Regulation of post-Golgi LH3 trafficking is essential for collagen homeostasis

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Post-translational modifications are necessary for collagen precursor molecules (procollagens) to acquire final shape and function. However, the mechanism and contribution of collagen modifications that occur outside the endoplasmic reticulum and Golgi are not understood. We discovered that VIPAR, with its partner proteins, regulate sorting of lysyl hydroxylase 3 (LH3, also known as PLOD3) into newly identified post-Golgi collagen IV carriers and that VIPAR-dependent sorting is essential for modification of lysines in multiple collagen types. Identification of structural and functional collagen abnormalities in cells and tissues from patients and murine models of the autosomal recessive multisystem disorder Arthrogryposis, Renal dysfunction and Cholestasis syndrome caused by VIPAR and VPS33B deficiencies confirmed our findings. Thus, regulation of post-Golgi LH3 trafficking is essential for collagen homeostasis and for the development and function of multiple organs and tissues.
In vertebrates, procollagen-lysine hydroxylation is catalysed by three lysyl hydroxylase isoenzymes (LH1–3), encoded by Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase (PLOD1-3) genes. LH3/PLOD3 is the only isoenzyme that also generates hydroxylysine-linked carbohydrates because of its galactosyl- and glucosyl galactosyl-transferase (GT and GGT) activities, critical for procollagen intermolecular crosslinking and stabilization of fibrils into the supramolecular collagen structure. Deficiency of LH3 affects assembly and secretion of multiple collagen types and leads to abnormal basement membrane formation. All LH enzymes are thought to exert their function in the endoplasmic reticulum (ER); however, LH3 is also found in the extracellular space, both in soluble form and anchored to the external side of the plasma membrane. While the earlier collagen modification steps have been extensively studied, the regulatory mechanism and contribution of LH3 modifications to collagen homeostasis outside ER and Golgi are not well understood.

We find that LH3 interacts with a trafficking protein, VIPAR. Deficiencies of VIPAR and its partner VPS33B cause arthropathy, renal dysfunction and cholestasis syndrome (ARC), a multisystem disorder with characteristics developmental and functional defects of the musculoskeletal system, kidneys, liver, skin and platelets that shows some overlap with a clinical phenotype seen in a patient with inherited LH3 deficiency. The LH3–VIPAR interaction, together with the engagement of first RAB10 and then RAB25, appears to be essential for LH3 trafficking and delivery to newly identified Collagen IV Carriers (CIVC) in inner medullary collecting duct cells (mIMCD3). We found that VPS33B and VIPAR deficiencies result in a reduction of LH3-dependent post-translational modification of collagen IV in these cells accompanied by an abnormal deposition of the extracellular matrix (ECM) and disruption of cell polarity in three-dimensional (3D) cyst models of VPS33B, VIPAR, and LH3 kd cells.

LH3-specific collagen modification levels are reduced in ARC patients’ urine, as well as in collagen I from cultured skin fibroblasts. In addition, structural defects in collagen I are found in tail tendons from VPS33B- and VIPAR-deficient mice. Taken together, these findings establish a role for VPS33B/VIPAR in the intracellular trafficking of LH3 and collagen homeostasis.

**Results**

**LH3 is a novel VIPAR N-terminal interactor.** We identified LH1 and LH3 isoenzymes as potential interactors of the coexpressed His₆-C-Myc₄-tagged VPS33B and His₆-StrepPl₁-tagged VIPAR in human embryonic kidney 293 (HEK293) cells using a pull-down assay and analysis of the purified sample by electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS; Supplementary Fig. 1a,d). While the LH1 interaction was not confirmed in vitro, the interaction with LH3 was further supported by co-immunoprecipitation of transiently expressed VPS33B and VIPAR with endogenous LH3 and colocalization of the endogenous proteins (Fig. 1a–e).

VPS33B and VIPAR are homologous to yeast Vps33p and Vps16p—core constituents of the multiprotein HOPS (Homo- typic fusion and vacuole Protein Sorting) and CORVET (class C core vacuole/endosome tethering) tethering complexes. Metazoans have two homologues of each Vps33p and Vps16p, and recent evidence suggested that only VPS33A and VPS16 homologues are members of the conventional mammalian HOPS and CORVET complexes, while functions of VIPAR and VPS33B remain unknown. Our in silico analysis showed that human VPS33B (UniProt Q9H267) is structurally similar to homologous VPS33A, whereas VIPAR (UniProt Q9H9C1) is a 57-kDa protein characterized by a long disordered region of ~130 amino acids at its N terminus, followed by a globular alpha-solenoid divergent in sequence but structurally related to the C terminus of VPS16 (Supplementary Fig. 2a). Further homology modelling using the human VPS33A–VPS16 and fungal VPS33–VPS16 crystal structures as references agreed with this predicted domain organization of VIPAR (Supplementary Fig. 2b), suggesting an extended interaction platform defined by the concave side of VIPAR alpha-solenoid domain embracing the globular VPS33B. This interface is structurally similar to that observed in the VPS33A–VPS16 complex, but is characterized by numerous unique complementary electrostatic and hydrophobic contacts (Supplementary Fig. 2c). Analytical gel filtration analysis showed that VPS33B and VIPAR co-elute in a single peak (Supplementary Fig. 1b), supporting the predicted strong macromolecular interactions between the two proteins.

The observation is further supported by the largely improved recombinant expression yields for VPS33B and VIPAR when the two proteins are coexpressed in HEK293 cells compared with production of single proteins (Supplementary Fig. 1c).

Pull-down experiments using recombinant short fragments of human VIPAR corroborated this structural organization, indicating that the flexible N-terminal region of the protein is dispensable for VPS33B interaction (Fig. 1b,c). Using the same pull-down strategy, we established that the flexible N terminus of VIPAR is necessary and sufficient for LH3 interaction (Fig. 1b,c).

The amino-acid sequence of VIPAR N terminus is not conserved in VPS16. Comparative bioinformatics predictions suggested that the presence of transmembrane segments in this region is unlikely and, in parallel, recombinant VIPAR and its fragments behaved as fully soluble cytoplasmic proteins during extraction and purification. Therefore, as previous studies suggested LH3 to be a membrane-associating (but not membrane-crossing) protein facing the organellar lumen, we concluded that the VIPAR–LH3 interaction requires an intermediate transmembrane mediator that is yet to be identified.

**VIPAR–LH3 interaction and its significance for LH3 secretion.** We then examined the intracellular localization of LH3–VIPAR interaction and whether VIPAR and VPS33B deficiencies affected LH3 distribution. First, we found that in human skin fibroblasts there was a higher level of colocalization of endogenous LH3 with the trans-Golgi network (TGN) protein TGN46 staining than with the ER markers PDI and Calreticulin (Supplementary Fig. 3). We then identified colocalization of both endogenous LH3 and VIPAR with TGN46 and the TGN-specific clathrin adaptor AP1 in HeLa cells (Fig. 1d,e). LH3-mCherry partially colocalized with Golgin97 TGN marker (Supplementary Fig. 4a) in murine inner medullary collecting duct (mIMCD3) cell line previously used to model apical–basolateral polarity defects of ARC syndrome.

As LH3 is normally secreted into the ECM and found in circulation, we tested whether VPS33B and VIPAR are necessary for extracellular secretion of LH3 by measuring intracellular and extracellular LH3 GGT activity in mIMCD3 cell lines: wild-type (wt) and stable knockdown (kd) clones including control shRNA without significant changes in the intracellular measurements, suggesting that the extracellular secretion of LH3 was not impaired by VIPAR or VPS33B deficiencies.

VPS33B–VIPAR mediate LH3 delivery to collagen IV carriers. Consistent with collagen IV being the major kidney constituent protein modified by LH3, in control shRNA cells the majority...
HeLa rather than mIMCD3 cells were used because of anti-LH3 and anti-VIPAR unsuitability for immunostaining of mouse cells. Colocalization for [near but distinct from the ER (Fig. 2c)]. LH3 in discrete cytoplasmic membrane-bound structures located correlative light and electron microscopy (CLEM) images identified colocalize with the ER marker calreticulin (Supplementary Fig. 4b), microscopic level some of the LH3-collagen IV puncta appeared to after LH3 transfection (Fig. 2a,b). Although at the light microscopic level some of the LH3-collagen IV puncta appeared to colocalize with the late endosome and lysosome marker CD63 (Figs 2a and 3a). The punctate collagen IV staining was unchanged in LH3 colocalization with TGN38 and CD63 was detected was lost in VIPAR shRNA cells (Fig. 2a) where an increase in LH3 colocalization with TGN38 and CD63 was detected (Figs 2a and 3a). LH3-collagen IV colocalization was rescued by re-introducing CFP-VIPAR and YFP-VPS33B (Fig. 2a). Pioneering studies of procollagen transport have elucidated ER to Golgi trafficking steps12–16,27–29; however, the post-Golgi collagen IV compartment has not been characterized. Therefore, we performed CLEM with 3D reconstruction to describe the sites of LH3 and collagen IV colocalization. Approximately 20 cytoplasmic

Figure 1 | LH3 interacts with the N terminus of VIPAR at the TGN. (a) Co-immunoprecipitation of endogenous LH3 with myc-VIPAR in HEK293 cells. HEK293 cells were transfected with HA-VPS33B and/or myc-tagged VIPAR. After anti-HA (left) or anti-myc (right) immunoprecipitation, samples were immunoblotted using anti-LH3, anti-HA or anti-myc antibodies. Experiment was repeated three times. (b) Schematic representation of the fragments of VIPAR used to investigate its interactions with VPS33B and LH3: full-length (FL), the flexible N terminus of VIPAR (N), two C-terminal constructs (C− and C+) including the putative alpha-solenoid region (oval cartoon), with two different starting points based on secondary structure predictions. (c) HEK293 cells were co-transfected with myc-tagged VIPAR constructs represented in b and HA-VPS33B. Immunoprecipitation was performed with anti-myc antibody. Immunoprecipitates and inputs were blotted with anti-LH3, anti-HA or anti-myc antibodies. Experiment repeated three times. In a,c representative blots are shown. Uncropped western blots are shown in Supplementary Fig. 11. (d) Colocalization analysis of endogenous LH3, VIPAR and TGN46 in HeLa cells. (VIPAR/LH3, n = 9; LH3/TGN46, n = 51; VIPAR/TGN46, n = 22 of pooled data set of two to three independent experiments). Colocalization for d,e was measured by the Pearson’s coefficient. Error bars represent s.d. Figures show representative data and images. Scale bars, 10 μm. HeLa rather than mIMCD3 cells were used because of anti-LH3 and anti-VIPAR unsuitability for immunostaining of mouse cells.

(~90%) of LH3-mCherry-positive puncta colocalized with intracellular collagen IV, while a small proportion of LH3 colocalized with the late endosome and lysosome marker CD63 (Figs 2a and 3a). The punctate collagen IV staining was unchanged after LH3 transfection (Fig. 2a,b). Although at the light microscopic level some of the LH3-collagen IV puncta appeared to colocalize with the ER marker calreticulin (Supplementary Fig. 4b), correlative light and electron microscopy (CLEM) images identified LH3 in discrete cytoplasmic membrane-bound structures located near but distinct from the ER (Fig. 2c).
membrane-bound CIVC per cell were identified in wt mIMCD3 cells. Most CIVCs were 400 nm in their largest diameter (range 200–900 nm), and were consistently circular in cross-section (Fig. 2c,d and Supplementary Fig. 9b). The content of individual CIVCs varied slightly but generally was marginally more electron-dense than the cytoplasm, containing one or more intact small vesicle(s), one or more small electron-dense non-membranous granule(s), and occasionally a small membranous whorl. The presence of collagen IV in LH3 containing CIVCs was further confirmed by CLEM of LH3-mCherry coupled to immunoperoxidase staining for collagen IV. The oxidation of DAB delineated collagen IV-positive dark areas (detectable by EM) that overlapped with LH3 positive puncta (detectable by light microscopy) (Supplementary Fig. 9). DAB-positive staining for collagen IV but
not LH3-mCherry, was visible in structures resembling the ER (Supplementary Fig. 9).

None of the LH3-positive puncta were found to localize to these characteristic CIVCs in the VIPAR kd cells where LH3 was found exclusively in larger membrane-bound structures of irregular shape that contained highly electron-dense material resembling degraded membranes, consistent with the CD63-positive endosomes containing LH3 identified with light microscopy (Fig. 3b and Supplementary Fig. 9c). Taken together, the data above suggest that VIPAR and VPS33B deficiencies result in a block of LH3 delivery to CIVCs and an increase in its delivery to late endosomes for degradation and/or secretion.

**RAB10 and RAB25 are involved in post-Golgi LH3 sorting.** RAB GTPases cycle between active GTP-bound and inactive GDP-bound states and regulate specific steps in intracellular trafficking by recruiting ‘effector’ molecules. Since previous data suggested that VPS33B and VIPAR in complex regulate apical–basolateral polarity and may act as an effector for RAB11A associated with recycling endosomes, we examined whether RAB11A, RAB25 and RAB10 proteins are required for LH3 delivery to CIVCs because of their involvement in recycling endosome function and in apical–basolateral polarity regulation.

Low level of colocalization was found between RAB11A and LH3-mCherry, while overexpression of RAB11A-dominant-
negative mutant did not significantly affect LH3-collagen colocalization (Supplementary Fig. 6a). On the contrary, both RAB10 and RAB25 were found to be present in puncta positive for overexpressed CFP-VIPAR and LH3-mCherry (Supplementary Fig. 6b,c). Furthermore, VPS33B alone and when coexpressed with VIPAR co-immunoprecipitated with both RAB25 and RAB10 (Fig. 4a), suggesting that the potential RAB10 and RAB25 involvement in LH3 trafficking is mediated by VPS33B.

Puncta positive for LH3-mCherry colocalized with RAB10 and RAB25 in wt and control shRNA, while in VIPAR shRNA cells (which also had reduced VPS33B expression; Supplementary Fig. 5b) LH3-mCherry colocalization with RAB10 and RAB25 was reduced (RAB10; Fig. 4b) or lost (RAB25; Fig. 4c). Neither RAB10 nor RAB25-positive puncta that colocalized with LH3-mCherry also colocalized with collagenv IV, suggesting that these interactions may occur before LH3 delivery to CIVCs. RAB10 and VIPAR-positive puncta colocalized with the Golgin97 TGN marker (Supplementary Fig. 6b). Overexpression of RAB10-dominant-negative mutant RAB10 (T23N) prevented LH3-mCherry colocalization with collagen IV and RAB25 (Fig. 5a), while overexpression of RAB25-dominant-negative mutant (T26N) resulted in a loss of LH3-mCherry colocalization with collagen IV but not with GFP-RAB10 (Fig. 5b), placing RAB10 function proximal to RAB25. Taken together, these data provide evidence for the sequential involvement of RAB10 and RAB25 in LH3 transport to CIVCs (Supplementary Fig. 7).

**Molecular and cellular defects in the kd mIMCD3 cells.** We questioned whether defective sorting of LH3 to CIVCs in VIPAR shRNA and VPS33B shRNA cells affects collagen IV modification and whether all three kd’s result in similar morphological changes. Our mIMCD3 cell culture experiments suggested that higher than 75% kd of LH3 resulted in cell death; thus, we accepted a lower kd level for LH3 shRNA than for VIPAR shRNA and VPS33B shRNA (Supplementary Fig. 5b). LH3-deficient cells and tissues are known to accumulate abnormally modified collagen and are unable to correctly form basement membranes, resulting in severe developmental defects and embryonic lethality in a complete LH3 knockout (ko) mouse.5,7,8 Similarly to LH3 kd cells, an increase in the intracellular collagen IV was observed also in VPS33B and VIPAR kd (Fig. 6a,b). A corresponding decrease in E-cadherin levels was observed in LH3 kd cells as previously described in VPS33B and VIPAR kd20 (Fig. 6a,c); in addition, in 3D culture all three kd mIMCD3 cell lines were unable to form spheres with the well-defined lumen and displayed abnormal deposits of ECM (Fig. 6c,d). Using mass spectrometry we detected a similar reduction in the levels of LH3-mediated post-translational modifications in collagen IV in VPS33B, VIPAR and
LH3 kd mIMCD3 cells, confirming that correct LH3 localization in CIVCs is necessary for collagen modification (Fig. 6e and Supplementary Table 1).

To study the effect of VPS33B, VIPAR and LH3 deficiencies on gene regulation, we used RNA expression arrays and subsequent in silico analysis (Fig. 6f, Fig. 7 and Supplementary Fig. 10). After identification of differentially expressed genes34, we used the online software DAVID to determine Gene Ontology (GO) annotations that were over-represented among the list of differentially expressed genes35. Similar results were obtained
across the kd cells, with several enriched GO terms being related to cell–cell adhesion (Supplementary Table 2). Furthermore, a strong correlation between gene expression changes across the kd’s was found when the similarity of the transcriptomes was analysed (Fig. 7). Lists of the top 100 differentially expressed genes were compiled for each kd cell line and a strong statistically significant overlap was observed, with 50% of the genes in each list differentially expressed in all three kd cell lines (Fig. 6f). Gene

Figure 5 | RAB10 and RAB25 are sequentially involved in post-Golgi LH3 trafficking. (a) Confocal fluorescence photomicrographs of control shRNA mIMCD3 cells co-transfected with LH3-mCherry and GFP-RAB10 or its dominant-negative form GFP-RAB10(T23N) and immunostained for collagen IV (top rows) or RAB25 (bottom row). (upper graph: + RAB10, n = 11; + RAB10(T23N), n = 12 of pooled data set of two independent experiments; P = 0.0002 using Mann–Whitney, two-tailed). (Lower graph: + RAB10(T23N), n = 8 of pooled data set of two independent experiments.) (b) Confocal fluorescence photomicrographs of mIMCD3 cells co-transfected with LH3-mCherry and GFP-RAB25 or its dominant-negative form GFP-RAB25(T26N) and immunostained for collagen IV (top rows). Confocal fluorescence photomicrographs of control shRNA cells, co-transfected with LH3-mCherry, GFP-RAB10 and untagged dominant-negative RAB25(T26N) (immunostained overexpressed, bottom row). Colocalization between collagen IV and LH3-mCherry for both a, b as well as between LH3-mCherry and overexpressed RAB25 (a), and LH3-mCherry and GFP-RAB10 (b) was measured by the Pearson’s coefficient (upper graph: + RAB25, n = 6; + RAB25(T26N), n = 9 of pooled data set of two independent experiments; P = 0.0022 using Mann–Whitney, two-tailed). (Lower graph: + RAB25(T26N), n = 8 of pooled data set of two independent experiments). In ab error bars represent s.d. Figures show representative images. Scale bars, 10 µm. ***P ≤ 0.001.

Figure 6 | Knockdown mIMCD3 cells share molecular and cellular phenotypes. (a) Total cell lysates from mIMCD3 cell lines were analysed by western blot with antibodies against collagen IV, E-cadherin and β-actin. Uncropped western blots are shown in Supplementary Fig. 13a. (b) mIMCD3 cell lines were grown on transwell supports for 2 weeks and immunostained with anti-collagen IV antibody. Figures show representative images from three independent experiments of n = 3 views of multiple cells each. Scale bars, 10 µm. (a, b) Repeated three times. Representative images are shown. (c) mIMCD3 cell lines cultured for 3 days in 3D collagen I gels, immunostained with anti-E-Cadherin and anti-Claudin-1 antibodies. Scale bars, 10 µm. (d) EM of 3D cultured mIMCD3 cells (top panels), scale bars, 20 µm. Higher magnification of the areas of abnormal extracellular matrix deposits in kd cells (bottom panels). Representative images of n = 9 (Control shRNA), n = 7 (VIPAR shRNA), n = 9 (VPS33B shRNA) and n = 8 (LH3 shRNA) spheres are shown. Scale bars, 1 µm. (e) LC-MS-MS analysis for the relative quantification of the degree of Lys-O-GalGlc, Lys-O-Gal and Lys-OH in collagen IV from mIMCD3 cell lines. Two different control lines (wt and Control shRNA) and two different VIPAR shRNA clones were used. LH3 shRNA cells were used as a positive control. The top 100 genes based on P value were selected and compared across the three kds.
set enrichment analysis\(^{36,37}\) was then used to highlight the cellular processes perturbed in the kd cell lines by looking for gene sets with a larger than expected number of differentially expressed genes. Interestingly, the overlapping ‘Axon guidance’ and ‘Semaphorin interactions’ gene sets were the most significantly enriched sets for all three kd cell lines (Supplementary Table 3).

Collagen defects in ARC patients and murine models. ARC syndrome caused by VPS33B or VIPAR deficiencies results in abnormal kidney and liver function, extremely dry skin (ichthyosis), defective platelet \(\alpha\)-granule biogenesis, osteopaenia and recurrent bone fractures, and death in infancy in the majority of patients. We generated inducible \(\text{Vps33b}^{\text{fl/fl}}\)-ERT\(^2\) \(\text{mice because of embryonic lethality of constitutive Vps33b}^{\text{ko}}\) at E7.5 and recently described haematological defects that accurately model abnormalities found in ARC patients’ platelets\(^{38}\). Neither \(\text{Vps33b}^{\text{fl/fl}}\)-ERT\(^2\) nor the \text{de novo} generated inducible \(\text{Vipas39}^{\text{fl/fl}}\)-ERT\(^2\) \(\text{ko mice showed visceral abnormalities; however, both ko’s developed dry, scaly skin and hair loss 4 weeks after induction. Cultured osteoblasts derived from tamoxifen-induced \(\text{Vps33b}^{\text{fl/fl}}\)-ERT\(^2\) and \(\text{Vipas39}^{\text{fl/fl}}\)-ERT\(^2\) mice demonstrated successful ko (Supplementary Fig. 5c). As it was previously shown that LH3-mediated glycosylation of procollagen I is crucial for crosslink formation, fibrillogenesis and bone mineralization\(^{59}\), we euthanized the animals to analyse collagen I structure and function in the tail tendons. Atomic force microscopy (AFM) and scanning electron microscopy (SEM) of collagen I from \(\text{Vipas39}^{\text{fl/fl}}\)-ERT\(^2\) and \(\text{Vps33b}^{\text{fl/fl}}\)-ERT\(^2\) male and female pooled mouse tendons showed swelling and distortion of the fibrils, lack of cohesion, crimping and disordered fibrils compared with control mice that displayed normal D-band with a consistently regular and aligned fibrils (Fig. 8a,b and Supplementary Fig. 8). Although the fibrillar D-band period did not statistically vary \((P=0.32 \text{ using one-way analysis of variance})\) between control \((D=67.8 \pm 1.0)\) nm and \(\text{Vipas39}^{\text{fl/fl}}\)-ERT\(^2\) \((D=65.0 \pm 2.3)\) nm and \(\text{Vps33b}^{\text{fl/fl}}\)-ERT\(^2\) \((D=64.1 \pm 1.9)\) nm when measured by two-dimensional (2D) Fast Fourier transform (FFT) of AFM images \((N=7)\), localized variations in the shape of the banding were apparent (Fig. 8a) in the case of both \(\text{Vipas39}^{\text{fl/fl}}\)-ERT\(^2\) and \(\text{Vps33b}^{\text{fl/fl}}\)-ERT\(^2\). These data suggest a disparity in the quaternary collagen I structure in the ko mice.

Cultured skin fibroblasts derived from five ARC patients with different VPS33B and VIPAS39 mutations were analysed, and consistent procollagen I accumulation was found in patients’ fibroblasts compared with controls (Fig. 8c). Defects in LH3-dependent lysine modifications were identified in patients’ fibroblasts grown in the presence of ascorbic acid compared with the age-matched control (Fig. 8d). The difference in collagen lysine hydroxylation (also performed by LH1 and LH2) was less pronounced when compared with LH3-specific modifications, suggesting that the defect is LH3-specific. In addition, testing urine available from three different ARC patients with known mutations in \(\text{VPS33B}\) showed a substantial decrease in all LH3-dependent post-translational lysine modifications compared with age-matched controls (Fig. 8e).

Discussion

We have demonstrated that LH3 targeting from the TGN to the newly described collagen IV-containing organelles is regulated by VIPAR and its interacting proteins. In silico modelling and co-immunoprecipitation experiments allowed us to identify the N terminus of VIPAR as a novel indirect interactor of LH3, while the VIPAR C terminus stably interacts with its molecular partner VPS33B. Contradicting reports provide room for controversy regarding the potential roles for different homologues of yeast VPS33 and VPS16 in HOPS and/or CORVET function in endocytosis and autophagy\(^{20,23,40–42}\). Our study proposes that VIPAR and VPS33B, in association with RAB10 and RAB25, are involved in a novel post-Golgi LH3 trafficking pathway. VIPAR, in complex with VPS33B, may have a role in RAB10/RAB25 conversion on post-Golgi vesicles, similar to HOPS and CORVET regulation of RAB5/RAB7 conversion in endocytosis\(^{43,44}\), and may assist membrane tethering by interacting with SNARE proteins. To confirm the importance of this new pathway to collagen homeostasis, we have demonstrated that VPS33B and VIPAR deficiencies result in abnormal LH3-dependent post-translational modification of collagen IV in a murine kidney cell line, and procollagen I in ARC patients’ skin fibroblasts.

Furthermore, a reduction in LH3-specific modifications was detected in ARC patients’ urine, and structural defects in collagen I were found in tail tendons from VPS33B- and VIPAR-deficient mice. The collagen abnormalities identified in skin fibroblasts and tendons consistent with the characteristic ARC features of ichthyosis, arthrogryposis, osteopaenia and bone fractures suggest an important role for VPS33B- and VIPAR-dependent LH3 trafficking in connective tissue.

Gene expression analysis provided insight into the possible regulatory mechanisms responsible for the cell morphology defects and detected high similarity between the profiles in VPS33B-, VIPAR- and LH3-deficient cells with dysregulation of ‘axon guidance’ and ‘semaphorin interactions’ overlapping gene sets. Semaphorins initiate signals to the cytoskeleton that regulate the organization of actin filaments and the microtubule network\(^{45}\). The genes incorporated into this set have recently been implicated in cancer progression as they can influence cell behaviour by activating plexins or inhibiting the interactions of growth factors\(^{46}\). In addition, a recent report suggested that LH3
might be involved in recruitment of matrix metalloproteinase 9, which plays a prominent role in ECM remodelling and TGF-beta activation17.

We therefore present evidence that regulation of LH3-collagen modification by this novel VIPAR-dependent trafficking pathway is crucial for cell differentiation and tissue morphogenesis. Further work is required to demonstrate why collagen IV carriers are the preferred location for the LH3-dependent collagen IV modification and whether they have a role in homeostasis of other basement membrane components.

Methods

DNA constructs. Mouse-specific pRS plasmid shRNA constructs (VIPAR: T1594651, LH3: T1541624) and pRS control shRNA (TR30012) were obtained from Origene (Cambridge Biosciences, UK).

Plasmids generated previously20 containing tagged full-length VIPAS39, VPS33B and endosomal markers cDNA were used for immunofluorescence and co-immunoprecipitation experiments. Short constructs of VIPAR used for co-immunoprecipitation experiments (Fig. 1b,c) were cloned in pCMV-Myc vector using the primer sequences and restriction enzymes described in Supplementary Table 4. Full-length human PLOD3 cDNA was obtained from Source Biosciences and was cloned into pmCherry N1 vector using the primer sequences and restriction enzymes described in Supplementary Table 4. GFP-Rab10 was a gift from D. Cutler (MRC Laboratory for Molecular Cell Biology, UCL, UK). Rab25- and Rab10-dominant-negative mutants GFP-Rab25(T26N) and GFP-Rab10(T23N) were created using Quick-change XL site-directed Mutagenesis Kit (Agilent Technologies, UK). GFP-LC3 plasmid was a gift from R. Ketteler (MRC Laboratory for Molecular Cell Biology, UCL, UK). Plasmids of the UPE series for affinity-tagged recombinant expression of VPS33B and VIPAR in HEK293 cells were provided by U-protein Express BV (U-PE, the Netherlands) and cloned using the conditions reported in Supplementary Table 4. pEGFP-Rab11a and the dominant-negative Rab11a plasmid pEGFP-Rab11aDN, containing the S25N missense mutation, were gifts from F. Barr (University of Oxford, UK). Rab25 cDNA was custom-synthesized by Eurofins MWG Operon (London, UK) and cloned into pEGFP-C3 vector backbone using EcoRI and Kpn1 restriction enzymes.

Mammalian cell culture and transfection. All cell culture reagents were from Sigma-Aldrich, UK, unless otherwise stated. Human skin fibroblasts, HEK293 and HeLa cells (not authenticated, both gift from E.R. Maher, Department of Medical Genetics, University of Cambridge, UK) were maintained in high-glucose DME supplemented with 2% fetal bovine serum (FBS), and human lung fibroblasts (MRC Laboratory for Molecular Cell Biology, UCL, UK) in 10% FBS. All cell lines were regularly tested for the lack of mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza, UK). For microscopy experiments, cells were seeded either on eight-well tissue culture-treated μ-Slides (IBIDI, Thistle Scientific, UK) or on glass coverslips. For live imaging IBIDI 35-mm cell culture-treated dishes were used.

For protein extraction, cells were cultured in plastic multwell plates (Corning, UK) or 75-cm² flasks (Corning). For collagen IV analysis, mIMCD3 cells were plated onto 0.4-μm-pore Transwell-permeable supports (Corning) at 1 × 10⁵

Figure 8 | Abnormal collagen modification and structure in VPS33B- and VIPAR-deficient mouse models and patients. (a) AFM of mouse tail tendon collagen I from control, VIPAS39/ERT2 and VPS33B/ERT2 male and female mice. Scale bar, 400 nm. (b) SEM of mouse tail tendon collagen I from control, VIPAS39/ERT2 and VPS33B/ERT2 male and female mice. Scale bar, 20 μm. For a, b representative images from three independent experiments with three animals per group are shown. (c) Total cell lysates of human skin fibroblasts derived from ARC patients and age-matched controls were immunoblotted with antibodies against procollagen I and β-actin, and densitometry was carried out. Levels of procollagen I in ARC patient fibroblasts were measured relative to control I in the figure. Uncropped western blots are shown in Supplementary Fig. 13b. (d) LC-MS-MS analysis for the relative quantification of Lys-O-GalGluc, Lys-O-Gal and Lys-OH in collagen I from human skin fibroblasts derived from ARC patients with different VPS33B and VIPAS39 mutations and an age-matched control. Error bars represent s.d. from two experimental repeats. (e) LC-MS-MS of urine analysed for LH3-dependent collagen lysine modifications in three different ARC patients and age-matched controls (n = 19). Error bars represent s.d.
Collagen elution and digestion with Pronase E. Collagens IV and I were immunoprecipitated as described above. After three washes with NP-40 lysis buffer containing 200 µl FAPS elution solution (50% formic acid, 25% acetonitrile, 15% isopropanol and 10% water) to remove the affinity-purified collagen. Samples were then dried with a centrifugal evaporator (Costar 2-18 HCl) and rehydrated with 100 µl of phosphate buffer (pH 7.4). Subsequently, collagen was digested with 50 µl of 5 mg ml⁻¹ Pronase E in phosphate buffer for 6 h at 37 °C by shaking. Samples were further digested with 100 µl of Pronase E for 16 h and additional 50 µl for 6 h.

Tandem mass spectrometry measurement of lysine modifications. A Waters Acuity Ultra Performance Liquid Chromatography (UPLC) coupled to a Xevo TQ-S Triple Quadrupole Mass Spectrometer (Waters Corp, UK) and stable isotope internal standards were used to develop a rapid 5-min test for the quantification of gelatin/glycolylated hydroxylysine (lysine-GalGaGa), galactosyl hydroxylysines (lysine-O-Gal and hydroxylysines (lysine-Oh)). Lys-O-GalGlc and Lys-Oh-Gal standards were obtained as a kind gifts from Professor Ruggero Tenni (University of Pavia, Italy). A stable isotope-labelled lysine (13C6N-lysine) was used for quantification of all targeted metabolites. The instrument was operated in a negative ion mode and standards (10 mM) were infused into the electrospray source at a flow rate of 25 µl min⁻¹ to determine parent and product ion m/z; optimum cone and collision energies of the MRM assay (MS-operating parameters are shown in Supplementary Table 1).

The capillary voltage was maintained at 3.7 kV, with source temperature held constant at 80 °C and nitrogen gas used as the nebulizing gas at 50 l min⁻¹. The collision energy was set at 30 l/min for peptides below 1000 Da and 36 l/min for peptides above 1000 Da. The masses were determined for each amino acid in the scan mode over the mass range of m/z 200–240. Product ions were determined over a mass range of m/z 50–950 following collision-induced dissociation and using argon as the collision gas.

UPLC liquid chromatography parameters. A UPLC profile was created and optimized to these transitions using an ACQUITY UPLC 2.1 x 50 mm BEH C8 1.7-µm column (Waters Corp). Column temperature was maintained at 55 °C. The peptide elution gradient consisted of a total of 5 min starting from 5 to 67% acetonitrile, 33% methanol over 4 min and back to 5% acetonitrile for 1 min for re-equilibration. Flow rates were 0.8 ml min⁻¹ at a column temperature of 40 °C.

FMOC-derivatization of amino acids for UPLC-MS/MS analyses. The collagen-derived amino acids Lys, Lys-OH, Lys-O-GalGlc and 13C6N-Lys were derivatized before mass spectrometry with Fluorenylmethylcarbonyl chloride (FMOC-C) following the established protocols. Calibration curves were used for quantification of collagen-derived amino acids over the range 190 nmol to 100 µmol. The degree of collagen modification was determined by rationing the collagen-derived amino acid response to the 13C6N-Lys internal standard for each collagen-derived amino acid described. For the relative quantification to the amount of collagen, these values were attuned to the degree of the Lysine response. All data were analysed using MassLynx and Targetlyn (Waters Corp), GraphPad Prism and Microsoft Excel. All samples for each group were analysed in triplicate. Two-tailed Mann–Whitney P value < 0.05 was considered significant.

Immunofluorescence and confocal microscopy. HeLa cells and fibroblasts were fixed with 4% paraformaldehyde in PBS for 20 min and then permeabilized with 0.1% Triton X-100 in PBS. Cells were blocked with 2% BSA in PBS with 0.05% Tween 20. Solvent-treated CD3D spheres were marked and then fixed in 4% PFA for 20 min. Samples were blocked with 2% BSA with 0.5% Saponin in PBS and stained for E-cadherin and Claudin-1, with DAPI counterstaining. Coverslips were mounted in ProLong Gold anti-fade solution (Life Technologies). Cells in μ-Slides were imaged in PBS. Confocal microscopy images with scan format of 1,024 × 1,024 pixels were captured using an inverted Leica TCS 35S SP5 AOBS confocal microscope with a ×63 oil immersion objective (numerical aperture (N.A.) 1.4) and a ×3.5 optical zoom; the pinhole was set to 1 Airy unit. Single transfections of cyan fluorescent protein (CFP)- or yellow fluorescent protein (YFP)- were used to control the leak-through between the channels. A series of optical sections was collected at 0.25 µm z-spacing, processsed using ImageJ and single x-y images were collected at 4 × 1024 × 1,024 pixels were also obtained in the middle plane of 3D spheres with a ×40 oil immersion objective (N.A. 1.25). Representative images are shown in all experiments. Colocalization was determined using the Fiji plugin JACoP and was represented by Pearson’s coefficient calculated on the 3D projection of entire cells, after Costes randomization and automatic threshold calculation.

Electron microscopy. Cells in 2D and 3D cultures were fixed using 2% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium cacodylate before being incubated with 1% osmium tetroxide and 1.5% potassium ferricyanide at 4 °C. They were further treated with 1% tannic acid before being serially dehydrated in ethanol and embedded in Epon (TAAB). Ultrathin sections (70 nm) were taken using a Leica UC7 ultramicrotome (Leica Microsystems, Austria) and collected on formvar-coated slot (1 x 100 mm) grids. Sections were lead citrate-stained and their magnification using a 30 kV Spirit Biotwin (FEI, the Netherlands) and Morada CCD camera using imaging platform for transmission electron microscopy (ITEM software; EMSIS, Germany).
Correlative light and electron microscopy. Control and VIPAR shRNA-treated mIMCD3 cells were grown and transfected with LH3-mCherry as described above. Cells were fixed for 5 h with 4% paraformaldehyde and imaged on an inverted Leica TCS SPE AOBS confocal microscope. Dissolved inorganic carbon (DIC) and fluorescent (568 nm) images were taken using a ×20 dry objective to identify transfected cells in relation to the grid, and confocal stacks using a × 40 objective (N.A. 1.4) to localize individual LH3-mCherry puncta within the transfected cell in x, y, and z planes. Cells were then fixed and prepared for EM as described above. Once embedded, the cells of interest were relocated on to the block surface, and 70-nm silver ultrathin sections were collected on formvar-coated slot grids and then stained with lead citrate. Regions of interest in each cell were identified by overlapping DIC and fluorescent images over low-magnification images of early sections. These areas were then imaged at higher magnification for every section before being aligned digitally in Adobe Photoshop. High-magnification correlation of light microscopy and electron microscopy data was performed using characteristic features in the DIC images. Temporal colour-coding of the fluorescence data was performed to confirm the correlation of puncta in 2 between the light and EM data. EM width was used to measure the diameter of LH3-positive structures at their predicted educator.

CLEM on LH3 coupled to DAB-staining for collagen IV. Immunoperoxidase and DAB (–TAAB, UK) staining for CLEM was performed following the established protocols2,5. Briefly, control mIMCD3 cells were transfected with LH3-mCherry, fixed with 4% paraformaldehyde (PFA) in PBS for 50 min at room temperature and imaged as described above. Cells were then washed three times with PBS and incubated with biotin (0.05% Rsa, 200 mM citrate) for 30 min and stained with collagen IV antibody (1:2 dilution) for 2 h at room temperature. Subsequently, cells were washed three times with buffer A and incubated with horseradish peroxidase-conjugated anti-Rabbit IgG (DAKO, Denmark) 1:400 for 30 min. After three washes in PBS, cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate supplemented with 5% sucrose for 50 min at room temperature. Cells were rinsed three times with 50 mM Tris–HCl buffer, pH 7.6.

The peroxidase reaction was developed by incubating cells in the dark with the DAB reaction mix (0.07% H2O2, 0.075% DAB in Tris–HCl buffer) for 10 min. The peroxidase reaction was monitored by light microscopy. To stop the reaction cells were rinsed three times with Tris–HCl buffer. Cells were then prepared for EM, imaged and analysed as described above.

Microarray analysis of kd mIMCD3 cell lines. The transcriptome of VPS33B, VIPAR, and LH3 shRNA mIMCD3 cells was investigated by hybridizing RNA to Affymetrix GeneChip Mouse Gene 2.0 ST arrays following the recommended Affymetrix protocols. The data were pre-processed with standard RMA normalization and summarization using the oligo R package54. Principal component analysis and various distance metrics were used to ensure that the data were of high quality and clustered into the expected experimental groups. Microarray analysis was also carried out in wild-type and control shRNA mIMCD3 cells and results from both sets were compared with the kd cell lines. Differentially expressed genes were identified by a linear model to each sample using the limma package55 R. P values were adjusted for multiple testing using the Benjamini–Hochberg correction and a cutoff of false discovery rate (FDR) < 0.05 was applied.

GO annotation analysis was carried out using the online software DAVID56 by inputting the lists of significantly differentially expressed genes. The Mus musculus genome was used as the background. The GO term annotations were used, in which very broad GO terms are filtered out. As a simple way to compare the similarity of the gene expression across the microarrays, pairwise correlations were computed across the microarrays and plotted in a correlogram. Lists of the top 100 most differentially expressed genes in each kd were also compared for overlap, shown in a Venn diagram (Fig. 6B). The statistical significance of the overlap between each pair of kd’s was computed based on the hypergeometric distribution for the probability of k or more (k = 62, 62 or 66) genes overlapping when 100 genes are selected from a background of 12,055 genes. P values were highly significant (P = 0.1 < × 10−12). To approach the question of which biological processes are perturbed, gene set enrichment analysis57,58 was used to identify gene sets that have a larger than expected number of differentially expressed genes. The squared regularized t-statistic was used as the individual gene statistic, and then a summary statistic was computed for each gene set based on the mean for all the genes in that set. Significance was assessed by randomly sampling gene sets of the same size and computing an approximate P value.

Structural bioinformatic. Secondary structure prediction on VPS33B, VIPAR and LH3 was performed by comparing the results of multiple web servers including HHpred59 and Predictprotein60. Intrinsic disorder and flexibility of the VIPAR N terminus were further evaluated using multiple protein disorder web servers57. The presence of potential transmembrane helices in this region was excluded by thorough evaluation of the results from several servers for transmembrane protein prediction including TMHMM61 and HMMTOP62. Through comparison of multiple structural prediction algorithms enabled unambiguous identification of the most prominent features of the proteins analysed.

The 3D models of the VIPAR C terminus and full-length VPS33B were created using HHpred and MODELLER60. The model of the VPS33B–VIPAR complex was generated by superposing the structural models of VIPAR and VPS33B to the available crystal structures of human VPS33A-VPS35 (PDB ID: 4BX9)23 and Chlamydomonas thermophila VPS35–VPS33 (PDB ID: 4KMO)64 using the software COOT65. The model was further optimized by geometry idealization using PHENIX66. Final model quality was assessed using PROCHECK, PDBSUM67 and the Omevar server68.

Recombinant tagged VPS33B–VIPAR expression and purification. Recombinant tagged VPS33B–VIPAR complexes were produced in HEK293 cells stably expressing Epstein-Barr virus nuclear Antigen 1 (ref. 6). Initial attempts to produce isolated VPS33B or VIPAR resulted in low recombinant protein yields that completely and transiently improved by introducing a VPS33B–VIPAR expression plasmids. Cultures were harvested 5–6 days later by centrifugation at 1,000 g. Cells were resuspended in cold hypotonic solution (10 mM HEPES, 10 mM KCl, 5 mM MgCl2, pH 8.5) in 1/10 of the total culture volume and lysed by adding 0.3% NP-40. After high-speed centrifugation, the soluble fractions were collected and analysed by western blotting for evaluation of protein production. After extensive small-scale expression scouting for the most suitable combinations of purification tags, constructs pUPE.02.09-VIPAR (bearing N-terminal His(StrepII)-tag) and pUPE.02.13-VPS33B (bearing N-terminal His(StrepGY)-tag) were selected for large-scale expression. Large-scale expression cultures were performed in shake flasks, and the cells were harvested 6 days post-transfection. After lysis and high-speed centrifugation, the soluble fractions were collected, NaCl was added to reach a final concentration of 300 mM and the sample was incubated with Strep-tag Sepharose beads (GE Healthcare, USA) for 2 h. The beads were then packed into a column, washed extensively with buffer, and then eluted by supplementing the elution buffer with 2.5 mM imidazole. The DAB reaction mix (0.07% H2O2, 0.075% DAB in Tris–HCl buffer) for 10 min. The peroxidase reaction was monitored by light microscopy. To stop the reaction cells were rinsed three times with Tris–HCl buffer. Cells were then prepared for EM, imaged and analysed as described above.

In-solution tryptic digestion and LC-MS/MS analyses. The recombinant tagged VPS33B–VIPAR complex with the captured protein was first denatured with 20 µl of 100 mM Tris–HCl, pH 7.2, 6 M urea and 5 M dithiothreitol for an hour at room temperature before being carboxymethylated for 45 min with 6 µl of 100 mM iodoacetamide and 5 M iodoacetamide. The final product was the digested with Vivaspin centrifugal filter devices (Sartorius, Germany) and injected into a Superdex 200 10/300 GL gel filtration column (GE Healthcare). The VPS33B–VIPAR complex eluted as a single peak. Samples were concentrated to 3 mg ml−1 and stored at −80 °C for future usage. During the co-purification of overexpressed VIPAR and VPS33B and VIPAR, we consistently observed additional bands in SDS–PAGE analysis. The purified sample was therefore subjected to mass spectrometry analysis.

Data analysis of samples analysed by T-TOF MS/MS. ProteinLynx GlobalServer version 2.5 was used to process all data acquired. Protein identifications were obtained by searching the UniProt reference human proteome with the sequence of pig trypsin (P00761) added. Protein identification from the MS/MS spectra for each sample was processed using a hierarchical approach where more than three fragment ions per peptide, seven fragment ions per protein and more than two peptides per protein had to be matched. Carboxymethylation of cysteines was used as a fixed modification.

Animal models. For Vipas39fl/fl-ERT2 mouse generation, targeted ES cells for Vipas39 were obtained from the KOMP Repository (www.komp.org), a NCRR-supported mouse strain repository (U42-RR024244). The mouse generation, targeted ES cells for which were generated were created by the UCSD consortium from funds provided by the trans-NIH KnockOut Mouse Project (KOMP; Grant #5U01HG004080). The chimeric mice containing the ‘Knockout First, Conditional Ready’ Vipas39lsl-lacZ; KOMP/lsl allele, with LoxP sites flanking Vipas39lsl-recombinase-expressing mice (Jackson Laboratories, USA) to introduce tazoxifen-inducible Cre recombinase expression. Vipas39fl/fl-ERT2 mouse further mated to obtain Vipas39fl/fl-ERT2/-mice. The removal of Vipas39 exon 10 was induced by intraperitoneal injections of 100 mg kg−1 per day tamoxifen for five consecutive days on 6–8-week-old mice.
Controls used were either Vips33bfl/fl-ERT2 mice not induced with tamoxifen or Vips33bfl/fl-ERT2 mice without Cre recombinase that had been treated with tamoxifen. Vips33bfl/fl-ERT2 mouse generation was performed in a similar manner to Vips33bfl/fl-ERT2 mice38. Controls used were either Vps33bfl/fl-ERT2 mice not induced with tamoxifen or Vps33bfl/fl-ERT2 mice without Cre recombinase that had been treated with tamoxifen. All lines were performed 5–6 weeks post induction, adding some potential variability because of different mouse age and induction length. All procedures were undertaken with United Kingdom Home Office approval (licence number PPL 70/7470) in accordance with the Animals Scientific Procedures Act of 1986.

Detection of Vps33b and Vipar expression in murine osteoblasts. Osteoblasts were isolated from control, Vps33bfl/fl-ERT2 and Vips33bfl/fl-ERT2 murine long bones using the following protocol37.

Long bones were isolated from one to two adult mice for each group and epiphyses were cut off before the bone marrow was flushed out with PBS using a 5-ml syringe and a 27-gauge needle. Diaphyses were cut using scissors into pieces of 1–2 mm² and washed thoroughly with PBS before incubation in 4 ml collagenase II solution at 37°C in a shaking incubator for 2 h, with vigorously shaking manually every 30 min. Bone fragments were then washed three times with cCM media (DMEM supplemented with 10% FBS, 100 U ml⁻¹ penicillin, 50 μg ml⁻¹ streptomycin sulfate, 50 μg ml⁻¹ gentamicyn, 1.25 μg ml⁻¹ fungizone and 100 μg ml⁻¹ ascorbate). About 20–30 fragments were transferred to a 25-cm² flask and evenly distributed with 5 ml of cCM. The media (freshly prepared each time) was changed three times per week for 2 weeks. Osteoblasts migrated from the bone fragments were lysed as above and whole-cell lysates were immunoblotted with anti-VIPAR and anti-VPS33B antibodies.

Scanning electron microscopy. A minimum of three tails from individual mice per ko condition were selected, with no less than three tendon extract samples for each mouse. Whole tendons were immersed in 2.5% glutaraldehyde solution for 24 h before being cut into short (~0.5 cm) lengths. These were then fixed for 24 h in 3% glutaraldehyde (Agar Scientific, UK) in 0.1 M sodium cacodylate solution. Samples were then dehydrated using an ethanol series before critical point drying with hexamethyldisilazane (HDMS). Samples were mounted using carbon-adhesive tabs to aluminium stubs (Agar Scientific) and coated in Au/Pd. Imaging for qualitative analysis was performed using a Philips XL30 FEG-SEM (FEI), with an accelerating voltage of 5 kV.

Atomic force microscopy. A minimum of three tails from individual mice per ko condition were selected, with no less than three tendon extract samples for each mouse. Whole tendons were immersed in 2.5% glutaraldehyde solution for 24 h before being cut into short (~0.5 cm) lengths. These were then fixed for 24 h in 3% glutaraldehyde (Agar Scientific, UK) in 0.1 M sodium cacodylate solution. Samples were then dehydrated using an ethanol series before critical point drying with hexamethyldisilazane (HDMS). Samples were mounted using carbon-adhesive tabs to aluminium stubs (Agar Scientific) and coated in Au/Pd. Imaging for qualitative analysis was performed using a Philips XL30 FEG-SEM (FEI), with an accelerating voltage of 5 kV.

GGT activity measurements. GGT activity measurements on mLMCD3 cell lines (wild-type, control shRNA, VPS33B shRNA and VIPAR shRNA) media and cell lysate was measured with a method13 based on the transfer of [14C]glucose from UDP-[14C]glucose (139 Ci mol⁻¹) to galactosylhydroxylysyl residues in a calf skin gelatin substrate. After collecting media, cells were first washed in PBS and then homogenized in a Teflon-glass homogenizer with 0.1 M Glycine, 0.02 M Tris-HCl, pH 7.8 and 1% (v/v) IGEPAL CA-630. Homogenates were sonicated three times for 5 s and centrifuged at 14,000 g for 15 min. Supernatants were collected and used in the assay. Proteins were first precipitated and then hydrolysed overnight in 2 N NaOH at 105°C. The reaction was neutralized and fluorescent FMOC-labelled amino acids were analysed by Nova-Pak C18-HPLC column (Waters, Milford, MA). For the GGT activity measurements 1.70 μM corresponds approximately to 1 μg of LH3 based on the measurements performed using the purified recombinant Lh3 (ref. 11).

Patients' samples. The research, using skin fibroblast cell lines and urine samples from patients with ARC syndrome and age-matched control individuals, was approved by the UCL research ethics committee (REC 13/LO/1068) and all relevant institutional ethics review boards. Informed consent was obtained.

Data availability. Microarray data described in this study has been deposited in the NCBI GEO database under accession code GSE81376 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE81376). The authors declare that all other data supporting the findings of this study are available within the article and its supplementary information files or are available from the corresponding author upon request.

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Additional information
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