Evidence for OTUD-6B Participation in B Lymphocytes Cell Cycle after Cytokine Stimulation

Zhongping Xu¹,4, Yufang Zheng², Yufei Zhu¹,4, Xiangyin Kong¹,3,4*, Landian Hu¹,3,4*

¹ The Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS) and Shanghai Jiao Tong University School of Medicine (SJTUSM), Shanghai, People’s Republic of China, ² Department of Physiology and Biophysics, School of Life Sciences, Fudan University, Shanghai, People's Republic of China, ³ State Key Laboratory of Medical Genomics, Ruijin Hospital, Shanghai Jiaotong University, Shanghai, People’s Republic of China, ⁴ Graduate School of the Chinese Academy of Sciences, Beijing, People’s Republic of China

Abstract

Deubiquitinating enzymes (DUBs) are important regulators of cell proliferation. Here we identified a functional deubiquitinating enzyme, ovarian tumor domain-containing 6B (OTUD-6B). Mutation of the conserved Cys residue abolished its deubiquitinating activity in vitro. Otud-6b expression was induced with cytokine stimulation in both mouse Ba/F3 cells and primary B lymphocytes followed a rapid decrease. This rapid decrease was partially facilitated by tristetraprolin (TTP) destabilization of Otud-6b mRNA through AU-rich motifs. Enforced expression of OTUD-6B in Ba/F3 cells could block cell proliferation by arresting cells in G1 phase. In addition, cyclin D2 level was down-regulated when OTUD-6B WT was overexpressed. Therefore, down-regulation of Otud-6b expression after prolonged cytokine stimulation may be required for cell proliferation in B lymphocytes.

Introduction

The ubiquitin-mediated proteolytic pathway is involved in multiple cellular processes including cell cycle regulation [1], transcriptional activation [2], and antigen presentation [3]. In addition to ubiquitination, the importance of deubiquitinating enzymes (DUBs) has been demonstrated recently [4,5,6]. DUBs can either recycle ubiquitin as components of the 26S proteasome [7] or rescue proteins from the degradation pathway by deubiquitination [7,8]. There are five sub-families of DUBs classified by their sequence diversity: the ubiquitin C-terminal hydrolases (UCHs), the ubiquitin-specific peptidases (USPs/UBPs), the ovarian tumor (OTU) domain proteins, the Josephin or Machado-Joseph disease (MJD) proteins, and the JAMM (Jab1/MPN domain-associated metalloisopeptidase) domain proteins. The JAMM proteins are zinc metalloisopeptidases, while the other four families are cysteine peptidases [9].

B cell fate is essentially associated with the adaptive immune system [10], and B cell fate is modulated by cytokines during its maturation, homeostasis, and proliferation through target genes expression [11,12]. Recently, mouse Dub-1, Dub-1a, Dub-2, and Dub-2a were reported to be hematopoietic-specific DUBs in B lymphocytes. Their expression levels were rapidly induced upon cytokine stimulation, which is probably due to the cytokine-inducible enhancer in the 5' UTR [13,14]. Interestingly, the expression levels of those DUBs were sharply down-regulated following the fast induction and little is known about this fast down-regulation. These four DUBs belong to the USP17 gene family, members of which form part of highly polymorphic tandem repeat sequences on mouse chromosome 7 [15]. There is no other DUB reported to present this induction-decline expression pattern. More recently, microarray data have shown that many OTU family members were rapidly up-regulated or down-regulated in human esophageal epithelial cells and lymphocytes when stimulated by different cytokines, such as ovarian tumor domain containing 6B (OTUD-6B), a novel DUB of the OTU family members. OTUD-6B was originally named as CGI-77 and has deubiquitinating enzyme activity in vitro [16]. It was up-regulated on human esophageal epithelial cells after interleukin-13 (IL-13) stimulation [17]. BAFF, a cell-activating factor of the TNF family, could also induce Otud-6b expression on mouse B cells after 4 hours stimulation [18]. However, granulocyte colony-stimulating factor (G-CSF) could effectively down-regulate OTUD-6B expression when human leukocytes were stimulated for 16 hours [19]. Although these experiments showed that OTU family members were regulated by cytokines, little is known about the mechanism and function of such regulations.

Here we report that Otud-6b, a functional DUB of the OTU family, can be induced by IL-3, IL-4, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation in B lymphocytes. However, prolonged stimulation with these cytokines effectively decreased the expression of Otud-6b. This is the first OTU family member to be reported to have such cytokines response. To further investigate the down-regulation mechanism, we knocked down several proteins involved in mRNA regulation and found that tristetraprolin (TTP) was responsible for Otud-6b mRNA rapid degradation. Enforced expression of OTUD-6B
could block cell growth and arrest cells in G1 phase. Apoptosis assays showed that overexpression of OTUD-6B in Ba/F3 cells increased the number of cells in subG1 and pro-apoptotic stages. In addition, cyclin D2 expression level was down-regulated when OTUD-6B WT was overexpressed in Hela and Ba/F3 cells, while overexpression of OTUD-6B C188S, which abolished its deubiquitinating activity, had no effect on cyclin D2 level. Therefore, OTUD-6B may participate in cell cycle regulation in B lymphocytes after cytokine stimulation.

## Results

### OTUD-6B is a Functional Deubiquitinating Enzyme

Human OTUD-6B, also named as DUBA5 and CGI-77, is located on Chr8: 92151719-92168498 [20]. Specific primers were designed to amplify OTUD-6B cDNA from Raji cells by RT-PCR (Figure 1A). The sequence of OTUD-6B cDNA clone was identical to GeneBank NM_016023. The full-length OTUD-6B cDNA is 3306 bp and contains a 972-bp ORF. The mouse homolog Otud-6b cDNA is 3311 bp long and consists of seven exons encoding a 325-amino acid mouse Otud-6b protein. The protein homology between human OTUD-6B and mouse Otud-6b is about 87% (Figure S1). We analyzed the expression pattern of Otud-6b mRNA in mouse tissues by RT-PCR using the Otud-6b specific primers. RT-PCR results revealed that Otud-6b mRNA is expressed in various mouse tissues, including brain, heart, lung, kidney, ovary, spleen, and B lymphocytes (Figure 1B), which indicated that Otud-6b is probably a widely expressed housekeeping gene.

Next we investigated whether OTUD-6B is a functional deubiquitinating enzyme. Sequence alignment on human OTU family members indicated that the Cys188 is the putative conserved Cys residue in OTUD-6B [21]. Therefore, we mutated this site into a Ser to generate an OTUD-6B C188S mutant. In vitro deubiquitinating enzyme assay showed that GST-OTUD-6B WT fusion protein could deubiquitinate Ub-Met-β-gal to an extent comparable to GST-CYLD, which is a reported functional DUB [22,23], while the OTUD-6B C188S mutant failed to cleave the Ub-Met-β-gal substrate (Figure 1C). Immunoblot confirmed that all GST fusion proteins were synthesized effectively (Figure S2). These results demonstrated that OTUD-6B is a functional deubiquitinating enzyme *in vitro*.

### Cytokines could Induce Otud-6b Expression in B lymphocytes Followed by a Rapid Decline

As microarray data have showed that OTUD-6B expression levels could be regulated upon cytokine stimulation [17,18,19,20]. To investigate the response of Otud-6b expression levels to cytokine stimulation in B lymphocytes, we first examined that in Ba/F3 cells, a mouse pro-B cell line. The mRNA levels of Otud-6b

---

**Figure 1. OTUD-6B is a functional deubiquitinating enzyme.** A. Molecular cloning of the cDNA of human OTUD-6B was amplified from total RNA of Raji cells and subjected to 1% agarose gel analysis. B. The expression of mouse Otud-6b mRNA in Ba/F3 cells [5], CTLL-2 cells and various tissues (brain, heart, liver, lung, kidney, ovary, and spleen) was analyzed by RT-PCR using Otud-6b-specific primers. G3PDH was used as control. Ba/F3 cells were kindly supplied by prof. Xin yuan Liu (Shanghai Institute of Biochemistry and Cell Biology, SIBS, CAS). CTLL2 cells were provided by the Cell Bank, Shanghai Institute of Biochemistry and Cell Biology, SIBS, CAS. C. Ub-Met-β-gal fusion protein was prepared from MC1061 cells. The supernatant was incubated with purified GST (lane 1), OTUD-6B WT (lane 2), OTUD-6B C188S (lane 3), GST (lane 4), GST-CYLD WT (lane 5), and GST-CYLD CS (lane 6) fusion protein at 4 °C with rotation for 4 hours. Both OTUD-6B WT and GST-CYLD WT could cleave ubiquitin from the Ub-Met-β-gal fusion protein.

doi:10.1371/journal.pone.0014514.g001
showed a dose-dependent response after 2 hours incubation with different concentrations of IL-3, IL-4, IL-13, and GM-CSF (0, 0.01, 0.1, 1, 10, 100, and 1000 pM) [Figure 2A]. We also tested the time course response for Otud-6b mRNA expression in Ba/F3 cells under 10 pM IL-3, IL-4, IL-13, or GM-CSF stimulation [Figure 2B]. Our results showed that Otud-6b mRNA expression levels were increased from 0 to 2 hours but decreased rapidly after 4–6 hours with those cytokine stimulation. On the other hand, IL-2 could not induce Otud-6b expression in Ba/F3 cells [Figure 2A]. Therefore, we analyzed Otud-6b mRNA expression in those cells transfected with pcDNA3.1(+) and OTUD-6B WT expressing cells [Figure 2E&F]. The concentration course experiments showed that both Otud-6b mRNA and protein could be detected after 1 hour stimulation and declined after 4 hours [Figure 2G]. This could be also induced in mouse primary B cells with IL-3 stimulation. Although this cytokine stimulation pattern has been reported for Dsb-1 and Dsb-2a, Otud-6b is the first OTU family member found to be regulated by cytokines in B lymphocytes.

OTUD-6B Overexpression Slows Proliferation and Increases the Rate of Apoptosis

During our cytokine stimulation experiments, we observed an interesting rapid down-regulation of Otud-6b by prolonged cytokine stimulation. We wanted to understand why Otud-6b is so rapidly down-regulated after prolonged stimulation. Therefore, we enforced OTUD-6B expression in Ba/F3 cells to overturn the down-regulation. Interestingly, overexpression of OTUD-6B could affect cell proliferation and cell cycle in Ba/F3 cells. While cells transfected with pcDNA3.1(+) and OTUD-6B C188S mutant vector doubled 2–3 times after 48 hours of culture, OTUD-6B WT vector transfected cells showed a substantial reduction in the proliferation rate [Figure 3A]. PI staining was also performed on those cells. There were only about 28% of OTUD-6B WT transfected cells in S and G2/M phase while there were 47% control cells and 46% OTUD-6B C188S transfected cells in those phases [Figure 3B]. Therefore, there were more OTUD-6B WT transfected cells arrested in G1 phase (72% versus 53% in control and 54% in OTUD-6B C188S transfected cells, Figure 3B). Moreover, PI-Annexin V assays were also performed on Ba/F3 cells 32 hours after transfection. The ratio of PI+/AnnexinV(+) cells in OTUD-6B WT expressing cells was 16.5% ± 2.5% (p < 0.03), which was significantly higher than that of pcDNA3.1(+) vector and OTUD-6B C188S transfected cells [Figure 3C]. Even though the expression levels of wild-type and mutant OTUD-6B were similar [Figure 3D], these findings indicated that overexpression of OTUD-6B WT in Ba/F3 cells can block cell proliferation and lead to apoptosis, while down-regulation of Otud-6b in Ba/F3 cells has no such effect [Figure S3].

Cyclin D2 was Down-regulated when OTUD-6B was Overexpressed

Next we checked the cell cycle regulator(s) in OTUD-6B expressing cells. Real-time PCR of the cell cycle regulators was performed on the RNAs from Tet-On advanced HA-OTUD-6B Hela cells with or without DOX induction. OTUD-6B mRNA level in DOX(+) cells was about 7 times higher than that in DOX(−) cells and cyclin D2, a G1/S cell cycle regulator, was down-regulated about 70% in DOX(+) cells compared to DOX(−) cells [Figure 4A]. There is no significant difference on the mRNA levels of the other regulators (cyclin D1, cyclin D3, p21, p27, p15, p16, cdk4, cdk6, cdk2, cyclin E, Rb, and c-Myc genes). We also confirmed this down-regulation of cyclin D2 through RT-PCR and immunoblot [Figure 4B & Figure 4C]. Moreover, down-regulation of cyclin D2 was correlated with increased HA-OTUD-6B WT expression [Figure 4D & Figure 4E]. OTUD-6B C188S had no effect on cyclin D2 [Figure 4F]. Similar results were also obtained in Ba/F3 cells [Figure 4G]. Over all, these data indicated that in our system, cyclin D2 level is down-regulated in OTUD-6B overexpressing cells and such down-regulation is dependent on OTUD-6B’s catalytic activity even though we did not observe any deubiquitinating activity of OTUD-6B on cyclin D2 (data not shown). Down-regulation of OTUD-6B also has no effect on cyclin D2 level in those cells [Figure S6].

TTP Destabilizes Otud-6b mRNA through its 3’UTR

Otud-6b mRNA was rapidly down-regulated after prolonged cytokine induction. In order to investigate the mechanism of such rapid decay, we investigated several possible regulation pathways for mRNA degradation. MicroRNAs are reported to be involved in directing mRNA degradation of the target genes [25]. Therefore, we analyzed Otud-6b mRNA sequence and found that there are several AU-rich sequence motifs in the 3’UTR of Otud-6b mRNA. We then checked Otud-6b mRNA sequences for that of TTP. Interestingly, overexpression of TTP could only be detected after 4 hours and declined after 6 hours of stimulation [Figure 2D]. Such difference on mRNA kinetics between IL-3 and IL-4 stimulation is probably due to different downstream signaling pathways induced by those two cytokines [24].

To confirm the induction effect of Otud-6b in B lymphocytes, we next conducted similar experiments in primary B cells from C57BL/6 mice. Similar time course and concentration course experiments were performed on those primary B cells with IL-3 stimulation. The time course experiments showed that both Otud-6b mRNA and protein were induced after 1 hour stimulation and declined after 3 hours’ stimulation [Figure 2E]. While the response with IL-4 stimulation was slower as endogenous Otud-6b protein expression could only be detected after 4 hours and declined after 6 hours of stimulation. The time course response for Otud-6b mRNA expression in Ba/F3 cells was 16.5% in OTUD-6B WT expressing cells was 16.5% in OTUD-6B WT expressing cells, 2.5% in OTUD-6B C188S transfected cells, and 54% in OTUD-6B C188S transfected cells. The ratio of PI+/AnnexinV(+) cells in OTUD-6B WT expressing cells was 16.5% ± 2.5% (p < 0.03), which was significantly higher than that of pcDNA3.1(+) vector and OTUD-6B C188S transfected cells [Figure 3C]. Even though the expression levels of wild-type and mutant OTUD-6B were similar [Figure 3D], these findings indicated that overexpression of OTUD-6B WT in Ba/F3 cells can block cell proliferation and lead to apoptosis, while down-regulation of Otud-6b in Ba/F3 cells has no such effect [Figure S3].

Cytochrome D2 was Down-regulated when OTUD-6B was Overexpressed

Next we checked the cell cycle regulator(s) in OTUD-6B expressing cells. Real-time PCR of the cell cycle regulators was performed on the RNAs from Tet-On advanced HA-OTUD-6B Hela cells with or without DOX induction. OTUD-6B mRNA level in DOX(+) cells was about 7 times higher than that in DOX(−) cells and cyclin D2, a G1/S cell cycle regulator, was down-regulated about 70% in DOX(+) cells compared to DOX(−) cells [Figure 4A]. There is no significant difference on the mRNA levels of the other regulators (cyclin D1, cyclin D3, p21, p27, p15, p16, cdk4, cdk6, cdk2, cyclin E, Rb, and c-Myc genes). We also confirmed this down-regulation of cyclin D2 through RT-PCR and immunoblot [Figure 4B & Figure 4C]. Moreover, down-regulation of cyclin D2 was correlated with increased HA-OTUD-6B WT expression [Figure 4D & Figure 4E]. OTUD-6B C188S had no effect on cyclin D2 [Figure 4F]. Similar results were also obtained in Ba/F3 cells [Figure 4G]. Over all, these data indicated that cyclin D2 level is down-regulated in OTUD-6B overexpressing cells and such down-regulation is dependent on OTUD-6B’s catalytic activity even though we did not observe any deubiquitinating activity of OTUD-6B on cyclin D2 (data not shown). Down-regulation of OTUD-6B also has no effect on cyclin D2 level in those cells [Figure S6].
Figure 2. *Otud-6b* expression is induced by cytokines following by a rapid decline. A. *Otud-6b* RNA level in Ba/F3 cells after starvation and two hours stimulation with different concentrations (0, 0.01, 0.1, 1, 10, 100, and 1000 pM) of mouse IL-3, IL-4, IL-13, and GM-CSF. B. *Otud-6b* RNA level in Ba/F3 cells after starvation and stimulation with 10 pM mouse IL-3, IL-4, IL-13, and GM-CSF for the indicated times (0, 0.5, 1, 2, 4, 8, and 16 hours). C&D. Ba/F3 cells were starved and stimulated with 10 pM mouse IL-3 or IL-4 for the indicated time. *Otud-6b* expression of the Ba/F3 cell lysates was
In order to confirm TTP could regulate Otud-6b mRNA, we investigated the specificity and interaction of TTP with Otud-6b mRNA in Ba/F3 cells. We first performed protein-mRNA complex immunoprecipitation assay on HA-TTP expressing Ba/F3 cells. Otud-6b mRNA could be detected by RT-PCR from TTP-RNA complex immunoprecipitated with anti-HA antibody.

Figure 3. OTUD-6B overexpression slows proliferation and increases the rate of apoptosis. A. Ba/F3 cells were transfected with pcDNA3.1(+), OTUD-6B WT, and OTUD-6B C188S vectors. Thirty-two hours later, Ba/F3 cells were seeded at 2×10⁵ cells per ml and analyzed at 16-hour intervals by trypan blue staining. B. Ba/F3 cells were transfected with pcDNA3.1(+), OTUD-6B WT, and OTUD-6B C188S vectors. Thirty-two hours later, cells were stained using PI and analyzed by flow cytometry. Cell cycle profile from three independent experiments was calculated statistically. C. PI-Annexin V analysis among pcDNA3.1(+), OTUD-6B WT, and OTUD-6B C188S-expressing Ba/F3 cells. All experiments were repeated three times independently. D. Ba/F3 cells were transfected with pcDNA3.1(+), OTUD-6B WT, and OTUD-6B C188S vectors. Thirty-two hours later, cell extracts were analyzed by immunoblot with anti-HA antibody. GAPDH was used as a loading control.

doi:10.1371/journal.pone.0014514.g002
Immunoblot of OTUD-6B, cyclin D2, and p27 with gradient concentrations (0, 0.1, 0.5, 1, 2 ug/ml) of DOX stimulation. Quantitation was performed with Bandscan 5.0 from 3 independent results. F & G. Immunoblot (left panel) for cyclin D1, cyclin D2, and cyclin D3 on Hela and Ba/F3 cells overexpressing pcDNA3.1(+), HA-OTUD-6B WT, and HA-OTUD-6B C188S. Quantitation (right panel) of cyclin D1, cyclin D2, and cyclin D3 on Hela and Ba/F3 cells was performed with Bandscan 5.0 from three independent results.

doi:10.1371/journal.pone.0014514.g004

Figure 4. Cyclin D2 level is down-regulated with OTUD-6B overexpression. A & B & C. Real-time PCR, RT-PCR, and immunoblot of OTUD-6B, GAPDH, cyclin D1, cyclin D2, cyclin D3, p21, p27, p15, p16, cdk4, cdk6, cdc2, cyclin E, Rb, and c-Myc genes from total RNA samples and protein extracts of DOX(−) and DOX(+) Hela cells. Hela cells were provided by the Cell Bank, Shanghai Institute of Biochemistry and Cell Biology, SIBS, CAS, D & E. Immunoblot of OTUD-6B, cyclin D2, and p27 with gradient concentrations (0, 0.1, 0.5, 1, 2 ug/ml) of DOX stimulation. Quantitation was performed with Bandscan 5.0 from 3 independent results. F & G. Immunoblot (left panel) for cyclin D1, cyclin D2, and cyclin D3 on Hela and Ba/F3

D&E. Immunoblot of OTUD-6B, cyclin D2, and p27 with gradient concentrations (0, 0.1, 0.5, 1, 2 ug/ml) of DOX stimulation. Quantitation was performed with Bandscan 5.0 from 3 independent results.
Therefore, it is important to further investigate which substrate(s) or pathway OTUD-6B regulates to control the level of cyclin D2. Many pathways have been reported to regulate cyclin D2 level in different tissues. For example, cyclin D2 is a direct target gene of Myc [39] and PU.1 transcription factor [40]. Its expression can also be induced by colony-stimulating factor-1 receptor through Src, MAPK/ERK kinase, and c-Myc pathways in macrophages [41]. On the other hand, GSK3 beta can suppress cyclin D2 expression by tumor suppressor PTEN [42]. Due to the complexity of cyclin D2 signaling pathway, we had not found the direct substrate of OTUD-6B in Cell Proliferation.

Figure 5. TTP is involved in Otud-6b mRNA rapid degradation after induction. A. The sequence of Otud-6b 3’UTR. AUUUA pentamer or AUUUUA hexamer are underlined. Each rectangle indicates an AU-rich sequence site. The AU-rich sequence with U surrounding it is indicated as a black rectangle. B. pSUPER Mock, pSUPER siTTP-1 or 2 and HA-TTP vectors co-expressing Ba/F3 cells were seeded at 2 x 10^5 cells per ml. Cell extracts were immunoblotted with anti-HA antibody. GFP was used as a loading control. C. Otud-6b RNA level in Ba/F3 cells transfected with pSUPER siTTP-1,2 vectors after starvation and stimulation with 10 pM mouse IL-3 for the indicated times (0, 0.5, 1, 2, 4, 8, 12, 16, and 24 hours). D. Ba/F3 cells were transfected with pSUPER Mock, pSUPER siTTP-1 or 2 vectors. 24 later, cells were stimulated by 10 pM IL-3 and incubated with 15 ug/ml Act.D for the time indicated. The qRT-PCR was performed to detect Otud-6b mRNA remaining at each time point. E. Semi-quantitative RT-PCR analysis of Otud-6b mRNA remaining at each time point after Act.D adding. F. RT-PCR for Otud-6b mRNA in protein-mRNA immunoprecipitation complex. The mRNAs of Ba/F3 cells transfected with HA-TTP when response to IL-3 was immunoprecipitated with anti-HA and IgG serum. As a control, Input (10%) denoted that the RNA sample contained Otud-6b mRNA and didn’t contain genomic DNA. G. TTP transfection had little effect on pGL3 control luciferase expression. Wild-type TTP transfection resulted in a significant decrease in full-length Otud-6b 3’UTR luciferase reporter expression in 293T cells, while the TTP Zn-FM did not alter Otud-6b 3’UTR-mediated expression. Different region of Otud-6b 3’UTR was inserted into downstream of luciferase coding region. The truncate 3’UTR vectors were constructed depending on the AU-rich sequence distribution. 293T cells were provided by the Cell Bank, Shanghai Institute of Biochemistry and Cell Biology, SIBS, CAS.

doi:10.1371/journal.pone.0014514.g005
OTUD-6B yet. While preparing this article, we noticed that recent work reported by Mathew E. Sowa et al. used a CompPASS method in a global proteomic analysis on DUBs, including OTUD-6B, and their associated protein complexes [43]. However, likely because of the complexity of the substrate(s) and the transient nature of DUB-substrate interaction, they also failed to identify the right substrate(s) for OTUD-6B. However, further investigations on identifying OTUD-6B substrates will still be needed to understand the mechanism of cell cycle regulation by OTUD-6B.

Materials and Methods

Cell Lines and Cell Culture

Ba/F3 cells were cultured in RPMI1640 medium supplemented with 10% (v/v) FBS (Invitrogen) and 1 ng/ml recombinant mouse IL-3 (R&D systems) [5]. Recombinant mouse IL-2, IL-3, IL-4, IL-15, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from R&D systems. Before addition of the cytokines for induction, Ba/F3 cells were cultured without serum and IL-3 for two hours. Hela cells were cultured in DMEM medium with 10% FBS. 293T cells were cultured in DMEM medium supplemented with 10% FBS. Raji cells were cultured in RPMI-1640 medium supplemented with 10% FBS. Ba/F3 cells were kindly supplied by prof. Xin yuan Liu (Shanghai Institute of Biochemistry and Cell Biology, SIBS, CAS). Hela cells, 293T cells, Raji cells, and CTL12 cells were provided by the Cell Bank, Shanghai Institute of Biochemistry and Cell Biology, SIBS, CAS.

Mouse Primary B Cells Isolation and Induction

Dynabeads® Mouse pan B (B220) (Invitrogen) can be used directly on whole blood samples from six 4-week C57BL/6 mice according to the manufacturer’s procedures. Mouse B cells can then be lysed on the beads using DETACH BEAD CD19 (Invitrogen) to detach the cells from the beads. Isolated mouse B cells will be bead and antibody free and in perfect shape for downstream IL-3 induction.

OTUD-6B Cloning and Semi-quantitative RT-PCR of Otud-6b

OTUD-6B cDNA was amplified from the total RNA of Raji cells by RT-PCR with specific primers. RT-PCR was performed at 94°C for 1 min, 50°C for 30 s, and 72°C for 3.5 min for a total of 40 cycles. The amplified cDNA was subcloned into the pGEMT-easy vector (Promega) and transformed into E.coli DH5α (Clontech). Total RNA was isolated by using Trizol (Invitrogen). Semi-quantitative RT-PCR was performed using a pair of specific primers for Otud-6b or Otud-6a (Table S3) at 94°C for 1 min, 50°C for 30 s, and 72°C for 1 min for a total of 35 cycles. For cell cycle regulators screens with RT-PCR, OTUD-6B, GAPDH, cyclin D1, cyclin D2, cyclin D3, p21, p27, p15, p16, cdk4, cdk6, cdc2, cyclin E, Rb, and c-Myc genes were performed with the specific primers (Table S1) at 94°C for 1 min, 53°C for 30 s, and 72°C for 30 s for a total of 30 cycles.

Site-directed Mutagenesis

The OTUD-6B (C188S) mutant was generated by using a QnikChange TM site-directed mutagenesis kit (Stratagene) to replace Cys188 with Ser according to the manufacturer’s instructions.

Deubiquitination Assay

The deubiquitination assay has been previously described [5]. In brief, ubiquitin-β-galactosidase fusion protein (Ub-Met-β-gal) was expressed from a pACYC184-based plasmid in MC1061 cells. Purified GST, GST-OTUD-6B WT, GST-OTUD-6B C188S, GST-CYLD WT, and GST-CYLD CS fusion protein was incubated with Ub-Met-β-gal supernatant at 4°C for rotation for 4 hours, then the total protein was analyzed by immunoblot with anti-beta-gal antibodies (Sigma).

Trypan Blue Staining

2×10⁷ Ba/F3 cells transfected with pcDNA3.1(+), OTUD-6B WT, and OTUD-6B C188S vectors and collected after different time (0 hour, 16 hours, 32 hours, and 48 hours), and then washed with 4°C PBS, and stained with 0.1 ml of 0.4% Trypan Blue for 5 min at room temperature. Then cells were observed and counted under a microscope.

Cell Cycle Analysis and Apoptosis Assay

Ba/F3 cells were transfected with pcDNA3.1(+), OTUD-6B WT, and OTUD-6B C188S vectors. Thirty-two hours later, cells were washed with 4°C PBS and fixed with 70% ethanol at 4°C overnight. Then cells were treated with 200 μLN ase in 37°C water for 30 minutes and stained with propidium iodide (PI, Sigma). Cell cycle distribution was measured by flow cytometric assay, MODFIT LT of FACS Calibur (Becton Dickinson). PI-Annexin V assay was performed according to the manufacturer’s instructions and analyzed with CELLQUEST PRO (Becton Dickinson).

Tet-on Systems and Cell Cycle Regulators Screen

Doxycycline (DOX, Sigma)-inducible OTUD-6B expressing Hela cells were prepared as in Ref [44]. In brief, Hela cells stably transfected with pTet-On (Clontech) HA-OTUD-6B WT were treated with or without 2.5 μg/ml DOX. Total RNAs from both DOX (−) and DOX (+) Hela cells were collected and subjected to real-time PCR (Table S2) by an iCycler IQ thermal cycler system (Bio-Rad). Cycling parameters for real-time PCR were the same as mentioned before. Cell extracts were also collected and subjected for immunoblot with antibodies against OTUD-6B, c-Myc (Santa Cruz), GAPDH, cyclin D1, cyclin D2, cyclin D3, p21, p27, p15, p16, cdk4, cdk6, cdc2, cyclin E, and Rb antibodies (CST).

Act.D Chase Assay for mRNA Stability Measurement

For mRNA stability measurement, Ba/F3 cells were incubated with 15 μg/ml actinomycin D (Act.D) to inhibit transcription. At the indicated time points after the addition of Act.D, cells were harvested and total RNA was extracted. The expression levels of Otud-6b at each time points were measured by qRT-PCR and normalized to the according G3pdh levels. The remaining mRNA was determined by comparison with the expression level of the Otud-6b at the zero time point (designated 1) when Act.D was added.

Luciferase Assay

The pcDNA3.1, HA-TTP, HA-TTP Zn-FM constructs were generated as previously described [29]. The pGL3 luciferase constructs contain the Otud-6b 3’-UTR nucleotides (1221–2950, 1221–2720, and 2721–2950 of mouse Otud-6b cDNA). 293T cells were transfected with 1 μg of luciferase constructs and 500 ng of TTP expression constructs or pcDNA3.1. Cells were lysed 24 h in 100 μl of luciferase lysis buffer (Promega), and 20 μl of each sample was read in a luminometer according to the manufacturer’s protocol.

Immunoprecipitation of mRNA-protein Complex

2×10⁷ Ba/F3 cells transfected with HA-TTP vector were used for the immunoprecipitation [45]. 24 hours later, Ba/F3 cells were...
stimulated with 10 μM IL-3 for 3 hours, inducing Otud-6b mRNA to high levels. The cells were then lysed for 10 min on ice in RNA immunoprecipitation (RIP) buffer [10 mM Tris-HCl at pH 7.6, 1.5 mM MgCl2, 100 mM NaCl, 1 mM DTT, 0.5% Nonidet P-40, 0.5% Triton X-100, 100 U/ml RNase inhibitor (Promega), 10 μg/ml protease inhibitor cocktails (Sigma)]. The cell lysate was centrifuged at 12000 g for 15 min at 4°C and the supernatant of the cytoplasmic lysate was collected for RNA IP assay. Protein-RNA complexes were incubated with 1 μg anti-HA antibody and rotated for 12 hours at 4°C. Precleared pro-A Sepharose beads (Amersham) was used to pull-down anti-HA antibody or pre-immune serum by incubating overnight at 4°C with continuous rotation in RIP buffer. The beads were pelleted and the supernatant was removed. These beads and their bound complexes were recovered by centrifugation and washed six times with RIP buffer. Otud-6b mRNA was detected with RT-PCR using the specific primers.

Statistical Analysis
Average values were expressed as mean ± SD. The statistical significance was assessed by Student’s t test using SPSS 10.0 statistical software or Excel statistics. P<0.05 was considered statistically significant.

Supplementary information associated experiments
OTUD-6B antibody production, OTUD-6B recombinant protein, Otud-6b enhancer luciferase plasmids, and Otud-6b siRNA design were shown in the (Materials and Methods S1).

Supporting Information
Materials and Methods S1
Supplementary experimental materials and methods. Found at: doi:10.1371/journal.pone.0014514.s001 (0.04 MB DOC)

Figure S1
Amino acid alignment of human OTUD-6B and OTUD-6A, and mouse Otud-6b by CLUSTAL 2.01.12. Found at: doi:10.1371/journal.pone.0014514.s002 (0.30 MB TIF)

Figure S2
Immunoblot for GST-DUB fusion protein. Purified GST (lanes 1 and 4), GST-OTUD-6B WT (lane 2), GST-OTUD-6B C188S (lane 3), GST-CYLD WT (lane 5), and GST-CYLD CS (lane 6) were analyzed by immunoblot with an anti-GST monoclonal antibody. Found at: doi:10.1371/journal.pone.0014514.s003 (0.17 MB TIF)

Figure S3
IL-2 doesn’t induce Otud-6b expression in Ba/F3 cells. A. Otud-6b RNA level in Ba/F3 cells after starvation and two hours stimulation with different concentrations (0, 0.01, 0.1, 1, 10, 100, and 1000 μM) of mouse IL-2. B. Otud-6b RNA level in Ba/F3 cells after starvation and stimulation with 10 μM mouse IL-2 for the indicated times (0, 0.5, 1, 2, 4, 8, 12, 16, and 24 hours). Found at: doi:10.1371/journal.pone.0014514.s004 (0.13 MB TIF)

Figure S4
Characterization of the rabbit antibody for endogenous OTUD-6B. A. Purified GST-OTUD-6A, GST-OTUD-6B WT, and GST-OTUD-6B C188S fusion protein was immunoblotted with anti-OTUD-6B antibody. Then, membranes were stripped and immunoblotted with anti-GST antibody. B. Ba/F3 cells were cultured with IL-3, then transfected with pcDNA3.1(+), HA-OTUD-6B WT, and HA-Otud-6b WT vectors. Immunoblot was performed with anti-OTUD-6B, anti-HA, and anti-GAPDH antibodies. Found at: doi:10.1371/journal.pone.0014514.s005 (0.24 MB TIF)

Figure S5
Knockdown of Otud-6b has no effect on cell growth. A & B. Ba/F3 cells were transfected with pSUPER mock, pSUPER Otud-6b siRNA-1 or 2 vectors, then stimulated with 10pM IL-3 for 2 hours after starvation. Total RNAs were analyzed by RT-PCR, and cell extracts were immunoblotted with anti-HA antibody. GAPDH was used as a loading control. C. pSUPER Mock and pSUPER Otud-6b siRNA-1 or 2 expressing Ba/F3 cells were seeded at 2×105 cells per ml and stimulated by 10 pM IL-3 and then analyzed by trypan blue exclusion assay 16 hours later. Found at: doi:10.1371/journal.pone.0014514.s006 (0.31 MB TIF)

Figure S6
Knockdown of OTUD-6B has no effect on cyclin D2 level. A. The levels of Cyclin D2 had no difference when OTUD-6B was knocked down in Hela cells. Hela cells transfected with pSUPER mock, pSUPER-OTUD-6B siRNA-1 or 2. Twenty four hours later, cell lysates were collected and then subjected to immunoblot with anti-OTUD-6B, anti-cyclin D2 antibodies. GAPDH was used as a loading control. B. The levels of Cyclin D2 had no difference when Otud-6b was knocked down in Ba/F3 cells. Ba/F3 cells were stimulated with 10 μM IL-3 for 2 hours. Found at: doi:10.1371/journal.pone.0014514.s007 (0.45 MB TIF)

Figure S7
Knockdown of AUF1, BRF1 and Dicer1 has no effect on Otud-6b mRNA level. A, B, and C. Ba/F3 cells were transfected with pSUPER mock, pSUPER AUF1, BRF1, and Dicer1 siRNA vectors. AUF1, BRF1, and Dicer1 mRNAs were analyzed by RT-PCR. GAPDH was used as a loading control. D and E. Otud-6b mRNA level in Ba/F3 cells transfected with pSUPER AUF1, BRF1, and Dicer1 siRNA vectors after starvation and stimulation with 10 pM mouse IL-3 for the indicated times (0, 0.5, 1, 2, 4, 8, 12, 16, and 24 hours). F. Ba/F3 cells were transfected with pSUPER Mock, AUF1, BRF1, and Dicer1 siRNA vectors. 24 hours later, cells were stimulated by 10 μM IL-3 and incubated with 15 ug/ml Act.D for the time indicated. The qRT-PCR was performed to detect Otud-6b mRNA remaining at each time point. Found at: doi:10.1371/journal.pone.0014514.s008 (0.70 MB TIF)

Figure S8
Scrambled vector has no effect on the Otud-6b mRNA rapid degradation after induction. A. pSUPER Scramble, pSUPER siTTP-1 or 2 and HA-TTP vectors co-expressing Ba/F3 cells were seeded at 2×105 cells per ml. Scrambled siRNA sequence is 5'- GAGGAGCCGACGCCTTAATA-3'. Cell extracts were immunoblotted with anti-HA antibody. GFP was used as a loading control. B. Otud-6b RNA level in Ba/F3 cells transfected with pSUPER scramble, siTTP-1,2 vectors after starvation and stimulation with 10 pM mouse IL-3 for the indicated times (0, 0.5, 1, 2, 4, 8, 12, 16, and 24 hours). Found at: doi:10.1371/journal.pone.0014514.s009 (0.31 MB TIF)

Figure S9
The Otud-6b gene contains a cytokine-inducible enhancer element. A. Alignment of the cytokine-inducible enhancer regions of mouse Dub-1a, Dub-1, Dub-2a, and the promoter region of mouse Otud-6b. The putative enhancer region of mouse Otud-6b contains one ETS and one GATA TF binding site. Mutation sites in motifs are indicated with arrows. B. Luciferase activity was assayed in Ba/F3 cells that were transfected with the indicated constructs. The cells were starved and then treated with or without 10 pM mouse IL-3 stimulation. Luciferase assays were performed after 16 hours of treatment. All ratios are from three independent experiments. P value was calculated by student’s t test. Found at: doi:10.1371/journal.pone.0014514.s010 (0.41 MB TIF)

Figure S10
P27 and cyclin E levels in Ba/F3 cells and Hela cells when overexpression of OTUD-6B WT and OTUD-6B CS. A and B. Immunoblot for p27 and cyclin E on Ba/F3 cells and Hela cells overexpressing pcDNA3.1(+), HA-OTUD-6B WT, and HA-OTUD-6B C188S. C. Cell cycle profile of PI-stained Hela cells when overexpressing pcDNA3.1(+), HA-OTUD-6B WT, and HA-OTUD-6B C188S. Found at: doi:10.1371/journal.pone.0014514.s011 (0.51 MB TIF)
Table S1 Primers used for RT-PCR in cell cycle regulators screen.
Found at: doi:10.1371/journal.pone.0014514.s012 (0.04 MB DOC)

Table S2 Primers used for real time PCR in cell cycle regulators screen.
Found at: doi:10.1371/journal.pone.0014514.s013 (0.04 MB DOC)

Table S3 Subcloning primers used in this study.
Found at: doi:10.1371/journal.pone.0014514.s014 (0.05 MB DOC)

References

1. DeSalle LM, Pagano M (2001) Regulation of the G1 to S transition by the ubiquitin pathway. FEBS Lett 490: 179–189.
2. Muratani M, Taneys WP (2003) How the ubiquitin-proteasome system controls transcription. Nat Rev Mol Cell Biol 4: 192–201.
3. Loureiro J, Poehl HL (2006) Antigen presentation and the ubiquitin-proteasome system in host-pathogen interactions. Adv Immunol 92: 225–305.
4. Song L, Rape M (2008) Reverse the curse- the role of deubiquitination in cell cycle control. Curr Opin Cell Biol 20: 156–163.
5. Zhu Y, Carroll M, Papa FR, Hochstrasser M, D’Andrea AD (1996) DUB-1, a deubiquitinating enzyme with growth-suppressing activity. Proc Natl Acad Sci U S A 93: 3275–3279.
6. Burrows JF, McGrattan MJ, Rascle A, Humbert M, Baek KH, et al. (2004) DUB-3, a cytokine-inducible deubiquitinating enzyme that blocks proliferation. J Biol Chem 279: 13993–14000.
7. Amerik AY, Hochstrasser M (2004) Mechanism and function of deubiquitinating enzymes. Biochem Biophys Acta 1695: 189–207.
8. Hanna J, Hathaway NA, Tone Y, Crosas B, Elsasser S, et al. (2006) Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasome degradation. Cell 127: 99–111.
9. Komander D, Clague MJ, Urbe S (2009) Breaking the chains: structure and function of the deubiquitinas. Nat Rev Mol Cell Biol 10: 550–563.
10. Caligaris-Cappio F, Ferrari M (1996) B cells and their fate in health and disease. Immunol Today 17: 205–208.
11. Milne CD, Paige CJ (2006) IL-7: a key regulator of B lymphopoiesis. Semin Immunol 18: 20–30.
12. Acosta-Rodriguez EV, Merino MC, Moates CL, Motran CC, Gruppi A (2007) Cytokines and chemokines shaping the B-cell compartment. Cytokine Growth Factor Rev 18: 73–83.
13. Baek KH, Kim MS, Kim YS, Shin JM, Choi HK (2004) DUB-1A, a novel deubiquitinating enzyme subfamily member, is polyubiquitinated and cytokine-inducible in B-lymphocytes. J Biol Chem 279: 2560–2576.
14. Zhu Y, Lambert K, Cordless C, Copeland NG, Gilbert DJ, et al. (1997) DUB-2 gene is specifically induced by the betac subunit of interleukin-3 receptor. Mol Cell Biol 16: 4088–4092.
15. Zhu Y, Pless M, Inhorn R, Mathey-Prevot B, D’Andrea AD (1996) The murine DUB-1 gene encodes a protein that binds to the APE subunit of interleukin-3 receptor. Mol Cell Biol 16: 4088–4092.
16. Knag H (1993) The role of mRNA degradation in the regulated expression of bacterial photosynthesis genes. Mol Microbiol 1: 1–7.
17. Chen CY, Gherzi R, Ong SE, Chan EL, Rajmakers R, et al. (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell 107: 451–464.
18. Levy NS, Chung H, Furneaux H, Levy AP (1998) Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J Biol Chem 273: 6417–6423.
19. Share G, Kamen R (1986) A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46: 659–667.
20. Hau HH, Wahl RI, Ogilvie RL, Williams DA, Reilly CS, et al. (2007) Tristetraprolin recruits functional mRNA decay complexes to ARE sequences. J Cell Biolom 100: 1477–1492.
21. Nakayama KI, Hatakeyama S, Nakayama K (2001) Regulation of the cell cycle at the G1-S transition by proteolysis of cyclin E and p27Kip1. Biochem Biophys Res Commun 282: 853–860.
22. Sherr CJ (1996) Cancer cell cycles. Science 274: 1672–1677.
23. Whitfield ML, George LK, Grant GD, Perou CM (2006) Common markers of proliferation. Nat Rev Cancer 6: 99–106.
24. Bouchard C, Dittrich O, Kiernar H, Dohmann K, Menckel A, et al. (2001) Regulation of cyclin D2 gene expression by the Myc/Max/Mad network. J Cell Biol 150: 1477–1492.
25. Iwai Y, Aru J, Masuda H, Iwade H, Kato Y, et al. (2000) The PHD finger domain interacts with the cyclin D1 promoter. Genes Dev 14: 2525–2537.
26. Nakayama KI, Hatakeyama S, Nakayama K (2001) Regulation of the cell cycle at the G1-S transition by proteolysis of cyclin E and p27Kip1. Biochem Biophys Res Commun 282: 853–860.
27. Sherr CJ (1996) Cancer cell cycles. Science 274: 1672–1677.
28. Whitfield ML, George LK, Grant GD, Perou CM (2006) Common markers of proliferation. Nat Rev Cancer 6: 99–106.
29. Bouchard C, Dittrich O, Kiernar H, Dohmann K, Menckel A, et al. (2001) Regulation of cyclin D2 gene expression by the Myc/Max/Mad network. J Cell Biol 150: 1477–1492.
30. Iwai Y, Aru J, Masuda H, Iwade H, Kato Y, et al. (2000) The PHD finger domain interacts with the cyclin D1 promoter. Genes Dev 14: 2525–2537.
31. Sherr CJ (1996) Cancer cell cycles. Science 274: 1672–1677.
32. Whitfield ML, George LK, Grant GD, Perou CM (2006) Common markers of proliferation. Nat Rev Cancer 6: 99–106.
33. Bouchard C, Dittrich O, Kiernar H, Dohmann K, Menckel A, et al. (2001) Regulation of cyclin D2 gene expression by the Myc/Max/Mad network. J Cell Biol 150: 1477–1492.
34. Iwai Y, Aru J, Masuda H, Iwade H, Kato Y, et al. (2000) The PHD finger domain interacts with the cyclin D1 promoter. Genes Dev 14: 2525–2537.
35. Sherr CJ (1996) Cancer cell cycles. Science 274: 1672–1677.
36. Whitfield ML, George LK, Grant GD, Perou CM (2006) Common markers of proliferation. Nat Rev Cancer 6: 99–106.
37. Bouchard C, Dittrich O, Kiernar H, Dohmann K, Menckel A, et al. (2001) Regulation of cyclin D2 gene expression by the Myc/Max/Mad network. J Cell Biol 150: 1477–1492.
38. Iwai Y, Aru J, Masuda H, Iwade H, Kato Y, et al. (2000) The PHD finger domain interacts with the cyclin D1 promoter. Genes Dev 14: 2525–2537.
39. Sherr CJ (1996) Cancer cell cycles. Science 274: 1672–1677.