

# Role of Ubiquitin-Like Protein FAT10 in Epithelial Apoptosis in Renal Disease

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Dysregulated apoptosis of renal tubular epithelial cells (RTEC) is an important component of the pathogenesis of several renal diseases, including HIV-associated nephropathy (HIVAN), the most common cause of chronic kidney failure in HIV-infected patients. In HIVAN, RTEC become infected by HIV-1 in a focal distribution, and HIV-1 infection has been shown to induce apoptosis *in vitro*. In microarray studies that used a novel renal tubular epithelial cell line from a patient with HIVAN, it was found that the ubiquitin-like protein FAT10 is one of the most upregulated genes in HIV-infected cells. Previously, FAT10 was shown to induce apoptosis in murine fibroblasts. The expression of FAT10 in HIVAN and the ability of FAT10 to induce apoptosis in human RTEC therefore were studied. This study revealed that FAT10 expression is induced after infection of RTEC by HIV-1 and that expression of FAT10 induces apoptosis in RTEC *in vitro*. Moreover, it was found that inhibition of endogenous FAT10 expression abrogated HIV-induced apoptosis of RTEC. Immunohistochemical studies demonstrated increased FAT10 expression in a murine model of HIVAN, in HIVAN biopsy samples, and in autosomal dominant polycystic kidney disease, another renal disease that is characterized by cystic tubular enlargement and epithelial apoptosis. These results suggest a novel role for FAT10 in epithelial apoptosis, which is an important component of the pathogenesis of many renal diseases.

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Dysregulated epithelial apoptosis and proliferation contribute to the pathogenesis of several progressive renal diseases, including HIV-associated nephropathy (HIVAN) and autosomal dominant polycystic kidney disease (ADPKD) (1,2). In both of these diseases, patients develop tubulointerstitial disease, characterized by the presence of tubular cysts in multiple nephron segments and interstitial fibrosis. In ADPKD, tubular cysts progressively enlarge, eventually leading to compression of the remaining normal renal parenchyma (2). In HIVAN, cyst development ceases at the “microcystic” stage (3).

HIV-1 infection of the renal epithelium and subsequent epithelial expression of viral genes are crucial determinants of HIVAN pathogenesis (4,5). Epithelial apoptosis that is induced by expression of HIV-1 genes is an important contributor to progression of renal failure (4,6,7). In patients with HIVAN, HIV-1 infection occurs in epithelial cells from any segment of the nephron, including the glomerulus, proximal tubule, thick

ascending limb of Henle, and collecting duct (8). Although HIV-1 infection of renal tubular epithelium is known to occur, the host genes that are differentially expressed after infection by HIV-1 that subsequently leads to proliferation and apoptosis remain largely undefined.

There clearly are genetic factors that contribute to HIVAN susceptibility. HIVAN occurs almost exclusively in individuals of African ancestry, and black individuals with HIVAN are far more likely to have HIV-negative relatives with ESRD than are black individuals without HIVAN (9). Moreover, in the HIV-1 transgenic model of HIVAN, the penetrance of the HIVAN phenotype is dramatically influenced by the genetic background of the mice (10,11). Despite the importance of host factors, most *in vitro* studies of HIVAN pathogenesis have used cell lines that were derived either from animals or from humans from racial groups with a very low susceptibility to developing HIVAN.

In preliminary studies using a proximal tubular cell line derived from a patient with HIVAN, we used oligonucleotide microarrays to determine the genes that are differentially expressed after transduction with replication-incompetent HIV-1. In these studies, one of the most robustly upregulated genes was FAT10. FAT10 is a ubiquitin-like protein (UBL) that contains two ubiquitin-like domains and is capable of inducing

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ubiquitin-independent degradation of proteins *via* the proteasome (12). Because FAT10 has putative roles in regulating apoptosis and cell-cycle progression (13,14), we studied FAT10 expression in the HIV-1 transgenic model of HIVAN and in HIVAN biopsy specimens. Given that ADPKD is also characterized by renal tubular dilation and dysregulated apoptosis (15,16), we examined the expression of FAT10 in ADPKD. We report here that FAT10 expression is increased in renal epithelial cells in HIVAN and ADPKD, that *in vitro* expression of FAT10 induces apoptosis in human renal tubular epithelial cells (RTEC), and that inhibition of FAT10 expression prevents HIV-induced apoptosis in RTEC.

## Materials and Methods

### Tissue Specimens

The HIV-1 transgenic mouse model of HIVAN has been characterized extensively. Heterozygous transgenic mice develop proteinuria, progressive renal failure, and histologic renal disease that is identical to HIVAN (4,17,18). HIVAN biopsy tissue was collected at the time of diagnostic renal biopsy under a protocol that was approved by the Mount Sinai Institutional Review Board. ADPKD tissue sections were obtained from nephrectomy specimens that were procured by National Disease Research Interchange and Polycystic Kidney Disease Foundation. All tissues were fixed in 4% paraformaldehyde/PBS and embedded in paraffin.

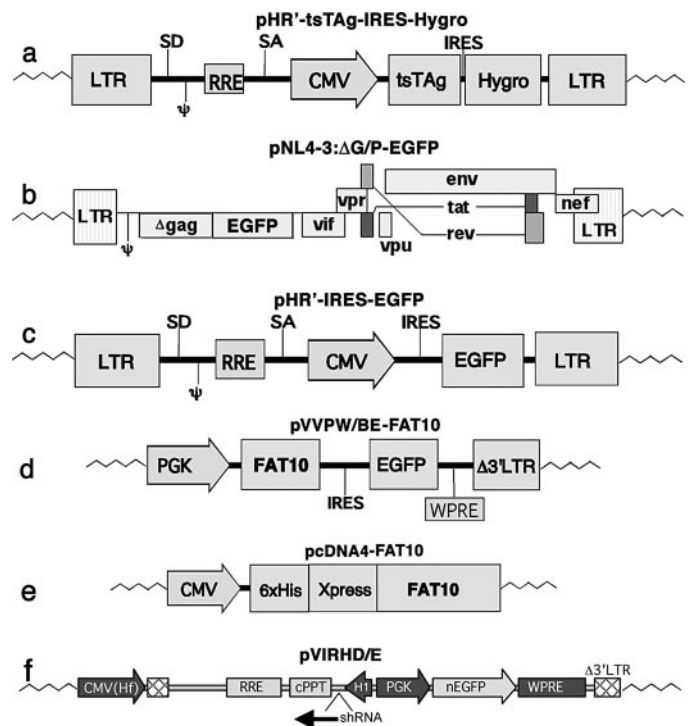
### Generation of the Human Proximal Tubular Cell Line

Primary human proximal tubular cells (HPT) were grown from a HIVAN biopsy specimen by mincing of a portion of a fresh cortical renal biopsy sample and plating onto collagen-coated tissue culture plates in media selective for growth of proximal tubular cells according to previously published methods (19). A subpopulation of these cells (subsequently named HPT-1) was conditionally immortalized by infection with vesicular stomatitis virus (VSV)–temperature-sensitive SV40 large T-antigen (tsTag)-IRES-Hygro, a VSV-glycoprotein (VSV-G) pseudotyped replication defective lentivirus encoding the SV40 temperature-sensitive large T antigen under the control of the immediate early cytomegalovirus (CMV) promoter (Figure 1a). For all subsequent studies, HPT-1 cells were expanded at 33°C until they reached 80% confluence and subsequently cultured at 37°C for 14 d to induce T antigen degradation and cellular differentiation.

### Viral Transduction

To conditionally immortalize proximal tubular epithelial cells (PTEC), we developed a lentiviral vector encoding the tsTag allele tsA58U19 (20). tsTag was subcloned into pIRES-hyg (Clontech, Palo Alto, CA). A fragment that included the CMV promoter tsTag and hygromycin-resistance genes was subsequently subcloned into pHR-CMV-IRES2-EGFP-ΔB (gift of Dr. James C. Mulloy, Memorial Sloan Kettering Cancer Center, New York, NY), resulting in the vector pHR'-tsTag-IRES-hygro (Figure 1a).

pNL4-3:ΔG/P-EGFP, a *gag/pol*-deleted HIV-1 construct that contains enhanced green fluorescence protein (EGFP) in the *gag* open reading frame (Figure 1b) (21) and pHR'-IRES-EGFP (EGFP control; Figure 1c), were used to generate VSV-G-pseudotyped virus for infection of HPT-1 cells as described previously (21). A multiplicity of infection (MOI) of 0.5 was used to infect HPT-1 cells because higher MOI resulted in excessive cytotoxicity at 3 and 7 d after infection.



**Figure 1.** Constructs used in these studies. pHR'-temperature-sensitive SV40 large T-antigen (tsTag)-IRES-hygro (a) was used to produce lentivirus to conditionally immortalize proximal tubular epithelial cells (PTEC). pNL4-3:ΔG/EGFP (P-EGFP; b) encodes a *gag/pol*-deleted HIV-1 provirus with enhanced green fluorescent protein (EGFP) cloned into the *gag* open reading frame. pNL4-3:ΔG/P-EGFP and pHR'-IRES-EGFP (c) were used to generate VSV-G-pseudotyped lentiviruses for infection of human proximal tubular-1 (HPT-1) cells. (d) pVIRHD/E is a lentiviral vector that allows production of short hairpin RNA (shRNA) in transduced cells. (e) pVVPW/BE-FAT10 is a bicistronic vector that expresses FAT10 and EGFP as separate proteins. (f) pcDNA4-FAT10 expresses FAT10 with N-terminal histidine and Xpress epitope tags.

### Characterization of HPT-1 Cells

HPT-1 cells were grown and differentiated on glass coverslips that had been coated with type 1 rat tail collagen (BD Biosciences, San Jose, CA). Cells were fixed in 4% paraformaldehyde/PBS. Endogenous alkaline phosphatase activity was detected by incubating cells in 10 mM Tris (pH 9.0) with 0.3 mM 5-bromo,4-chloro,3-indolylphosphate and nitroblue tetrazolium (Roche, Indianapolis, IN). For immunocytochemical detection of aquaporin-1 and cytokeratin, HPT-1 cells were permeabilized with 0.1% Triton X-100. Primary antibodies that were used to characterize HPT-1 cells included anti-aquaporin-1 (AQP11-S), anti-aquaporin-2 (AQP21-A; Alpha Diagnostics, San Antonio, TX), anti-cytokeratin (BT-571; BTL, Stoughton, MA), anti-vimentin (V2258; Sigma, St. Louis, MO), anti-smooth muscle myosin (MAB3568; Chemicon, Temecula, CA), and Tamm-Horsfall protein (55140; Cappel Laboratories, Westchester, PA). Primary antibodies were detected using Cy3-labeled goat anti-rabbit IgG or Cy3-labeled goat anti-mouse IgG (KPL, Gaithersburg, MD) diluted 1:100.

### Analysis of FAT10 RNA Expression in HPT-1 Cells and Kidneys from HIV-1-Transgenic Mice

Total RNA was harvested from HPT-1 cells after infection with VSV-NL4-3:ΔG/P-EGFP or VSV-HR'-IRES-EGFP and from kidneys

from adult transgenic mice with severe histologic renal disease and proteinuria, transgenic mice without significant histologic renal disease, and normal mice. Ten micrograms of RNA from each sample was resolved on 1.2% agarose/formaldehyde gel and transferred to a 0.45- $\mu$ m Biodyne membrane (Pall Corp., East Hills, NY).

For analysis of FAT10 expression in HPT-1 cells, a portion of the second exon of human FAT10 was amplified by PCR using cDNA from VSV-NL4-3: $\Delta$ G/P-EGFP-infected HPT-1 cells using HotStar Master Mix kit (Qiagen, Valencia, CA) and the primers 5'-GATCTTAAAGC-CACGGAGAA-3' (sense) and 5'-CATCCACCCAAATCTTACT-3' (antisense) with the thermocycler parameters 1 min at 94°C, 1 min at 58°C, and 1 min at 72°C for 40 cycles. For analysis of FAT10 expression in murine kidneys, the murine FAT10 coding sequence was amplified using cDNA from HIV-transgenic kidneys using primers 5'-CGGGAT-CCCACCATGGCTTCTGTCCGCACCTGTG-3' (sense) and 5'-GGAAT-TCGGTATCCCCCAGTGCAGTGTGTGTC-3' (antisense) and the following thermocycler parameters: 15 min at 95°C for one cycle; 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C for four cycles; and 30 s at 94°C, 1 min at 64°C, 1 min at 72°C for 31 cycles.

PCR products were purified using Qiaquick columns (Qiagen) and labeled with dCTP<sup>32</sup> (Perkin-Elmer, Wellesley, MA) using Ready to Go dCTP Labeling Beads (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase and ribosomal RNA bands were analyzed to ensure equal loading of RNA.

#### Generation of FAT10 Expression Vectors

The FAT10 coding sequence was amplified from plasmid MGC-21200 (ATCC) using primers 5'-CGGGATCCACCATGGCTCCCAATGCTTC-CTGCC-3' (sense; *Bam*H1 adapter in italics) and 5'-GGAATTCGGT-TACCTCCAATAACAATAAGATGCCAG-3' (antisense; *Eco*R1 adapter in italics) and the following thermocycler parameters: 15 min at 95°C for one cycle; 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C for four cycles; and 30 s at 94°C, 1 min at 64°C, and 1 min at 72°C for 31 cycles. The PCR product was cloned into VVPW/BE (see below) and pcDNA4/HisMaxC (Invitrogen) using *Bam*H1 and *Eco*R1 (New England Biolabs, Beverly, MA), resulting in the pVVPW/BE-FAT10 (Figure 1d) and pcDNA4-FAT10 (Figure 1e) vectors. pVVPW/BE-FAT10 expresses FAT10 in a bicistronic transcript with EGFP expressed as a separate protein, whereas pcDNA4-FAT10 expresses FAT10 with an N-terminal Xpress epitope tag.

#### Generation of the Plasmid VVPW/BE

The lentiviral vector VVPW/BE was derived by the insertion of the BE (BiP/EGFP) cassette, which contained an internal ribosome entry site from the human heat-shock protein BiP upstream of the EGFP gene, into VVPW (22). For generation of this cassette, the NotI site at the 3' end of the EGFP reporter gene in pHR'PGK/EGFP vector (23) first was

removed after NotI digestion, fill-in with Klenow polymerase, and self-ligation. In parallel, an NcoI linker was introduced in place of the KpnI site at the 3' of BiP in ZQ-30 to 1-S-IRES plasmid (provided by Dr. Christian LeGuern). The BiP element, digested with NotI and NcoI, and the EGFP reporter gene, excised with NcoI and XhoI, then were introduced between the NotI and XhoI sites of VVPW in a triple ligation to generate VVPW/BE.

#### Generation of Short Hairpin RNA Vectors

We constructed two short hairpin RNA (shRNA) vectors to knock down expression of FAT10. One vector (pVIRHD/E-FAT10.1) is targeted against the first exon of FAT10, and the other (pVIRHD/E-FAT10.2) is targeted against the second exon. The VIRHD/E/siLuc lentivector expressing the anti-luciferase siRNA was used as negative control. Each vector was constructed by annealing complementary 61-mer oligonucleotides that contained the 19 nucleotide sequence that corresponded to the target FAT10 sequence in the sense and antisense orientation separated by a nine-nucleotide spacer region, allowing generation of a shRNA. Oligonucleotide sequences are provided in Table 1. Annealed oligonucleotides were inserted under the control of the human H1 promoter in the self-inactivating pVIRHD/E lentiviral vector (Figure 1f) derived from VVPW/EGFP (22). The resulting lentiviral vectors, which also express the reporter EGFP from the constitutive murine phosphoglycerate kinase promoter, were packaged into VSV-G-pseudotyped virions using the methods described above in the Viral Transduction section (Gusella *et al.*, unpublished observations, 2005).

#### Generation of Anti-FAT10 Antiserum

Rabbit polyclonal antiserum against FAT10 was raised against the peptide APNASCLCVHVRSE, which spans the splice junction between the first and second exons of FAT10. This peptide was conjugated to keyhole limpet hemocyanin before inoculation. Peptide synthesis, inoculations, and harvesting of antiserum were performed by Open Biosystems (Huntsville, AL). Antiserum that was used in these studies was collected 70 d after inoculation.

#### Immunohistochemistry

Paraformaldehyde-fixed paraffin-embedded tissue was stained for FAT10 using FAT10 antiserum at a 1:100 dilution followed by incubation with biotinylated rabbit anti-goat IgG at a 1:200 dilution (KPL). The Vector ABC Elite Kit and aminoethylcarbazole (Vector Labs, Burlingame, CA) were used according to a previously published protocol (8). For ensuring specificity of the antiserum, staining controls included using preimmune serum from the same rabbit that was used to generate the antiserum and incubating the antiserum with 10  $\mu$ g/ml of the immunizing peptide for 60 min before use in immunohistochemistry.

Table 1. Oligonucleotides used for generating shRNA vectors<sup>a</sup>

pVIRHD/E-FAT10.1	
sense	5'-GATCCTGCTTCTGCCTCTGTGTGTTGATATCCGCACACAGAGGCAGGAAGCATTTCCTGG-3'
antisense	5'-AATTCACAAAAAATGCTTCTGCCTCTGTGTGCGGATATCAACACACAGAGGCAGGAAGCAG-3'
pVIRHD/E-FAT10.2	
sense	5'-GATCCGAACATGTCCGGTCTAAGATTGATATCCGCTCTAGACCCGGACATGTTCTTTTTGG-3'
antisense	5'-AATTCACAAAAAAGAACATGTCCGGTCTAAGACGGATATCAATCTTAGACCCGGACATGTTCCG-3'
pVIRHD/E/siLuc	
sense	5'-GATCCGTGCGTTGCTAGTACCAACTTCAAGAGAGTTGGTACTAGCAACGCACCTTTTTTGG-3'
antisense	5'-AATTCACAAAAAAGTGCCTTGTAGTACCAACTCTCTTGAAGTTGGTACTAGCAACGCACG-3'
antisense	5'-GATCCTGCTTCTGCCTCTGTGTGTTGATATCCGCACACAGAGGCAGGAAGCATTTCCTGG-3'

<sup>a</sup>shRNA, short hairpin RNA.

### FAT10 shRNA Inhibition Studies

Separate populations of HPT-1 cells were transduced with VSV-pseudotyped VIRHD/e-FAT10.1 and VIRHD/E-FAT10.2 at an MOI of 10. After expansion and differentiation,  $10^7$  cells were transfected with pcDNA4-FAT10 (Figure 1e) using Lipofectamine 2000 (Invitrogen). Lysate from pcDNA4-FAT10-transfected HPT-1 cells was used for detection of Xpress-tagged FAT10 by Western blotting using Anti-Xpress-HRP (Invitrogen) diluted 1:2000.

### Flow Cytometry

Differentiated HPT-1 cells were transfected with VVPW/BE-FAT10 or VVPW/BE (control) using Lipofectamine 2000 (Invitrogen) at a DNA:lipid ratio of 1:3 according to the manufacturer's protocol. Twenty-four hours after transfection,  $1.2 \times 10^6$  HPT-1 cells were stained using the Vybrant apoptosis #6 kit (Invitrogen) according to the manufacturer's protocol. Cells then were analyzed by flow cytometry at the Mount Sinai Flow Cytometry Shared Research Facility using a FACSVantage flow cytometer (BD Biosciences). Transfected cells were identified by the presence of EGFP fluorescence. Apoptotic cells were identified by the presence of annexin V staining in the absence of propidium staining, and necrotic cells were identified by the presence of propidium staining.  $\chi^2$  testing was used to compare the number of apoptotic and apoptotic plus necrotic cells that were transfected with VVPW/BE-FAT10 to cells that were transfected with VVPW/BE. A two-sided  $P < 0.05$  was used to define statistical significance.

To study the effect of FAT10 inhibition on HIV-induced apoptosis, we infected separate populations of HPT-1 cells that had been stably transduced with VIRHD/e-FAT10.1, VIRHD/E-FAT10.2, or VIRHD/E/siLuc with VSV-NL4-3:ΔG/P-EGFP as described in the section Viral Transduction. Seven days after infection, we analyzed apoptosis in each population as described above.

## Results

### Development of a Tubular Epithelial Cell Line HPT-1 from a Patient with HIVAN

Host genetic factors strongly influence susceptibility to HIVAN. Therefore, to maximize the relevance of our studies to HIVAN pathogenesis, we developed a PTEC line from a patient with HIVAN for use in these studies. We chose PTEC because they are the predominant epithelial cell type in the kidney, we have shown them to be infected by HIV-1, and they are important in disease progression in HIVAN (8).

Primary PTEC that were grown from a HIVAN biopsy were conditionally immortalized by stable transduction with a lentivirus encoding the SV40 temperature-sensitive large T antigen under the control of the CMV promoter (Figure 1a). The resulting cell population (subsequently referred to as HPT-1) grows indefinitely under permissive conditions (33°C), but at 37°C, T antigen degrades (Figure 2a) and the cells are induced to differentiate maximally. HPT-1 cells express PTEC markers such as cytokeratin, alkaline phosphatase, and aquaporin-1 (Figure 2, b through d). HPT-1 cells do not express nonepithelial proteins such as vimentin or smooth muscle myosin, Tamm Horsfall protein, or aquaporin-2 (markers for thick ascending limb of Henle and collecting duct, respectively [data not shown]). HPT-1 cells are not latently infected by HIV-1 as determined by PCR analysis (data not shown).

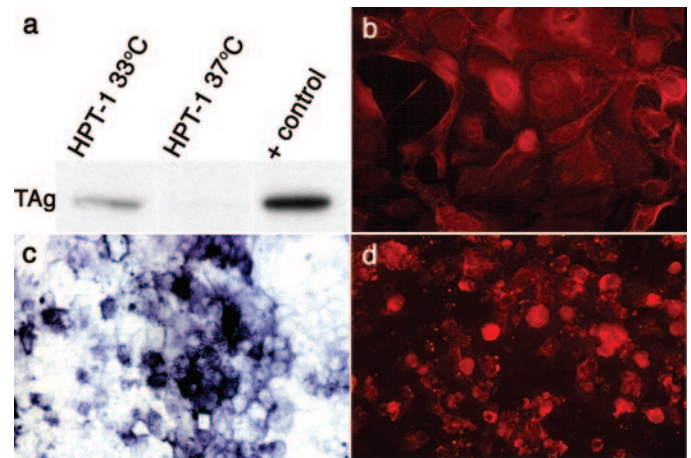


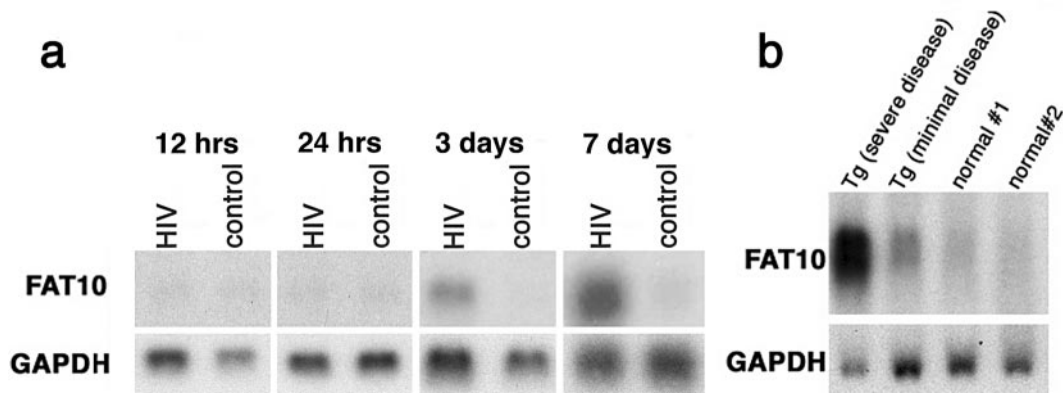
Figure 2. HPT-1 cells express tsTag when grown at 33°C, but at 37°C, T-antigen degrades (a) and the cells differentiate, expressing the epithelial marker cytokeratin (b) and the proximal tubular markers alkaline phosphatase (c) and aquaporin-1 (d). Magnifications:  $\times 400$  in b;  $\times 200$  in c and d.

### FAT10 mRNA Expression after In Vitro HIV-1 Transduction of Human Proximal Tubular Cells

In preliminary studies using oligonucleotide microarrays, we found that FAT10 was one of the most robustly upregulated genes in HIV-infected HPT-1 cells (24). To determine the specificity of the increased expression of FAT10, we performed Northern blotting using RNA from HPT-1 cells that were infected with VSV-NL4-3:ΔG/P-EGFP (Figure 1b), a gag/pol-deleted virus that was derived from the same proviral construct that was used to create the HIV-1-transgenic HIVAN model (17,21). VSV-HR'-IRES-EGFP, a lentivirus that expresses EGFP (Figure 1c), was used as a control. Because infection of RTEC *in vitro* by HIV-1 is inefficient (6,25), both viruses were pseudotyped with VSV-G to maximize the efficiency of infection. FAT10 expression was markedly increased in HPT-1 cells that were infected with VSV-pNL4-3:ΔG/P-EGFP at 3 and 7 d after infection but not at 12 and 24 h after infection (Figure 3a). FAT10 was not expressed in control-infected cells at any time point (Figure 3a) or in noninfected cells (data not shown).

### FAT10 RNA Expression in Kidneys from HIV-1-Transgenic Mice

We next studied FAT10 expression in kidneys from the HIV-1-transgenic model of HIVAN. These mice develop proteinuria, renal failure, and histologic renal disease that is identical to HIVAN (18). Kidneys were obtained from transgenic mice with severe proteinuria and histologic disease, transgenic mice without overt proteinuria or histologic disease, and normal adult mice. FAT10 expression was markedly increased in kidneys from HIV-transgenic mice with severe renal disease, with lesser FAT10 levels detectable in kidneys from HIV-transgenic mice without overt renal disease (Figure 3b). FAT10 expression was minimally detectable in kidneys from normal adult mice (Figure 3b).



**Figure 3.** FAT10 mRNA expression is increased in HIV-infected HPT-1 cells and in kidneys from HIV-transgenic mice. (a) Infection with *gag/pol*-deleted HIV-1 induced expression of FAT10 at 3 and 7 d after infection. Expression of FAT10 mRNA was markedly increased in severely diseased kidneys from HIV-transgenic mice. (b) Lesser expression of FAT10 was present in kidneys from transgenic mice without overt nephropathy, and there was minimal expression in kidneys from normal adult mice.

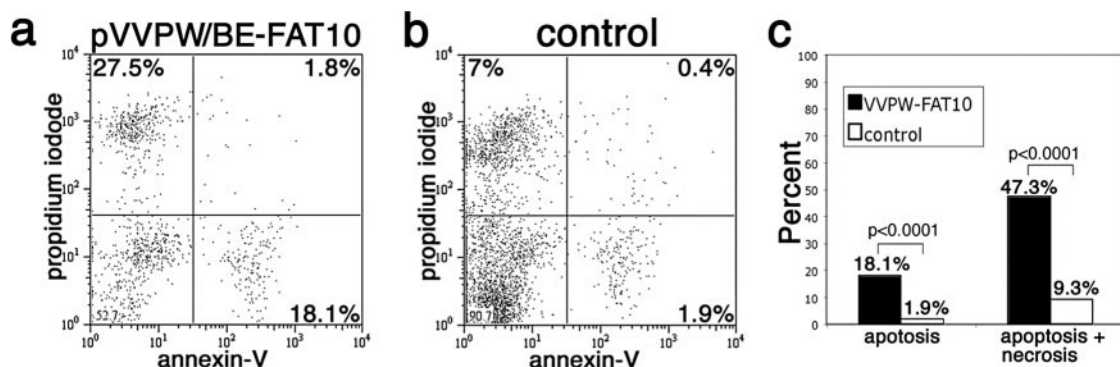
#### FAT10 Expression Induces Apoptosis in HPT-1 Cells

Because FAT10 expression and epithelial apoptosis are increased in HIVAN, we studied whether FAT10 is capable of inducing apoptosis in HPT-1 cells. HPT-1 cells were transfected with a plasmid encoding FAT10 and EGFP in a dicistronic vector (pVVPW/BE-FAT10; Figure 1d), allowing identification of transfected cells by EGFP fluorescence. Twenty-four hours after transfection with pVVPW/BE-FAT10, 18.1% of EGFP-expressing cells were apoptotic as determined by annexin V staining (Figure 4, a and c), whereas only 1.9% of HPT-1 cells that were transfected with the control vector (VVPW/BE) were apoptotic (Figure 4, b and c). Moreover, 47.3% of pVVPW/BE-FAT10-transfected cells were either apoptotic or necrotic (positive for propidium and/or annexin V staining), but only 9.3% of vector-transfected cells were apoptotic or necrotic (Figure 4c). Levels of apoptosis and apoptosis plus necrosis were significantly greater in the cells that expressed FAT10 ( $P < 0.0001$ ).

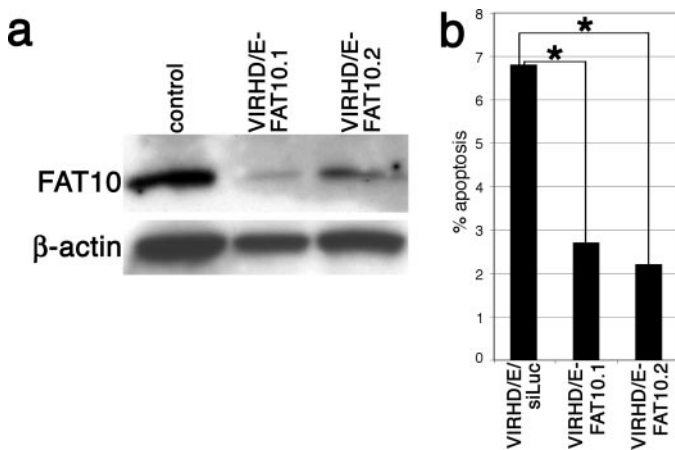
#### Inhibition of FAT10 Prevents HIV-Induced Apoptosis of RTEC

To determine whether FAT10 has a direct causal role in HIV-induced apoptosis of RTEC, we studied whether specific inhibition of endogenous FAT10 using shRNA vectors could prevent apoptosis of RTEC after infection with HIV-1. We generated two lentiviral vectors, VIRHD/e-FAT10.1 and VIRHD/E-FAT10.2, to stably transduce HPT-1 cells with shRNA directed against two separate regions of the FAT10 transcript. To determine the efficacy of these vectors in suppressing FAT10 protein expression, we stably transduced HPT-1 cells with the shRNA vectors and subsequently transfected them with the FAT10 expression vector pcDNA4-FAT10. Both shRNA vectors effectively inhibited expression of FAT10 protein (Figure 5a).

We then tested whether preventing upregulation of endogenous FAT10 could prevent apoptosis in HPT-1 cells after infec-



**Figure 4.** FAT10 expression induces apoptosis in HPT-1 cells. Twenty-four hours after transfection with VVPW/BE-FAT10, EGFP-expressing HPT-1 cells were examined by flow cytometry. A total of 18.1% of cells that were transfected with pVVPW/BE-FAT10 were apoptotic as determined by annexin V staining (a and c), whereas only 1.8% of vector-transfected cells became apoptotic (b and c;  $P < 0.0001$ ). A total of 47.3% of HPT-1 cells that were transfected with FAT10 were either apoptotic (annexin V positive, propidium negative) or necrotic (positive for propidium) in contrast to vector-transfected cells (9.3%; c;  $P < 0.0001$ ).



**Figure 5.** (a) Stable transduction of HPT-1 cells with FAT10 shRNA constructs (VIRHD/E-FAT10.1 and VIRHD/E-FAT10.2) markedly reduces FAT10 protein production after transfection with a FAT10 expression vector as compared with HPT-1 cells that do not express FAT10 shRNA. (b) HPT-1 cells in which endogenous FAT10 expression is suppressed by stable transduction with FAT10 shRNA (VIRHD/E-FAT10.1 and VIRHD/E-FAT10.2) are resistant to apoptosis after infection with VSV-NL4-3-ΔG/P-EGFP as compared with HPT-1 cells that express the luciferase shRNA vector VIRHD/E/siLuc; \* $P < 0.0001$ .

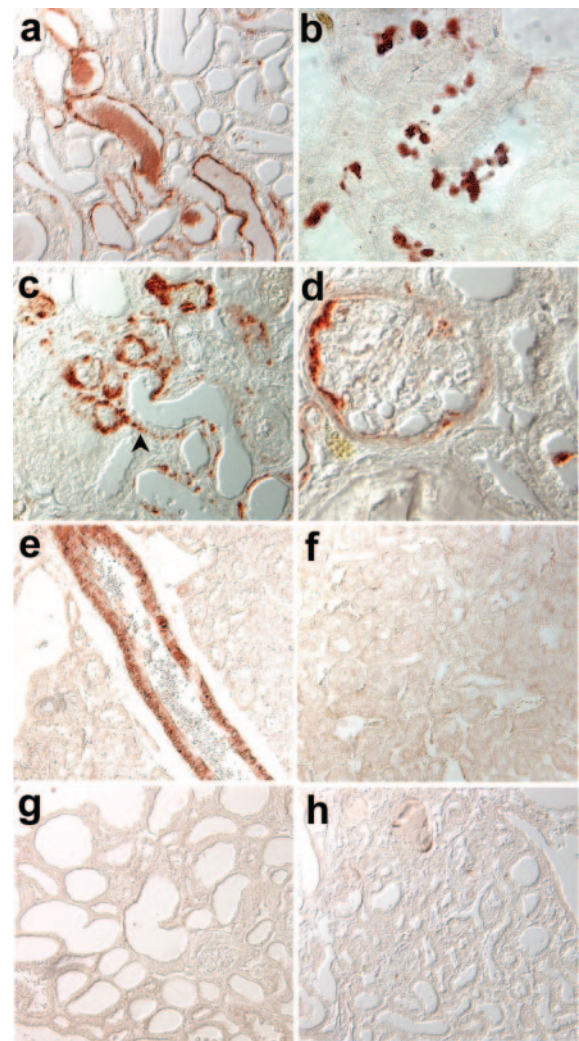
tion by HIV-1. We infected HPT-1 cells that had been stably transduced with VIRHD/e-FAT10.1, VIRHD/E-FAT10.2, or VIRHD/E/siLuc with VSV-NL4-3:ΔG/P-EGFP. Cells that expressed either anti-FAT10 shRNA construct demonstrated significantly lower levels of apoptosis as compared with cells that expressed the shRNA control vector VIRHD/E/siLuc ( $P < 0.0001$ ; Figure 5b).

#### FAT10 Protein Expression Is Increased in Kidneys from HIV-1-Transgenic Mice

We used FAT10 antiserum to perform immunohistochemical analysis of FAT10 expression and localization in kidneys from HIV-1-transgenic mice and in normal controls. FAT10 staining was focal with expression in RTEC in some nephrons, (Figure 6a), sloughing (often apoptotic) epithelial cells (Figure 6b) and cells just beneath the epithelial layer (Figure 6c). Focal expression of FAT 10 also was present in glomeruli (Figure 6d) and in vascular smooth muscle cells (Figure 6e). In kidneys from normal adult mice, FAT10 was not detected in glomeruli, tubules, or interstitium (Figure 6f) but was detected in vascular smooth muscle cells (data not shown). Preimmune serum controls were negative (Figure 6g), and preincubation of the antiserum with the peptide that was used to generate the antiserum abolished all staining (Figure 6h). Immunostaining with other polyclonal antibodies to FAT10 donated by Dr. C. Lee [26] and Dr. S. Weissman [13]) resulted in similar staining (data not shown).

#### FAT10 Expression in HIVAN and ADPKD

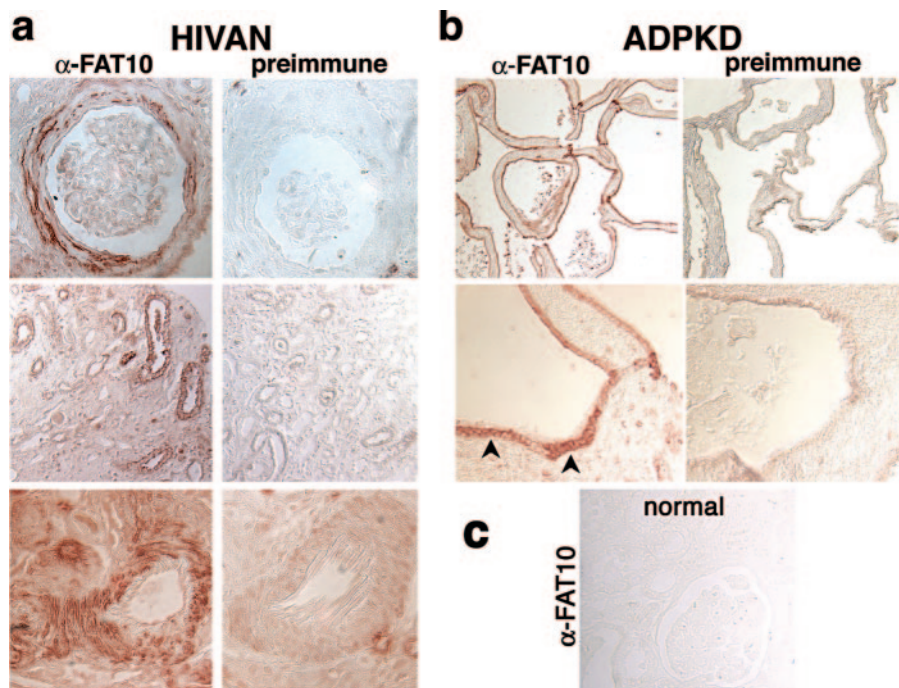
To determine whether FAT10 expression is increased in patients with HIVAN, we performed immunohistochemistry us-



**Figure 6.** FAT10 protein expression in kidneys from HIV-1-transgenic mice with HIV-associated nephropathy (HIVAN). FAT10 is detected in tubular epithelial cells (a), in tubular epithelial cells that have detached from the tubular basement membrane (b), and in cells beneath the tubular epithelium (c, arrow). Focal expression of FAT10 was also detected in glomeruli (d) and in vascular smooth muscle cells (e). No FAT10 protein was detected in normal adult murine kidneys (f), with the exception of some vascular smooth muscle cells (data not shown). Preimmune serum controls using kidneys from transgenic mice are negative (g), and preincubation of antiserum with 10 ng/ml immunizing peptide abolished FAT10 staining in transgenic kidneys (h). Magnifications:  $\times 200$  in a, c, and e through h;  $\times 400$  in b and d.

ing HIVAN biopsy specimens. FAT10 was expressed in areas of periglomerular fibrosis surrounding Bowman's capsule (Figure 7a, top left), in tubular epithelial cells (Figure 7a, middle left), and in arterial smooth muscle cells (Figure 7a, bottom left). Preimmune serum controls using serial sections were negative (Figure 7a, right).

Because ADPKD, like HIVAN, is characterized by tubular cystic dilation and dysregulated RTEC apoptosis, we performed immunohistochemistry to investigate whether FAT10 is



**Figure 7.** FAT10 expression in HIVAN kidney biopsies and in autosomal dominant polycystic kidney disease (ADPKD). (a) FAT10 is expressed in fibrotic scars surrounding sclerotic glomeruli (top left), in tubular epithelial cells (middle left), and in vascular smooth muscle cells (bottom left). Preimmune controls performed on serial sections (right) were negative. (b) FAT10 expression is increased in cystic epithelium in ADPKD. FAT10 protein was detected in the epithelium lining cysts (top) and in areas of aberrant epithelial proliferation (bottom left, arrowheads). Preimmune serum controls demonstrated no staining (bottom). (c) FAT10 was not detected in normal human nephrectomy specimen. Magnifications:  $\times 400$  in a, top and bottom;  $\times 200$  in a, middle, b, top, and c;  $\times 100$  in b, bottom.

expressed in kidneys from patients with ADPKD. FAT10 was strongly expressed in RTEC that lined cysts (Figure 7b), particularly in areas where the epithelium had become hyperplastic (Figure 7b, bottom left, arrowheads). Preimmune controls were negative (Figure 7b, right), and FAT10 expression was not detected in normal adult human kidneys (Figure 7c).

## Discussion

HIVAN and ADPKD are important causes of renal disease. HIVAN is the third-leading cause of ESRD in black individuals who are aged 20 to 64 yr and is the most common cause of chronic kidney failure in HIV-1-infected patients (1). ADPKD is the most common lethal inherited disease in humans, occurring in 1 in 800 live births and affecting 4 to 6 million people worldwide (2).

HIVAN is caused by expression of HIV-1 genes in infected renal epithelial cells (4,5,27,28). Host genetic factors are important in modulating the predisposition to developing HIVAN in patients and in HIV-1-transgenic mice (9–11,29). Most previous studies of the response of renal epithelial cells to HIV-1 infection have used cell lines from rodents or hosts that are not susceptible to developing HIVAN (white individuals). In these studies, we used RTEC from a patient with HIVAN to maximize the likelihood that the *in vitro* cellular response to infection will be relevant to HIVAN pathogenesis.

UBL have important roles in the pathogenesis of several

diseases, including malignancy, neurodegenerative diseases, immune disorders, and infectious diseases (30). FAT10 is a 165–amino acid UBL that contains two ubiquitin-like domains, each of which is approximately one third identical to ubiquitin (14). FAT10 first was cloned by Fan *et al.* (31), who discovered the gene by cDNA hybridization selection while analyzing the human HLA-F locus for novel genes. FAT10 initially was reported to be expressed in mature B lymphocytes and dendritic cells, suggesting a role for the gene in antigen processing/presentation and immune response (32). Other investigators have found, however, that a variety of transformed cell lines express FAT10 when they are exposed to TNF- $\alpha$  and IFN- $\gamma$  but not IFN- $\alpha$  (14). This is in contrast to the UBL ISG-15, which is induced by IFN- $\alpha$  but not IFN- $\gamma$  (33). Although both the cytokine-mediated induction of FAT10 *in vitro* and its genomic localization suggest that FAT10 may have a role in immunity, the *in vivo* role of FAT10 remains obscure.

The first evidence that FAT10 is a proapoptotic molecule came from Raasi *et al.*, who reported that attempts to produce cell lines that would constitutively express FAT10 protein were unsuccessful as a result of increased cell death (34). The same group later found that inducible expression of FAT10 in mouse fibroblasts results in caspase-dependent apoptosis that is abrogated by deletion of the c-terminal diglycine motif of FAT10 (14). Because this motif is necessary for covalent attachment of ubiquitin and UBL to target proteins, this study suggested that

FAT10 must become covalently attached to as-yet-unidentified protein(s) to induce apoptosis. Further supporting a role for FAT10 in apoptosis, Coyle *et al.* (35) reported that induction of apoptosis in hepatoma cells by exposure to TGF- $\beta$  results in strong upregulation of FAT10. Because TGF- $\beta$  is upregulated in renal specimens from patients with HIVAN (36) and has been implicated as a mediator in the pathogenesis of many forms of progressive renal failure, FAT10 may be involved in TGF- $\beta$ -induced apoptosis.

FAT10 conjugation to proteins was shown recently to target proteins for ubiquitin-independent proteasomal degradation (12). Although the proteins to which FAT10 becomes covalently conjugated remain unknown, FAT10 has been shown to interact noncovalently with two proteins, MAD2 (13) and NEDD8 ultimate buster 1L (NUB1L) (37). MAD2 is a mitotic spindle assembly checkpoint protein whose inhibition can lead to genomic instability and subsequent apoptosis (38). NUB1L has been reported to bind noncovalently to another UBL, NEDD8. NUB1L shortens the half-life of NEDD8 by facilitating its degradation *via* the proteasome (39). Because NEDD8 modifies the function of several proteins, including ubiquitin ligases (40) and p53 (41), dysregulated FAT10 expression could lead to apoptosis by altering intracellular levels of NEDD8. Therefore, increased apoptosis that is caused by increased FAT10 expression may result from covalent and/or noncovalent interactions of FAT10 with several cellular proteins.

Ubiquitin and UBL are critical co-factors in the HIV-1 life cycle. HIV-1 Tat, for example, must be ubiquitylated to maximally transactivate transcription from the HIV-1 LTR promoter (42), and the Gag late protein p6 must be ubiquitylated to allow budding of HIV-1 virions from the cell membrane (43). P6 also was shown recently to be capable of modification by the UBL SUMO-1 (44). Other HIV-1 proteins, such as Vif and Vpu, induce ubiquitylation and subsequent degradation of host proteins (45–47). Whether HIV-1 proteins are targets of FAT10 conjugation or induce FAT10 conjugation to host proteins is currently under investigation.

## Conclusion

These studies demonstrate that HIV-1 expression in RTEC induces expression of FAT10 and that FAT10 expression induces apoptosis in RTEC. Moreover, inhibition of FAT10 expression results in suppression of HIV-induced apoptosis of RTEC. Because FAT10 expression is increased in HIVAN and ADPKD, it may have an important role in the dysregulation of apoptosis that occurs in the renal epithelium in these diseases.

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