Regulation of apoptosis by Bat3-enhanced YWK-II/APLP2 protein stability

Wei Wu¹, Wei Song¹, Shuchun Li¹*, Songying Ouyang¹, Kin Lam Fok², Ruiying Diao², Shiyong Miao¹, Hsiao Chang Chan¹* and Linfang Wang¹**

¹State Key Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Peking Union Medical College, Tsinghua University 5 Dong Dan San Tiao, Beijing 100005, China
²Epithelial Cell Biology Research Center, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, Hong Kong

*Present address: Minzu University of China, Institute of Chinese Minority Traditional Medicine, Beijing 100081, China
**Authors for correspondence (wang.linfang@imicams.ac.cn; hsiaocchan@cuhk.edu.hk)

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Summary
YWK-II protein/APLP2 is a member of an evolutionarily conserved protein family that includes amyloid precursor protein (APP) and amyloid precursor-like protein-1 (APLP1). We have previously demonstrated that YWK-II/APLP2 functions as a novel G protein-coupled receptor for Müllerian inhibiting substance (MIS) in cell survival. However, factors regulating the stability and turnover of YWK-II/APLP2 have not been identified. Here we present evidence that human leukocyte antigen-B-associated transcript 3 (Bat3), an important regulator involved in apoptosis, can interact with YWK-II/APLP2 and enhance its stability by reducing its ubiquitylation and degradation by the ubiquitin–proteasome system. Coexpression of different Bat3 domain deletion constructs with YWK-II/APLP2 reveals that the proline-rich domain of Bat3 is required for its binding to YWK-II/APLP2. In addition, we find that the protein levels of YWK-II/APLP2 could be enhanced by nuclear export of Bat3 under apoptotic stimulation. We also find elevated levels of Bat3 and YWK-II/APLP2 in human colorectal cancer with a positive correlation between the two. Taken together, these results have revealed a previously undefined mechanism regulating cell apoptosis and suggest that aberrant enhancement of YWK-II/APLP2 by nuclear export of Bat3 may play a role in cancer development by inhibiting cell apoptosis.

Key words: YWK-II protein/APLP2, Bat3, Stability, Ubiquitin, Nuclear export, Apoptosis, Colon cancer

Introduction
YWK-II protein was initially identified as the target antigen to a monoclonal antibody raised against human sperm proteins, which is synonymous with amyloid beta (A4) precursor-like protein 2 (APLP2) and thus is referred to as YWK-II protein (YWK-II)/APLP2 (Huang et al., 2000; Yan et al., 1990). YWK-II/APLP2 appears to be a multifunctional protein but its exact role remains elusive. We have previously shown that YWK-II/APLP2 may serve as a G protein-coupled receptor for Müllerian inhibiting substance (MIS) and activate the Erk signaling pathway, which promotes cell survival through an anti-apoptotic pathway suppressing p53 (Yin et al., 2007). Injection of anti-YWK-II/APLP2 antibody into the seminiferous tubule of mouse testis, which highly expresses YWK-II/APLP2 protein, resulted in a significant reduction in sperm count with elevated cell apoptosis (Yin et al., 2007). Expression of YWK-II/APLP2 has also been found increased in the process of apoptosis induced by trophic factor withdrawal in rat pheochromocytoma PC12 cells, further supporting its role in apoptosis (Araki and Wurtman, 1998). Interestingly, increased levels of YWK-II/APLP2 expression have been found in invasive ductal carcinoma (Abba et al., 2004). Together with the demonstrated role of YWK-II/APLP2 in apoptosis or cell survival, it suggests that YWK-II/APLP2 may play a role in tumor development.

In a yeast two-hybrid screening using a segment of YWK-II/APLP2 as bait, we found interaction of YWK-II/APLP2 with human leukocyte antigen-B-associated transcript 3 (Bat3). Bat3 was initially identified within the class III region of the major histocompatibility complex (MHC) on human chromosome 6 (Banerji et al., 1990). Depletion of Bat3 in mouse was found to result in embryonic lethality with defects in the development of the brain, testis, lung, and kidney. In all these cases, the developmental defects were associated with widespread aberrant apoptosis and cellular proliferation (Desmots et al., 2005). The Xenopus homolog of Bat3, Scythe, has been reported to be associated with Reaper (Rpr), a central regulator of apoptosis in Drosophila melanogaster, and the interaction between Scythe and Reaper is necessary for Reaper-induced apoptosis (Thress et al., 1999). Recent studies have also shown that Bat3 regulates the stability and localization of apoptosis-inducing factor (AIF) after apoptotic stimuli, and p53 has been identified as a Bat3 target after DNA damage, indicating its important role in apoptosis (Desmots et al., 2008; Sasaki et al., 2007). Bat3 has also been implicated in regulating apoptotic cell death in various tumor or cancer cells (Tsukahara et al., 2009). However, there is
no study reporting the interaction between Bat3 and YWK-II/APLP2. Given that both YWK-II/APLP2 and Bat3 have been implicated in cell apoptosis, their interaction, as indicated in the yeast two-hybrid screening, may represent an important regulatory mechanism governing the process. We therefore undertook the present study to investigate the detailed interaction of Bat3 with YWK-II/APLP2 and the potential role of this interaction in cell apoptosis and tumorigenesis.

**Results**

**Bat3 interacts with YWK-II/APLP2 in vivo**

We first confirm the interaction between Bat3 and YWK-II/APLP2 observed in the yeast two-hybrid screening using co-immunoprecipitation assays. Myc-tagged YWK-II/APLP2 (APLP2-Myc) and FLAG-tagged Bat3 (Bat3–FLAG) were coexpressed in human embryonic kidney HEK-293T cells. As shown in Fig. 1A, cell lysates were immunoprecipitated with anti-Myc antibody and analyzed by western blotting using anti-FLAG antibody. The results showed that APLP2–Myc was co-precipitated with Bat3–FLAG. The interaction between Bat3 and YWK-II/APLP2 was further confirmed in reverse immunoprecipitation experiment (Fig. 1B).

We next sought evidence for endogenous interaction between endogenous Bat3 and endogenous YWK-II/APLP2 in HEK-293T cells. Immunoprecipitation assays shown in Fig. 1C demonstrated that endogenous YWK-II/APLP2 pulled down with anti-YWK-II/APLP2 antibody resulted in co-precipitation of endogenous Bat3, whereas no Bat3 was found in the immunocomplex precipitated with a nonspecific immunoglobulin G (IgG) control. Thus, YWK-II/APLP2 formed a complex with Bat3 in vivo.

As shown in Fig. 1D, Bat3 contains several highly conserved domains, including an N-terminal ubiquitin-like domain, a proline-rich domain, and a C-terminal Bcl2-associated athanogene (BAG) domain. To map the domain of Bat3 that mediates the interaction with YWK-II/APLP2, we prepared a series of FLAG-tagged Bat3 deletion constructs including Bat3-ΔUBL (aa 1–88 deleted), Bat3-NT (aa 1–482), Bat3-CT (aa 1–482 deleted), and Bat3-Proline-rich (aa 387–675 deleted). These Bat3 deletion constructs were co-transfected with Myc-tagged YWK-II/APLP2 into HEK-293T cells. Co-immunoprecipitation with anti-Myc antibody results shown in Fig. 1Db revealed that both Bat3-NT and Bat3-CT bind readily to YWK-II/APLP2 with stronger affinity in Bat3-NT construct. However, Bat3-Proline-rich failed to bind to YWK-II/APLP2 in this assay, suggesting that the proline-rich domain of Bat3 is required for its binding to YWK-II/APLP2. Together, the results of these experiments demonstrate that Bat3 physically interacts with YWK-II/APLP2 in vivo and the proline-rich domain of Bat3 is necessary for the interaction.

**Bat3 modulates YWK-II/APLP2 turnover**

Since Bat3 has been proposed as a nucleus–cytoplasm shuttling protein and implicated in stabilizing its binding proteins, we speculated that Bat3 might modulate YWK-II/APLP2 expression/turnover (Desmots et al., 2008; Tsukahara et al., 2009). To test this, we expressed different amounts of FLAG-tagged Bat3 in HEK-293T cells and determined the expression of YWK-II/APLP2 by western blotting. As shown in Fig. 2A, endogenous YWK-II/APLP2 levels rose with increasing exogenous Bat3. The upregulation of YWK-II/APLP2 can be resulted from an increase in expression or a decrease in protein turnover. Therefore, we next used cycloheximide (CHX) chase assays, in which CHX is added to the culture medium to block protein synthesis. As shown in Fig. 2B, the levels of YWK-II/APLP2 were maintained at a higher level in the presence of Bat3, indicating that Bat3 can modulate the turnover of YWK-II/APLP2.

**Fig. 1.** Bat3 interacts with YWK-II/APLP2 in vivo. (A,B) HEK-293T cells were transfected with empty vector (–), Bat3-FLAG, APLP2-Myc or Bat3-FLAG plus APLP2-Myc plasmids as indicated. (A) After immunoprecipitation with anti-Myc antibody, Bat3–FLAG was detected by western blotting using anti-FLAG antibody. (B) After immunoprecipitation with anti-FLAG beads, APLP2–Myc was detected by western blotting using anti-Myc antibody. (C) HEK-293T cell lysates were immunoprecipitated using anti-YWK-II/APLP2 antibody or control IgG. The immunoprecipitated samples were analyzed by western blotting using anti-FLAG antibody. (D) Schematic representation of Bat3 constructs (a). HEK-293T cells were transfected with APLP2-Myc and a series of Bat3-FLAG deletion mutants (b). Lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FLAG antibody.
used as an inhibitor of protein synthesis, to determine whether YWK-II/APLP2 protein stability was affected by enhanced Bat3. As shown in Fig. 2B, the half-life of YWK-II/APLP2 was dramatically increased in cells with exogenous Bat3 expression compared with the empty vector control. To confirm that the observed increase in YWK-II/APLP2 stability was indeed caused by Bat3, we tested whether Bat3 knockdown could decrease the half-life of YWK-II/APLP2. As the kinetics of YWK-II/APLP2 stabilization tested under CHX treatment shown (Fig. 2C), the half-life of YWK-II/APLP2 in HEK-293T cells was markedly increased in cells with exogenous Bat3 expression compared with the empty vector control.

Fig. 2. Bat3 modulates YWK-II/APLP2 turnover. (A) HEK-293T cells were transfected with empty vectors or different doses of expression vectors for Bat3-FLAG (0.1, 0.3, 1 or 3 µg). 48 h later extracts were immunoblotted with YWK-II/APLP2, FLAG or Bat3 antibody; beta-actin was used as a loading control. (B) HEK-293T cells were transfected with empty vectors or Bat3-FLAG vectors (2 µg). 48 h later cells were treated with cycloheximide (CHX, 50 µg/ml) and extracted at different time points and immunoblotted with anti-YWK-II/APLP2, FLAG or Bat3 antibody; beta-actin was used as a loading control. The right panel shows averaged ratio values of triplicate measurements of YWK-II/APLP2 quantification in the indicated groups. The values are normalized to the expression of GAPDH. (C,D) HEK-293T cells (C) or HCT116 cells (D) were transfected with control siRNA (si-CNT) or siRNA for Bat3 (si-Bat3). 72 h later cells were treated with CHX (50 µg/ml) for the indicated times and extracts were immunoblotted with anti-YWK-II/APLP2 antibody; GAPDH was used as a loading control. The right panels in C and D show averaged ratio values of triplicate measurements of YWK-II/APLP2 quantification in the indicated groups. The values are normalized to the expression of GAPDH.
shortened from 50 minutes to 25 minutes by Bat3 knockdown. Interestingly, the turnover of YWK-II/APLP2 in a colon cancer cell line, HCT116, was also found to be dependent on Bat3, with its half-life greatly shortened from 50 minutes to 25 minutes by Bat3 knockdown (Fig. 2D). Taken together, these data indicate that Bat3 can markedly increase the stability of YWK-II/APLP2 in normal and cancer cells.

**Bat3 reduces YWK-II/APLP2 ubiquitylation**

The two main routes for regulating intracellular protein turnover are the ubiquitin–proteasome and autophagy–lysosome pathways (Ciechanover, 2005). To determine which pathway is responsible for YWK-II/APLP2 turnover, HEK-293T cells were treated with MG132 (a proteasome inhibitor) or chloroquine (a lysosome inhibitor). The levels of YWK-II/APLP2 were markedly increased after treatment with MG132 or chloroquine (Fig. 3A). We used CHX chase analysis to confirm that YWK-II/APLP2 stability was indeed regulated by the two pathways, as shown in Fig. 3B, the half-life of YWK-II/APLP2 was increased after treatment with MG132 or chloroquine.

Proteins targeted for degradation by the 26S proteasome are typically covalently modified with a polyubiquitin chain. Since Bat3, with a ubiquitin-like domain, has been reported to be involved in protein degradation, we were interested in testing whether Bat3 affects YWK-II/APLP2 degradation, particularly through regulating its ubiquitylation (Sasaki et al., 2008; Thress et al., 2001). We first performed ubiquitylation assay on YWK-II/APLP2. As shown in the left panel of Fig. 3C, coexpression of hemagglutinin (HA)-tagged ubiquitin (HA-Ub) with YWK-II/APLP2–FLAG (APLP2–FLAG) in HEK-293T cells induced the formation of high-molecular-mass ladder-like bands that could be recognized by anti-HA antibody in the YWK-II/APLP2 immunoprecipitates. Furthermore, the ubiquitylated YWK-II/APLP2 became more pronounced after MG132 treatment due to inhibition of its degradation by ubiquitin–proteasome pathway. To rule out the possibility that these ladder-like bands were YWK-II/APLP2-associated proteins rather than YWK-II/APLP2 itself, a re-immunoprecipitation assay was performed to pull down ubiquitylated proteins with anti-FLAG antibody. The protein mixture was first heated in the presence of 1% sodium dodecyl sulfate (SDS) and 5 mM dithiothreitol (DTT) to dissociate protein-protein interactions before immunoprecipitation, and was pulled down with anti-FLAG antibody. The immunoprecipitates immunoblotted with anti-HA antibody showed that ubiquitylated YWK-II/APLP2 was increased upon MG132 treatment, confirming the involvement of ubiquitin–proteasome pathway in degradation of YWK-II/APLP2 (Fig. 3C, right panel). To study the effect of Bat3 on the ubiquitylation status of YWK-II/APLP2, Myc-tagged YWK-II/APLP2 (APLP2–Myc) was expressed in HEK-293T cells with or without FLAG-tagged Bat3 (Bat3–FLAG), followed by treatment with MG132. YWK-II/APLP2 was then immunoprecipitated by anti-Myc antibody, and the extent of ubiquitylation was analyzed. Inhibition of proteasome activity by MG132 enhanced the YWK-II/APLP2–Myc protein content (Fig. 3Da, lanes 7 and 8). The weak ubiquitylation of YWK-II/APLP2 detected in cells transfected with APLP2–Myc alone (Fig. 3Da, lane 3) was markedly elevated in the presence of MG132 (Fig. 3Da, lane 7). Expression of Bat3 effectively suppressed the ubiquitylation of YWK-II/APLP2 in both MG132-treated and untreated groups (Fig. 3Da, lanes 8 and 4). Next, we tested whether Bat3 knockdown could induce YWK-II/APLP2 polyubiquitylation. HEK-293T cells were transfected with control or Bat3 siRNA in combination with FLAG-tagged YWK-II/APLP2 and HA-tagged ubiquitin. As shown in Fig. 3Db, Bat3 knockdown significantly enhanced the level of polyubiquitylated YWK-II/APLP2 in both MG132-treated and untreated groups. Taken together, these data suggest that Bat3 antagonizes YWK-II/APLP2 ubiquitylation, thereby suppressing YWK-II/APLP2 degradation.

**Uprogulation and positive correlation between Bat3 and YWK-II/APLP2 in human colorectal tumor tissues**

Given that YWK-II/APLP2 had been demonstrated to have anti-apoptotic activity, the currently observed Bat3-suppressed YWK-II/APLP2 degradation prompted us to speculate that abnormal Bat3-dependent YWK-II/APLP2 turnover might be involved in tumor formation and/or cancer development (Yin et al., 2007). To test this, we analyzed the correlation of Bat3 and YWK-II/APLP2 in colorectal carcinoma from patients with matched normal colorectal tissues. As shown in Fig. 4A, of the eleven pairs of matched normal and carcinoma tissue, all carcinoma samples exhibited higher levels of Bat3 and 9 of the 11 cases showed higher levels of YWK-II/APLP2 compared with corresponding normal tissues. These findings demonstrate a positive correlation between Bat3 and YWK-II/APLP2 in human colorectal tumor samples.

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**Nuclear export of Bat3 enhances protein stability of YWK-II/APLP2**

To confirm whether nuclear export of Bat3 increases the protein stability of YWK-II/APLP2 by reducing the ubiquitylation-mediated protein turnover, we investigated the half-life of YWK-II/APLP2 after overexpression of a nuclear localization signal mutated Bat3 construct (Bat3ΔNLS/ΔRNAi). Endogenous Bat3 was first depleted by si-Bat3 and followed by co-transfection of either Bat3 or Bat3ΔNLS/ΔRNAi 24 hours after endogenous Bat3 knockdown. As shown in Fig. 5A, the half-life of YWK-II/APLP2 in Bat3ΔNLS/ΔRNAi group was increased from 45 minutes to 60 minutes compared with the half-life in Bat3 group. We further used ubiquitylation assay to determine the effect of overexpression of Bat3ΔNLS/ΔRNAi on the ubiquitylation level of YWK-II/APLP2. As shown in Fig. 5B, forced expression of Bat3 in the cytoplasm by Bat3ΔNLS/ΔRNAi significantly reduced the ubiquitylation level of YWK-II/APLP2 in both MG132-treated and untreated groups when compared to wild-type Bat3. These data provide compelling evidence indicating that nuclear export of Bat3 stabilizes YWK-II/APLP2 by reducing ubiquitylation.
Fig. 3. Bat3 reduces YWK-II/APLP2 ubiquitylation. (A) HEK-293T cells were treated for 14 h with MG132 (5, 10 or 20 μM), chloroquine (100 μM) or vehicle. Cell lysates were immuno-blotted with anti-YWK-II/APLP2 antibody, and beta-actin as a loading control. (B) The half-life of YWK-II/APLP2 was analyzed using a cycloheximide (CHX) chase procedure following a 4-hour pretreatment with MG132 (25 μM) or chloroquine (100 μM). Cell lysates were immuno-blotted with antibody against YWK-II/APLP2 and GAPDH (loading control). (C) HEK-293T cells were co-transfected with APLP2-FLAG and HA-ubiquitin plasmids. At 48 h post-transfection, cells were mock-treated or treated with 25 μM MG132 for 6 h. Cell lysates were immunoprecipitated by anti-FLAG antibody or control IgG and the immunoprecipitates were either denatured and re-dissolved in SDS sample buffer (1% SDS, 5 mM DTT; right panel) or left untreated (left panel). The SDS sample buffer was diluted ten times then subjected to a second immunoprecipitation with anti-FLAG M2 agarose beads. The immunocomplex was subjected to western blotting with anti-HA antibody. HA-ubiquitin denotes ubiquitin conjugates of YWK-II/APLP2. (D) a: HEK-293T cells were transfected with empty vector (-), HA-ubiquitin, APLP2-Myc, or Bat3-FLAG plasmids. 48 h later cells were treated with 25 μM MG132 for 6 h or vehicle, and cells were then boiled in the presence of 1% SDS and 5 mM DTT. Myc-tagged APLP2 was immunoprecipitated from preheated cell extracts using anti-Myc antibody. Immunoprecipitates were separated by SDS-PAGE, and blotted with anti-HA antibody. Expression of APLP2-Myc, Bat3-FLAG, or endogenous Bat3 in cell lysates was determined using the corresponding antibodies. (b) HEK-293T cells were transfected with control or BAT3-targeted RNAi in combination with FLAG-tagged APLP2 and HA-tagged ubiquitin plasmids. 72 h later cells were treated with 25 μM MG132 or vehicle for 6 h, cell lysates were prepared and the ubiquitin assay was carried out as in a.
Apoptotic stimulation-induced elevation of YWK-II/APLP2 is associated with Bat3 nuclear export

We further examined the association of YWK-II/APLP2 levels with Bat3 nuclear export in response to apoptotic stimuli in the colon cancer cell line. HCT116 cells were treated with 5 mM camptothecin (CPT), a topoisomerase inhibitor known to induce apoptosis, for 12 h and the levels of YWK-II/APLP2 in HCT116 cells were observed to increase gradually from 0 h to 12 h after treatment with the progression of apoptosis that was marked by the cleaved PARP (Fig. 6A). Interestingly, the levels of Bat3 did not change notably during the process (Fig. 6A). However, the CPT-enhanced levels of YWK-II/APLP2 were abolished when Bat3 in HCT116 cells was depleted by siRNA, but remained unaffected in control (Fig. 6B). These results indicate that the elevated levels of YWK-II/APLP2, which may be important for cell survival, in response to the apoptotic stimulus are dependent on Bat3.

We further hypothesized that the elevated levels of YWK-II/APLP2 in response to apoptotic stimuli are due to its suppressed ubiquitlation by Bat3, which requires binding of Bat3 to YWK-II/APLP2, and therefore, Bat3 nuclear export since YWK-II/APLP2 is known to mainly localize to the plasma membrane and intracellular compartments (Kaden et al., 2009). In fact, Bat3 could be exported to the cytoplasm and bind with its interacting proteins in cells exposed to specific apoptotic stimuli (Desmots et al., 2008; Pogge von Strandmann et al., 2007). To confirm nuclear export of Bat3 in response to apoptotic stimuli, HCT116 cells were treated with 5 mM CPT and harvested at different time points. Bat3 levels in the cytoplasmic and nuclear fractions at each time point were analyzed by immunoblotting. The quality of the cytoplasmic and nuclear fractionation was determined by the presence of cytoplasmic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and nuclear lamin A/C. At time point 0 h, Bat3 was distributed nearly equally between cytoplasmic and nuclear fractions. CPT treatment promoted the export of Bat3 to the cytoplasm in HCT116 cells with corresponding decrease in its levels in the nuclear fraction (Fig. 6C). Similar results were also obtained in a cervical cancer cell line, HeLa, showing gradual increase of Bat3 in the cytoplasmic fraction with corresponding decrease in the nucleus faction in response to UV exposure (Fig. 6D). To further demonstrate the correlation between nuclear export of Bat3 and upregulation of YWK-II/APLP2 in response to apoptotic stimuli, we constructed certain Bat3 mutant, Bat3^NES/DNAi (the nuclear exporting signal of Bat3 was mutated). Cells were transfected with si-Bat3 and co-transfected 24 h later with different plasmids, including Bat3^NES/DNAi, Bat3^RNAi, or

| Patient number | Nucleus to cytoplasm shuttling of Bat3 | Upregulation of APLP2 | Simultaneous occurrence of both | Grade | Stage | Sex | Age | Position on tissue array |
|----------------|--------------------------------------|-----------------------|-------------------------------|-------|-------|-----|-----|-------------------------|
| 1              | +                                    | +                     | #                             | 1     | II    | F   | 42  | A6                      |
| 2              | +                                    | −                     | −                             | 1–2   | II    | M   | 77  | B8                      |
| 3              | −                                    | +                     | −                             | 1     | II    | M   | 34  | C2                      |
| 4              | −                                    | +                     | −                             | 1     | II    | F   | 63  | C3                      |
| 5              | −                                    | −                     | −                             | 2     | I     | M   | 74  | C4                      |
| 6              | −                                    | +                     | −                             | 2     | II    | M   | 34  | C8                      |
| 7              | +                                    | +                     | #                             | 2     | II    | M   | 79  | D4                      |
| 8              | +                                    | −                     | −                             | 2     | III   | F   | 51  | D5                      |
| 9              | −                                    | +                     | −                             | 2     | III   | F   | 67  | D8                      |
| 10             | −                                    | −                     | +                             | 2     | II    | M   | 66  | D9                      |
| 11             | −                                    | +                     | −                             | 2     | III   | F   | 44  | E1                      |
| 12             | +                                    | −                     | −                             | 2     | III   | M   | 61  | E6                      |
| 13             | −                                    | +                     | −                             | 1–2   | II    | F   | 71  | E8                      |
| 14             | −                                    | +                     | −                             | 2     | II    | M   | 58  | E9                      |
| 15             | −                                    | +                     | −                             | 2     | II    | M   | 71  | E10                     |
| 16             | +                                    | +                     | #                             | 2     | II    | F   | 51  | E11                     |
| 17             | +                                    | −                     | −                             | 2     | III   | F   | 60  | F5                      |
| 18             | +                                    | +                     | #                             | 2     | II    | F   | 80  | F8                      |
| 19             | +                                    | +                     | #                             | 2     | III   | M   | 30  | F10                     |
| 20             | +                                    | −                     | −                             | 2     | IV    | M   | 50  | F12                     |
| 21             | +                                    | −                     | −                             | 2     | II    | M   | 79  | G3                      |
| 22             | −                                    | +                     | −                             | 1     | II    | M   | 49  | G4                      |
| 23             | −                                    | +                     | −                             | 2     | II    | M   | 77  | G7                      |
| 24             | +                                    | +                     | #                             | 2     | II    | F   | 67  | G10                     |
| 25             | −                                    | −                     | +                             | 3     | II    | F   | 92  | H8                      |
| 26             | +                                    | +                     | #                             | 3     | II    | M   | 75  | H12                     |
| 27             | −                                    | +                     | −                             | 3     | II    | F   | 72  | I5                      |
| 28             | −                                    | −                     | +                             | 3     | II    | F   | 74  | I7                      |
| 29             | +                                    | +                     | #                             | 3     | III   | M   | 72  | I8                      |
| 30             | −                                    | +                     | −                             | 3     | III   | M   | 47  | I9                      |
| Total          |                                    |                      |                               | 15/102 | 24/102 |    | 9/102*       |               |

+ positive; −, negative; #, double positive.

*P<0.001 (Pearson Chi-square test): the cytoplasm shuttle of Bat3 is highly co-related with the up-regulation of APLP2 in 9 out of 102 cancer patients.
null vectors. At 48 h post-transfection, the cells were treated with CPT and the level of YWK-II/APLP2 was detected. As shown in Fig. 5E, the enhanced levels of YWK-II/APLP2 resulted from overexpression of wild-type Bat3 was abolished in Bat3 NES mutant group. Taken together, these results suggest that Bat3 nuclear export is required for the enhanced levels of YWK-II/APLP2 in response to apoptotic stimuli.

Fig. 4. Upregulation and positive correlation between Bat3 and YWK-II/APLP2 in human colorectal tumor tissues. (A) Eleven pairs of fresh frozen human colorectal tumor tissues and corresponding normal tissues were lysed, and tissue lysates were blotted with the indicated antibodies. (N, normal tissue; T, tumor tissue). Beta-actin or GAPDH were used as a loading control. (B) Expression of Bat3 and YWKII/APLP2 were studied by immunohistochemical staining in colon adenocarcinoma tissue arrays. Corresponding representative images from Bat3 and YWKII/APLP2 arrays are shown. (C) Localization of Bat3 was determined from the tissue arrays in B. Bat3 in cytoplasm was found in 15 out of 102 specimens. Of the 15 specimens with elevated cytoplasmic Bat3, 9 of them showed a simultaneous upregulation of YWKII/APLP2. Enlarged: enlarged views of parts of the images beneath. Original magnification: 200×.

Fig. 5. Nuclear export of Bat3 enhances stability of YWK-II/APLP2 protein. (A) HCT116 cells were transfected with si-Bat3 and co-transfected 24 h later with null, Bat3RNAi or Bat3RNAi/DNLS. At 48 h post-transfection, cells were treated with cycloheximide (CHX; 50 μg/ml) and extracted at different time points and immunoblotted with anti-YWK-II/APLP2 or Bat3 antibody. Beta-actin was used as a loading control. Graph shows averaged ratio values of triplicate measurement of YWK-II/APLP2 quantification in the indicated groups. The values are normalized to the expression of β-actin. (B) HEK-293T cells were transfected with si-Bat3 oligoduplexes and co-transfected 24 h later with the indicated plasmids including null, HA-ubiquitin, APLP2-Myc, Bat3RNAi-FLAG, Bat3RNAi/DNLS-FLAG, and MG132.
Fig. 6. See next page for legend.
Nuclear export of Bat3 suppresses the apoptosis induced by knockdown of YWK-II/APLP2

To further demonstrate the physiological significance of the interaction between Bat3 and YWK-II/APLP2, and to determine whether this interaction promotes colon tumorigenesis, we examined the effect of either Bat3 or YWK-II/APLP2 knockdown on cell survival of HCT116 cells in response to apoptotic stimulus. HCT116 cells were transiently transfected with either Bat3 or YWK-II/APLP2 siRNA and treated with CPT for inducing apoptosis. As shown in Fig. 7A, knockdown of either Bat3 or YWK-II/APLP2 significantly inhibited cell survival denoted by higher LDH release. To demonstrate the correlation between Bat3 and YWK-II/APLP2 in apoptosis, we used Annexin V/propidium iodide (PI) staining assay to determine whether Bat3 can rescue the apoptosis induced by knockdown of YWK-II/APLP2. HCT116 cells were transfected with either si-APLP2 or si-Bat3 and co-transfected 24 h later with Bat3NLS or null vectors. At 48 h post-transfection, the cells were treated with Vulcade (an inhibitor of proteasome, can induce cell apoptosis) for 24 h and the apoptotic ratio was detected using Annexin V/PI staining assay. The result in Fig. 7B showed that knockdown of either Bat3 or YWK-II/APLP2 enhanced the cell apoptosis and overexpression of Bat3NLS could partially rescue the apoptosis induced by knockdown of YWK-II/APLP2. Taken together, these results suggest that the elevated levels of YWK-II/APLP2 induced by nuclear export of Bat3 can inhibit cancer cell apoptosis.

Depletion of Bat3 suppresses the growth of colon tumor xenograft

To further access the role of Bat3 in tumor formation, we performed xenograft assay. HCT116 cells were stably infected with lentivirus vector (plentilox3.7) expressing control or Bat3 shRNA and inoculated subcutaneously into the dorsal flanks of athymic nude mice. As shown in Fig. 8, knockdown of Bat3 significantly suppressed the tumor formation of HCT116 cells in nude mice. Taken together, our results suggest that depletion of Bat3 suppresses colorectal tumor formation in vivo.

Fig. 6. Apoptotic stimulation-induced elevation of YWK-II/APLP2 is associated with Bat3 nuclear export. (A) HCT116 cells were treated with CPT (5 mM) for different time. Cell lysates were immunoblotted for YWK-II/APLP2, Bat3 or PARP. (B) HCT116 cells were transfected with control (si-CNT) or Bat3-specific siRNA (si-Bat3). 72 h later cells were treated with CPT (5 mM) and harvested at the indicated time. Cell lysates were blotted for YWK-II/APLP2, Bat3, and GAPDH as a control. The right panel shows averaged ratio values of triplicate measurements of YWK-II/APLP2 in the indicated groups. The values are normalized to the expression of GAPDH. (C) HCT116 cells were treated with CPT (5 mM) for the indicated times. Both the nuclear and cytoplasmic fractions were collected and the fractions were immunoblotted for Bat3. The nucleus-localized protein Lamin A/C and cytoplasm-localized protein GAPDH demonstrate separation of the fractions and proper sample loading. The right panel shows averaged ratio values of triplicate measurements of Bat3 in the indicated groups. The values of Bat3 in cytoplasm or nucleus are normalized to the expression of GAPDH or Lamin A/C. (D) Following UVB irradiation (30 mJ/cm²), HeLa cells were harvested at the indicated times, fractionated, immunoblotted and quantified as in C. (E) HCT116 cells were transfected with Bat3-specific siRNA oligoduplexes and co-transfected 24 h later with null, Bat3SARNAI or Bat3SARNAI/ANES. At 48 h post-transfection, the cells were treated by CHX and the halflife of YWKII/APLP2 was examined as in Fig. 2D.

Fig. 7. Nuclear export of Bat3 suppresses the apoptosis induced by knockdown of YWK-II/APLP2. (A) HCT116 cells were transfected with YWK-II/APLP2 or Bat3-specific siRNA. At 48 h post-transfection, cells were treated with different doses of CPT for 2 days. LDH activities in the media were measured using a Cytotoxicity detection kit (Roche). LDH release (% cytotoxicity) was calculated using the following equation: [(OD490 experimental release – OD490 spontaneous release)/(OD490 maximum release – OD490 spontaneous release)]×100. The spontaneous release is the amount of LDH released from the cytoplasm of untreated cells, whereas the maximum release is the amount released by total lysates of untreated cells by Triton X-100. Data are means ± s.e.m. of four separate experiments (*P<0.05 versus control groups). (B) HCT116 cells were transfected with control (si-control), YWK-II/APLP2(si-APLP2) or Bat3 (si-Bat3) RNA oligoduplexes, and co-transfected 24 h later with null or Bat3NLS. At 48 h post-transfection, the cells were treated with vulcade (1 mM). Percentage apoptosis was assayed at 24 h post-treatment by Annexin V/PI staining. Data shown are one trial representative of three independent experiments.

Discussion

The present study has revealed a previously undefined mechanism involving interaction between YWK-II/APLP2 and Bat3 in regulating cellular response to apoptotic stimuli, aberrance of which may contribute to tumorigenesis or cancer development. The present findings suggest that the previously demonstrated anti-apoptotic activity of YWK-II/APLP2 or its effect on cell survival may be enhanced by Bat3 through suppression of YWK-II/APLP2 ubiquitylation and thus degradation.

The ability of Bat3 to stabilize its interacting proteins has been previously documented. The ubiquitin-like domains of Bat3 have been shown to interact with the Xrpn10c subunit of the proteasome, and those domains are required for normal development of the Xenopus embryo (Minami et al., 2007). In addition, Bat3 has been reported to increase heat shock protein (Hsp) 70-2 protein stability
The present study has also demonstrated that the apoptotic stimulation-enhanced YWK-II/APLP2 levels are associated with the nuclear export of Bat3. Bat3 contains a nuclear localization signal (NLS) and a nuclear export signal (NES), indicating that it is subject to nuclear export. It has been reported that Bat3 translocates from the nucleus to the cytoplasm during endoplasmic reticulum stress-induced apoptosis (Desmots et al., 2008). The interaction between YWK-II/APLP2 and Bat3 observed in the present study provides a novel mechanism regulating cell apoptosis. While the proline-rich domain of Bat3 is shown to be necessary for the interaction, the detailed mechanism by which Bat3 reduces the ubiquitylation of YWK-II/APLP2, as well as its other interacting proteins, remains to be elucidated.

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Interaction between Bat3 and YWK-II in apoptosis

Western blot analysis
Protein samples were prepared in NP-40 lysis buffer (1% NP-40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail (Roche Diagnostics)), and 20 μg total protein was immunoblotted according to standard procedures. Antibodies recognizing the following proteins were used in this study: APLP2 (Calbiochem), Bat3 and Lamin A/C (both from Abcam), β-actin, Caspase3, GAPDH and Myc (all from Santa Cruz Biotechnology), PARP (Cell Signaling Technology), and FLAG (M2, Sigma).

Immunoprecipitation
HEK-293T cells were transfected with empty vector (p3xFLAG-CMV-14; Sigma), Bat3-FLAG, or APLP2-Myc. Cells were lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40) supplemented with protease inhibitor cocktail (Roche Diagnostics) for 30 min on ice and then cleared by centrifugation. Extracts were incubated with the appropriate antibodies overnight at 4°C with rotation, and then incubated for an additional 2 h with protein A/G agarose beads (Roche Diagnostics). In some experiments, anti-FLAG (Sigma, M2) or anti-Myc agarose beads (Santa Cruz Biotechnology) were used to pull down FLAG-tagged or Myc-tagged proteins. The samples were fractionated by SDS-PAGE and subjected to immunoblotting as described above.

In vivo ubiquitination assay
In vivo ubiquitination assays were performed as described previously (Zhang et al., 2005). HEK-293T cells were co-transfected with HA-ubiquitin and the indicated YWK-II/APLP2 and Bat3 expression constructs. At 48 hours post-transfection, cells were incubated for 6 h with 25 mM of MG132 or vehicle. Cells were then boiled in the presence of 1% SDS and 5 mM DTT. The boiled extract was diluted ten times containing sasodacothione and Then incubated to immunoprecipitation using anti-FLAG or anti-Myc antibody as indicated. Immunocomplexes were analyzed by SDS-PAGE followed by immunoblotting with an anti-HA antibody to detect ubiquitin conjugates.

Cell death measurement
LDH activities in the conditioned media were measured using a Cytotoxicity detection kit (Roche, USA), according to the manufacturer’s instructions and absorbance was measured at 490 nm using a microplate reader (Biotek, USA).

Apoptosis
Treated cells were stained by using Annexin V/PI staining kit according to the manufacturer’s guidelines (Invitrogen) and the cells were immediately analyzed by using flow cytometry (BD, FACScalibur).

Tissue microarray
Colon adenocarcinoma tissue array was purchased from US Biomax and stained by standard immunohistochemical protocol. Briefly, slide was deparaffinized by serial xylene–ethanol treatment. Antigen was retrieved by boiling in sodium citrate buffer for 10 minutes. Slide was blocked in 5% normal goat serum (Sigma) for 1 h at room temperature followed by probing with 1:50 mouse anti-Bat3 Ab (AbCAM). Signal was detected by Ultravision ONE detection system (Thermo Scientific).

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Institutional Animal Care and Use committee of the University of Wuhan. 6-week-old male nude mice were inoculated subcutaneously in both flanks with 5×10^6 HCT116 cells stably infected with either control or Bat3 targeting shRNA virus. After 7 days tumors were measured with calipers. Statistical analysis was done using paired Student’s t-tests, and P<0.05 was used as threshold for statistically significant different tumor growth rates.

Statistical methods
To compare two groups, two-tailed t-tests were used. A probability P<0.05 was considered to be statistically significant.

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