Introduction

Ubiquitin (Ub) is a highly conserved 8 kDa protein that covalently attaches to the lysine (Lys) residues of target proteins. Ubiquitylation is performed by Ub-activating enzymes (E1s), Ub conjugating enzymes (E2s), and Ub ligases (E3s) [1,4,5]. Proteins are reliant on the quantity of attached Ub moieties and can be modified through mono-ubiquitination or polyubiquitination. The former means that a single Ub moiety is attached to one or several lysine residues on the substrate; whereas the latter means that the substrate protein is modified by a chain of over four Ub moieties linked to one of the seven lysine residues in Ub [2]. The activity and degradation of proteins that control key processes, such as cell cycle progression, differentiation, and apoptosis, are regulated by the Ub/proteasome system [3]. The Ub chain assembly factor (E4), a new class of ubiquitylation regulator of p53 and only catalyzes p53 monoubiquitination or polyubiquitination.

The loss of p53 has a vital role in carcinogenesis, and the regulation of p53 expression and its stability are essential to maintain normal cell growth [7]. Hdm2 is a critical negative regulator of p53 and only catalyzes p53 monoubiquitination or multiple monoubiquitination. Hdm2 does not polyubiquitinate p53 [7–8]. In 2011, Wu et al. showed that UBE4B physically interacts with p53 and Hdm2 and negatively regulates p53 stability and function [7]. However, no one has determined whether UBE4B promotes p53 degradation in breast cancer. In this study, UBE4B promoted the degradation and ubiquitination of p53 to inhibit the apoptosis of cancer cells and promote tumorigenesis. Our results indicate that UBE4B regulates p53 in breast cancer and could be a viable target for developing new therapeutic strategies for breast cancer treatment.
primary antibodies. The membranes were then incubated for 1 h with the HRP-conjugated secondary antibodies at room temperature and shaken slightly. Immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrates (Thermo, USA).

Immunoprecipitation

The cultured cells were lysed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer composed of 50 mM HEPES (pH 7.2), 250 mM NaCl, 10% glycerol, 1% NP-40, 1.0 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol, and 10 mM phenylmethylsulfonyl fluoride supplemented with Protease Inhibitor Cocktail Tablets (Roche). The cell lysates were incubated for 8 h at 4°C on agarose with the indicated primary antibodies and then washed according to the manufacturer’s protocol. The samples were separated using SDS-PAGE and western blot analysis.

In vivo ubiquitylation assay

The cells were treated with 20 μM MG132 proteasome inhibitor for 6 h. The cells were washed with PBS and lysed in 0.5 ml of HEPES buffer (20 mM HEPES, pH 7.2, 50 mM NaCl, 1 mM NaF, 0.5% Triton-X 100) supplemented with 0.1% SDS and protease-inhibitor cocktail (Roche, Germany). The lysates were centrifuged to obtain the cytosolic proteins. Briefly, individual samples were incubated for 6 h with primary antibodies and then with protein A/G-agarose beads (Santa Cruz) for another 8 h at 4°C. The beads were then washed three times with HEPES buffer. The proteins were released from the beads by boiling in a 40 μl of 2× SDS-PAGE sample buffer for 10 min. The samples were subjected to western blot analysis.

Apoptosis assays

MCF-7 cells were cotransfected with a p53 expression plasmid combined with an empty vector, UBE4B, and Hdm2 for 48 h. Another group of MCF-7 cells were transfected with UBE4B siRNA, Hdm2 siRNA, or control siRNA. The cells were then treated with cisplatin (10 μM, 24 h, Sigma–Aldrich, USA). After
incubation, the cells were washed with PBS solution and stained in the dark with Annexin V and propidium iodide (PI) for 10 min at room temperature. Apoptotic cells were then determined via flow cytometry.

Nude mouse experiments
A total of 20 female 6-week-old nude mice weighing 20 g to 22 g were purchased from the animal center of Shandong University. The mice were fed a standard rodent diet and provided with water in an aseptic laminar flow room at 25°C. Then, 100 μl of the cell suspensions (containing 1×10^7 logarithmic growth phase tumor cells) were subcutaneously injected into the mice. Tumor growth was observed every week for 6 weeks by measuring the length and width using a Vernier caliper. Tumor volume was calculated from the lengths and widths using the following formula: tumor volume = (length × width^2)/2. The Animal Ethics Review Committee of Shandong University approved the animal research of this study.

Statistical analysis
Data are presented as mean ± standard deviation. A t-test was used to analyze all results. For all tests, differences with \( P < 0.05 \) were considered statistically significant.

Results
UBE4B and p53 are expressed in breast cancer tissues and UBE4B enhances p53 ubiquitination
We detected UBE4B protein expression in normal breast tissues and breast cancer tissues. UBE4B expression was higher in breast cancer tissues than in normal breast tissues (Fig. 1a). We then examined the protein expression of UBE4B and p53 in the breast cancer tissues using western blot analysis. The levels of p53 and UBE4B mainly showed an opposite state (Fig. 1b). HA-tagged ubiquitin (HA-Ub) was coexpressed in MCF-7 cells with plasmids encoding p53 or in combination with either UBE4B, Hdm2, or UBE4B and Hdm2. p53 was immunoprecipitated and analyzed via western blot analysis using anti-HA antibodies to detect ubiquitinated p53. The p53 protein was heavily ubiquitinated in the presence of Hdm2 or UBE4B and was more heavily ubiquitinated by the coexpression of Hdm2 and UBE4B. The level of p53 ubiquitination was evidently decreased when p53 and UBE4B-siRNA were coexpressed (Fig. 1c). The same condition was observed in the SK-BR-3 cells (Fig. 1d).

Interactions of UBE4B with Hdm2 and with p53 in vivo
The interaction of UBE4B with Hdm2 and with p53 was tested in MCF7 cells. Immunoprecipitation was performed on the cultured MCF7 cells using anti-UBE4B antibodies. The physiologic interaction between UBE4B and p53 was evaluated using
western blot analysis with the respective antibodies (Fig. 2a). Immunoprecipitation in MCF7 cells was conducted using the anti-p53 antibody (Fig. 2b). The results showed that UBE4B interacts with p53. Similar experiments were conducted to determine whether UBE4B interacts with Hdm2 (Figs. 2c and 2d). The results validated the hypothesis that UBE4B interacts with Hdm2. The combined results proved that UBE4B interacts with both p53 and Hdm2.

UBE4B negatively regulates p53 and promotes p53 degradation in the presence of Hdm2.

p53−/− MCF-7 cells (p53 KO) were cotransfected with Flag-UBE4B, Myc-Hdm2 with p53 were cotransfected into p53−/− MCF-7 cells (p53 KO), and analyzed via western blot analysis using Flag-specific, Myc-specific, and p53-specific antibodies. (b) MCF-7 cells were cotransfected with UBE4B-siRNA or Hdm2-siRNA, and then analyzed via western blot analysis using p53-specific, UBE4B-specific, or Hdm2-specific antibodies. (c) The increasing amounts of UBE4B plasmid were transfected into WT p53-expressing MCF-7 cells. The transfected cells were analyzed via western blot analysis using anti-p53, anti-UBE4B and anti-Hdm2 antibodies, with actin as the internal reference gene. (d) MCF-7 cells were transfected with control siRNA or UBE4B siRNA. After 30 h, the cells were transfected further with Hdm2 expression plasmid and analyzed via western blot analysis using p53-specific, UBE4B-specific, and Hdm2-specific antibodies.

Figure 3. UBE4B negatively regulates p53 and promotes p53 degradation in the presence of Hdm2. (a) Plasmids expressing Flag-UBE4B, Myc-Hdm2 with p53 were cotransfected into p53−/− MCF-7 cells (p53 KO), and analyzed via western blot analysis using Flag-specific, Myc-specific, and p53-specific antibodies. (b) MCF-7 cells were cotransfected with UBE4B-siRNA or Hdm2-siRNA, and then analyzed via western blot analysis using p53-specific, UBE4B-specific, or Hdm2-specific antibodies. (c) The increasing amounts of UBE4B plasmid were transfected into WT p53-expressing MCF-7 cells. The transfected cells were analyzed via western blot analysis using anti-p53, anti-UBE4B and anti-Hdm2 antibodies, with actin as the internal reference gene. (d) MCF-7 cells were transfected with control siRNA or UBE4B siRNA. After 30 h, the cells were transfected further with Hdm2 expression plasmid and analyzed via western blot analysis using p53-specific, UBE4B-specific, and Hdm2-specific antibodies.

doi:10.1371/journal.pone.0090154.g003

UBE4B promotes p53 degradation

PLOS ONE | www.plosone.org 4 February 2014 | Volume 9 | Issue 2 | e90154

UBE4B inhibits p53-dependent apoptosis and promotes tumorigenesis

The decrease in breast cancer cells apoptosis induced by UBE4B overexpression in contrast to that of p53 indicated that UBE4B inhibits cancer cell apoptosis (Fig. 4a).
UBE4B and Hdm2 more significantly prevented apoptosis than cotransfected with UBE4B-siRNA, Hdm2-siRNA, or control siRNA (Fig. 4b). To test the in vivo tumorigenic efficacy of UBE4B, we established a xenograft animal model in nude mice. We subcutaneously injected MCF-7 expressing vector encoding UBE4B or an empty vector into the mice. We found that UBE4B-overexpressing tumors have significantly faster growth rates than tumors with normal UBE4B expression. By contrast, the tumors formed by cells transfected with empty vector were much smaller (Fig. 4c).

Figure 4. UBE4B inhibits p53-dependent apoptosis and promotes tumorigenesis. (a) p53/−/− MCF-7 cells (p53 KO) were cotransfected with a p53 expression plasmid in combination with an empty vector, Flag-UBE4B, and Myc-Hdm2 (* P<0.05). (b) MCF-7 cells were transfected with UBE4B siRNA, Hdm2 siRNA, or control siRNA. (a and b) The cells were treated with cisplatin after 48 h, and all of cells were then analyzed via flow cytometry (* P<0.05). (c) Six-week-old female nude mice weighing 20 g to 22 g were subcutaneously injected with 1×10⁶ MCF-7 cells expressing vector encoding UBE4B or an empty vector. Tumor growth was observed every week for 6 weeks by measuring their length and width using a Vernier caliper. Tumor volume = length × width²/2 (* P<0.05).

doi:10.1371/journal.pone.0090154.g004
Discussion

The p53 protein and its gene, TP53, were identified in 1979 [9–11]. p53 plays a critical role in cancer prevention because it suppresses tumorigenesis by inducing cell cycle arrest and apoptosis through its transcriptional activity. p53 is a tumor suppressor gene most frequently inactivated in cancer [12]. Aside from its role in tumorigenesis, p53 also affects the effectiveness of platinum therapy [13]. Breast cancer is the most common cancer in women worldwide [14]. Breast cancer is over 100 times more common in women than in men. The efficacy of p53 activity varies between different stages of breast cancer [15]. Breast cancer cells with wild-type p53 often have high levels of the oncogenic protein Hdm2, which suggests that Hdm2 may block p53 function [16–18]. The Hdm2 oncoprotein is a well-studied E3 Ub that targets the p53 tumor suppressor protein for proteasomal degradation [19]. The ubiquitination of p53 by Hdm2 generally inhibits p53 function. Hdm2 only catalyzes p53 monoubiquitination or multiple monoubiquitination in vitro and in vivo. Hdm2 does not polyubiquitinate p53 in vitro [20,21]. In 2011, Wu et al. reported that UBE4B is essential for Hdm2-mediated p53 polyubiquitination and degradation [7], which suggests that increased UBE4B activity may be oncogenic by reducing the abundance of p53. In this study, we revealed the physiological significance of UBE4B in p53 degradation in breast cancer.

The UBE4B gene is located in the 1p36 region and encodes an E3/E4 Ub ligase [6] that promotes the degradation of a pathologic form of ataxin-3 [22]. UBE4B levels reportedly influenced neuroblastoma tumor cell proliferation, and UBE4B was overexpressed in various brain tumors [7,23]. In this paper, we first reported UBE4B protein expression in breast cancer tissues and we determined that breast cancer tissues have significantly higher levels of UBE4B than normal tissues. The amount of p53 was inversely proportional to that of UBE4B. Furthermore, the experiments determined that UBE4B ubiquitinates p53 and promotes the Hdm2-mediated ubiquitination of p53 in the breast cancer cell line. In addition, UBE4B physically interacted with Hdm2 and p53 in vitro, and UBE4B negatively regulates p53. Finally, UBE4B inhibits p53-dependent apoptosis and promotes tumorigenesis in breast cancer. All of the results illustrate that UBE4B is essential for Hdm2-induced p53 degradation and UBE4B may promote the formation of breast cancer.

Overall, we have identified how UBE4B can reduce the amount of p53 protein and promote the Hdm2 mediated ubiquitination of p53 in breast cancer. These findings proved further that UBE4B is an oncogene and may promote the formation of breast cancer tumors. UBE4B will ultimately provide the most effective way to take advantage of the ever-increasing list of new biological therapies for breast cancer.

Author Contributions

Conceived and designed the experiments: Ying Zhang YL HG. Performed the experiments: Ying Zhang YL. Analyzed the data: Ying Zhang YL. Contributed reagents/materials/analysis tools: Ying Zhang YL. Wrote the paper: Ying Zhang Yongyang Zhang. Tumor specimens: HG.

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