The Novel Human DNA Helicase hFBH1 Is an F-box Protein*

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We have identified a novel DNA helicase in humans that belongs to members of the superfamily I helicase and found that it contains a well conserved F-box motif at its N terminus. We have named the enzyme hFBH1 (human F-box DNA helicase 1). Recombinant hFBH1, containing glutathione S-transferase at the N terminus, was expressed in Sf9 cells and purified. In this report, we show that hFBH1 exhibited DNA-dependent ATPase and DNA unwinding activities that displace duplex DNA in the 3'- to 5'-direction. The hFBH1 enzyme interacted with human SKP1 and formed an SCF (SKP1/Cullin/F-box) complex together with human Cullin and ROC1. In addition, the SCF complex containing hFBH1 as an F-box protein displayed ubiquitin ligase activity. We demonstrate that hFBH1 is the first F-box protein that possesses intrinsic enzyme activity. The potential role of the F-box motif and the helicase activity of the enzyme are discussed with regard to regulation of DNA metabolism.

DNA helicases are ubiquitous enzymes that play essential roles in various DNA transactions involved in replication, repair, and recombination (1, 2) and have been implicated in a number of human genetic disorders (3, 4). A trademark of these enzymes is a set of well conserved amino acid sequences termed "helicase motifs" (5, 6). Helicases are generally believed to generate single-stranded DNA by catalyzing the melting of stable helical DNA structures utilizing energy derived from the hydrolysis of nucleoside triphosphates. Analysis of the genome of Saccharomyces cerevisiae indicates that there are 134 open reading frames containing with helicase-like features that represent ~2% of its genome (7). The presence of such a large number of helicases or helicase-like proteins in cells, many of unknown function in vivo, most likely reflects a complexity of nucleic acid metabolic reactions and the distinct structural template requirements for a given helicase. Alternatively, many helicases may play roles that functionally overlap, making it difficult to determine a specific role for a given helicase. For example, each mutant cell of the two yeast helicases, Sgs1 and Srs2, grows with wild-type kinetics, but the combination of an sgs1 mutation with loss of the helicase srs2 resulted in a severe growth defect (8, 9). A majority of sgs1 and srs2 mutant cells stops dividing stochastically as large budded DNA (9). Such double mutant cells are unable to replicate DNA at restrictive temperature and unable to efficiently transcribe rDNA (8). This suggests that these two helicases provide a redundant but essential activity for DNA replication and rDNA transcription. Despite well conserved motifs specific to helicase family proteins, there are several proteins for which no helicase activity has been demonstrated, indicating that helicase motifs cannot, without additional information, be used to define a protein as a helicase. For example, the chromatin-remodeling factor SWI2/SNF2 containing these motifs lacks helicase activity, although its ability to hydrolyze ATP is stimulated by DNA (10, 11), suggesting that ATP hydrolysis provides the energy required to alter protein-DNA structure rather than duplex DNA or RNA structure. This suggests that proteins with helicase motifs may have functions that do not involve unwinding of nucleic acids.

As a continued effort to understand the role of Schizosaccharomyces pombe DNA helicase I that we reported previously (12), we identified a human homolog of this fission yeast enzyme and named it hFBH1 (human F-box DNA helicase 1) because it contains a well conserved functional F-box motif (13). In this report, we present data that hFBH1 is not only an ATPase/DNA helicase but also is an F-box protein that can form an SCF complex with human SKP1 and Cullin 1. SCF complexes constitute a new class of E3 ligase that plays important roles in cell cycle regulation and signal transduction by catalyzing ubiquitin-mediated proteolysis (14–18). The substrate specificity of an SCF complex is governed by the interchangeable F-box protein subunit, which recruits a specific set of substrates for ubiquitination to the SCF core complex composed of SKP1, Cdc53, ROC1, and the E2 enzyme Cdc34. The polyubiquitinated proteins are rapidly captured by the 26 S proteasome, an abundant, self-compartmentalized protease particle (19). To date, hFBH1 is the first example of an F-box protein that contains intrinsic enzymatic activity, suggesting that a helicase can play a role in a certain aspect of DNA metabolism that requires ubiquitin-dependent proteolysis. The potential biological role of this interesting human DNA helicase will be discussed.

MATERIALS AND METHODS

Enzymes, Antibodies, and Nucleotides—The following proteins were obtained commercially: restriction endonucleases, the Klenow fragment

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of Escherichia coli DNA polymerase I, and polynucleotide kinase from KOSCO Inc. (Taejoen). The antibodies were obtained commercially: mouse monoclonal anti-FLAG antibodies from Sigma and mouse monoclonal anti-GST-horseradish peroxidase and rabbit polyclonal anti-human SKP1 from Santa Cruz Biotechnology. All of the secondary antibodies were from Amersham Biosciences. The DNA-dependent ATPase activity were pooled and dialyzed against buffer T100. The dialyze (0.16 mg/ml, 7 ml) was loaded onto a Hitrap heparin column (1 ml; Amersham Biosciences). The column was eluted with a linear gradient (20-500 mU) of NaCl in buffer T (10 ml). The mRNA levels in B. subtilis or human cells were quantified via the use of the quantitative real-time PCR method. The background level detected in the absence of added DNA helicase was less than 2% of the input substrate, and this value was subtracted from the amount of displaced products formed in the presence of the DNA helicase.

Constructions of Yeast Two-hybrid Vectors and Yeast Two-hybrid Analyses—To examine in vivo interaction of hFBH1 with SKP1 in yeast

**Expression and Purification of Recombinant hFBH1**—To express hFBH1 as GST fusion protein in insect cells, the full-length cDNA of hFBH1 was cloned into pFastBac1 (Invitrogen) as follows. A PCR-amplified GST gene from pGEX vector (Amersham Biosciences) was first cloned into the BsuII and EcoRI sites of pFastBac. An N-terminal portion of hFBH1 was amplified using a pair of primers (5'-GAA TCT AGG GAC GTA CTA GGC GAA GAG CAG TCG-3' and 5'-GGG TTC A1TG GCC AAA AGC AAT TCT G 19-3'), and the remaining C-terminal portion was amplified using a pair of primers (5'-CGC ATG ACC TCT GCT TCA ATG CTG GGA GAA GAG CAG TCG-3'), specific to the hFBH1 cDNA, were carried out using Glutathione-Sepharose 4B beads. A mixture (200 μl) containing beads (10 μl) and purified GST-hFBH1 (400 ng) was adjusted to 300 μl NaCl and incubated for 4 h at 4 °C with occasional rocking. The mixture was spun down, and the supernatant was examined for the presence of ATPase and helicase activities. M2 anti-FLAG beads (Sigma) were used as a negative control. Coimmunoprecipitation was carried out using cell extracts obtained 48 h after transfection. The cells were incubated in 300 μl of immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mg phenylmethylsulfonyl fluoride) for 30 min at 4 °C. The cell extracts were cleared by centrifuging at 15,000 × g for 15 min and incubated for 4 h at 4 °C with 20 μl of appropriate resin as indicated. After quick spin, the sedimented resin was washed twice with 1 ml of immunoprecipitation buffer and 1 ml of phosphate-buffered saline once. The proteins were released from the resin by adding 1× SDS-PAGE sample buffer and subjected to SDS-PAGE. Western blot analyses were performed with antibodies as indicated in each experiment.

**Preparation of Helicase Substrates**—The DNA substrate used for standard helicase assays (see Fig. 2C) were exactly as described previously (12). In brief, the substrate consists of 5′X174 single-stranded cDNA with a 20-nucleotide oligonucleotide (5′-GGC TGC GTA CCC GCC GA3′) annealed to it. To prepare DNA substrates to measure direction of hFBH1 translocation (see Fig. 3), a 22-mer oligonucleotide (5′-CGA ATT CAG CCG CCT TTA CCG GAC GCT CGA CAT TAA TGG TTT C3′; 10 pmol) containing a sequence complementary to 4X174 single-stranded cDNA at nucleotides 703–754 was annealed to 4X174 single-stranded cDNA (2 pmol). The annealed oligonucleotide was labeled at its 5′-end by incorporating [α-32P]dATP using T4 polynucleotide kinase. The partial duplex region of the 5′-end labeled DNA substrate was cleaved by HpaII to generate a linear DNA in which a labeled 23-nucleotide fragment present at the 5′-end of the template was labeled. The cleavage by HpaII of the 3′-end labeled DNA substrate resulted in generation of linear DNA with a labeled 29-nucleotide fragment at the 5′-end of the template. These two substrates were used to measure the translocation direction of the hFBH1 enzyme.

**ATPase and Helicase Assays**—DNA-dependent ATPase activity was measured in a reaction mixture (20 μl) containing 25 mM Tris-HCl, pH 7.8, 7.2 mM dithiothreitol, 2 mM MgCl2, 0.25 mM bovine serum albumin, 250 μg cold ATP, 20 μg [α-32P]ATP (5000 Ci/mmol), and 50 ng of M13 single-stranded DNA when necessary. After incubation at 37 °C for 15 min, an aliquot (1 μl) was spotted onto a polyethylenimine-cellulose plate (J. T. Baker) and developed in a 0.5 M LiCl/1.0 M formic acid. The products were analyzed using a PhosphorImager (Molecular Dynamics).

Helicase activity was measured in a reaction mixture (20 μl) containing 25 mM Tris-HCl (pH 7.8), 2 mM MgCl2, 2 mM dithiothreitol, 2 mM ATP, 0.25 mM bovine serum albumin, and the 3′-P-labeled partial duplex DNA substrate (15 fmol). The reactions were incubated at 37 °C for 10 min and stopped with 4 μl of 6× stop solution (60 mM EDTA, pH 8.0, 40% (w/v) sucrose, 0.6% SDS, 0.25% bromphenol blue, 0.25% xylene cyanol). The reaction products were subjected to electrophoresis for 1.5 h at 150 volts through 10% polyacrylamide gels containing 0.1% SDS in 0.5 × TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). The gel was dried on DEAE-cellulose paper and autoradiographed. Labeled DNA was quantified using a PhosphorImager.
two-hybrid analyses, we constructed four bait vectors as follows. The cDNAs encoding full-length (1-969 aa), N-terminal (1-345 aa), and C-terminal (285-969 aa) fragments of hFBH1 were inserted into pAS2-1 (CLONTECH) for GAL4 DNA binding fusion (hFBH1/pAS2-1, N-hFBH1/pAS2-1, and C-hFBH1/pAS2-1, respectively). ΔFhFBH1 lacking the F-box motif was prepared by an internal deletion of amino acids 141-185 and was fused to the GAL4 DNA-binding domain (ΔFhFBH1/pAS2-1).

To construct hFBH1/pAS2-1, an EcoRI-AviII fragment was isolated from GST-hFBH1/pFastBac1 described above, and a C-terminal fragment was amplified using a pair of primers (5'-GAG GAA GAG CAG 2890-3' and 5'-AGG CCT AGG GAG GCC 264-3') described (28). This and the C-terminal fragment isolated from hFBH1/pAS2-1 were ligated into pAS2-1 vector digested with EcoRI and SalI to construct hFBH1/pAS2-1. For construction of ΔFhFBH1/pAS2-1, we first amplified the N-terminal region using a pair of primers (5'-GAG TTA TGC ACA GGA AGC AAT TCT G3' and 5'-AGG CCT AGG GAG GCC 264-3'). The amplified C-terminal fragment was restricted with AviII and SalI. The two fragments were simultaneously ligated into pAS2-1 vector digested with EcoRI and SalI to construct hFBH1/pAS2-1. For construction of ΔFhFBH1/pAS2-1, we first amplified the N-terminal region using a pair of primers (5'-GAG TTA TGC ACA GGA AGC AAT TCT G3' and 5'-AGG CCT AGG GAG GCC 264-3').

The full-length cDNA of ROC1 into the PCRII vector was ligated into pEG for expression as GST fusion proteins. The full-length cDNAs encoding human SKP1 and CUL1 were amplified from a human cDNA library (CLONTECH) using pairs of primers (upstream and downstream primers). The amplified products were digested with SalI and EcoRI and ligated into the SalI and EcoRI sites of pcDNA3 (Invitrogen), followed by insertion of the full-length cDNA into the pcDNA3 vector. The ligated cDNA was transformed into E. coli strain DH5α, and the transformed colony was selected on LB plates containing ampicillin and 100 μg/ml X-Gal. The transformed plasmid was purified and sequenced. The transformation was performed as described (22).

**RESULTS**

**Cloning and Analysis of a cDNA Encoding a Human F-box DNA Helicase—**Using the TBLASTN homology search program, we identified a human EST cDNA clone (GenBank™ accession number AA349988) that possessed a significant homology to the *S. pombe* DNA helicase 1 (Ref. 12; GenBank™ accession number AA58077). Screening of human cDNA libraries using the EST clone as a probe resulted in the isolation of two partial clones lacking the 5' region of the cDNA. Using a 5' RACE-PCR method, we isolated the missing 5' one-third of the cDNA. The 5' cDNA amplified by reverse transcription-PCR, together with the two partial cDNAs, was used to reconstitute the full-length cDNA (GenBank™ accession number AF380349). The open reading frame of the cloned cDNA encoded a polypeptide of 969 amino acids with all seven conserved helicase motifs (Fig. 1A), and the protein had 28% identity and 44% similarity at the amino acid level to the *S. pombe* homolog and 95% identity to the mouse protein (data not shown). Interestingly, it was noted that all three homologs (humans, mice, and *S. pombe*) included a well conserved F-box motif (Fig. 1B) that is required to form a complex with SKP1, a key component of the SCF complexes involved in ubiquitin-dependent proteolysis (16). In addition, all three homologs contained the seven well conserved helicase motifs (Fig. 1C).

Therefore, we named the human enzyme hFBH1 (human F-box DNA helicase 1).

The hFBH1 Has Both DNA-dependent ATPase and DNA Unwinding Activities—To determine whether hFBH1 contained helicase activity, we expressed the enzyme as a GST-fused recombinant protein (GST-hFBH1) in insect cells as described under “Materials and Methods.” The expression of recombinant GST-hFBH1 was monitored by Western blot analyses using two antibodies: anti-GST antibody, which detects only a polypeptide with an intact N terminus, and anti-hFBH1 polyclonal antibody h-hFBH1, which was raised against the N-terminal 345-aa region of hFBH1. The full-length GST-hFBH1 protein was detected by both anti-GST and anti-hFBH1 antibodies in the crude extracts prepared from Sf9 insect cells infected with the recombinant baculovirus expressing hFBH1 (Fig. 2A, lanes 2, 5, and 8), whereas it was not detected in control extracts prepared from uninfected cells (Fig. 2A, lanes 1, 4, and 7).

Because the crude extracts prepared from the baculovirus-infected cells displayed elevated levels of single-stranded DNA-dependent ATPase activity compared with control extracts (data not shown), we purified GST-hFBH1 by monitoring ATP hydrolysis in the presence and absence of single-stranded DNA as a cofactor. The hFBH1 enzyme purified according to the procedure described under “Materials and Methods” was also assayed by SDS-PAGE and was detected by both antibodies (Fig. 2A, lanes 3, 6, and 9). To demonstrate that the ATPase and helicase activities detected are intrinsic to hFBH1, we examined whether they were specifically depleted by glutathione-coupled beads (Fig. 2, B and C). The addition of buffer alone (Fig. 2, B, lanes 2 and 6, and C, lane 3) or unrelated antibodies such as anti-FLAG monoclonal antibody (Fig. 2, B,
lanes 4 and 8, and C, lane 5) failed to deplete either ATPase (Fig. 2B) or DNA unwinding activity (Fig. 2C). In contrast, the addition of glutathione beads efficiently depleted the ATPase and helicase activities in the solution (Fig. 2, lanes 3 and 7, and C, lane 4), demonstrating that both activities are intrinsic to the purified hFBH1 enzyme. When we examined the unwinding direction of hFBH1, it displaced the 23-mer oligonucleotide annealed to the 5'-H11032-3'-end region of the template (Fig. 3, lanes 6 and 9), indicating that the enzyme translocated in the 3'-H11032 to 5'-H11032 direction. The enzyme was not dependent on fork structures for its unwinding activity (data not shown).

The hFBH1 Enzyme Interacts with SKP1, Forming a SCF Complex—Because the F-box protein, a component of a SCF complex, interacts with SKP1, we investigated whether the hFBH1 enzyme physically interacts with the human SKP1 protein. For this purpose, we constructed plasmids expressing bait proteins that contained different regions of hFBH1 fused to a GAL DNA-binding domain; these included the full-length protein, F-box-deleted protein, the N-terminal 1–345-aa fragment, and the C-terminal 285–969-aa fragment of hFBH1 (hFBH1, ΔFhFBH1, N-hFBH1, and C-hFBH1, respectively; Fig. 4A). The F-box motif is present in the full-length hFBH1 and N-hFBH1 constructs as shown Fig. 1 (A and B). The human SKP1 gene was fused to a GAL4 activation domain to prepare a prey plasmid as described under “Materials and Methods.” The yeast transformants that did not express hFBH1 (vector) or those that expressed either ΔFhFBH1 lacking the F-box motif or the C-terminal fragment of hFBH1 failed to activate transcription of the reporter gene in combination with the human Skp1 expression vector (Fig. 4A). Such transformants were unable to grow on selective media containing 10 mM 3-aminotriazole as shown in Fig. 4A. In contrast, transformants obtained with the full-length hFBH1 and the N-terminal fragment containing the F-box motif grew in the presence of 10 mM 3-aminotriazole (Fig. 4A). Consistent with this, we detected significant β-galactosidase activity in the extracts derived from the growing cells (Table I), suggesting that interaction between the hFBH1 and SKP1 proteins occurs in vivo.

To further verify that hFBH1 formed a complex in vivo with SKP1 in an F-box-dependent manner, we transfected transiently a plasmid expressing GST-tagged human SKP1 (GST-SKP1) together with a plasmid expressing either FLAG-tagged full-length (FLAG-hFBH1) or F-box-deleted hFBH1 (FLAG-ΔFhFBH1, lacking amino acids 141–185) proteins into the human 293T cell line and prepared extracts for coimmunoprecipitation experiments (Fig. 4, B and C). FLAG-hFBH1 and GST-hSKP1 were coimmunoprecipitated by anti-FLAG antibodies (Fig. 4B, lane 2). In the absence of FLAG-hFBH1, GST-hSKP1 was not precipitated (Fig. 4B, lane 1). Moreover, the coimmunoprecipitation did not occur when the F-box motif was deleted (Fig. 4B, lane 3). This was also the case when coimmunoprecipitation was performed using glutathione beads (Fig. 4C). These results demonstrate that hFBH1 interacts with...
hSKP1 in vivo and that the interaction between SKP1 and hFBH1 is dependent on the presence of the functional F-box motif.

Because we confirmed that hFBH1 interacts with SKP1 and that the interaction required a functional F-box motif, we examined whether the hFBH1-SKP1 complex contained Cullin (CUL1), another protein that interacts directly with SKP1 to form an SCF complex. For this purpose, we constructed an additional plasmid expressing GST-tagged human CUL1 (GST-CUL1). Shown are crude extracts from uninfected cells (lanes 1, 4, and 7; 20 µg each) and infected cells (lanes 2, 5, and 8; 20 µg each) with recombinant GST-hFBH1 baculovirus and purified GST-hFBH1 (lane 3; 200 ng; lanes 6 and 9, 60 ng). Molecular size markers are shown (in kDa). The position of purified GST-hFBH1 is indicated by an arrow. B and C, purified enzymes (200 µl, 0.4 µg) were incubated with either glutathione-coupled beads (GTT) or anti-FLAG antibody resin (α-FLAG) at 4 °C for 4 h to form an immunocomplex. Buffer indicates immunodepletion without any resin. After the resins were spun down, an aliquot of the supernant (3 µl) was examined for ATPase (B) or helicase (C) activities. Helicase and ATPase activities were measured in standard reaction mixtures described under “Materials and Methods.” The helicase substrate is the standard substrate described previously (12). The amount of ATP hydrolyzed or the substrate unwound is presented below the autoradiograms. ss, single-stranded.

![Image of Fig. 2](http://www.ncbi.nlm.nih.gov)
along with the plasmids expressing GST-hCUL1 and HA-ROC1, the complex immunoprecipitated by FLAG antibody resin failed to produce long chains of polyubiquitin (Fig. 6B, lane 4), similar to negative control (Fig. 6B, lane 1). In contrast, the immunoprecipitated complex with FLAG-hFBH1 displayed ubiquitin ligase activity (Fig. 6B, lane 3). Formation of ubiquitin polymers by immunoprecipitated complexes containing GST-hCUL1 and HA-ROC1 is more efficient than that by those containing hFBH1 (Fig. 6B, compare lanes 2 and 3). This reflects the fact that glutathione beads against GST-hCUL1 precipitated a large number of SCF complexes in the extracts, whereas FLAG antibody precipitated only a subset of the SCF complexes that contains hFBH1 only. When E1 or E2 was removed from the reaction mixture, no polyubiquitin chain was detected (Fig. 6B, lanes 5 and 6, respectively), indicating that the formation of polyubiquitin chain by SCF<sub>hFBH1</sub> is dependent on E1 and hCdc34. This result confirms that SCF<sub>hFBH1</sub> contains ubiquitin ligase activity and interacts with the ubiquitination machinery.

**DISCUSSION**

In this report, we demonstrate that hFBH1 is a DNA helicase that translocates in the 3’ to 5’ direction and interacts with SKP1 in a manner that requires a functional F-box motif. Besides, hFBH1 interacted with CUL1/ROC1 and formed a SCF complex that contained all known four subunits (SKP1, hFBH1, CUL1, and ROC1). Moreover, SCF<sub>hFBH1</sub> displayed ubiquitin ligase activity in a F-box motif-dependent fashion. The polyubiquitination activity of the SCF<sub>hFBH1</sub> complex was dependent upon functional E1 and E2 enzymes, demonstrating that the complex interacts with the ubiquitination machinery. Our study showed that hFBH1 is the first example of an F-box protein that harbors an intrinsic enzymatic activity, catalyzing a helicase reaction. In addition, hFBH1 is the first F-box protein implicated in nucleic acid metabolism. Therefore, our findings raise the possibility that the role of hFBH1 is most likely
to recruit a target protein, most likely involved in some aspect of DNA metabolism, to the protein degradation pathway. Thus, a protein involved in DNA metabolism would be marked by hFBH1 for degradation via the highly regulated ubiquitin degradation pathway.

As an initial attempt to evaluate the role of the F-box motif of the enzyme in this regard, we decided to identify a protein(s) that interacts specifically with hFBH1 using the yeast two-hybrid screen. This screen resulted in the isolation of human MAT1 (data not shown), which is a regulatory subunit of the Cdk-activating kinase (29, 30). It is unlikely that MAT1 is a direct substrate of SCF<sub>hFBH1</sub> E3 ligase activity because a substrate phosphorylation is a prerequisite step prior to recognition by an F-box protein (31, 32). MAT1 in Cdk-activating kinase may play a role leading to the phosphorylation of hFBH1 instead of acting as a substrate for the E3 ligase activity of the SCF<sub>hFBH1</sub> complex. Therefore, the level of hFBH1 itself is likely to be regulated by posttranslational modifications that involve both Cdk-activating kinase and E3 ligase activities. Because it has been reported that F-box proteins can be degraded autocatalytically in a ubiquitination-dependent fashion (33), the F-box motif of hFBH1 may regulate the level of hFBH1 through auto-ubiquitination during cell cycle progression. However, we were not able to demonstrate this, because the endogenous levels of hFBH1 were too low to be detected with the antibodies that we have (data not shown). We also explored whether hFBH1 is ubiquitinated in vivo. Although the hFBH1 was indeed ubiquitinated in vivo (data not shown), the efficiency of hFBH1 ubiquitination was independent of the intact F-box motif, suggesting that ubiquitination occurred in trans by some other ubiquitinating activity. Reconstitution of the SCF complex containing hFBH1 with the purified proteins will help to address this question.

Although the biological function in vivo of hFBH1 is unclear at present, mutational studies of the <i>S. pombe</i> fdh1<sup>+</sup> gene (encoding the <i>S. pombe</i> homolog of hFBH1) have provided some clues. When the <i>S. pombe</i> fdh1<sup>+</sup> gene was deleted, the cells showed elongated cell morphology and uneven distribution of chromosomes in dividing cells. In addition, deletion of either the F-box motif or a single amino acid change in the ATP-binding motif resulted in phenotypes similar to those of the null mutation. These observations suggest that both the F-box and ATPase/helicase activity of hFBH1 are required for the physiological function of the enzyme. Currently, both genetic and biochemical studies are underway using an <i>S. pombe</i> model system to delineate the precise biological role of hFBH1.

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