00272] Items

[00273] 1. A method for identifying a compound that activates the proteolytic activity of an iRhom polypeptide on a substrate comprising:

a) contacting a cell expressing an iRhom polypeptide with a test compound; and

b) determining an increase in stability of the iRhom polypeptide relative to an appropriate control, wherein an increase in stability of the iRhom polypeptide indicates that the compound activates the proteolytic activity of the iRhom polypeptide on a substrate.

[00274] 2. The method of item 1, wherein an increase in stability of the iRhom polypeptide is determined by analyzing the half-life of the iRhom polypeptide following exposure to the compound.

[00275] 3. The method of item 1, wherein the substrate is an EGFR ligand or an EGF-like substrate.

[00276] 4. The method of item 1, wherein an increase in stability of the iRhom polypeptide is determined by detecting an increase in secretion of an EGFR ligand.

[00277] 5. The method of item 4, wherein the EGFR ligand is selected from the group consisting of AREG, HB-EGF, TGFα and EPGN.

[00278] 6. The method of item 1, wherein an increase in stability of an iRhom polypeptide is determined by detecting an increase in the level of soluble EGFR ligands.

[00279] 7. The method of item 6, wherein the EGFR ligand is selected from the group consisting of AREG, HB-EGF, TGFα and EPGN.

[00280] 8. The method of item 1, wherein an increase in stability of an iRhom polypeptide is determined by detecting an increase in EGFR signaling activity.

[00281] 9. The method of item 1, wherein the compound inhibits an interaction between the iRhom polypeptide and a proteasome.

[00282] 10. The method of item 1, wherein the compound inactivates the cytoplasmic domain of an iRhom polypeptide.

[00283] 11. The method of item 10, wherein the inactivation of the cytoplasmic domain is transient.

[00284] 12. The method of item 1, wherein the compound cleaves and/or deletes the cytoplasmic domain of an iRhom polypeptide such that the polypeptide has proteolytic activity or altered biological activity.

[00285] 13. The method of item 12, wherein mouse iRhom2 is cleaved between amino acid residues 1 and 268 or human iRhom2 is cleaved between amino acid residues 1 and 298.

[00286] 14. The method of item 12, wherein mouse iRhom1 is cleaved between amino acid residues 1 and 272 or human iRhom1 is cleaved between amino acid residues 1 and 316.

[00287] 15. The method of item 1, wherein the compound activates the peptidase domain of an iRhom polypeptide.

[00288] 16. The method of any one of item 1-15, wherein the iRhom polypeptide is iRhom1 or iRhom2.

[00289] 17. The method of item 15, wherein the iRhom polypeptide is a human or mouse iRhom polypeptide.

[00290] 18. The method of item 1, wherein the compound is selected from the group consisting of a small molecule, a peptide or a polypeptide decoy.

[00291] 19. The method of item 18, wherein the compound is attached to a cell penetrating peptide.

[00292] 20. The method of item 1, wherein the compound accelerates migration of keratinocytes.

[00293] 21. The method of item 1, wherein the compound accelerates proliferation of fibroblasts.

[00294] 22. The method of item 1, wherein the compound is cell-permeable.

[00295] 23. The method of item 1, wherein the compound is an ubiquitin protease inhibitor.

[00296] 24. A method for identifying a compound that inhibits the proteolytic activity of an iRhom polypeptide on a substrate comprising:

a) contacting a cell expressing an iRhom polypeptide with a test compound; and

b) determining a decrease in secretion of a physiological target of the iRhom polypeptide relative to an appropriate control or a decrease in EGFR activity relative to an appropriate control, wherein a decrease in secretion of a physiological target of the iRhom polypeptide or a decrease in EGFR activity indicates that the compound inhibits the proteolytic activity of the iRhom polypeptide on a substrate.

[00297] 25. The method of item 24, wherein the substrate is an EGFR ligand or an EGF-like substrate.

[00298] 26. The method of item 24, wherein the inhibition of proteolytic activity of the iRhom polypeptide is determined by detecting a decrease in secretion of a physiological target of the iRhom polypeptide.

[00299] 27. The method of item 26, wherein the physiological target of the iRhom polypeptide is an EGFR ligand.

[00300] 28. The method of item 27, wherein the EGFR ligand is selected from the group consisting of AREG, HB-EGF, TGFα and EPGN.

[00301] 29. The method of item 24, wherein the inhibition of proteolytic activity of the iRhom polypeptide is determined by detecting a decrease in the level of soluble EGFR ligands.

[00302] 30. The method of item 29, wherein the EGFR ligand is selected from the group consisting of AREG, HB-EGF, TGFα and EPGN.

[00303] 31. The method of item 24, wherein the inhibition of proteolytic activity of an iRhom polypeptide is determined by detecting a decrease in EGFR activity.

[00304] 32. The method of item 24, wherein the compound inhibits the peptidase domain of an iRhom polypeptide.

[00305] 33. The method of item 24, wherein the compound affects the activity of an iRhom polypeptide by inactivating one or more amino acid residues in the proteolyic site of the iRhom polypeptide peptidase domain.

[00306] 34. The method of item 33, wherein the iRhom polypeptide is mouse iRhom2 and the compound inactivates one or more amino acid residues in the proteolytic site of mouse iRhom2 selected from the group consisting of Histidine 635 on TM helix 2, Glutamine 695 on TM helix 4, Cysteine 701 on transmembrane helix 4 and Histidine 744 on TM helix 6.

[00307] 35. The method of item 33, wherein the iRhom polypeptide is human iRhom2 and the compound inactivates one or more amino acid residues in the proteolytic site of human iRhom2 selected from the group consisting of Histidine 664 on TM helix 2, Glutamine 724 on TM helix 4, Cysteine 730 on transmembrane helix 4 and Histidine 773 on TM helix 6.

[00308] 36. The method of any one of items 24-33, wherein the iRhom family member is iRhom1 or iRhom2.

[00309] 37. The method of item 33, wherein the iRhom family member is a human or mouse iRhom family member.

[00310] 38. The method of item 24, wherein the compound is selected from the group consisting of a small molecule, a peptide, or a polypeptide decoy.

[00311] 39. The method of item 38, wherein the compound is attached to a cell penetrating peptide.

[00312] 40. The method of item 24, wherein the compound is cell-permeable.

[00313] 41. A method for identifying a compound capable of accelerating wound healing or tissue repair in a subject comprising: a) contacting a cell expressing an iRhom polypeptide and an EGFR ligand with a test compound; b) determining an increase in stability of the iRhom polypeptide relative to an appropriate control; and c) determining an increase in secretion of an EGFR ligand by the cell relative to an appropriate control, wherein an increase in stability of the iRhom polypeptide and an increase in secretion of an EGFR ligand by the cell indicates that the compound is capable of accelerating wound healing.

[00314] 42. The method of item 41, wherein the iRhom family member is iRhom1 or iRhom2.

[00315] 43. The method of item 41, wherein the iRhom family member is a human or mouse iRhom family member.

[00316] 44. A method for identifying a compound capable of reducing tumor growth and/or progression or treating cancer in a subject comprising: a) contacting a cell expressing an iRhom polypeptide with a test compound; and b) determining a decrease in secretion of a physiological target of the iRhom polypeptide relative to an appropriate control or a decrease in EGFR activity relative to an appropriate control, wherein a decrease in secretion of a physiological target of the iRhom polypeptide or a decrease in EGFR activity indicates that the compound is capable of reducing tumor growth and/or progression or treating cancer in the subject.

[00317] 45. The method of item 44, wherein the tumor is a solid tumor.

[00318] 46. The method of item 44, wherein the cancer is an epithelial cancer.

[00319] 47. The method of item 44, wherein the cancer is cancer of the esophagus, lung, brain, colon, kidney, prostate, skin, liver, pancreas, stomach, uterus, ovary, breast, lymph glands or bladder.

[00320] 48. A method for identifying a compound that inhibits the cytoplasmic domain of an iRhom polypeptide comprising: a) contacting a cell expressing an iRhom polypeptide with a test compound; b) determining an increase in stability of the iRhom polypeptide relative to an appropriate control, wherein an increase in stability of the iRhom polypeptide indicates that the compound inhibited the cytoplasmic domain of the iRhom polypeptide.

[00321] 49. The method of any one of items 44-48, wherein the iRhom family member is iRhom1 or iRhom2.

[00322] 50. The method of any one of items 44-48, wherein the iRhom family member is a human or mouse iRhom family member.

[00323] 51. The method of item 41, wherein the compound is selected from the group consisting of a small molecule, a polypeptide decoy, an miRNA molecule, an siRNA molecule, an shRNA molecule, a dsRNA molecule, an antisense molecule, a ribozyme specific for *Rhbdf2*; or a polynucleotide encoding the miRNA, siRNA, shRNA, dsRNA; or a biological equivalent of each thereof.

[00324] 52. An isolated polypeptide comprising a variant iRhom polypeptide.

[00325] 53. An isolated polypeptide comprising human iRhom2 with a deletion of the cytosolic N-terminal domain.

[00326] 54. An isolated polypeptide comprising mouse iRhom2 with a deletion of the cytosolic N-terminal domain.

[00327] 55. An isolated mouse iRhom2 polypeptide comprising a mutation at one or more amino acid residues in the proteolytic site of mouse iRhom2 selected from the group consisting of Histidine 635 on TM helix 2, Glutamine 695 on TM helix 4, Cysteine 701 on TM helix 4 and Histidine 744 on TM helix 6.

[00328] 56. An isolated human iRhom2 polypeptide comprising a mutation at one or more amino acid residues in the proteolytic site of human iRhom2 selected from the group consisting of Histidine 664 on TM helix 2, Glutamine 724 on TM helix 4, Cysteine 730 on TM helix 4 and Histidine 773 on TM helix 6.

[00329] 57. An isolated nucleic acid molecule encoding the polypeptide of any one of claims 52-56.

[00330] 58. A vector comprising the nucleic acid molecule of claim 57.

[00331] 59. A host cell expressing the vector of claim 58.

[00332] 60. A method for identifying a compound capable of promoting hair growth in a subject comprising: a) contacting a cell expressing an iRhom polypeptide with a test compound; and b) determining a decrease in secretion of a physiological target of the iRhom polypeptide relative to an appropriate control or a decrease in EGFR activity relative to an appropriate control, wherein a decrease in secretion of a physiological target of the iRhom polypeptide or a decrease in EGFR activity indicates that the compound is capable of promoting hair growth in the subject.

[00333] **SEQUENCES**

[00334] **Mouse iRhom2 protein sequence full-length SEQ ID NO:1**

MASADKNGSNLPSVSGSRLQSRKPPNLSITIPPPESQAPGEQDSMLPERRKNPAYLKS
VSLQEPRGRWQEGAEKRPGFRRQASLSQSIRKSTAQWFGVSGDWEGKRQNWHRRS
LHHCSVHYGRLKASCQRELELPSQEVPSFQGTESPKPCKMPKIVDPLARGRAFRHPD
EVDRPHAAHPPLTPGVLSLTSFTSVRSGYSHLPRRKRISVAHMSFQAAAALLKGRSVL
DATGQRCRHVKRSFAYPSFLEEDAVDGADTFDSSFFSKEEMSSMPDDVFESPPLSAS
YFRGVPHSASPVSPDGVHIPLKEYSGGRALGPGTQRGKRIASKVKHFAFDRKKRHYG
LGVVGNWLNRSYRRSISSTVQRQLESFDSHRPYFTYWLTFVHIIITLLVICTYGIAPVG
FAQHVTTQLVLKNRGVYESVKYIQQENFWIGPSSIDLIHLGAKFSPCIRKDQQIEQLV
RRERDIERTSGCCVQNDRSGCIQTLKKDCSETLATFVKWQNDTGPSDKSDLSQKQPS
AVVCHQDPRTCEEPASSGAHIWPDDITKWPICTEQAQSNHTGLLHIDCKIKGRPCCIG
TKGSCEITTREYCEFMHGYFHEDATLCSQVHCLDKVCGLLPFLNPEVPDQFYRIWLS
LFLHAGIVHCLVSVVFQMTILRDLEKLAGWHRISIIFILSGITGNLASAIFLPYRAEVGP
AGSQFGLLACLFVELFQSWQLLERPWKAFFNLSAIVLFLFICGLLPWIDNIAHIFGFLS
GMLLAFAFLPYITFGTSDKYRKRALILVSLLVFAGLFASLVLWLYIYPINWPWIEYLT
CFPFTSRFCEKYELDQVLH

[00335] ***Rhbdf2^{cub}*** **mouse mutation SEQ ID NO:2**

MSSMPDDVFESPPLSASYFRGVPHSASPVSPDGVHIPLKEYSGGRALGPGTQRGKRIA
SKVKHFAFDRKKRHYGLGVVGNWLNRSYRRSISSTVQRQLESFDSHRPYFTYWLTF
VHIIITLLVICTYGIAPVGFAQHVTTQLVLKNRGVYESVKYIQQENFWIGPSSIDLIHLG
AKFSPCIRKDQQIEQLVRRERDIERTSGCCVQNDRSGCIQTLKKDCSETLATFVKWQN
DTGPSDKSDLSQKQPSAVVCHQDPRTCEEPASSGAHIWPDDITKWPICTEQAQSNHT
GLLHIDCKIKGRPCCIGTKGSCEITTREYCEFMHGYFHEDATLCSQVHCLDKVCGLLP
FLNPEVPDQFYRIWLSLFLHAGIVHCLVSVVFQMTILRDLEKLAGWHRISIIFILSGITG
NLASAIFLPYRAEVGPAGSQFGLLACLFVELFQSWQLLERPWKAFFNLSAIVLFLFICG
LLPWIDNIAHIFGFLSGMLLAFAFLPYITFGTSDKYRKRALILVSLLVFAGLFASLVLW
LYIYPINWPWIEYLTCFPFTSRFCEKYELDQVLH

[00336] **Mouse iRhom1 protein sequence full-length SEQ ID NO:3**

MSEARRDSTSSLQRKKPPWLKLDIPAAVPPAAEEPSFLQPLRRQAFLRSVSMPAETAR
VPSPHHEPRRLVLQRQTSITQTIRRGTADWFGVSKDSDSTQKWQRKSIRHCSQRYGK
LKPQVIRELDLPSQDNVSLTSTETPPPLYVGPCQLGMQKIIDPLARGRAFRMADDTAD
GLSAPHTPVTPGAASLCSFSSSRSGFNRLPRRRKRESVAKMSFRAAAALVKGRSIRDG
TLRRGQRRSFTPASFLEEDMVDFPDELDTSFFAREGVLHEEMSTYPDEVFESPSEAAL
KDWEKAPDQADLTGGALDRSELERSHLMLPLERGWRKQKEGGPLAPQPKVRLRQE
VVSAAGPRRGQRIAVPVRKLFAREKRPYGLGMVGRLTNRTYRKRIDSYVKRQIEDM
DDHRPFFTYWLTFVHSLVTILAVCIYGIAPVGFSQHETVDSVLRKRGVYENVKYVQQ
ENFWIGPSSEALIHLGAKFSPCMRQDPQVHSFILAARE REKHSACCVRNDRSGCVQTS
KEECSSTLAVWVKWPVHPSAPDLAGNKRQFGSVCHQDPRVCDEPSSEDPHEWPEDI
TKWPICTKSSAGNHTNHPHMDCVITGRPCCIGTKGRCEITSREYCDFMRGYFHEEAT
LCSQVHCMDDVCGLLPFLNPEVPDQFYRLWLSLFLHAGILHCLVSVCFQMTVLRDLE
KLAGWHRIAIIYLLSGITGNLASAIFLPYRAEVGPAGSQFGILACLFVELFQSWQILAR
PWRAFFKLLAVVLFLFAFGLLPWIDNFAHISGFVSGLFLSFAFLPYISFGKFDLYRKRC
QIIIFQVVFLGLLAGLVVLFYFYPVRCEWCEFLTCIPFTDKFCEKYELDAQLH

[00337] Mouse iRhom1 N-terminal truncated protein sequence (equivalent to *Rhbdf2*^{cub} mutation- No cytosolic domain but retains transmembrane peptidase domain) SEQ ID NO:4

MSTYPDEVFESPSEAALKDWEKAPDQADLTGGALDRSELERSHLMLPLERGWRKQK
EGGPLAPQPKVRLRQEVVSAAGPRRGQRIAVPVRKLFAREKRPYGLGMVGRLTNRT
YRKRIDSYVKRQIEDMDDHRPFFTYWLTFVHSLVTILAVCIYGIAPVGFSQHETVDSV
LRKRGVYENVKYVQQENFWIGPSSEALIHLGAKFSPCMRQDPQVHSFILAARELREKH
SACCVRNDRSGCVQTSKEECSSTLAVWVKWPVHPSAPDLAGNKRQFGSVCHQDPR
VCDEPSSEDPHEWPEDITKWPICTKSSAGNHTNHPHMDCVITGRPCCIGTKGRCEITS
REYCDFMRGYFHEEATLCSQVHCMDDVCGLLPFLNPEVPDQFYRLWLSLFLHAGIL
HCLVSVCFQMTVLRDLEKLAGWHRIAIIYLLSGITGNLASAIFLPYRAEVGPAGSQFGI
LACLFVELFQSWQILARPWRAFFKLLAVVLFLFAFGLLPWIDNFAHISGFVSGLFLSF
AFLPYISFGKFDLYRKRCQIIIFQVVFLGLLAGLVVLFYFYPVRCEWCEFLTCIPFTDKF
CEKYELDAQLH

[00338] Human iRhom2 protein sequence full-length SEQ ID NO:5

MASADKNGGSVSSVSSSRLQSRKPPNLSITIPPPEKETQAPGEQDSMLPEGFQNRRLK
KSQPRTWAAHTTACPPSFLPKRKNPAYLKSVSLQEPRSRWQESSEKRPGFRRQASLS
QSIRKGAAQWFGVSGDWEGQRQQWQRRSLHHCSMRYGRLKASCQRDLELPSQEAP
SFQGTESPKPCKMPKIVDPLARGRAFRHPEEMDRPHAPHPPLTPGVLSLTSFTSVRSG
YSHLPRRKRMSVAHMSLQAAAAALLKGRSVLDATGQRCRVVKRSFAFPSFLEEDVVD
GADTFDSSFFSKEEMSSMPDDVFESPPLSASYFRGIPHSASPVSPDGVQIPLKEYGRAP
VPGPRRGKRIASKVKHFAFDRKKRHYGLGVVGNWLNRSYRRSISSTVQRQLESFDSH
RPYFTYWLTFVHVIITLLVICTYGIAPVGFAQHVTTQLVLRNKGVYESVKYIQQENFW
VGPSSIDLIHLGAKFSPCIRKDGQIEQLVLRERDLERDSGCCVQNDHSGCIQTQRKDCS
ETLATFVKWQDDTGPPMDKSDLGQKRTSGAVCHQDPRTCEEPASSGAHIWPDDITK
WPICTEQARSNHTGFLHMDCEIKGRPCCIGTKGSCEITTREYCEFMHGYFHEEATLCS
QVHCLDKVCGLLPFLNPEVPDQFYRLWLSLFLHAGVVHCLVSVVFQMTILRDLEKL
AGWHRIAIIFILSGITGNLASAIFLPYRAEVGPAGSQFGLLACLFVELFQSWPLLERPW
KAFLNLSAIVLFLFICGLLPWIDNIAHIFGFLSGLLLAFAFLPYITFGTSDKYRKRALILV
SLLAFAGLFAALVLWLYIYPINWPWIEHLTCFPFTSRFCEKYELDQVLH

[00339] Human truncated iRhom2 (equivalent to *Rhbdf2*^{cub} mutation- No cytosolic domain but retains transmembrane peptidase domain) SEQ ID NO:6

MSSMPDDVFESPPLSASYFRGIPHSASPVSPDGVQIPLKEYGRAPVPGPRRGKRIASKV
KHFAFDRKKRHYGLGVVGNWLNRSYRRSISSTVQRQLESFDSHRPYFTYWLTFVHVI
ITLLVICTYGIAPVGFAQHVTTQLVLRNKGVYESVKYIQQENFWVGPSSIDLIHLGAK
FSPCIRKDGQIEQLVLRERDLERDSGCCVQNDHSGCIQTQRKDCSETLATFVKWQDD
TGPPMDKSDLGQKRTSGAVCHQDPRTCEEPASSGAHIWPDDITKWPICTEQARSNHT
GFLHMDCEIKGRPCCIGTKGSCEITTREYCEFMHGYFHEEATLCSQVHCLDKVCGLL
PFLNPEVPDQFYRLWLSLFLHAGVVHCLVSVVFQMTILRDLEKLAGWHRIAIIFILSGI
TGNLASAIFLPYRAEVGPAGSQFGLLACLFVELFQSWPLLERPWKAFLNLSAIVLFLFI
CGLLPWIDNIAHIFGFLSGLLLAFAFLPYITFGTSDKYRKRALILVSLLAFAGLFAALV
LWLYIYPINWPWIEHLTCFPFTSRFCEKYELDQVLH

[00340] Human iRhom1 protein sequence full-length SEQ ID NO:7

MSEARRDSTSSLQRKKPPWLKLDIPSAVPLTAEEPSFLQPLRRQAFLRSVSMPAETAH
ISSPHHELRRPVLQRQTSITQTIRRGTADWFGVSKDSDSTQKWQRKSIRHCSQRYGKL
KPQVLRELDLPSQDNVSLTSTETPPPLYVGPCQLGMQKIIDPLARGRAFRVADDTAEG
LSAPHTPVTPGAASLCSFSSSRSGFHRLPRRRKRESVAKMSFRAAAAALMKGRSVRDG
TFRRAQRRSFTPASFLEEDTTDFPDELDTSFFAREGILHEELSTYPDEVFESPSEAALKD
WEKAPEQAD
LTGGALDRSELERSHLMLPLERGWRKQKEGAAAPQPKVRLRQEVVSTAGPRRGQRI
AVPVRKLFAREKRPYGLGMVGRLTNRTYRKRIDSFVKRQIEDMDDHRPFFTYWLTF
VHSLVTILAVCIYGIAPVGFSQHETVDSVLRNRGVYENVKYVQQENFWIGPSSEALIH
LGAKFSPCMRQDPQVHSFIRSARE REKHSACCVRNDRSGCVQTSEEECSSTLAVWVK
WPIHPSAPELAGHKRQFGSVCHQDPRVCDEPSSEDPHEWPEDITKWPICTKNSAGNH
TNHPHMDCVITGRPCCIGTKGRCEITSREYCDFMRGYFHEEATLCSQVHCMDDVCGL
LPFLNPEVPDQFYRLWLSLFLHAGILHCLVSICFQMTVLRDLEKLAGWHRIAIIYLLSG
VTGNLASAIFLPYRAEVGPAGSQFGILACLFVELFQSWQILARPWRAFFKLLAVVLFL
FTFGLLPWIDNFAHISGFISGLFLSFAFLPYISFGKFDLYRKRCQIIIFQVVFLGLLAGLV
VLFYVYPVRCEWCEFLTCIPFTDKFCEKYELDAQLH

[00341] Human iRhom1 N-terminal truncated protein sequence (equivalent to *Rhbdf2*^{cub} Mutation- No cytosolic domain but retains transmembrane peptidase domain)

SEQ ID NO:8

MLPLERGWRKQKEGAAAPQPKVRLRQEVVSTAGPRRGQRIAVPVRKLFAREKRPYG
LGMVGRLTNRTYRKRIDSFVKRQIEDMDDHRPFFTYWLTFVHSLVTILAVCIYGIAPV
GFSQHETVDSVLRNRGVYENVKYVQQENFWIGPSSEALIHLGAKFSPCMRQDPQVH
SFIRSARE REKHSACCVRNDRSGCVQTSEEECSSTLAVWVKWPIHPSAPELAGHKRQ
FGSVCHQDPRVCDEPSSEDPHEWPEDITKWPICTKNSAGNHTNHPHMDCVITGRPCCI
GTKGRCEITSREYCDFMRGYFHEEATLCSQVHCMDDVCGLLPFLNPEVPDQFYRLW
LSLFLHAGILHCLVSICFQMTVLRDLEKLAGWHRIAIIYLLSGVTGNLASAIFLPYRAE
VGPAGSQFGILACLFVELFQSWQILARPWRAFFKLLAVVLFLFTFGLLPWIDNFAHIS
GFISGLFLSFAFLPYISFGKFDLYRKRCQIIIFQVVFLGLLAGLVVLFYVYPVRCEWCEF
LTCIPFTDKFCEKYELDAQLH

Table II: Primers used to amplify *exons* of *mcub* candidate genes *Epgn, Ereg, Areg,* and *Btc* to compare V/Le strain DNA sequence with other strains. The Mcub allele originated from the V/Le strain

Genomic sequence of exons and flanking sequence					
		Primer sequence	**Exon**	**Product size**	
Forward	Epgn1F	AAAACCCTCCACCC TTCC	Exon1	182	SEQ ID NO:9
Reverse	Epgn1R	CGGGGAACAACTCT GAATCC			SEQ ID NO:10
Forward	Epgn2F	AGAGCCACTTTGGG TCTGTC	Exon2	222	SEQ ID NO:11

Reverse	Epgn2R	ACGCAACGTTCTGTCAAAAG			SEQ ID NO:12
Forward	Epgn3F	TGTATTGAACATGTATTATGAAGTTGG	Exon3	245	SEQ ID NO:13
Reverse	Epgn3R	CAACTGTGCTGTGTGCATCC			SEQ ID NO:14
Forward	Epgn4F	CCTTGTGCCTTAGCAAATGT	Exon4	351	SEQ ID NO:15
Reverse	Epgn4R	AGACACACACATCAACAATAGGG			SEQ ID NO:16
Forward	Epgn5-1F	TCCATCACCATCAACACACAG	Exon5_1	546	SEQ ID NO:17
Reverse	Epgn5-1R	CCCTGAAATTCCATAAGGACAG			SEQ ID NO:18
Forward	Epgn5-2F	TCAATCAAGTCCAAGTAATGCC	Exon5_2	530	SEQ ID NO:19
Reverse	Epgn5-2R	ATTCCACATGCTGTTGTTCC			SEQ ID NO:20
Forward	Epgn5-3F	TCACACTTGGTATCCATCGC	Exon5_3	549	SEQ ID NO:21
Reverse	Epgn5-3R	AAAAGAAATAAGCCCATGGTTC			SEQ ID NO:22
Forward	Ereg1F	CTAGTAAGTCCTCGCGTGCC	Exon1	319	SEQ ID NO:23
Reverse	Ereg1R	GTCCTATGACAAATGCACCG			SEQ ID NO:24
Forward	Ereg2F	AACGAAAGTTGCTTTCCTGG	Exon2	225	SEQ ID NO:25
Reverse	Ereg2R	TTCATTGGCATGTACTTATATTGAC			SEQ ID NO:26
Forward	Ereg3F	GCTATACAGCCAGAGTCCCAG	Exon3	244	SEQ ID NO:27
Reverse	Ereg3R	CAGAAGAGGCGAGGAAAGTG			SEQ ID NO:28
Forward	Ereg4F	GGTTTCTTATATTGCAGTGGGG	Exon4	355	SEQ ID NO:29
Reverse	Ereg4R	CGCAGATTCCACTTTGTTCC			SEQ ID NO:30
Forward	Ereg5-1F	TGTCTGGGCATTTTACATAAGC	Exon5_1	612	SEQ ID NO:31
Reverse	Ereg5-1R	TGTGCCAAGCCATAATTCAG			SEQ ID NO:32
Forward	Ereg5-2F	TGACATTCCTGCGAGCTTAG	Exon5_2	566	SEQ ID NO:33
Reverse	Ereg5-2R	CAAGATCTGGTTAGCGCCC			SEQ ID NO:34
Forward	Ereg5-3F	TCTGAAGACAAATT	Exon5	598	SEQ ID

		TGAACCCC	_3		NO:35
Reverse	Ereg5-3R	TTCCATGGGTATGG ATAATGAAG			SEQ ID NO:36
Forward	Ereg5-4F	GGTGCCCAGATTCA AAATG	Exon5 _4	716	SEQ ID NO:37
Reverse	Ereg5-4R	TAGTGCACCAGAAT GCCTTG			SEQ ID NO:38
Forward	Ereg5-5F	AAGTGCCAAGGGAC CAATC	Exon5 _5	560	SEQ ID NO:39
Reverse	Ereg5-5R	TACCCCAAGGTTCT TGCATC			SEQ ID NO:40
Forward	Ereg5-6F	GAAAACAGGCGTTT TAGATATTGG	Exon5 _6	561	SEQ ID NO:41
Reverse	Ereg5-6R	CACGGAGTGGATCT ACTGGAC			SEQ ID NO:42
Forward	Ereg5-7F	CACTATACTGAGAA CCAAAGCAACC	Exon5 _7	549	SEQ ID NO:43
Reverse	Ereg5-7R	AAGTGCTGTTTATG CTCCCC			SEQ ID NO:44
Forward	Ereg5-8F	CAAATTTGTCACGT TTGGTTC	Exon5 _8	605	SEQ ID NO:45
Reverse	Ereg5-8R	CCGACACTACTTTC AAGCCC			SEQ ID NO:46
Forward	Areg1F	TCCAGCGGGTCTAT AAAAGC	Exon1	387	SEQ ID NO:47
Reverse	Areg1R	GACGTTTGGGGAAT GTCAAG			SEQ ID NO:48
Forward	Areg2F	TGTCTACACAGCAA TGCTCAC	Exon2	362	SEQ ID NO:49
Reverse	Areg2R	CTTTGCAGAGGTAA AACCCG			SEQ ID NO:50
Forward	Areg3F	AAAAGAGAACGTGG GCAGTC	Exon3	339	SEQ ID NO:51
Reverse	Areg3R	CCTTGGTTTACACA CTGGAAAAG			SEQ ID NO:52
Forward	Areg4F	GTATGTTTGAGATG CAGAAAACC	Exon4	284	SEQ ID NO:53
Reverse	Areg4R	TGACAGTAGAGTCT GATAGGAATACTG			SEQ ID NO:54
Forward	Areg5F	GTCACAGGAGGAAA GTCCCC	Exon5	283	SEQ ID NO:55
Reverse	Areg5R	CTTCTTTTGCCTTGA GATAAGTG			SEQ ID NO:56
Forward	Areg6F	TGGTGGTTTTCTATA AGAATATTTAGC	Exon6	462	SEQ ID NO:57
Reverse	Areg6R	AAGCCCTTGTAAAT GATTGTCC			SEQ ID NO:58

Forward	Btc1F	AAGGTTCACAGGAC TCCAGC	Exon1	395	SEQ ID NO:59
Reverse	Btc1R	AAGTGGTGTGCCTC TCCG			SEQ ID NO:60
Forward	Btc2F	AAAAGGATTAAGCA TTTGGGG	Exon2	232	SEQ ID NO:61
Reverse	Btc2R	TGGGTCATGACACT AAAAGGAC			SEQ ID NO:62
Forward	Btc3F	TTTCCAATGCTGTCA AACCC	Exon3	249	SEQ ID NO:63
Reverse	Btc3R	GAATCAATGAATAA AACGGGG			SEQ ID NO:64
Forward	Btc4F	TGGGAAGAGGAGA AAAGCAAG	Exon4	278	SEQ ID NO:65
Reverse	Btc4R	GAAAACAGTGCTAA TTACTTCCAAAG			SEQ ID NO:66
Forward	Btc5F	ATTAGACATGGTGA AGCCCC	Exon5	241	SEQ ID NO:67
Reverse	Btc5R	TTTGCATACTGACA GGAACTCAC			SEQ ID NO:68
Forward	Btc6-1F	TGCTGAGCAGACAG TCACG	Exon6 _1	541	SEQ ID NO:69
Reverse	Btc6-1R	TTGGGCTCATGTGC GAG			SEQ ID NO:70
Forward	Btc6-2F	TGCTTTGGTCAGTC AACCAG	Exon6 _2	582	SEQ ID NO:71
Reverse	Btc6-2R	TTTTCCCACTCTTGT GGTCC			SEQ ID NO:72
Forward	Btc6-3F	TTCCCATTGGCTGCT GTAAG	Exon6 _3	533	SEQ ID NO:73
Reverse	Btc6-3R	CCAACTGAACCACA CTTTGC			SEQ ID NO:74
Forward	Btc6-4F	TCCATGAAGTCAAT CCTGAAG	Exon6 _4	533	SEQ ID NO:75
Reverse	Btc6-4R	GAGGCAGGCAAATC TCTGTG			SEQ ID NO:76
Forward	Btc6-5F	TGGTTGGTTGGTTG GTTAGG	Exon6 _5	567	SEQ ID NO:77
Reverse	Btc6-5R	TGAAGCACAAAAGC CAACTC			SEQ ID NO:78
Genotyping *Mcub* mutation of *Areg* gene					
Forward	Mcub-1F	CGGTGGAACCAATG AGAACT		197	SEQ ID NO:79
Reverse	Mcub-1R	CTCACCCTCCTAGC ATGAGC			SEQ ID NO:80

		after *HphI* digestion	
		wildtype	118 bp

				+ 79 bp	
			mutant	197 bp (uncut)	
cDNA primers (within *Areg* exons)					
Forward	ARwex1F	CGGTGGAACCAATG AGAACT		236	SEQ ID NO:81
Reverse	ARwex2R	GTCGTAGTCCCCTG TGGAGA			SEQ ID NO:82
Forward	ARwex1F	CGGTGGAACCAATG AGAACT		385	SEQ ID NO:83
Reverse	ARwex3R	TTGCCTCCCTTTTTC TTCCT			SEQ ID NO:84
Forward	ARwex1F	CGGTGGAACCAATG AGAACT		535	SEQ ID NO:85
Reverse	ARwex4R	CCACACCGTTCACC AAAGTA			SEQ ID NO:86
Forward	ARwex1F2	GTTGCTGCAGAGAC CGAGAC		265	SEQ ID NO:87
Reverse	ARwex2R	GTCGTAGTCCCCTG TGGAGA			SEQ ID NO:88
Forward	ARwex2F	CTGGCAGTGAACTC TCCACA		331	SEQ ID NO:89
Reverse	ARwex4R	CCACACCGTTCACC AAAGTA			SEQ ID NO:90

[00342] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for identifying a compound that modulates activity of an iRhom polypeptide comprising:

contacting a cell expressing an iRhom polypeptide with a test compound; and

determining an effect of the test compound on activity of the iRhom polypeptide.

2. The method of claim 1, wherein determining the effect of the test compound on activity of the iRhom polypeptide comprises determining stability of the iRhom polypeptide compared to an appropriate control.

3. The method of claim 1, wherein determining the effect of the test compound on activity of the iRhom polypeptide comprises determining a level of secretion of a physiological target of the iRhom polypeptide compared to an appropriate control.

4. The method of claim 1, wherein determining the effect of the test compound on activity of the iRhom polypeptide comprises determining a level of EGFR activity relative to an appropriate control.

5. The method of claim 1, wherein determining the effect of the test compound on activity of the iRhom polypeptide comprises determining a level of a soluble EGFR ligand compared to an appropriate control.

6. The method of any one of claims 1-5, wherein activity of the iRhom polypeptide is proteolytic activity to cleave a substrate.

7. The method of claim 1, wherein determining the effect of the test compound on activity of the iRhom polypeptide comprises detecting proteolytic activity of the iRhom polypeptide to proteolytically cleave a substrate compared to an appropriate control.

8. The method of claim 2, wherein a decrease in stability of the iRhom polypeptide indicates that the compound decreases proteolytic activity of the iRhom

polypeptide, and wherein an increase in stability of the iRhom polypeptide indicates that the compound increases proteolytic activity of the iRhom polypeptide.

9. The method of claim 3, wherein an increase in secretion of the physiological target of the iRhom polypeptide indicates that the compound increases proteolytic activity of the iRhom polypeptide, and wherein a decrease in secretion of the physiological target of the iRhom polypeptide indicates that the compound inhibits proteolytic activity of the iRhom polypeptide.

10. The method of claim 4, wherein an increase in EGFR activity indicates that the compound increases proteolytic activity of the iRhom polypeptide to proteolytically cleave a substrate, and wherein a decrease in EGFR activity indicates that the compound inhibits proteolytic activity of the iRhom polypeptide.

11. The method of claim 5, wherein an increase in the soluble EGFR ligand indicates that the compound increases proteolytic activity of the iRhom polypeptide, and wherein a decrease in a soluble EGFR ligand indicates that the compound inhibits proteolytic activity of the iRhom polypeptide.

12. The method of any of claims 1-11, wherein a decrease in proteolytic activity of the iRhom polypeptide indicates that the compound is capable of one or more of: reducing tumor growth, reducing tumor progression, treatment of cancer and promoting hair growth in a subject, and an increase in proteolytic activity of the iRhom polypeptide indicates that the compound is capable of accelerating wound healing in a subject.

13. The method of any one of claims 1-12, wherein the cell expresses an EGFR ligand.

14. The method of claim 6, 7 or 10, wherein the substrate is an EGFR ligand or an EGF-like substrate.

15. The method of claim 3 or 9, wherein the physiological target is an EGFR ligand.

16. The method of claim 5, 11, 13, 14 or 15, wherein the EGFR ligand is selected from the group consisting of: AREG, HB-EGF, TGFα and EPGN.

17. The method of any one of claims 1-16, wherein the cell is in vitro.

18. The method of any one of claims 1-17, wherein the iRhom polypeptide is iRhom1 or iRhom2.

19. The method of any one of claims 1-18, wherein the iRhom polypeptide is a human or mouse iRhom.

20. The method of any one of claims 1-19, wherein the test compound is selected from the group consisting of: a small molecule, a peptide, a polypeptide decoy, an miRNA molecule, an siRNA molecule, an shRNA molecule, a dsRNA molecule, an antisense molecule, a ribozyme specific for *Rhbdf2*; or a polynucleotide encoding an miRNA, siRNA, shRNA, dsRNA; a combination of any two or more thereof; and a biological equivalent of any one or more thereof.

21. A method for identifying a compound that modulates activity of an iRhom polypeptide substantially as shown or described herein.

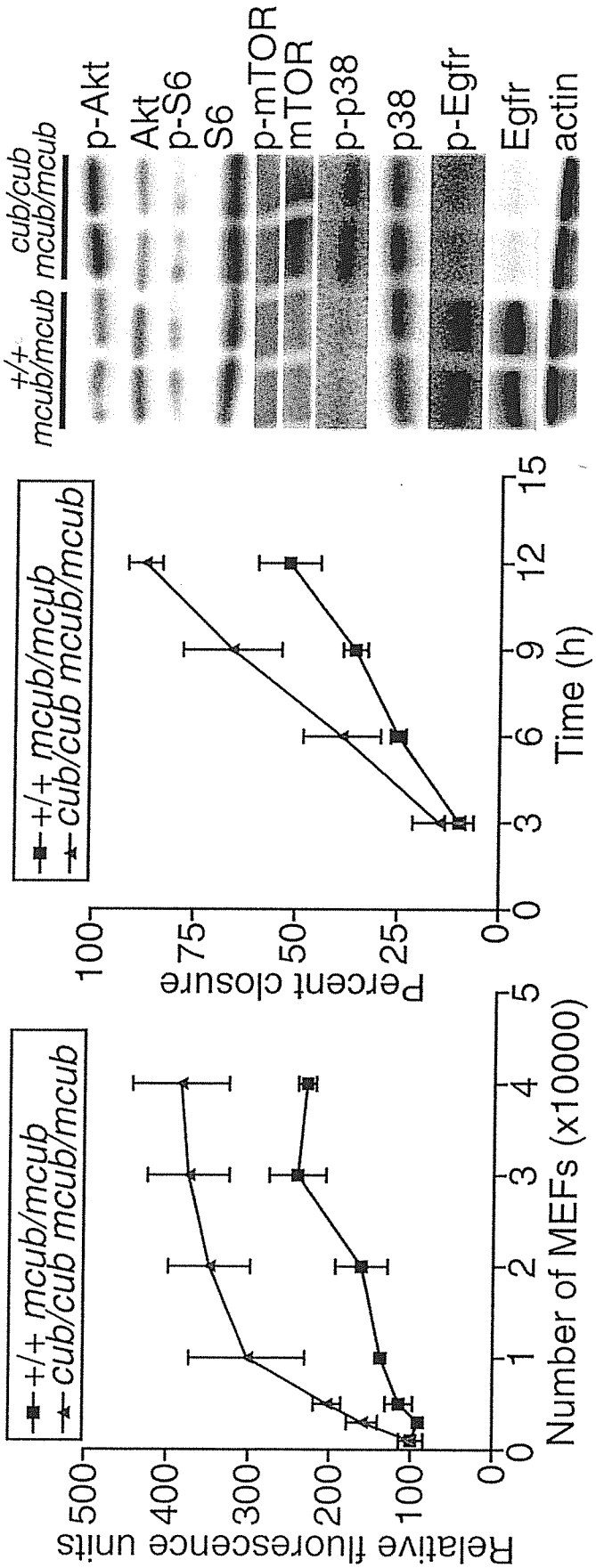


FIG. 1A

FIG. 1B

FIG. 1C

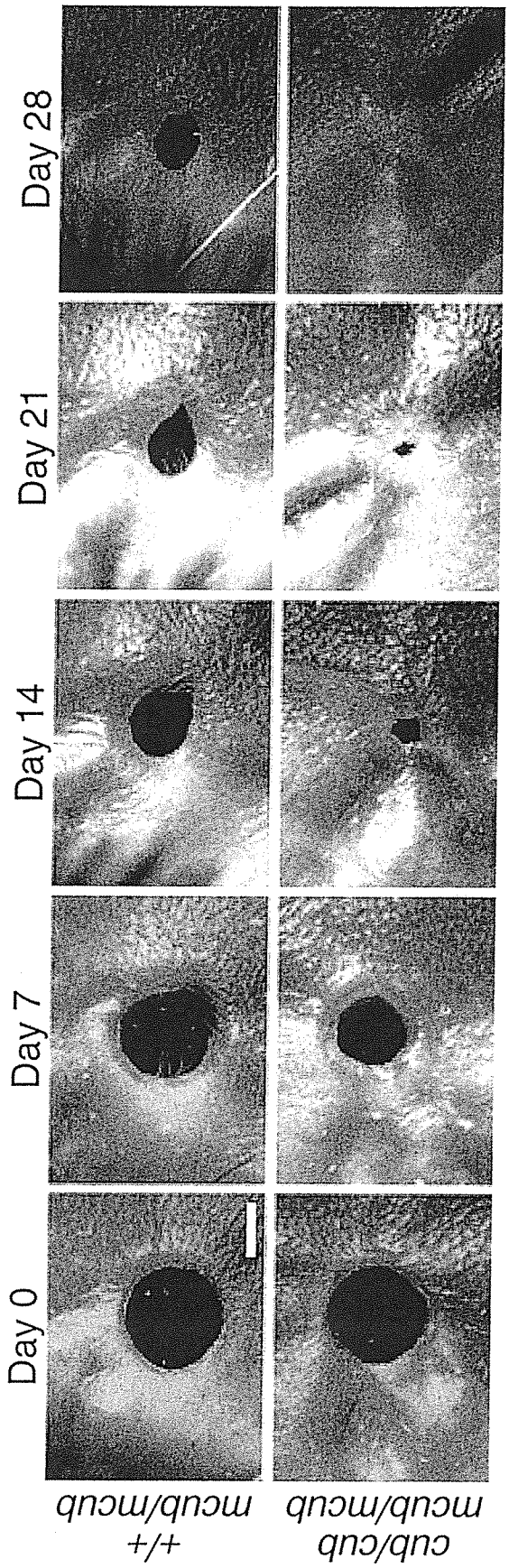


FIG. 1D

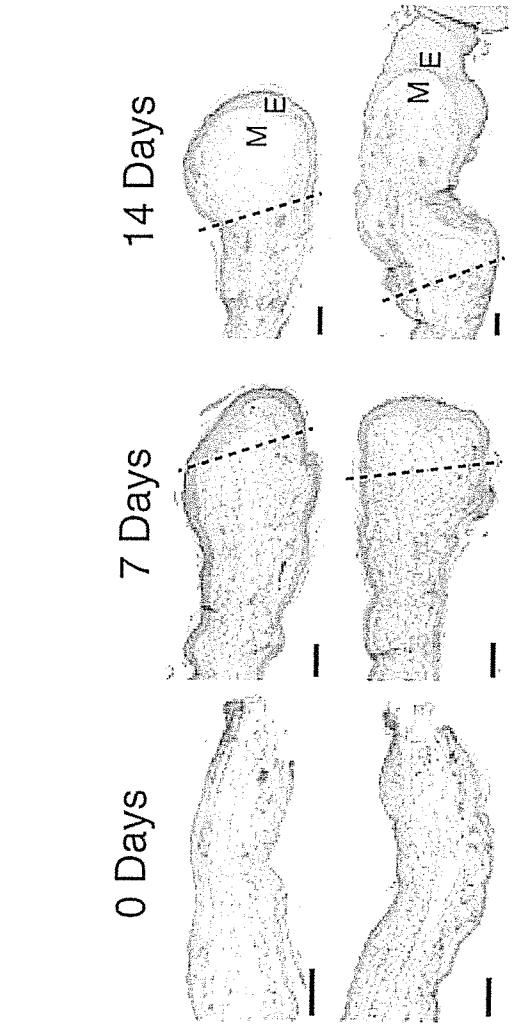

14 Days
7 Days
0 Days
M
E
+/+
mcub/mcub
cub/cub
mcub/mcub

FIG. 1F

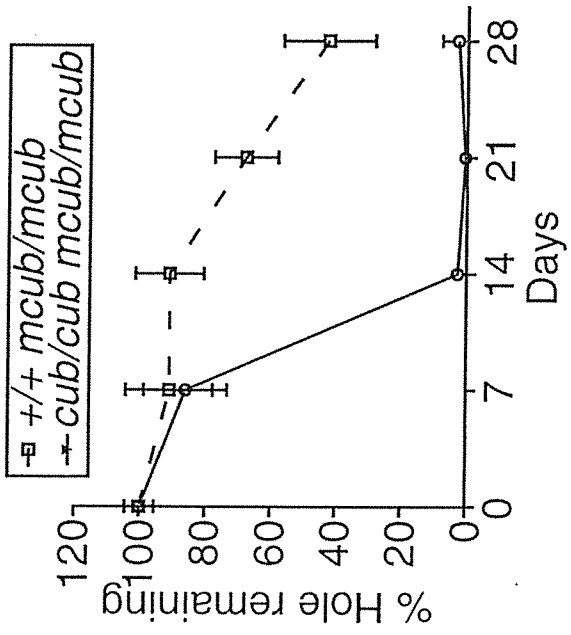

+/+ mcub/mcub
cub/cub mcub/mcub
% Hole remaining
120
100
80
60
40
20
0
0
7
14
21
28
Days

FIG. 1E

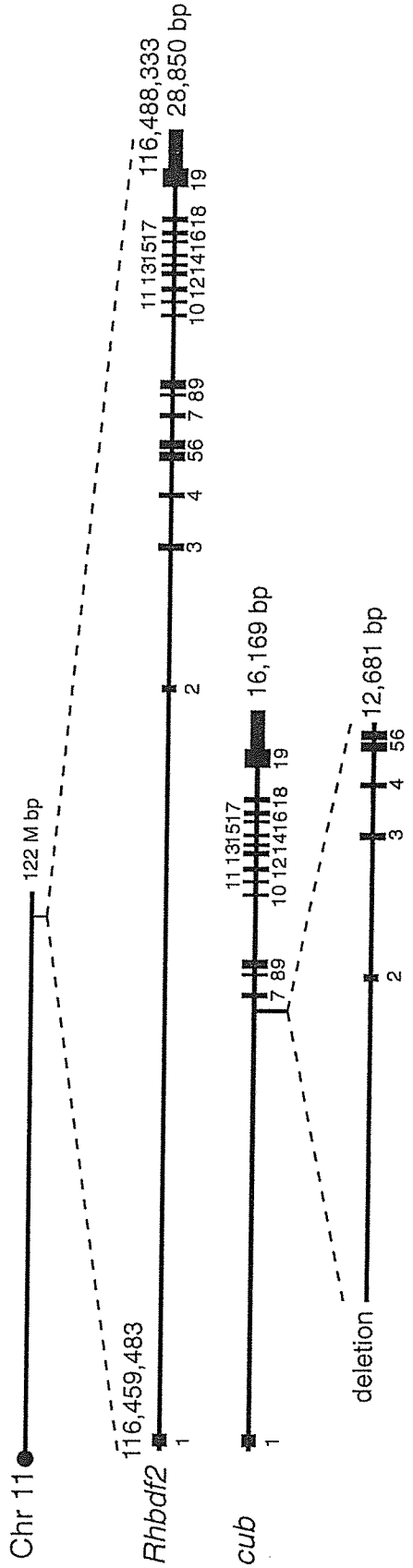

Chr 11
122 M bp
116,459,483
116,488,333
28,850 bp
Rhbdf2
cub
16,169 bp
12,681 bp
deletion

FIG. 2A

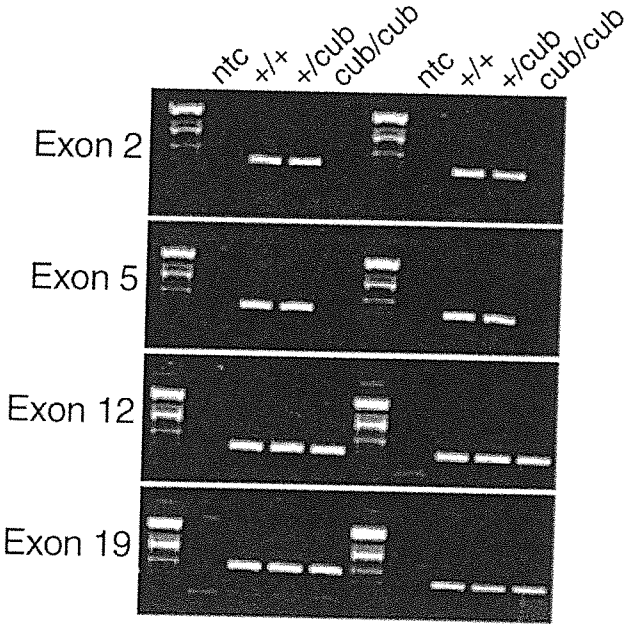

ntc
+/+
+/cub
cub/cub
ntc
+/+
+/cub
cub/cub
Exon 2
Exon 5
Exon 12
Exon 19

FIG. 2B

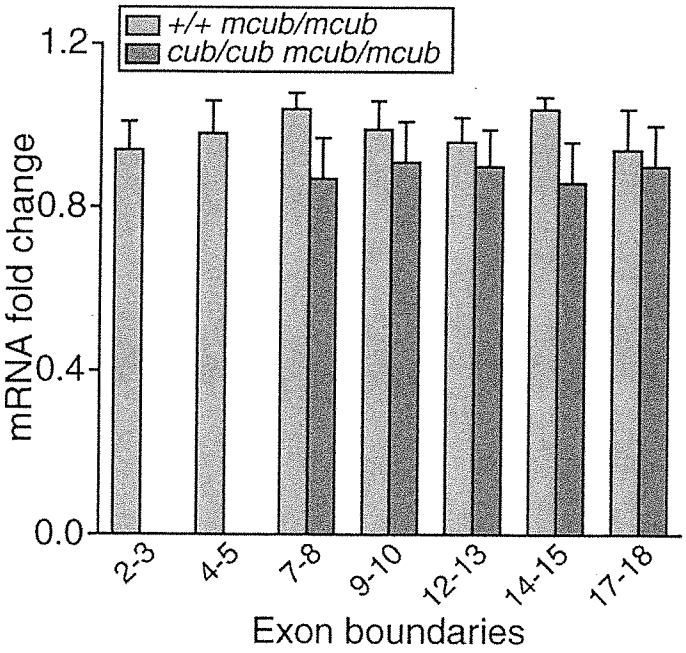

+/+ mcub/mcub
cub/cub mcub/mcub
1.2
0.8
0.4
0.0
mRNA fold change
2-3
4-5
7-8
9-10
12-13
14-15
17-18
Exon boundaries

FIG. 2C

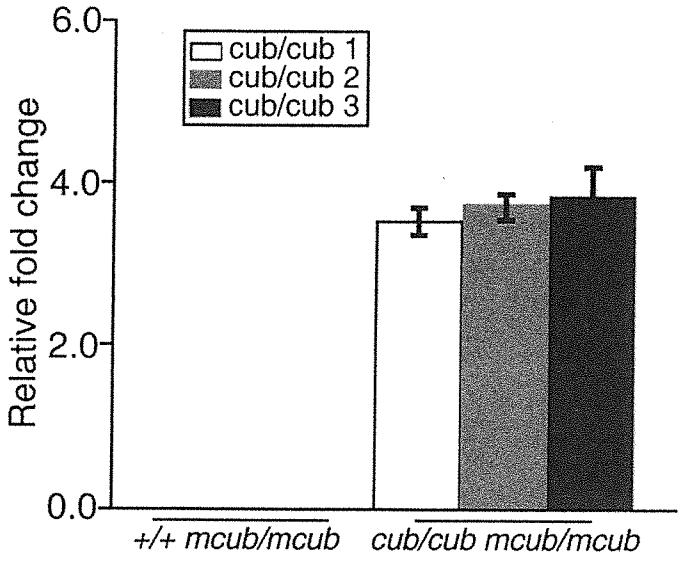

6.0
4.0
2.0
0.0
Relative fold change
cub/cub 1
cub/cub 2
cub/cub 3
+/+ mcub/mcub
cub/cub mcub/mcub

FIG. 2D


HuWt
HuCub

FIG. 2F

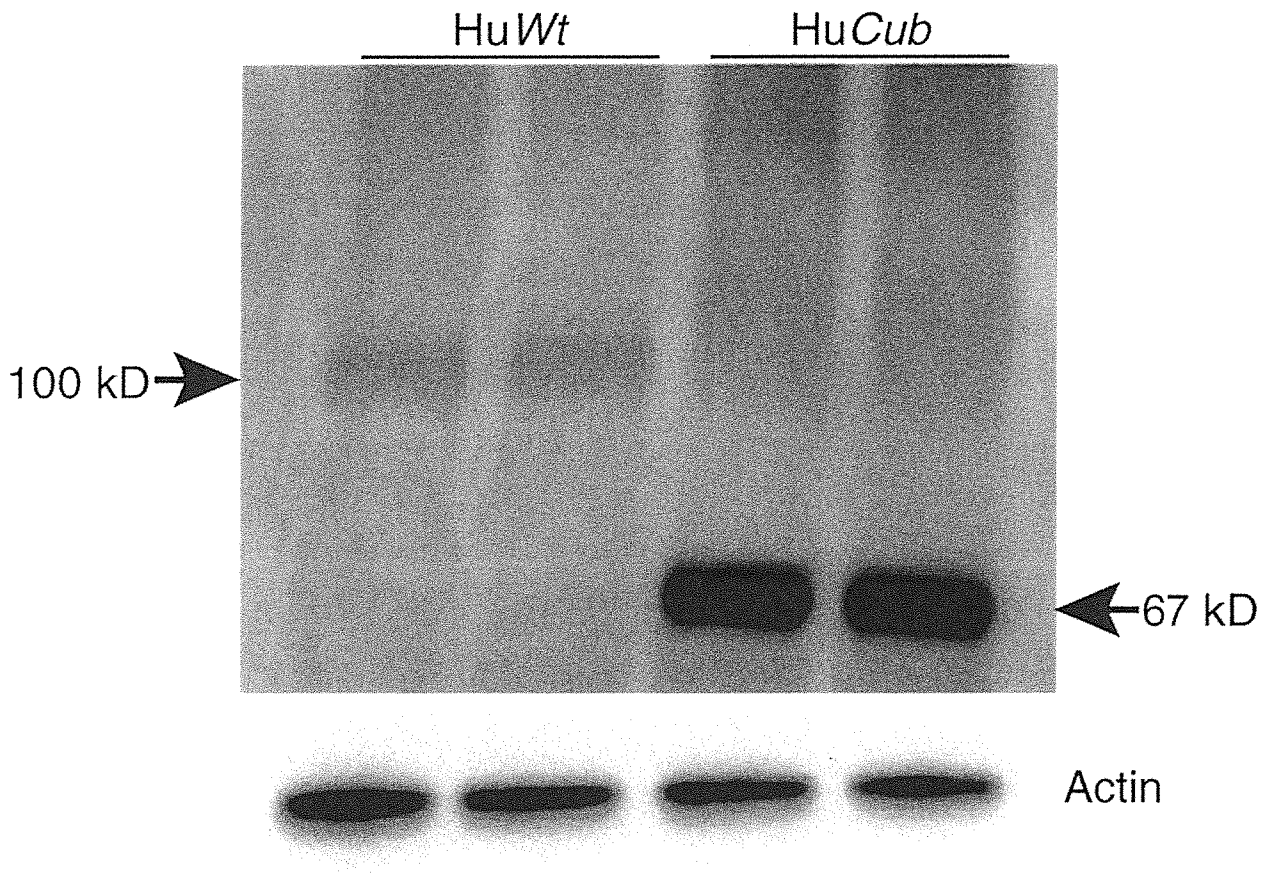


FIG. 2E

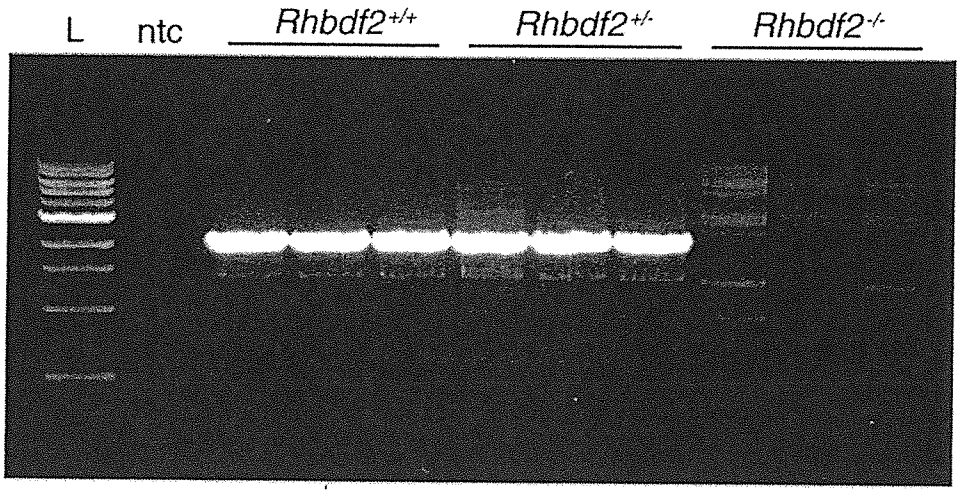


FIG. 3A

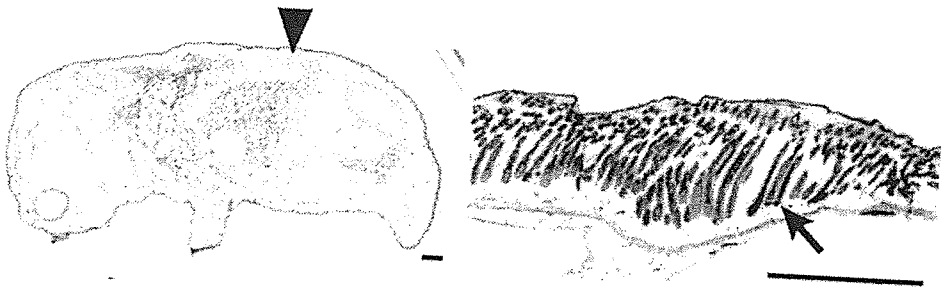


FIG. 3B

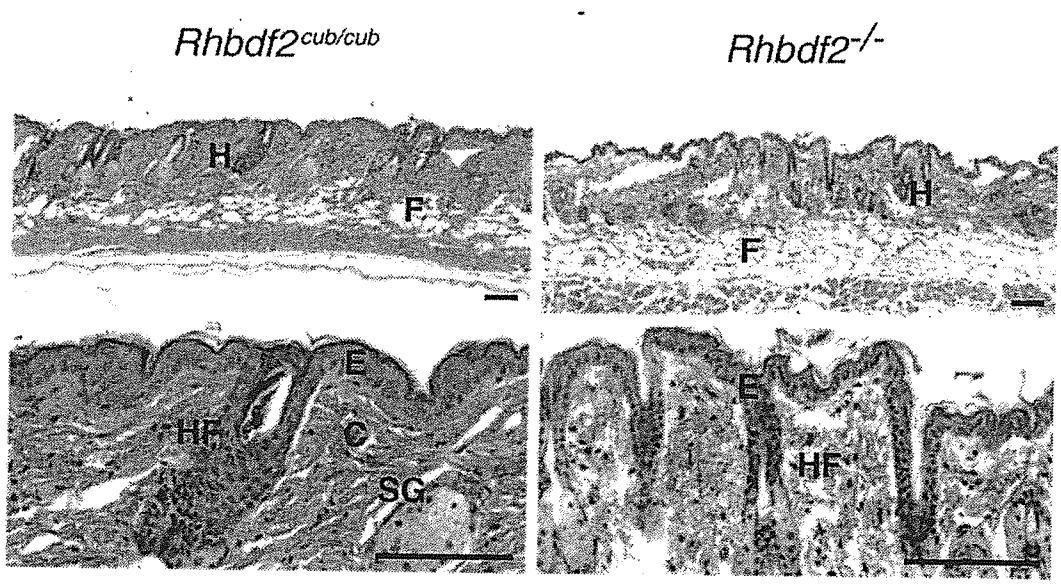


FIG. 3D

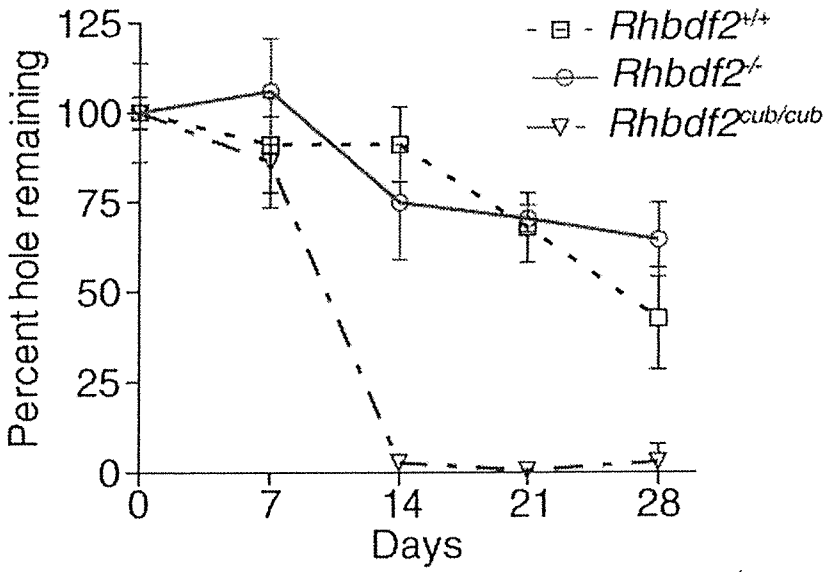

125
100
75
50
25
0
Percent hole remaining
0
7
14
21
28
Days
Rhbdf2+/+
Rhbdf2-/-
Rhbdf2cub/cub

FIG. 3C

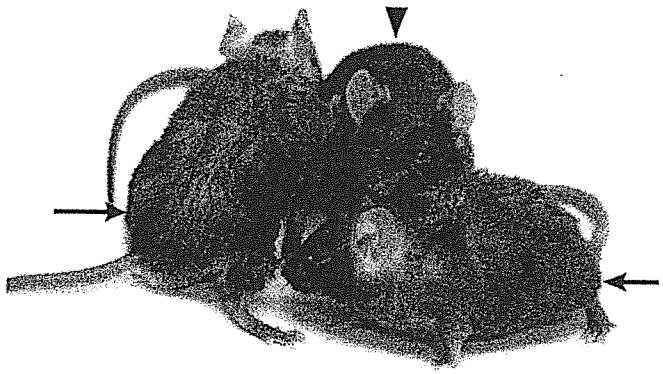


FIG. 3E

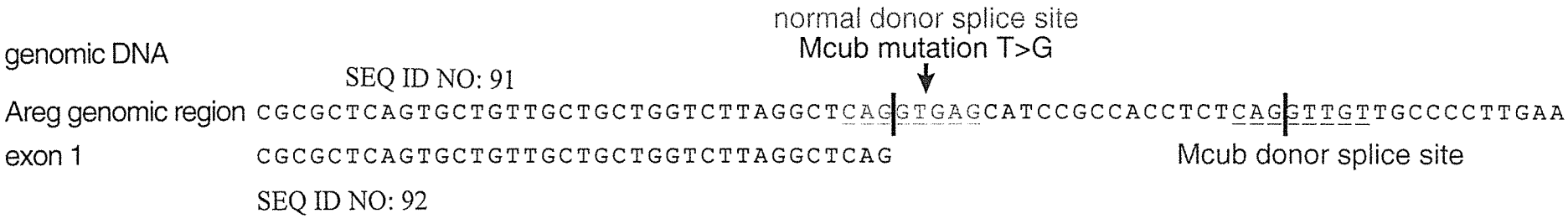

normal donor splice site
Mcub mutation T>G
genomic DNA
SEQ ID NO: 91
Areg genomic region CGCGCTCAGTGCTGTTGCTGCTGGTCTTAGGCTCAGGTGAGCATCCGCCACCTCTCAGGTTGTTGCCCCTTGAA
exon 1 CGCGCTCAGTGCTGTTGCTGCTGGTCTTAGGCTCAG
Mcub donor splice site
SEQ ID NO: 92

FIG. 4A

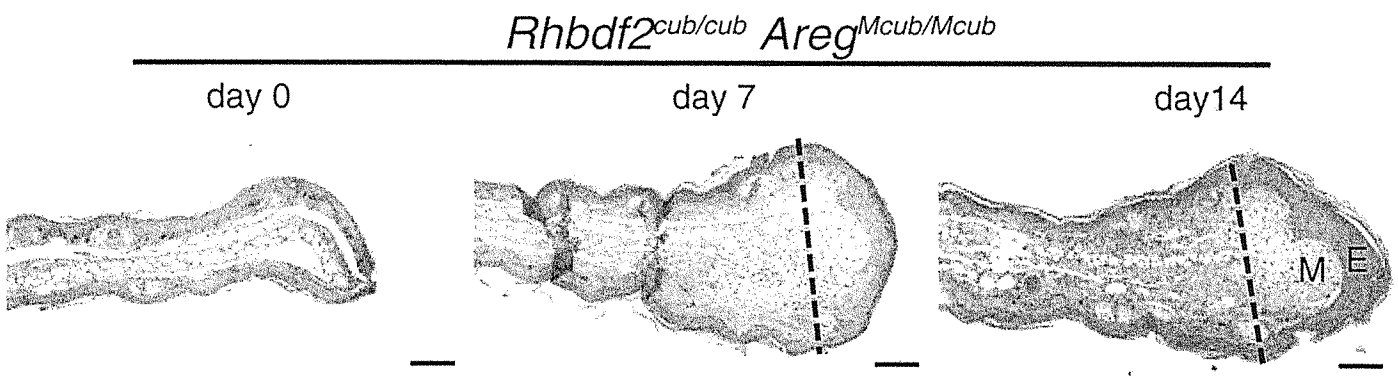

Rhbdf2cub/cub Aregmcub/Mcub
day 0
day 7
day14
M
E

FIG. 4C

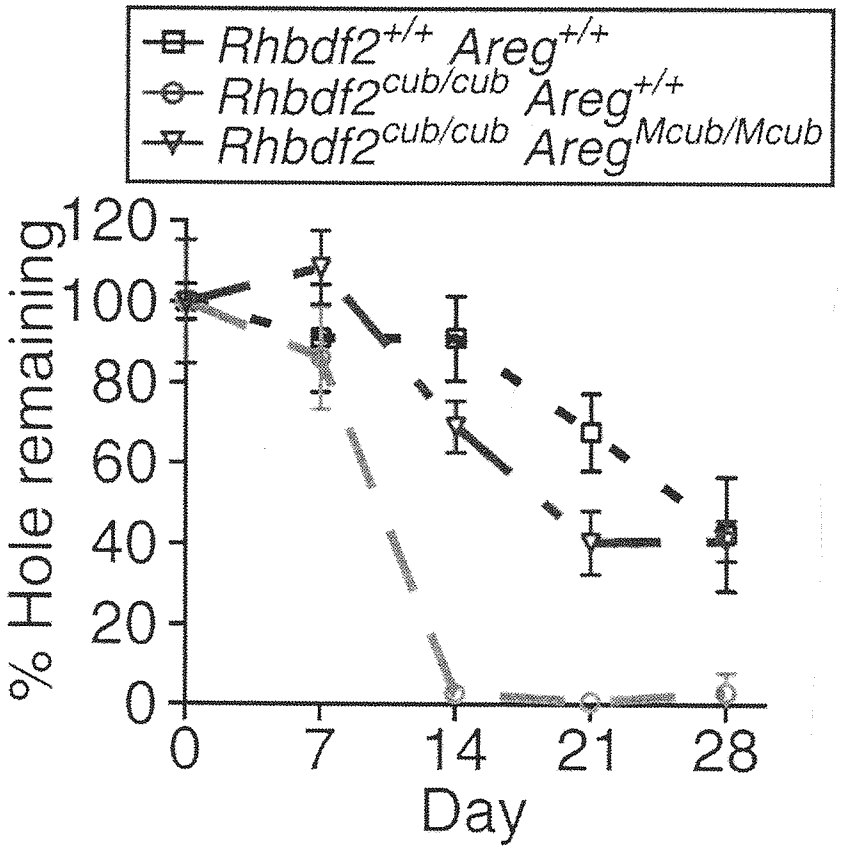

Rhbdf2+/+ Areg+/+
Rhbdf2cub/cub Areg+/+
Rhbdf2cub/cub AregMcub/Mcub
% Hole remaining
120
100
80
60
40
20
0
0
7
14
21
28
Day

FIG. 4B

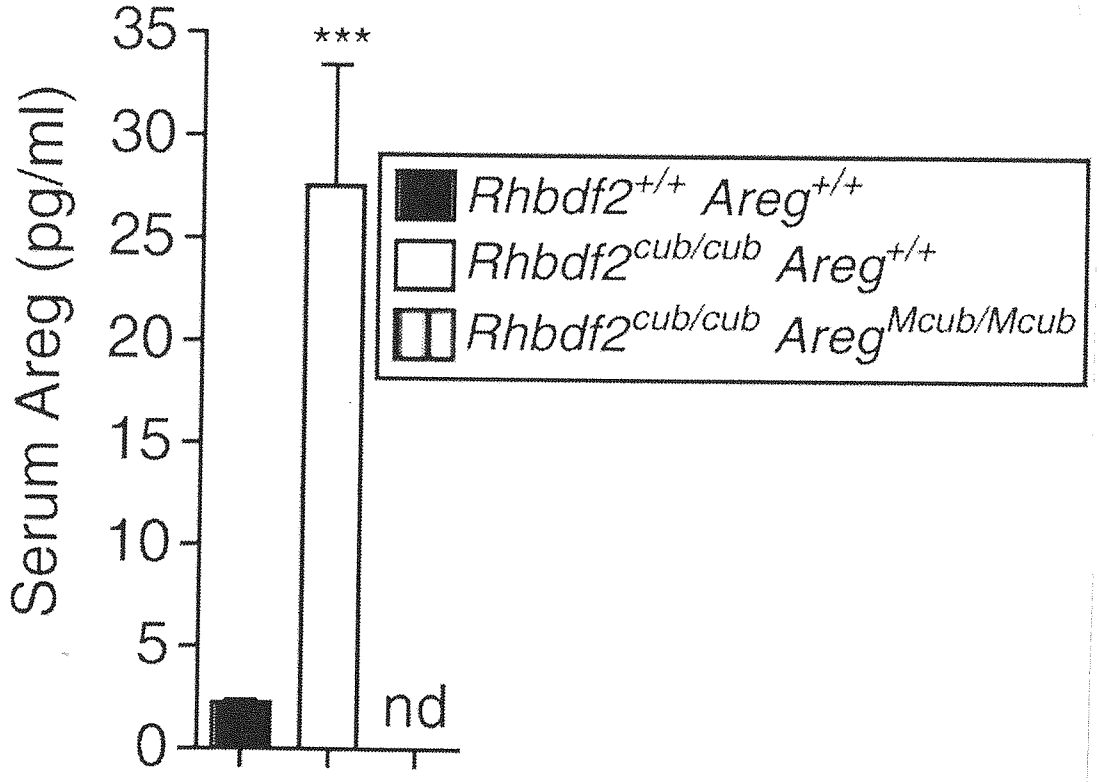

Serum Areg (pg/ml)
35
30
25
20
15
10
5
0
***
nd
Rhbdf2+/+ Areg+/+
Rhbdf2cub/cub Areg+/+
Rhbdf2cub/cub AregMcub/Mcub

FIG. 4D

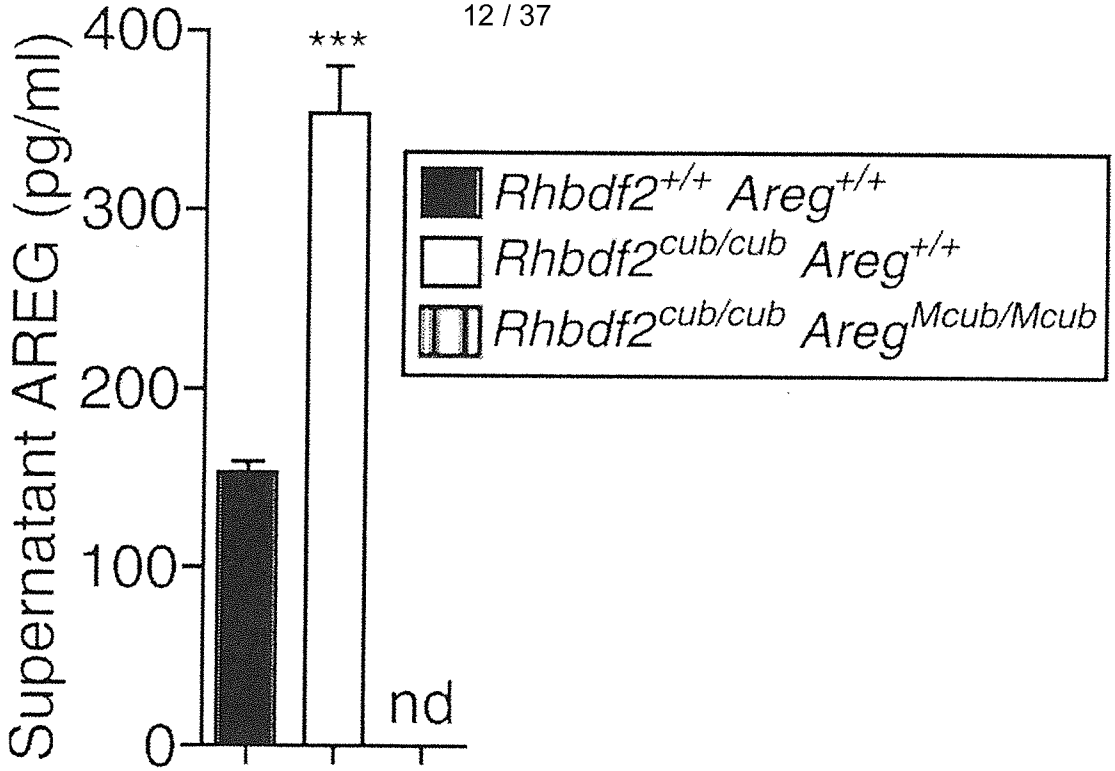

Supernatant AREG (pg/ml)
400
300
200
100
0
***
nd
Rhbdf2+/+ Areg+/+
Rhbdf2cub/cub Areg+/+
Rhbdf2cub/cub AregMcub/Mcub

FIG. 4E

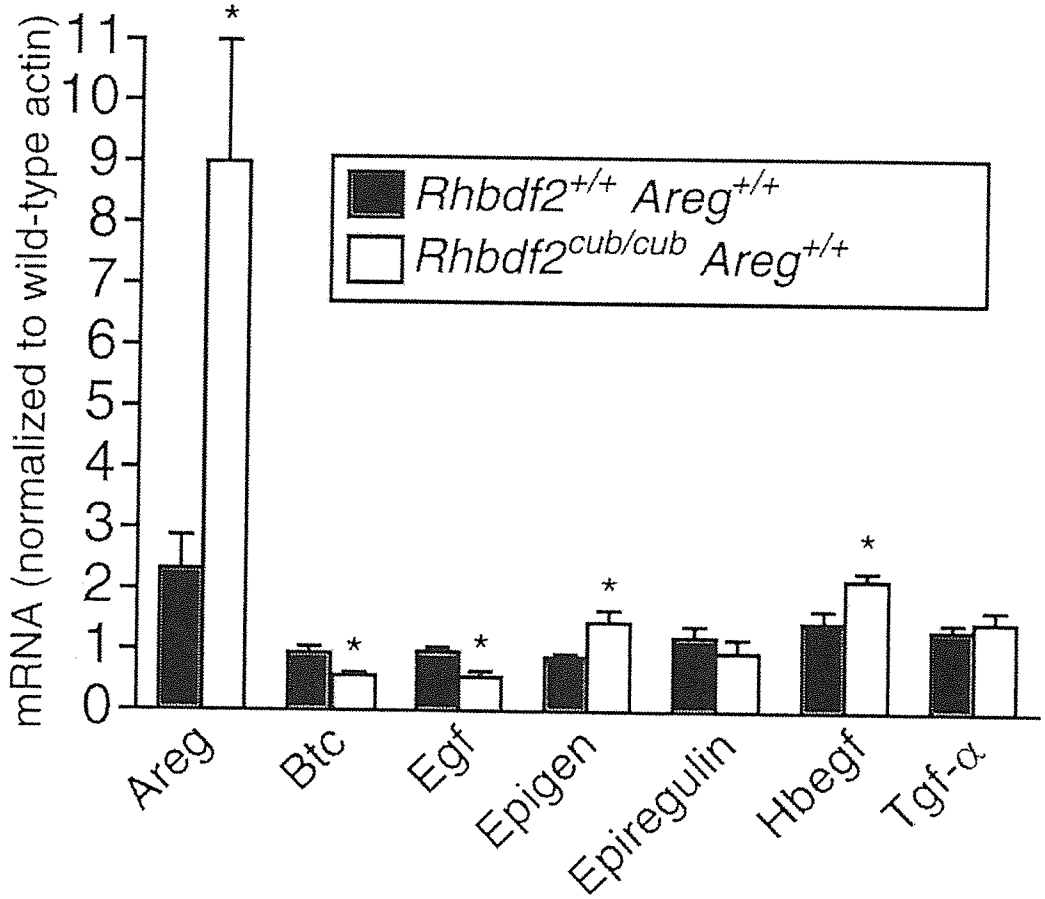

mRNA (normalized to wild-type actin)
11
10
9
8
7
6
5
4
3
2
1
0
*
*
*
*
*
Rhbdf2+/+ Areg+/+
Rhbdf2cub/cub Areg+/+
Areg
Btc
Egf
Epigen
Epiregulin
Hbegf
Tgf-α

FIG. 4F

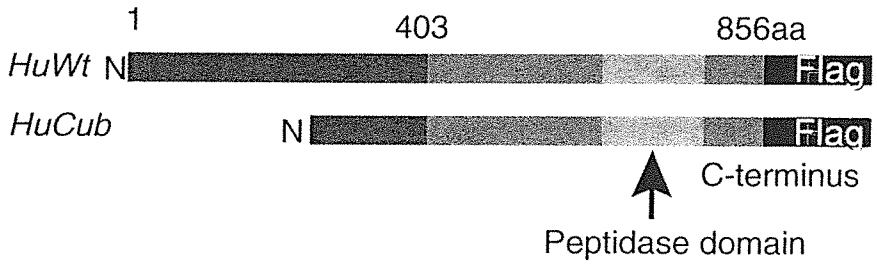

1
403
856aa
HuWt N
Flag
HuCub
N
Flag
C-terminus
Peptidase domain

FIG. 5A

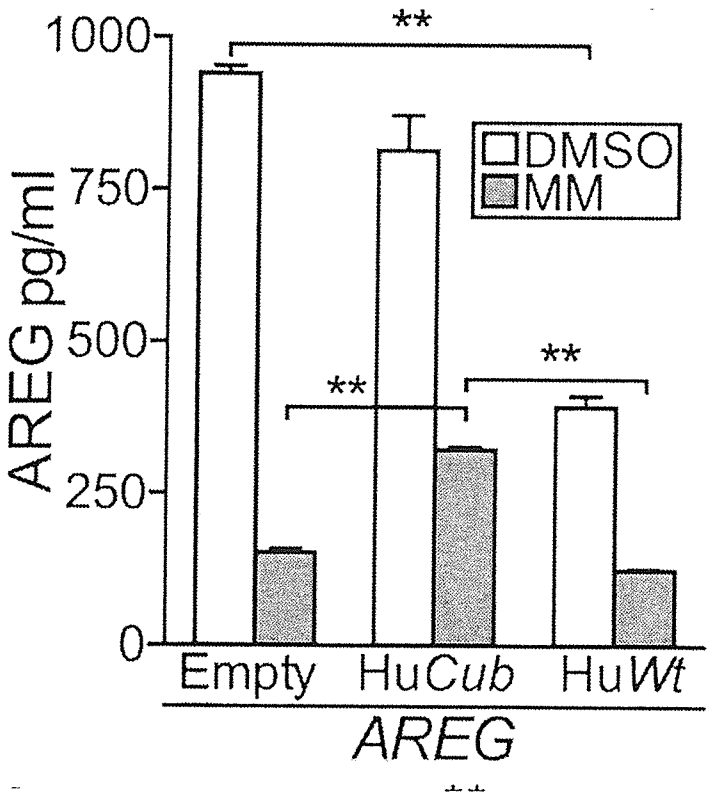

1000
750
500
250
0
AREG pg/ml
**
**
**
DMSO
MM
Empty
HuCub
HuWt
AREG

FIG. 5B

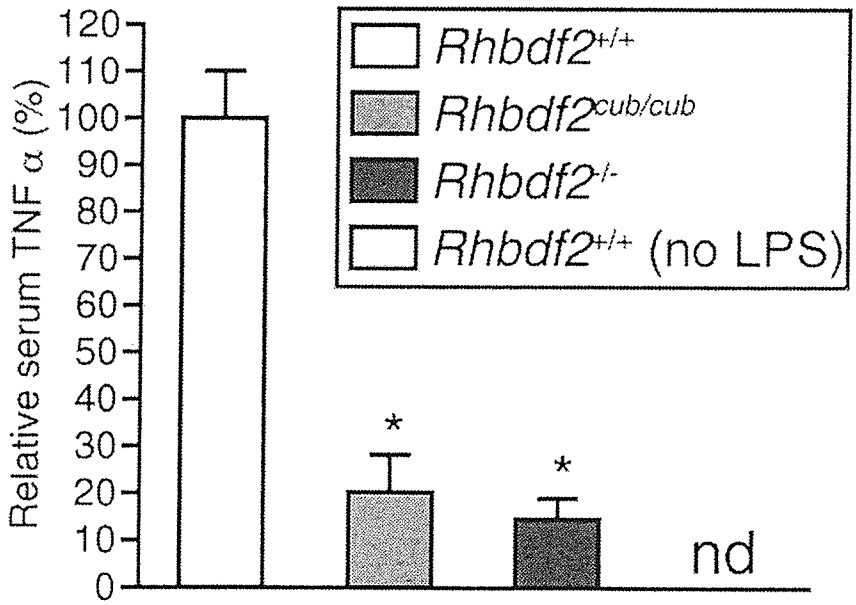

Relative serum TNF α (%)
120
110
100
90
80
70
60
50
40
30
20
10
0
Rhbdf2+/+
Rhbdf2cub/cub
Rhbdf2-/-
Rhbdf2+/+ (no LPS)
*
*
nd

FIG. 5C

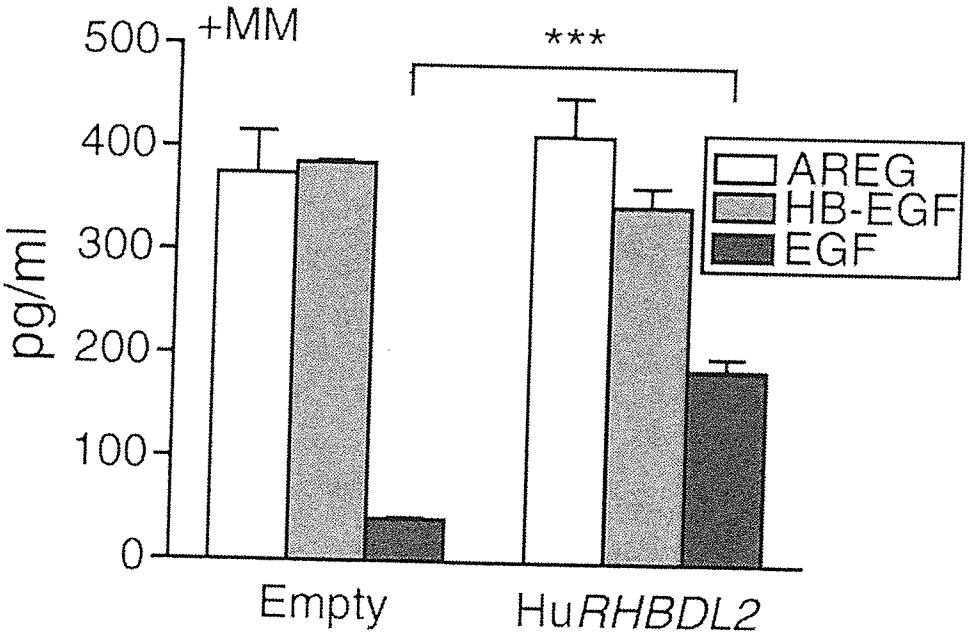

+MM
***
500
400
300
200
100
0
pg/ml
AREG
HB-EGF
EGF
Empty
Hu*RHBDL2*

FIG. 5D

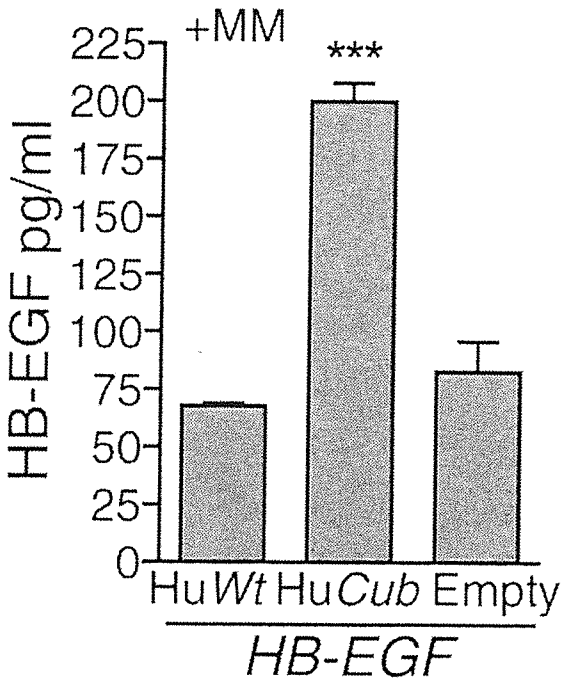

+MM
***
225
200
175
150
125
100
75
50
25
0
HB-EGF pg/ml
Hu*Wt*
Hu*Cub*
Empty
HB-EGF

FIG. 5E

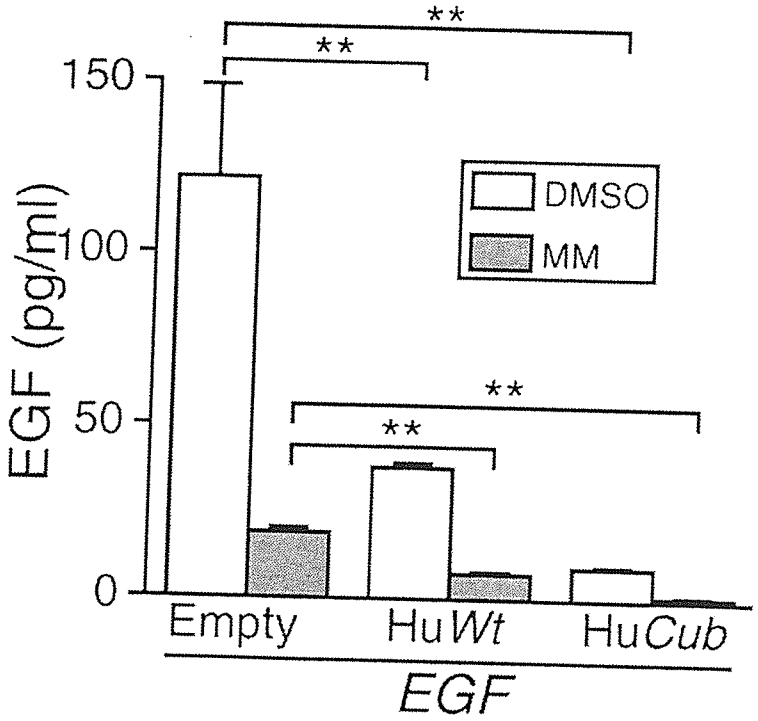

**
**
150
100
50
0
EGF (pg/ml)
DMSO
MM
**
**
Empty
HuWt
HuCub
EGF

FIG. 5F

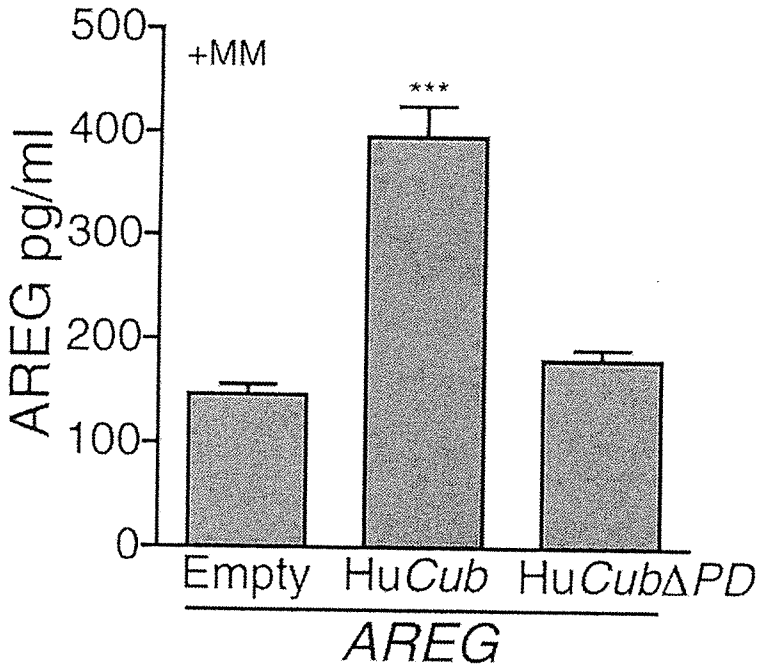

500
400
300
200
100
0
AREG pg/ml
+MM
***
Empty
HuCub
HuCubΔPD
AREG

FIG. 5G

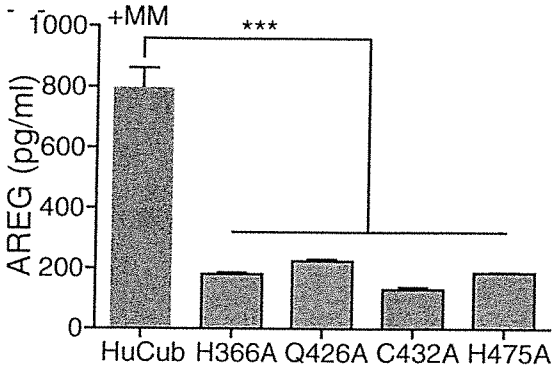


FIG. 5H

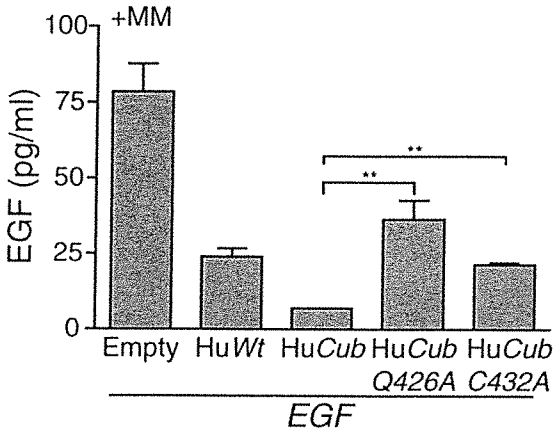


FIG. 5I

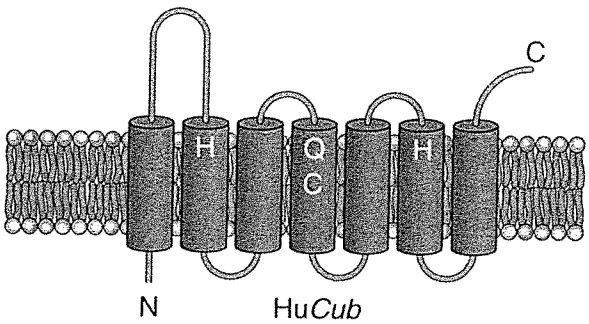


FIG. 5J

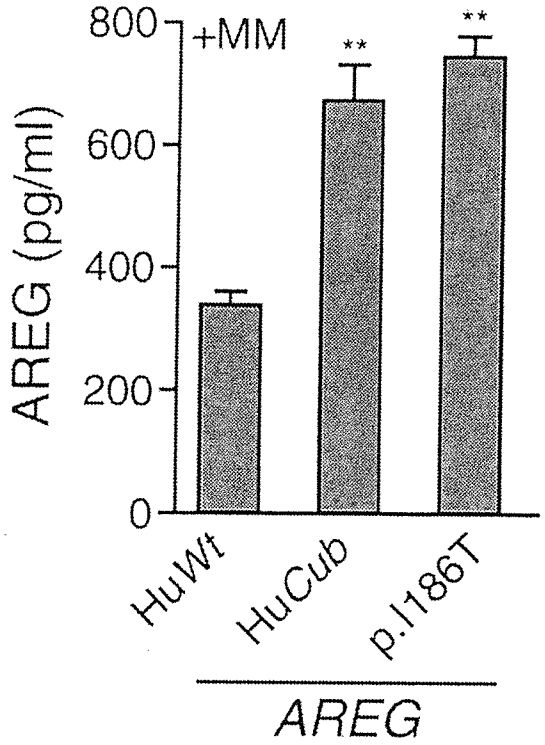


FIG. 6A

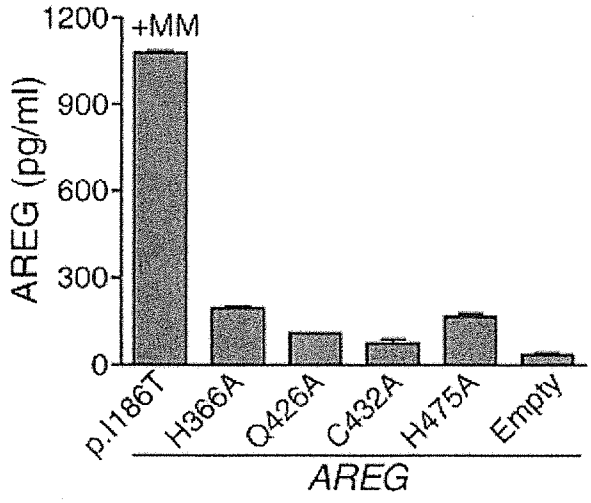


FIG. 6C

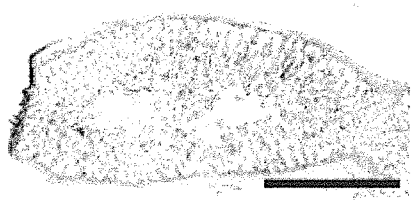


FIG. 6D

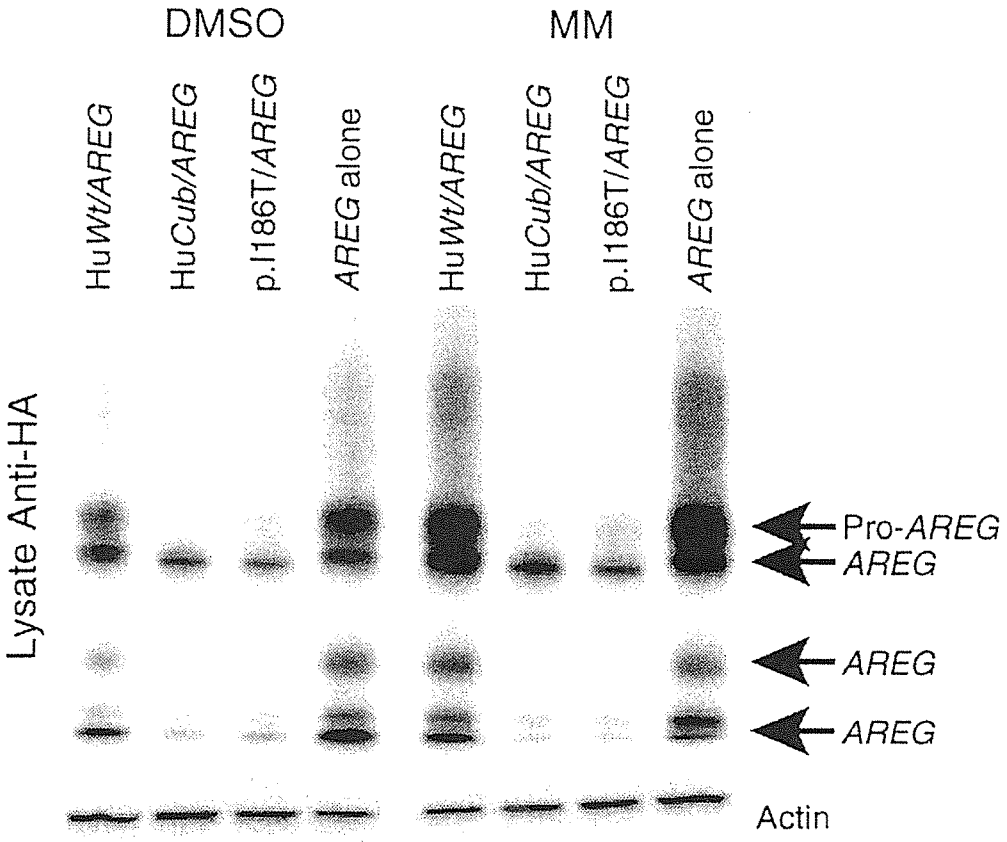

DMSO
MM
HuWt/AREG
HuCub/AREG
p.I186T/AREG
AREG alone
HuWt/AREG
HuCub/AREG
p.I186T/AREG
AREG alone
Lysate Anti-HA
Pro-AREG
AREG
AREG
AREG
Actin

FIG. 6B

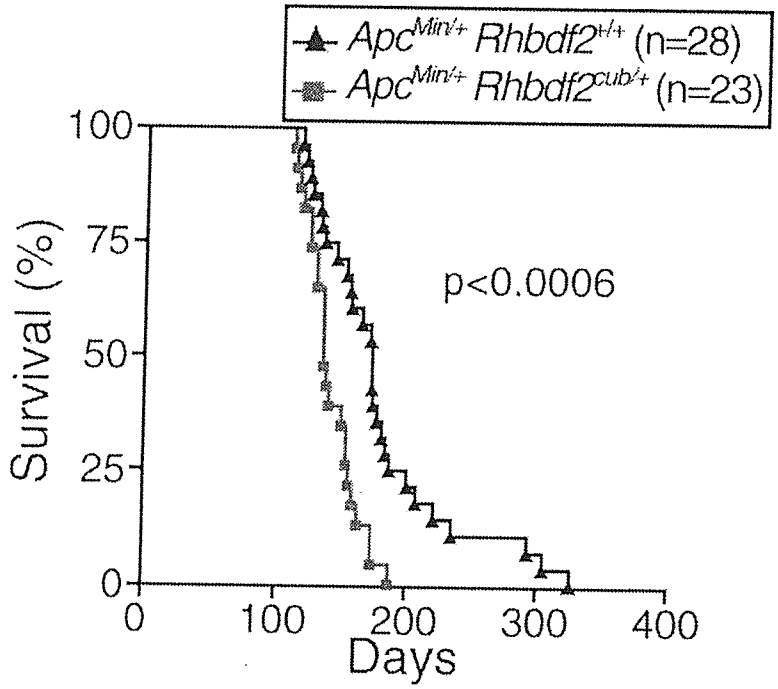

Apc^Min/+ Rhbdf2^+/+ (n=28)
Apc^Min/+ Rhbdf2^cub/+ (n=23)
Survival (%)
100
75
50
25
0
0
100
200
300
400
Days
p<0.0006

FIG. 6E

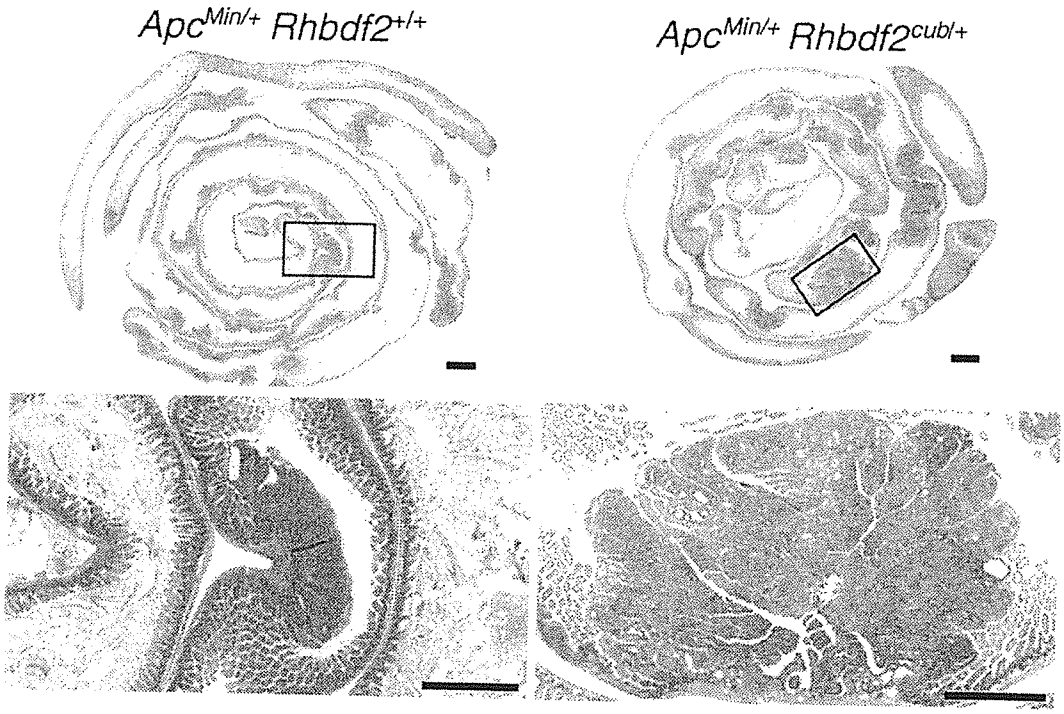

Apc^Min/+ Rhbdf2^+/+
Apc^Min/+ Rhbdf2^cub/+

FIG. 6F

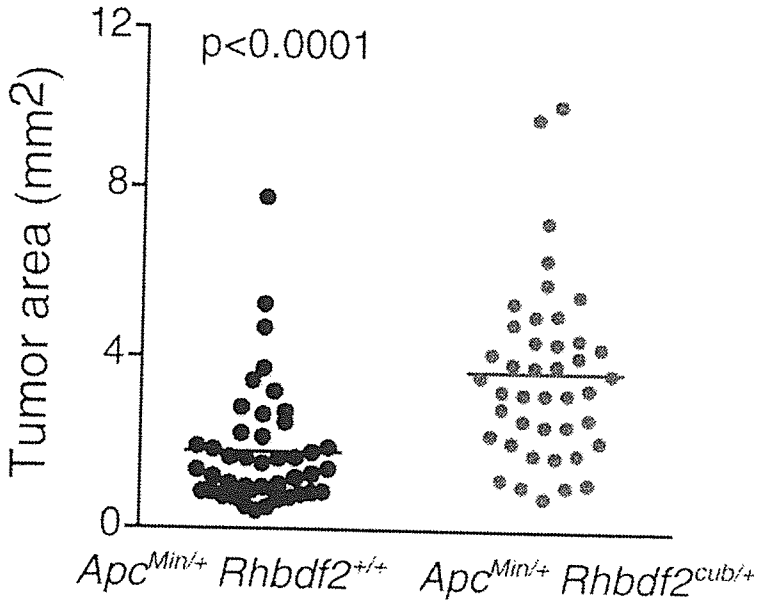

12
8
4
0
Tumor area (mm2)
p<0.0001
ApcMin/+ Rhbdf2+/+
ApcMin/+ Rhbdf2cub/+

FIG. 6G

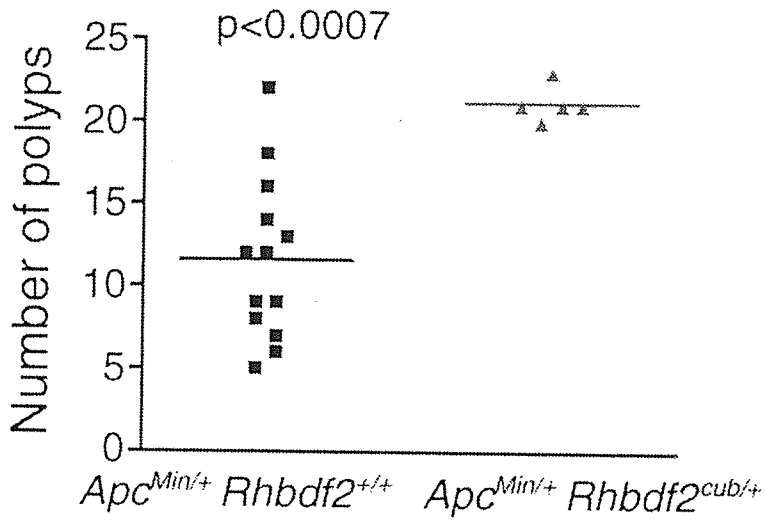

25
20
15
10
5
0
Number of polyps
p<0.0007
ApcMin/+ Rhbdf2+/+
ApcMin/+ Rhbdf2cub/+

FIG. 6H

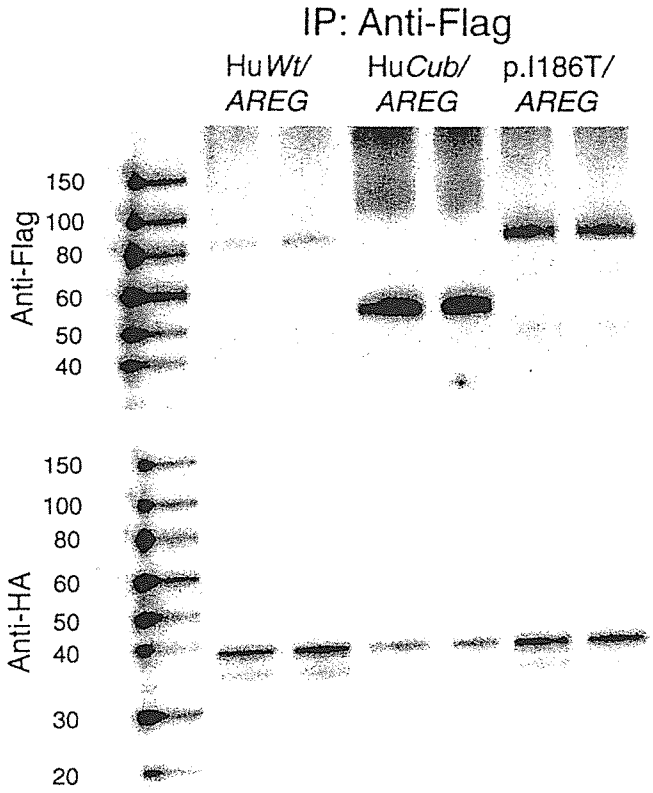

IP: Anti-Flag
HuWt/ AREG
HuCub/ AREG
p.I186T/ AREG
Anti-Flag
150
100
80
60
50
40
Anti-HA
150
100
80
60
50
40
30
20

FIG. 7A

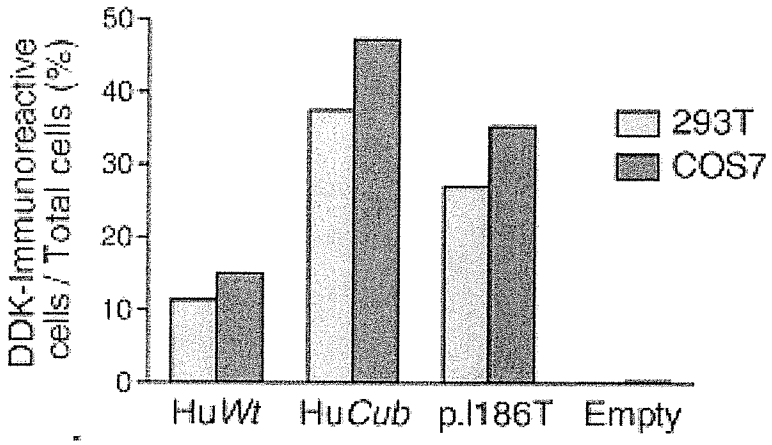

DDK-Immunoreactive cells / Total cells (%)
50
40
30
20
10
0
293T
COS7
HuWt
HuCub
p.I186T
Empty

FIG. 7C

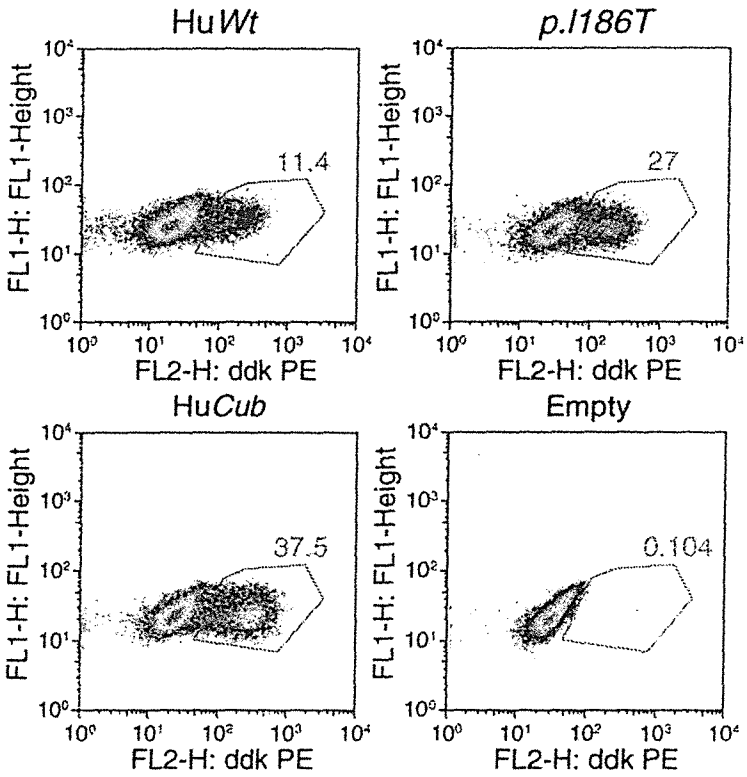

HuWt
p.I186T
HuCub
Empty
11.4
27
37.5
0.104
FL1-H: FL1-Height
FL2-H: ddk PE

FIG. 7B

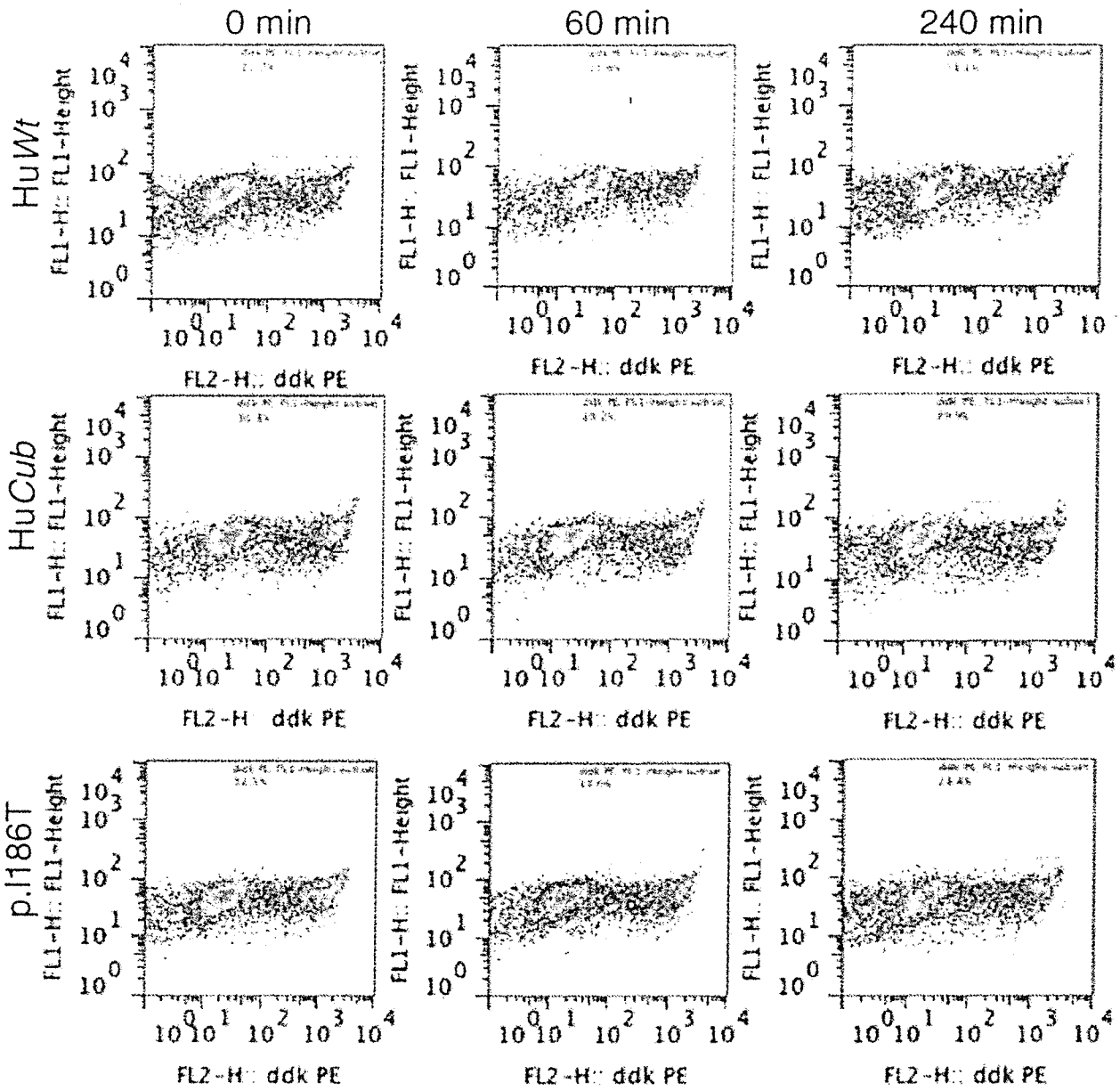

0 min
60 min
240 min
HuWt
HuCub
p.I186T
FL1-H:: FL1-Height
FL2-H:: ddk PE

FIG. 7D

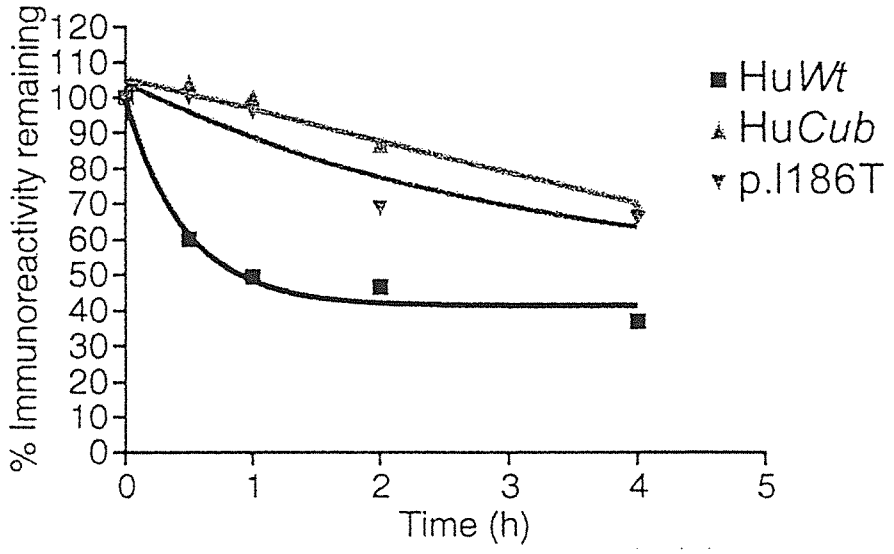

% Immunoreactivity remaining
120
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Time (h)
HuWt
HuCub
p.I186T

FIG. 7E

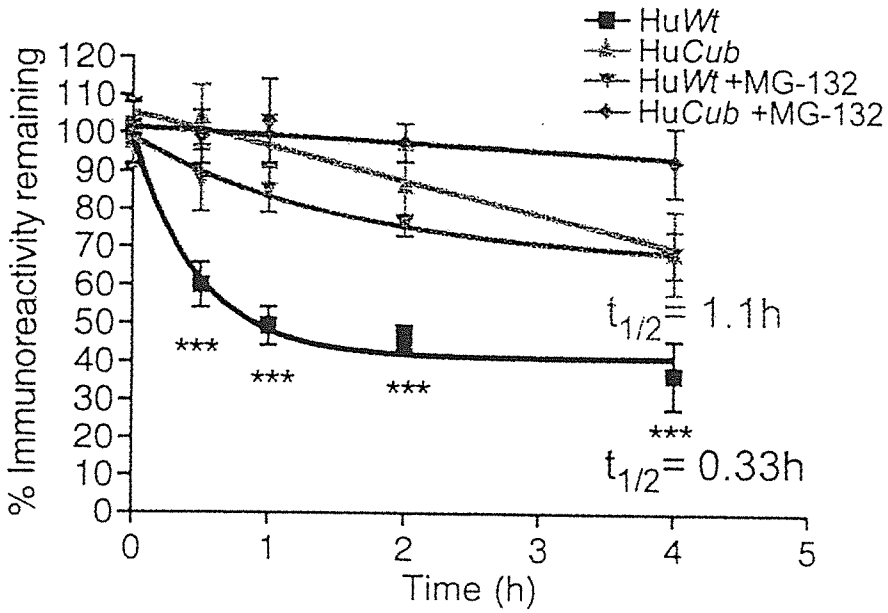

% Immunoreactivity remaining
120
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Time (h)
HuWt
HuCub
HuWt +MG-132
HuCub +MG-132
***
***
***
***
t1/2= 1.1h
t1/2= 0.33h

FIG. 7F

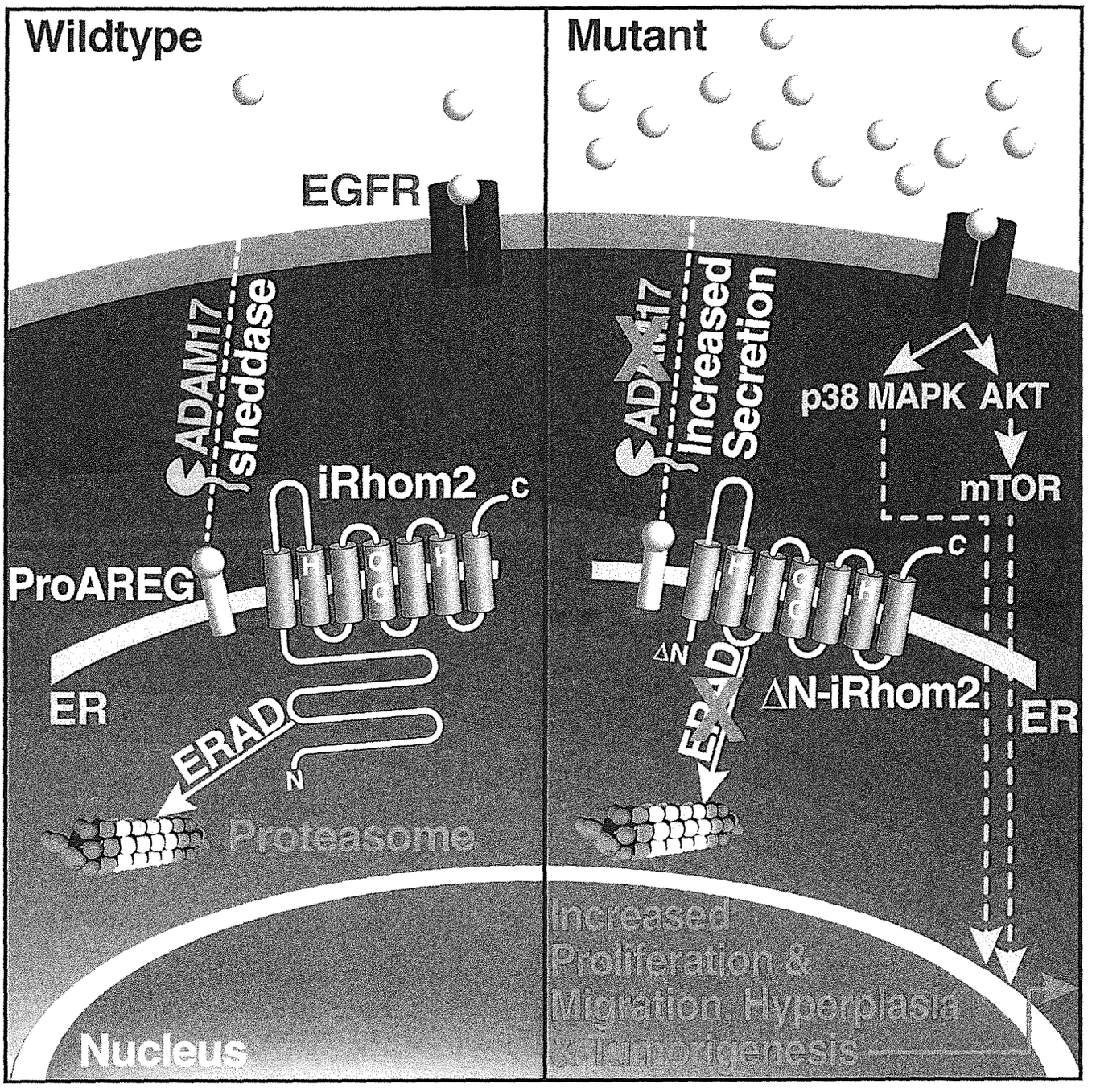

Wildtype
Mutant
EGFR
ADAM17
sheddase
iRhom2
C
ProAREG
ER
ERAD
N
Proteasome
Nucleus
ADAM17
Increased
Secretion
p38 MAPK
AKT
mTOR
ΔN
ΔN-iRhom2
ER
Increased
Proliferation &
Migration, Hyperplasia
& Tumorigenesis

FIG. 8

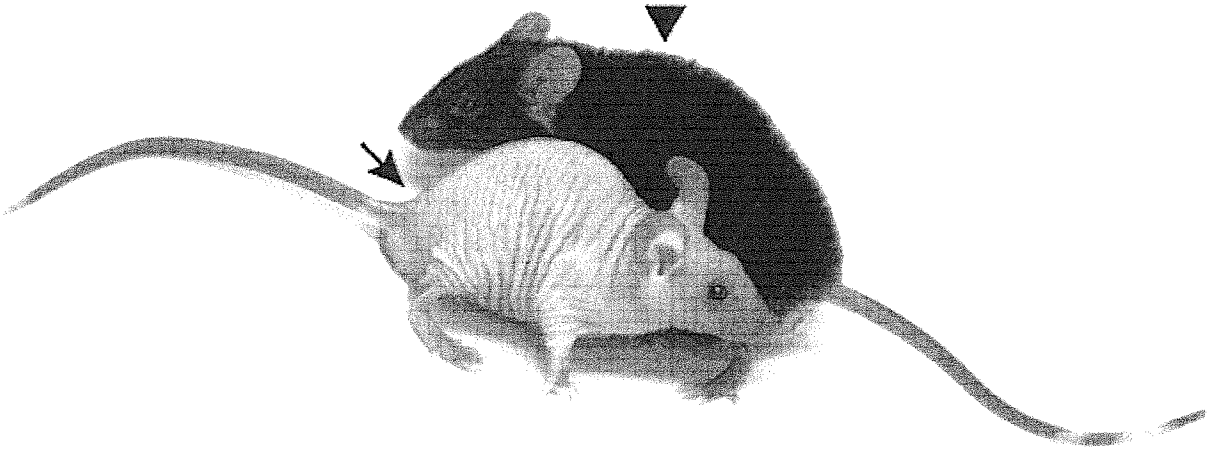


FIG. 9A

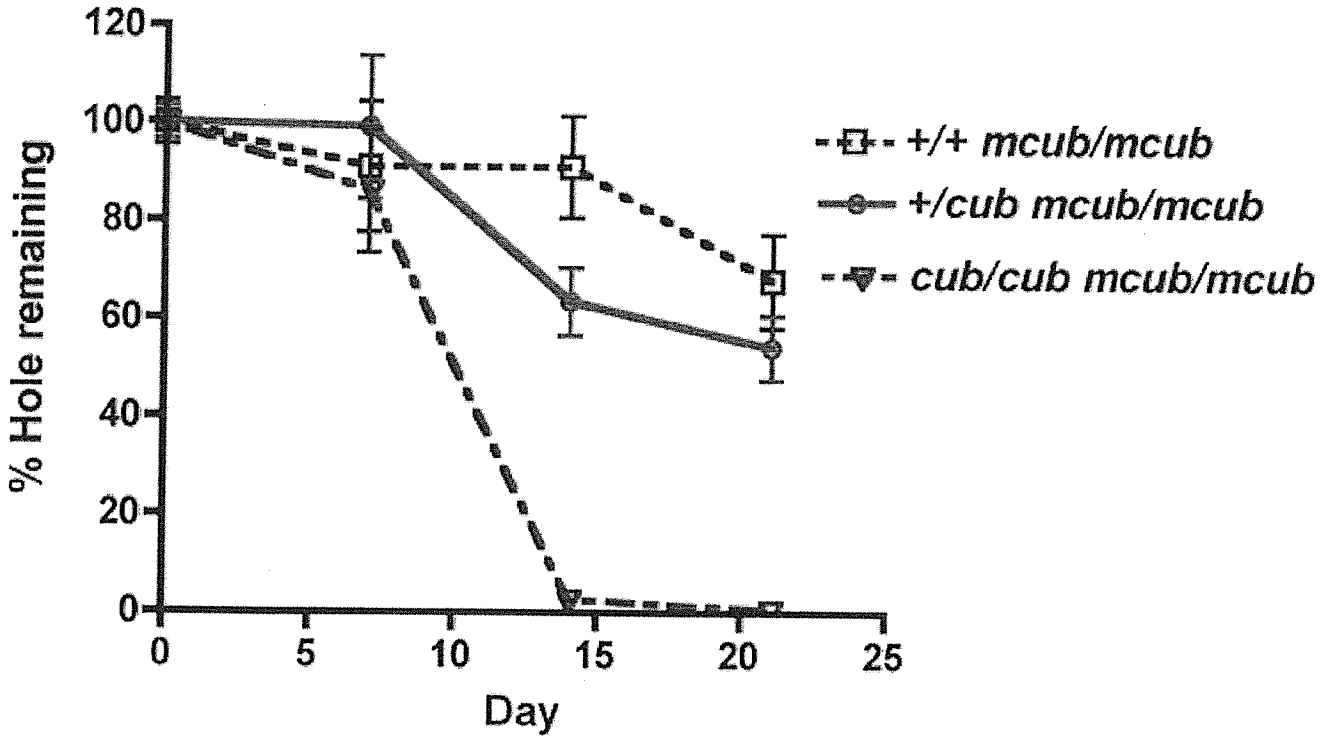

120
100
80
60
40
20
0
% Hole remaining
0
5
10
15
20
25
Day
+/+ mcub/mcub
+/cub mcub/mcub
cub/cub mcub/mcub

FIG. 9B

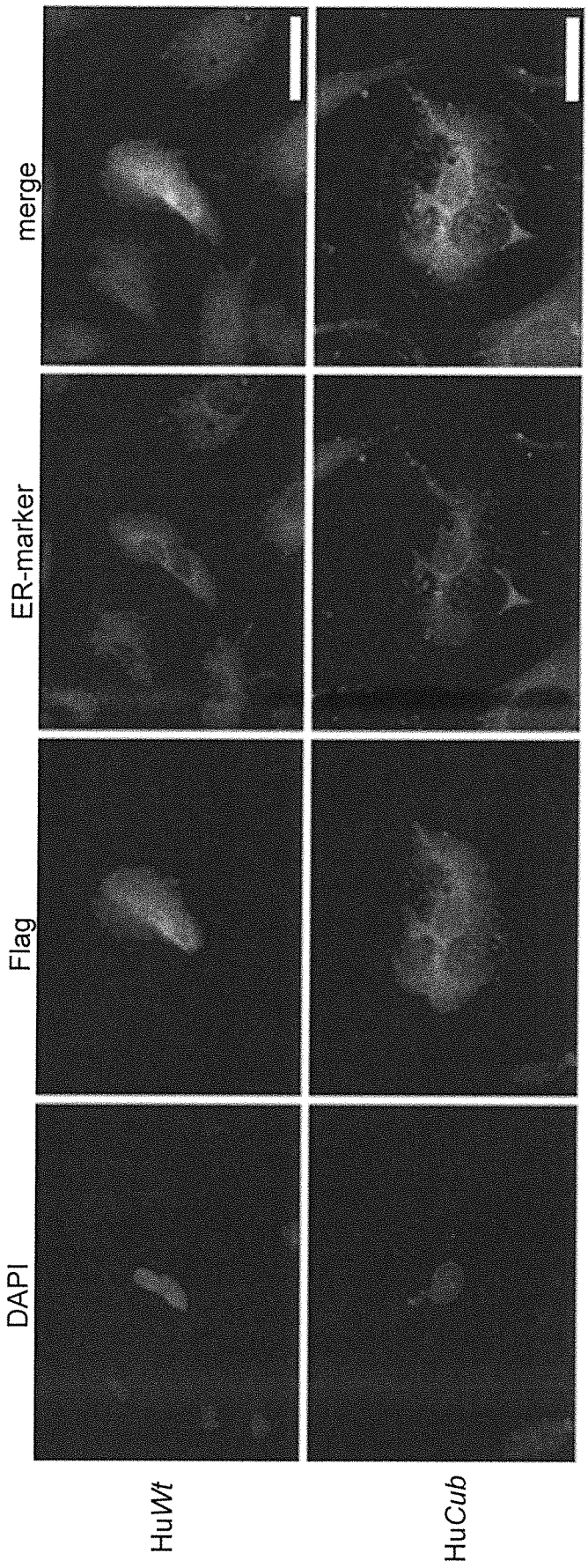


FIG. 10A

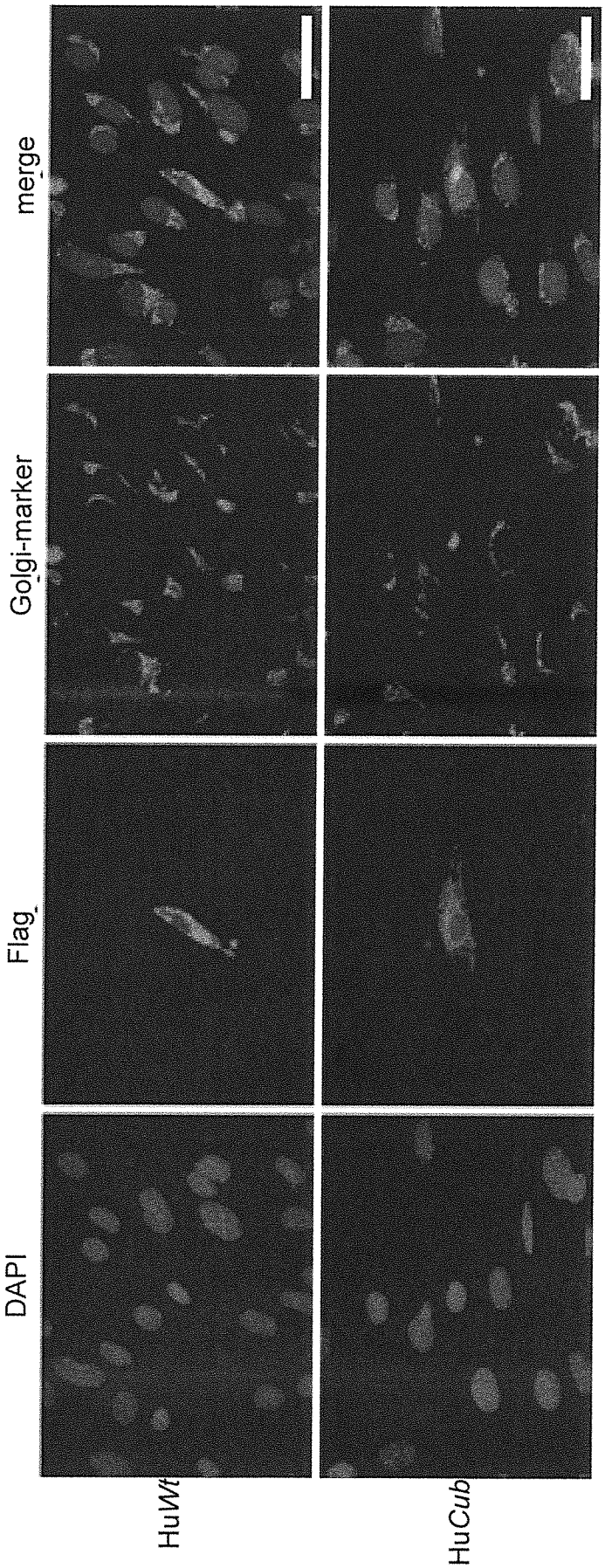


FIG. 10B

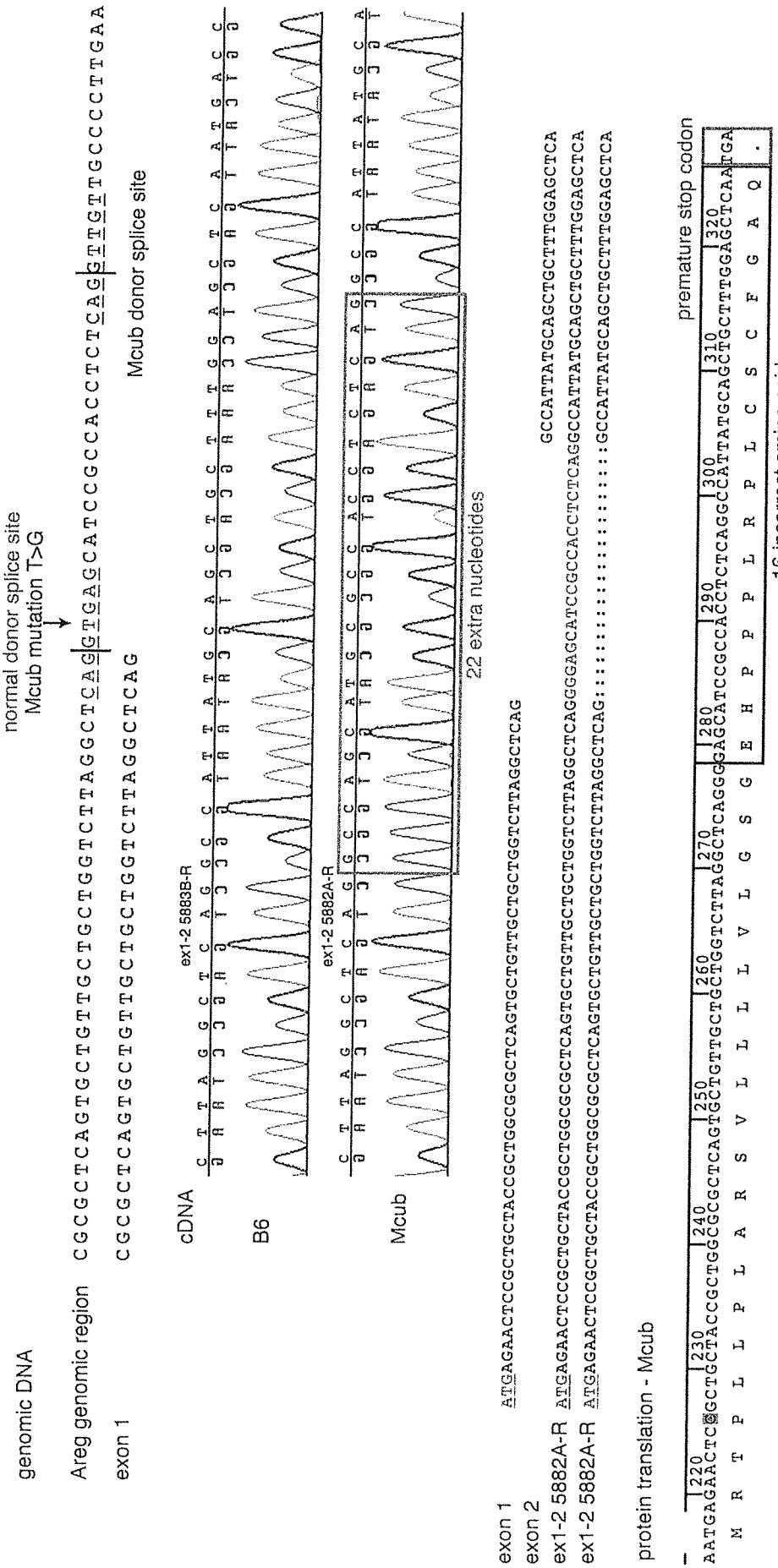

genomic DNA
normal donor splice site
Mcub mutation T>G
Areg genomic region
exon 1
Mcub donor splice site
cDNA
ex1-2 5883B-R
B6
ex1-2 5882A-R
Mcub
22 extra nucleotides
exon 1
exon 2
ex1-2 5882A-R
ex1-2 5882A-R
protein translation - Mcub
premature stop codon
16 incorrect amino acids

FIG. 11

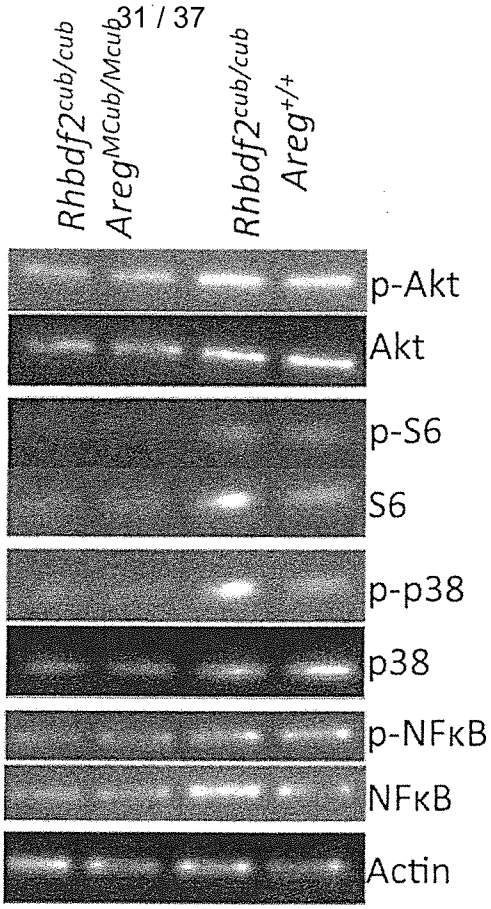


FIG. 12A

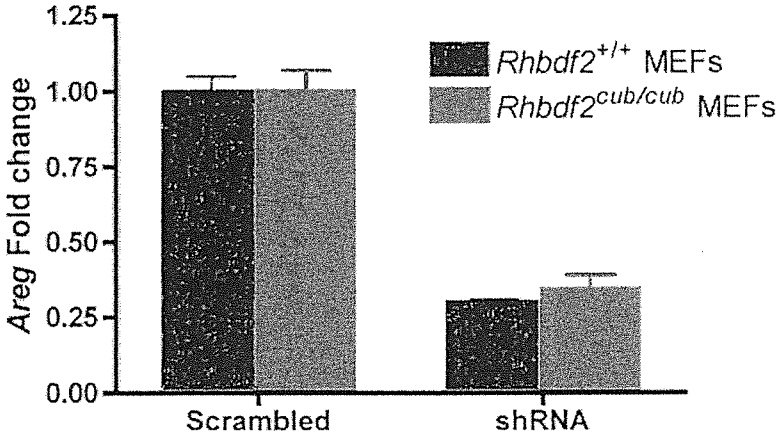


FIG. 12B

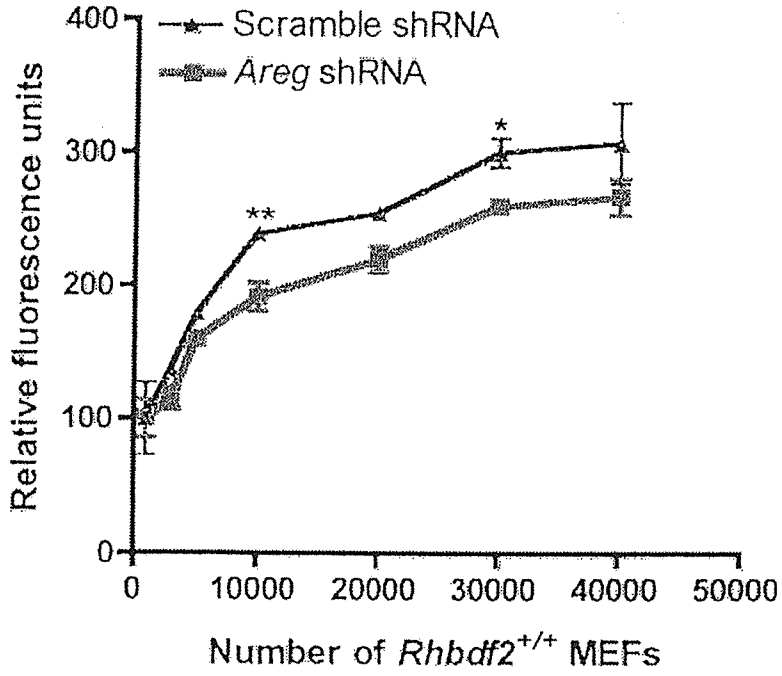


FIG. 12C

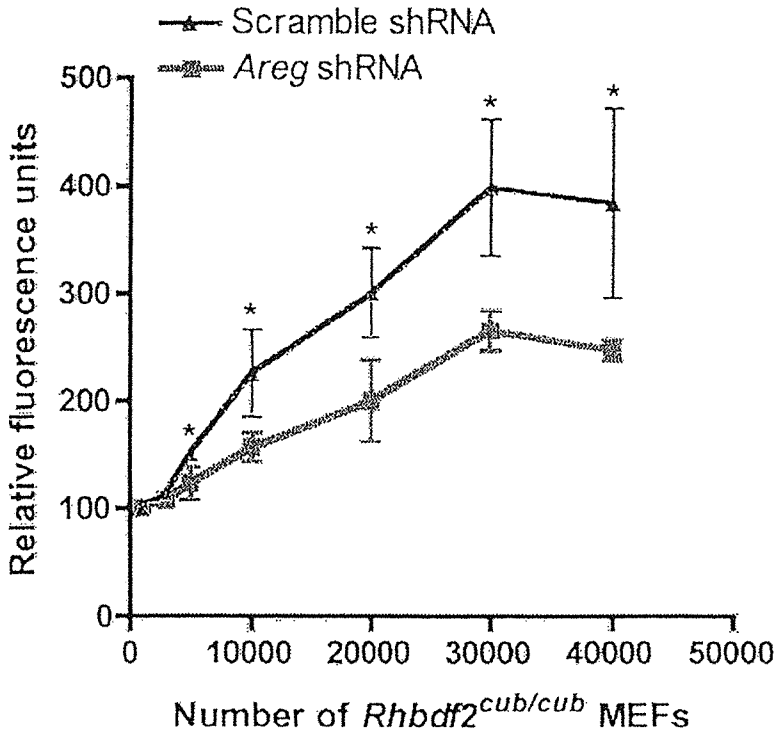


FIG. 12D

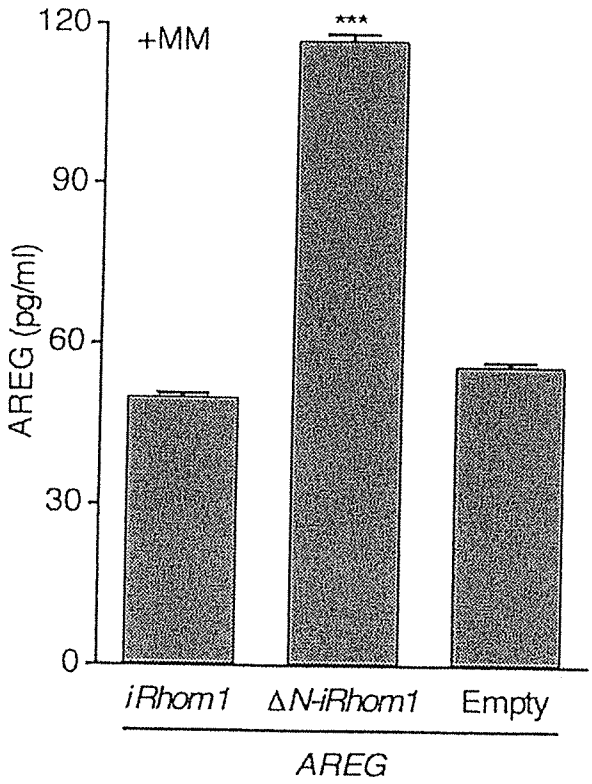


FIG. 13A

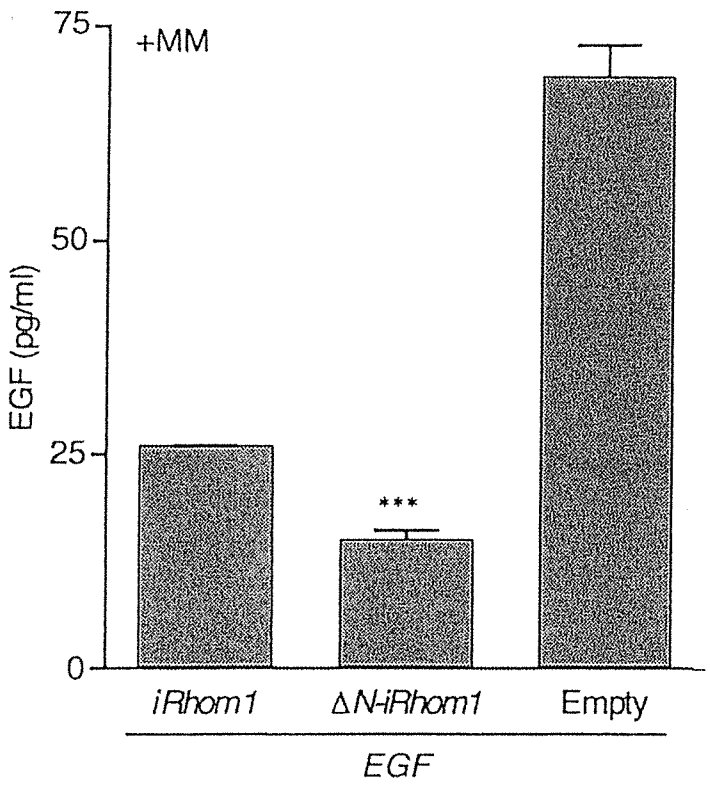


FIG. 13B

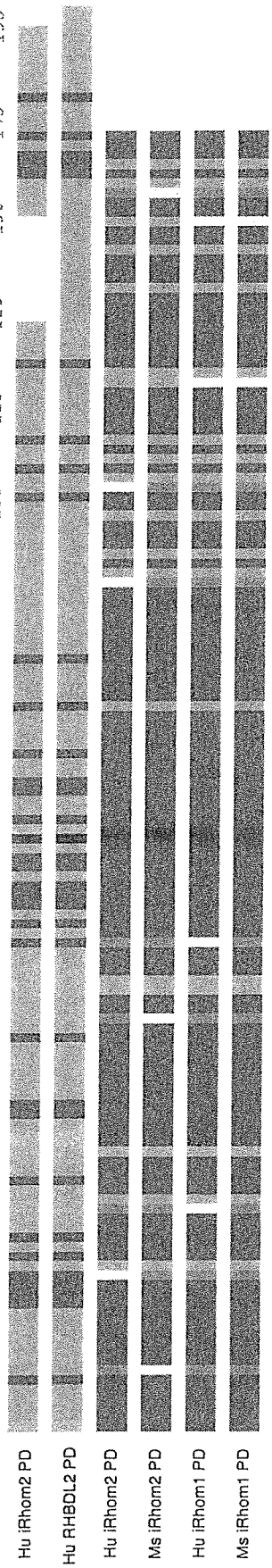

10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
Hu iRhom2 PD
Hu RHBDL2 PD
Hu iRhom2 PD
Ms iRhom2 PD
Hu iRhom1 PD
Ms iRhom1 PD

FIG. 13C

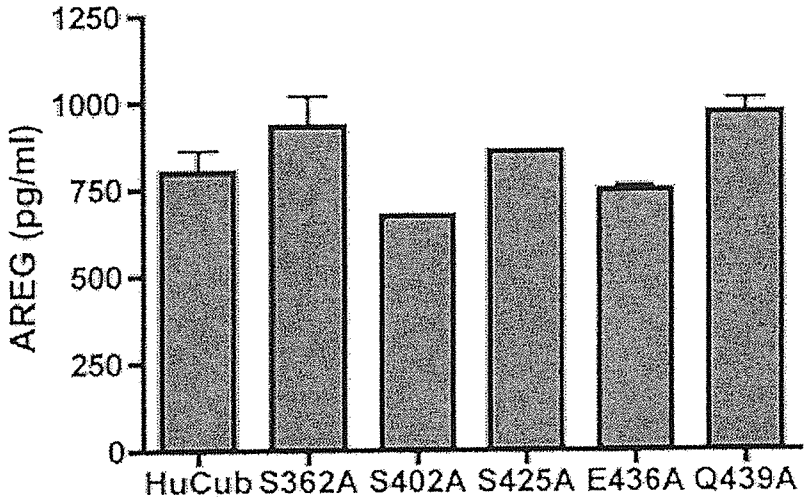

1250
1000
750
500
250
0
AREG (pg/ml)
HuCub
S362A
S402A
S425A
E436A
Q439A

FIG. 13D

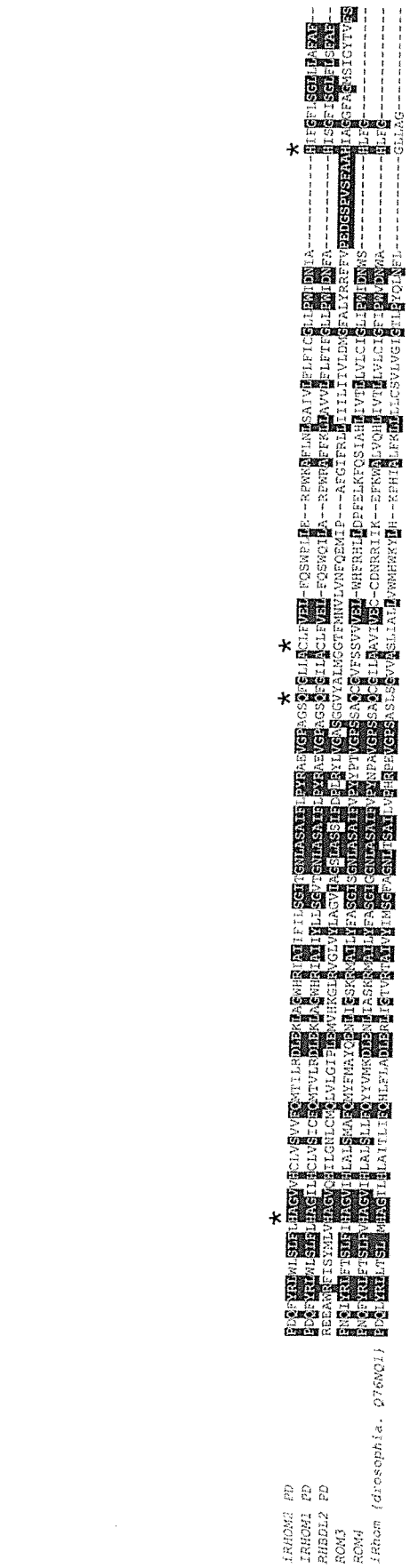

IRHOM2 PD
IRHOM1 PD
RHBDL2 PD
ROM3
ROM4
iRhom (drosophila. Q76NQ1)

FIG. 13E

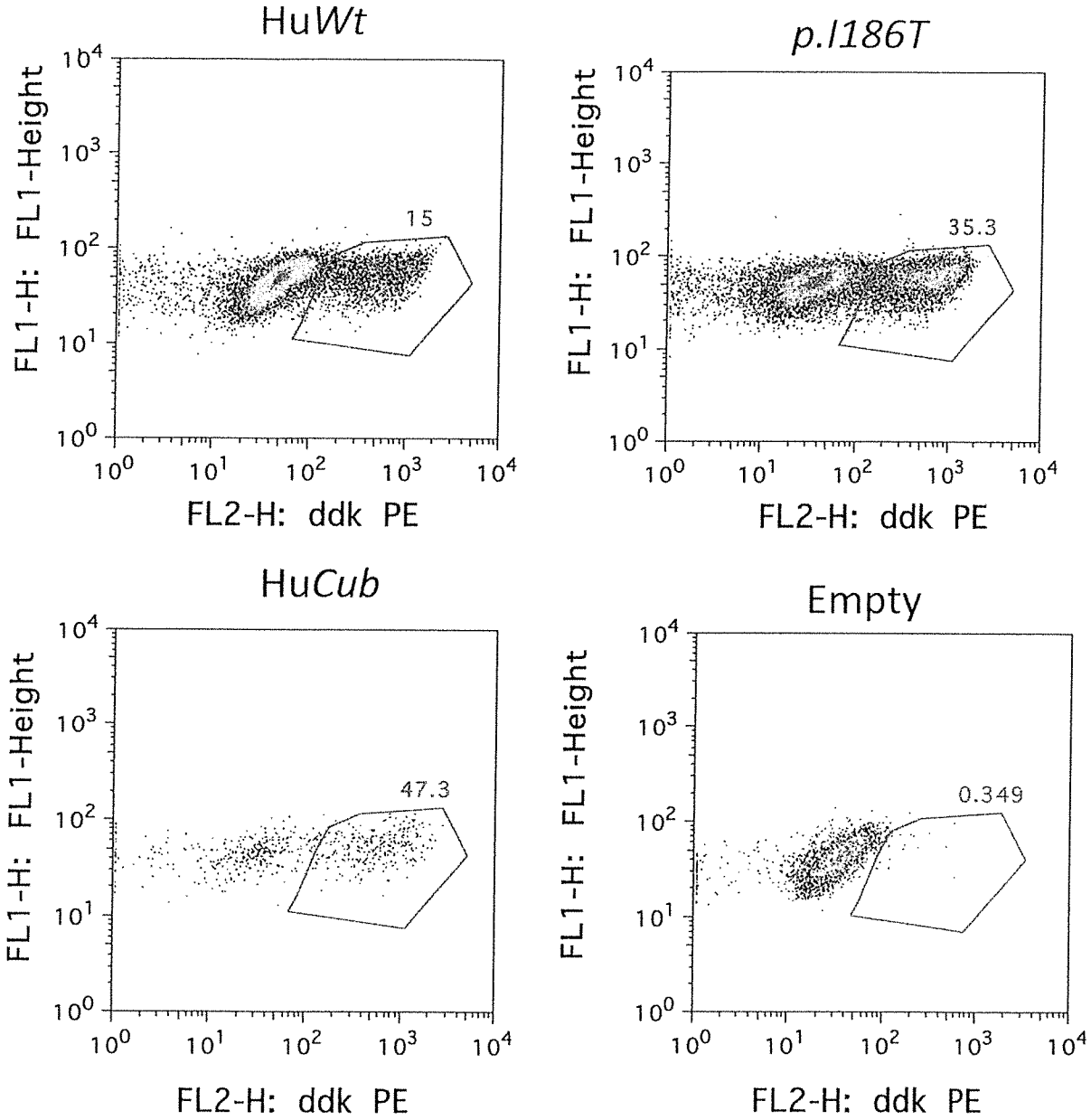

HuWt
15
p.I186T
35.3
HuCub
47.3
Empty
0.349
FL1-H: FL1-Height
FL2-H: ddk PE
10^0
10^1
10^2
10^3
10^4

FIG. 14

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference JLA-11352/47	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US2015/030140	International filing date *(day/month/year)* 11 May 2015	(Earliest) Priority Date *(day/month/year)* 09 May 2014
Applicant THE JACKSON LABORATORY		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☒ **Certain claims were found unsearchable** (see Box No. II).

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. 8

☐ as suggested by the applicant.

☒ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☐ none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/030140

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, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of 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/US2015/030140

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 21
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 21 has been held as omnibus claim, as it refers to an invention "substantially as shown or described herein".

3. ☒ Claims Nos.: 12-14, 16-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/030140

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/37 (2015.01)
CPC - C12Q 1/37 (2015.07)
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)
IPC(8) - C12Q 1/00, 1/34, 1/37; G01N 33/00, 33/15 (2015.01)
CPC - C12Q 1/00, 1/34, 1/37; G01N 2333/00, 2333/90 (2015.07)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C12Q 1/00, 1/34, 1/37; G01N 2333/00, 2333/90 (2015.07) (keyword delimited)
USPC - 435/4, 18, 23, 183, 195

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, PubMed, Google

Search terms used: irhom irhom1 irhom2 egfr identify compounds modulates activity proteolytic activity cleave cleavage substrate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2014/043223 A1 (HOSPITAL FOR SPECIAL SURGERY) 20 March 2014 (20.03.2014) entire document	1 --- 2-5,15
Y	WO 2002/10767 A2 (THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND) 07 February 2002 (07.02.2002) entire document	2
Y	WO 2006/004593 A2 (TANOX, INC) 12 January 2006 (12.01.2006) entire document	3,5,15
Y	US 2011/0052570 A1 (KLAGSBRUN et al) 03 March 2011 (03.03.2011) entire document	4
A	US 2005/0142546 A1 (FREEMAN) 30 June 2005 (30.06.2005) entire document	1-11,15
P, X	HOSUR et al. "Rhbdf2 mutations increase its protein stability and drive EGFR hyperactivation through enhanced secretion of amphiregulin," Proceedings of the National Academy of Sciences, 13 May 2014 (13.05.2014), Vol. 111, Pgs. E2200-E2209. entire document	1-11,15

☐ 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	Date of mailing of the international search report
29 July 2015	14 AUG 2015
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)