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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:*Upf1*^{D45B} 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:*Upf1*^{D45B} 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. melenogaster,* 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 PKCl 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 RNeasy 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). *Upf1* and *Rp49* ^{32}P-labeled probes were 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 Adh^{n4} allele contains a premature stop codon located 258 bp upstream from the boundary between exon 3 and exon 4 while Adh^{fn6} 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* ovaric cDNA library was screened using that *Upf1*-probe. Hybridization of the Hybond-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:Upf1^{D45B} Negative Dominant and UAS:Vhl^{n1} and UAS:Vhl^{n2} 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,* Vhl^{n1}F and Vhl^{n1}R for Vhl^{n1}, and Vhl^{n2}F and Vhl^{n2}R for Vhl^{n2} 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 transposase containing plasmid into a w^{1118} *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 w^{1118};*β2*::GAL4 with w^{1118}; Adh^{n4}*/Cy;* UAS*:*$Upf1^{D45B}$*/TM3* and w^{1118}; Adh^{fn6}*/Cy;* UAS*:*$Upf1^{D45B}$*/TM3*, respectively. The *β2*::GAL4 line drives the transgene expression in the testis. The levels of *Adh* and *Upf1* mRNA from testes of w^{1118}; Adh^{n4}*/β 2*::GAL4*;* UAS*:*$Upf1^{D45B}$/+ and w^{1118}; Adh^{fn6}*/β2*::GAL4*;* UAS*:*$Upf1^{D45B}$/+ flies and the levels of *Vhl* from testes of w^{1118}; UAS*:*Vhl^{n1}*/β2*::GAL4*;* UAS*:*$Upf1^{D45B}$/+ and w^{1118}; UAS*:*Vhl^{n2}*/β2*::GAL4*;* UAS*:*$Upf1^{D45B}$/+ 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 transcripted 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*, respectively (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

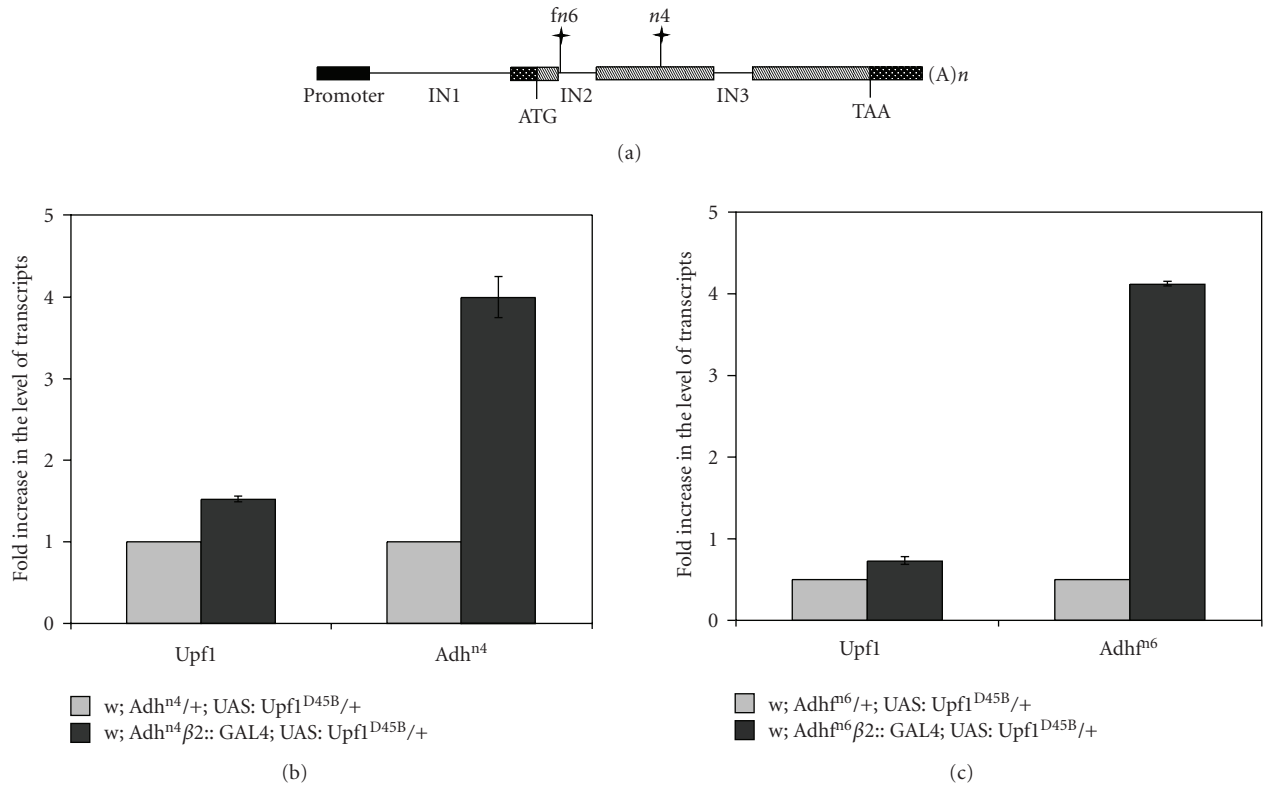

Figure 1: Schematic map of the *Adh* gene. (a) Rectangles represent exons; black box represents promoter region; hatched boxes indicate coding regions; punctated boxes represent untranslated regions. The horizontal lines represent introns, IN1-IN3. The position of the mutations are indicated with vertical lines marked with a star; Adh^{fn6} is the mutation affecting splicing while Adh^{n4} is a nonsense mutation. (b) and (c) Compared levels of *Adh* and *Upf1* transcripts in flies that were heterozygous for *Adh* alleles and for $Upf1^{D45B}$. A 3.8- and 8.8-fold accumulation of nonsense-containing *Adh* mRNA was observed in w^{1118}; $Adh^{n4}/\beta 2$::GAL4; UAS:$Upf1^{D45B}$/+ and w^{1118}; $Adh^{fn6}/\beta 2$::GAL4; UAS:$Upf1^{D45B}$/+ mutant flies with respect to the endogenous levels of *Adh* and *Upf1* control mRNAs. The experiment was repeated three times.

contained exon 1 and part of its downstream intronic sequence (700 bp) and exon 2 and part of its flanking introns (390 bp at intron 1 and 134 bp at intron 2). The PCR fragments were amplified using the primers listed in Table 1. The different constructs were cloned into the pcDNA3.1 vector (Invitrogen). The c.163delG mutation was introduced by site-directed mutagenesis using the QuickChange II kit (Stratagene) with the generation of a stop codon in exon1 (V66X).

Construct 2 (Figure 3(b)). The entire coding sequence of the *VHL* gene was amplified by PCR using as template the cDNA obtained by reverse transcription of RNA extracted from HEK293 cells. The c.239delG stop codon mutation was introduced by site-directed mutagenesis.

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

Table 1: Primer's list used in this study.

Name	Sequence 5′-3′	Utilization
Upf1F	TTGGAATCATCACGCCTTACGA	Upf1 probe
Upf1R	CATGCCAACCGGAACTGGCATG	Upf1 probe
Upf1Fmut	ATGTCTTGCGTGTGTTCTAACGAACGT	Mutagenesis
Upf1Rmut	ACGTTCGTTAGAACACACGCAAGACAT	Mutagenesis
Vhl^{n1}F	CCCTCAAGCCCTTCAGGAGGTGCGGGTGAAC	Vhl^{n1} mutagenesis
Vhl^{n1}R	GGGAGTTCGGGAAGTCCTCCACGCCCACTTG	Vhl^{n1} mutagenesis
Vhl^{n2}F	GGATGCACGTACGCTGCAGAGGATCTTTCA	Vhl^{n2} mutagenesis
Vhl^{n2}R	TGAAAGATCCTCTGCAGCGTACGTGCATCC	Vhl^{n2} mutagenesis
Vhl^{n1}F construct1(EcoRI)	CGCGCGGAATTCATGCCCCGGAGGGCGGAGAACT	Construct1
Vhl^{n1}R construct1(XhoI)	CGCGCGCTCGAGATGGTGAAACCCCGTCTCTACT	Construct1
Vhl^{n1}F1 construct1(XhoI)	CGCGCGCTCGAGTCAGGGGAAATGGAGAAAATAG	Construct1
Vhl^{n1}R1 construct1(XbaI)	CGCGCGTCTAGAGAGAATAGGATACAAAAAGATTGGA	Construct1
c.163delGF	ACTGGGCGCCGAGGAGGAGATGAGGCCGGGCGGCCGC	Construct1 mutagenesis
c.163delGR	GCGGCCGCCCGGCCTCATCTCCTCCTCGGCGCCCAGT	Construct1 mutagenesis
Vhl^{n2}F construct2(EcoRI)	CGCGCGGAATTCATGCCCCGGAGGGCGGAGAACT	Construct2
Vhl^{n2}R construct2(XhoI)	CGCGCGCCTCGAGTCAATCTCCCATCCGTTGATGTGC	Construct2
c.172delGF	CAGGTCATCTTCTGCAATCGCATCCGCGCGTCGTGCT	Construct2 mutagenesis
c.172delGR	AGCACGACGCGCGGATGCGATTGCAGAAGATGACCTG	Construct2 mutagenesis
pVHLF	GGCCGCCGCATCCA	VHL QPCR
pVHL R	CATCGTGTGTCCCTGCATCTC	VHL QPCR
pGFP F	GCAACTACAAGACCCGC	GFP QPCR
pGFP R	GTCGGCCATGATATAGACG	GFP QPCR
rp49_F	CACACCGGAAACTCAATGGAT	rp49 QPCR
rp49_R	GGTCATCTTGAAGCTGGAAGG	rp49 QPCR
Act4A_F	GCTTCGCTGTCTACTTTCCA	Act4A QPCR
Act4A_R	CAGCCCGACTACTGCTTAGA	Act4A QPCR
Adh_RT_F	GGCGGTCCCGGTGGTA	RT-PCR, Adh QPCR
Adh_RT_R	CTGGTAGATGGCATTGAATCC	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 ovaric 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:$Upf1^{D45B}$. We observed that the ubiquitous expression of the dominant-negative $UPF1^{D45B}$ 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:$Upf1^{D45B}$ 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 $Upf1^{D45B}$ is able to modulate NMD pathway, we used QPCR to test its effect on mRNA levels of Adh^{n4} and Adh^{fn6}, 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

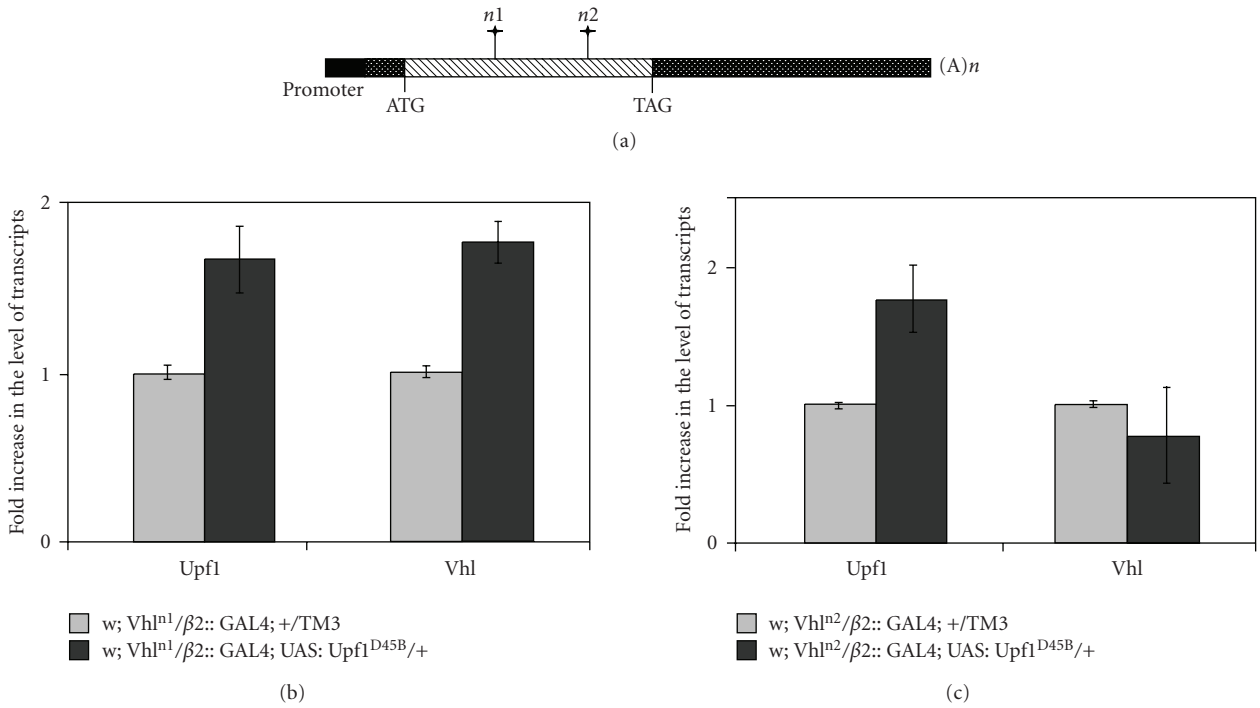

Figure 2: QPCR to measure the levels of transgenic Vhl lines. (a) Schematic map of the *Vhl* gene. Black rectangles represent the promoter region; hatched box represents the coding region; punctated boxes represent untranslated regions. The position of the point mutations *n*1 and *n*2 are indicated with vertical lines marked with a star. (b) and (c) We compared the levels of *Vhl* and *Upf1* transcripts in flies that were heterozygous for *Vhl* nonsense alleles and for $Upf1^{D45B}$. No increase of *Vhl* transcript in transgenic UAS:Vhl^{n2} line was observed under the inhibition of NMD pathway whereas the UAS:Vhl^{n1} transgenic line showed a 1.8 accumulation of nonsense-containing *Vhl* mRNA. The experiment was repeated three times.

[20], that they were 25% and 10% lower compared to the wild type strain (data not shown), because of increased turnover of the mutant transcript by the NMD pathway.

Since the ubiquitous expression of $UPF1^{D45B}$ protein caused larval lethality, we reasoned that the choice of a tissue in which the absence of the NMD pathway is not essential for *Drosophila* viability or development is fundamental to carry out functional analysis on NMD targets.

Interestingly, we observed that the expression of $UPF1^{D45B}$ protein in *Drosophila* testis, driven by a testis-specific GAL4 driver line (β2::GAL4), had no effect on testis development and/or larval lethality. Thus, we retained that the use of β2::GAL4 driver line could allow a detailed analysis of adult-onset phenotypes induced by loss of UPF1 activity.

Further, we tested whether the *Upf1* mutation has a dominant-negative effect on the regulation of Adh^{n4} and Adh^{fn6} transcripts by inducing the expression of UAS:$Upf1^{D45B}$ transgene in *Drososphila* testis. w^{1118}; Adh^{n4}/*Cy;* UAS:*Upf1*^{D45B}/*TM3* and w^{1118}; Adh^{fn6}/*Cy;* UAS:*Upf1*^{D45B}/*TM3* males were crossed to β2::GAL4 females and the levels of *Adh* and *Upf1* mRNAs from testes of w^{1118}; Adh^{n4}/β2::GAL4; UAS:$Upf1^{D45B}$/+ and w^{1118}; Adh^{fn6}/β2::GAL4; UAS:$Upf1^{D45B}$/+ 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 $UPF1^{D45B}$ mutation could abolish NMD pathway function.

Together these results strong point out that $Upf1^{D45B}$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:$Upf1^{D45B}$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 Vhl^{n1}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 Vhl^{n2} allele contains the c.254delC mutation corresponding to the c.239delG in human *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:Vhl^{n1} and UAS:Vhl^{n2}.

To verify whether the two different *Vhl* mutant transcripts were targeted by NMD pathway, w^{1118}; UAS:Vhl^{n1}/*Cy;* UAS:*Upf1*^{D45B}/*TM3* and w^{1118}; UAS:Vhl^{n2}/*Cy;* UAS:*Upf1*^{D45B}/*TM3* males were crossed to w^{1118};β2::GAL4 females. The mRNA levels of both wild type and mutated forms of *Vhl* and *Upf1* from testis of w^{1118}; UAS:Vhl^{n1}/β2::GAL4;

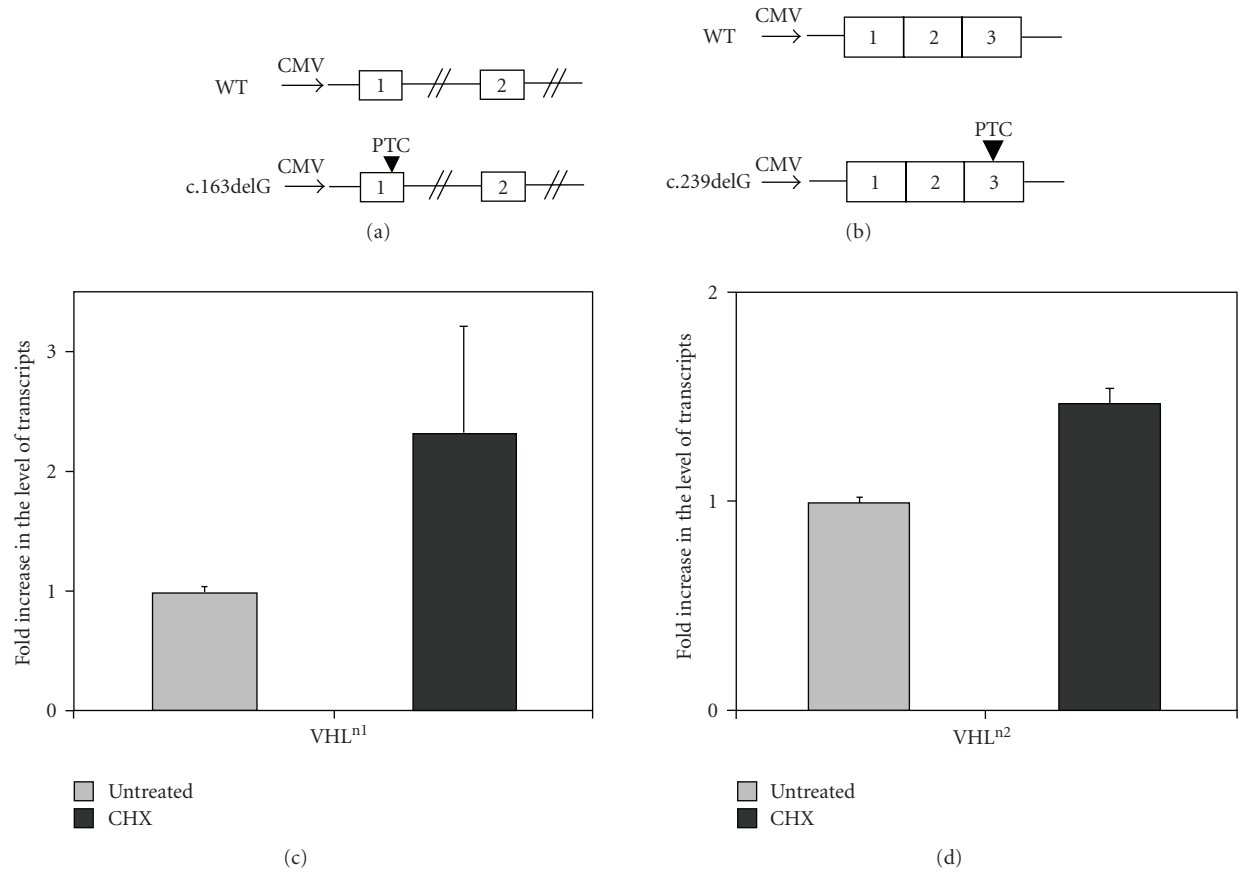

Figure 3: Effect of CHX treatment on the level of the c.163delG and c.239delG *VHL* mRNA. (a) Scheme of the WT (upper panel) and c.163delG constructs, which contained the exons 1-2 (marked in the boxes by numbers), a part of the intronic sequence between exons 1-2 and a piece of intron downstream the 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.

UAS:$Upf1^{D45B}$/+ and w^{1118}; UAS:$Vhl^{n2}/\beta 2$::GAL4; UAS:$Upf1^{D45B}$/+ flies were measured by QPCR. We observed an increased of *Vhl* transcript in UAS:$Vhl^{n1}/\beta 2$::GAL4; UAS:$Upf1^{D45B}$/+ transgenic line. Conversely, the UAS:$Vhl^{n2}/\beta 2$::GAL4;UAS:$Upf1^{D45B}$/+ flies did not show any difference in *Vhl* expression compared to the *Upf1* wild type line (Figures 2(b) and 2(c)).

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].

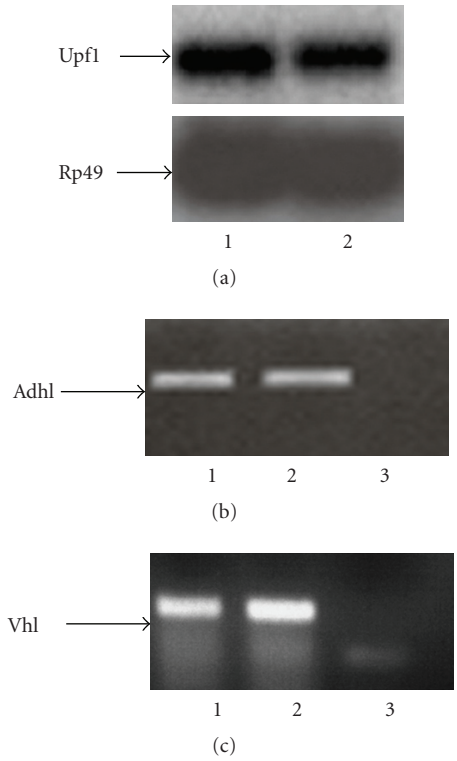

Figure 4: *Upf1*, *Adh,* and *Vhl* expression in *Drosophila* testes and ovaries. (a) Northern blot analysis of poly (A+) RNAs isolated from wild type adult flies. *Lane 1*: poly(A+) RNA from ovaries of adult females; *lane 2*: poly(A+) RNA from testes of adult males. Arrowheads on the left mark the position of the detected mRNAs. (b) RT-PCR analysis of *Adh* transcripts in wild type adult flies. *Lane 1*: total RNA from ovaries of adult females; *lane 2*: total RNA from testes of adult males; *lane 3:* negative control. (c) RT-PCR analysis of *Vhl* transcripts in wild type adult flies. *Lane 1:* total RNA from testes of adult males; *lane 2*: total RNA from ovaries of adult females; *lane 3:* negative control.

In the present study we generated *Upf1* mutant fly line, UAS:$Upf1^{D45B}$, to investigate whether two nonsense alleles of the *Vhl* gene are NMD targets. In agreement with others we observed that the ubiquitous expression of $UPF1^{D45B}$ 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 $\beta 2$::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* dominant-negative mutant suggested that the UAS:$Upf1^{D45B}$ line could be used as genetic system to screen nonsense mutations substrate of NMD pathway.

We investigated if the expression of two specific *Vhl* nonsense transcripts, Vhl^{n1} and Vhl^{n2}, is modulated by NMD mechanism. The observed effect of NMD on the degradation of these two *Vhl* nonsense transcripts was different. In particular we showed the involvement of NMD in the degradation of Vhl^{n1} transcript, whereas Vhl^{n2} transgenic line showed no difference in *Vhl* expression compared to the wild type. These results suggest that the NMD of *Vhl* transcript carrying the c.172delG mutation is effective, whereas the c.254delC is not.

Similar results were observed in HEK293 cell line transfected with *VHL* constructs harboring the corresponding human *VHL* mutations after CHX treatment. Indeed while the c.239delG mutation transcript level showed only a marginal increase following the NMD inhibition, a fairly good increase of mRNA expression of c.163delG mutant transcript was observed.

Our data confirm that some frameshift mutations located in the last portion of *VHL* gene could escape NMD. In fact in both mammalian cells and *Drosophila* the c.239delG and the c.254delC transcripts, respectively, are immune to NMD.

Not all nonsense codons trigger NMD. In mammalian cells a splicing-dependent signal seems to be involved in PTC definition. Remarkably, stop codons located at least 50–55 nt upstream of an exon-exon boundary are generally defined as premature, whereas most PTCs downstream of this point do not elicit decay [24]. Notably, although the splicing and NMD machineries are conserved in *D. melanogaster*, the NMD in this organism was shown to occur independently of EJC components and PTC-containing transcripts that derive from intronless genes were found to be NMD-competent [11, 25].

Recent data demonstrated that the position of nonsense codons relative to the cytoplasmic poly(A)-binding protein 1 (PABPC1) is also a critical determinant for PTC definition both in *Drosophila* and in human [26]. Interestingly, if PABPC1 is in close proximity to the PTC, it seems to function as an NMD repressor. The observation that the Vhl^{n1} but not the Vhl^{n2} mRNA abundance is subject to NMD regulation might be attributable to the spatial rearrangements of the 3′UTR that close PABPC1 to the PTC. Probably, proximity of the PABPC1 protein to the PTC generated by c.172delG mutation inhibits NMD.

Another possible explanation for the difference observed between the two mutants should be that whereas the c.163delG mutation is located sufficiently upstream of an exon-exon junction and therefore is able to trigger NMD, the c.239delG mutation is located in the 3′-most exon. Therefore it is not surprising that this mutation is not able to trigger NMD in mammalian cells.

The possibility that *VHL* truncating mutations may recognised by NMD was recently verified [6]. In that study, inhibition of NMD in two sporadic RCC cell lines (786O and KTCL26) with endogenous frameshift *VHL* mutations that generated stop codons at residues 104 and 147 amino acidic composition residues, respectively, did not produce

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 need 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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References

[1] P. L. Choyke, G. M. Glenn, J. P. Wagner, et al., "Epididymal cystadenomas in von Hippel-Lindau disease," *Urology*, vol. 49, no. 6, pp. 926–931, 1997.

[2] E. R. Maher, J. R. W. Yates, R. Harries, et al., "Clinical features and natural history of von Hippel-Lindau disease," *Quarterly Journal of Medicine*, vol. 77, no. 283, pp. 1151–1163, 1990.

[3] T. J. Manski, D. K. Heffner, G. M. Glenn, et al., "Endolymphatic sac tumors: a source of morbid hearing loss in von Hippel-Lindau disease," *Journal of the American Medical Association*, vol. 277, no. 18, pp. 1461–1466, 1997.

[4] B. M. Shehata, C. A. Stockwell, A. A. Castellano-Sanchez, S. Setzer, C. L. Schmotzer, and H. Robinson, "Von Hippel-Lindau (VHL) disease: an update on the clinico-pathologic and genetic aspects," *Advances in Anatomic Pathology*, vol. 15, no. 3, pp. 165–171, 2008.

[5] C. Gallou, D. Chauveau, S. Richard, et al., "Genotype-phenotype correlation in von Hippel-Lindau families with renal lesions," *Human Mutation*, vol. 24, no. 3, pp. 215–224, 2004.

[6] R. O. Kai, E. R. Woodward, P. Killick, C. Lim, F. Macdonald, and E. R. Maher, "Genotype-phenotype correlations in von Hippel-Lindau disease," *Human Mutation*, vol. 28, no. 2, pp. 143–149, 2007.

[7] S. Ruiz-Llorente, J. Bravo, A. Cebrian, et al., "Genetic characterization and structural analysis of VHL Spanish families to define genotype-phenotype correlations," *Human Mutation*, vol. 23, no. 2, pp. 160–169, 2004.

[8] R. Pulak and P. Anderson, "mRNA Surveillance by the Caenorhabditis elegans smg genes," *Genes and Development*, vol. 7, no. 10, pp. 1885–1897, 1993.

[9] J. Rehwinkel, J. Raes, and E. Izaurralde, "Nonsense-mediated mRNA decay: target genes and functional diversification of effectors," *Trends in Biochemical Sciences*, vol. 31, no. 11, pp. 639–646, 2006.

[10] Y. Cui, K. W. Hagan, S. Zhang, and S. W. Peltz, "Identification and characterization of genes that are required for the accelerated degradation of mRNAs containing a premature translational termination codon," *Genes and Development*, vol. 9, no. 4, pp. 423–436, 1995.

[11] D. Gatfield, L. Unterholzner, F. D. Ciccarelli, P. Bork, and E. Izaurralde, "Nonsense-mediated mRNA decay in *Drosophila*: at the intersection of the yeast and mammalian pathways," *The EMBO Journal*, vol. 22, no. 15, pp. 3960–3970, 2003.

[12] F. He, A. H. Brown, and A. Jacobson, "Upf1p, Nmd2p, and Upf3p are interacting components of the yeast nonsense-mediated mRNA decay pathway," *Molecular and Cellular Biology*, vol. 17, no. 3, pp. 1580–1594, 1997.

[13] J. Lykke-Andersen, M.-D. Shu, and J. A. Steitz, "Human Upf proteins target an mRNA for nonsense-mediated decay when downstream of a termination codon," *Cell*, vol. 103, no. 7, pp. 1121–1131, 2000.

[14] M. M. Metzstein and M. A. Krasnow, "Functions of the nonsense-mediated mRNA decay pathway in *Drosophila* development," *PLoS Genetics*, vol. 2, no. 12, article e180, 2006.

[15] N. Amrani, R. Ganesan, S. Kervestin, D. A. Mangus, S. Ghosh, and A. Jacobson, "A faux 3′-UTR promotes aberrant termination and triggers nonsense-mediated mRNA decay," *Nature*, vol. 432, no. 7013, pp. 112–118, 2004.

[16] S. Brogna, "Nonsense mutations in the alcohol dehydrogenase gene of *Drosophila melanogaster* correlate with an abnormal 3′ end processing of the corresponding pre-mRNA," *RNA*, vol. 5, no. 4, pp. 562–573, 1999.

[17] G. M. Rubin and A. C. Spradling, "Genetic transformation of *Drosophila* with transposable element vectors," *Science*, vol. 218, no. 4570, pp. 348–353, 1982.

[18] X. Sun, H. A. Perlick, H. C. Dietz, and L. E. Maquat, "A mutated human homologue to yeast Upf1 protein has a dominant-negative effect on the decay of nonsense-containing mRNAs in mammalian cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 17, pp. 10009–10014, 1998.

[19] A. H. Brand and N. Perrimon, "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes," *Development*, vol. 118, no. 2, pp. 401–415, 1993.

[20] S. Brogna, K. Bourtzis, L. M. Gomulski, et al., "Genomic organization and functional characterization of the alcohol dehydrogenase locus of Ceratitis capitata (Medfly)," *Insect Molecular Biology*, vol. 15, no. 3, pp. 259–268, 2006.

[21] C. E. Stebbins, W. G. Kaelin Jr., and N. P. Pavletich, "Structure of the VHL-elonginC-elonginB complex: implications for VHL tumor suppressor function," *Science*, vol. 284, no. 5413, pp. 455–461, 1999.

[22] M. J. Nyhan, G. C. O'Sullivan, and S. L. McKenna, "Role of the VHL (von Hippel-Lindau) gene in renal cancer: a multifunctional tumour suppressor," *Biochemical Society Transactions*, vol. 36, no. 3, pp. 472–478, 2008.

[23] J. K. Maranchie, A. Afonso, P. S. Albert, et al., "Solid renal tumor severity in von Hippel Lindau disease is related to

germline deletion length and location," *Human Mutation*, vol. 23, no. 1, pp. 40–46, 2004.

[24] E. Nagy and L. E. Maquat, "A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance," *Trends in Biochemical Sciences*, vol. 23, no. 6, pp. 198–199, 1998.

[25] A. L. Silva and L. Romão, "The mammalian nonsense-mediated mRNA decay pathway: to decay or not to decay! Which players make the decision?" *The FEBS Letters*, vol. 583, no. 3, pp. 499–505, 2009.

[26] I. Behm-Ansmant, D. Gatfield, J. Rehwinkel, V. Hilgers, and E. Izaurralde, "A conserved role for cytoplasmic poly(A)-binding protein 1 (PABPC1) in nonsense-mediated mRNA decay," *The EMBO Journal*, vol. 26, no. 6, pp. 1591–1601, 2007.