The forkhead transcription factor FOXM1 has a key role in DNA damage response, and its deregulated overexpression is associated with genotoxic drug resistance in breast cancer. However, little is known about the posttranslational mechanisms by which FOXM1 expression is regulated by genotoxic agents and how they are deregulated in resistant cells. Initial co-immunoprecipitation studies verified previous proteomic analysis finding that the OTUB1 is a novel FOXM1-interacting protein. Western blot analysis showed that both OTUB1 and FOXM1 expression reduced upon genotoxic agent treatment in MCF-7 cells, but remained relatively constant in resistant cells. FOXM1 expression reduced upon OTUB1 depletion by siRNA and increased with OTUB1 overexpression in MCF-7 cells, arguing that OTUB1 positively regulates FOXM1 expression. In agreement, co-immunoprecipitation experiments demonstrated that FOXM1 expression is associated with OTUB1 binding but inversely correlates with conjugation to the protein degradation-associated Lys-48-linked ubiquitin-chains. Overexpression of wild-type (WT) OTUB1, but not the OTUB1(C91S) mutant, disrupted the formation of Lys48-linked ubiquitin-conjugates on FOXM1. Importantly, knockdown of OTUB1 by siRNA resulted in an increase in turnover of FOXM1 in MCF-7 cells treated with the protein synthesis inhibitor cycloheximide, whereas overexpression of WT OTUB1, but not the OTUB1(C91S) mutant, significantly enhances the half-life of FOXM1. In addition, proliferative and clonogenic assays also show that OTUB1 can enhance the proliferative rate and epirubicin resistance through targeting FOXM1, as OTUB1 has little effect on FOXM1-deficient cells. The physiological relevance of the regulation of FOXM1 by OTUB1 is further underscored by the significant correlations between FOXM1 and OTUB1 expression in breast cancer patient samples. Cox-regression survival analysis indicates that OTUB1 overexpression is linked to poorer outcome in particular in patients treated with chemotherapy. Collectively, these data suggest that OTUB1 limits the ubiquitination and degradation of FOXM1 in breast cancer and has a key role in genotoxic agent resistance.

Oncogene (2016) 35, 1433–1444; doi:10.1038/onc.2015.208; published online 6 July 2015

INTRODUCTION

Breast cancer is one of the most prevalent causes of death in women worldwide. Genotoxic anti-cancer agents, including anthracyclines, platinum compounds, methylating agents and ionizing irradiation, are used widely to treat breast cancer patients who are not suitable for hormonal therapy and those with advanced or metastatic cancer. These genotoxic agents are also often used in the adjuvant setting, particularly after surgery, to prevent the return of the disease. However, resistance to these agents often emerges in patients, and this leads to suboptimal efficacy and disease relapse.1 The cellular response to DNA damage is a key determinant of the efficacy of these genotoxic agents, and these reactions include initiation of DNA damage repair response, cell cycle-checkpoint activation and induction of apoptosis or senescence. These processes ultimately govern cell fate and sensitivity to radiotherapy or chemotherapy. Conversely, a deregulated DNA damage response can lead to resistance to these anticancer agents.

Substantial evidence has accumulated to indicate that the Forkhead box M1 (FOXM1) transcription factor has a central role in cell proliferation, migration, invasion, angiogenesis, stem cell renewal, DNA damage repair and cellular senescence, which impact tumour initiation, progression, metastasis, angiogenesis and drug sensitivity. Recent research also indicates that deregulated FOXM1 overexpression confers genotoxic and other chemotherapeutic agent resistance in cancer.2–7 There is already good evidence that FOXM1 acts as a mediator of DNA damage response as well as a modulator of genotoxic agent sensitivity.4–6,8–10 Even though deregulated FOXM1 overexpression is considered key to the development of genotoxic agent resistance, the specific mechanisms involved in FOXM1 deregulation remain unknown. Therefore, a better understanding of the mechanisms that regulates FOXM1 expression in response to genotoxic agents and how FOXM1 is deregulated in resistant cancer cells is of importance for designing new therapeutic approaches directed to this degradation pathway.

Epirubicin is an anthracycline genotoxic drug commonly used for treating breast cancer.11 FOXM1 is downregulated by epirubicin at the transcriptional levels in breast cancer cells.8,12,13 However, the fact that FOXM1 protein expression
OTUB1 regulates FOXM1 expression in response to epirubicin in breast cancer cells

To explore the potential regulation of FOXM1 by OTUB1 in response to genotoxic agents, we investigated the expression of OTUB1 and FOXM1 in the epirubicin-sensitive MCF-7 and epirubicin-resistant MCF-7EpiR breast carcinoma cell lines following epirubicin treatment. Western blot results showed that both OTUB1 and FOXM1 expressed at higher levels in the resistant MCF-7EpiR cells compared with the parental MCF-7 cells. Moreover, both OTUB1 and FOXM1 decreased in expression levels in response to epirubicin in the sensitive cells, but were moderately induced in the resistant cells (Figure 2a). Consistently, the expression of the FOXM1 downstream target cyclin B1 also displayed a similar expression pattern as FOXM1 and OTUB1. Furthermore, epirubicin treatment also induced the accumulation of cleaved-PARP, indicative of apoptosis, in the sensitive MCF-7 but not in the resistant MCF-7EpiR cells, confirming the differential drug sensitivity of these MCF-7 cells. Tubulin was also immunoblotted for as a control to ensure equal loading. Next, we studied the expression of OTUB1 and FOXM1 in response to another DNA-damaging agent, γ-irradiation. To this end, MCF-7 and MCF-7EpiR cells were harvested for western blot analysis at 24 h after exposure to various doses of γ-irradiation (Figure 2b). The results again revealed that the expression levels of OTUB1 and FOXM1 were higher in the resistant compared with the sensitive MCF-7 cells. Although γ-irradiation caused an induction in OTUB1 and a corresponding increase in FOXM1 expression in a dose-dependent manner in the MCF-7EpiR cells, the induction of OTUB1 by γ-irradiation was not matched by a similar induction of FOXM1 expression in the MCF-7 cells, which likely reflects the low baseline levels of OTUB1 in MCF-7 cells. Nevertheless, the correlations between the kinetics of OTUB1 and FOXM1 expression in the sensitive and resistant MCF-7 cells in response to DNA-damaging agents support the notion that OTUB1 restricts the downregulation of FOXM1 expression in response to DNA damage and genotoxic agents.

FOXM1 expression is positively regulated by OTUB1 and negatively by the proteasome pathway

To investigate the idea that OTUB1 modulates FOXM1 expression further, we examined the effects of silencing OTUB1 and FOXM1 on the expression of endogenous OTUB1 and FOXM1 protein in MCF-7 cells. The results showed that in MCF-7 cells OTUB1 depletion culminated in the downregulation of FOXM1, but not vice versa (Figure 2c), suggesting that OTUB1 positively regulates FOXM1 expression. In agreement, OTUB1 overexpression also caused an increase in FOXM1 levels (Figure 2d). It is noteworthy that depletion of FOXM1 had little effect on OTUB1 mRNA levels (Supplementary Figure S2). Together these results suggest that OTUB1 regulates FOXM1 expression primarily at the translational or posttranslational levels (Figure 2d). The results also indicate that OTUB1 has a role in modulating the steady-state level of the FOXM1 protein.

Given that OTUB1 has been shown to restrict the ubiquitination and proteasomal degradation of target proteins in response to DNA damage, we asked whether FOXM1 is also downregulated through protein degradation by the ubiquitin–proteasome pathway and tested the effect of the proteasome inhibitor MG132 treatment on the expression of endogenous FOXM1 in the absence or presence of epirubicin in MCF-7 cells (Figure 2e). Treatment with MG132 essentially prevented the downregulation in FOXM1 expression levels by epirubicin, indicating that the downregulation of FOXM1 expression in response to epirubicin is, at least partially, due to proteasomal degradation. It is also notable that MG132 enhanced the expression of FOXM1 in untreated cells, suggesting that FOXM1 is continuously downregulated by proteasomal degradation at the steady state level.
OTUB1 binding promotes FOXM1 expression and attenuates Lys-48 linked ubiquitination in MCF-7 breast cancer cells.

We next examined whether OTUB1 binding is associated with the suppression of FOXM1 ubiquitination and downregulation in the epirubicin-sensitive and -resistant MCF-7 cells. To this end, we studied by co-immunoprecipitation (co-IP) the levels of OTUB1 and ubiquitination chains associated with FOXM1 in MCF-7 and MCF-7EpiR cells in response to epirubicin (Figure 3a and Supplementary Figure S3). The result showed that epirubicin treatment caused a prominent decrease in FOXM1 levels, which was associated with decreased interactions with OTUB1 in the sensitive MCF-7 cells. By contrast, FOXM1 protein levels and its association with OTUB1 remained relatively constant in the resistant cells upon epirubicin treatment. There was also an apparent loss of polyubiquitin chains in the FOXM1 complexes upon epirubicin treatment in the MCF-7 cells, but this is likely the result of a decline in endogenous FOXM1 levels upon epirubicin treatment. However, when we quantified the FOXM1 and Lys-48-linked polyubiquitin conjugates from the same immunoprecipitates, we found that the relative levels of Lys-48-linked polyubiquitin chains co-precipitated with FOXM1 actually increased with epirubicin treatment in MCF-7 cells (Figure 3b; Supplementary Figure S4). This induction in

Figure 1. FOXM1 complexes with OTUB1 in MCF-7 and MCF-7EpiR cells. Co-immunoprecipitation (co-IP) was performed with an IgG antibody control, a FOXM1 (α or anti-FOXM1) or an OTUB1 (αOTUB1) antibody on lysates from MCF-7 and MCF-7EpiR cells; Inputs (1/20 of IP), and IP products with IgG and specific antibodies were resolved on western blot and probed for (a) FOXM1 and (b) OTUB1. FOXM1* represents a FOXM1 species associated with its SUMOylation. (c) MCF-7 and MCF-7EpiR cells were treated with epirubicin (1 μM) for 0, 6 and 24 h. Co-IP was performed with an IgG antibody control and a FOXM1 antibody (αFOXM1); Inputs (1/10 of IP), and IP products with IgG and a FOXM1 antibody (αFOXM1) were resolved on western blot and probed for OTUB1. (d) Lysates prepared from epirubicin-treated MCF-7 and MCF-7EpiR cells as in (c) were precipitated with an IgG antibody control, a FOXM1 (αFOXM1) and an OTUB1 (αOTUB1) antibody and probed for FOXM1 expression.
Lys48-linked polyubiquitination by epirubicin in MCF-7 cells was also associated with a decline in the FOXM1-OTUB1 interaction and FOXM1 expression in the MCF-7 cells. Quantification of the immunoblot signals indicated the kinetics for the disassociation of FOXM1-OTUB1 complexes occurred at a faster rate than the downregulation of FOXM1 expression, indicating that OTUB1 disassociation precedes FOXM1 ubiquitination and degradation in response to epirubicin in MCF-7 cells (Figure 3b). By comparison, the relative levels of total and Lys48-linked polyubiquitin chains, FOXM1 protein and FOXM1-OTUB1 complexes remained relatively constant in the resistant cells. It is noteworthy that the kinetics for Lys63-linked polyubiquitin downregulation was similar to that of FOXM1 expression, indicating that the Lys63-linked polyubiquitin chains are less likely to be involved in FOXM1 degradation in response to epirubicin (Supplementary Figure S5). These results are consistent with the notion that OTUB1 binds to FOXM1 and limits its ubiquitination and degradation in MCF-7 breast cancer cells.

Polyubiquitin chains conjugated to FOXM1 are suppressed by OTUB1

We next investigated whether the polyubiquitin chains detected are directly conjugated to FOXM1. To confirm direct covalent linkage of Ubiquitin to FOXM1, we expressed a GFP-FOXM1 in the presence or absence of a His-tagged Ubiquitin, and pulled down the His-tagged ubiquitinated proteins under denaturing conditions after incubation with the proteasome inhibitor MG132 (Figure 4a). The purified His-tagged Ubiquitin-conjugated proteins were then immunoblotted with an anti-FOXM1 antibody, which detected the Ubiquitin-conjugated forms of FOXM1 as smears above the predicted GFP-FOXM1 molecular weight of 160kDa, indicative of polyubiquitin chains. We next studied by co-immunoprecipitation whether overexpression of OTUB1 can repress the conjugation of K48-linked polyubiquitin chains, which are known to promote target protein degradation, on FOXM1 in MCF-7 cells. The results showed that the FOXM1 immunoprecipitates contained higher levels of Lys48-linked polyubiquitin chains in both untreated and epirubicin-treated MCF-7 cells when compared with the epirubicin-resistant MCF-7EpiR cells and two MCF-7 cell lines, one stably overexpressing OTUB1 and the other Flag-OTUB1 (Figure 4b and Supplementary Figure S6). The results also demonstrated that the levels of Lys48-linked polyubiquitin conjugates associated with FOXM1 increased upon epirubicin treatment. We next investigated whether the deubiquitinase activity of OTUB1 is required for FOXM1 stabilization and K48-polyubiquitin chain formation. To achieve this, we transfected
MCF-7 with either the empty expression vector, wild-type (WT) OTUB1, or the OTUB1(C91S) mutant and studied the levels of K48-polyubiquitin chains associated with FOXM1 by immunoprecipitation (Figure 4c). The FOXM1 co-immunoprecipitation analysis showed that overexpression of OTUB1, but not the OTUB1(C91S) mutant, substantially depleted the levels of K48-polyubiquitin chains and induced FOXM1 expression, particularly following epirubicin treatment (Figure 4c). These results suggest that the deubiquitinase activity of OTUB1 is required for the suppression of Lys48-linked FOXM1 polyubiquitination chains and for enhancing FOXM1 stability.

OTUB1 enhances FOXM1 stability in response to epirubicin treatment

To examine the role of OTUB1 in regulating FOXM1 protein stability, MCF-7 cells were transfected with either control vector, OTUB1(WT) or OTUB1(C91S), and subjected to treatment with epirubicin and then the translation inhibitor cycloheximide (Figure 5). Inhibition of de novo protein synthesis by cycloheximide caused a decline in FOXM1 protein levels in MCF-7 cells treated with epirubicin. Under these conditions, the rate of FOXM1 loss was reduced in MCF-7 cells transfected with OTUB1(WT) compared with cells transfected with the empty vector control. By contrast, the rates for the decline in FOXM1 levels were similar in MCF-7 cells transfected with the OTUB1(C91S) mutant and the empty vector. These findings indicate that the degradation of FOXM1 is impaired by overexpression of WT OTUB1 but not a mutant that lacks catalytic activity. Conversely, knockdown of OTUB1 by siRNA smart pool resulted in decreased half-life of FOXM1 in MCF-7 cells treated with epirubicin compared with cells transfected with non-silencing control siRNA. Together, these results suggest that the suppression of FOXM1 degradation by OTUB1 in response to epirubicin requires its deubiquitinating catalytic activity, further confirming that FOXM1 is a novel target of the deubiquitinase OTUB1. As an internal control, we measured the turnover of cyclin B1, a target of FOXM1 and not OTUB1, in response to epirubicin treatment. Taken together, the results provided strong evidence that OTUB1 suppresses FOXM1 ubiquitination and degradation.
OTUB1 promotes cell proliferation and epirubicin resistance through targeting FOXM1

Recent evidence demonstrates that FOXM1 can enhance cancer cell proliferation and protect cells from genotoxic agent-induced cell death by enhancing DNA damage repair.\(^5,8,12\) To test the possible function of OTUB1 in the regulation of breast cancer cell proliferation and DNA-damaging agent resistance, we transiently transfected MCF-7 cells with an OTUB1 expression plasmid and OTUB1 siRNA pool, and studied their effects on MCF-7 cell proliferation. The result showed that overexpression of OTUB1...
OTUB1 regulates FOXM1 degradation
U Karunarathna et al

Figure 5. OTUB1 suppresses the degradation of FOXM1 in epirubicin-treated MCF-7 cells. (a) MCF-7 cells transfected with control non-silencing siRNA or Smart Pool siRNA targeting OTUB1 were treated with 1 μM epirubicin for 16 h. These epirubicin-treated MCF-7 cells were then treated with cycloheximide, and protein lysates prepared from 0 to 8 h following cycloheximide treatment. Protein expression levels of FOXM1, OTUB1, β-tubulin and cyclin B1 in these MCF-7 lysates were examined by western blotting. Densitometry was used to quantify the FOXM1 and β-tubulin levels from which independent background readings were subtracted. Western blots are representative of three independent experiments. The relative expression levels shown (right panels) are means ± s.e.m. of the ratios of FOXM1 to β-tubulin levels relative to those at 0 h. Statistical significance was determined by Student’s t-test (*P < 0.05; significant; ns, non-significant). In (b) and (c), MCF-7 cells transfected with control pcDNA3 and (b) Flag-OTUB1(WT) or (c) the mutant Flag-OTUB1(C91S) were treated and analysed as in (a).

significantly enhanced MCF-7 cell proliferation, whereas OTUB1 depletion decreased the rates of proliferation of MCF-7 cells (Supplementary Figure S7). Furthermore, we also found that OTUB1 overexpression enhanced the resistance of MCF-7 cells to epirubicin (Supplementary Figure S7). Conversely, OTUB1 silencing potentiated the anti-proliferative function of epirubicin in MCF-7 cells (Supplementary Figure S7). We next tested whether FOXM1 has a role in the cell-proliferative and epirubicin-resistant functions of OTUB1. To this end, we transfected WT and Foxm1−/− mouse embryo fibroblasts (MEFs) with either the empty expression vector, OTUB1(WT), or the OTUB1(C91S) mutant and examined their effects on cell proliferation and epirubicin resistance by SRB assay. Overexpression of OTUB1, but not the OTUB1(C91S) mutant, significantly enhanced the cell proliferation as well as the viability of WT MEFs in response to epirubicin (Figure 6). By contrast, there were no significant differences in cell proliferation rates and epirubicin sensitivity in FOXM1-deficient MEFs transfected with vector control, OTUB1 and the OTUB1(C91S) mutant. In addition, clonogenic assays showed that at 0, 20 and 40 nm epirubicin, the colony formation capacity of WT MEFs transfected with WT OTUB1 was significantly enhanced when compared with MEFs transfected with empty expression vector or the OTUB1(C91S) mutant (Figure 7). Similar to the cell proliferation data, there were no appreciable differences in clonogenicity in Foxm1−/− MEFs transfected with empty vector control, WT OTUB1 and the mutant OTUB1(C91S). These results indicate that FOXM1 is a key target of OTUB1 and that FOXM1 mediates the oncogenic and DNA damage resistant functions of OTUB1.

Correlation between OTUB1 and FOXM1 expression in breast cancer patient samples
To establish further the physiological significance and clinical relevance of the regulation of FOXM1 by OTUB1 in breast cancer, FOXM1 and OTUB1 expression was assessed by immunohistochemistry in 116 breast cancer patient samples (Figure 8). OTUB1 was predominantly expressed in cytoplasm, consistent with its function as a deubiquitinating enzyme. Immunohistochemical analysis results showed that OTUB1 expression significantly correlated with FOXM1 expression (Chi-square test, P = 0.034). There was also significant correlation between OTUB1 and oestrogen receptor (ERα) observed in our cohort (Supplementary Figure S8), consistent with the previous finding that OTUB1 could deubiquitinate and inhibit the degradation of ERα. However, there were no significant correlations between OTUB1 and other clinicopathological parameters including progesterone status,
Figure 6. Overexpression of OTUB1 promotes cell proliferation, and epirubicin resistance in WT but not Foxm1-deficient MEFs. WT and Foxm1-deficient MEFs were transiently transfected with either the control pcDNA3 and Flag-OTUB1(WT) or the mutant Flag-OTUB1(C91S).

(a) Twenty-four hours after transfection, protein lysates were prepared from these cells and then analysed for the expression of FOXM1, OTUB1 and β-Tubulin. (b) Twenty-four hours after transfection, aliquots of the transfected cells were split into 96 well plates and their proliferation analysed at the times indicated by SRB assays. Cell proliferation assays revealed that while untreated WT MEFs cells transiently transfected with Flag-OTUB1(WT) but not Flag-OTUB1(C91S), proliferated faster than the control pcDNA3-Flag cells, there was no difference in the Foxm1-deficient MEFs (c) The transfected cells were also treated with a range of doses of epirubicin (0-500 nM) and their proliferative rates assayed by SRB assay at 48 and 72 h after epirubicin treatment. Statistical significance was determined by Student’s t-test (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.005; ns, non-significant) by comparing the proliferation rates of cells transfected with Flag-OTUB1(WT) or Flag-OTUB1(C91S) with the control pcDNA3-Flag transfected cells.
histological type, lymph node involvement and tumour stage (Supplementary Figure S8). Intriguingly, survival analysis showed no significant correlation between OTUB1 expression and patients’ survival (Supplementary Figure S9). In agreement with our survival data, analysis of OTUB1 transcript expression in a previously published cohort (3455 breast cancer patients)\textsuperscript{19} also revealed OTUB1 mRNA expression level was not significantly associated with poor survival (\(P = 0.42\) for overall survival, Kaplan–Meier analysis) (Supplementary Figure S10). However, in another published cohort of 1926 cases of lung cancer,\textsuperscript{19} OTUB1 mRNA expression level was a significant poor prognostic marker (\(P = 0.0025\) for overall survival, Kaplan–Meier analysis) (Supplementary Figure S11). The discrepancy between breast cancer and other cancer types is likely because OTUB1 substrates, in particular ER\(\alpha\), have a particularly essential role in breast cancer initiation, progression and treatment. Although ER\(\alpha\) signalling promotes breast cancer proliferation, expression of ER\(\alpha\) is also associated with good prognosis because these patients are likely to benefit from endocrine therapy. Moreover, in our tissue microarray cohort, tumour stage and lymph node involvement were significantly associated with poor survival (\(P = 0.007\) and \(P = 0.017\), respectively, for overall survival; \(P = 0.001\) and \(P = 0.01\), respectively, for disease-specific survival, refer to cox regression univariate analysis table) (Supplementary Figures S11 and S12). Therefore, to remove the interferences of the clinicopathological parameters on the survival analysis of OTUB1, multivariate analyses using Cox regression model were performed. In multivariate analysis, OTUB1 expression became significantly associated with poor survival after being adjusted for the clinicopathological parameters (\(P = 0.032\), relative risk = 3.822 for overall survival and \(P = 0.032\), relative risk = 3.822 for disease-specific survival) (Supplementary Figure S12). As resistance to chemotherapy is known to be associated with poorer survival, the results from cox regression analysis supported a role of OTUB1 in chemotherapy resistance. This is consistent with our previous study showing that FOXM1 overexpression confers resistance to genotoxic chemotherapy.\textsuperscript{9} Collectively, these data suggest that OTUB1 overexpression is associated with chemotherapeutic drug resistance, and this is likely to be mediated by promoting FOXM1 protein expression through suppressing its ubiquitination and degradation.

**DISCUSSION**

FOXM1 has an essential function in enhancing DNA damage repair and genotoxic agent resistance.\textsuperscript{5,8–10} Previous research has also demonstrated that OTUB1 can modulate DNA damage response by promoting protein deubiquitination.\textsuperscript{17} We show, in here, that OTUB1 can promote deubiquitination and stabilization of FOXM1 in response to the genotoxic agent epirubicin in breast cancer. Here, we also report that FOXM1 is conjugated with Lys48-linked polyubiquitin chains. FOXM1 expression is downregulated in response to epirubicin treatment in MCF-7 cells. This finding is consistent with the established concept that proteins with Lys48-linked polyubiquitin chains are generally targeted for proteasome-dependent degradation.\textsuperscript{20–27} Different OTU family proteins have certain specificity towards distinct types of ubiquitin linkages.\textsuperscript{28} For example, OTUB1 preferentially targets polyubiquitin chains joined by Lys48 bonds, while OTUB2 has specificity towards chains associated through Lys63.\textsuperscript{28}

In concordance, our data demonstrate that OTUB1 overexpression represses the conjugation of Lys48-linked polyubiquitin chains on FOXM1 and impairs its degradation, thus promoting resistance to genotoxic agents. Our previous study shows that SUMOylation inhibits FOXM1 activity, promotes its ubiquitination
and enhances its cytoplasmic translocation as well as degradation.\textsuperscript{10} Thus, deubiquitination of FOXM1 may not only result in the stabilization of FOXM1 but may also promote its nuclear retention.\textsuperscript{10}

In addition to its function as a deubiquitinase, previous studies have shown that OTUB1 can suppress ubiquitination by inhibiting the E2 ubiquitin-conjugating enzymes independently of its catalytic activity. For example, OTUB1 suppresses RNF168-dependent polyubiquitination independently of its catalytic activity. Accordingly, OTUB1 binds to and inhibits UBC13 (UBE2N), the cognate E2 enzyme for RNF168.\textsuperscript{17} OTUB1 also suppresses UBC5 E2 enzyme and stabilizes the p53 protein.\textsuperscript{14,29} However, we demonstrate here that OTUB1 suppresses the ubiquitination and degradation of FOXM1 through its deubiquitinase enzymatic activity. Our data show that WT OTUB1, but not the OTUB1(C91S) catalytic dead mutant, can enhance the stability of FOXM1 in response to

**Figure 8.** Positive correlation between FOXM1 and OTUB1 expression in breast cancer patients. (a) FOXM1 and OTUB1 expression was assessed by immunohistochemistry using tissue microarray constructed from 116 breast cancer patient samples. OTUB1 was expressed predominantly in the cytoplasm. Representative staining images of one patient with high FOXM1 and OTUb1 expression and one with low expression are shown. Images (magnification ×20; Insets (magnification ×100). Positive correlation between FOXM1 and OTUB1 expression was observed. (b) KIF20A staining were detected in both nuclear and cytoplasmic compartments and were correlated with FOXM1 staining. Statistical analysis revealed that OTUB1 were significantly correlated with FOXM1 expression ($P = 0.034$, Chi-Square test).
eproliferative and clonogenic assays also show that OTUB1 can interact with the emerging oncogenic role of OTUB1 in cancer, the OTUB1 also regulates steady-state expression of FOXM1. Consistently, the oncogenic and genotoxic functions of OTUB1 depend on the target of OTUB1 but also present evidence to demonstrate that here, we not only show that the potent oncogene FOXM1 is a target of OTUB1 but also present evidence to demonstrate that the oncogenic and genotoxic functions of OTUB1 depend on the expression of a functional FOXM1, suggesting that OTUB1 promotes cell proliferation and epirubicin resistance predominantly through targeting FOXM1.

Collectively, these results provide strong evidence to suggest that not only OTUB1 deubiquitinase activity prevents the proteasomal degradation of FOXM1 upon epirubicin treatment, OTUB1 also regulates steady-state expression of FOXM1. Consistent with the emerging oncogenic role of OTUB1 in cancer, the proliferative and clonogenic assays also show that OTUB1 can enhance the proliferative rate and genotoxic agent resistance of breast cancer cells and that FOXM1 is a crucial substrate of OTUB1 in cancer cell proliferation and genotoxic agent resistance.

**MATERIALS AND METHODS**

**Cell culture, plasmids and transfection reagents**

The MCF-7 cell line used originated from the American Type Culture Collection and was acquired through CRUK cell bank (London, UK). MCF-7Ep3 cells and MEFs have previously been described.15 Cells were cultured in DMEM (Sigma-Aldrich, Poole, UK) supplemented with 10% (v/v) foetal calf serum and 2 mM glutamine at 37°C. Epirubicin Hydrochloride (2 mg/ml (3.4 mM) in 0.9% sodium chloride, Medac, Germany) was obtained from Imperial College Healthcare (London, UK). The pcDNA3-Flag-OTUB1 and -OTUB1(C91S) plasmids used in this study have previously been described.16 The eGFP-FOXM1 and pcDNA3-FOXM1 expression plasmids have also been described.17 For ubiquitination studies, cells were treated with 10 μM MG132 (M7449; Sigma-Aldrich) for 6 h before collection for analysis. Cells were transfected using FuGENE 6 transfection reagent (Promega, Southampton, UK) and XtremeGENE HP reagent (Roche Diagnostics, Welwyn Garden City, UK) as recommended by the manufacturers.

**NI-NTA pull-down assays**

His-tagged proteins were purified by nickel magnetic agarose beads (Qiagen, Manchester, UK) under denaturing conditions as described.30 For details, see also Supplementary Materials and Methods.

**Quantitative real-time PCR (qRT–PCR)**

Total RNA was extracted with the RNeasy Mini Kit (Qiagen). Complementary DNA generated by Superscript III reverse transcriptase and oligo-dT primers (Invitrogen, Paisley, UK) was analysed by qRT–PCR as described.8,12 See also Supplementary Materials and Methods.

**Gene silencing with siRNAs**

For gene silencing, cells were transiently transfected with siRNA SMART-pool reagents purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA) using Oligofectamine (Invitrogen, Life Technologies Ltd, Paisley, UK) according to the manufacturer’s instructions. siRNAs On Target Smart Pool used were: siRNA FOXM1 (L-009762-00), siRNA OTUB1 (L-021061-00) and the non-silencing control siRNA (D-001810-10-05).

**Measure of FOXM1 protein turnover**

The turnover rate of endogenous FOXM1 in MCF-7 cells was determined using cycloheximide (01810; Sigma-Aldrich) inhibition of protein synthesis. For details, see Supplementary Materials and Methods.

**Immunoprecipitation and western blotting**

Western blotting was performed on whole-cell extracts by lysing cells in buffer or precipitates in the presence of N-ethyl-amide (10 mM) (Sigma UK, Poole, UK) as previously described.38,39 Using the primary antibodies and the species-specific secondary antibodies, the expression levels were visualized using X-ray film (Digilumi, Amersham) for 1/3000 dilution of each antibody. Densitometry was performed on the X-ray film using GelPro Analyser 5.0 (MediaCybernetics) and Western analysis was done with ImageJ.

**Statistical analysis**

All statistics were determined using SPSS 16.0 and Microsoft Excel (Imperial College London, Software Shop, UK). Also, see Supplementary Materials and Methods.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.
ACKNOWLEDGEMENTS

EW-F Lam and AR Gomes were supported by grants from Cancer Research UK. EW-F Lam and S Zona by grants from Breast Cancer Campaign. U Karunarathna is the recipient of a BBSRC studentship. P Khongkow and M Kongsema were supported by grants from the Royal Thai Government Scholarship. US Khoo received funding from Committee on Research and Conference Grants from the University of Hong Kong (CRGCI) (201007176118). We also thank for help with this work.

REFERENCES

1 Savaresi DM, Hsieh C, Stewart FM. Clinical impact of chemotherapy dose escalation in patients with hematologic malignancies and solid tumors. J Clin Oncol 1997; 15: 2981–2995.
2 Myatt SS, Lam EW. The emerging roles of forkhead box (Fox) proteins in cancer. Nat Rev Cancer 2007; 7: 847–859.
3 Myatt SS, Lam EW. Targeting FOXM1. Nat Rev Cancer 2008; 8: 242.
4 Kwok JM, Myatt SS, Marson CM, Coombes RC, Constantinidou D, Lam EW. Thiostrepton selectively targets breast cancer cells through inhibition of forkhead box M1 expression. Mol Cancer Ther 2008; 7: 2022–2032.
5 Kwok JM, Peck B, Monteiro LJ, Sweneden HD, Millour J, Coombes RC et al. FOXM1 confers acquired cisplatin resistance in breast cancer cells. Mol Cancer Res 2010; 8: 24–34.
6 McGovern UB, Francis RE, Peck B, Guest SK, Wang J, Myatt SS et al. Gefitinib (Iressa) represses FOXM1 expression via FOXO3a in breast cancer. Mol Cancer Ther 2009; 8: 582–591.
7 Khongkow P, Gomes AR, Gong C, Man EP, Tsang JW, Zhou L et al. Paclitaxel targets FOXM1 to regulate KIF20A in mitotic catastrophe and breast cancer paclitaxel resistance. Oncogene 2016; 35: 990–1002.
8 Monteiro LJ, Khongkow P, Kongsima M, Morris JR, Man C, Weekes D et al. The Forkhead Box M1 protein regulates BRIP1 expression and DNA damage repair in epirubicin treatment. Oncogene 2013; 32: 4634–4645.
9 Khongkow P, Karunarathna U, Khongkow M, Gong C, Gomes AR, Yague E et al. FOXM1 targets NB51 to regulate DNA damage-induced senescence and epirubicin resistance. Oncogene 2014; 33: 4144–4155.
10 Myatt SS, Kongsima M, Man CW, Kelly DJ, Gomes AR, Khongkow P et al. SUMOylation inhibits FOXM1 activity and delays mitotic transition. Oncogene 2014; 33: 4316–4329.
11 Krasaviz M, Bell R, Dang C. Epirubicin is it like doxorubicin in breast cancer? A clinical review. Breast 2012; 21: 142–149.
12 Millour J, de Olano N, Horimoto Y, Monteiro LJ, Langer JK, Alique R et al. and p53 regulate FOXM1 expression via E2F in breast cancer epirubicin treatment and resistance. Mol Cancer Ther 2011; 10: 1046–1058.
13 de Olano N, Koo CY, Monteiro LJ, Pinto PH, Gomes AR, Alique R et al. The p38 MAPK-MK2 axis regulates E2F1 and FOXM1 expression after epirubicin treatment. Mol Cancer Res 2012; 10: 1189–1202.
14 Sun XX, Challagundla KB, Dai MS. Positive regulation of p53 stability and activity by the deubiquitinating enzyme Ot ubiquitin 1. EMBO J 2012; 31: 517–529.
15 Edelmann MJ, Iphofer A, Akutsu M, Altum M, di Gloria K, Kramer HB et al. Structural basis and specificity of human otubain 1-mediated deubiquitination. Biochem J 2009; 418: 379–390.
16 Wang T, Yin L, Cooper EM, Lai MY, Dickey S, Pickart CM et al. Evidence for bidentate substrate binding as the basis for the K48 linkage specificity of otubain 1. J Mol Biol 2009; 386: 1011–1023.
17 Nakada S, Tai I, Pariser S, Al-Hakim A, Lemura S, Jiang YC et al. Non-canonical inhibition of DNA damage-dependent ubiquitination by OTUB1. Nature 2010; 466: 941–946.
18 Stanisic V, Malovannaya A, Qin J, Lonard DM, O’Malley BW. OTU Domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) deubiquitinating estrogen receptor (ER) alpha and affects ERalpha transcriptional activity. J Biol Chem 2009; 284: 16135–16145.
19 Gyorgy B, Lanczyz A, Elkind AC, Denkert C, Budczes J, Li Q et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat 2010; 123: 725–731.
20 Chau V, Tobias JW, Bachmair A, Marriott D, Ecker DJ, Gonda DK et al. A multi-ubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science 1989; 243: 1576–1583.
21 Deshaies RJ, Joazeiro CA. RING domain E3 ubiquitin ligases. Annu Rev Biochem 2009; 78: 399–434.
22 Finley D. Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu Rev Biochem 2009; 78: 477–513.
23 Xu P, Duong DM, Seyfried NT, Cheng D, Xie Y, Robert J et al. Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. Cell 2009; 137: 133–145.
24 Komander D, Rape M. The ubiquitin code. Annu Rev Biochem 2012; 81: 203–229.
25 Cohen MM, Amiot EA, Day AR, Lebourgeois GP, Pyrce EN, Glickman MH et al. Sequential requirements for the GTGase domain of the mitofusin Fzo1 and the ubiquitin ligase SCFMdm30 in mitochondrial outer membrane fusion. J Cell Sci 2011; 124: 1403–1410.
26 Lipkowitz S, Weissman AM. RINGs of good and evil: RING finger ubiquitin ligases at the crossroads of tumour suppression and oncogenesis. Nat Rev Cancer 2011; 11: 629–643.
27 Weissman AM, Shabek N, Ciechanover A. The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation. Nat Rev Mol Cell Biol 2011; 12: 605–620.
28 Mevissen TE, Hospenthal MK, Geurink PP, Elliott PR, Akutsu M, Arnaudo N et al. OTU deubiquitinasen reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis. Cell 2013; 154: 169–184.
29 Li Y, Sun XX, Elferich J, Shinde U, David LL, Dai MS. Monoubiquitination is critical for ovarian tumor domain-containing ubiquitin aldehyde binding protein 1 (OTUB1) to suppress Ubch5 enzyme and stabilize p53 protein. J Biol Chem 2014; 289: 5097–5108.
30 Zhou Y, Wu J, Fu X, Du W, Zhou L, Meng X et al. OTUB1 promotes metastasis and serves as a marker of poor prognosis in colorectal cancer. Mol Cancer 2014; 13: 258.
31 Liu X, Jiang WJ, Wang YG, Chen H. Colon cancer bears overexpression of OTUB1. Pathol Res Pract 2014; 210: 770–773.
32 Iliopoulos D, Shaaban AM, Yi Y, Shah KV, Sieber OM, Jansen HC et al. High expression of OTUB1 correlates with poor survival in early-stage colorectal cancer. Nat Genet 2009; 41: 774–777.
33 Fox EM, Davis RJ, Shupnik MA. ERbeta in breast cancer—onlooker, passive player, or active protector? Steroids 2009; 73: 1039–1051.
34 Malkin D, Li FQ, Strong LC, Fraumeni JF, Jr., Nelson CE, Kim DH et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 1990; 250: 1233–1238.
35 Singh S, Simon M, Meybohm J, Jantke I, Jonat W, Maass H et al. Human breast cancer: frequent p53 allele loss and protein overexpression. Hum Genet 1993; 90: 635–640.
36 Salghetti SE, Kim SY, Tansey WP. Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. EMBO J 1999; 18: 717–726.
37 Hui RC, Francis RE, Guest SK, Costa JR, Gomes AR, Myatt SS et al. Doxorubicin activates FOXO3a to induce the expression of multidrug resistance gene ABCB1 (MDR1) in K562 leukemic cells. Mol Cancer Ther 2008; 7: 670–678.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/