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# FAT10 modifies p53 and upregulates its transcriptional activity

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ABSTRACT

FAT10, also known as diubiquitin, has been implicated in the regulation of diverse cellular processes, including mitosis, immune response, and apoptosis. We seek to identify FAT10-targeted proteins, an essential step in elucidating the physiological function of FAT10. To this end, human FAT10 or its non-conjugatable derivative, FAT10ΔGG, was overexpressed in HEK293 cells. We observed a number of high molecular weight FAT10 conjugates in cells expressing wild-type FAT10, but not in FAT10ΔGG. The FAT10 conjugates are inducible by TNF-α and accumulated significantly when cells were treated with proteasome inhibitor, MG132. Among them, tumor suppressor p53 was found to be FATylated. The p53 transcriptional activity was found to be substantially enhanced in FAT10-overexpressing cells. In addition, overexpressing FAT10 in HEK293 cells also reduced the population of p53 which cross reacted with monoclonal anti-p53 antibody, PAB240, known to recognize only the transcriptionally inactive p53. FAT10 in the nucleus was found co-localized with p53 and altered its subcellular compartmentalization. Furthermore, overexpressing FAT10 led to a reduction in the size of promyelocytic leukemia nuclear bodies (PML-NBs) and altered their distribution in the nucleus. Based on these observations, a potential mechanism which correlates FATylation of p53 to its translocation and transcriptional activation is discussed.



## Introduction

The ubiquitin-like modifiers (UBLs) is a family of proteins homologous to ubiquitin, which can covalently modify target substrates [1]. Besides the relatively well-studied SUMO and NEDD8, there are at least seven other UBLs: FAT10[1], ISG15, FUB1, UBL5, URM1, ATG8, and ATG12. The major substrates and enzymatic pathways for FAT10, FUB1, and UBL5 remain to be elucidated [2].

FAT10, also known as diubiquitin, is an 18 kDa protein sharing 29% and 36% sequence identity with ubiquitin at the N- and C-termini, respectively [3]. FAT10 is originally identified as a gene encoded in the major histocompatibility complex class I locus and is inducible with TNF-α and interferon-γ [4]. It is regulated in a cell-cycle dependent fashion with the highest expression at the S phase [5], and is negatively regulated by p53 [6]. Although the functions of FAT10 are unclear, it has been implicated to play important roles in many cellular processes. Upregulation of FAT10 gene expression was observed in hepatocellular carcinoma and several epithelial cancers, including gastrointestinal and gynecological cancers [7]. Overexpression of FAT10 induces apoptosis in a caspase-dependent manner [8]. FAT10 also plays a role in the regulation of chromosomal stability [9]. It has also been shown to interact non-covalently with MAD2, a spindle-assembly checkpoint protein [9,10]. In addition, FAT10 can interact non-covalently with NEDD8 Ultimate Buster-1L (NUB-1L) based on results from yeast two-hybrid screening [11]. Several studies demonstrated that wild-type, but not non-conjugatable FAT10 forms a 35 kDa conjugate resistant to boiling in the present of SDS and β-mercaptoethanol, indicating that FAT10 can form covalent linkage to target proteins [8,12]. However, to date, no covalently-conjugated target protein has been identified. Intriguingly, knockout of the FAT10 gene in mice caused minimal phenotypic changes, though lymphocytes of FAT10-deficient mice were more susceptible to spontaneous apoptotic death [13].

Using our proteomic approach [14–16] we demonstrated that p53 can form a covalent conjugate with FAT10. This is the first identified FAT10 target protein. Overexpressing FAT10 leads to p53 conformational change and transcription activation. Notably, we observed that the upregulation of p53 activity by overexpress-

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[1] *Abbreviations used:* FAT10, HLA-*F* Adjacent Transcript *10*; PML-NB, promyelocytic leukemia nuclear body; UBLs, ubiquitin-like modifiers.

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ing FAT10 may be mediated via its effect on PML nuclear body functions.

## Materials and methods

### *Antibodies and plasmids*

The monoclonal anti-Myc (9E10), monoclonal anti-p53 (DO-1), polyclonal anti-p53 (FL-393), anti-ubiquitin, agarose conjugated anti-p53, TRITC conjugated anti-p53, FITC-conjugated anti-PML and FITC conjugated anti-Myc antibodies were purchased from Santa Cruz; monoclonal anti-β-actin antibody from Sigma; monoclonal anti-p53 (PAB 240) antibody from Abcam. The preparation of purified anti-FAT10 antibody was previously described [7]. The cDNAs encoding FAT10GG (aa 1–165), and FAT10ΔGG (aa 1–163) were amplified by PCR. A 6× His-tag sequence immediately upstream of the start codon of the FAT10 cDNA sequence was designed in the amplifying primers. The PCR amplified cDNAs were inserted into the pTRE2hyg2-Myc vector (Clontech) as *Nhe*I/*Cla*I fragments to generate pTRE2hyg2-Myc-His-FAT10GG and pTRE2hyg2-Myc-His-FAT10ΔGG plasmids, respectively. The engineering of pTRE2hyg2-Myc-His-SUMO-1/2/3GG plasmids had been described previously [14,15]. The pTER2hyg2-Myc-Luciferase (LUC) control plasmid was purchased from Clontech. The p53 response reporter plasmids, pRGC-Luc and pG13-Luc were generous gifts from Dr. Seung J. Baek (The University of Tennessee).

### *Cell culture, transfection, and cell lines*

The stable HEK293 Tet-On cell lines overexpressing Myc-His-FAT10GG, Myc-His-FAT10ΔGG, Myc-His-SUMO-1/2/3GG, Myc-His-SUMO-1ΔGG, Myc-LUC and pTRE2hyg2-Myc vector were established using the protocol previously described [14]. FuGENE6 (Roche) was used for transient transfection.

### *Purification of 6× His-tagged SUMO substrates*

The 6× His-tagged proteins were purified under denaturing conditions using Ni–NTA agarose according to the manufacturer's instructions (Qiagen) with some modifications. The detailed method has been previously described [15].

### *Immunoprecipitation, immunoblotting, and immunofluorescence*

Cells were lysed in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 20 mM *N*-ethylmaleimide, 1 mM PMSF, 10 μg/ml pepstatin, 20 μg/ml leupeptin, 10 μg/ml aprotinin] to obtain whole cell extracts (WCE). The nuclear and cytosolic protein separation was carried out using NE-PER kit from PIERCE. Immunoprecipitation and immunoblotting were carried out as described [14,15]. For immunofluorescence, HEK293 Tet-On cells harboring pTRE2hyg2-Myc-His-FAT10GG, pTRE2hyg2-Myc-His-FAT10ΔGG or pTRE2hyg2-Myc-LUC were seeded onto 2-well Lab-Tek chamber slide (Nalge Nuc International) and induced with 2 μg/ml Dox for 48 h. Cells were fixed in 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. The fixed cells were first stained with TRITC conjugated anti-p53 antibody and washed for five times with 1× PBS, then stained with either FITC conjugated anti-Myc antibody or FITC conjugated anti-PML antibody and washed with 1× PBS for five times. The fluorescent images were captured on a Zeiss LSM-5 Pascal laser scanning confocal microscope (Carl Zeiss, Jena, Germany). Images were analyzed with the 3D for LSM software package from Zeiss.

### *p53 transcriptional transactivation activity assay*

The transfection with p53 response reporter plasmid (pRGC-Luc) was used to assess the transcriptional activity of p53 *in vivo* as previously described [16]. The luciferase activity was determined with the Luciferase Reporter Assay Kit (BioVision) using a Turner Design Luminometer (Promega). The values obtained were normalized with protein concentration in each sample.

## Results

### *Establishing cell lines stably expressing FAT10 and its non-conjugatable form*

Stable cell lines overexpressing Myc and 6× His tagged wild-type FAT10 (Myc-His-FAT10GG, abbreviated as FAT10GG) and its non-conjugatable mutant (Myc-His-FAT10ΔGG, abbreviated as FAT10ΔGG) were established in HEK293 cells by transfection with pTRE2hyg2-Myc-His-FAT10GG and pTRE2hyg2-Myc-His-FAT10ΔGG, respectively. Fig. 1A shows that FAT10GG, FAT10ΔGG and luciferase (LUC) control were successfully expressed in stably transfected HEK293 cell lines. The expression of FAT10GG and FAT10ΔGG was inducible with 2 μg/ml of doxycycline (Dox). The successful expression of FAT10GG in HEK cells allowed examination of associated cellular response. Interestingly, the FAT10GG and some of its conjugates, but not FAT10ΔGG and Luciferase (LUC), are also inducible with TNF-α. When cells were treated with proteasome inhibitor, MG132, both FAT10GG and FAT10ΔGG as well as FAT10 conjugates were accumulated. The ubiquitinylated proteins were also accumulated as predicted when cells were treated with MG132 (Fig. S1). More importantly, many high molecular weight conjugates were found in cells expressing FAT10GG, but not in cells expressing FAT10ΔGG or LUC. Even without the treatment of MG132, the FAT10GG can form high molecular weight conjugates (see Fig. 1B, obtained with longer exposure time than that shown in Fig. 1A). Together, these indicate that FAT10 formed covalent conjugates with its target proteins, because these conjugates are resistant to boiling in the presence of SDS and β-mercaptoethanol. It should be pointed out that Fig. 1A shows that, the FAT10GG and its conjugates expression level is apparently higher than that of FAT10ΔGG for unknown reason. Nevertheless this observation is consistent with a previous report [8]. To demonstrate that the observed conjugates were not caused by elevated FAT10GG expression relative to that of FAT10ΔGG, we utilized the advantage of the adjustable Tet-On cell line by reducing the Dox concentration to 0.5 μg/ml for the FAT10GG overexpressing cells, to yield a similar expression level for FAT10GG and FAT10ΔGG. The results as shown in Fig. 1B, reveal that the observed FAT10 conjugated proteins are not an artifact due to differential expression of FAT10GG vs FAT10ΔGG, but due to the ability of FAT10GG and not FAT10ΔGG to form protein conjugates.

### *Overexpressing FAT10GG enhances p53 transcriptional activity*

Raasi et al. reported that overexpressing FAT10 induces apoptosis [8]. We investigate whether the transcriptional activity of p53, a pivotal protein of apoptosis, is affected in cells overexpressing FAT10GG. The luciferase reporter plasmids, containing binding sites for p53 at the promoter regions, were used to analyze the transcriptional activity of p53. When stable cell lines were transfected with the pRGC-Luc p53 reporter plasmid, the luciferase activity in cells expressing FAT10GG was found to be elevated about 10-fold over those expressing FAT10ΔGG or mock vector (Fig. 2), demonstrating that overexpressing FAT10GG upregulates p53 transcriptional activity in HEK293 cells. Similar results were