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Fam83h is Associated with Intracellular Vesicles and ADHCAI

ABSTRACT

Defects in *FAM83H* on human chromosome 8q24.3 cause autosomal-dominant hypocalcified amelogenesis imperfecta (ADHCAI). *FAM83H* does not encode a recognizable signal peptide, so we predicted that the Fam83h protein functions within the cell. We tested this hypothesis by constitutively expressing mouse Fam83h with green fluorescent protein (GFP) fused to its C-terminus in HEK293 and HeLa cell lines. Green fluorescent signal from the Fam83h-GFP fusion protein was associated with perinuclear vesicles, usually in the vicinity of the Golgi apparatus. No signal was observed within the nucleus. In addition, we identified *FAM83H* nonsense mutations in Hispanic (C1330C>T; p.Q444X) and Caucasian (c.1192C>T; p.Q398X) families with ADHCAI. We conclude that Fam83h localizes in the intracellular environment, is associated with vesicles, and plays an important role in dental enamel formation. *FAM83H* is the first gene involved in the etiology of amelogenesis imperfecta (AI) that does not encode a secreted protein.

KEY WORDS: ADHCAI, Golgi, enamel, tooth, amelogenesis imperfecta.

INTRODUCTION

Amelogenesis imperfecta (AI) is the collective designation for a diverse group of inherited diseases exhibiting defects in dental enamel in the absence of significant non-dental symptoms (Witkop and Sauk, 1976). Because the phenotype is primarily restricted to enamel, significant research efforts have been devoted to identifying the suite of extracellular matrix proteins that control enamel biomineralization, and to determining if the genes encoding them contribute to the etiology of AI. The major proteins expressed in secretory stage enamel are amelogenin (Snead *et al.*, 1983, 1985), enamelin (Hu *et al.,* 1997b), ameloblastin (Cerny *et al.,* 1996; Krebsbach *et al.*, 1996; Hu *et al.*, 1997a), and enamelysin (MMP-20) (Bartlett *et al*., 1996). In addition, kallikrein 4 (KLK4) (Simmer *et al.*, 1998), amelotin (Iwasaki *et al.*, 2005; Moffatt *et al.*, 2006), and apin (Moffatt *et al.*, 2008) are expressed during the maturation stage. AI-causing mutations in *AMELX*, *ENAM*, *MMP20*, and *KLK4* have been identified (Hu *et al.*, 2007; Wright *et al.*, 2009a); however, mutational analyses of AI kindreds with these candidate genes successfully identified the causative mutation in only 25% of cases (Kim *et al*., 2006). So when a genome-wide scan mapped autosomal-dominant hypocalcified amelogenesis imperfecta (ADHCAI) to a 2.1-Mb region of chromosome 8q24.3 (Mendoza *et al.*, 2007) that contained no AI candidate genes, it started an informal race to identify the disease-causing gene. A gene-by-gene search determined that defects in *FAM83H* cause ADHCAI (OMIM #130900) (Kim *et al*., 2008), and subsequent reports have brought to 14 the number of novel disease-causing mutations in *FAM83H* (Lee *et al.*, 2008; Hart *et al.*, 2009; Wright *et al*., 2009b). Remarkably, all 14 of the *FAM83H* mutations are in the last exon (exon 5), and cause premature termination of the Fam83h protein (Fig. 1).

FAM83H (family with sequence similarity 83 member H on chromosome 8q24.3) is a gene that was originally discovered during computer analyses of the human genomic sequence. Expressed sequence tags (ESTs) indicate that *Fam8h3* is expressed in many tissues. How could defects in a gene that is broadly expressed cause a phenotype that is restricted to enamel? The pattern of *FAM83H* mutations that cause ADHCAI may provide a clue. Premature translation termination codons in the last coding exon generally escape non-sense-mediated decay (Shyu *et al.*, 2008), suggesting that truncated proteins are translated, and that the phenotype is not due to haploinsufficiency (a simple 50% reduction in the amount of Fam83h protein). Human Fam83h has 1179 amino acids. All of the reported *FAM83H* mutations shorten the protein so that as little as 286 or as many as 693 amino acids of the normal protein are synthesized. Apparently, half the normal amount of Fam83h does not cause ADHCAI (since no mutations predicted to cause haploinsufficiency have been observed), but synthesis of reduced amounts
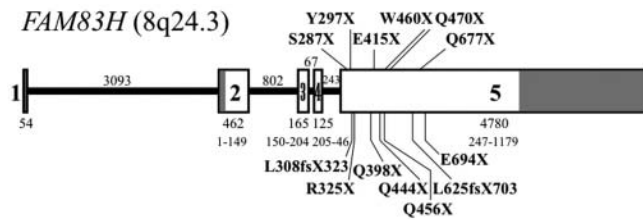
Figure 1. *FAM83H* gene diagram showing mutations identified in families with ADHCAI. The 5 exons are indicated by numbered boxes. The non-coding regions of exons 1, 2, and 5 are shaded. The number of nucleotides in each exon is shown immediately under the box. The range of amino acids in the Fam83h protein encoded by each exon is below the number of nucleotides. Introns are represented by a solid line, with the number of nucleotides in the intron shown above. The 14 disease-causing mutations in *FAM83H* are labeled in bold above and below the 5′ part of exon 5.

of wild-type protein, along with truncated Fam83h, does. ADHCAI may be caused by the dominant-negative effects of expressing truncated Fam83h. Truncated Fam83h might interact with wild-type Fam83h (expressed from the unmutated allele) or with another protein, forming a non-functional dimer or multimer.

Knowing where Fam83h normally functions is an important early step in understanding its functional role in dental enamel formation. In this study, we report *FAM83H* mutations in two families with ADHCAI and test the hypothesis that Fam83h functions within the cell by expressing Fam83h fused to green fluorescent protein (GFR) in cell lines and then localizing it by fluorescent microscopy.

MATERIALS & METHODS

Protocol Approval

The study protocol and participant consent forms were reviewed and approved by the Institutional Review Board at the University of Michigan.

Recruitment and Examination of Participants

All participants were given oral and radiographic examinations to characterize their dental phenotype and affection status. The proband of family 1 was a Hispanic boy, who was the only person recruited from this family. The medical history revealed no health concerns or allergies. The proband of family 2 was a Caucasian girl. The five-person nuclear family was recruited.

Mutation Analysis

Based upon the clinical diagnosis of ADHCAI, we limited our mutation analysis to exon 5 of the *FAM83H* gene. Genomic DNA was isolated from 2 mL of peripheral whole blood by means of the QIAamp® DNA Blood Maxi Kit (Qiagen, Valencia, CA, USA). PCR amplifications used Platinum® DNA polymerase (Invitrogen, Carlsbad, CA, USA). Five primer pairs were used to amplify overlapping segments of exon 5: Set 1 (748 bp, 57°C annealing T), ACCTCACATCCCTGCGTCCTC, CGGGTCCCACTGGTA

CTGCT; Set 2 (725 bp, 57°C annealing T), GTGTCGC GGCAGACGTTCCTC: GAGCGGAATGAGTCCTGC TTGG; Set 3 (610 bp, 60°C annealing T), AGCAGGACTCAT TCCGCTC, GACTCATTATGGAGCACCTGG; Set 4 (726 bp, 64°C annealing T), GCCTGCCTTCCCGCTTCC, CTAGCTCG GGGCTGTTGTGGG; and Set 5 (709 bp, 64°C annealing T), GCC AACGCCTTGTACAGCAGC, TGCTGAGGGGGAGAA AGGCACTG. The PCR amplifications were performed in 50-µL reaction mixes containing 21.5 µL sterile water, 1 µL each of 10 pmol of sense and antisense primers, 1 µL 0.5 µg/µL template, 0.5 µL FailSafe PCR Enzyme Mix, and 25 µL FailSafe PCR 2x Premix J (Epicenter Biotechnologies, Madison, WI, USA). The reactions had a five-minute denaturation at 94°C, followed by 40 cycles at 94°C for 30 sec, primer annealing at 57-64°C for 30 sec, and product extension at 72°C for 60 sec. In the final cycle, the 72°C extension was for 7 min. PCR amplification products were run in a 1.2% agarose gel, excised with a razor blade, purified *via* the QIAquick Gel Extraction Kit (Qiagen), and characterized by direct DNA sequencing. The same oligonucleotide primers used to generate the PCR amplification products were used to prime the sequencing reactions. The sequencing results were checked against the *Homo sapiens* chromosome 8 genomic contig reference assembly AC105219 and cDNA reference sequence NM_198488.3.

Fabrication of the Fam83h-GFP Expression Construct

A full-length mouse *Fam83h* cDNA clone (BY722193) in pBluescriptIIKS+ (Stratagene, La Jolla, CA, USA) was obtained from K.K. DNAFORM (Yokohama, Japan). Restriction sites were introduced before the Fam83h translation initiation codon (BglII) and replacement of the translation termination codon (SalI) by PCR, which generated a 3651-bp amplicon (primer set: aagatcTGGCCCCAAC**ATG**GCCCGTC, agtcgacTTTTTT-GCTTTTGAATGTGCCC). The PCR conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, and extension at 72°C for 4 min. The final extension was carried out at 72°C for 7 min. The products were resolved by electrophoresis on a 1.0% agarose gel buffered with 1×TAE and visualized by ethidium bromide. The amplification product was extracted from the gel and ligated into pCR2.1-TOPO (Invitrogen). The Fam83h coding region was excised by double digestion with BglII and SalI and ligated into phrGFP-C (Stratagene), which had been restricted with BamHI and SalI. Proper construction of the recombinant expression plasmid was verified by DNA sequencing.

Intracellular Localization of Fam83h

HEK293 or HeLa cells were cultured with DMEM with 10% FBS, 1% 100 mM sodium pyruvate, and 1% GluTA MAX-1 in a Lab-Tek chamber slide with cover (Nalge Nunc International, Rochester, NY, USA) and used to seed glass coverslips to a density of 2×10^5 cells/mm², one day prior to transfection. The phrGFP-Fam83h plasmid in Lipofectamine2000 (Invitrogen) was diluted with DMEM without serum to a final concentration of 1.2 µg/µL, and incubated for 20 min at room temperature. The phrGFP-Fam83h/Lipofectamine2000 complexes were then

added to the seeded coverslips. After 24 hrs, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Golgi apparatus was stained with 1 µM BODIPY TR complexed to BSA (Invitrogen), mounted with ProLong Gold anti-fade reagent with DAPI (Invitrogen) for staining of the nucleus, and photographed by means of a Leica DM 5000B fluorescence microscope.

RESULTS

Evaluation of Participants

The proband of family 1 was a Hispanic boy (9 yrs, 5 mos) with age-appropriate dental develop-ment, but his dentition exhibited rough hypoplastic enamel, having a brownish discoloration (Fig. 2, top). Both the primary and perma-nent dentitions were affected. The surface roughness and sensitivity hindered effective oral hygiene, and the proband presented with heavy plaque and calculus accu-mulation and generalized gingivi-tis. The enamel layer was thin (contributing to spacing of the teeth) and did not contrast with dentin on radiographs. The thin-ness of the enamel layer appeared to be the result of rapid attrition following eruption of the teeth. The proband's teeth were highly sensitive to thermal changes. Removal of the surface enamel on the permanent maxillary central incisors exposed a thin layer of whiter enamel, which bound com-posite restorative materials ade-quately (data not shown). The biological mother of the proband was not available for examination, but reportedly has similar prob-lems with her teeth, indicating a dominant pattern of inheritance. The stepmother reported that the proband was otherwise healthy. Mutation analysis of genomic DNA from the proband identified a *FAM83H* nonsense mutation (g.2452C>T; c.1330C>T; p.Q444X), which was previously shown to cause ADHCAI in a Turkish family (Hart *et al*., 2009).

The dental phenotype of the proband in family 2 was similar to that of family 1 (Fig. 2, bottom). The proband was a Caucasian
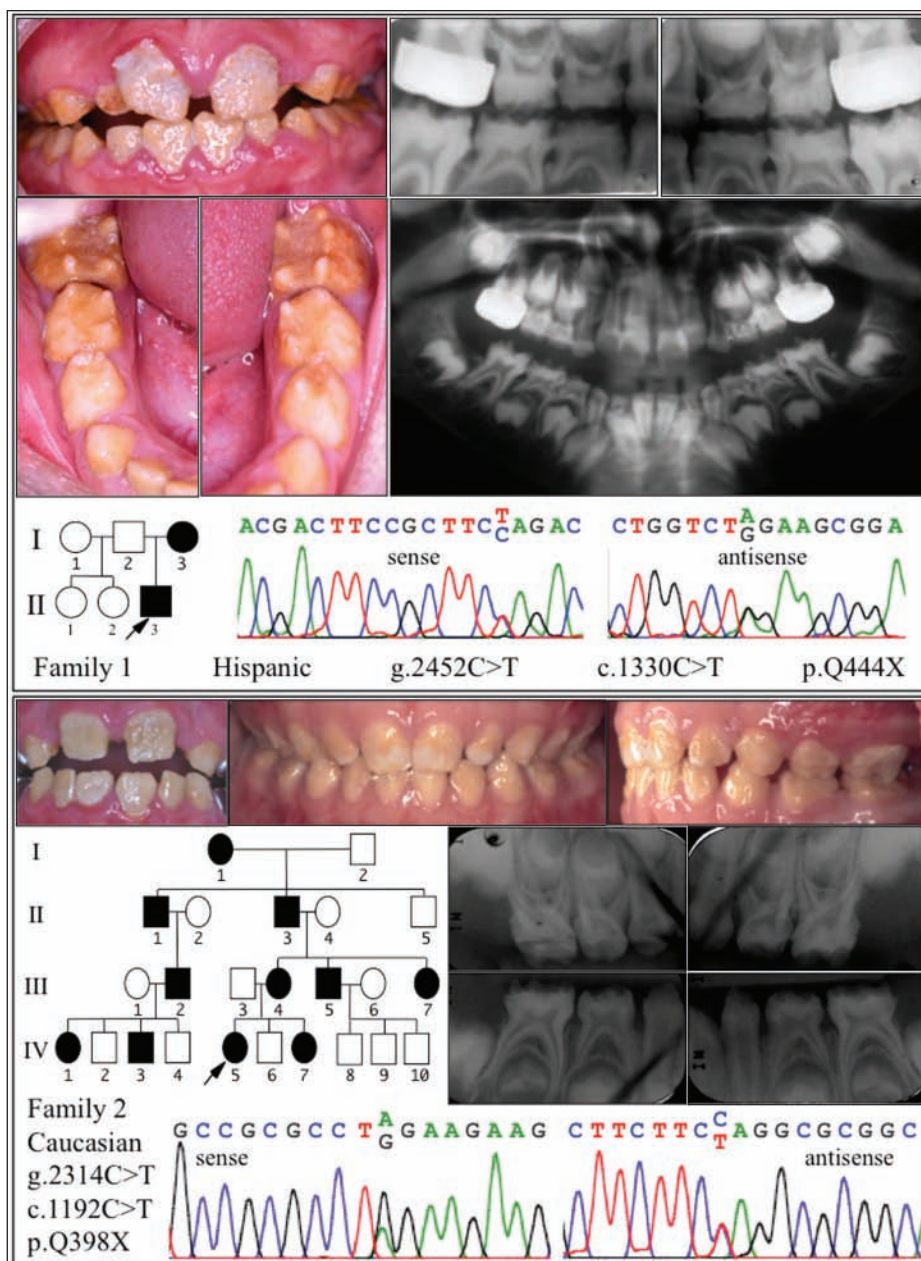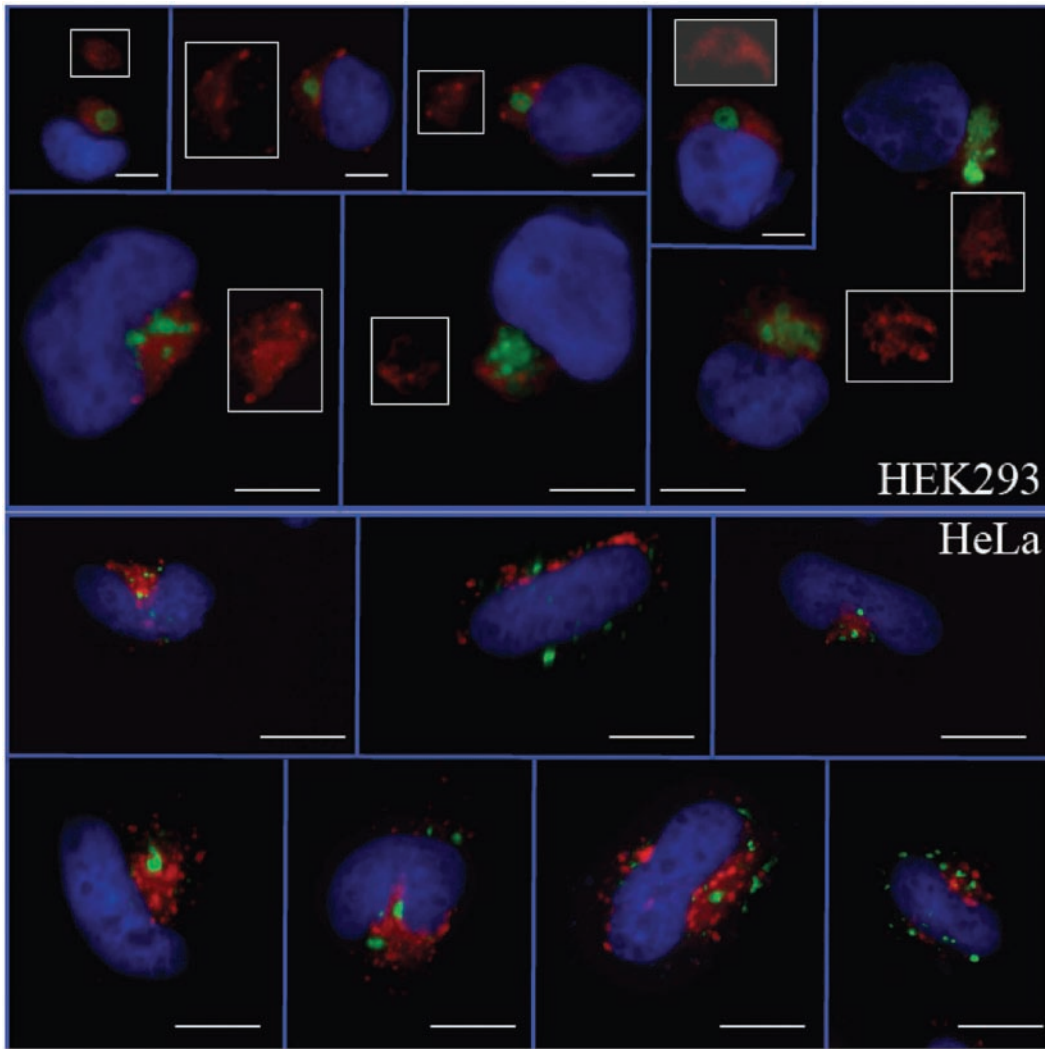

Figure 2. Phenotypic features and mutation identification. **(top)** Oral photographs and radiographs of the proband of family 1, who was in the mixed-dentition stage at age 9 yrs and 5 mos. Note the plaque accumulation and associated gingivitis in the anterior region. Both the primary and secondary dentitions were affected. The enamel was generally brown and rough, and cannot be distinguished from dentin on radiographs. DNA sequencing chromatograms reveal a C-to-T transition in one of the two *FAM83H* alleles, changing codon 444 from a glutamine (Q) to a stop (X) codon. **(bottom)** Oral photographs and radiographs of the proband of family 2. The permanent maxillary central incisors (left) at 8 yrs and 10 mos were rough and stained. The primary dentition (center and right), shown at 5 yrs of age, was also affected. The enamel and dentin are not distinguishable on radiographs taken at 4 yrs and 10 mos. DNA sequencing chromatograms show an A-to-G transition in one of the two *FAM83H* alleles, changing codon 398 from a glutamine (Q) to a stop (X) codon.

girl (8 yrs, 10 mos) with no known systemic abnormalities. The individual had dental hypersensitivity. The enamel layer was yellowish brown, thin, and did not contrast with dentin on radiographs. The enamel was rough, covered with plaque and calculus, and associated with generalized gingivitis. Wear

Figure 3. HEK293 **(top)** and HeLa **(bottom)** cells transfected with a construct expressing Fam83h-GFP. In HEK293 cells, Fam83h (green) is always associated with the Golgi (red) and never appears inside the nucleus (blue). Typically, the Golgi stain is weaker or negative where the GFP localizes (white-outlined insets). In HeLa cells, the association between Fam83H-GFP and the Golgi is not as strict. The green fluorescence is always associated with small vesicle-like structures that only sometimes overlap with the Golgi. Bars = 4 μm.

Intracellular Localization of Fam83h

The full-length mouse Fam83h protein fused to green fluorescent protein, expressed in a human kidney epithelial cell line (HEK293), suggests that Fam83h is associated with perinuclear vesicles in the vicinity of the Golgi apparatus, perhaps in the trans-Golgi network or in the late endosomes (Fig. 3, top). The localization pattern varies with cell type: The association with the Golgi is less strict in HeLa cells (Fig. 3, bottom). A more definitive localization requires the development of Fam83h-specific antibodies to immunolocalize Fam83h in ameloblasts at the ultrastructural level.

DISCUSSION

The human FAM83 family of genes is comprised of 8 members, designated *FAM-83A* through *FAM83H*. Fam83h (1179 amino acids) is the largest; Fam83a (434 amino acids) is the smallest.

facets on the proband's primary teeth suggested more than average enamel attrition. The primary dentition displayed islands of normal-looking enamel, particularly near the incisal edges or cusp tips. The enamel phenotype in this family can be traced back for over four generations, with virtually all affected individuals having extensive dental histories. Participant I1, at age 88 yrs, was reported to be healthy, other than having AI. Mutation analysis of genomic DNA from the proband identified a *FAM83H* nonsense mutation (g.2314C>T; c.1192C>T; p.Q398X), which was previously identified as a spontaneous nonsense mutation in a Korean family with ADHCAI (Kim *et al.*, 2008) and also in a Turkish family (Hart *et al.*, 2009). The highly varied ethnic backgrounds of the three AI families with this *FAM83H* mutation indicate that they arose independently.

Alignment of the human Fam83 proteins shows that homology common to the entire group begins near the N-terminus and ends at Ser[281] (Appendix). This conserved N-terminal region also corresponds closely to the part of Fam83h (Arg[4] to Leu[284]) that is included in the conserved domain database (CDD) for the phospholipase D (PLD) superfamily (Marchler-Bauer *et al.*, 2007). The homology with PLD, however, is trace, and may be indicative of a conserved three-dimensional fold. The N-terminal domain that shows trace similarity to PLD bears a much greater resemblance to the other members of the FAM83 family and is the shared element that gives the group its identity. The 14 *FAM83H* mutations that cause ADHCAI delete the sequence on the C-terminal side of the PLD-like domain, with the closest truncation terminating at Ser[287].

A BLASTP search of GenBank identified *FAM83H* homologues going back to fish. Alignment of selected vertebrate

sequences (human NM_198488; mouse AK035531; cattle XM603315; opossum XM_001370407; bird XM_423955; frog BC078004; and zebrafish NM_001045090) showed homology throughout the entire protein, although the homology for the C-terminal segment starting with Ser[287] was lower than the homology for the PDL-like region (Appendix). Thus, the Fam83h gene might have arisen early in vertebrate evolution by duplication of a gene in the Fam83 family. It appears that ameloblasts are dependent upon the Fam83h protein for proper dental enamel formation.

While the protein sequence of Fam83h on the C-terminal side of the PLD-like domain is not homologous with other proteins, it does have some notable features. This part of Fam83h contains a tandem repeat region that varies in length between species. The repeated segment is 11 amino acids in length, with at least 7 copies in humans (E[866] to S[942]) and 12 copies in the mouse (G[848] to K[979]) (Fig. 4). Tandem repeat sequences of variable length are also observed in amelogenin (Takagi *et al.*, 1984; Hu *et al.*, 1996) and enamelin (Hu *et al.*, 1998). Large-scale characterization of phosphorylated sequences in the mouse liver and in a human (HeLa) cell line identified multiple serine residues that are sometimes phosphorylated in Fam83h ([mouse] Ser[5], Ser[7], Ser[8], Ser[9], Ser[512], Ser[513], Ser[515], Ser[522], Ser[832], Ser[834], Ser[841], Ser[915], Ser[926], Ser[937], Ser[948], and Ser[1041]; and [human] Ser[523], Ser[731], Ser[914], and Ser[1003]). Most of the phosphorylation sites are in the area deleted by *FAM83H* truncation mutations in ADHCAI kindreds, and some are in the tandem repeat region. The finding that Fam83h is phosphorylated suggests that it is regulated by kinases.

Phospholipase D (PLD) is a key facilitator of membrane vesicle trafficking. There are two human PLD genes: *PLD1* (3q26) and *PLD2* (17p13.1). Our fluorescent images suggest that Fam83h is associated with intracellular vesicles in the perinuclear region, usually closely associated with the Golgi complex. This localization pattern is similar to that of PLD1, but not PLD2. GFP-tagged PLD1 localizes mainly to the perinuclear region, including the trans-Golgi apparatus, multivesicular endosomes, and late endosomes, but not to other organelles, while PLD2 localizes to the plasma membrane (Du *et al.*, 2004; Hiroyama and Exton, 2005). Phospholipase D catalyzes the hydrolysis of phosphatidylcholine (a membrane phospholipid) to phosphatidic acid and choline. Because the homology with PLD is low, it seems unlikely that Fam83h could catalyze this reaction, but this cannot be ruled out. At present we speculate that Fam83h might be a peripheral membrane protein that plays a role in intracellular trafficking or cytoskeletal re-organization.

ACKNOWLEDGMENTS

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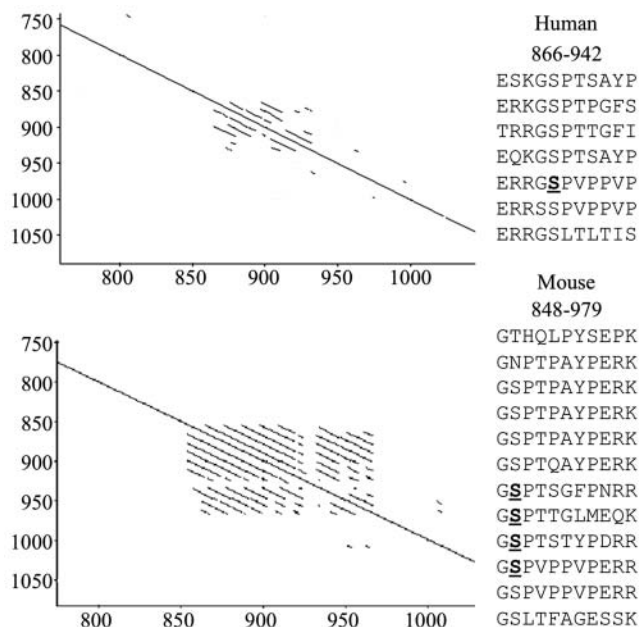
Figure 4. Tandem repeats in human **(top)** and mouse **(bottom)** Fam83h. Self-diagonal plots highlight repeated sequences in the Fam83h protein. In this type of analysis, a protein is aligned against itself. The solid line along the diagonal indicates the protein's identity with itself. Amino acid identities to upstream or downstream sequences are marked by lines above or below the diagonal. Inspection of the sequences corresponding to lines off the diagonal showed the repeats are 11 amino acids in length. The human Fam83h sequence between E[866] and S[942] and the mouse Fam83h sequence between Gly[848] and Lys[979] are shown at the right. Phosphorylated serines in the repeat region are underlined and in bold.

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