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Vps41, a protein involved in lysosomal trafficking, interacts with caspase-8

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Caspase-8 is a member of the cysteine-aspartic acid protease (caspase) family which plays a central role in apoptosis and development. We screened caspase-8 interacting proteins from mouse T-cell lymphoma and 7.5-day embryo cDNA libraries by yeast two-hybrid system and obtained eleven positive clones, including Vacuolar protein sorting 41 (Vps41), a protein involved in trafficking of proteins from the late Golgi to the vacuole. The interaction of Vps41 with caspase-8 was confirmed by co-immunoprecipitation (co-IP) and co-localization studies in HEK293T cells. Co-IP experiments also showed that Vps41 binds to the p18 subunit of caspase-8 through its WD40 region and RING-finger motif. Furthermore, we found that overexpression of Vps41 promotes Fas-induced apoptosis in A549 human lung adenocarcinoma cells. The cleavage of caspase-3, a caspase-8 downstream effector, was increased when cells were transfected with Vps41-overexpressing plasmid. Together, these results suggest a novel interaction of caspase-8 with Vps41 and provide a potential role of Vps41 beyond lysosomal trafficking.

Key words: caspase-8, Vps41, yeast two-hybrid, protein interaction, apoptosis

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INTRODUCTION

Caspase-8 was first described as a FADD-homologous interleukin 1β converting enzyme (ICE)–like protease (Muzio *et al.*, 1996), and plays a crucial role in apoptosis as an apical caspase. In FasL-induced apoptosis it is recruited to the cytoplasmic domain of Fas through the adaptor protein FADD. The proteolytic activity of the C-terminal domain of caspase-8 appears after its cleavage initiated by the formation of the Fas-FADD-caspase-8 complex. Caspase-8 initiates the downstream apoptotic process through activating caspase-3 which cleaves PARP subsequently (Boldin *et al.*, 1996; Cohen, 1997; Juo *et al.*, 1998). In the mitochondrial apoptosis pathway caspase-8 cleaves Bid, a pro-apoptotic member of the Bcl-2 family, which induces permeabilization of the outer mitochondrial membrane and helps mitochondria release cytochrome *c*, a crucial member of the apoptosome complex in mitochondrial apoptosis (Bossy *et al.*, 1998; Li *et al.*, 1998; Bratton *et al.*, 2000; Fischer *et al.*, 2003). Therefore, caspase-8 mediates both death receptor-mediated apoptosis and intrinsic apoptosis.

VPS (vacuolar protein sorting) is a class of proteins originally identified in vesicular trafficking in yeast (Radisky *et al.*, 1997). Although more than 60 VPS genes have been identified, their functions in mammalian cells are only partly known.

Vps41 is a highly conserved protein. In yeast, it was indicated to be a subunit of both AP-3 (heterotetrameric adaptor protein complex 3) and HOPS (homotypic fusion and protein transport) complexes, both of which control protein trafficking from the late Golgi into the vacuole, an organelle analogous to the lysosome of mammalian cells (Conibear *et al.*, 1998; Rehling *et al.*, 1999; Wurmser *et al.*, 2000; Brett *et al.*, 2008; Cabrera *et al.*, 2010). There are two isoforms of hVps41, both of which contain a WD40 domain, a CLH domain, as well as a RING-finger motif which mediates membrane association (McVey Ward *et al.*, 2001). These two isoforms differ in that one of them lacks amino acids 83–107 of the amino terminus (Ruan *et al.*, 2010). In mammalian cells Vps41 was found to be recruited by Arf-like GTPase Arl8b to lysosomes, and the two proteins then recruit other HOPS complex members to lysosomes. Vps41-silenced cells displayed a dramatic delay in delivery of proteins to lysosomes (Garg *et al.*, 2011). These results indicate that the function of Vps41 in mammalian cells is similar to that in yeast, which is mediating the trafficking of proteins to vacuoles/lysosomes. Recent study has shown that Vps41 blocks downstream events in the apoptotic cascade including activation of caspase-9 and caspase-3, and PARP cleavage in SH-SY5Y neuroblastoma cell line (Ruan *et al.*, 2010). This result was then explained that Vps41 could enhance the clearance of misfolded and aggregated proteins, such as neurotoxic α-synuclein (Harrington *et al.*, 2012). Until present, all studies on Vps41 in mammalian cells have been based on its function of protein trafficking to lysosomes.

In this study, we investigated proteins capable of interacting with caspase-8 by yeast two-hybrid screening and found Vps41 to be a novel caspase-8-interacting partner. We confirmed this interaction in mammalian cells. This result supplies a novel insight of apotential role of Vps41 beyond lysosomal trafficking.

MATERIALS AND METHODS

Cell culture and transfection. Human embryonic kidney HEK293 cells and human lung adenocarcinoma A549 cells were cultured in DMEM supplemented with

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Abbreviations: FITC, fluorescein isothiocyanate; HOPS, homotypic fusion and protein transport; IP, immunoprecipitation; PBS, phosphate-buffered saline; PI, propidium iodide

Table 1. Primer sequences used to generate truncated forms of hVps41 and hCaspase-8

Name	forward primer (5' to 3')	reverse primer (5' to 3')
WD40	ttaaggatccatggcggaagcagtggag	ccgactcgagatgcttgtggatcaactg
CLH	ggtcggatccgattttgattcagagaaagc	cagtctcgagttaaacatgtgtgccaatgttg
RING	acacggatccgacccaattctactgattc	caatctcgagtcacatctccaaaattgcacttc
DED	cataggatccatggacttcagcagaaatc	aataaagctttcattcaaggctgctgcttctc
p18	aattggatccaatggggaggagttgtgtg	ccggaagcttatcagtctcaacaggtatac
p10	atatggatcctcagaggagcaaccctat	ccagaagctttcaatcagaagggaagac

10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. Transient transfection of cells was performed using Lipofectamine 2000 (Invitrogen) according to the instructions provided by the manufacturer.

Yeast two-hybrid screening. Caspase-8 was used as a bait in the yeast two-hybrid screening experiments. Bait gene encoding mCaspase-8 was amplified by PCR and then cloned into the GAL4-DNA binding expression vector pGBKT7 DNA-BD (Clontech). The bait did not exhibit any intrinsic activation function in the yeast strain AH109 (Clontech). This bait was used to screen both mouse T lymphoma and 7.5-day embryo cDNA libraries constructed in the pACT2 DNA-AD vector (Clontech) and pretransformed into the AH109 strain. The transformants were then transformed with pACT2-cDNA libraries and plated on yeast dropout medium lacking tryptophan (Trp), leucine (Leu), histidine (His) and adenosine (Ade). Colonies obtained on the dropout media (Trp−, Leu−, His− and Ade−) were checked for the activity of β-galactosidase according to the Clontech protocol, thereby selecting the plasmids encoding proteins capable of a two-hybrid interaction with caspase-8.

Plasmid constructs. The full-length hVps41 was amplified by PCR using the forward primer 5'-att gga tcc atg gcg gaa gca gtg gag -3' and reverse primer 5'-ccg gct cga gct att ttt tca tct cca aaa ttg c-3' from cDNA of HEK293T cells and cloned into the BamHI-XhoI sites of pRK5-Flag vector. The full length hCaspase-8 was amplified using the forward primer 5'-cat agg atc cat gga ctt cag cag aaa tc-3' and reverse primer 5'-cca gaa gct ttc aat cag aag gga aga c-3' by PCR from cDNA of HEK293T cells and cloned into the BamHI-Hind- sites of pRK5-HA vector. Truncated forms of hVps41: WD40 (amino acids 1–546), CLH (amino acids 560–721) and RING (amino acids 722–852) were cloned to pRK5-Flag vector, while truncated forms of hCaspase-8: DED (amino acids 1–190), p18 (amino acids 197-374) and p10 (amino acids 375–479) were cloned to pRK5-HA vector, using appropriate primers (Table 1).

Co-immunoprecipitation. As many as 5×10^6 cells were lysed for 30 min on ice in lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% Triton X-100 and 10 μl/ml protein inhibitor cocktail) after 48-h transfection. The supernatant was incubated with 1 μg anti-Flag (Sigma), anti-HA (Sigma) or normal mouse immunoglobulin G (IgG) (Millipore) antibody (IP antibody) at 4°C overnight. Thereafter incubation with 20 μl protein G Sepharose beads (Upstate) was performed for 2 h at 4°C. After washing three times with lysis buffer, the immunoprecipitates were incubated at 95°C for 5 min and probed with appropriate antibodies by western blotting.

Immunofluorescence. HEK293T cells were cultured on a cover slip and transfected with pRK5-Flag-hVps41 and pRK5-HA-hCaspase-8. Sixteen hours later, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 1 h. After washing with PBS twice, cells were permeabilized in 0.5% TritonX-100 in PBS for 45 min at room temperature, and then washed twice with PBS. Subsequently, the cells were incubated with 3% BSA in PBS for 2 h to block nonspecific binding sites and thereafter incubated with appropriate antibodies diluted in 3% BSA in PBS at 4°C overnight. Rabbit anti-Flag (Cell Signal Technology), rabbit anti-HA (Sigma) and mouse anti-HA (Sigma) antibodies were diluted at 1:200, and anti-clathrin (Abcam) antibody was diluted at 1:75. After incubation the cells were washed three times with PBS on shaking table for 8 min each time, then incubated with Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit antibodies (Invitrogen) diluted in 3% BSA in PBS at 1:1000 for 1 h at room temperature in the dark. After washing three times, immunofluorescence was observed under a confocal laser scanning microscope.

Induction and analysis of apoptosis. A549 cells were cultured in a 24-wells plate, transfected with pRK5-Flag-hVps41 plasmid 0.5 μg/well for 24 h, and then treated with 200 ng/ml anti-Fas antibody (Millipore). After incubation for 6 or 24 h, cells were collected, washed with binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) and stained with annexin V–fluorescein isothiocyanate (FITC) in binding buffer for 10 min on ice, then propidium iodide (PI) was added and cells were analyzed immediately on a FACSCalibur flow cytometer using CellQuest software. Cells in the early stage of apoptosis were stained positive for annexin V-FITC and negative for PI, while those in the late stage of apoptosis were stained positive for both annexin V-FITC and PI.

Table 2. Proteins interacting with caspase-8 in yeast two-hybrid screen

No.	gene identified	NCBI accession number
2	Mib2 (Mib2 mindbomb homolog 2)	NM_145124.2
3	Lztr1 (leucine-zipper-like transcriptional regulator)	NM_025808.3
4	Rpsa (ribosomal protein SA)	NM_011029.4
6, 8	Eif3 (eukaryotic translation initiation factor 3)	NM_016876.3
9	Vps41 (vacuolar protein sorting 41)	NM_172120.4
11	Immt (inner membrane protein, mitochondrial)	NM_029673.2
14, 16	Patz1 (POZ/BTB and AT hook containing zinc finger 1)	NM_019574.3
28	Guk1 (guanylate kinase 1)	NM_001159410.1
29	Nsun5 (NOL1/NOP2/Sun domain family, member 5)	NM_145414.2
30	Abhd16a (abhydrolase domain containing 16A)	NM_178592.3
31	Cryba4 (crystalline)	NM_021351.1

RESULTS

Eleven clones were obtained through yeast two-hybrid screening

To identify proteins that interact with caspase-8, cDNA libraries derived from mouse T lymphoma and 7.5-day embryo were screened using a yeast two-hybrid system with the bait plasmid pGBKT7–mCaspase-8 which expresses the bait protein caspase-8. Thirty-one candidates were obtained by the screening. We cloned these 31 DNA fragments to pACT2 vector and re-transformed them into the caspase-8 bait yeast strain AH109 independently to confirm their interactions with caspase-8 in yeast by two-hybrid system and finally obtained 11 positive clones. These clones were sequenced, and blast searches were performed against GenBank to identify putative caspase-8-interacting proteins. The blast result showed diverse proteins, including Lztr1 (leucine-zipper-like transcriptional regulator), Vps41 (vacuolar protein sorting 41), and Guk1 (guanylate kinase 1) (Table 2).

Vps41 binds to caspase-8 in HEK293T cells

Eleven proteins interacting with caspase-8 were identified in yeast two-hybrid screen. To confirm their interaction with caspase-8 in mammalian cells, we cloned these 11 genes into mammalian expression vector pRK5-Flag. After a 48-h transfection with the plasmids encoding putative interacting proteins, HEK293T cells were lysed and immunoprecipitated with either anti-Flag antibody or normal mouse immunoglobulin G as negative control. The co-immunoprecipitates were subsequently immunoblotted with anti-caspase-8 antibody (Calbiochem). For the cells transfected with pRK5-Flag-hVps41, endogenous caspase-8 was detected in the co-immunoprecipitates (Fig. 1a, upper panel), and expression of the recombinant Flag-hVps41 was detected with anti-Flag antibody (Fig. 1a, lower panel). This result demonstrates that Vps41, a protein involved in lysosomal trafficking, binds to endogenous caspase-8 in HEK293T cells.

In order to determine which domains play crucial roles in the interaction of Vps41 with caspase-8, a series of plasmids were constructed. The WD40, CLH and RING domain of Vps41 were cloned individually into pRK5-Flag vector, whereas DED, p18 and p10 domain of caspase-8 were cloned into pRK5-HA vector according to the structural information of these two proteins provided by Swiss-prot (Fig. 1b).

The pRK5-Flag-hVps41 plasmid was co-transfected with pRK5-HA-DED, pRK5-HA-p18, pRK5-HA-p10 or pRK5-HA vector in HEK293T cells, and cell lysates were subjected to immunoprecipitation with anti-HA antibody. Co-IP results revealed that the p18 subunit of caspase-8, but not the DED or p10 domain, interacts with Vps41 (Fig. 1c). We further focused on the domains of Vps41 that may interact with caspase-8. In yeast, the N-terminus of Vps41 is required for binding to adaptor protein complex AP-3, an important complex involved in trafficking of proteins from Golgi and endosomal compartments to both the vacuole/lysosome and the plasma membrane, whereas the C-terminal CLH domain directs homo-oligomerization of Vps41 (Ruan *et al.*, 2010). The RING-finger motif has been shown to mediate membrane association in mammalian cells (Bossy *et al.*, 1998). The pRK5-HA-p18 plasmid was co-transfected with pRK5-Flag-hVps41, pRK5-Flag-WD40, pRK5-Flag-CLH, pRK5-Flag-

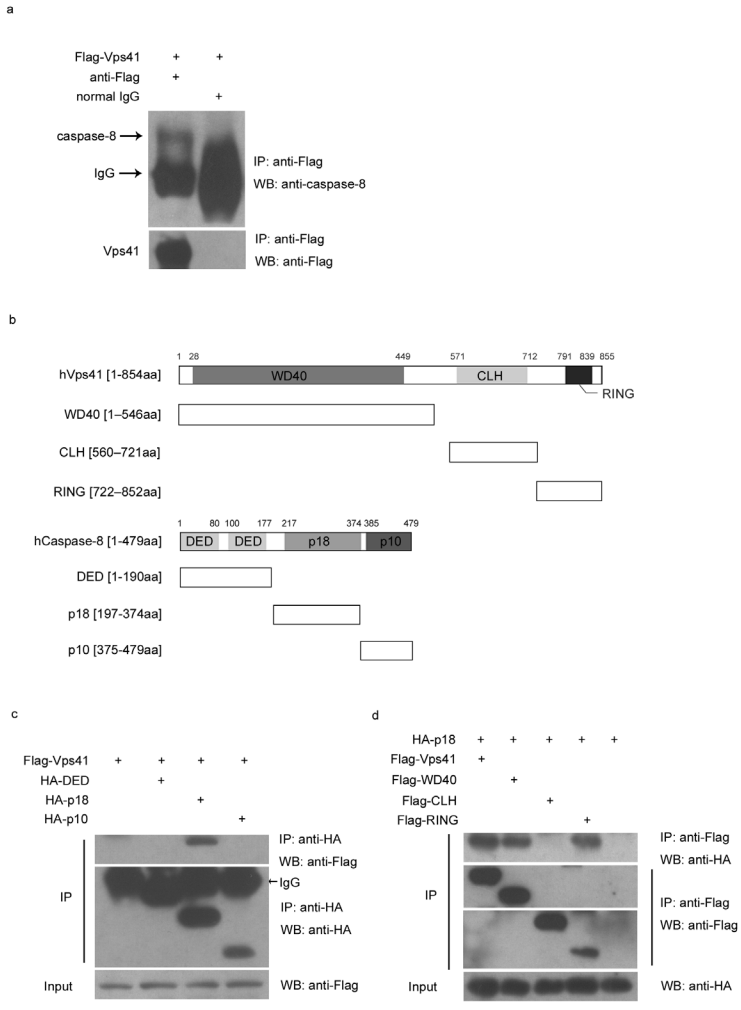

Figure 1. Vps41 interacts with caspase-8 in HEK293T cells.
(a) Flag-Vps41 was transfected into HEK293T cells. Twenty-four hours later, cells were lysed and protein complexes were co-immunoprecipitated by either anti-Flag antibody or normal IgG. Caspase-8 and Vps41 were dectected by western blotting with anti-caspase-8 and anti-Flag antibodies, respectively. **(b)** Structural information of hVps41 and hCaspase-8 and their truncated forms cloned into the expression vector and used in this study. **(c)** Flag-Vps41 was co-transfected with pRK5-HA vector or pRK5-HA-DED, pRK5-HA-p18, pRK5-HA-p10 into HEK293T cells, co-IP was performed with anti-HA antibody, then Vps41 was detected with anti-Flag antibody and the domains of caspase-8 were detected with anti-HA antibody by western blotting. The input of Vps41 is also shown at the bottom. **(d)** HA-p18 was co-transfected with pRK5-Flag-Vps41, pRK5-Flag-WD40, pRK5-Flag-CLH, pRK5-Flag-RING or pRK5-Flag vector into HEK293T cells. Co-IP was performed with anti-Flag antibody, then the p18 subunit was detected with anti-HA antibody, and full-length Vps41 or its domains were detected with anti-Flag antibody. The input of p18 is shown at the bottom.

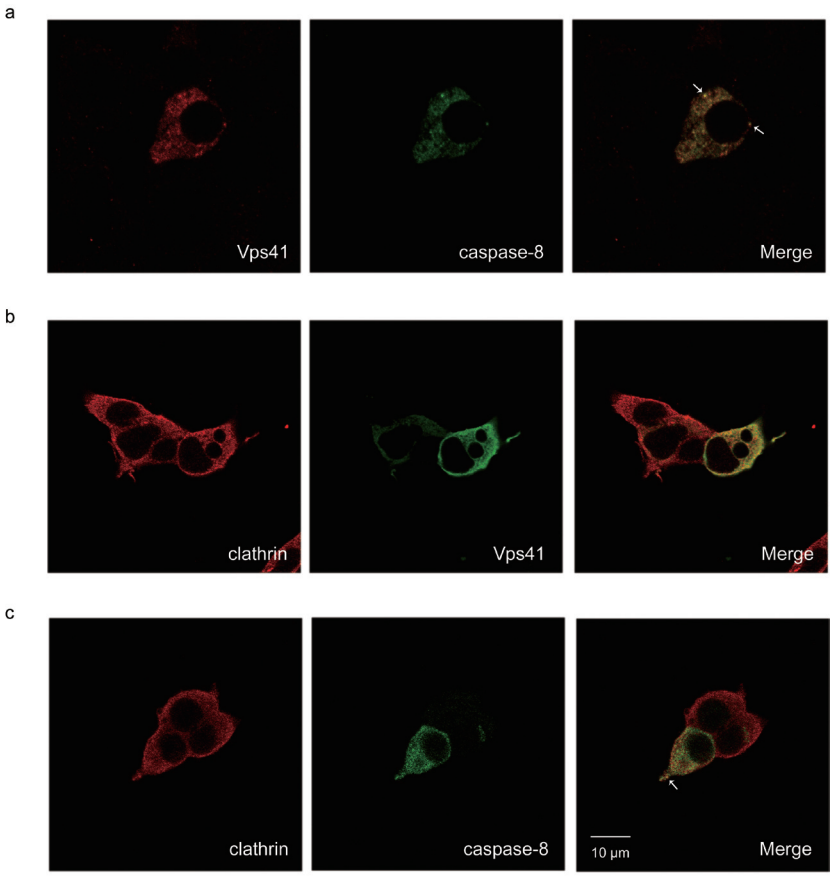

Figure 2. Vps41 and caspase-8 co-localize with clathrin in HEK293T cells.
HEK293T cells were transfected with pRK5-Flag-hVps41 and pRK5-HA-hCaspase-8. Cells were fixed after 16 h and incubated with **(a)** mouse anti-Flag and rabbit anti-HA antibodies **(b)** mouse anti-clathrin and rabbit anti-Flag antibodies **(c)** mouse anti-clathrin and rabbit ant-HA antibodies at 4°C overnight, followed by staining with Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit antibodies. Co-localization is shown in the right panel.

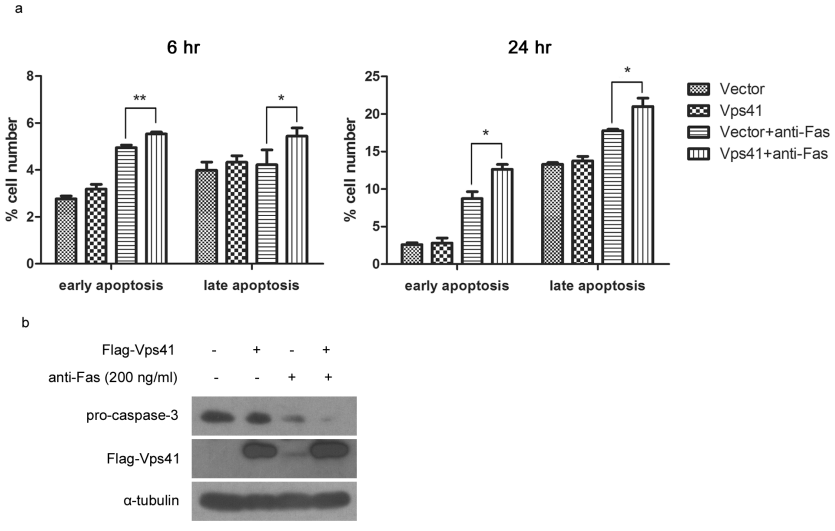

Figure 3. Vps41 promotes apoptosis induced by anti-Fas antibody in A549 cells.
A549 cells were transfected with vector or pRK5-Flag-Vps41. **(a)** Twenty-four hours later, cells were treated or not with 200 ng/ml anti-Fas antibody in 5% FBS culture medium for 6 or 24 h and apoptosis was analyzed by double staining with annexin V and PI (values are mean ± S.D., $*p<0.05$, $**p<0.01$, Student's *t*-test). **(b)** Twenty-four hours later, cells were treated or not with 200 ng/ml anti-Fas antibody in 5% FBS culture medium for 24 h. Pro-caspase-3 was detected by western blotting with anti-caspase-3 antibody.

RING or pRK5-Flag control vector in HEK293T cells, followed by immunoprecipitation with anti-Flag antibody. Co-IP results showed that when cells were transfected with plasmids encoding WD40, RING or full-length Vps41, HA-p18 was detected in the co-immunoprecipitates by immunoblotting (Fig. 1d). These observations reveal that Vps41 binds to the p18 subunit of caspase-8 through both its WD40 region and the RING-finger motif.

Vps41 co-localizes with caspase-8 in HEK293T cells

To determine the cellular localization of Vps41 interacting with caspase-8, confocal microscopy was performed on HEK293T cells expressing Flag-hVps41 and HA-hCaspase-8 simultaneously. The result demonstrated co-localization of these two proteins in the cytoplasm (Fig. 2a). Since Vps41 is believed to cover transport vesicles and help the docking onto lysosomes, we were interested in whether caspase-8 also localizes to vesicles. We examined the localization of Vps41 and caspase-8 to determine whether they co-localize with the vesicle marker clathrin. Immunofluorescent co-localization assay revealed that Vps41 is co-localized with clathrin and caspase-8 is partially co-localized with clathrin (Fig. 2b, 2c), which indicates that Vps41 may interact with caspase-8 on vesicles in HEK293T cells.

Vps41 promotes apoptosis induced by anti-Fas antibody in A549 cells

To investigate whether Vps41 plays a role in caspase-8-mediated apoptosis, we chose a Fas-sensitive cell line A549 to perform apoptosis induction and analysis since caspase-8 is known as an apical caspase in Fas-induced apoptosis. We transfected A549 cells with hVps41 and treated the cells with anti-Fas antibody for 6 or 24 h to induce apoptosis. Flow cytometric data revealed that among the A549 cells transfected with Vps41, early apoptotic cells (annexin V+/PI-) were more fragment ($5.54 \pm 0.07\%$) compared to the control ($4.95 \pm 0.11\%$) (n=3,

$p < 0.01$) and late apoptotic cells (annexin V+/PI+) were also more fragment ($5.44 \pm 0.35\%$) compared to the control cells ($4.22 \pm 0.63\%$) (n=3, $p < 0.05$) after a 6-h anti-Fas treatment. Also after 24-h anti-Fas treatment early apoptotic cells were more abundant among those transfected with Vps41 ($12.63 \pm 0.65\%$) compared to the control ($8.75 \pm 0.91\%$) (n=3, $p < 0.05$), as were late apoptotic cells were increased ($21.00 \pm 1.11\%$) vs. ($17.79 \pm 0.18\%$) (n=3, $p < 0.05$) (Fig. 3a). We also estimated the protein level of pro-caspase-3 after anti-Fas treatment for 24 h and found pro-caspase-3 reduced in cells transfected with Vps41 compared to control cells transfected with vector (Fig. 3b). As caspase-3 is a downstream effector of caspase-8, these results show that Vps41 may promote apoptosis and this promotion is probably through regulating caspase-8 activity in A549 cells.

DISCUSSION

In previous studies, Vps41 was shown to control protein trafficking. It covers transport vesicles and helps the dock onto lysosomes. In this study we first validated a direct interaction of Vps41 with caspase-8, a pro-apoptosis protease. Co-IP experiments showed that the p18 domain, which is known as a subunit of activated caspase-8, interacts with Vps41. However, as the cleavage reaction by caspase-8 is an instanteneous process that is difficult to be detected by co-IP, and we did not find any cleavage band of Vps41 in apoptotic cells induced by anti-Fas antibody (unpublished data), we believe that Vps41 is not a substrate of caspase-8.

In recent studies, over-expression of hVps41 was shown to enhance the clearance of misfolded and aggregated α-synuclein, and to block downstream events in the apoptotic cascade including activation of caspase-9 and caspase-3, and PARP cleavage in SH-SY5Y neuroblastoma cells. Vps41 was supposed to be a useful target for developing therapeutic strategies for human Parkinson's disease (Ruan *et al*., 2010; Garg *et al*., 2011). However, our experiments showed that in the A549 human lung adenocarcinoma cell line, Vps41 appeared to have a promoting effect on Fas-induced apoptosis. This result may have some relationship with the direct interaction of Vps41 with caspase-8, and is also consistent with the observation that a Vps41 gene mutation which resulted in premature termination of protein synthesis was found in gastric cancer (1/30) and colorectal cancer (1/40), while DNA from normal tissues from the same patients showed no evidence of the mutation (An *et al*., 2012). The latter results indicate that a defect of this protein may be associated with tumorigenesis itself or with apoptosis residence of tumor cells. Correspondingly, another research showed that Vps41 gene was significantly amplified in 12 of 13 (92%) primary effusion lymphoma (PEL) cell lines based on Affymetrix arrays (Roy *et al*., 2011). Together with the finding mentioned previously that overexpression of Vps41 protected SH-SY5Y cells from apoptosis, these results indicate that Vps41 may play different roles in different tissues. The function or effect of Vps41 in human diseases requires further research. Our result that Vps41 interacts directly with caspase-8 supplies a novel insight of a potential role of Vps41 beyond lysosomal trafficking and suggests a possible mechanism of Vps41 participation in the regulation of caspase-8-mediated apoptosis.

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