Research Article

VHL Frameshift Mutation as Target of Nonsense-Mediated mRNA Decay in Drosophila melanogaster and Human HEK293 Cell Line

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There are many well-studied examples of human phenotypes resulting from nonsense or frameshift mutations that are modulated by Nonsense-Mediated mRNA Decay (NMD), a process that typically degrades transcripts containing premature termination codons (PTCs) in order to prevent translation of unnecessary or aberrant transcripts. Different types of germline mutations in the VHL gene cause the von Hippel-Lindau disease, a dominantly inherited familial cancer syndrome with a marked phenotypic variability and age-dependent penetrance. By generating the Drosophila UAS:Upf1D45B line we showed the possible involvement of NMD mechanism in the modulation of the c.172delG frameshift mutation located in the exon 1 of Vhl gene. Further, by Quantitative Real-time PCR (QPCR) we demonstrated that the corresponding c.163delG human mutation is targeted by NMD in human HEK 293 cells. The UAS:Upf1D45B line represents a useful system to identify novel substrates of NMD pathway in Drosophila melanogaster. Finally, we suggest the possible role of NMD on the regulation of VHL mutations.

1. Background

Germline mutations in the VHL gene cause the von Hippel-Lindau disease (VHL; MIM# 193300), a dominantly inherited familial cancer syndrome with retinal and central nervous system hemangioblastomas, renal cell carcinoma, pheochromocytoma, pancreatic endocrine tumors, and endolymphatic sac tumors [1–4]. The VHL mutation pattern includes missense, nonsense, frameshift, and splice site mutations. Genotype-phenotype correlation studies showed that the incidence of renal involvement in VHL disease was increased in families with nonsense or frameshift mutations that disrupted the structural integrity of VHL protein, whereas missense mutations associated with a higher risk of pheochromocytoma [5–7].

We hypothesize that the genic localization of VHL variations and nonsense mutations of VHL activating NMD pathway may play an important role in the determination of a specific phenotype.

NMD is an evolutionarily conserved mRNA surveillance pathway that protects cells from potentially harmful effects of truncated proteins that would otherwise be translated from mRNAs bearing PTC. The process serves as a general surveillance mechanism to abolish aberrant transcripts resulting not only from rare mutations but also from mistakes in RNA processing [8], regulating the expression of about 3%–10% of the transcriptome in S. cerevisiae, D. melanogaster, and human cells. These natural NMD targets play a role in different biological processes such as transcription, cell proliferation, cell cycle, telomere maintenance, cellular transport and organization, and metabolism [9]. The high evolutionary conserved Upf proteins, Upf1, Upf2, and Upf3, constitute the core of NMD machinery [10–14]. The key molecular component is Upf1, an RNA helicase that recognizes aberrant translation termination events [15].

Although conserved in all eukaryotes that have been analysed so far, NMD employs different molecular mechanisms, depending on the species, to discriminate between natural
and premature stop codons and to degrade the targeted mRNAs. In mammalian cells, termination codons that lie upstream of an exon-exon boundary are generally recognized as premature and target the mRNA for degradation by NMD.

The Drosophila melanogaster intron-less Vhl gene maps at polytene chromosomal position 47E5-6 (http://flybase.org/). The human and fly proteins show a high degree of amino acid similarity spread throughout the entire length of VHL with 67% and 76% of similarity in the functional domains of PKC± and elongin C binding domains, respectively.

Here, by establishing a Drosophila NMD mutant, we showed the involvement of NMD mechanism in the modulation of a novel human VHL frameshift mutation and we confirmed this data in the HEK 293 human cell line using a molecular strategy based on the minigene constructs.

2. Methods

2.1. Fly Strains and Culture Conditions. Flies were cultured at 25°C on standard cornmeal-sucrose-yeast-agar medium containing propionic acid as mold inhibitors. Detailed description of mutations and chromosome rearrangements used in the present study could be found at FlyBase: http://flybase.bio.indiana.edu. The stocks used in the present work were supplied by Bloomington Stock Center.

2.2. Northern Blotting Assays. Total RNAs from testes and ovaries of adult flies were isolated using RNaseasy Kit (Qiagen) and poly(A)+ RNAs were prepared with oligo(dT)-coupled beads (Oligotex, Qiagen). RNAs were separated in denaturing formaldehyde agarose gel (5–20 µg/lane) and blotted onto positively charged nylon membranes (Amersham) in denaturing formaldehyde agarose gel (5–20 µg/lane). Autoradiography was carried out for both 16 and 80°C using intensifying screens. Filters were stripped and hybridization was repeated with a Rp49 32P-labeled probes generated by random priming using standard methods. Hybridization was carried out overnight at 65°C in hybridization solution (formamide 50%, SSC 5x, Denhardt’s 5x, SDS 0.5x, EDTA pH 8.0 10 mM, Salmon Sperm DNA 100 g/mL). After hybridization, the membranes were washed four times at 65°C. Autoradiography was carried out for both 16 and 48 hours at −80°C using intensifying screens. Filters were stripped and hybridization was repeated with a Rp49 specic probe.

2.3. Genetics Mutants. The Adh alleles were previously characterized by Brogna [16]. The Adh11 allele contains a premature stop codon located 258 bp upstream from the boundary between exon 3 and exon 4 while Adh116 is a small deletion that eliminates the 5′ splicing signal of intron 2 leading to an in-frame premature stop codon (Figure 1(a)).

2.4. Isolation of Full Length Upf1 cDNA. Drosophila genomic DNA was isolated from adults using Genomic DNA Extraction Kit (Qiagen). As probe for subsequent screens, a fragment of 700 bp was PCR amplified from genomic DNA as template (Upf1F and Upf1R primers in Table 1). The identity of cloned PCR product was confirmed by sequencing. For the isolation of the Upf1 cDNA full-length, a Drosophila melanogaster ovarian cDNA library was screened using that Upf1-probe. Hybridization of the Hybiont-N+ filters (Amersham) was carried out at 65°C according to the manufacturer’s instructions. The positive Upf1 cDNA clone was cloned into the pGEM-T-Easy vector (Promega) and verified by DNA sequencing.

2.5. UAS:Upf1ΔD45B Negative Dominant and UAS:Vhl1 and UAS:Vhl2 Nonsense Mutant Lines. The entire coding sequence of the Vhl gene was amplified by PCR with Pfu Polymerase (Promega) using cDNA obtained by reverse transcription of total RNA extracted from adult flies. The PCR fragment was inserted into the pcDNA3 vector and verified by direct sequencing. The Upf1 and Vhl mutations were introduced in the cloned cDNAs by site-directed mutagenesis with the QuickChange II kit (Stratagene) using the following oligonucleotides: Upf1Fmut and Upf1Rmut for Upf1, VhlΔ21F and VhlΔ21R for Vhl1, and VhlΔ21F and VhlΔ21R for Vhl2 mutations (Table 1), respectively. Subsequently, mutated Upf1 and Vhl cDNAs were sequenced and inserted into pUAST vector by site directed cloning.

P element transformation was performed by microinjection of pUAST:Upf1 or pUAST:Vhl together with a Δ2-3 transposable containing plasmid into a w1118 Drosophila melanogaster strain [17]. Multiple lines were obtained for each injected construct. The expression of different upstream activating sequences (UASs) constructs was tested using a pGAL4 line that drives ubiquitous expression.

The Upf1 dominant negative activity of the transgenic lines selected was tested by crossing virgin females w1118;β2::GAL4 with w1118; AdhΔ24/Cy; UAS:Upf1ΔD45B/TM3 and w1118; AdhΔ24/Cy; UAS:Upf1ΔD45B/TM3, respectively. The β2::GAL4 line drives the transgene expression in the testis. The levels of Adh and Upf1 mRNA from testes of w1118; AdhΔ24/β2;GAL4; UAS:Upf1ΔD45B/+ and w1118; AdhΔ24/β2;GAL4; UAS:Upf1ΔD45B/+ flies and the levels of Vhl from testes of w1118; UAS:VhlΔ21/β2;GAL4; UAS:Upf1ΔD45B/+ and w1118; UAS:VhlΔ21/β2;GAL4; UAS:Upf1ΔD45B/+ flies were analysed by QPCR.

2.6. Reverse Transcription PCR (RT-PCR) and Quantitative Real-Time PCR (QPCR). Total RNA from testis of 50 adult flies was obtained using RNeasy Mini Kit and reverse transcribed using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. RT-PCR was performed using pVHLF and pVHLR and Adh_RT_F and Adh_RT_R primers for Vhl and Adh (Table 1). QPCR was carried out in triplicates using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and run on ABI 7900HT Sequence Detection System with defaults parameters. The geometric mean of two reference genes (Rp49 and Act4A) was used to normalize the relative quantities. The calculations were made using the Comparative CT method as reported (User Bulletin #2, Applied Biosystems).

2.7. Minigenes Construct. Construct 1 (Figure 3(a)). We ligated two PCR fragments from DNA of healthy individual carrying wild type VHL alleles. The PCR fragments
Figure 1: Schematic map of the Adh gene. (a) Rectangles represent exons; black box represents promoter region; hatched boxes indicate coding regions; punctated boxes represent untranslated regions. The horizontal lines represent introns, IN1-IN3. The position of the mutations are indicated with vertical lines marked with a star; Adhfn6 is the mutation effecting splicing while Adhn4 is a nonsense mutation.

(b) and (c) Compared levels of Adh and Upf1 transcripts in flies that were heterozygous for Adh alleles and for Upf1D45B. A 3.8- and 8.8-fold accumulation of nonsense-containing Adh mRNA was observed in w; Adhn4/β2::GAL4; UAS:Upf1D45B/+ and w1118; Adhfn6/β2::GAL4; UAS:Upf1D45B/+ mutant flies with respect to the endogenous levels of Adh and Upf1 control mRNAs. The experiment was repeated three times.

2.8. Transfection, RNA Extraction, and QPCR on HEK 293 Cell Lines. HEK 293 cells were grown at 37°C in DMEM supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (Invitrogen). They were seeded at 1 × 10^6 cells per 100 mm diameter petri dish 24 hours before transfection, performed by Fugene HD (Roche) with either 1 μg of VHL wt or mutant constructs. A GFP plasmid was used as a reference for transfection efficiency in each cell line. The mRNA levels of the different constructs were normalized to the mRNA level of GFP. Then, the ratio between the normalized mRNA levels transcribed from the mutant and the wt constructs following CHX treatment was calculated and compared with this ratio in untreated cells. The experiments were repeated at least three times.

3. Results

3.1. RT-PCR Analysis Reveals that Upf1, Adh, and Vhl Are Expressed in Ovaries and Testes of Adult Flies. To test whether Drosophila testis expresses detectable levels of the endogenous Upf1, Adh, and Vhl genes, we performed Northern blot and RT-PCR analysis on poly(A)^+ and total RNA extract from ovaries and testes of adult flies. As shown in Figure 4 these analyses revealed that Upf1, Adh, and Vhl are normally expressed in ovaries and testes of adult flies.

3.2. Upf1 Dominant-Negative Mutant Abolish Degradation of Nonsense Transcripts in Drosophila melanogaster. The yeast R779C and mammals R844C Upf1 mutations convert the conserved arginine to cysteine at residue 799 and 844 respectively, within the RNA helicase domain, conferring a
| Name       | Sequence 5′-3′                        | Utilization       |
|------------|--------------------------------------|-------------------|
| Upf1F      | TTGGAATCATCACGCCTTACGA               | Upf1 probe        |
| Upf1R      | CATGCCAACCGGAACCTGGCATG             | Upf1 probe        |
| Upf1Fmut   | ATGTCTTGCGTGTGTTTCTAAGAACGT         | Mutagenesis       |
| Upf1Rmut   | AGATGCACTATCGTCAGAGATGTTTCTA        | Mutagenesis       |
| VhlnF      | CCCTAAAGCCCTTACAGGGTGGTTGCAAC       | VhlnF mutagenesis |
| VhlnR      | GGGAGTTGGGAAAGTCTCCTACCGCCACCTTG    | VhlnR mutagenesis |
| VhlF       | AGATGCACTATCGTCAGAGATGTTTCTA        | VhlF mutagenesis  |
| VhlR       | TGAAGATCTCTCGACGTACGTTGCCATCC       | VhlR mutagenesis  |
| Vhl F1     | CGCGCGGATTATCCGCCCCGAGGGGGAGGAACCT  | Construct1        |
| Vhl R1     | CGCGGCTCTCGAGATGGTGAAACCCCGTCTCTACT | Construct1        |
| c.163delGF | GGGGCGCGGGGCGGCTACTCCTCGGCGCGCGCTAGT| Construct1        |
| c.163delGR | GGGGCGCGGGGCGGCTACTCCTCGGCGCGCGCTAGT| Construct1        |
| Vhl F2     | CGCGGCTCTCGAGATGGTGAAACCCCGTCTCTACT | Construct2        |
| Vhl R2     | CGCGCGGATTATCCGCCCCGAGGGGGAGGAACCT  | Construct2        |
| c.172delGF | CAGGTCAATATTCTTCGCAATCGATGGCCTCGGTCTGCT | Construct2 mutagenesis |
| c.172delGR | AGCAAGACGCGGAGATGCAGTTGCAGGATGAGATGCTGCT | Construct2 mutagenesis |
| pVHLF      | GGCCGCCGCGCATCCA                     | VHL QPCR          |
| pVHL R     | CATCGTGTGTGCGCATCTCT                 | VHL QPCR          |
| pGFP F     | GCAAATCAAGACCAGGC                   | GFP QPCR          |
| pGFP R     | GTGGCCCATGATATAGACG                  | GFP QPCR          |
| rp49 F     | CACACGGGAAACTCAATGGAT                | rp49 QPCR         |
| rp49 R     | GTTCATCTTGAACTCGGAAGG               | rp49 QPCR         |
| Act4A F    | GCTTCGCTGTCTACCTTCATCCA             | Act4A QPCR        |
| Act4A R    | CGCCCGCATCTACTGCTTTAGA              | Act4A QPCR        |
| Adh RT F   | GGGGTTCCGCGGCTGTA                   | RT-PCR, Adh QPCR  |
| Adh RT R   | CTGGTAGATGGCATTGAGATCC              | RT-PCR, Adh QPCR  |

dominant-negative effect on yeast and human Upf1p activity in nonsense-mediated mRNA decay pathway [18]. Because of the high identity of Drosophila UPF1 protein with the human and yeast Upf1 protein (67% and 53%, resp.) we introduced the same mutation into the fly Upf1 cDNA and we tested whether this mutation in vivo exerts a dominant negative effect on the regulation of alcohol dehydrogenase gene, Adh, a specific substrate of NMD, by utilizing the heterologous GAL4-UAS binary expression system [19].

By screening of a Drosophila melanogaster adult ovarian cDNA library with a Upf1 probe we isolated one positive clone that consisted of an Upf1 full-length containing an open reading frame of 3530 bp with a 5′-untranslated region of 372 bp and a 3′-untranslated region of 802 bp, respectively.

Using directed mutagenesis we generated Upf1 cDNA that carries the R822C substitution that mimics the yeast R779C and mammals R844C Upf1 mutations. This mutated Upf1 cDNA was cloned into P element expression vectors under the control of yeast GAL4-UAS. P element-mediated germ line transformation was used to generate an UAS:Upf1 transgenic fly line that we called UAS:Upf1D45B. We observed that the ubiquitous expression of the dominant-negative UPF1D45B protein, driven by actin-GAL4 driver line (P{Act5C – GAL4}17bFO1), caused 100% larval lethality (0/1445). Consistently, the observed phenotype and the efficacy of UAS:Upf1D45B transgene were confirmed by isolation and characterization of a loss-of-function mutation in the Drosophila Upf1 gene that causes lethality during larval development [14].

To verify whether Upf1D45B is able to modulate NMD pathway, we used QPCR to test its effect on mRNA levels of Adh<sup>ns4</sup> and Adh<sup>ns6</sup>, two nonsense mutations of the alcohol dehydrogenase gene (Adh) (Figure 1(a)), known to be targeted by NMD in S2 cells and in vivo [14, 20]. First, we measured the levels of Adh mRNA in both Adh mutant strains and we detected, in agreement with previous data
heterozygous for nonsense alleles and for Upf1D45B. No increase of Vhl transcript in transgenic UAS:VhlD45B line was observed under the inhibition of NMD pathway whereas the UAS:VhlD45B transgenic line showed a 1.8 accumulation of nonsense-containing Vhl mRNA. The experiment was repeated three times.

Together these results strongly point out that Upf1D45B line represents a useful system to conduct a functional study to identify possible substrates of NMD pathway in Drosophila melanogaster.

### 3.3. Vhl Gene Is a Target of NMD in Drosophila melanogaster.

The UAS:Upf1D45B line was used to investigate whether Vhl alleles carrying PTCs are degraded by NMD pathway. We constructed two different Vhl nonsense mutants by using site-directed mutagenesis strategy. The VhlD45B allele contains the c.172delG frameshift mutation located in the exon 1 of Vhl gene. This mutation corresponds to the human c.163delG pathogenic mutation that we recently identified in a sporadic case of human cerebellar hemangioblastomas (Muscarella, submitted). The VhlD45B allele contains the c.254delC mutation corresponding to the c.239delG frameshift mutation located in the exon 1 of Vhl gene (Figure 2(a)) [21].

P element-mediated germ line transformation was used to generate two independent transgenic fly lines carrying Vhl mutations, UAS:VhlD45B and UAS:VhlD45B.

To verify whether the two different Vhl mutant transcripts were targeted by NMD pathway, w1118; UAS:VhlD45B/Cy; UAS:Upf1D45B/TM3 and w1118; UAS:VhlD45B/Cy; UAS:Upf1D45B/TM3 males were crossed to β2::GAL4 females and the levels of Adh mRNAs from testes of w1118; AdhD45B/β2::GAL4; UAS:Upf1D45B/+ flies were measured by using QPCR. Consistently with [11] we observed a 3.8- and 8.8-fold accumulation of Adh mRNA when compared to the control (Figures 1(b) and 1(c)), implying that the expression of Upf1D45B mutation could abolish NMD pathway function.
Figure 3: Effect of CHX treatment on the level of the c.163delG and c.239delG VHL mRNA. (a) Scheme of the WT (upper panel) and c.163delG constructs, which contained the exons 1-2 (marked in the boxes by numbers), a part of the intronic sequence between exons 1-2 and a piece of intron downstream the exon 2. The CMV promoter is marked by a thick horizontal arrow. (b) Scheme of the WT and c.239delG construct containing the entire coding sequence of the VHL gene. (c) and (d) QPCR analysis of VHL transcripts before and following CHX treatment. The level of mRNA transcribed from VHL construct carrying either the wild type sequence or the c.163delG and c.239delG mutations was normalized to the mRNA level of GFP. The ratio between these normalized levels following CHX treatment was calculated and compared with the ratio in untreated cells. The fold increase in the level of VHL c.163delG and c.239delG transcripts is shown as mean ± SEM.

3.4. VHL Mutations Are Targeted by NMD in Human Cell Lines. To investigate the stability of VHL wild type and certain human nonsense transcripts, we measured by QPCR the mRNA levels of constructs carrying wild type, c.163delG and c.239delG (Figures 3(a) and 3(b)) VHL mutations transfected into HEK 293 cells in absence and presence of cycloheximide (CHX), a widely used inhibitor of NMD. The analysis showed that the fold increase differs between the two mutants, with a modest increase in the level observed (1.4 ± 0.10) for c.239delG and a higher increase of 2.0 (2.4 ± 0.8) for c.163delG (Figures 3(c) and 3(d)).

4. Discussion
In humans, the role of NMD as a modifier of the phenotypic consequences of PTC is becoming more apparent. There are a consistent number of genetic diseases in which NMD partially mitigates the consequences of mutation owing to phenotypic variability.

The VHL is a well-known tumor suppressor gene, involved in cell cycle, regulation of hypoxia inducible genes and proper fibronectin assembly in extracellular matrix [22]. Germline mutations of the VHL gene lead to the development of the von Hippel-Lindau disease, a rare dominantly inherited familial cancer syndrome with a marked phenotypic variability and age-dependent penetrance.

The number of mutations in VHL gene is enormous and includes missense, nonsense, frameshift, and splice site mutations. In the past few years many different approaches using several molecular gene parameters have been used to make a possible correlation between VHL gene mutation and tumor phenotype [5–7, 23].
In the present study we generated Upf1 mutant fly line, UAS:Upf1D45B, to investigate whether two nonsense alleles of the Vhl gene are NMD targets. In agreement with others we observed that the ubiquitous expression of UPF1D45B protein, induced by a specific actin-GAL4 driver line, causes 100% larval lethality [14].

NMD pathway modulates the activity of specific native transcripts, whose misregulation would perturb the development or function of select cells or tissues and leading to lethality. Since Upf1 gene is broadly expressed and active throughout development, identification of the tissue target of Upf1 lethality will be an important first step to select the cellular substrates of NMD gene regulation.

Since the expression of UAS-transgene can be targeted to a specific tissue using the GAL4/UAS binary expression system, for our experiments we have chosen the β2::GAL4 line that drives the transgene expression in a region of testis in which the perturbation of NMD pathway is not essential for Drosophila viability and development.

The observed abolishing effect of degradation of Adh nonsense transcript, an NMD substrate, by Upf1 domi-
major changes in VHL mRNA expression (1.6- and 0.4-fold increase following emetine treatment, unpublished observations). The authors concluded that the NMD is not effective on the modulation of VHL because of the small length of VHL gene that consists of only three exons [6].

5. Conclusions

Our experiments confirm the utility of Drosophila melanogaster as an easy experimental system for understanding the NMD mechanism with a relevant potential applicability. Further molecular investigations on a greater number of Drosophila transgenic lines harbouring mutations that result in truncated proteins in different regions of Vhl gene or whatever also gene will be needed to get more indications on the correlations between mutation position, activation of NMD in Drosophila, and specification of a definite phenotype.

Finally, a larger number of mutations need to be tested to definitely establish whether the NMD is involved in the pathogenesis of von Hippel Lindau disease.

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