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The Mammalian Gene *ZNF268* Is Regulated by hUpf1

Chengang Zhu, Zhouzhou Zhao, Mingxiong Guo, Huanjie Shao, Hongling Qiu, Di Wang, Junhua Xu, Lu Xue, and Wenxin Li*

State Key Laboratory of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, P. R. China; fax: +86-27-68752146; E-mail: liwxlab@whu.edu.cn

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Abstract—Nonsense-mediated mRNA decay (NMD), also called RNA surveillance, is a process that degrades mRNAs with premature translation termination codons. In *Saccharomyces cerevisiae*, it has also been shown that NMD can regulate gene expression at the transcriptional level. To date, there has been no example where promoters are regulated by the NMD pathway in higher eukaryotes. Taking advantage of our previous research on *ZNF268* transcription control, we studied the relationship between the *ZNF268* promoter and the NMD pathway. We showed by transient transfection that the *ZNF268* promoter activity was influenced by hUpf1, not hSmg6, in HeLa cells. This result was confirmed by the analysis of the steady state mRNA of *ZNF268* after depletion of endogenous hUpf1 or hSmg6 in HeLa cells. Direct mutational analysis revealed that the C/EBP site in the promoter region is important for hUpf1 function on *ZNF268* promoter. Together our results demonstrated that the mammalian gene *ZNF268* is regulated by hUpf1 via its promoter.

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Key words: NMD, hUpf1, *ZNF268*, promoter regulation

Several decades ago, it was discovered that mutations truncating coding sequences reduce the mRNA level without lowering the rate of mRNA synthesis in yeast [1]. This process, by which transcripts containing premature termination codons (PTCs) are detected and degraded within cells, has been called nonsense-mediated mRNA decay (NMD), and is present in many organisms including yeast [2], worms [3], plants [4], and mammals [5]. At first, NMD is thought to identify and degrade mRNAs containing PTCs that cause premature termination of translation, which protect cells from the accumulation of potentially deleterious truncated proteins [6]. Now, studies have shown that NMD is involved in many processes, for example, efficient translation [7], telomere balance [8-10], and even response to oxidative stress [11].

Besides the degradation of abnormal mRNAs with PTCs, up-stream open reading frames (uORF), etc., NMD also controls the expression of some wild-type genes. High-density oligonucleotide arrays (HDOAs) data showed that several hundred wild-type *Saccharomyces cerevisiae* mRNAs altered their steady-state levels in Upf protein mutants. Nine mRNAs, with CKI (combined-knockout index) score of $\geqslant +0.44$ and confirmed by northern blot, were randomly selected for measurement of half-lives. Interestingly, none of them have an altered half-life [12]. This raised the possibility that the Upf proteins may cause a change in the mRNA half-life of a small subset of the Upf-dependent mRNAs. Further, experiments demonstrated that the promoter sequences of STN1, EST1, EST2, and EST3 are sufficient to confer NMD-dependent control on their mRNA levels [8]. Unfortunately, to date, such promoter regulation by the NMD pathway has not been demonstrated outside of yeast.

The *ZNF268* gene, cloned and characterized from a 3-5-week-old human fetus cDNA library in our laboratory, is a typical KRAB-containing zinc finger gene [13]. Our previous studies implied that *ZNF268* may play a role of importance in the development of human fetal liver [14]. Detailed transcription control studies revealed that *ZNF268* promoter is atypical and requires an intragenic element located within the first exon [15]. Taking advantage of our previous research, we studied the relationship between the *ZNF268* promoter and NMD as an example of mammalian gene promoters. Interestingly, it was found that the *ZNF268* promoter activity was influenced by hUpf1, not hSmg6, both of which are critical players of the NMD pathway, in HeLa cell line. Semi-quantitative

Abbreviations: NMD) nonsense-mediated mRNA decay; PTCs) premature termination codons.

* To whom correspondence should be addressed.

RT-PCR demonstrated that endogenous *ZNF268* gene was similarly affected. Further, direct mutational analysis indicated that the C/EBP site in *ZNF268* promoter is important for hUpf1 to affect the promoter function. Our results thus provided the first mammalian example where the NMD pathway affects mRNA levels at the transcriptional level with the promoter.

MATERIALS AND METHODS

Cell culture, transient transfection, dual luciferase assay, and western blot. HeLa cells were grown in DMEM medium under standard conditions. Cells were transiently transfected with plasmids using Sofast (Sunma, China) according to manufacturer's instructions, and then harvested after 48 h. For knockdown of hUpf1, we used a mixture with the same amount of pSUPERpuro-hUpf1/I and pSUPERpuro-hHup1/II [16]. For knockdown of hSmg6, we used a mixture with the same amount of pSUPERpuro-hSmg6/I and pSUPERpuro-hSmg6/II [16].

For dual luciferase assay, 300 ng of indicated expression construct and 300 ng firefly luciferase reporter construct and 50 ng internal control Renilla luciferase reporter construct, pRL-TK (Promega, USA), were used for each transfection. The dual-luciferase activity was analyzed with a Turner BioSystems TD-20/20 luminometer (Turner Designs, USA) using the Dual-Luciferase® reporter assay system (Promega). Triplicate samples were measured for each construct, and the average values of the ratio of firefly luciferase light units to Renilla luciferase light units were used for data analysis. The results showed the mean values of three independent experiments with standard errors.

For western blot, 10 μg cell lysates were separated by 10% SDS-PAGE and analyzed by using antibodies against hUpf1 or β-actin. The color reaction was carried out by SuperSingal® West Pico (Pierce, USA) according to manufacturer's instructions.

RNA analysis. Total RNA was prepared using TRIZOL reagent (Gibco, Invitrogen Corporation, USA) according to the manufacture's instruction. To remove genomic DNA contamination, DNase treatment was performed as recommended by the kit manufacturers. The first strand cDNA of all the samples were reversely transcribed using RNA PCR Kit (TaKaRa, Japan) with oligo dT-adaptor primer containing 500 ng of total RNA.

For semi-quantitative RT-PCR, one-tenth of the purified DNA/RNA volume were amplified using EX Taqase PCR system (TaKaRa) with the primer pairs of 5′-AAGTCCAAAGCCAAGGTGC-3′ and 5′-TTGCGATTTCTTATTGACGG-3′ (424 bp) for *ZNF268* cDNA amplification (96°C for 15 sec; 56°C for 30 sec; 72°C for 45 sec, for 35 cycles). To ensure the integrity of cDNA during PCR, β-actin was amplified as a positive control with the primer pairs of 5′-GCTCGTCGTCGACAACGGCTC-3′ and 5′-CAAACATGATCTGGGTCATCTTCTC-3′ (353 bp). The PCR products were separated on a 1.5% agarose gel.

RESULTS

To address the relationship between *ZNF268* promoter and NMD, transient transfection on *ZNF268* promoter activity was studied after inhibition of hUpf1 or hSmg6 in HeLa cell line. To this end, we obtained a dominant-negative clone of human Upf1, called R844C, which contains an arginine-to-cysteine mutation in its RNA helicase domain that abrogates NMD [17]. Two RNA interfering constructs of pSUPERpuro-hUpf1/I and pSUPERpuro-hUpf1/II were used for knockdown of hUpf1 [16]. Another two interfering constructs of pSUPERpuro-hSmg6/I and pSUPERpuro-hSmg6/II were used for

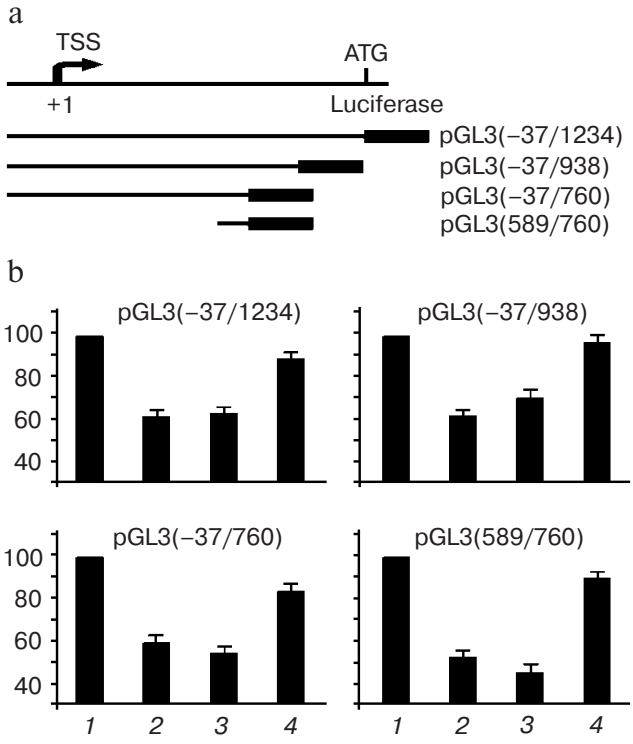

Fig. 1. Transient transfection assay analysis of the effect on the *ZNF268* promoter activity by hUpf1 or hSmg6. a) Diagrams of the four promoter constructs—pGL3(–37/1234), pGL3(–37/938), pGL3(–37/760), or pGL3(589/760). The transcription start site (TSS) and the initial ATG are indicated. b) The four promoter plasmids were transfected into HeLa cells with overexpression of a dominant negative form of hUpf1 (R844C) (*2*) or knockdown of endogenous hUpf1 (*3*) or knockdown of hSmg6 (*4*). Column *1* represents cells transfected with the same amount of control plasmid (pSuper.puro). All signals were normalized against the control set to 100. Triplicate samples were measured for each construct, and the results show the mean values of three independent experiments with standard errors.

knockdown of hSmg6 [16]. In our previous work, several *ZNF268* promoter reporter constructs in pGL3 vector with different promoter length were generated [15]. Four plasmids with full promoter activity, named pGL3(−37/1234), pGL3(−37/938), pGL3(−37/760), and pGL3(589/760), were used in this study. These promoter reporter plasmids were transfected into HeLa cells with or without simultaneous inhibition of hUpf1 or hSmg6 through transient transfection. The dual luciferase assay showed that the activity of *ZNF268* promoter was reduced to 60% after inhibiting the function of hUpf1 either by overexpression of R844C or knockdown of hUpf1, on all promoter reporter constructs. However, the promoter activity fluctuated little after knockdown of hSmg6 (Fig. 1b).

To investigate if this NMD effect also functions on the endogenous *ZNF268* gene, the steady state mRNA of *ZNF268* was measured by semiquantitative RT-PCR after inhibition of the function of hUpf1 or hSmg6 in HeLa cells. HeLa cells were transiently transfected with RNAi constructs for depleting hUpf1 or hSmg6 or the construct overexpressing R844C. After 48 h, total RNA was isolated and analyzed by semiquantitative RT-PCR (Fig. 2a). A fraction of cells was analyzed by western blot (Fig. 2b), which confirmed the changes in the levels of hUpf1. To control for the mRNA levels, the *ZNF268* mRNA amount was normalized against the mRNA for β-actin. When R844C was overexpressed in HeLa cell line, the steady state mRNA of *ZNF268* was reduced compared to wild type HeLa cells. Down regulation of hUpf1 by RNAi had a similar effect as overexpression of R844C. However, when hSmg6 was depleted by RNAi, the amount of *ZNF268* mRNA remained unchanged. These results were consistent with the luciferase activity result (Fig. 1b) and indicated that the *ZNF268* promoter sequences were sufficient to confer hUpf1-dependent control on *ZNF268* mRNA level.

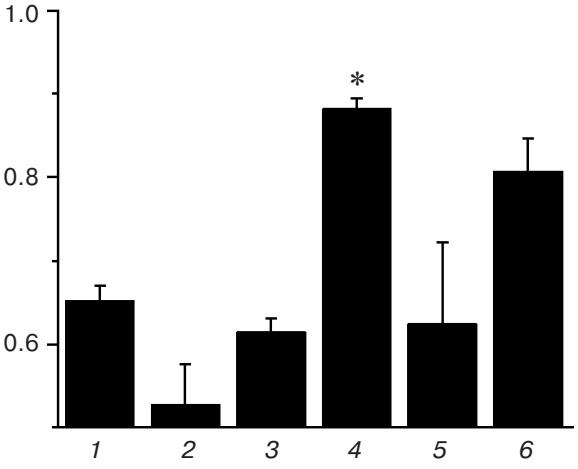

Fig. 3. Transient transfection assay analysis of the effect of hUpf1 on the activity of pGL3(−37/938) plasmids containing various mutations. Promoter plasmids, pGL3(−37/938) without (*1*), or with mutations in the AP1 (*2*), CREB (*3*), C/EBP (*4*), Ets (*5*), or P53 sites (*6*), were transfected into HeLa cells with knockdown of hUpf1. Control cells were transfected with the same amount of pSuper.puro. The ratio reflected the promoter activity change after knockdown of hUpf1. When the C/EBP mutation was present in pGL3(−37/938), the transcription inhibitory effect of hUpf1 was drastically reduced (indicated by an asterisk). Triplicate samples were measured for each construct and the results show the mean values of three independent experiments with standard errors.

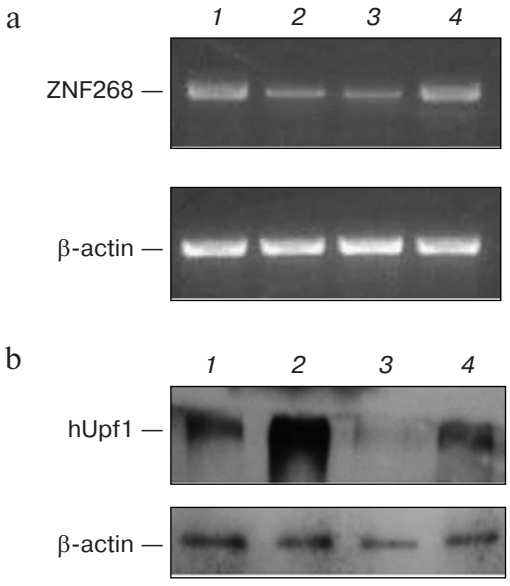

Fig. 2. Altering hUpf1 but not hSmg6 affects the expression of endogenous *ZNF268* in HeLa cells. a) HeLa cells were transfected with control plasmid (lane *1*) or R844C (lane *2*), pSUPERpuro-hUpf1/I and pSUPERpuro-hHup1/II (lane *3*), or pSUPERpuro-hSmg6/I and pSUPERpuro-hSmg6/II (lane *4*). Total RNA was isolated and analyzed by semiquantitative RT-PCR. b) Aliquots of cell extracts were analyzed for hUpf1 and β-actin by western blot. This experiment was repeated three times.

The present data suggests that the promoter of *ZNF268* gene is subject to control by hUpf1 through either an indirect or direct mechanism at the transcription level. In our previous work, we have identified some important trans-factors involved in *ZNF268* transcription by using several pGL3(−37/938) plasmids containing different mutations, including those at the binding sites for transcription factors AP1, CREB, C/EBP, Ets, and P53 [15]. To investigate whether the NMD effect was mediated by one or more of these trans-factors, we transfected these plasmids into HeLa cells with or without altering hUpf1 function. The dual luciferase assay showed that mutations in the C/EBP site abolished the effect of hUpf1 on the promoter activity, while the pGL3(−37/938) promoter with mutations in the binding sites for other transcription factors retained the sensitivity to hUpf1 (Fig. 3), indicating that the NMD effect was mediated through the C/EBP sites, likely by altering the function of C/EBP.

DISCUSSION

This study focused on the question whether the *ZNF268* promoter is influenced by NMD. Toward this end, the promoter activity was measured after depleting the function of hUpf1 or hSmg6 in HeLa cell line. The dual luciferase assay showed that the promoter activity was influenced by hUpf1, not hSmg6. This finding was confirmed by semiquantitative RT-PCR analysis of endogenous *ZNF268* mRNA. We have further shown that the C/EBP site in *ZNF268* promoter is important for hUpf1 function. Our results demonstrate that the *ZNF268* promoter sequences are sufficient to confer hUpf1-dependent control on *ZNF268* mRNA levels and provide the first mammalian example of NMD control of promoter activity.

Human Upf1 is a vital component of the NMD pathway [18]. However, we are not sure whether the transcription inhibitory effect was cause by loss of NMD function or rather by loss a putative NMD-independent function of hUpf1. In our experiments, hSmg6 seemed to have nothing to do with the regulation of *ZNF268*. Considering that there are only very low levels of hSmg6 in HeLa cells [9, 10], our finding may simply reflect the lack of hSmg6 in HeLa cells. Human UPF1 is a member of the superfamily I group of helicase, like its counterpart of yeast UPF1 [19], and has a DNA helicase activity [20]. Both the human and yeast enzymes contain two unique putative zinc finger motifs near their N-terminal ends [19]. *In vivo*, hUpf1 interacts with the third subunit of pol δ, p66, which implies a role for hUpf1 in DNA replication or repair [21]. More and more evidence indicates that a small amount of hUpf1 could be present in the nucleus [21-23], and thus may directly affect gene transcription. Our dual luciferase assay showed that the C/EBP site in the *ZNF268* promoter is important for the function of hUpf1. Further studies are needed to investigate how hUpf1 functions through the C/EBP site.

The first purpose served by NMD is thought to identify and degrade mRNAs containing PTCs. This function of NMD is well documented in yeast, drosophila, and mammals. Interesting, NMD in drosophila seems like a mosaic of the yeast and mammalian pathways, which reflect the evolutionary continuity of NMD [24]. Several years ago, it was discovered that the promoter can be regulated by NMD in yeast. Here, we provided the first example of hUpf1 regulating a gene via the promoter in mammals. The findings that Upf can affect promoter activity in both human and yeast suggest that the mechanism of nonsense-mediated mRNA decay is highly conserved. Consistent with this hypothesis, it has been reported that some of the genes involved in NMD are conserved between yeast [25-27], *C. elegans* [28], and mammals [25-27].

It has been reported that loss of Ufp1 function and NMD is tolerated by lower eukaryotes such as yeast, but the analogous function appears essential for basic cellular viability in mice [29]. A hypothesis on this phenomenon is that the NMD pathway may take an increased burden of dealing with faulty transcripts in higher eukaryotes due to the increased number of genes, increased number of introns, or increased complexity of RNA processing in mammals [29]. The fact that the human *ZNF268* gene is controlled by NMD makes a good example of this. Human *ZNF268* gene is a typical Kruppel-associated box/C2H2 zinc finger gene whose homolog has been found only in higher mammals and not in lower mammals such as mouse [15]. This provides evidence that new genes like *ZNF268* are possible to be involved in the nonsense-mediated mRNA decay pathway. The controlling network becomes so important and intricate that the NMD pathway is indispensable to the development of higher eukaryotes.

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