

# Tumor suppressor pp32 represses cell growth through inhibition of transcription by blocking acetylation and phosphorylation of histone H3 and initiating its proapoptotic activity

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## Abstract

pp32 belongs to a family of leucine-rich acidic nuclear proteins, which play important roles in many cellular processes including regulation of chromatin remodeling, transcription, RNA transport, transformation and apoptosis. pp32 is described as a new tumor suppressor. It is unknown as to how pp32 works in tumor suppression. We found that overexpression of pp32 in human Jurkat T cells inhibits cell growth, and silenced pp32 promotes growth. We first showed that hyperacetylation and hyperphosphorylation of histone H3 are required for T-cell activation. Phosphorylation of histone H3 precedes acetylation during T-cell activation. pp32 specifically binds to histone H3 and blocks its acetylation and phosphorylation. pp32 directly initiates caspase activity and also promotes granzyme A-mediated caspase-independent cell death. Taken together, pp32 plays a repressive role by inhibiting transcription and triggering apoptosis.

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**Keywords:** pp32; growth inhibition; histone H3 acetylation; histone H3 phosphorylation; transcription; apoptosis

**Abbreviations:** aa, amino acid; ChIP, chromatin immunoprecipitation; CRE, cAMP-response element; EGF, epidermal growth factor; ER, endoplasmic reticulum; GFP, green fluorescent protein; Gzm, granzyme; HAT, histone acetyltransferase; IE, immediate-early; IL-2, interleukin 2; Lys, lysine; MAPK, mitogen-activated protein kinase; NAP, nucleosome assembly protein; PCAF, p300/CBP-associated factor; PFP, pore-forming protein; pI, isoelectric point; PKC, protein kinase C; PMA, phorbol myristate acetate; PP2A, protein phosphatase 2A; RNAi, RNA interference; Ser, serine

## Introduction

pp32 (also known as PHAPI, I<sub>1</sub><sup>PP2A</sup>) was confirmed as a tumor suppressor gene.<sup>1</sup> Malek *et al.*<sup>2</sup> described two related nuclear phosphoproteins, pp32 and pp35, in a neoplastic B-lymphoblastoid cell line A20. The pp32 gene located on chromosome 15 encodes a 249 aa acidic protein with four leucine-rich repeats (a motif implicated in binding the nuclear export factor crm1) in the N-terminal region and a C-terminal acidic domain (aa 168–249) in which a nuclear localization signal (KRKR, aa 236–239) is embedded.<sup>3</sup> Cotransfection of pp32 with oncogene pairs such as E6 + E7, ras + c-jun, ras + c-myc, ras + E1A and ras + mutant p53 suppresses transformed focus formation. Constitutive expression of pp32 abolishes ras-mediated transformation *in vitro* and tumorigenesis *in vivo*, whereas ablation of endogenous pp32 expression augments ras-mediated oncogenesis. Clinical studies show pp32 gene mutation and underexpression in prostate and breast cancers.<sup>4</sup> Deletion and truncation analysis defines a region of pp32 spanning aa 150–174 as being absolutely required for inhibition of transformation. Closely related homologs of pp32 (pp32r1, pp32r2 and APRIL) exist; the first two lack the NLS and do not act as tumor suppressors.<sup>1</sup>

Although the molecular mechanisms of pp32 in tumor suppression remain unknown, some intriguing connections to fundamental cellular processes have been made. They suggest that pp32 is a component of nuclear and endoplasmic reticulum (ER)-associated complexes possibly involved in modulating chromatin structure, transcriptional regulation and apoptosis. pp32 shuttles between the nucleus and the cytoplasm by binding the nuclear export protein crm1 through leucine-rich regions (LRR). pp32 is homologous to the regulatory  $\beta$ -chain of protein phosphatase 2A (PP2A), a phosphatase whose activity is increased during apoptosis, and inhibits PP2A activity.<sup>5</sup> Its role as a PP2A inhibitor might provide a mechanism for affecting multiple distinct oncogenic pathways. Two studies have shown that pp32, along with its homolog APRIL, and the protein product of the leukemia-translocated gene SET are contained in an ~150 kDa nuclear complex.<sup>6</sup> SET (also known as PHAPII, TAF-1 $\beta$  or I<sub>2</sub><sup>PP2A</sup>) is an evolutionarily conserved ATP-independent nucleosome assembly protein (NAP) that also inhibits PP2A and facilitates transcription.<sup>7</sup> SET and other NAP family members physically associate with the p300/CREB-binding protein family of transcriptional coactivators and with core histones.<sup>8</sup> One study showed that the pp32-containing nuclear complex inhibits histone acetylation by binding to and masking histone acetyltransferase (HAT) targets.<sup>6</sup> SET also associates with the methylated silencing gene region on chromatin and represses active demethylation of DNA.

Post-translational modifications of core histone proteins play a crucial role in genome function and gene expression. These modifications include acetylation, phosphorylation, methylation, ADP-ribosylation and monoubiquitination.<sup>9</sup> The totality of modifications, both in kind and number, dictates a particular biological outcome, called the 'histone code'.<sup>10</sup> A role has been established for the acetylation and phosphorylation of core histones in transcriptional processes and remodeling chromatin structure. Several HATs and histone deacetylases were found in eukaryotic cells, and maintain a balance between acetylation and deacetylation. Acetylation of histone tails disrupts higher-order chromatin folding and maintains the unfolded structure of the transcribed nucleosome to transcriptional coactivators and transcription to occur. It is generally accepted that acetylated histones are mostly associated with active genomic regions. By contrast, deacetylation mainly leads to repression and silencing.<sup>11</sup> Lysine (Lys) 9, 14, 18 and 23 can be acetylated at the N-terminal tails of histone H3. Besides acetylation, histones undergo phosphorylation on specific serine (Ser) and threonine residues in association with different cellular processes.<sup>12</sup> Histone H3 phosphorylation at Ser 10 is closely related to induction of immediate-early (IE) genes, chromatin remodeling and chromatin condensation during mitosis and meiosis.<sup>13</sup> Ser 10 phosphorylation of histone H3 is also correlated with the transcriptional activation of mitogen-induced genes.<sup>14</sup> Ser 28 phosphorylation of histone H3 also appears in response to stimulation of the mitogen-activated protein kinase (MAPK) pathways involving activation of extracellular signal-regulated kinases and p38 MAPK at a time when IE genes are expressed. A recent report has shown that Ser 10 and 28 phosphorylation of histone H3 is excluded from regions of highly condensed chromatin and is present in increased levels following stimulation of the ras-MAPK pathway in ras-transformed cells.<sup>15</sup> These two phosphorylation events act separately to promote gene expression. It is unknown as to how acetylation and phosphorylation of histone H3 crosstalk in tumor suppressor-mediated gene repression.

We recently identified a larger 270–420 kDa ER-associated complex (SET complex) containing pp32 and SET in a novel caspase-independent cell death pathway induced by the cytotoxic T-lymphocyte protease, granzyme A (Gzma).<sup>16,17</sup> SET, but not pp32, is a Gzma substrate. pp32 interacts weakly with SET in cell lysate co-immunoprecipitation experiments, but the interaction is greatly strengthened in the presence of Gzma. In the presence of Gzma, the purified SET complex activates in isolated nuclei the novel type of DNA damage induced in Gzma-mediated cell death (single-stranded DNA nicking).<sup>18,19</sup> A Gzma-activated DNase was identified as NM23H1, a nucleoside diphosphate kinase implicated in suppression of tumor metastasis, and SET is its specific inhibitor.<sup>16</sup> Therefore, pp32 may participate in the regulation of caspase-independent apoptosis induced by cytotoxic T-lymphocytes. Intriguingly, pp32 may also play a role in caspase-dependent apoptosis.<sup>20</sup> pp32 promotes caspase-9 activation in the apoptosome formation following the initiation of the mitochondrial apoptotic pathway and subsequent activation of the effector caspase-3. In this study, we overexpressed and silenced tumor suppressor pp32 in Jurkat T cells and showed that pp32 inhibits cell growth *in vivo*.

pp32 specifically binds to histone H3 and exerts its repressive role by blocking acetylation and phosphorylation of histone H3. And pp32 also triggers apoptosis by initiating caspase activation.

## Results

### pp32 overexpression inhibits cell growth

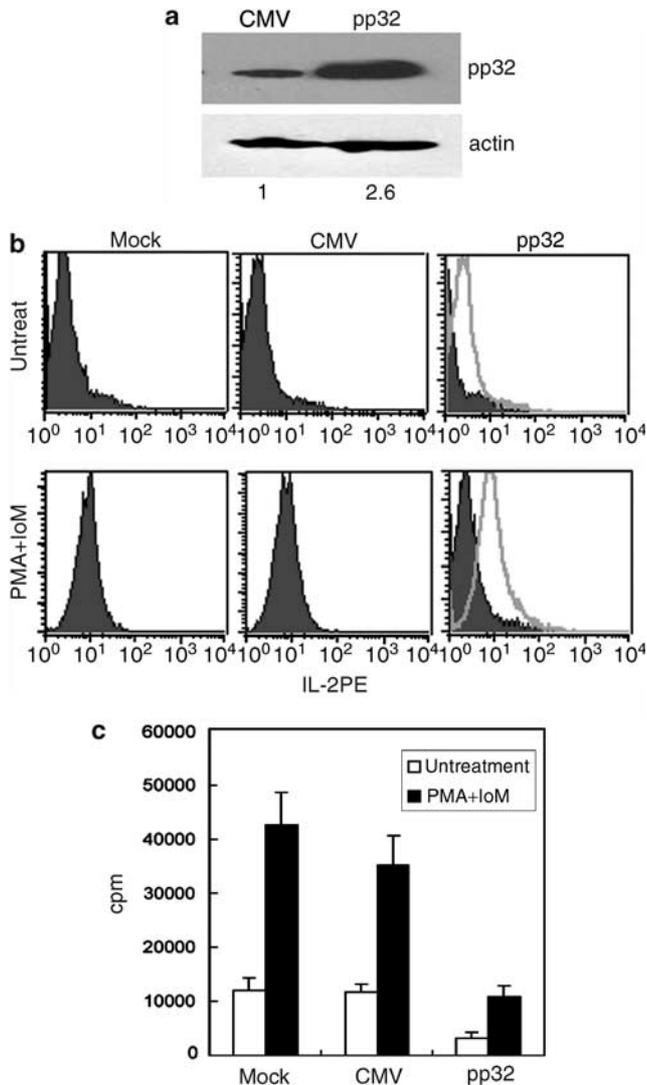
We wanted to determine whether pp32 represses cell growth without oncogene transfection. pCMV-pp32 vector was transfected into human lymphoma Jurkat T cells. pp32 was overexpressed in pCMV-pp32-transfected Jurkat cells 2.6-fold higher than that treated with pCMV empty vector control (Figure 1a). T cells require both a Ca<sup>2+</sup> signal and a protein kinase C (PKC)/ras signal for activation. Phorbol myristate acetate (PMA) activates PKC and ionomycin opens membrane Ca<sup>2+</sup> channels, which mimic antigen receptor engagement plus an accessory signal to induce interleukin 2 (IL-2) production and proliferation in T cells. After 3 days of transfection of pp32, Jurkat cells were treated with or without PMA plus ionomycin and brefeldin A for 24 h. Intracellular IL-2 was assayed through flow cytometry. pp32 overexpression decreased IL-2 production with or without treatment of PMA plus ionomycin, but more dramatically reduced with PMA and ionomycin treatment (Figure 1b). Jurkat cells post-3 days pp32 transfection were incorporated by <sup>3</sup>H-TdR with or without PMA plus ionomycin stimulation for 24 h. pp32-overexpressed Jurkat cells proliferated at an extremely lower level than the pCMV empty vector control ( $P < 0.001$ ) (Figure 1c). Similar results were found in pp32-overexpressed cells without treatment of PMA plus ionomycin ( $P < 0.001$ ).

### pp32 silencing augments cell growth

pp32 was silenced by RNA interference (RNAi). Two 21-nucleotide siRNA duplexes for pp32 were separately transfected into Jurkat cells. After 3 days, pp32 expression was detected with immunoblot. Two siRNA duplexes can knock down pp32 expression by more than 90% as compared with a control siRNA for green fluorescent protein (GFP) or mock-transfected cells. Silenced pp32 by one siRNA duplex is shown in Figure 2a. After 3 days of pp32 silencing, Jurkat cells were stimulated with PMA and ionomycin as well as brefeldin A for 24 h. IL-2 production increased in pp32-silenced Jurkat cells with or without treatment of PMA and ionomycin (Figure 2b). The increase in IL-2 production was less profound in Jurkat cells with treatment of PMA plus ionomycin than without the treatment. Proliferation of pp32-silenced Jurkat cells increased more than two-fold than that treated with siRNA for GFP control with stimulation of PMA plus ionomycin (Figure 2c). The unstimulated pp32-silenced cells showed a proliferation trend similar to the stimulated cells. It suggests that IL-2 may be a more sensitive factor in reflecting T-cell activation in an early stage.

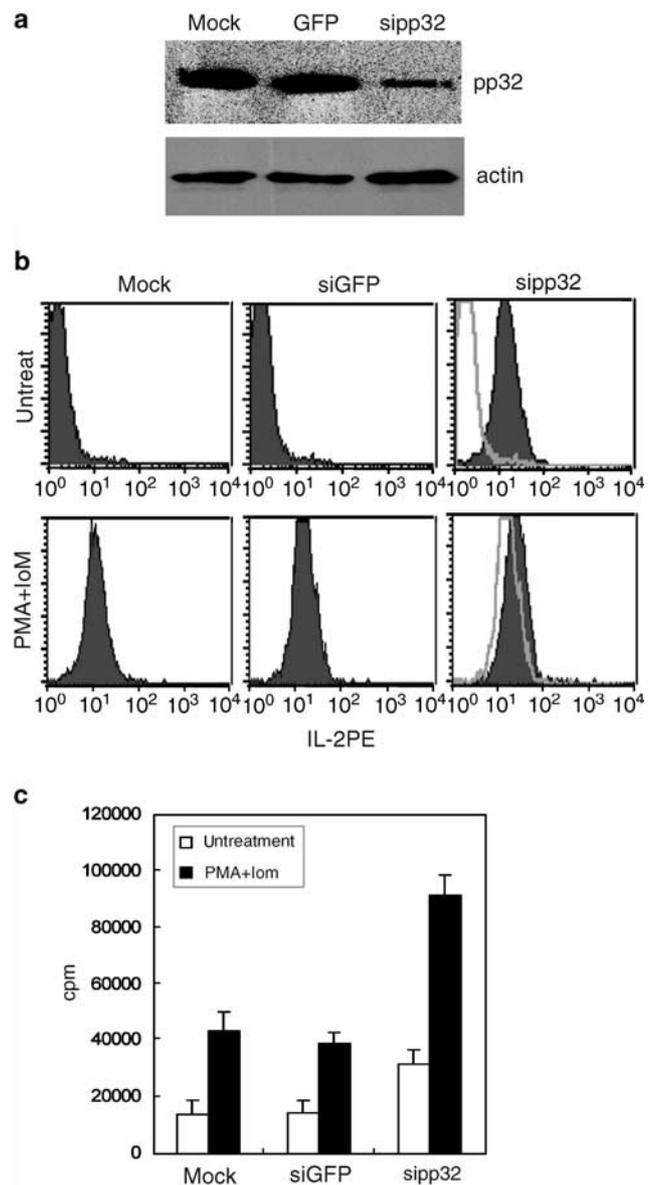
### pp32 specifically blocks acetylation and phosphorylation of histone H3

Chromatin has important structural and regulatory roles in the control of gene expression and silence in eukaryotes.<sup>21</sup>



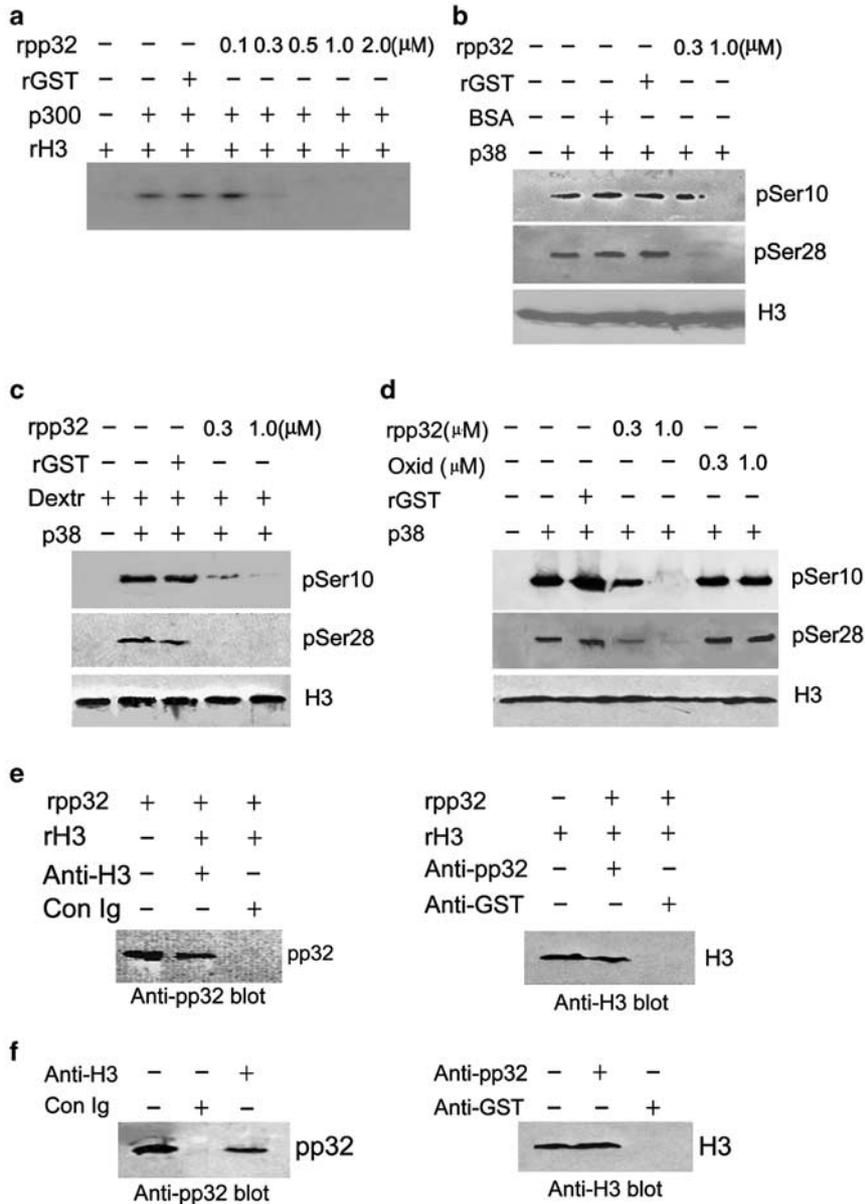
**Figure 1** pp32 overexpression decreases IL-2 production and cell proliferation. (a) pp32 is overexpressed in Jurkat cells after a 3 day transfection.  $\beta$ -Actin was probed as a loading control. The ratio of pp32 to  $\beta$ -actin signal was calculated by densitometry. (b) IL-2 production reduces in pp32-overexpressed Jurkat cells without or with the treatment of PMA plus ionomycin. The results are representative of four independent experiments. (c) pp32 overexpression significantly inhibits cell proliferation poststimulation of PMA plus ionomycin by <sup>3</sup>H-TdR incorporation assay. Similar results were found without the stimulation of PMA plus ionomycin

Regulation of chromatin structure and transcription can be achieved through interaction between histones and chromatin-remodeling factors and/or post-transcriptional modification of N-terminal histone tails.<sup>22</sup> It is unclear how histone codes crosstalk in modulation of gene expression. pp32 is a subunit of the inhibitor of acetyltransferase (INHAT) complex, which binds to histones and represses p300/CBP- and p300/CBP-associated factor (PCAF)-mediated histone acetylation.<sup>23</sup> We showed here that rpp32 blocks p300-induced histone H3 acetylation in a dose-dependent manner. rpp32 (0.3  $\mu$ M) can completely block 1  $\mu$ M H3 acetylation (Figure 3a). To determine whether pp32 affects phosphorylation of histone H3, p38 kinase was used to phosphorylate rH3. rpp32



**Figure 2** pp32 silencing increases IL-2 production and cell proliferation. (a) pp32 expression is silenced by more than 90% in Jurkat cells after treatment with siRNA1 duplexes against pp32.  $\beta$ -Actin expression is unchanged in mock, GFP control or silenced pp32 cells. The other duplex siRNA2 has a similar silencing effect on pp32 (not shown). (b) IL-2 production increases in pp32-silenced Jurkat cells without or with PMA and ionomycin treatment. IL-2 rises dramatically in pp32-silenced cells without stimulation of PMA and ionomycin. The results are representative of four independent experiments. (c) pp32 silencing significantly augments cell proliferation after treatment of PMA plus ionomycin. Similar results were found without the stimulation of PMA plus ionomycin

inhibited phosphorylation of histone H3 at Ser 10 and Ser 28 (Figure 3b). rpp32 (0.3  $\mu$ M) can block Ser 28 phosphorylation of histone H3, while complete blockade of Ser 10 phosphorylation required 1  $\mu$ M rpp32. rGST had no effect on histone H3 phosphorylation as a negative control. pp32 is an acidic protein, while histone H3 is a basic protein. To verify the blockade specificity of histone H3 phosphorylation by pp32, a negatively charged reagent dextran sulfate was added to



**Figure 3** pp32 blocks acetylation and phosphorylation of histone H3. (a) rpp32 blocks p300- or PCAF-mediated histone H3 acetylation (and not shown). rGST (1  $\mu$ M) has no inhibitory effect as a negative control. (b) rpp32 inhibits p38 kinase-mediated histone H3 phosphorylation at Ser 10 and Ser 28. rGST (1  $\mu$ M) has no repressive effect on histone H3 phosphorylation. Histone H3 was probed as a loading control. (c) Negatively charged dextran sulfate cannot influence rpp32-mediated inhibition of histone H3 phosphorylation at Ser 10 and Ser 28. (d) Glucose oxidase of similar *pI*/protein with pp32 cannot repress p38 kinase-mediated histone H3 phosphorylation at Ser 10 and Ser 28. (e) rpp32 binds directly to rH3. rpp32 and rH3 were co-incubated and immunoprecipitated with anti-pp32 mAb or anti-H3 antisera or control antibody. (f) pp32 associates with histone H3 *in vivo*. Anti-pp32 mAb can precipitate native histone H3, and anti-H3 antisera can specifically pull down native pp32 in Jurkat nuclear extracts

neutralize the positively charged rH3 in the reaction. rpp32 still abolished Ser 10 and Ser 28 phosphorylation of histone H3 in the dextran sulfate-containing buffer (Figure 3c). And dextran sulfate had no effect on pp32-mediated inhibition of histone H3 acetylation induced by p300/CBP (data not shown). Glucose oxidase is an acidic protein, whose isoelectric point (*pI*) is around 4, similar to pp32. Glucose oxidase had no effect on the inhibition of histone H3 phosphorylation both at Ser 10 and Ser 28 mediated by p38 kinase, or on the inhibition of histone H3 acetylation mediated by p300/CBP (Figure 3d and

not shown). These results indicate that pp32 specifically inhibits acetylation and phosphorylation of histone H3.

We next wanted to look at the association between pp32 and histone H3. rpp32 co-precipitated rH3 by immunoprecipitation experiments (Figure 3e). Dextran sulfate did not influence the direct association between rpp32 and rH3 (not shown). Glucose oxidase cannot precipitate rH3 (not shown). To determine pp32 and histone H3 interaction in cells, Jurkat nuclei were sonicated and harvested the supernatants. Salmon sperm DNA/protein A agarose slurry was used to

precipitate the supernatants, and immunoprecipitation was carried out. Anti-H3 can precipitate pp32 in Jurkat nuclear extracts. Control rabbit Ig was used as a negative control. In an alternative way, anti-pp32 can precipitate histone H3. Anti-GST did not pull down histone H3 as a negative control (Figure 3f). Together, pp32 specifically binds to histone H3 *in vitro*, and associates with H3 *in vivo*.

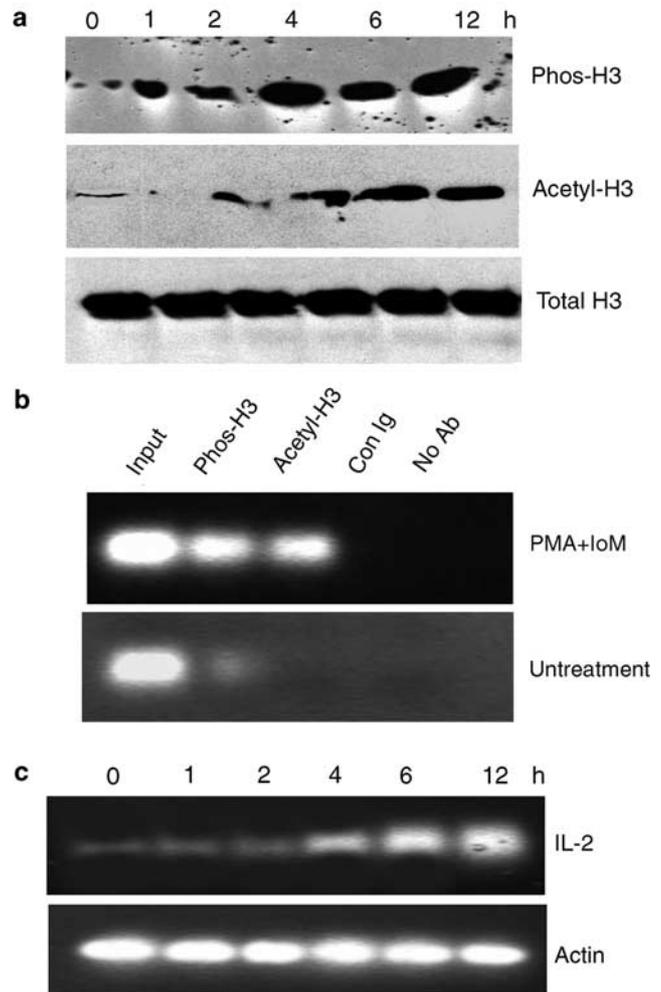
### Cell growth requires hyperacetylation and hyperphosphorylation

T-cell activation is accompanied by visible changes in chromatin structure. An unstimulated T cell has a small, extremely compact nucleus with a dense heterochromatin. Within a few hours after activation, its nucleus increases five- to 10-fold in volume and euchromatin appears. Little is known about the mechanisms that undergo these rapid and signal-dependent changes in chromatin. Total acetylation and phosphorylation were visualized in Jurkat cells with immunoblot upon PMA plus ionomycin stimulation. Total acetylation and phosphorylation of histone H3 increased with the time course of stimulation (Figure 4a). Phosphorylation precedes acetylation and peaks at 4 h, whereas acetylation reaches its maximal level at 6 h, complete acetylation and phosphorylation were almost undetectable in unstimulated Jurkat cells.

IL-2 plays a crucial role in T-cell activation. The IL-2 promoter is activated in T cells upon engagement of the T-cell receptor /CD3 complex. PMA plus ionomycin can bypass these receptors to initiate IL-2 production. The kinetics of IL-2 production parallels that of chromatin remodeling in T cells.<sup>24</sup> IL-2 promoter activation needs open chromatin. The cAMP-response element (CRE) site of the IL-2 promoter is important for IL-2 production, which recruits transcriptional cofactors and remodels chromatin.<sup>25</sup> To verify whether histone H3 modification influences IL-2 promoter activation, Jurkat cells were stimulated with PMA plus ionomycin for 12 h, then fixed with formaldehyde to crosslink DNA to proteins, sonicated and the DNA-protein complexes were precipitated with specific antibodies for total acetylation and phosphorylation as above. The DNA was extracted and amplified with primers flanking the -180 CRE site of the IL-2 promoter. The CRE region of the activated IL-2 promoter bound to phosphorylated and acetylated histone H3, while they were undetectable in nonactivated IL-2 promoter (Figure 4b). To further determine whether IL-2 generation is required for hyperacetylation and hyperphosphorylation of histone H3, we analyzed IL-2 mRNA expression in Jurkat cells with the time course as treated above. IL-2 mRNA expressed dramatically at 4 h and dynamically rose during activation (Figure 4c). IL-2 mRNA was undetectable in the unstimulated cells.  $\beta$ -Actin was the internal control. IL-2 mRNA expression parallels the total acetylation and phosphorylation of histone H3 in the process of T-cell activation. These data indicate that IL-2 promoter activation is required for hyperacetylation and hyperphosphorylation of histone H3.

### Overexpression of pp32 reduces acetylation and phosphorylation of histone H3

Jurkat cells were treated with PMA plus ionomycin for 12 h post-3 days overexpression of pp32. Jurkat nuclear extracts



**Figure 4** Hyperacetylation and hyperphosphorylation of histone H3 are required for T-cell activation. (a) Total phosphorylation and acetylation of histone H3 increase during the time course of Jurkat activation through treatment of PMA plus ionomycin. Total histone H3 is unchangeable at all time points. Histone H3 phosphorylation increases faster than its acetylation. Jurkat nuclei were extracted in lysis buffer with 100  $\mu$ M sodium orthovanadate, 10 mM sodium butyrate, 0.1  $\mu$ M okadaic acid and protease inhibitor cocktails after stimulation of PMA plus ionomycin, and probed with anti-Phos-H3, anti-Acetyl-H3 or anti-H3 antibodies. (b) Phosphorylated and acetylated histone H3 are present on nucleosomes associated with the CRE region of IL-2 promoter activation. Formaldehyde crosslinked chromatin was prepared from 12 h PMA plus ionomycin or nontreated Jurkat cells and immunoprecipitated with the anti-Phos-H3 or anti-acetyl-H3 antibody. The recovered DNAs were amplified with the primers for the CRE site of IL-2 promoter. Rabbit Ig (Con Ig) or no antibody (No Ab) was used as a negative control. The amount of input DNA used for the template in the PCR was one-tenth that utilized for the immunoprecipitation. The results shown represent one out of four independent experiments. (c) IL-2 mRNA expression parallels with hyperacetylation and hyperphosphorylation of histone H3 during T-cell activation. IL-2 mRNA was measured in Jurkat cells with stimulation by PMA plus ionomycin at the times indicated as above.  $\beta$ -Actin was as an internal control. The data are representative of three independent experiments

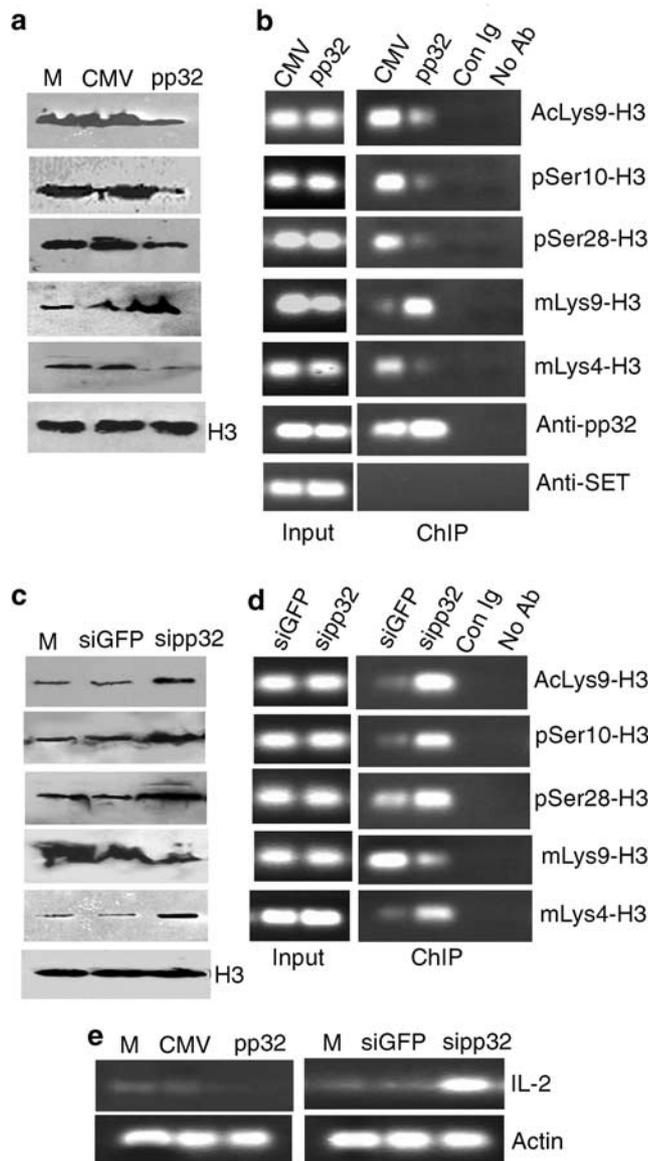
were prepared and analyzed histone H3 acetylation at Lys 9 and Ser 10 and 28 phosphorylation were analyzed by immunoblot. pp32 overexpression decreased histone H3 acetylation at Lys 9 as well as phosphorylation at Ser 10 and 28 (Figure 5a). Phosphorylation at Ser 10 and 28 showed similar reduced levels after pp32 overexpression. Histone H3

Lys 9 methylation is associated with gene silencing and repression, which is characteristic of the heterochromatic state,<sup>26</sup> whereas histone H3 Lys 4 methylation represents active and permissive chromatin regions served as a marker of euchromatin. As expected, heterochromatic marker Lys 9 methylation of histone H3 was presented at a high level in pp32-overexpressed cells. Mock- or pCMV empty vector-transfected Jurkat cells showed low levels of Lys 9 methylation, while euchromatic marker Lys 4 methylation of histone H3 was almost undetectable in overexpressed pp32 cells. Mock- or empty vector-treated Jurkat cells showed comparable levels. Total histone H3 showed no changes in mock-, pCMV- or pCMV-pp32-treated cells. To further determine histone H3 modification state of the CRE region for the IL-2 promoter, chromatin immunoprecipitation (ChIP) was performed from pp32-overexpressed Jurkat cells with PMA and ionomycin treatment for 12 h. The CRE site of the IL-2 promoter bound to less-acetylated and phosphorylated

histone H3 in pp32-overexpressed cells compared with those in mock- or empty vector-treated cells (Figure 5b). Lys 9 methylation was presented at a high level and Lys 4 methylation was undetectable in the CRE site of the IL-2 promoter. To further define the direct role of pp32 in inhibiting histone H3 acetylation and phosphorylation at the CRE site of the IL-2 gene, we performed ChIP experiments with anti-pp32 or anti-SET antibody in pp32-overexpressed Jurkat cells as treated above. pp32, not SET, bound to the CRE region of the IL-2 gene (Figure 5b). The same amount of DNA was used and the results are representative of at least three independent experiments. These data indicate that pp32 specifically associates with the CRE region of the IL-2 gene to modulate IL-2 promoter activation.

### pp32 silencing augments acetylation and phosphorylation of histone H3

pp32 was silenced with siRNA against pp32 for 3 days in Jurkat cells, and then stimulated with PMA plus ionomycin for 12 h. Acetylation and phosphorylation of histone H3 were analyzed by immunoblot as above. pp32 knockdown increased acetylation at Lys 9 and phosphorylation at Ser 10 and 28 of histone H3. A control siRNA for GFP showed comparable levels of acetylated and phosphorylated histone H3 (Figure 5c). Histone H3 methylation at Lys 9 remained at a very low level, while histone H3 methylation at Lys 4 remained a high level. Total histone H3 remained at the same levels in either pp32-silenced or mock- or siRNA- to GFP-treated cells. The Histone H3 modification state of the CRE region for IL-2 promoter was assayed by ChIP with PMA plus ionomycin treatment post-pp32 silencing. The CRE site of IL-2 promoter with pp32 knockdown bound more acetylated and phosphorylated histone H3 than siRNA for GFP control (Figure 5d). This region showed undetectable histone H3 Lys 9 methylation and



**Figure 5** pp32 influences *in vivo* acetylation and phosphorylation of histone H3 via pp32 overexpression or silencing. (a) Overexpressed pp32 inhibits Lys 9 acetylation and Ser 10 and Ser 28 phosphorylation of histone H3. Euchromatic marker Lys 4 methylation of histone H3 is almost undetectable in overexpressed pp32 cells, while heterochromatic marker Lys 9 methylation of histone H3 is present at a high level. Total histone H3 is unchanged in mock-, pCMV- or pCMV-pp32-treated cells. Nuclear extracts were obtained as above and probed with the indicated antibodies. (b) The CRE region of IL-2 promoter in pp32-overexpressed cells binds less-acetylated or phosphorylated histone H3. The nucleosomes of the CRE region remain in a heterochromatic state with more Lys 9 methylation and less Lys 4 methylation of histone H3. pp32, not SET, directly bound to the CRE site of the IL-2 gene. Rabbit Ig (Con Ig) or no antibody (No Ab) was used as a negative control. The amount of input DNA used for the template in the PCR was one-tenth that utilized for the immunoprecipitation. Results shown are representative of one out of three independent experiments. (c) Silenced pp32 promotes Lys 9 acetylation and Ser 10 and Ser 28 phosphorylation of histone H3. Lys 4 methylation of histone H3 is present in a high level in pp32-silenced cells, while Lys 9 methylation of histone H3 is at a very low level. Total histone H3 remained at similar levels. (d) The CRE region of the IL-2 promoter in pp32-silenced cells binds more acetylated and phosphorylated histone H3. The nucleosomes of the CRE region occurs in a euchromatic state with less Lys 9 methylation and more Lys 4 methylation of histone H3. Rabbit Ig (Con Ig) or no antibody (No Ab) was used as a negative control. (e) pp32 blocks IL-2 mRNA expression. IL-2 mRNA was measured in pp32-overexpressed or -silenced Jurkat cells with stimulation by PMA plus ionomycin for 12 h. The data are representative of three independent experiments

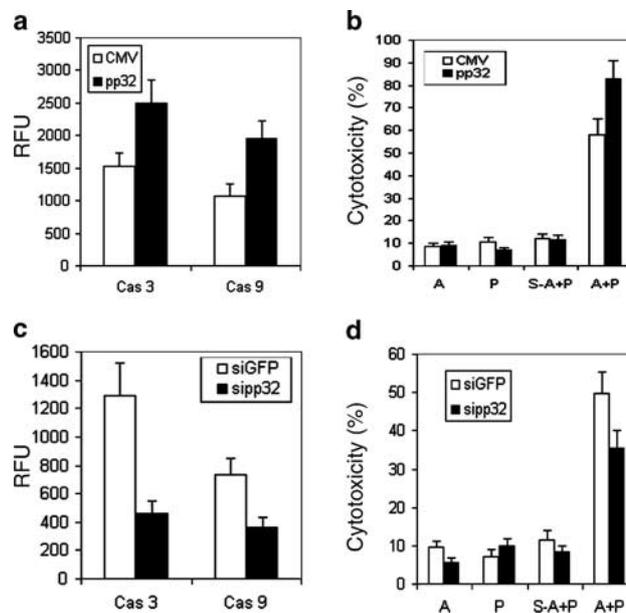
underwent more Lys 4 methylation. IL-2 production by T cells is regulated at multiple levels, including transcription, mRNA stability and rate of protein secretion. To analyze the stages at which pp32 acts, we measured the levels of IL-2 mRNA transcripts by RT-PCR in pp32-overexpressed or -silenced Jurkat cells for 3 days, and stimulated with PMA plus ionomycin for 12 h. Total RNA was extracted and RT-PCR was performed to detect IL-2 mRNA abundance. pp32 overexpression blocked IL-2 mRNA expression (Figure 5e), while pp32 silencing dramatically increased IL-2 mRNA expression, IL-2 mRNA was comparable in mock-, pCMV vector- or siGFP-treated cells.  $\beta$ -Actin was used as an internal control. The data represent three independent experiments. These results indicate that pp32 specifically acts on the transcription level of the IL-2 gene.

### pp32 overexpression triggers caspase activity and enhances cell death

pp32 is associated with GzmA substrate SET in the presence of GzmA.<sup>19</sup> GzmA degrades SET and abolishes its inhibition of DNase NM23H1 to yield DNA nicks in GzmA-induced caspase-independent cell death. However, pp32 is not cleaved by GzmA in its recombinant form or in cell lysates and even in GzmA-loaded cells (not shown). pp32 is associated with GzmA and not its substrate. This indicates that pp32 plays roles in GzmA-mediated caspase-independent cell death. A recent report showed that pp32 induces caspase-9 activation to promote apoptosome formation in caspase-dependent apoptosis.<sup>20</sup> Jurkat cells with 3 days pp32 overexpression were treated with UV irradiation and incubated overnight. Cytosolic caspase activity was analyzed by degrading their specific fluorogenic substrates. pp32 overexpression augmented caspase-3 and -9 activity compared with pCMV empty vector-treated cells in UV-induced apoptosis ( $P < 0.001$ ) (Figure 6a). To verify further whether pp32 enhances GzmA-induced cell death, GzmA was added with perforin (pore-forming protein (PFP)) <sup>51</sup>Cr-labeled Jurkat cells that were transfected with pCMV-pp32 or pCMV vector for 3 days. GzmA plus PFP induced  $71 \pm 9\%$  specific lysis above the PFP background in pp32-transfected cells, 1.5-fold higher than that in pCMV-transfected control cells ( $46 \pm 5\%$ ) ( $P < 0.001$ ). There were no substantial differences between the background lysis induced by GzmA or PFP alone, or by PFP and inactive S-AGzmA (Figure 6b). Together, pp32 triggers both caspase-dependent and -independent cell death.

### pp32 silencing reduces caspase activity and protects cells from death

To verify further proapoptotic roles of pp32, cytosolic caspase activity from Jurkat cells was assayed in UV-induced apoptosis after pp32 silencing. pp32 silencing decreased caspase-3 and -9 activity more than that in a control siRNA for GFP ( $P < 0.001$ ) (Figure 6c). pp32-silenced Jurkat cells are less susceptible to cytolysis by GzmA plus PFP than that in cells transfected with siGFP control. GzmA plus PFP induced  $21 \pm 2\%$  cytotoxicity in pp32-silenced Jurkat cells above the PFP background in control cells, whereas  $38 \pm 3\%$  specific lysis occurred in siGFP-transfected control ( $P < 0.001$ ). The



**Figure 6** pp32 affects caspase activity after UV treatment and cytotoxicity induced by GzmA through pp32 overexpression or silencing. (a) pp32-overexpressed Jurkat cells increase UV-induced caspase-3 and -9 activity. Cytosolic caspase-3 and -9 activity was measured 12 h after UV irradiation by the fluorogenic caspase assay. (b) pp32 overexpression enhances GzmA-mediated cytotoxicity. pp32-overexpressed or pCMV-transfected Jurkat cells were labeled with <sup>51</sup>Cr and loaded 1  $\mu$ M GzmA plus sublytic PFP for 4 h. Cell death after treatment with GzmA alone (A), PFP alone (P) or inactive S-AGzmA plus PFP (S-A + P) are comparable on the pCMV-pp32- or pCMV-transfected cells. Data represent six independent experiments. (c) pp32-silenced Jurkat cells decrease UV-induced caspase 3 and -9 activity. Cytosolic caspase-3 and -9 activity was measured as above. (d) pp32 silencing inhibits GzmA-mediated cytotoxicity. pp32-silenced or siGFP-treated Jurkat cells were labeled with <sup>51</sup>Cr and loaded 1  $\mu$ M GzmA plus sublytic PFP for 4 h. Cell death after treatment with GzmA alone (A), PFP alone (P) or inactive S-AGzmA plus PFP (S-A + P) are comparable on the pp32-silenced or GFP control cells. Data represent six independent experiments

background lysis caused by GzmA or PFP alone, or by PFP plus S-AGzmA was around 10% (Figure 6d).

## Discussion

Human T-cell line Jurkat was used to visualize cell growth by assessing IL-2 production and proliferation after pp32 overexpression or silencing. Our *ex vivo* and *in vivo* data demonstrate that pp32 inhibits cell growth through suppression of transcription by blocking acetylation and phosphorylation of histone H3 and initiating its proapoptotic activity to promote apoptosis. This first elucidated that the tumor suppressor pp32 exerts its inhibitory role as a suppressor by bridging transcription and apoptosis. A previous report showed that overexpressed pp32 did not affect NIH3T3 cell growth on measuring the growth curve by counting cells. Here, we showed that overexpressed pp32 inhibits IL-2 production and proliferation in Jurkat cells. Bai *et al.* further showed that pp32 overexpression abolished ras-mediated transformation *in vitro* and tumorigenesis *in vivo*. Ablation of pp32 by antisense treatment was not tumorigenic but augmented ras-mediated oncogenesis, whereas other pp32 family members pp32r1 and pp32r2 are oncogenic and may

cause clinical tumors.<sup>1</sup> There is no evidence to show that oncogenic pp32r1 or pp32r2 can inactivate the suppressive role of pp32 to result in cancers. Most prostate and breast cancers express high levels of pp32r1 and pp32r2, but an undetectable level of pp32. However, in some cancers, pp32 is highly expressed, such as highly malignant prostatic adenocarcinomas.<sup>27,28</sup> A fairly recent study has shown hyperphosphorylated Rb, the retinoblastoma gene product, associates with pp32.<sup>29</sup> Rb is an important tumor suppressor and cell cycle regulator, that controls the G1/S transition, whose active form is a hypophosphorylated state.<sup>30</sup> pp32 and hyperphosphorylated Rb association is mediated through the LRR of pp32, which serves as adapter sites for protein-protein interactions. pp32 also binds to crm1 to mediate nucleocytoplasmic shuttling. Rb binding of the pp32 LRR in the nucleus prevents binding to crm1, which may abolish pp32 shuttling. Cancer cells increased hyperphosphorylated Rb sequester pp32 in the nucleus, leading to apoptotic resistance, increased free E2F1 and proliferation.

pp32, along with its homolog APRIL, TAF1 $\alpha$  and TAF1 $\beta$ /SET, formed a complex called INHAT. INHAT binds to histones and masks them for histone acetylation. INHAT complex and its individual components have distinct but overlapping histone targets and HAT repression specificities.<sup>23</sup> Besides blockade of histone acetylation, SET binds with a higher affinity to histones H3 and H4. SET also represses demethylation of ectopically methylated DNA, leading to gene silencing. pp32 showed a stronger interaction with histone H3 and H2B. We showed that the association between pp32 and histone H3 is specific *in vivo* and *ex vivo* not by charge. *In vitro* results showed that pp32 blocks acetylation and phosphorylation of histone H3 in a dose-dependent manner, which is confirmed by *in vivo* data from pp32-overexpressed and -silenced cells. ChIP assays demonstrated that activation of IL-2 promoter requires both acetylation and phosphorylation of histone H3. This suggests that acetylation and phosphorylation of histone H3 are linked to regulation of transcription. Our data are consistent with Choung *et al.*'s observation. They showed that Ser 10 phosphorylation and Lys 14 acetylation of histone H3 are coupled in epidermal growth factor (EGF)-induced IE gene expression. However, Thomson *et al.* reported an opposite result by assessing c-jun promoter with stimulation of quiescent cells through subinhibitory anisomycin. They showed enhanced histone H3 and H4 acetylation on c-jun nucleosome that was not phosphorylated.<sup>31</sup> This suggests that crosstalk between phosphorylation and acetylation of histones may depend on the precise manner in which a particular gene is connected up to the signaling pathway.

We found that phosphorylation of histone H3 precedes acetylation during T-cell activation, consistent with the previous observation that histone H3 phosphorylation occurs earlier than acetylation in EGF-induced gene transcription. pp32 inhibits both Ser 10 and Ser 28 phosphorylation of histone H3 *ex vivo* and *in vivo*. These two site Ser phosphorylations appear and disappear within the same time in pp32-overexpressed or -silenced Jurkat cells. The CRE region of the activated IL-2 promoter binds to both Ser 10 and Ser 28 phosphorylated histone H3 as well as acetylated histone H3. This region simultaneously recruits more Lys 4

methylation of histone H3 and undetectable Lys 9 methylation in its activation state. This suggests that IL-2 gene expression requires a euchromatic nucleosome, which is obtained by the modulation of hyperphosphorylation and hyperacetylation of histone H3. The spatial patterns and their crosstalk of phosphorylation and acetylation still need to be further elucidated. A recent study has shown that 12-*O*-tetradecanoylphorbol-13-acetate stimulation of 10T1/2 mouse fibroblasts, the distribution of Ser 10-phosphorylated histone H3 among the modified histone H3 isoforms, is distinct from that of Ser 28-phosphorylated forms. Ser 10 phosphorylation resides mainly in monomodified isoforms, while phosphorylated Ser 28 mostly exists in trimodified isoforms.<sup>15</sup> Both Ser 10 and Ser 28 phosphorylation of histone H3 are localized in chromatin regions participating in dynamic acetylation.

pp32, a component of the GzmA-associated SET complex, is not cleaved by GzmA, and pp32 does not inhibit the DNase activity of NM23H1.<sup>17</sup> GzmA, the most abundant Gzm in CTLs, associates with the SET complex and induces caspase-independent cell death.<sup>16</sup> Wang Lab demonstrated that pp32 augments the activity of the apoptosome, leading to more caspase 9 activity *ex vivo*.<sup>20</sup> They did not obtain any *in vivo* data because of failure to knock down pp32 with RNAi. We succeeded in silencing pp32 expression in Jurkat cells. We found that silenced pp32 reduces caspase activation and overexpressed pp32 promotes caspase activity. These *ex vivo* and *in vivo* data confirmed that pp32 can initiate caspase cascade to induce caspase-dependent apoptosis. We loaded GzmA with sublytic PFP into pp32-overexpressed or -silenced Jurkat cells to determine the role of pp32 in GzmA-mediated caspase-independent apoptosis. We showed that silenced pp32 decreases GzmA-mediated cell death and overexpressed pp32 increases it. This indicates that pp32 is a proapoptotic protein that triggers apoptosis by linking caspase-dependent and -independent pathways. The proapoptotic activity of pp32 may also contribute to excessive death of cerebellar Purkinje cells in the neural degenerative disease spinocerebellar ataxia type 1 (SCA1).<sup>32</sup> pp32 associates with Ataxin 1, a polyglutamine domain protein mutated in SCA1. Albeit widely expressed and present during adulthood, pp32 is particularly abundant in the developing cerebellum characterized by massive cell death.<sup>33</sup> pp32 is also considerably expressed in self-renewing stem-like populations and cell types known for rapid proliferation, such as intestinal crypt epithelial and prostatic epithelial cells competent in cell renewal.<sup>34</sup> However, a recent study showed that pp32-null mice have no apparent phenotypes for the most part compared with their wild-type littermates.<sup>35</sup> This may be due to the compensatory role of pp32 closely related family members, which continue to be expressed in pp32-deficient mice. Mice lacking two or more pp32 family members need to be generated to test their functions of pp32 and related family members in development and tumorigenesis.

## Materials and Methods

### Antibodies and reagents

Anti-pSer/ThrH3, anti-AcH3, anti-pSer10H3 and anti-H3 antibodies were from Cell Signaling. Anti-pSer28H3, anti-AcLys9H3, anti-mLys4H3,

anti-mLys9H3 antibodies, p38 kinase, rH3, p300, PCAF and the ChIP kit were from Upstate (NK, USA). Anti- $\beta$ -actin mAb, brefeldin A, PMA, ionomycin and dextran sulfate were from Sigma-Aldrich. Ac-DEVD-AFC, Ac-LEHD-AFC and glucose oxidase were from Calbiochem. Anti-IL-2-PE was purchased from BD Pharmingen. Anti-pp32 and anti-SET were prepared as described previously.<sup>36</sup> Anti-GST was from Clontech. Oligofectamine was from Invitrogen. Jurkat cell line was purchased from ATCC and was grown in 10% FBS RPMI1640.

### Recombinant proteins, plasmids and purified perforin

GzmA, S-AGzmA, perforin, rpp32, rSET and rGST were prepared as described previously.<sup>17</sup> Mammalian expression plasmid pCMV-pp32 for transfection was a gift from G Pasternack (the Johns Hopkins University).

### pp32 silencing by RNAi

Two siRNA duplexes were synthesized by Dharmacon Research: siRNA1 (sense, 5'-AAG AAG CUU GAA CUA AGC GAU-3'; antisense, 5'-AUC GCU UAG UUC AAG CUU CUU-3') and siRNA2 (sense, 5'-AAC CUC ACG CAU CUA AAU UUA-3'; antisense, 5'-UAA AUU UAG AUG CGU GAG GUU-3'). Control duplexes for GFP were prepared with sense (5'-GGC UAC GUC CAG GAG CGC ACC-3') and antisense (5'-UGC GCU CCU GGA CGU AGC CUU-3'). siRNA was deprotected and annealed to form duplex siRNA as described.<sup>37</sup> siRNA (100 nM final concentration) was transfected into Jurkat cells in DMEM using oligofectamine at 37°C for 8 h with gentle shaking. An equal volume of 20% FBS in DMEM was added and cells were incubated overnight, washed and incubated at 37°C for an additional 3 days before use.

### Intracellular staining of IL-2 and detection for IL-2 mRNA

Jurkat cells with pp32 overexpression or silencing for 3 days were stimulated with 20 ng/ml PMA plus 100 ng/ml ionomycin and 20  $\mu$ M brefeldin A at 37°C for 12 h. Cells were fixed and stained with anti-IL-2-PE and analyzed by Flow Cytometry. Total RNA was extracted using TRIzol reagent (Gibco BRL). Total RNA (500 ng) was used for cDNA synthesis by reverse transcription (RT-PCR kit, Promega). PCR was performed according to the manufacturer's instruction using the following primers: IL-2 – 5'-CAC TAC TCA CAT TAA CCT CAA CTC CTG-3'; reverse 5'-CTG GGA AGC ACT TAA TTA TCA AGT TAG TG-3', and  $\beta$ -actin – 5'-tGA CGG GGT CAC CCA CAC TGT GCC CAT-3'; reverse 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3'.

### ChIP assay

Jurkat cells (10<sup>6</sup>) with stimulation of PMA plus ionomycin post-pp32 overexpression or silencing were treated with 1% formaldehyde for 10 min, washed, lysed and sonicated as according to the manufacturer's instruction. The DNA-protein complexes were immunoprecipitated with desired antibodies and extracted by protein A agarose beads. DNA was extracted postreversing of the DNA-protein crosslinks. The DNA was amplified with the CRE site of the IL-2 promoter (forward 5'-CTA AGT GTG GGC TAA TGT AAC-3' and reverse 5'-TGT AAA ACT GTG GGG GT-3') as described.<sup>25</sup> PCR products were run on a 1.5% agarose gel.

### pp32-mediated HAT inhibition assay

p300 or PCAF (10 nM) was preincubated with different doses of rpp32 at 4°C for 10 min, and then added to the reaction mixture of 0.5  $\mu$ M rH3 plus

2 nM <sup>14</sup>C-acetyl CoA (Sigma-Aldrich) and incubated at 37°C for 30 min. Reaction products were separated by SDS-PAGE and analyzed by autoradiography after drying.

### Phosphorylation inhibition assay

Different concentrations of rpp32 were added to the reaction mixture of 0.5  $\mu$ M rH3, 0.1  $\mu$ M p38 kinase and 200  $\mu$ M ATP in 50  $\mu$ l kinase buffer (25 mM Tris, pH 7.5, 5 mM  $\beta$ -glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM MgCl<sub>2</sub>), incubated at 30°C for 40 min. The samples were resolved by 15% SDS-PAGE. Phosphorylated H3 was detected by immunoblot with H3-phospho-specific antibodies.

### Caspase activity assay

Jurkat cells with pp32 overexpression or silencing were treated with 10 mJ/cm<sup>2</sup> of UV light with UV Stratalink and incubated overnight. Cells were lysed in buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) plus protease inhibitors by three freeze-and-thaw cycles. Caspase activity was detected by incubation of 20  $\mu$ g cytosolic protein with 10  $\mu$ M Ac-DEVD-AFC for caspase-3 or Ac-LEHD-AFC for caspase-9 fluorogenic substrate at 37°C for 30 min and measured with a Fluorescence Spectrometry Reader.

### In vitro cleavage assay

In all, 0.5  $\mu$ M rpp32 or rSET or Jurkat lysates (2  $\times$  10<sup>5</sup> cell equivalent) were incubated at 37°C for 2 h with the indicated dose of GzmA in 20  $\mu$ l buffer of 50 mM Tris, pH 7.5, 1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>. Reaction products were electrophoresed for immunoblot.

### Cytotoxicity assay

pp32-overexpressed or -silenced or control-transfected Jurkat cells were radiolabeled with <sup>51</sup>Cr at 37°C for 1 h and washed before loading with GzmA with PFP as reported.<sup>51</sup> After 4 h, <sup>51</sup>Cr released into the supernatant of pelleted cells was counted using a Packard Topcount. Specific cytotoxicity was calculated using the formula [(sample release – spontaneous release)/(total release – spontaneous release)]  $\times$  100%.

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