Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin

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Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin (HPFH) is characterized by persistent high levels of fetal hemoglobin (HbF) in adults. Several contributory factors, both genetic and environmental, have been identified1 but others remain elusive. HPFH was found in 10 of 27 members from a Maltese family. We used a genome-wide SNP scan followed by linkage analysis to identify a candidate region on chromosome 19p13.12–13. Sequencing revealed a nonsense mutation in the KLF1 gene, p.K288X, which ablated the DNA-binding domain of this key erythroid transcriptional regulator2. Only family members with HPFH were heterozygous carriers of this mutation. Expression profiling on primary erythroid progenitors showed that KLF1 target genes were downregulated in samples from individuals with HPFH. Functional assays suggested that, in addition to its established role in regulating adult globin expression, KLF1 is a key activator of the BCL11A gene, which encodes a suppressor of HbF expression3. These observations provide a rationale for the effects of KLF1 haploinsufficiency on HbF levels.

Hemoglobin (Hb) is composed of two α-like and two β-like globin chains, encoded by genes in the HBA and HBB clusters, respectively. Developmental regulation of globin genes results in the expression of stage-specific Hb variants (Supplementary Fig. 1). Persistent expression of HbF ameliorates the symptoms of β-thalassemia and sickle cell disease, and reactivation of the HBG1 and HBG2 genes in adults is therefore of substantial interest for the clinical management of β-type hemoglobinopathies. After birth, HbF is gradually replaced by adult hemoglobin (HbA)4. Residual amounts of HbF continue to be synthesized throughout life. In most adults, HbF contributes <2% to adult hemoglobin (HbA)5,6. In HPFH1 carriers, HbF levels remain high enough to cause clinical symptoms5,6.

We performed a genome-wide linkage analysis on 27 family members to identify candidate loci for the HPFH modifier. We carried out whole-genome multipoint parametric linkage analysis using the Merlin program12 with two software packages, easyLINKAGE13 and dChip14. The analyses resulted in one significant linkage peak with log\(_10\) odds (LOD) scores of 2.7 and 4.2, respectively, on chromosome 19p13.12–13 (Fig. 1b and Supplementary Fig. 2). We performed these analyses using an autosomal dominant model, assuming a penetrance of 90% and 1% phenocopy rate. We found no evidence of significant linkage to the previously reported trans-acting HPFH loci at 2p16.1 (BCL11A)8,10 and 6q23.3 (HBS1L-MYB)6,8,9. We further investigated these two loci by genotyping the five individual SNPs previously associated with increased HbF levels. These analyses ruled out involvement of the HBS1L-MYB locus and revealed that heterozygosity at SNP rs766432 in the BCL11A locus might have contributed to the increased HbF levels but was not the main determinant (Supplementary Table 1). Individuals with HPFH had a consistent haplotype at 19p13.12–13, and the inferred haplotypes revealed that all such individuals shared one copy of an identical chromosome segment, presumably containing HBS1L-MYB (6q23.3)6,8,9 and BCL11A (2p16.1)10,11. Together, these loci account for <50% of the variation in HbF, indicating that additional loci are involved5.

Genetic analysis of families in which HPFH is found is a particularly powerful approach by which to identify modifiers of HbF levels8. Here we describe a Maltese pedigree with HPFH. The proband (II-5; Fig. 1a) was referred to the clinic because of microcytosis. She presented with high HbF levels (19.5%). We recruited additional family members, and 10 of 27 tested showed HPFH (Fig. 1a and Supplementary Table 1), suggesting that inheritance of the trait was autosomal dominant. We excluded linkage to the HBB locus, indicating that a trans-acting factor was involved.

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The putative HPFH locus (Supplementary Fig. 2). Recombination events delineating the linkage region are indicated with arrows. The distal boundary is determined by a recombination event in individuals IV-3 and IV-5 (Supplementary Fig. 2, white arrow). The proximal boundary is determined by individuals III-12, III-18, IV-6 and IV-7 (Supplementary Fig. 2, black arrow). These results narrowed the region down to a 663-kb interval between rs7247513 and rs12462609.

We identified a set of common differentially regulated genes (Supplementary Table 2). Cluster analysis with this set of genes separated the samples from individuals with HPFH from samples from those without (Fig. 2a), consistent with the notion that KLF1 activity is compromised in the family members with HPFH. Deregulation of these KLF1 target genes could explain the mild hypochromic microcytic indices shown by the individuals with HPFH (Supplementary Table 2).
Of note, the embryonic Hbb-γ and HBE1 genes were highly upregulated (Supplementary Table 2), whereas expression of the fetal globin repressor BCL11A was downregulated in individuals with HPFH (Supplementary Table 2 and Supplementary Fig. 3). We could not measure the expression of fetal and adult globins quantitatively on the microarrays owing to saturation effects. However, quantitative RT-PCR (qPCR) confirmed the downregulation of BCL11A and showed that the expression of HBG1/HBG2 genes was increased in the samples from individuals with HPFH (Fig. 2b).

Next, we investigated the effects of KLF1 knockdown in HEPS derived from healthy donors. We obtained efficient knockdown of KLF1 with two out of five lentiviral shRNA constructs tested (Fig. 3a). Quantitative S1 nuclease protection assays showed that KLF1 knockdown led to a significant increase in HBG1/HBG2 expression (Fig. 3b-d), which was confirmed by qPCR (Fig. 3e). In addition, we found that BCL11A expression was diminished after KLF1 knockdown, both at the protein (Fig. 3a) and at the mRNA level (Fig. 3e). Thus, the effects of KLF1 insufficiency on HBG1/HBG2 and BCL11A expression in HEPS from healthy donors were similar to those observed in KLF1 p.K288X heterozygotes, supporting the causative role of this mutation in the HPFH phenotype.

To further investigate this idea, we transduced HEPS with lentiviral vectors that expressed either the KLF1 p.K288X truncation mutant or full-length KLF1. The transgenic proteins were expressed at physiological levels in control HEPS (Supplementary Fig. 4a). This did not affect HBG1/HBG2 expression (Supplementary Fig. 4b,c), indicating that the truncated form of KLF1 does not act as a dominant-negative factor. In HPFH HEPS, lentivirus-mediated expression of full-length KLF1 resulted in considerable downregulation of HBG1/HBG2 mRNA, whereas expression of truncated KLF1 had no effect (Fig. 4). Levels of BCL11A protein were increased after transduction with full-length KLF1 lentivirus, whereas no such changes were observed after transduction with either GFP or truncated KLF1 lentiviral vectors (Fig. 4a).

The endogenous truncated KLF1 protein was not or at best barely detectable in HEPS from individuals with HPFH. This suggested that RNA transcribed from the KLF1 p.K288X allele was subject to nonsense-mediated decay, further emphasizing that it was dysfunctional. Consistent with this notion, we found that KLF1 mRNA expression was lower in HEPS from individuals with HPFH than in those from healthy donors (Supplementary Fig. 3).

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KLF1 preferentially activates the HBB gene at the expense of HBG1/HBG2 gene expression by interacting directly with regulatory elements in the HBB promoter. The molecular analysis of the Maltese HPFH-affected family is consistent with this function of KLF1. In addition, our results also suggest a new potential mechanism by which KLF1 might tip the balance from HBG1/HBG2 to HBB expression: through activation of the gene encoding the HBG1/HBG2 repressor BCL11A. The promoter region of the BCL11A gene contains several putative KLF1 binding sites (CACC boxes; Fig. 5a). We performed chromatin immunoprecipitation (ChIP) assays to investigate whether KLF1 was bound to the BCL11A promoter in vivo. We used human fetal liver erythroid progenitors, which express high levels of HBG1/HBG2, and HEPs from adult peripheral blood in which the HBG1/HBG2 genes are suppressed. In adult HEPs, we observed strong binding of KLF1 to the BCL11A promoter (Fig. 5b). This was similar to the binding of KLF1 to the HBB promoter, which served as a positive control. Neither promoter seemed to be bound by KLF1 in fetal liver-derived erythroid progenitors. ChIP reactions with the unrelated CD71 antibody were negative in all cases. We conclude that in adult HEPs KLF1 is bound to the BCL11A promoter in vivo.

Diminished KLF1 activity, mediated either through mutation of one KLF1 allele (as occurs in the Maltese individuals with HPFH) or experimentally through shRNA-mediated knockdown in HEPs from normal donors, results in decreased BCL11A expression. Conversely, BCL11A levels were increased upon restoration of KLF1 activity in HEPs from Maltese family members with HPFH. This identifies KLF1 as a dual regulator of fetal-to-adult globin switching in humans (Supplementary Fig. 5). First, it acts on the HBB locus as a preferential activator of the HBB gene. Second, it activates expression of BCL11A, which in turn represses the HBG1/HBG2 genes. This dual activity ensures that, in most adults, HbF levels are <2% of total Hb.

In conclusion, we have identified haploinsufficiency for KLF1 as a cause of HPFH. We suggest that attenuation of KLF1 activity may be a fruitful approach to raise HbF levels in individuals with β-type hemoglobinopathies.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession code. The microarray expression data can be found at GEO under accession number GSE22109.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

F.G.G., A.E.F., G.P.P. and S.P. designed experiments. J.B., P.P., M.G., L.G., G.G., P.F., M.P., C.A.S., W.C., R.G., Z.O., N.G. and M.v.L. performed experiments. J.B., P.P., M.G. and G.G. analyzed results. P.J.x.S., F.G.G., A.E.F., G.P.P. and S.P. supervised data analysis. P.J.x.S., V.w.II. and M.B. provided expertise, analysis tools and infrastructure. A.I.M.H.V., J.H. and M.B. analyzed data. J.B., P.P., M.G., F.G.G., M.v.L., A.E.F., G.P.P. and S.P. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Quantitative S1 nuclelease protection assays. To measure globin mRNA levels directly, we used quantitative S1 nuclelease protection assays. The probe fragment for detection of HBG1/HBG2 mRNAs was amplified by PCR using the primers S1-HBG-S and S1-HBG-A (Supplementary Table 3). Sizes of probes/protected fragments are: HBG1/HBG2: 700 nt/218 nt; HBG1/HBG2: 350 nt/165 nt; HBB: 525 nt/155 nt (ref. 21). Quantification was performed using a Typhoon Trio Phosphorimage (GE Healthcare) and corrected for specific activity of the probes.

qPCR analysis. Total RNA (1 μg) isolated from HEps was converted to cDNA using SuperScript II reverse transcriptase according to the manufacturer’s instructions (Invitrogen). Expression of mRNAs was analyzed by qPCR. Amplification reactions were performed with primers designed with Primer Express v2.0 (Applied Biosystems). All amplifications used SYBR Green PCR Master Mix (Applied Biosystems). qPCR was performed with an Optical IQ Thermal Cycler (Bio-Rad Laboratories) with the following conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 62 °C for 45 s. All reactions were performed in triplicate. Gene expression levels were calculated with the 2−ΔΔC(T) method. Target gene expression was normalized to GAPDH expression, unless indicated otherwise.

Primers used are listed in Supplementary Table 3.

Statistical analysis. Statistical analysis of gene expression data obtained from quantitative S1 nuclelease protection assays and qPCRs was performed with Mann Whitney tests using STATA data analysis and statistical software (StataCorp LP).

KLF1 expression constructs. A human KLF1 cDNA clone (BC040000, Imagenes) was amplified by PCR with an att-specific set of primers (Invitrogen) to fuse the cDNA with a V5 tag at the carboxy terminus of the protein. Primers used were KLF1-F and KLF1-R1 (Supplementary Table 3). In parallel, part of the clone was amplified, truncating the protein at amino acid 288, with att-specific primers using a different reverse primer KLF1-R2. The PCR products were introduced into the lentiviral expression vector pRRLsin.sPPT. CMV/Wpre modified for Gateway cloning (Invitrogen). The final clones were verified by sequencing.

Lentiviral transduction of human erythroid progenitors. Lentivirus was produced by transient transfection of 293T cells according to standard protocols. Two days after transfection, the supernatant was collected, filtered and concentrated by centrifugation at 20,000 rpm for 2 h at 4 °C. HEps cultured for 1 week were transduced in 24-well plates. We plated 0.5 × 10^6 cells per well and sufficient amounts of virus to transduce ~80% of the cells. When appropriate, puromycin (1 μg/ml final concentration) was added to the cells after 2 d, and selection was performed for 2–3 d. At day 5–7 after transduction, cells were harvested and nuclear extracts were prepared. RNA was extracted with the Trizol reagent. For knockdown experiments, clones from The RNAi Consortium (TRCN; Sigma) were used. The nontarget SHC002 vector was used as a control (SHC002: 5′-CAACAA GTGAAGGACCAAC-3′). Five shRNA clones targeting KLF1 were tested: TRCN0000016273, TRCN0000016274, TRCN0000016275, TRCN0000016276 and TRCN0000016277. Efficient knockdown of KLF1 expression was observed with TRCN0000016276 (sh1) and TRCN0000016277 (sh2). Sequences are listed in Supplementary Table 3.

Protein blotting. Nuclear extracts were separated on denaturing polyacrylamide gels followed by semi-dry blotting to PVDF or nitrocellulose membranes. The membranes were probed with the following primary antibodies: BCL11A (sc-56013, Santa Cruz Biotechnology), NPM1 (ab10530, Abcam), KLF1 (ref. 26) and anti-V5-HRP ( BP961-25, Invitrogen). For detection, the appropriate secondary antibodies were used. The enhanced chemoluminescence kit (GE Healthcare) or the Odyssey Infrared Imaging System (Li-Cor Biosciences) was used to develop the membranes.

Chromatin immunoprecipitations. Fetal liver and adult HEps were cultured and used for ChIP reactions, which were performed as described with the KLF1 antibody and a CD71 antibody (347510, BD Biosciences) as a
negative control. qPCR was performed on the input and immunoprecipitated samples using primers for the RASSF1A, HBB and BCL11A genes. The relative fold enrichment was calculated as $2^{-(	ext{CT}_{x \text{ChIP} y} - \text{CT}_{\text{input} y}) - (\text{CT}_{\text{KLF1-ChIP HEP RASSF1A}} - \text{CT}_{\text{input HEP RASSF1A}})}$ (where ‘x’ is the antibody and ‘y’ the sample), that is, setting the relative fold enrichment of the RASSF1A amplicon by the KLF1 antibody in HEPs to 1. Primers used are listed in Supplementary Table 3.

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