Detection of mutations in **KLHL3** and **CUL3** in families with FHHt (familial hyperkalaemic hypertension or Gordon’s syndrome)

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Abstract
The study of families with rare inherited forms of hypo- and hyper-tension has been one of the most successful strategies to probe the molecular pathophysiology of blood pressure control and has revealed dysregulation of distal nephron Na\(^{+}\) reabsorption to be a common mechanism. FHHt (familial hyperkalaemic hypertension; also known as Gordon’s syndrome) is a salt-dependent form of hypertension caused by mutations in the regulators of the thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) co-transporter NCC [also known as SLC12A3 (solute carrier family 12 member 3)] and is effectively treated by thiazide diuretics and/or dietary salt restriction. Variation in at least four genes can cause FHHt, including **WNK1** [With No lysine (\(=K\)) 1] and **WNK4**, **KLHL3** (kelch-like family member 3), and **CUL3** (culin 3). In the present study we have identified novel disease-causing variants in **CUL3** and **KLHL3** segregating in 63% of the pedigrees with previously unexplained FHHt, confirming the importance of these recently described FHHt genes. We have demonstrated conclusively, in two unrelated affected individuals, that rare intronic variants in **CUL3** cause the skipping of exon 9 as has been proposed previously. **KLHL3** variants all occur in kelch-repeat domains and so probably disrupt WNK complex binding. We have found no evidence of any plausible disease-causing variants within **SLC4A8** (an alternative thiazide-sensitive sodium transporter) in this population. The results of the present study support the existing evidence that the **CUL3** and **KLHL3** gene products are physiologically important regulators of thiazide-sensitive distal nephron NaCl reabsorption, and hence potentially interesting novel anti-hypertensive drug targets. As a third of our non-**WNK** FHHt families do not have plausible **CUL3** or **KLHL3** variants, there are probably additional, as yet undiscovered, regulators of the thiazide-sensitive pathways.

Key words: diuretic, Gordon’s syndrome, hypertension, hyperkalaemia, pseudohypoaldosteronism, thiazide

INTRODUCTION

Hypertension is estimated to contribute 3.5-fold more to the total global disease burden of cardiovascular disease than smoking and 1.6-fold that of hypercholesterolaemia. Worldwide, 20% of deaths in men, 24% of deaths in women, 62% of strokes and 49% of heart disease are attributable to blood pressure [1–3]. The current limitations in anti-hypertensive therapeutics are perhaps not surprising since for most affected individuals the molecular mechanisms driving their hypertension remain undefined.

Although rare, Mendelian forms of hypo- and hypertension represent experiments of Nature that have informed our understanding of the molecular basis of human cardiovascular disease and hypertension. These complex genetic disorders can be used to explore the relationship of hypertension to other cardiovascular disease risk factors [4]. Hypertension is a leading cause of cardiovascular disease, responsible for 30% of strokes and 40% of heart failures [5]. Therefore, understanding the potential of Mendelian forms of hypertension may help in the development of new anti-hypertensive treatment strategies.
understanding of the physiology of the distal nephron. Remarkably, given the variety of physiological systems that affect arterial pressure, all of these Mendelian syndromes for which the molecular mechanism is understood converge around a common theme: distal nephron sodium wasting in hypotensive syndromes and excessive sodium reabsorption in hypertensive conditions [4].

Although the amiloride-sensitive ENaC (epithelial sodium channel) has classically dominated research interests, NaCl reabsorption via the thiazide-sensitive Na$^+$/Cl$^-$ co-transporter NCC [also known as SLC12A3 (solute carrier family 12 member 3)] is at least as important [5]. Thiazide diuretics are potent antihypertensive agents [6] and mimic the effects of loss-of-function mutations of NCC observed in the hypotensive monogenic syndrome of Gitelman [7]. Moreover, the heritable condition of FHHt (familial hyperkalaemic hypertension) results from increased sodium reabsorption via NCC and is effectively ameliorated by thiazide diuretics and/or dietary sodium restriction [8].

FHHt is a salt-sensitive hypertension characterized by hyperkalaemic acidosis and exquisite sensitivity to low-dose thiazide diuretics [8,9]. As in Liddle’s syndrome [10], significant inter-and intra-pedigree phenotypic variation is observed clinically [11]. Causative variants have been identified in WNK1 [With No lysine (=K) 1] and WNK4, KLHL3 (kelch-like family member 3), and CUL3 (cullin 3) [12–15], but not within the NCC itself [16]. Variants are inherited in an autosomal dominant or recessive manner depending on the gene involved and can also occur de novo [8,14].

The current model for the regulation of NCC is complex and involves a scaffold of at least 12 interacting proteins centred on a WNK signalling cascade, with intermediary STE20 (sterile 20) kinases [SPAK (STE20/SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase-1)] activated by WNKs which in turn activate NCC [17–19]. CUL3 and KLHL3 are both components of the cullin/Ring E3 ligase ubiquitination pathway and at least some variants of KLHL3 appear to affect NCC via the control of WNK1 ubiquitination [15,20].

We have identified previously three FHHt pedigrees carrying WNK4 mutations (D564H, E562K and Q565E) [21], but none carrying CUL3 or KLHL3 mutations. To assess whether our remaining pedigrees with FHHt and without WNK1/4 mutations had either CUL3 or KLHL3 mutations, we undertook NGS (next-generation sequencing) of these genes and also screened an alternative thiazide-sensitive sodium transporter (SLC4A8) hypothesized to be an additional candidate [14].

**DNA analysis**

CUL3, KLHL3 and SLC4A8 genes were sequenced in the affected proband of each family using NGS. PCR amplicons covering all coding exons and exon/intron boundaries were prepared from genomic DNA (Fluidigm Access Array$^\text{SM}$; the amplicons used are listed in Supplementary Table S1 at http://www.clinsci.org/cs/126/cs1260721add.htm) and sequenced on the Illumina HiSeq platform. Reads were aligned to the human reference sequence hg19 using the Burrows–Wheeler Aligner, and the Genome Analysis Toolkit was used for base recalibration, local realignment and variant calling, following published best practice guidelines, and as described previously [22]. Variants were filtered for rarity and protein consequence: variants altering the protein-coding sequence [missense and nonsense SNPs (single nucleotide polymorphisms), insertions or deletions, or intronic variants at the exon/intron boundary] that were absent from public databases [dbSNP, 1000 Genomes and the NHLBI ESP (National Heart, Lung, and Blood Institute Exome Sequencing Project) Exome Variant Server] were considered candidates. All candidates detected by NGS were confirmed in the proband and assessed for segregation in the pedigree using Sanger sequencing. Variants are reported using Human Genome Variation Society standard nomenclature (http://www.hgvs.org/mutnomen/). The reference sequences used for each gene and protein are listed in Supplementary Table S2 (at http://www.clinsci.org/cs/126/cs1260721add.htm).

**RNA studies**

The functional effects of putative splice variants were confirmed using RNA studies. Peripheral blood mononuclear cell RNA was isolated from 5 ml of whole blood using a PAXgene blood RNA kit (Qiagen) according to the manufacturer’s instructions. The RNA was then transcribed using a Promega AMV reverse transcriptase kit (catalogue number A3500) according to manufacturer’s instructions using either random primers (RT1) or a CUL3-specific primer (5'-TTATGCTACATATGGTATAC-TTTC-3'; RT2). The resulting cDNA was then PCR-amplified using exon-specific primers to amplify exons 8–10 of the CUL3 transcript (forward, 5'-TGGACCTTACCGGTGTCTCC-3' and reverse, 5'-TAGTGGCTGTTTCTATCCATG-3'). The PCR products were run on a 2% agarose gel to visualize them, excised, cleaned using a Promega PCR clean-up kit and Sanger-sequenced on a Beckman CEQ 8800 sequencer. The expected PCR product

**MATERIALS AND METHODS**

**Study population**

The present study was carried out in accordance with the Declaration of Helsinki (2013) of the World Medical Association. Study participants with an FHHt phenotype were identified through tertiary specialist hypertension clinics in the U.K. and Australia. Diagnosis of FHHt was confirmed by the authors. All affected patients were Caucasian and shared a phenotype of persistent hyperkalaemia (plasma potassium > 5.0 mmol/l in blood collected without stasis) and hypertension (> 140/90 mmHg for adults) following exclusion of the relevant co-morbidities and pharmacotherapies. Detailed phenotypes of the affected individuals are given in Supplementary Figure S1 (at http://www.clinsci.org/cs/126/cs1260721add.htm). All non-affected individuals demonstrated plasma potassium < 5 mmol/l. The disparity in ages prevented comparison of age-related blood pressure between affected and non-affected individuals. DNA was extracted using a standard method from venous blood acquired following informed consent (Princess Alexandra Hospital Human Research Ethics Committee ID EC00 167 in Australia and National Research Ethics Committee reference 12/EM/0317 in the U.K.).
sizes were 338 bp and 167 bp for the exon 8–10 and del9 transcripts respectively.

**Paralogue mapping**

For each gene we first identified paralogues using pre-defined Ensembl protein families (http://www.ensembl.org; release 70), and constructed a multiple sequence alignment using M-Coffee [23]. Reported Mendelian disease-causing variants (non-synonymous exonic SNPs causing a single non-terminal amino acid change) in paralogues of the FHHt genes were identified using the Human Gene Mutation Database Professional version (http://www.hgmd.cf.ac.uk; release 2012.3), and mapped to the equivalent residue of the FHHt gene in the multiple sequence alignment.

**Exon-directed array and identity by descent analysis**

Representative affected individuals in pedigrees 6, 7 and 8 were genotyped using the Illumina Infinium HumanExome BeadChip array. Pair-wise IBD (identity by descent) analysis was undertaken using PLINK version 1.0.7 [24] on the basis of a subset of 27,402 informative autosomal SNPs with a minor allele frequency >5%. A proportion of IBD (PI_HAT) < 0.05 was considered to indicate no excess of sharing (i.e. unrelated individuals).

**RESULTS**

Genetic analysis of 25 affected individuals from 16 families with FHHt who had already been screened and found negative for WNK1/4 mutations was performed. A total of 95% of the targeted bases were sequenced adequately for variant calling. The sequencing depth and coverage achieved by gene and exon analysis indicated no excess of sharing (i.e. unrelated individuals).

| Pedigree | Gene | HGVS coding DNA position | Zygosity | rs_identity | Protein effect | Conservation |
|----------|------|--------------------------|----------|-------------|---------------|--------------|
| 1 G      | CUL3 | Chr2:225388888            | Heterozygous |            | c.1377+1G>T   | 9/9, M (37/37), V (49/50) Australia |
| 2 G      | CUL3 | Chr2:225388888            | Heterozygous | rs199469660 | Exon 9/9 intron 8splicing | Australia |
| 3 G      | CUL3 | Chr2:225388888            | Heterozygous |            | c.1377+1G>T   | 9/9, M (37/37), V (49/50) Australia |
| 4 G      | CUL3 | Chr2:225388888            | Heterozygous | rs199469654 | Exon 9/9 intron 8splicing | Australia |
| 5 G      | CUL3 | Chr2:225388888            | Heterozygous |            | c.1377+1G>T   | 9/9, M (37/37), V (49/50) Australia |
| 6 G      | KLHL3| Chr5:136964078            | Homozygous |            | c.1499G>T     | P (9/9), M (37/37), V (49/50) Australia |
| 7 G      | KLHL3| Chr5:136964078            | Homozygous |            | c.1499G>T     | P (9/9), M (37/37), V (49/50) Australia |
| 8 G      | KLHL3| Chr5:136964078            | Homozygous |            | c.1499G>T     | P (9/9), M (37/37), V (49/50) Australia |

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Figure 1 Demonstration that the CUL3 variants result in splice variation leading to a loss of exon 9 in affected individuals from pedigree 1 (Ped1) and pedigree 2 (Ped2)
The affected individuals sequenced are highlighted by * in Supplementary Figure S3 (at http://www.clinsci.org/cs/126/cs1260721add.htm). (A) Reverse transcription–PCR of CUL3 from peripheral blood mononuclear cells demonstrated an additional (smaller) cDNA band only in the affected individuals. The size of the smaller band was consistent with a deficiency of exon 9 (difference in band size = 171 bp). PCR primers RT1 (random primers) and RT2 (a CUL3-specific primer) are detailed in the Materials and methods section. The molecular size is given on the left-hand side in bp. (B) Sanger sequencing of CUL3 cDNA from the smaller 167 bp band confirmed that exon 9 is skipped in individuals from both pedigrees. Sequence excerpts from the larger 338 bp band are shown for the wild-type (WT) individual for comparison, demonstrating the wild-type exon boundaries. Sequencing chromatograms are shown together with the DNA sequence and amino acid codons above.

childhood despite coming from different families and living on opposite sides of the globe. Although variants surrounding this exon 9/intron 9 acceptor splice site have been predicted to affect splicing of exon 9 [14], the present study has provided the first evidence of this effect in FHHt patients. Specifically, RNA from peripheral blood monocytes of the index case in pedigrees 1 and 2 contains exon 9-deficient transcripts from the mutated CUL3 allele (Figure 1).

Pedigrees 6, 7 and 8 all carry the same KLHL3 p.L387P mutation that segregates completely with an FHHt phenotype, raising the question whether these families have a common founder. IBD analysis (Illumina Infinium HumanExome Bead-Chip) revealed that these pedigrees were no more related than by chance (PI_HAT = 0.0440), indicating that the mutation has probably arisen independently in each lineage. Although the KLHL3 R528H mutation has also been reported in three pedigrees [15], it was not established whether they shared a common founder. Hence in our pedigrees, KLHL3 p.L378P is the most commonly identified FHHt-causing KLHL3 mutation with robust evidence of independent founder mutations.

To assess the pathogenicity of the KLHL3 variants associated with FHHt, we used a Paralogue Annotation approach [25]. KLHL3 is one of a family of evolutionarily related cytoskeletal BTB/kelch repeat proteins, variation in several of which cause Mendelian disease. Using multiple sequence alignment to identify structurally and functionally equivalent residues across the protein family, we observed that one of the KLHL3 variants reported previously to be associated with FHHt [14,15] (KLHL3 p.R384W) co-locates with a reported disease-causing variant in another member of the protein family KBTBD13 p.R248S {where KBTBD13 is kelch repeat and BTB [BR-C (Broad Complex), ttk (tramtrack) and bab (bric a brac)] (POZ) domain-containing 13}, which is associated with nemalin myopathy [26]. This suggests that the variants lie at a functionally important site conserved across the protein family that is intolerant of sequence variation. Similarly, two of the KLHL3 FHHt variants in our patients (L387P and A494T) are very close to the location of known disease-causing variants in GAN (gigaxonin) [27], suggesting that these too are probably functionally important sites. GAN p.G368 and p.G474 (at which substitutions are associated with giant axonal neuropathy [27]) are equivalent to KLHL3 p.G388 and p.G496, and are adjacent to rare variants found in our FHHt pedigrees.
DISCUSSION

In the present study we have identified disease-causing variants in CUL3 and KLHL3 in 63% of our pedigrees with FHHT who had been screened and found to be negative for WNK1/4 mutations, confirming recent reports of association between CUL3 and KLHL3 variants and FHHT [14,15]. In the case of CUL3 mutation at position c.1377 +1 we report a second variant allele associated with a similar thiazide-responsive FHHT phenotype, strengthening further the case for a functional role of aberrant CUL3 function on sodium reabsorption in the distal nephron. We have also demonstrated that the predicted exon 9 splicing effect produced by c.1377 +1G>T and c.1377 +1G>A is, in fact, observed.

We have found that KLHL3 p.L387P associated with FHHT in three unrelated pedigrees, making this the most commonly occurring single FHHT mutation not only within our FHHT consortium, which includes three FHHT pedigrees carrying different WNK4 mutations (D564H, E562K and Q565E) [21], but also among all KLHL3 mutations reported to date [14,15]. That KLHL3 variants in our pedigrees are restricted to kelch repeats, and that other FHHT-associated KLHL3 variants cluster in these domains provides further support for disruption of WNK complex binding as reported previously [20].

Accepting the limitations of bioinformatics tools to predict pathogenicity, we did not find evidence of probable disease-causing variants within an alternative thiazide-sensitive sodium bicarbonate exchanger, SLC4A8, hypothesized as an alternative genetic candidate for FHHT [14]. A third of our pedigrees with non-WNK FHHT therefore remain without a genetic diagnosis, which is somewhat greater than that reported in other pedigree collections [14,15]. This highlights the genetic heterogeneity of the FHHT phenotype and the likelihood that additional, as yet undiscovered, regulators of thiazide-sensitive pathways exist. It is also worth emphasizing that we set out to identify KLHL3 and CUL3 variants in subjects with a clinical diagnosis of FHHT on the basis of measurements routinely recorded in the clinic. Similar data are recorded for unaffected relatives, but because of the large disparity in ages it is often impossible to provide a comparison of age-related blood pressure between affected and non-affected individuals. Nevertheless, all non-affected individuals were normokalaemic with a plasma potassium <5 mmol/l, and we are confident that we have correctly assigned affected compared with non-affected status within our pedigrees.

Further detailed laboratory and clinical studies are required to establish whether the effects of the reported heterogeneity of variant KLHL3 on WNK1 immunoprecipitation and ubiquitination translate into differential effects on thiazide-sensitive distal nephron sodium trafficking and phenotype within FHHT [20]. For instance, do patients with KLHL3 A340V and A494T Gordon’s syndrome have the same CUL3/KLHL3/WNK/SPAK/NCC pathway abnormalities as those with KLHL3 L387P?

In conclusion we have identified disease-causing variants in CUL3 and KLHL3 in patients with FHHT screened previously and found to be negative for WNK1 and WNK4 mutations, but did not find evidence of such variants in the alternative candidate SLC4A8. Approximately one-third of our non-WNK patients with FHHT remain without a molecular diagnosis raising the possibility that there may be additional regulators of thiazide-sensitive distal nephron sodium trafficking which remain to be discovered.

CLINICAL PERSPECTIVES

- The present study was performed to ascertain whether pedigrees with FHHT, but without mutation in WNK1/WNK4, contain mutation in CUL3, KLHL3 or SLC4A8.
- The present study confirms recent findings of CUL3 and KLHL3 mutations in FHHT and identifies novel disease-causing variants. This strengthens the argument that these gene products are physiologically important regulators of distal nephron NaCl reabsorption via thiazide-sensitive pathways, and hence are potentially interesting novel anti-hypertensive drug targets.
- As only 63% of our non-WNK FHHT families were found to contain plausible CUL3 or KLHL3 variants, there are probably additional, as yet undiscovered, regulators of thiazide-sensitive pathways.

AUTHOR CONTRIBUTION

Mark Glover, James Ware, Ian Hall, Richard Gordon, Michael Stowasser and Kevin O’Shaughnessy designed the study and drafted the paper. Mark Glover, Martin Wolley, Shengxin Xu, William Van’t Hoff, Richard Gordon, Michael Stowasser and Kevin O’Shaughnessy collected the patient material. Mark Glover, James Ware, Kevin O’Shaughnessy and Amanda Henry performed and analysed CUL3, KLHL3 and SLC4A8 genetic sequencing. Louise Wain and Martin Tobin undertook the IBD analysis. Roddy Walsh, James Ware and Stuart Cook performed the paralogue mapping.

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SUPPLEMENTARY ONLINE DATA

Detection of mutations in KLHL3 and CUL3 in families with FHHt (familial hyperkalaemic hypertension or Gordon’s syndrome)

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Supplementary Figures S1–S5 and Tables S1 and S2 can be found on the following pages.

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Figure S1 Clinical features in FHHt-affected individuals stratified by genotype

(A) Age at diagnosis and serum $K^+$, $HCO_3^-$ and $Cl^-$ are shown for FHHt-affected individuals with mutations in CUL3 and KLHL3. (B) Systolic and diastolic blood pressure (BP) for FHHt-affected individuals of each mutation class is shown as a function of age.
Figure S2  Average depth of sequencing for (A) KLHL3, (B) CUL3 and (C) SLC4A8 genes enriched using the Fluidigm Access Array™ by exon.

Log_{10} normalized depth of coverage is shown against DNA position with numbered exons in each case.
Figure S3  Pedigree structure of the ten kindreds with FHHt and mutations in CUL3 and KLHL3 detailed in Table 1 of the main text

Affected, unaffected and phenotype-undetermined subjects are denoted by black, white and grey symbols respectively. Alongside each pedigree, Sanger sequence traces are shown showing the wild-type sequence and the corresponding CUL3 or KLHL3 variant segregating with the affected phenotype within each family. *The affected individual whose sequence is shown; c, the following co-ordinate is of coding DNA. Variants are described using standard Human Genome Variation Society nomenclature. Reference sequences for each gene are listed in Table S1. Pedigree 1: the affected individual carries a previously unreported variant disrupting a consensus splice site in CUL3. The lower chromatogram of CUL3 shows that in the affected individual the wild-type guanine at position c.1377 + 1 (the first base of intron 9 at the border with exon 9) is mutated to thymine in a heterozygous manner compared with the wild-type chromatogram shown above. The position of this variant is shown in more detail in Figure S5 and full genetic co-ordinates are detailed in Table 1 of the main text. Pedigree 2: the affected individual carries a previously reported variant co-locating with the variant observed in pedigree 1, which also disrupts the consensus splice site in CUL3. The lower chromatogram shows that in the affected individual the wild-type guanine at position c.1377 + 1 (the first base of intron 9 on the border with exon 9) is mutated to cytosine in a heterozygous manner compared with the wild-type chromatogram above. The position of this variant is shown in more detail in Figure S5 and full genetic co-ordinates are detailed in Table 1 of the main text. Pedigree 3: the affected individual carries a previously unreported splice region variant close to a consensus splice site at the border of exon 9 in CUL3. The lower chromatogram of CUL3 shows that in the affected individual the wild-type thymine, 12 bases into intron 8 from the exon 9 border position c.1207, is mutated to adenine in a heterozygous manner compared with the wild-type chromatogram above.
KLHL3 and CUL3 in Gordon’s syndrome

Figure S3 Continued

chromatogram above. The position of this variant is shown in more detail in Figure S5 and full genetic co-ordinates are detailed in Table 1 of the main text. Pedigree 4: the affected individual carries a previously reported variant disrupting a consensus splice site at the border of exon 9 in CUL3. The lower chromatogram of CUL3 shows that in the affected individual the wild-type guanine, one base into intron 8 from the exon 9 border position c.1207, is mutated to adenine in a heterozygous manner compared with the wild-type chromatogram above. The position of this variant is shown in more detail in Figure S5 and full genetic co-ordinates are detailed in Table 1 of the main text. Pedigree 5: the affected individual carries a previously reported FHHt-associated missense variant in KLHL3. The lower chromatogram of KLHL3 shows that in the affected individual the wild-type guanine is mutated to thymine in a homozygous manner compared with the wild-type chromatogram. This leads to exon 13 of KLHL3 encoding the amino acid mutation G500V. The position of this mutation is shown in more detail in Figure S4 and full genetic co-ordinates are detailed in Table 1 of the main text. Pedigree 6: the affected individual carries a previously reported FHHt-associated missense variant in KLHL3. The lower chromatogram of KLHL3 shows that in the affected individual the wild-type thymine is mutated to cytosine in a heterozygous manner compared with the wild-type chromatogram. This leads to exon 10 of KLHL3 encoding the amino acid change L387P. The position of this mutation is shown in more detail in Figure S4 and full genetic co-ordinates are detailed in Table 1 of the main text. Pedigree 7: the affected individual carries a previously reported FHHt-associated missense variant in KLHL3. The lower chromatogram of KLHL3 shows that in the affected individual the wild-type thymine is mutated to cytosine in a heterozygous manner compared with the wild-type chromatogram. This leads to exon 10 of KLHL3 encoding the amino acid change L387P. The position of this mutation is shown in more detail in Figure S4 and full genetic co-ordinates are detailed in Table 1 of the main text. Pedigree 8: the affected individual carries a previously reported FHHt-associated missense variant in KLHL3. The lower chromatogram of KLHL3 shows that in the affected individual the wild-type thymine is mutated to cytosine in a heterozygous manner compared with the wild-type chromatogram. This leads to exon 10 of KLHL3 encoding the amino acid mutation L387P. The position of this mutation is shown in more detail in Figure S4 and full genetic co-ordinates are detailed in Table 1 of the main text. Pedigree 9: the affected individual carries a previously reported FHHt-associated missense variant in KLHL3. The lower chromatogram of KLHL3 shows that in the affected individual the wild-type thymine is mutated to cytosine in a heterozygous manner compared with the wild-type chromatogram. This leads to exon 10 of KLHL3 encoding the amino acid mutation L387P. The position of this mutation is shown in more detail in Figure S4 and full genetic co-ordinates are detailed in Table 1 of the main text. Pedigree 10: the affected individual carries a previously reported FHHt-associated missense variant in KLHL3. The lower chromatogram of KLHL3 shows that in the affected individual the wild-type thymine is mutated to cytosine in a heterozygous manner compared with the wild-type chromatogram. This leads to exon 10 of KLHL3 encoding the amino acid mutation L387P. The position of this mutation is shown in more detail in Figure S4 and full genetic co-ordinates are detailed in Table 1 of the main text.
Figure S4  A schematic diagram illustrating the location of FHHt-associated variants in the KLHL3 protein
Variants identified in the present study are highlighted in red. KLHL3 variants cluster in the kelch domains. The domain structure of KLHL3 includes BTB (BR-C (Broad Complex), ttk (tramtrack) and bab (bric a brac)), BACK and C-terminal kelch domains.

Figure S5  CUL3 variants found in affected individuals with FHHt cluster at exon 9 splice sites and cause skipping of this exon
The 17 exons of CUL3 are depicted and the CUL3 variants associated with FHHt clustering at either end of the exon 9 borders are shown. Positions found mutated in the present study are coloured red.
Table S1 | Genomic DNA targets amplified by the Fluidigm Access Array™ for the CUL3, KLHL3 and SLC4A8 genes

| Gene     | Amplicon number | Location                  |
|----------|-----------------|---------------------------|
| KLHL3    | KLHL3_t1        | Chr5:136961441–136961585 |
| KLHL3    | KLHL3_t2        | Chr5:136957785–136957815 |
| KLHL3    | KLHL3_t3        | Chr5:136963975–136964126 |
| KLHL3    | KLHL3_t4        | Chr5:136972982–136973084 |
| KLHL3    | KLHL3_t5        | Chr5:136974641–136974839 |
| KLHL3    | KLHL3_t6        | Chr5:136975548–136975666 |
| KLHL3    | KLHL3_t7        | Chr5:136993819–136993969 |
| KLHL3    | KLHL3_t8        | Chr5:136997603–136997720 |
| KLHL3    | KLHL3_t9        | Chr5:137013233–137013343 |
| KLHL3    | KLHL3_t10       | Chr5:137027973–137028136 |
| KLHL3    | KLHL3_t11       | Chr5:137033975–137034097 |
| KLHL3    | KLHL3_t12       | Chr5:137045438–137045545 |
| KLHL3    | KLHL3_t13       | Chr5:137056153–137056273 |
| KLHL3    | KLHL3_t14       | Chr5:137071321–137071336 |
| CUL3     | CUL3_t1         | Chr2:225449660–225449727 |
| CUL3     | CUL3_t2         | Chr2:225422375–225422573 |
| CUL3     | CUL3_t3         | Chr2:225400244–225400358 |
| CUL3     | CUL3_t4         | Chr2:225379328–225379489 |
| CUL3     | CUL3_t5         | Chr2:225378240–225378355 |
| CUL3     | CUL3_t6         | Chr2:225367070–225367299 |
| CUL3     | CUL3_t7         | Chr2:225371574–225371720 |
| CUL3     | CUL3_t8         | Chr2:225370672–225370849 |
| CUL3     | CUL3_t9         | Chr2:225368368–225368539 |
| CUL3     | CUL3_t10        | Chr2:225367681–225367789 |
| CUL3     | CUL3_t11        | Chr2:225365079–225365204 |
| SLC4A8   | SLC4A8_t12      | Chr12:51888728–51888902  |
| SLC4A8   | SLC4A8_t22      | Chr12:51890770–51890963  |
| SLC4A8   | SLC4A8_t23      | Chr12:51897812–51897904  |
| SLC4A8   | SLC4A8_t24      | Chr12:51899618–51899714  |
| SLC4A8   | SLC4A8_t25      | Chr12:51901209–51901223  |
| CUL3     | CUL3_t12        | Chr2:225362469–225362566 |
| CUL3     | CUL3_t13        | Chr2:225360548–225360683 |
| SLC4A8   | SLC4A8_t11      | Chr12:51818770–51818819  |
| SLC4A8   | SLC4A8_t12      | Chr12:51834491–51834573  |
| SLC4A8   | SLC4A8_t13      | Chr12:51844659–51844806  |

Table S1 | Continued

| Gene     | Amplicon number | Location                  |
|----------|-----------------|---------------------------|
| SLC4A8   | SLC4A8_t4       | Chr12:51845907–51846043  |
| SLC4A8   | SLC4A8_t5       | Chr12:51847322–51847483  |
| SLC4A8   | SLC4A8_t6       | Chr12:51851134–51851323  |
| SLC4A8   | SLC4A8_t7       | Chr12:51852342–51852434  |
| SLC4A8   | SLC4A8_t8       | Chr12:51853734–51853892  |
| SLC4A8   | SLC4A8_t9       | Chr12:51854987–51855075  |
| SLC4A8   | SLC4A8_t10      | Chr12:51856093–51856240  |
| SLC4A8   | SLC4A8_t11      | Chr12:51857397–51857498  |
| SLC4A8   | SLC4A8_t12      | Chr12:51863397–51863572  |
| SLC4A8   | SLC4A8_t13      | Chr12:51864175–51864309  |
| SLC4A8   | SLC4A8_t14      | Chr12:51865070–51865316  |
| SLC4A8   | SLC4A8_t15      | Chr12:51868125–51868231  |
| SLC4A8   | SLC4A8_t16      | Chr12:51868828–51868990  |
| SLC4A8   | SLC4A8_t17      | Chr12:51879570–51879684  |
| SLC4A8   | SLC4A8_t18      | Chr12:51882482–51882644  |
| SLC4A8   | SLC4A8_t19      | Chr12:51883483–51883735  |
| SLC4A8   | SLC4A8_t20      | Chr12:51887487–51887556  |

Table S2 | Transcript and protein identities used for the variant descriptions in the present study

| Gene     | Ensembl transcript | Ensembl protein     |
|----------|--------------------|--------------------|
| WNK1     | ENST00000315939    | ENSP00000313059    |
| WNK4     | ENST00000246914    | ENSP00000246914    |
| CUL3     | ENST00000264414    | ENSP00000264414    |
| KLHL3    | ENST00000309755    | ENSP00000312397    |
| SLC4A8   | ENST00000453097    | ENSP00000405812    |
| GAN      | ENST00000248272    | ENSP00000248272    |
| SLC4A1   | ENST00000262418    | ENSP00000262418    |
| KBTBD13  | ENST00000293303    | ENSP00000293303    |
| KBTBD7   | ENST00000339077    | ENSP00000343273    |
| SLC4A4   | ENST00000340595    | ENSP00000344272    |
| KBTBD9   | ENST00000359039    | ENSP00000351933    |
| SLC4A11  | ENST00000380056    | ENSP00000369396    |
| CUL5     | ENST00000393094    | ENSP00000376808    |
| CUL4B    | ENST00000404115    | ENSP00000384109    |
| KBTBD13  | ENST00000432196    | ENSP00000388723    |

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