

# Transcriptomic characterization of fibrolamellar hepatocellular carcinoma

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**Fibrolamellar hepatocellular carcinoma (FLHCC) tumors all carry a deletion of ~400 kb in chromosome 19, resulting in a fusion of the genes for the heat shock protein, DNAJ (Hsp40) homolog, subfamily B, member 1, *DNAJB1*, and the catalytic subunit of protein kinase A, *PRKACA*. The resulting chimeric transcript produces a fusion protein that retains kinase activity. No other recurrent genomic alterations have been identified. Here we characterize the molecular pathogenesis of FLHCC with transcriptome sequencing (RNA sequencing). Differential expression (tumor vs. adjacent normal tissue) was detected for more than 3,500 genes ( $\log_2$  fold change  $\geq 1$ , false discovery rate  $\leq 0.01$ ), many of which were distinct from those found in hepatocellular carcinoma. Expression of several known oncogenes, such as *ErbB2* and *Aurora Kinase A*, was increased in tumor samples. These and other dysregulated genes may serve as potential targets for therapeutic intervention.**

pediatric cancer | liver cancer | protein kinase | genomics | fusion gene

Fibrolamellar hepatocellular carcinoma (FLHCC) is a rare liver cancer that usually occurs in adolescents and young adults (1–3). Effective therapeutic options are limited; FLHCC does not respond well to chemotherapy (4, 5), although there have been some encouraging retrospective studies assessing the use of 5-fluorouracil and IFN alpha-B2 (6). There have been mixed reports as to whether FLHCC does (7, 8) or does not (5) have a better prognosis than hepatocellular carcinoma (HCC). Surgical resection remains the mainstay of therapy with overall survival reported to be 30–45% at 5 years (4, 7, 9). Diagnosing FLHCC is a challenge because the symptoms are nonspecific and there are no useful biomarkers (3). Even with a biopsy, the diagnosis of FLHCC can be ambiguous (10). As a result, FLHCC is often diagnosed after the tumor is large and has metastasized.

FLHCC is often referred to as a variant of HCC, and the cell of origin is assumed to be the hepatocyte. FLHCC gets its name from the lamellar fibrous bands composed of type I, type III, and type V collagen (11) that run through the tissue. Unlike HCC, FLHCC is not associated with viral hepatitis (2, 9, 12), increased levels of alpha fetoprotein (AFP) (3, 5, 13, 14), or altered p53 or  $\beta$ -catenin (15). It has been proposed that FLHCC has a neuroendocrine origin based on increased expression of proprotein convertase subtilisin/kexin type 1 (PCSK1), NTS, DNER, and calcitonin-related polypeptide alpha (CALCA) (16, 17) or an origin in the biliary tree based on increased levels of EpCAM, mCEA, CA19-9, EMA, and CK7 (14). FLHCC has also been associated with features of the mammary gland. Some male FLHCC patients present with gynecomastia (18) and increased levels of aromatase, also known as cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1), or estrogen synthase (18–20). Increased expression of the breast cancer oncogene *v-erb-b2* avian erythroblastic leukemia viral oncogene homolog 2 (*ErbB2*) (16), has been observed in FLHCC tumors.

We recently described a ~400-kb deletion in chromosome 19 (21), which produced a chimeric gene incorporating the first

exon of *DNAJB1* and all but the first exon of *PRKACA*. This produced a chimeric RNA transcript and a translated chimeric protein that retains the full catalytic activity of wild-type PKA. This chimeric protein was found in 15 of 15 FLHCC patients (21) in the absence of any other recurrent mutations in the DNA (22), which strongly implies a causal link to malignancy. In normal tissue, PKA is involved in many cellular functions (23). It phosphorylates many targets, including transcription factors such as cAMP response element-binding protein (CREB) (24), GATA3, GATA6 (25), NF- $\kappa$ B (26),  $\beta$ -catenin [activating downstream elements of the *wnt* pathway (27)], the transcription coactivator yes-associated protein 1 (YAP) (28), and ETV1 (29) and indirectly activates XBP1 (30). Any or all of these molecular events could have significant effects on gene expression. Furthermore, decreased activity of the regulatory subunits PRKAR1A or PRKAR2B have been shown to lead to increased activity of PKA and tumorigenesis (31).

To characterize the molecular pathogenesis of FLHCC we performed whole-genome sequencing and RNA sequencing (RNA-seq) of FLHCC tumors and adjacent normal tissue. The differences in gene expression, as assayed by RNA-seq, between FLHCC and adjacent normal liver tissue were generally consistent across all patient samples, suggesting FLHCC has a unique gene expression profile. Although some of the changes were

## Significance

**Fibrolamellar hepatocellular carcinoma (FLHCC) is a rare pediatric liver cancer. A deletion of ~400 kb in one copy of chromosome 19 results in a chimeric protein, an activated protein kinase A. No other deletions, amplifications, mutations, or structural variants were found. This strongly implicates the chimera as the driving mutation. This paper examines gene expression in FLHCC. The results establish FLHCC as a single disease distinct from other cancers, including hepatocellular carcinoma. The results help explain some of the known pathophysiology: the collagen fibers that give fibrolamellar its name and the gynecomastia reported in young male patients. Finally, this work identifies oncogenes whose expression is increased and that may serve as targets for therapeutic intervention.**

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Data deposition: The sequence reported in this paper has been deposited in the database of Genotypes and Phenotypes (dbGaP) (accession no. [phs000709.v1.p1](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE600709)).

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similar to those in HCC or other tumors, many were distinct, suggesting FLHCC represents an independent pathology. In the FLHCC tissue there was increased expression of oncogenes associated with various cancers including pathways found in breast cancer: ErbB2 (EGF pathway) (32), Aurora Kinase A (AURKA) and E2F3 (cell cycle), and CYP19A1 (estrogen synthesis pathway). Although the transcription of CYP19A1 can be regulated by PKA, the pattern of altered gene expression did not match those of transcription factors known to be phosphorylated by PKA. PKA also increases the enzymatic activity of some of these proteins, such as ErbB2 and AURKA. These results suggest these oncogenes as potential targets for therapeutic intervention for FLHCC.

## Results

FLHCC gene expression patterns were distinct from those seen in adjacent normal liver tissue, and similar across patients, as observed in principal component analysis (Fig. 1A) and Euclidean distance measures (Fig. 1B). We assessed differential expression with the DESeq2 (33) analysis package with transcript annotations from GENCODE v74. Using a false discovery rate (FDR) threshold of 0.01 and a fold-change threshold of 2, we detected 3,439 protein-coding genes differentially expressed in FLHCC vs. normal tissue (1,408 up-regulated and 2,031 down-regulated; the complete list is given in [Dataset S1, FLHCC v normal](#)).

Significantly enriched pathway gene sets were identified with gene set variation analysis (GSVA) (34). Among gene sets from the “Canonical Pathways” collection in the Molecular Signatures Database (Broad Institute, c2.cp), we identified 364 pathway gene sets differentially enriched in FLHCC vs. normal ( $p_{Adj} \leq 0.05$ ; complete lists of pathways positively and negatively enriched are given in [Dataset S1, Pathway Increase and Pathway Decrease](#)).

Among those diminished most significantly ( $p_{Adj}$ ) in FLHCC were processes associated with various liver/hepatocyte functions including metabolism, synthesis, detoxification, and bile processes (Fig. 2A). Many pathways associated with cell proliferation were enriched in FLHCC (Fig. 2B).

**Relevance of Gene Expression Changes to FLHCC.** The increased expression of the catalytic subunit of PKA in FLHCC would be expected to alter the expression of many genes, which is consistent with our observations. Whereas some of these may drive the cancer, many may be epiphenomena: transcripts altered by the

increased activity of PKA (21), but not drivers of the tumor. Thus, we focused our analysis on select gene sets that are more likely to be relevant to FLHCC including estrogen biosynthesis (responsible for gynecomastia), extracellular matrix components (characteristic fibrous bands by histology), and the PKA pathway [mutation in the catalytic subunit PRKACA (21)]. Additionally, based on the differential expression analysis, we examined a number of gene sets that have been previously implicated in oncogenesis, including the EGF receptor pathway, cell cycle, glycolysis, and *wnt* signaling.

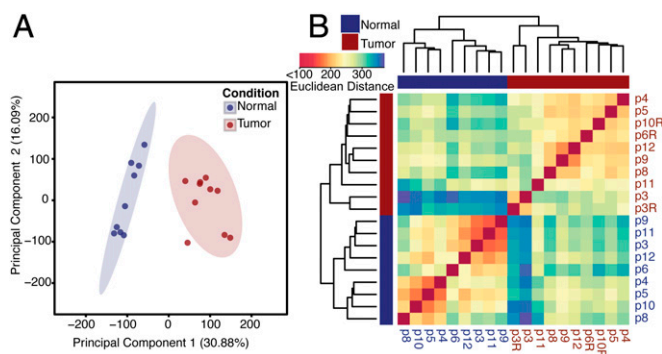
**Estrogen biosynthesis.** Gene sets associated with liver functions, including cytochrome p450 (Fig. 3A) and steroid biogenesis (Fig. 2), were, in general, down-regulated in FLHCC. The exception to the down-regulation of CYP450 genes was up-regulation of genes involved in estrogen synthesis. These included CYP19A1 (estrogen synthase) and CYP17A1 (steroid 17-alpha-monooxygenase), another key enzyme in the pathway of estrogen synthesis. An examination of other components of the estrogen biosynthesis pathway (GO:0008210 estrogen biosynthetic process; Fig. 3C and Table 1) revealed up-regulation of UDP glucuronosyl-transferase 2 family, polypeptide B4 (UGT2B11), the product of which catalyzes the glucuronidation of some catechol estrogens such as 4-hydroxy-estrone, and the solute carrier family 22 (organic anion/urate transporter), member 11 (SLC22A11), which transports estrone sulfate.

**Extracellular matrix and cell-cell interactions.** The fibrous bands in FLHCC are composed of type I, type III, and type V collagen (11). Differential gene expression analysis indicated significant increases in type I, III, and V collagen gene expression, as well as types IV, VI, VIII, IX, X, XI, XV, XVII, and XXII (Fig. 3D and [SI Appendix, Table S1](#)), resulting in a considerably altered collagen landscape in the FLHCC tissue. One of these, COL11A1, promotes tumor progression in ovarian cancer, where it is regulated by TGF- $\beta$ 1, which triggers the activation of SMAD2 (35). In FLHCC we observe increases in both TGF- $\beta$ 1 receptor and SMAD2 transcripts ([Dataset S1, FLHCC v normal](#)).

In contrast to the collagens, there are few changes in fibronectin or keratin gene expression. The only fibronectin family members increasing significantly were FNDC1 and FNDC3A ([SI Appendix, Table S2](#)). Although there has been one previous report of an increase in fibronectin in FLHCC (36), another found no difference (37). There are increases of gene expression of some keratins (KRT 7, 81, 83, 86, and 222), decreases of others (KRT 1, 19, 72, and 73), and no significant changes in the others ([SI Appendix, Table S3](#)). The increase in keratin 7 is consistent with a previous report of an increase in FLHCC, although we did not observe the previously reported increase of keratins 8, 18, and 19 in FLHCC (38).

Alterations of interactions with the extracellular matrix and other cells can be critical for transformation (39). A number of transcripts that encode proteins involved in cell-cell interactions ([SI Appendix, Table S4](#)) were up-regulated, including the gap junction proteins GJC1 and GJA5 and the integrins alpha-7 (ITGA7) and alpha5 (ITGAV). Two adhesion molecules that have been shown to be increased in HCC were also increased: cadherin-13 (CDH13) (40) and Cluster of Differentiation 90 (THY1) (41). Also up-regulated are transcripts for the roundabout homolog 1 (ROBO1) and its ligand (SLIT2), as well as Jagged-1 (JAG1), which interacts with the notch receptor and whose overexpression is associated with poor prognosis in breast cancer (42). Many of the mucins, which are frequently mutated or overexpressed in cancers (43), were up-regulated, especially Muc5B, Muc13, and Muc3a. Finally, Chitinase 3-like 1 (CHI3L1), which is also expressed in human gliomas (44) and promotes angiogenesis in colorectal cancer (45), is increased.

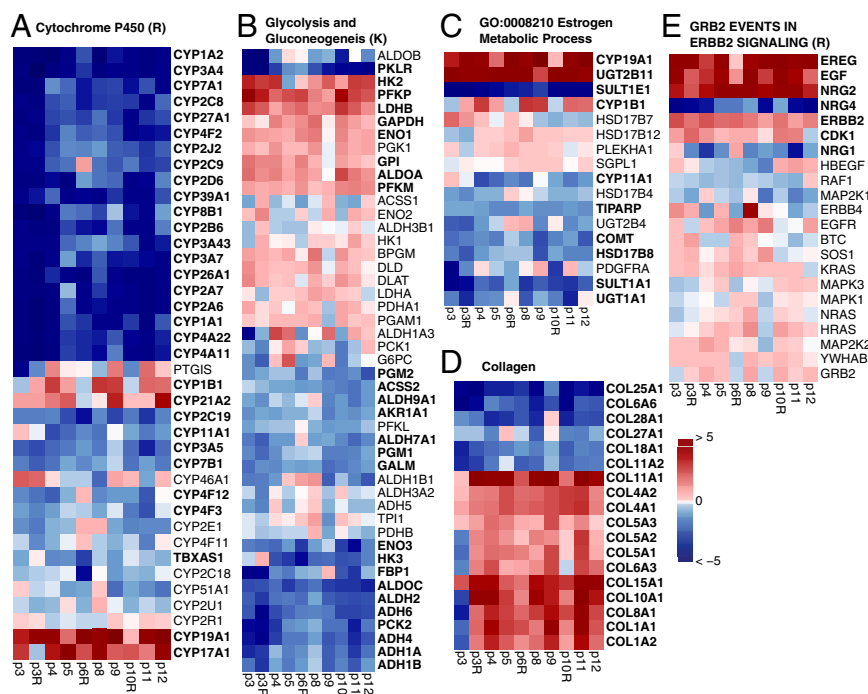
**PKA.** Some of the genes altered in FLHCC are involved in PKA-associated pathways (Table 2). Some, such as the A kinase anchor protein 12 (AKAP12) and PRKACA, are increased. The increase in



**Fig. 1.** Tumor gene expression patterns in FLHCC are distinct from adjacent normal tissue. (A) Principal component analysis of variance stabilized transformed RNA-seq read counts for whole transcriptomes. Ellipses indicate 95% confidence interval of group membership. Axis percentages indicate variance contribution. (B) Heat map depicting hierarchical clustering of sample-to-sample distance. Variance-stabilized transformed RNA-seq read counts for whole transcriptomes were used to calculate sample-to-sample Euclidean distances (color scale) for hierarchical clustering, complete method. R in patient labels indicates recurrence tumor sample.







**Fig. 3.** Gene expression differences in FLHCC vs. normal liver tissue for select gene collections of functional interest. For genes in each collection,  $\log_2$  fold change (FLHCC vs. adjacent normal liver) expression is plotted with heat-map color scale. Genes significantly differentially expressed (FDR 0.01,  $\log_2$  fold change  $>1$ ) are indicated in bold. Genes that did not pass read count filters (DESeq2) were removed from collection for plotting purposes. (A) Cytochrome P450 (Reactome, GSVA adjusted  $P$  value =  $6.86E-08$ ). (B) Glycolysis and gluconeogenesis (KEGG, GSVA adjusted  $P$  value = 0.014). (C) Estrogen metabolic process (GO:0008210, GSVA enrichment not assessed). (D) Collagen (genes selected by “collagen” gene description, Ensembl BIOMART annotation, differentially expressed FLHCC vs. normal liver, FDR 0.05). (E) GRB2 events in ERBB2 signaling (Reactome, GSVA adjusted  $P$  value = 0.0004).

**Gene Expression of Cancer Pathways in FLHCC.** In curating the FLHCC differential gene expression data, we observed significant changes in genes implicated in a variety of cancer-associated processes, including the EGF receptor pathway, cell cycle, glycolysis, and *wnt* signaling, some of which we discuss in more detail below.

**EGF/ErbB2 signaling.** Several gene sets significantly enriched in FLHCC (GSVA analysis) are associated with the EGF/ErbB2 signaling pathway (Fig. 3E and Table 3). These increases included many of the ligands for the pathway including neuregulin (NRG2; *SI Appendix, Table S7*), EGF, epiregulin (EREG), and amphiregulin (AREG) as well as some of the receptors of the pathway including EGFR and ErbB2.

**Cellular metabolism.** Oncogenic transformation is frequently associated with alterations of metabolism (51) and activation of PKA affects glycolysis (52). In FLHCC there were many changes in the pathway gene sets associated with carbohydrate metabolism (a representative sample is shown in Fig. 3B). One increased transcript is hexokinase 2 (HK2), which is required for both tumor initiation and maintenance in some mouse models (53). Other transcripts consistently increased included the platelet isoform of phosphofructokinase (PFKP), which plays a critical role in driving the proliferation of breast tumors (54), lactate dehydrogenase

B (LDHB), which is considered essential for triple-negative breast cancer, and KRAS-dependent lung adenocarcinomas (55).

Many genes that are involved in carbohydrate metabolism, but not annotated in this particular set of pathways, showed increased expression, such as NADPH Oxidase 1 (Nox1), which is required for the ras-dependent transformation of cells (56) working through phosphorylation of GATA-6 promoter (57). The promoter activity of GATA-6, in turn, is activated by PKA by phosphorylation on a unique serine (25).

Two proteins recently implicated in metastasis by allowing tumor cells to survive in anoxic conditions of the liver, creatine kinase (CK) and solute carrier family 6 (neurotransmitter transporter), member 8 (SLC6A8) (58), were up-regulated in FLHCC. Many other solute carriers involved in metabolism were also up-regulated (*SI Appendix, Table S8*), including SLC25A15, which transports ornithine from the cytosol into the mitochondria during the urea cycle, SLC38A1, important for uptake of nutrients, the bicarbonate transporter SLC4A11, the facilitative glucose transporter SLC2A1, the glucose transporter (SLC45A1), and the creatine transporter (SLC6A8). As mentioned in the estrogen section, SLC22A11 was also enriched.

**Cell cycle.** Many cancers demonstrate alterations in cell cycle regulation. In FLHCC, we observed increased gene expression of AURKA, cyclin E1 (CCNE1), cell division protein kinase 6

**Table 1. Transcripts up-regulated in retino/drug metabolism and steroid metabolism**

Gene	Name	$\Delta\log_2$	PA <sub>adj</sub>
UDP glucuronosyltransferase	UGT2B11	7.98	4.03E-45
Cytochrome P450, family 19, subfamily A, polypeptide 1 (aromatase – estrogen synthase)	CYP19A1	5.95	4.25E-26
Cytochrome P450, family 17, subfamily A, polypeptide 1	CYP17A1	2.62	2.60E-06
Pre-B-cell leukemia homeobox 1	PBX1	1.88	2.64E-30

**Table 2. PKA subunits and interacting partners**

Gene	Name	$\Delta\text{Log}_2$	pAdj
A kinase (PRKA) anchor protein 12	AKAP12	4.02	4.13E-20
Protein kinase, cAMP-dependent, catalytic, alpha	PRKACA	2.23	6.55E-23
Protein kinase, cAMP-dependent, regulatory, type II, alpha	PRKAR2A	0.49	4.58E-03
A kinase (PRKA) anchor protein 13	AKAP13	0.45	1.16E-02
A kinase (PRKA) anchor protein 17A	AKAP17A	-0.63	4.21E-05
Protein kinase, AMP-activated, beta 2 noncatalytic subunit	PRKAB2	-0.79	3.13E-04
A kinase (PRKA) anchor protein 1	AKAP1	-0.90	2.43E-05
A kinase (PRKA) anchor protein 3	AKAP3	-1.46	2.07E-06
Protein kinase, cAMP-dependent, regulatory, type II, beta	PRKAR2B	-2.05	2.96E-11

(CDK6), Cyclin O (CCNO), Cyclin-dependent kinase 2 inhibitor B (CDKN2B), and the transcription factor E2F3.

**Wnt signaling.** The *wnt* signaling pathway (59), and the receptor FZD10 in particular, has been implicated in many cancers such as colorectal cancer (60). Genes encoding many of the key elements in the KEGG *wnt* signaling pathway are up-regulated in FLHCC tissue (*SI Appendix, Table S9 and Fig. S2*). There is an increase in the transcripts of the *wnt* receptor FZD10 and DKK4, a secreted protein, which interacts with the *wnt* receptor. Up-regulation of DKK4 in colon cancer is associated with activation of the *wnt* signaling pathway (61).

#### Gene Expression in FLHCC and Other Cancers.

**FLHCC and HCC.** To contrast the gene expression profiles of FLHCC and HCC, HCC RNA-seq reads from TCGA (50 tumor/normal pairs) were processed using the workflow implemented for the FLHCC data with paired tumor-normal samples. We compared the differences in tumor vs. normal samples between FLHCC and HCC. Differential expression was observed in 3,708 genes (FDR  $\leq 0.01$ ,  $\text{log}_2 \geq 1$ ; *Dataset S1, FLHCC v HCC*). In hierarchical clustering analysis (Pearson correlation of fold-change, tumor vs. normal), the FLHCC cases formed a distinct cluster (Fig. 4), with a single exception: HCC patient TCGA23 (TCGA-DD-A1EC-01A-21R-A131-07), clustered with FLHCC rather than HCC. Upon further analysis, we found that TCGA23 RNA-seq alignments contained reads consistent with the FLHCC-associated DNAJB1-PRKACA chimeric transcript in tumor, but not in matched normal tissue. This chimera was not detected in any of the other TCGA HCC RNA-seq data (by Tophat Fusion and/or FusionCatcher). These results suggest that patient TCGA23's tumor was actually FLHCC rather than HCC. Unfortunately, pathology specimens are not available for histological confirmation.

**Neuroendocrine tumors.** FLHCC carries some hallmarks of neuroendocrine malignancies (16, 17). The RNA-seq results presented here are in agreement with recent observations that several genes implicated in neuroendocrine tumors (PCSK1 and CALCA) are significantly up-regulated in FLHCC. However, this pattern was not consistent for other genes associated with neuroendocrine disease (62) (Table 4 and Fig. 5). Many of the genes considered

hallmarks of neuroendocrine tumors, such as NCAM1, synaptophysin (63), or chromogranin A (64), were not expressed at all in FLHCC, and others, such as transthyretin (65), were significantly down-regulated in tumor samples.

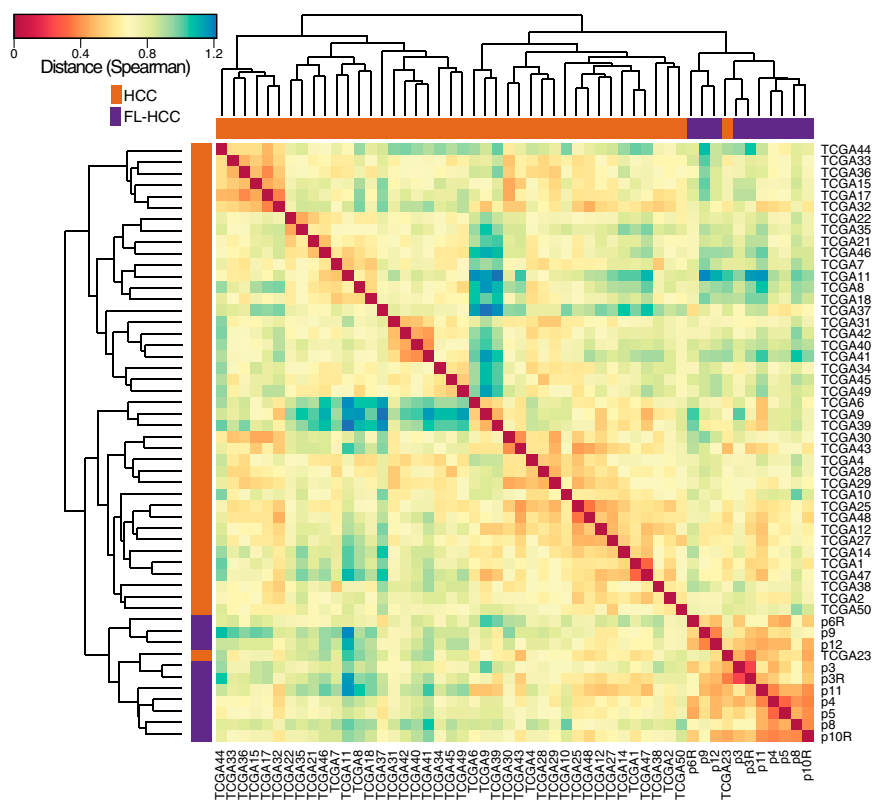
**Other KEGG cancer pathways sets.** Gene sets for a number of cancers, including bladder, pancreatic, and small-cell lung cancer, were differentially enriched in FLHCC [KEGG collection, Molecular Signatures Database, Broad Institute, c2.cp, canonical pathways, GSVA, FDR 0.05] (*SI Appendix, Fig. S3*). The genes in the small-cell lung cancer set that were most enriched in FLHCC tissue (*SI Appendix, Fig. S3A*) were the extracellular matrix molecules, especially integrins (ITGA3, ITGA6, ITGAV, ITGB1, and ITGA2), collagens (COL4A1 and COL4A2), and laminins (LAMA1, LAMA3, LAMA4, LAMA5, LAMB1, and LAMC1), members of the TNF receptor pathway (TRAF1, TRAF2, and TRAF5), and proteins implicated in apoptosis (BCL2L1, CASP9, and CYCS) and cell cycle (CCNE1, CDK6, CDKN2B, E2F1, and E2F3).

The enrichments of the altered FLHCC gene sets with bladder cancer and pancreatic cancer were predominantly in the EGF receptor family EGF and ErbB2 (*SI Appendix, Fig. S3 B and C*). In the set of genes that are associated with pancreatic cancer, the FLHCC samples showed additional enrichment with the transcripts involved in the cell cycle (E2F1, E2F3, CDKN2a, and CDK6) and the TGF beta pathway (SMAD2, TGFBR1, and TGFBR2).

**Validation of RNA-seq by quantitative PCR and proteomics.** We quantified the expression levels of several genes by quantitative RT-PCR (qPCR). Overall, qPCR fold-change (tumor vs. normal) values were very similar to those measured by RNA-seq (Fig. 6A and *SI Appendix, Fig. S4A*,  $R^2 = 0.98$ ). Protein levels were assayed by mass spectrometry and Western blot. By mass spectrometry there was a close correlation between the change of RNA-seq and the protein levels from FLHCC to adjacent normal tissue (Fig. 6B and *SI Appendix, Fig. S4B*,  $R^2 = 0.68$ ). By Western blot the increased intensity of the protein bands qualitatively matched the increases in transcripts by RNA-seq between the tumor and adjacent normal tissue (Fig. 6C).

**Table 3. Members of the EGFR pathway**

Gene	Name	$\Delta\text{Log}_2$	Padj
Epiregulin	EREG	6.06	4.28E-22
Neuregulin 2	NRG2	4.90	1.41E-42
Epidermal growth factor	EGF	4.54	7.23E-13
Amphiregulin	AREG	2.53	2.09E-03
V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2	ERBB2	2.15	6.44E-34
SHC (Src homology 2 domain containing) transforming protein 1	SHC1	1.13	4.16E-12
Breast cancer anti-estrogen resistance 1	BCAR1	1.00	7.67E-08
Epidermal growth factor receptor	EGFR	0.86	3.18E-03



**Fig. 4.** Distance clustering of FLHCC and HCC. Protein-coding gene expression  $\log_2$  fold change was calculated for each tumor–normal pair (10 FLHCC, 42 HCC after filtering by read depth ratios). Hierarchical clustering (complete method) was performed on spearman rank correlation matrix for all protein coding genes passing DESeq2 expression filter (ranked by moderated  $t$  statistic ranking). Side bar colors indicate tumor type on diagnosis: FLHCC (purple) and HCC (orange).

## Discussion

We have previously reported a heterozygous 400-kb deletion in FLHCC tumors that produces a fusion of the promoter and starting exons of the *DNAJB1* with the second-to-final exons of *PRKACA*. This generates not only a chimeric RNA transcript, but also a chimeric protein with kinase activity indistinguishable from the native *PRKACA* (21). Similar findings have been reported in other FLHCC patients (17). Whole-genome analysis showed no other recurrent mutations (22). Here we demonstrate that gene profiles are similar across patients, at the level of both individual genes and gene sets. Select RNA-seq results were validated by qPCR and supported changes in protein expression as tested by Western blot and mass spectrometry.

The ubiquity of the *DNAJB1-PRKACA* chimera and the consistency of gene expression in tumor tissue suggest that FLHCC is a single disease rather than a collection of diseases that happen to affect the same organ. Overall gene expression patterns, though generally similar, are not identical across patients. It remains to

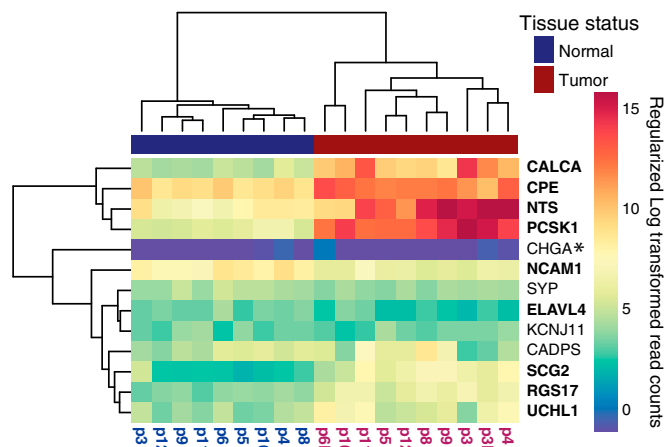
be determined which differences are epiphenomena that are inconsequential to FLHCC and which ones correlate with yet-to-be determined subvariants.

FLHCC was previously thought to be a variant of HCC (1, 2). Because they are both primary liver tumors they might be expected to have alterations in gene expression related to inflammation and, potentially, regeneration of the liver. Still, the gene expression changes in FLHCC tumors are more similar to each other than to the changes in HCC (Fig. 4). Although some transcripts are increased in both cancers, there are many that are not. This is consistent with the observations that known markers for HCC, such as elevated AFP, are not seen in FLHCC. FLHCC has been suggested to be of neuroendocrine origin as a consequence of the high levels of specific transcripts and products, such as PCSK1. However, many transcripts that are associated with neuroendocrine cancers are decreased in FLHCC, such as *TTR* and *ST6GAL1*, whereas others considered classic hallmarks of neuroendocrine tumors, such as synaptophysin and

**Table 4.** Genes implicated in neuroendocrine cancer

Gene	Gene name	$\Delta\log_2$	pAdj
Proteinase convertase subtilisin/kexin type 1	PCKS1	10.63	4.20E-100
Calcitonin-related polypeptide alpha	CALCA	7.49	1.54E-28
Transthyretin	TTR	-3.98	5.58E-07
ST6 beta-galactosamide alpha-2,6-sialyltransferase 1	ST6GAL1	-2.17	4.14E-22
Chromogranin A (parathyroid secretory protein 1)	CHGA	Not expressed	NA
ELAV like neuron-specific RNA binding protein 4	ELAVL4	Not expressed	
Synaptophysin	SYP	Not expressed	

NA, not assessed.



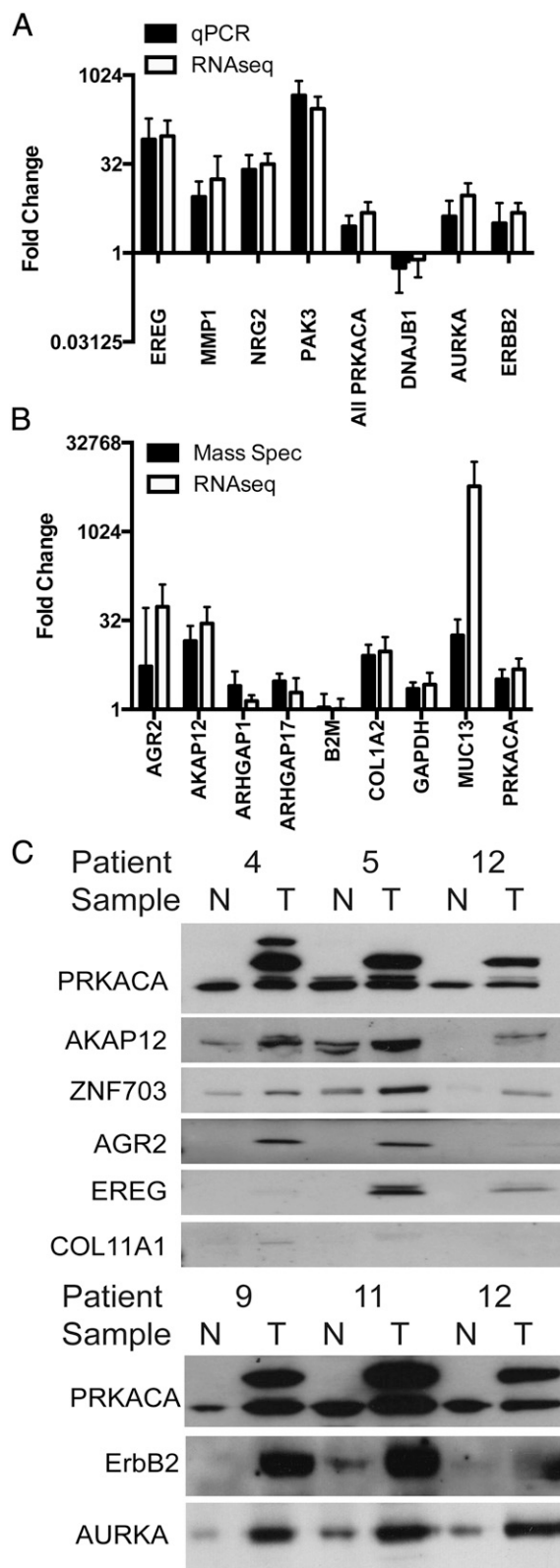
**Fig. 5.** Expression (variance stabilized read counts) of select genes implicated in neuroendocrine malignancies. Heat map displays variance-stabilized transformed RNA-seq read count values. Genes significantly differentially expressed (FDR 0.01,  $\log_2$  fold change >1) are indicated in bold. Asterisk indicates CHGA did not have sufficient reads to clear expression filters (DESeq2) but is included here for comprehensiveness.

chromogranin A, are not expressed in FLHCC (Table 4 and Fig. 5). Because the promoter region of PCSK1 is driven by PKA (66), its increased expression might be an epiphenomenon, a consequence of the chimera in FLHCC (21).

A number of the changes of FLHCC have the hallmarks of some forms of breast cancer (*SI Appendix, Table S13*). Some of transcripts that are considered oncogenic for breast cancers are also increased in FLHCC including AURKA CYP19A1 (67), the breast cancer anti-estrogen resistance 1 (BCAR1), and ZNF 703, a luminal B breast cancer oncogene (68), as well as members of the EGF receptor pathway, including EGFR, ErbB2, and their ligands NRG2, EREG, AREG, and NGF.

ErbB2 is considered an oncogene in many tumors including breast cancer, cervical cancer, childhood medulloblastoma, lung cancer, cholangiocarcinoma, colorectal cancer, oral squamous cell carcinoma, germ-cell testicular tumors, gastric cancer, ovarian cancer, pancreatic adenocarcinoma, prostate cancer, and salivary gland tumors (32, 69–71). BCAR1, which is increased twofold in FLHCC, is associated with increased expression of ErbB2 in breast cancer and is considered a critical element in the transformation by ErbB2 (72). The ErbB2-mediated metastatic invasion of breast cells requires signaling through SHC1 (73), which is also increased in FLHCC. Additionally, PKA, whose activity is increased in FLHCC, has previously been implicated in breast cancer through a number of links. PKA activates the EGF receptor pathway by enhancing the neuregulin activation of ErbB2-ErbB3 through phosphorylation of Thr-686 on ErbB2 (74). With NRG2 up-regulated 30-fold and ErbB2 up-regulated 4.4-fold, along with up-regulation of other ligands for the pathway including EGF (23-fold) and epiregulin (67-fold), chronic activation of PKA could result in substantial up-regulation of this pathway. PKA has also been implicated in breast cancer through phosphorylation of the estrogen receptor, which contributes to tamoxifen resistance (75).

AURKA is a serine-threonine kinase that is normally expressed during the prophase of mitosis, where it is critical for chromatin separation. However, it is also an oncogene whose overexpression throughout the cell cycle has been found in many cancers, including 94% of breast cancers (76), bladder cancer (77), colorectal cancer (78), neuroblastoma, and hepatocellular carcinoma (79). Inhibitors for AURKA have been explored for the treatment of many different tumors (80). The activity of AURKA is significantly enhanced by the PKA-dependent phosphorylation on



**Fig. 6.** (A) The transcriptional changes as assayed by RNA-seq were correlated with transcriptional changes as assayed by qPCR. Column plot of individual transcripts showing the expression as assayed by RNA-seq ( $n = 10$ ) and qPCR ( $n = 5-8$ ; error bars are SD). (B) Transcriptional changes as assayed by RNA-seq ( $n = 10$ ) were correlated with changes in the proteome as assayed by mass spectrometry ( $n = 4$ ). (C) Protein expression in FLHCC (T) and adjacent normal (N) liver was assayed by Western blot for transcripts that were increased in FLHCC as assayed by RNA-seq.



threonine 288 (81). Other oncogenes that affect the cell cycle that are increased in FLHCC include CCNE1, whose amplification is considered a driver of ovarian cancers (82); CDK6, which has been implicated in tumorigenesis both through phosphorylation of Rb (83) and a phosphorylation-independent effect on proliferation (84); E2F3, which is required for normal cell proliferation (85); and overexpression of E2F3, which has been associated with breast (86), prostate (87), and bladder cancer (88).

Our results are consistent with some previous reports on gene expression associated with FLHCC (*SI Appendix, Table S14*). The increase of CYP19A1 is consistent with reports of increased levels of aromatase in FLHCC as well as reports of gynecomastia in FLHCC patients (18, 20, 89). Increases in gene expression for AGR2, NTS, TCN1, SLC6A3, and PCSK1 in FLHCC are also consistent with previous reports (*SI Appendix, Table S14*). There are a number of proteins that were previously reported to increase in FLHCC but were not significantly altered on the RNA-seq. It remains to be determined whether this is a difference between the RNA levels and the protein levels, or a variability between patients, or some other factor.

One critical question is the link between expression of the chimera and the changes of gene expression. It is, as yet, not possible to determine whether the changes are the result of increased expression of the PRKACA as a consequence of the DNABJ1 promoter, or changes of activity of PRKACA as a consequence of loss of the normal amino terminus or gain of the new amino terminus. Protein kinase A activity in the FLHCC cell may be a result of increased chimera DNABJ1-PRKACA, altered activity of the chimera kinase, or decrease of the regulatory subunit protein kinase beta (PRKAR2B). The observation that patients with germ-line amplification of PRKACA that present with Cushing's syndrome and have not been reported to have aberrations in the liver (90) suggests that the amplification in expression is not sufficient for the FLHCC pathology.

In FLHCC, we observe expression changes in 3,500 genes. It is possible that these widespread gene expression changes could be accounted for by PKA effects on a transcription factor(s). There are several transcription factors known to be direct targets of PKA, including CREB, AP-2, GATA4, GATA6, YAP, ETV1, and NF- $\kappa$ B or indirect targets such as XBP1. PKA phosphorylates NF- $\kappa$ B on serine 276 of the p65 subunit (91). NF- $\kappa$ B has previously been shown to be activated in FLHCC (92). NF- $\kappa$ B target gene sets were included in our enrichment analysis for transcription factor sets. None of the annotated NF- $\kappa$ B sets (MSigDB, Broad Institute) were highly ranked among enriched TF target sets (GSVA analysis, FDR  $\leq 0.01$ ). From examination of the full dataset, the best NF- $\kappa$ B gene set has an FDR of  $\sim 0.3$ , with other NF- $\kappa$ B sets even higher. This suggests that, based on these annotations there is no enrichment of NF- $\kappa$ B target genes in tumor vs. normal.

By GSVA the gene expression patterns in FLHCC do not match the registered patterns of any of the MSigDB (Broad Institute) transcription factor gene sets clearly linked to PKA. There may be a critical transcription factor that we have not yet identified. Alternatively, the response to activation of a transcription factor in liver tissue may be different from that of cells grown in culture. It is also possible that, over the extended expression of the PKA, the cells compensate by regulating some of the pathways. As one such example, PKA can activate the transcription factor activator protein 2 (AP-2). In the mouse liver activation of AP-2 inhibits the transcription factors AP-1, CREB, and c-Myc (93). Thus, whereas PKA could be directly activating CREB, in the liver it may also be inhibiting indirectly through AP-2. It is important to note that in our study, tumor transcript levels were compared with adjacent normal liver. The identification of the cell of origin for FLHCC could affect the evaluation of the relevant transcription pathways.

Even though the overall patterns of transcription are not yet attributable to specific transcription factors, activation of PKA helps explain some of the changes observed in gene expression

patterns. CYP19A1, a driver for some forms of breast cancer (67), is increased in FLHCC. In the region upstream of the gene there are 10 tissue-specific promoters (94). Normally, CYP19A1 is regulated by the promoter I.4, independent of PKA. However, in breast cancer CYP19A1 is driven by three additional promoters, I.7, I.3, and II (95). The pII promoter is driven by GATA3 or GATA4, but only in the presence of activated PKA. Thus, the PKA may be increasing the expression of CYP19A1 through an effect on GATA4. Similarly, ErbB2, which is consistently up-regulated, is also not considered as being driven by PKA. However, PKA activity drives expression of ErbB2 through activation of the PKA activator protein-2, AP2 (96). Additionally, PKA directly phosphorylates ErbB2 on Thr-686, thereby gating the response of ErbB2-ErbB3 to neuregulin (74).

Additional critical issues include whether the activity of the chimeric kinase in the cell is altered (as a consequence of its structure, lifetime, interactions, or localization), whether the chimera is sufficient for transformation, and which of the changes in gene expression are driving the transformation. Although the activity of the solubilized DNABJ1-PRKACA chimera is indistinguishable from that of the native PRKACA (21), the absence of the normal amino terminus of PRKACA will result in the loss of myristoylation, which can affect activity (97). The replacement with the amino terminus of DNABJ1 may further alter localization and/or the proteins with which the kinase interacts. The issue of whether the chimera is sufficient for transformation may be addressed by expression in cell lines or mice. Even if some of the proteins that are increased in expression are not driving the tumor, they may still be useful as diagnostic or prognostic markers for FLHCC. Up-regulated oncogenes, along with the chimera DNABJ1-PRKACA, could be targets for therapy. The ability to simultaneously target both the presumed primary genetic driver for FLHCC, the chimera of DNABJ1-PRKACA, as well as critical downstream components could reduce the chances of developing drug resistance (98). A number of genes whose expression is increased in FLHCC, such as ErbB2 and AURKA, are known oncogenes for which therapeutics are actively under development. Alone or in combination with a potential inhibitor of the chimera protein kinase A, it remains to be seen which will be most efficacious against FLHCC.

## Methods

With institutional review board (IRB) approval (Rockefeller IRB SSI-0797 and Memorial Sloan-Kettering IRB protocol 13-010), 27 snap-frozen tissue samples were collected from nine patients for which patients and the family members had consented. A pathologist with specialization in liver tumors (U.K.B.) verified that all tumor samples were pure FLHCC, and microdissection was performed to exclude inflammatory cells. All tumor samples were free from necrosis and contained  $>80\%$  tumor cells. All samples that were from subsequent recurrences were marked with an R. Normal samples were confirmed to be free from cirrhosis, and stromal bands were removed from all samples to optimize hepatocyte RNA extraction.

**RNA-Seq and Read Mapping.** Total RNA was extracted from tissue using miRNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA-seq libraries were prepared using TruSeq Stranded Total RNA Sample Prep Kit with Ribo-Zero ribosomal RNA depletion (Illumina). The manufacturer's protocols were used to sequence ribosomal RNA-depleted libraries at  $2 \times 50$ -bp paired-end reads on an Illumina HiSeq. 2500 in high-output mode, to an average depth of  $86 \times 10^6$  paired-end reads per sample (range  $43 \times 10^6$  to  $139 \times 10^6$ ).

For mapping, following data quality assessments, reads derived from residual rRNA were removed by aligning [Bowtie2 v2.1.0 (99)] against ribosomal RNA references derived from GENCODE/Ensembl v74 annotations. Non-rRNA reads were then mapped to hg19 reference genome supplemented with GENCODE/Ensembl v74 gene annotations using STAR aligner, v2.3.0e (100).

**Differential Gene Expression Analysis: FLHCC.** Analyses and figure generation were conducted within the R statistical framework version 3.1.1, with specific software packages noted below.

RNA-seq reads were counted against Ensembl v74 gene annotations using htseq-count (101). Differential gene expression analysis was performed with



DESeq2 version 1.5.25 (102). Read counts for protein-coding genes were analyzed using standard DESeq2 expression filters and differential expression tests, incorporating variable factors for condition (tumor or normal) and patient. Thresholds for statistical significance are noted in *Results*.

**Gene Set Variation Analysis.** Gene set collections denoting Canonical Pathways (C2.CP canonical pathways) and Transcription Factor Targets (C3.TFT transcription factor targets) were downloaded from the Molecular Signatures Database version 4.0 ([www.broadinstitute.org/gsea/msigdb/collections.jsp](http://www.broadinstitute.org/gsea/msigdb/collections.jsp)). Gene set enrichment scores were calculated using the GSVA package version 1.8.0 (34) with RNA-seq parameters. Differential gene set enrichment was determined using the limma package (103). Thresholds for statistical significance are noted in *Results*. For gene-level heat-map figures, fold changes were calculated by taking gene level counts (with an added pseudocount of 0.5), normalizing to counts per million library reads, and taking the  $\log_2$  fold change of tumor vs. normal for each patient tumor-normal pair.

**Differential Gene Expression Analysis: FLHCC vs. HCC.** Raw RNA-seq reads (FASTQ files) for 50 HCC tumor-normal pairs were acquired from TCGA. Reads were mapped and counted with the same workflow described for FL-HCC samples above. Differential expression analysis was performed with voom-limma (103, 104), with an experimental design comparing the differences in FLHCC (tumor vs. normal) to differences in HCC (tumor vs. normal). Thresholds for statistical significance are noted in *Results*. For distance clustering of FLHCC and HCC, 10 FLHCC and 42 HCC (eight sample pairs were removed as read depth outliers) tumor-normal pairs, fold-change values for protein-

coding genes were calculated as described above, and hierarchical clustering (complete method) was performed on Spearman-rank correlations.

Real-time PCR was performed in triplicate on an Applied Biosystems QuantStudio 12K Flex instrument console using QuantiTect SYBR green-based PCR master mix (Qiagen) according to the manufacturer's instructions with the modification of total reaction volume being 25  $\mu$ L. Cycling conditions were initial activation step 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. QuantiTect primers were purchased from Qiagen, and primers for PRKACA and DNAJB1 were designed with MacVector's Primer Design (Primer3) (version 13.5.5) and purchased from Integrated DNA Technologies (*SI Appendix, Table S15*). Levels of RNA expression were determined using the QuantStudio 12K Flex Software version 1.2.2 (Applied Biosystems) and Microsoft Excel. Data were analyzed by the  $\Delta\Delta C_T$  method after normalization to human beta actin. Paired two-tailed *t* tests were used to identify statistical significance with  $P < 0.05$  as threshold for significant values.

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