

GABARAPL1 Negatively Regulates Wnt/ β -catenin Signaling by Mediating Dvl2 Degradation through the Autophagy Pathway

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Key Words

Wnt signaling • GABARAPL1 • Dvl2 • Autophagy

Abstract

Wnt signaling is critical for many biological processes and is tightly regulated. In this study, we found that GABARAPL1 (GABA_A receptor-associated protein like 1, GABARAPL1) interacts with Dvl2 by both yeast two-hybrid screening and immunoprecipitation experiments. Furthermore, we observed that p62 is required for the interaction of Dvl2 and GABARAPL1. Luciferase assays indicated that GABARAPL1 represses Wnt/ β -catenin signaling stimulated by Wnt1, Dvl2 and β -catenin. We further demonstrated that GABARAPL1 mediates degradation of Dvl2 and the effect is blocked by addition of 3-MA, a specific inhibitor of autophagy. Finally, we provided evidence that over-expression of GABARAPL1 inhibits proliferation and tumor growth of MCF7 cells *in vitro* and in nude mice. Taken together, our results suggested that GABARAPL1 as a tumor repressor inhibits Wnt signaling via mediating Dvl2 degradation through the autophagy pathway.

Introduction

Wnt signal plays a critical role in embryonic development, cell polarity and stem cell maintenance [1, 2]. Mutation and deregulated expression of the components in Wnt signaling are linked to various diseases, including cancers [3-5]. Three Wnt pathways, known as canonical Wnt/ β -catenin, non canonical planar cell polarity (PCP), and non canonical Wnt/calcium signaling pathways, have been well documented [6, 7]. In these pathways, Dishevelled (Dvl) functions as a hub to mediate different Wnt responses [8].

Dvl proteins have been identified in organisms from hydra to human and are ubiquitously expressed in the body during development. There are three members of Dvls in vertebrates [9-11]. All the three Dvl members have three highly conserved domains including DIX (Dishevelled and axin), PDZ (PSD-95, Disc-large and ZO-1), and DEP (Dishevelled, egl-10, pleckstrin). The DIX domain in the N-terminus of Dvl is necessary for the canonical Wnt/ β -catenin pathway [12]. The PDZ domain, located in the center of Dvl [13], mediates the interaction of Dvl with many different proteins including CK1, Frat1, PP2C, Idax, Nkd, Stbm, Dapper, and β -arrestin. A recent study suggested that the PDZ domain

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may serve as a regulatory domain to control the stability, localization and tertiary structure of Dvl proteins [11]. The DEP domain in the C-terminus of Dvl has been shown to be required for activation of the JNK pathway in establishment of planar cell polarity in *Drosophila* [14].

Although the function of different domains of Dvl has been extensively studied, it remains unclear for the detailed role of DEP domain in the canonical Wnt signal pathway as only several DEP domain interacting proteins (e.g. *prickle*, *Daam1* and *MuSK*) are just identified to date [15]. To reveal the role of DEP domain in Wnt signaling, a yeast two-hybrid assay was performed using DEP domain and C-terminal of Dvl2 as bait to screen an 11.5-day mouse embryo cDNA library in this study. We have obtained a clone coding GABARAPL1 (GABA_A receptor-associated protein like 1) protein [16]. GABARAPL1 belongs to the GABARAP protein family which contains 6 members in mammalian cells, including MAP1LC3A (microtubule-associated proteins-1 light chain 3A), MAP1LC3B, MAP1LC3C, GABARAP and GABARAPL2/GATE16 [17]. This family is homologous to the yeast Atg8 protein, which plays a critical role in autophagy together with LC3s and is regarded as an autophagy marker [18]. Recently, GABARAPL1 is reported to associate with autophagic vesicles [19].

Autophagy is a highly conserved intercellular process in all eukaryotes, from yeast to human [20]. Briefly, autophagy occurs under certain stresses or inductions. Subsequently, cytoplasm components or organelles are delivered to a double-membrane vesicle (autophagosome), and then are fused with lysosomes for protein degradation by lysosomal hydrolases. Autophagy functions to cleaning long-lived proteins (or aggregated proteins) and aberrant organelles. To date, autophagy has been found to play a variety of physiological and pathophysiological roles, including cell survival, cell death, tumor suppression, antigen presentation, pathogen clearance, anti-aging, and neurodegeneration [21-23].

Three types of autophagy are classified as: macroautophagy (usually referred to 'autophagy'), microautophagy and chaperone-mediated autophagy. Generally, macroautophagy and microautophagy are nonselective on degradation of substrates because cytoplasm components and organelles are engulfed randomly. In contrast, chaperone-mediated autophagy is of selection from cytoplasm to vacuole targeting [24]. During the process of selective autophagy, Atg8 family proteins, such as Atg8, LC3 and GABARAP, function

together in docking autophagosome formation, maturation and fusion with lysosome [17]. Recently, autophagy has been linked to regulate Wnt signaling [25]. It turned out that LC3 and GABARAP mediated degradation of Dvl2 and inhibited Wnt signaling. In this study, based on our findings that Dvl2 interacts with GABARAPL1, we proposed that GABARAPL1 negatively regulates Wnt/ β -catenin pathway via mediating Dvl2 degradation through autophagy pathway.

Materials and Methods

Plasmids and reagents

Plasmids Flag-Dvl2, Myc-Dvl2/DIX, Myc-Dvl2/PDZ, Myc-Dvl2/DEP, and the related deletions were gifts from Dr. Xi He (Harvard Medical School) [26]. GFP-p62 was kindly provided by Dr. Li Yu (School of Life Science, Tsinghua University). The cDNA sequence encoding DEP domain and C terminus of Dvl2 used as bait in the yeast two-hybrid screening was amplified by PCR from plasmid Flag-Dvl2 and inserted in frame with Gal4 DNA-binding domain into the pGBKT7 vector (pGBKT7/Gal4-DB-DEP+C). GFP-Dvl2 was constructed into pEGFP-N1 vector. Other plasmids involved in this paper were stored in the lab.

Full length GABARAPL1 was amplified by nest-PCR from human brain cDNA library and inserted into pEFneo-HA vector in the frame of HA tag. The sense and antisense primers for the first cycle are 5'-ACC CCA CCT TCT GCC CTC-3' and 5'-TCT TTC CCC AAG TCA CAC AC-3', and for the second cycle are 5'-TAT AGG ATC CAT GAA GTT CCA GTA CAA GGA G-3' and 5'-TAT AGA ATT CTC ATT TCC CAT AGA CAC TCT C-3'. The first cycle product was diluted of 1:10 and used as the template of the second cycle. GABARAPL1 cDNA sequence was also subcloned into the pGEX4T-2 vector for GST fusion protein purification.

A plasmid with a shRNA targeting p62 was constructed with a synthesized insert with two single-strand DNA fragments: 5'-AGC TTG CAT TGA AGT TGA TAT CGA TTT CAA GAG AAT CGA TAT CAA CTT CAA TGC TTT TTG-3' and 5'-AAT TCA AAA AGC ATT GAA GTT GAT ATC GAT TCT CTT GAA TCG ATA TCA ACT TCA ATG CA -3'. The underlined sequence presents the shRNA target of the human p62 mRNA (Genebank ID: 214830437) [27] inserted between the Hind III and EcoR I sites in the pBSU6 vector.

Anti- β -Actin (AC-15) and anti-FLAG (M2) antibodies were from Sigma (Sigma-Aldrich Co). Anti-Myc (9E10), anti-GFP (FL), and anti-HA (F-7) were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc). Fluorescent secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were purchased from Jackson ImmunoResearch Laboratories (Jackson ImmunoResearch Laboratories, Inc). Cyclohexanone (CHX), leupeptin and chloroquine were purchased from Amresco (AMRESCO Inc., OH). G418 (A1720) and 3-MA (M9281) were from Sigma.

Cell culture

HEK293T, MCF7, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. All the cells were kept at 37°C in a 5% CO₂-containing atmosphere. Media and serum were purchased from Gibco. For starvation assays, cells were cultured in Earle's Balanced Salt Solution (Sigma, E7510) for 4 h. For autophagy inhibition assays, cells were treated with 3-MA (10 mM) [28], leupeptin (0.25 mM) [29] or chloroquine (100 nM) [30] for 18 h. MCF7 cells were plated at a density of 5 × 10⁴ cells/cm² the day before transfection for the generation of stable cell lines under the selection medium containing 1 mg/ml of G418. Resistant clones were maintained in media containing 400 µg/ml of G418.

Luciferase assay

HEK293T cells were transiently transfected with indicated plasmids using Vigofect (Vigofect Inc. Beijing, China), according to the manufacture instructions. Briefly, 0.1 µg of reporter plasmid pGL3/LEF-1-luc together with 5 ng of the internal control plasmid pRL-TK were transfected into the cells cultured in a 24-well plate. For the expression of Wnt1 (0.1 µg), Dvl2 (0.1 µg), β-catenin (0.1 µg), and GABARAPL1 (0.4 µg), the indicated amount of plasmids was co-transfected in a 24-well plate. The pEFneo-HA empty plasmid was used to balance the total amount of DNA equally to 1 µg/well. Luciferase activity was assayed after 24 h transfection using a Dual-Luciferase reporter assay system (Vigofect Inc. Beijing, China). The luciferase activity was normalized by firefly against renilla luciferase activity and presented as mean via standard deviation (SD) [31].

Western blot and immunoprecipitation assay

Proteins were analyzed by SDS-PAGE and Western blot [32]. For immunoprecipitation experiments, HEK293T cells grown in 60 mm dishes were transfected with indicated plasmids and were lysed in cell lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 10 mM MgCl₂, 0.5% NP40, 10% glycerol, 1 mM DTT, 0.1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin) for 30 min, and then were centrifugated at maximum speed for 10 min. Supernatants of cell lysates were incubated with 2 µg of indicated antibodies for 4 h at 4°C, and then added 30 µl of protein G/A agarose beads for 2 h at 4°C. Beads were washed with cell lysis buffer four times and bound proteins were eluted with 2x loading sample buffer and analyzed by Western blot with indicated antibodies. For the protein degradation experiment, whole cell lysates were analyzed 24h after transfection.

In vitro binding assay

GST and GST-GABARAPL1 proteins were expressed in the *E. Coli* strain BL21 and purified by affinity chromatography. The purified GST-GABARAPL1 protein was linked to Glutathione Sepharose beads and incubated with lysates of HEK293T cells expressing Myc-Dvl2/DIX, Myc-Dvl2/PDZ, Myc-Dvl2/DEP at 4°C for 4 h. Beads were washed with cell lysis buffer and the precipitant was analyzed by Western blot.

Reverse transcriptase PCR (RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen). Reverse transcription-PCR (RT-PCR) analyses were done with an RT-PCR kit from Takara (TAKARA BIOTECHNOLOGY, DALIAN CO). Total RNA (0.5 µg) for each sample was used in the RT-PCR reaction. Primers used for Flag-Dvl2 were: 5'-ATG GAC TAC AAA GAC GAT GAC-3' and 5'-GCC ATG TTC ACT GCT GTC -3'. Primers used for HA-GABARAPL1 were: 5'-TAC GAC GTG CCC GAC TAC-3' and 5'-CC ATA GAC ACT CTC ATC ACT GTA G -3'. Primers used for the human β-Actin mRNA were: 5'-TCG TCG ACA ACG GCT CCG GCA TGT-3' and 5'-CCA GCC AGG TCC AGA CGC AGG AT-3'. The PCR was done for 25 reaction cycles and the PCR products were resolved on a 1% agarose gel stained with ethidium bromide. β-Actin was amplified as an internal control.

Immunostaining

HeLa cells were seeded on cover slips and were transfected with GFP-Dvl2 and HA-GABARAPL1. After 24 h transfection, cells were washed in PBS, fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.3% Triton X-100 in PBS for 10 min. After blocked with 10% FBS for 1 h cells were incubated with monoclonal mouse anti-HA antibodies at 37°C for 1 h, followed by incubation with anti-mouse antibodies conjugated with TRITC for 1 h and counterstained with DAPI for 10 min. Images were obtained with a confocal laser scanning microscope (OLYMPUS BX61).

³H-TdR incorporation assay

To detect the effect of GABARAPL1 on cells proliferation, 3 × 10⁴ MCF7 wild, mock and MCF7/GABARAPL1 cells were seeded into a 24-well plate, respectively. After starvation with serum free medium for 24 h, cells were cultured with fresh medium containing of 1 µCi ³H- thymidine for 4 h. Cells were harvested at indicated time points, and ³H-TdR incorporation was detected by a microplate scintillation & luminescence counter (Packard).

Tumorigenicity assay

To assess tumor formation potential of parental and stable cell lines transfected with GABARAPL1, 1.4 × 10⁶ wild, mock and MCF7/GABARAPL1 cells were injected into the nude mice (6 animals in each group), respectively. The size of the tumors was measured every 7 days. Tumor volume was calculated as described [33]. The mice were sacrificed 9 weeks after inoculation.

Results

GABARAPL1 interacts with Dvl2

To investigate the role of Dvl2 in Wnt signaling pathway, we performed a yeast two-hybrid screen using DEP domain and C-terminus of mDvl2 as a bait and found that GABARAPL1 is a novel Dvl2 interacting protein (data not shown). To address whether Dvl2 interacts with GABARAPL1 in mammalian cells, a co-

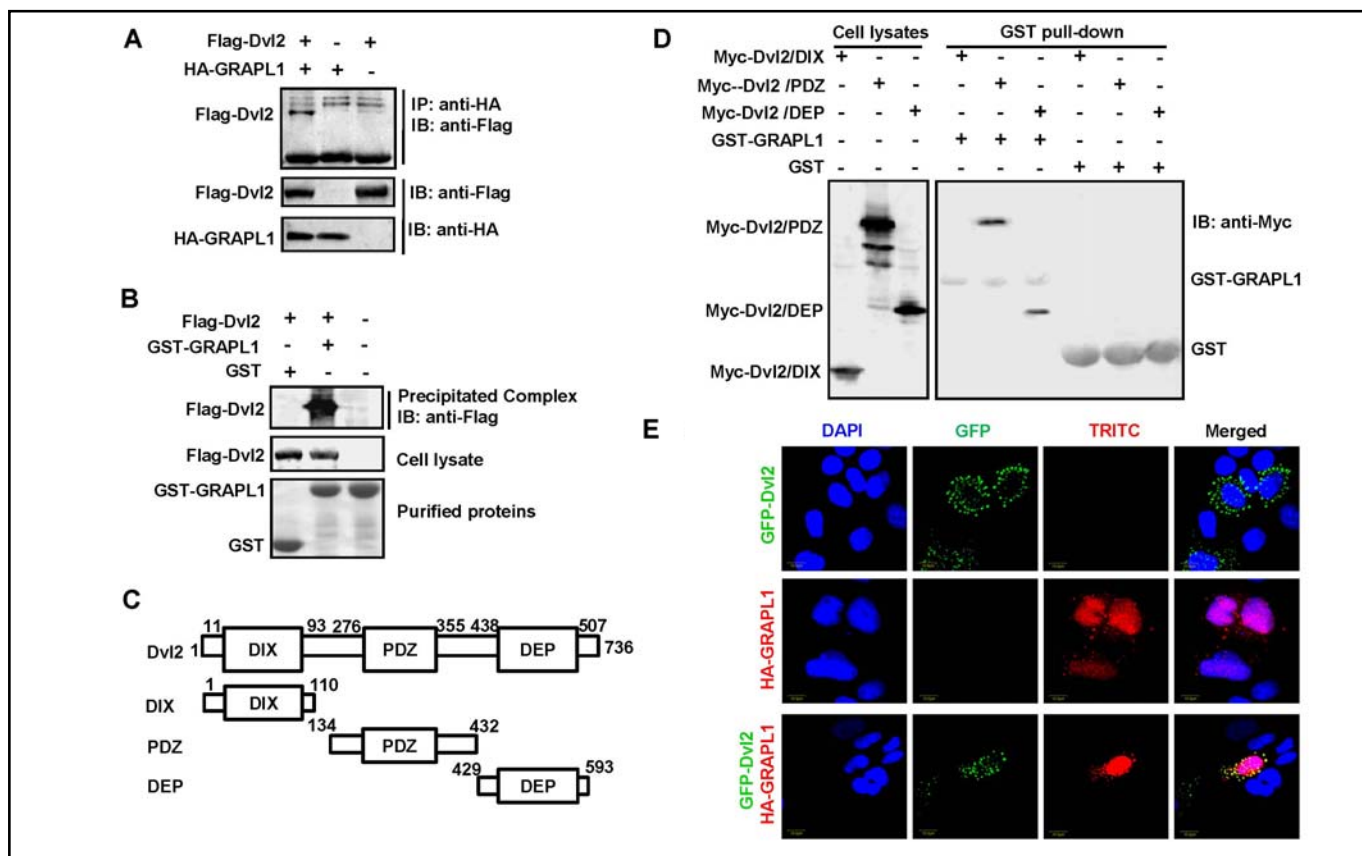


Fig. 1. GABARAPL1 interacts with Dvl2. (A) GABARAPL1 interacts with Dvl2 in the mammalian cells. HEK293T cells were co-transfected with Flag-Dvl2 and HA-GABARAPL1 (HA-GRAPL1). Immunoprecipitation was performed using an anti-HA antibody and the precipitants were detected by Western blot with an anti-Flag antibody. (B) GABARAPL1 interacts with Dvl2 *in vitro*. A GST pull-down assay was performed with GST or GST-GABARAPL1 (GST-GRAPL1) protein purified from *E.coli* and Flag-Dvl2 protein over-expressed in HEK293T cells. (C) Three main functional domains of Dvl2 (DIX, PDZ or DEP) are schematically presented. (D) PDZ or DEP domain of Dvl2 interacts with GABARAPL1. GST-GABARAPL1 (GST-GRAPL1) or GST was incubated with Myc tagged DIX, PDZ or DEP domains of Dvl2 over-expressed in HEK293T for the GST pull-down assay, respectively. (E) GABARAPL1 co-localizes with Dvl2 in HeLa cells. HeLa cells were co-transfected with HA-GABARAPL1 (HA-GRAPL1, red) and GFP-Dvl2 (green) and the cells were stained with an anti-HA (red) antibody. The co-localization of HA-GABARAPL1 (HA-GRAPL1) and GFP-Dvl2 is shown as yellow color in the merged images. The images were observed with a confocal microscope. Scale bar, 10 μ m.

immunoprecipitation assay was performed with Flag-Dvl2 and HA-GABARAPL1 co-expressed in HEK293T cells. A Western blot analysis indicated that Flag-Dvl2 was co-immunoprecipitated with HA-GABARAPL1 by using an anti-HA antibody (Fig. 1A). The interaction was confirmed by a GST pull-down assay using purified GST-GABARAPL1 protein (Fig. 1B). All these results indicated that GABARAPL1 interacts with Dvl2 *in vivo* and *in vitro*.

To map the interaction sites of the two proteins, Myc tagged DIX, PDZ or DEP domains of Dvl2 (Fig. 1C) over-expressed in HEK293T were incubated with GST-GABARAPL1 or GST protein, respectively. A GST pull-down analysis indicated that both PDZ and DEP domains of Dvl2 interact with GABARAPL1 (Fig. 1D).

The interaction of GABARAPL1 and Dvl2 was further validated by an immunostaining assay. The results showed that the two proteins were co-localized in a punctuated structure when HA-GABARAPL1 was co-expressed with GFP-Dvl2 in HeLa cells. The localization pattern of HA-GABARAPL1 appears to follow that of Dvl2, which distributed in a punctuated structure observed by many groups [34] (Fig. 1E). Taken together, we concluded that GABARAPL1 interacts with Dvl2 in mammalian cells.

p62 mediates the interaction of Dvl2 and GABARAPL1

p62 has been reported to directly bind to Atg8 family proteins to promote degradation of ubiquitinated and aggregated proteins through the autophagic mechanism

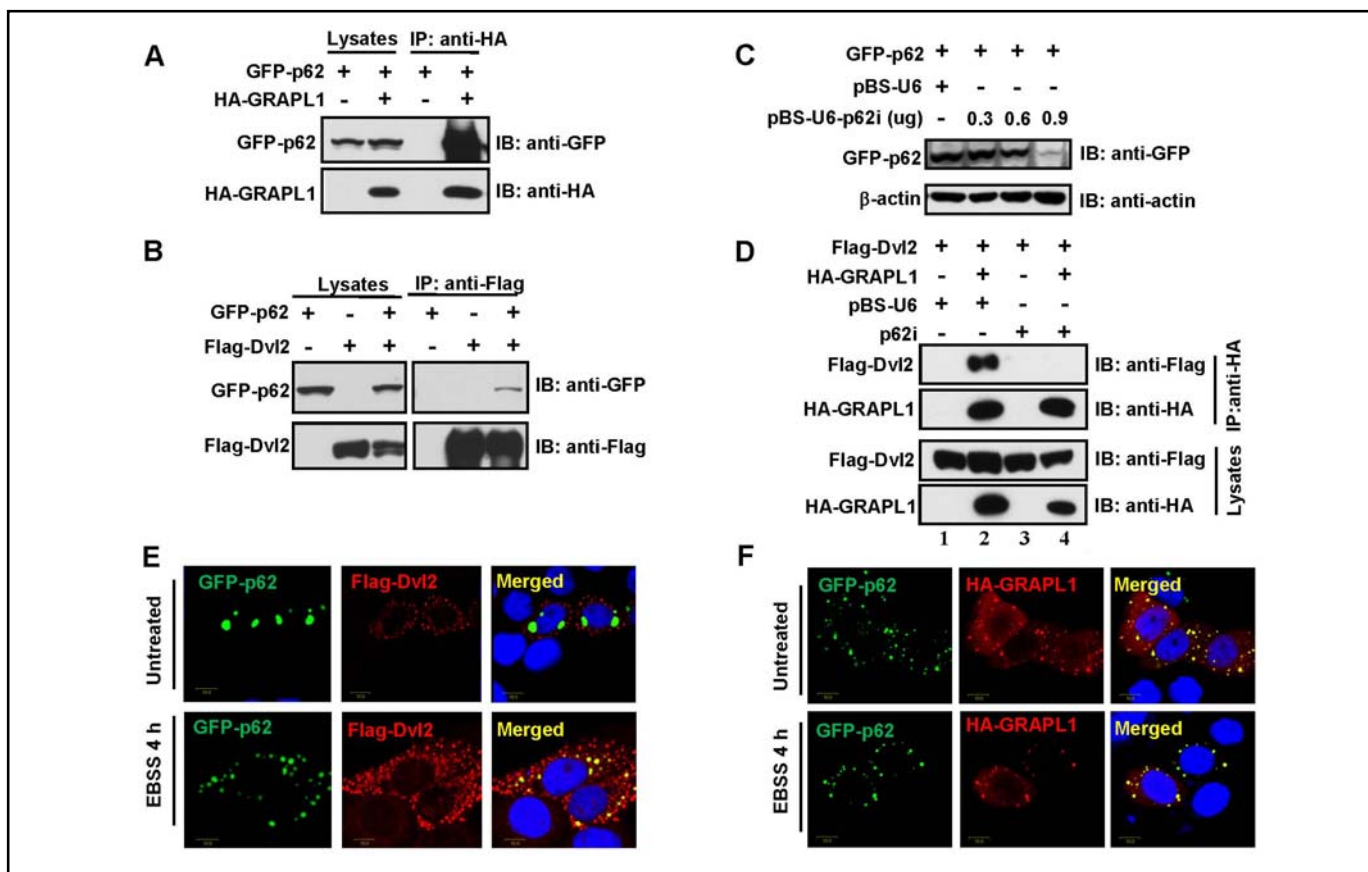


Fig. 2. p62 mediates the interaction of Dvl2 and GABARAPL1. (A) GABARAPL1 interacts with p62 in mammalian cells. HEK293T cells were co-transfected with GFP-p62 and HA-GABARAPL1. Immunoprecipitation was performed using an anti-HA antibody and the precipitant were analyzed by Western blot using an anti-GFP antibody. (B) Dvl2 interacts with p62 in mammalian cells. HEK293T cells were co-transfected with GFP-p62 and Flag-Dvl2. Immunoprecipitation was performed using an anti-Flag antibody and the precipitant was detected by Western blot using an anti-GFP antibody. (C) shRNA against p62 effectively depletes the expression of GFP-p62. HEK293T cells were co-transfected with GFP-p62 and different amount of shRNA-p62 or pBS-U6. The expression of GFP-p62 was observed by Western blotting, and β -actin was used as a loading control. (D) Depletion of endogenous p62 blocks the interaction of GABARAPL1 with Dvl2. HEK293T cells were co-transfected with the indicated plasmids. Immunoprecipitation was performed using an anti-HA antibody and the precipitant were detected by Western blot using an anti-Flag antibody. (E) p62 co-localized with Dvl2 upon starvation. Cells over-expressing GFP-p62 and Flag-Dvl2 were subjected to starvation treatment and stained with an anti-Flag (red) antibody. (F) p62 co-localized with GABARAPL1 upon starvation. Cells over-expressing GFP-p62 and HA-GABARAPL1 (HA-GRAPL1) were stained with an anti-HA antibody (red).

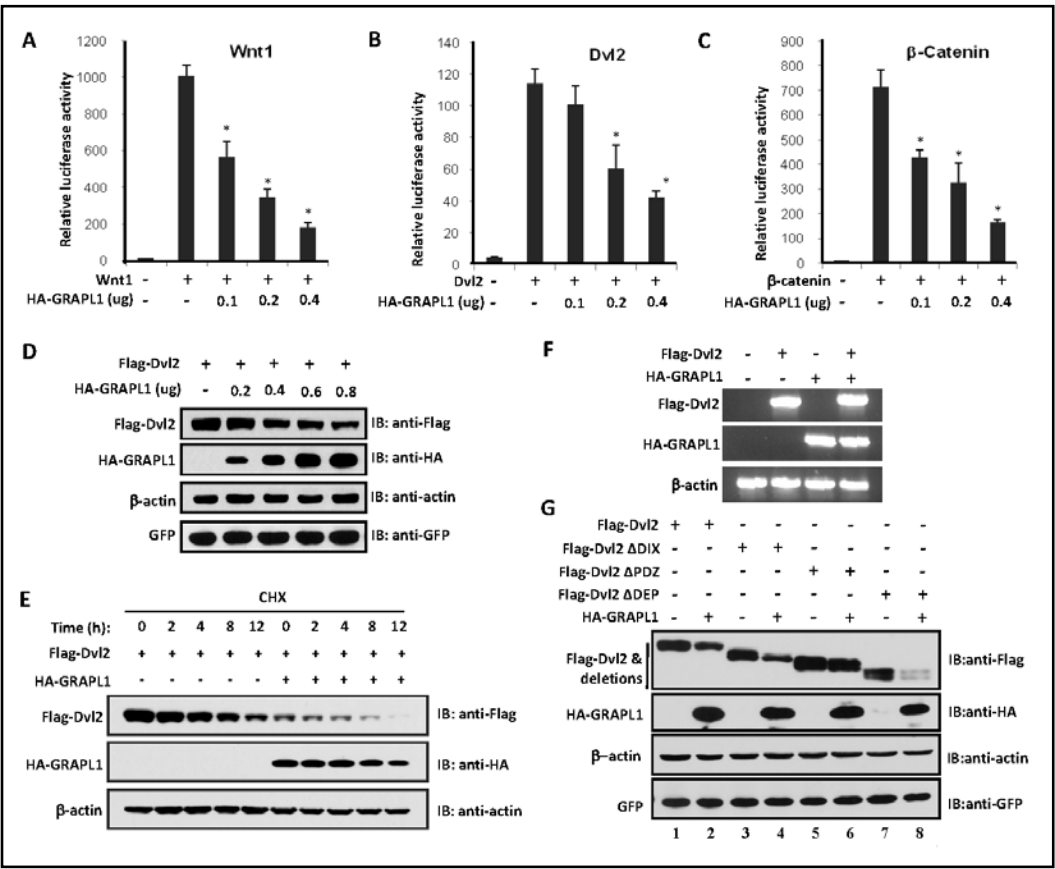
[27, 35]. Since GABARAPL1 is a member of Atg8 family proteins, we speculated that p62 interacts with GABARAPL1. To address this question, GFP-p62 and HA-GABARAPL1 were co-expressed in HEK293T cells for an immunoprecipitation assay. The result showed that an antibody against HA (for HA-GABARAPL1) precipitated down GFP-p62 protein very strongly (Fig. 2A), suggesting that GABARAPL1 interacts with p62. Furthermore we examined whether p62 interacts with Dvl2. An immunoprecipitation assay result showed that an anti-Flag antibody (for Flag-Dvl2) precipitated down with the GFP-p62 protein when Flag-Dvl2 and GFP-p62 were co-expressed in HEK293T cells (Fig. 2B). This result indicated that p62 interacts with Dvl2. Based on

the above results, we proposed that p62 might mediate the interaction of GABARAPL1 with Dvl2. To examine this hypothesis, we depleted the endogenous p62 protein by using a shRNA targeting the mRNA of p62 (Fig. 2C) and examined the interaction of Dvl2 and GABARAPL1. The result showed that when endogenous p62 was depleted, an antibody against Flag (for Flag-Dvl2) failed to precipitate down the HA-GABARAPL1 protein whereas Flag-Dvl2 and HA-GABARAPL1 remained a strong interaction in cells without depletion of p62 (Fig. 2D). Consistently, we observed that p62 co-localized with either Flag-Dvl2 (Fig. 2E) or HA-GABARAPL1 (Fig. 2F). All the data suggested that the interaction of Dvl2 and GABARAPL1 is mediated by p62.

Fig. 3. GABARAPL1

inhibits Wnt signaling pathway through promoting Dvl2 degradation. (A) GABARAPL1 inhibits Wnt1-mediated transcriptional activity. Luciferase assays were performed using HEK293T cells transfected with the indicated plasmids, a LEF-1-luc reporter and pRL-TK (as an internal control). Relative luciferase activities were normalized with the internal control. HA-GABARAPL1 (HA-GRAPL1) was transfected at an increasing amount. The relative activity of luciferase was compared with no transfection of HA-GABARAPL1 (*, $p<0.01$). (B) GABARAPL1 inhibits Dvl2-mediated transcriptional activity. The experiment was performed by transfection of Dvl2 and the indicated plasmids. The

relative luciferase activity was compared with no transfection of HA-GABARAPL1 (*, $p<0.01$). (C) GABARAPL1 inhibits β -catenin-mediated transcriptional activity. The experiment was performed by transfection of β -catenin and the indicated plasmids. The relative luciferase activity was compared with no expression of HA-GABARAPL1 (*, $p<0.01$). (D) GABARAPL1 degrades Dvl2 in mammalian cells. An increased amount of HA-GABARAPL1 (HA-GRAPL1) was co-transfected with Flag-Dvl2 in HEK293T cells, and Flag-Dvl2 protein level was analyzed by Western blotting. (E) Degradation of Dvl2 is accelerated by GABARAPL1. Flag-Dvl2 was transfected with or without HA-GABARAPL1 (HA-GRAPL1) in HEK293T. 24 hours later, cells were treated with CHX (50 μ g/ml) for 0, 1, 2, 4, 8 and 12h. Whole-cell lysates were analyzed by Western blot. (F) GABARAPL1 has no effect on the mRNA level of Dvl2. mRNA levels of Flag-Dvl2 were examined by an RT-PCR analysis in HEK293T cells under over-expression of HA-GABARAPL1 (HA-GRAPL1) and Flag-Dvl2. β -actin was used as a loading control. (G) The PDZ domain is critical for the degradation of Dvl2 by GABARAPL1. HEK293T cells co-transfected with HA-GABARAPL1 (HA-GRAPL1) and Flag-Dvl2 or its deletion mutants. The protein levels of Flag-Dvl2 or its deletion mutants were analyzed by Western blotting.



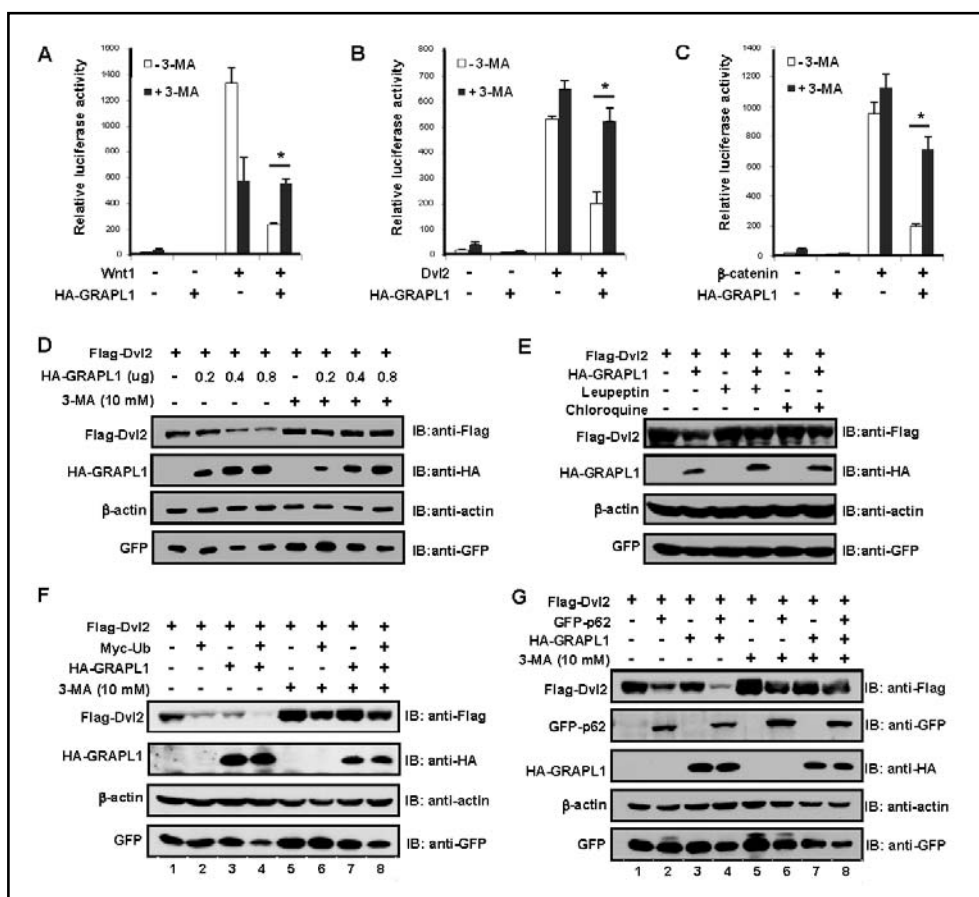
GABARAPL1 inhibits Wnt signaling through promoting Dvl2 degradation

The interaction of Dvl2 and GABARAPL1 promoted us to examine the role of GABARAPL1 on Wnt signaling. For this purpose, HEK293T cells were transfected with LEF-1-luc and GABARAPL1 in the presence of Wnt1, Dvl2 or β -catenin. Luciferase reporter results demonstrated that ectopical expression of HA-GABARAPL1 inhibits the transcriptional activities under either Wnt stimulation (Fig. 3A) or over-expression of Dvl2 (Fig. 3B). Interestingly, over-expression of HA-GABARAPL1 also leads to a strong inhibition of the β -catenin activated luciferase activity (Fig. 3C). The inhibitory role of HA-GABARAPL1 on the Wnt signaling is showed in a dose-dependent manner (Fig. 3A, B, C).

These results clearly indicated that GABARAPL1 is an inhibitor for Wnt signaling.

Since GABARAPL1 belongs to the Atg8 family which is associated with autophagy in mediating substrates to degradation, we speculated that GABARAPL1 might lead to Dvl2 degradation through the autophagy pathway. To examine this hypothesis, Flag-Dvl2 and HA-GABARAPL1 were co-transfected into HEK293T cells, and the protein levels were analyzed by Western blot. The result showed that an increasing amount of over-expressed HA-GABARAPL1 correlated to a decreasing Flag-Dvl2 protein level in the HEK293T cells (Fig. 3D). To confirm the degradation of Dvl2, we examined the half-life of Flag-Dvl2 in the absence or presence of HA-GABARAPL1 by blocking protein synthesis with

Fig. 4. GABARAPL1 promotes degradation of Dvl2 via autophagy. (A-C) GABARAPL1 inhibits Wnt signaling pathway via autophagy. HEK293T cells transfected with the indicated plasmids, a LEF-1-luc reporter and pRL-TK (as an internal control). Luciferase assays were performed in the presence or absence of 3-MA treatment. Relative luciferase activities were normalized with the internal control. The luciferase activities were boosted by Wnt1 (A), Dvl2 (B) and β -catenin (C). The data are presented as average with standard deviation (* $\Delta p < 0.01$). (D) Dvl2 degradation by GABARAPL1 is through autophagy pathway. An increased amount of HA-GABARAPL1 (HA-GRAPL1) was co-transfected with Flag-Dvl2 in HEK293T cells. 24 h after transfection, cells were treated with or without 3-MA, and Flag-Dvl2 protein level was analyzed by Western blotting. (E) Dvl2 degradation by GABARAPL1 is dependent on lysosome activities. HEK293T cells were transfected with the indicated plasmids. 24 h after transfection, cells were treated with or without leupeptin and chloroquine, and Flag-Dvl2 protein level was analyzed by Western blotting. (F) Over-expression of ubiquitin accelerates Dvl2 degradation mediated by GABARAPL1. HEK293T cells were transfected with the indicated plasmids. 36 h after transfection, cells were treated with or without 3-MA, and Flag-Dvl2 protein level was analyzed by Western blotting. (G) p62 promotes GABARAPL1-mediated Dvl2 degradation. p62 was over-expressed with Flag-Dvl2 with or without GABARAPL1 (HA-GRAPL1) in the presence or absence of 3-MA.

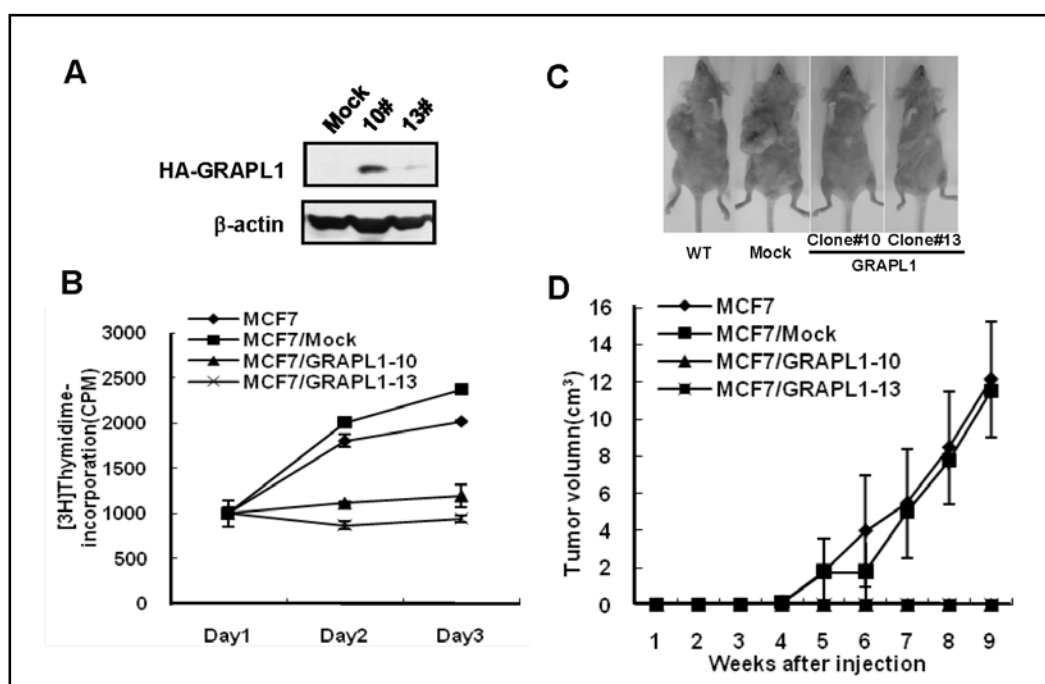


cyclohexanone (CHX). The result showed that over-expression of HA-GABARAPL1 significantly shortened the half-life of Flag-Dvl2 (Fig. 3E). We further observed that co-transfection of HA-GABARAPL1 and Flag-Dvl2 did not affect the mRNA levels from two independent vectors (Fig. 3F), suggesting that the negative correlation of the two protein levels is due to a protein degradation rather than a transcriptional mechanism. Furthermore, when the PDZ domain was deleted from Dvl2, HA-GABARAPL1 failed to promote degradation of the Flag-Dvl2 Δ PDZ protein (Fig. 3G, lanes 5 and 6), while deletion of DIX or DEP domain remains the ability of degradation by HA-GABARAPL1 (Fig. 3G, lanes 3 and 4, 7 and 8). These results suggested that GABARAPL1 induces degradation of the Dvl2 protein through the PDZ domain. This result is consistent with our observation that PDZ domain is critical for the interaction of Dvl2 and GABARAPL1 (Fig. 1D).

GABARAPL1 promotes degradation of Dvl2 via autophagy pathway

To address whether the inhibitory role of GABARAPL1 on Wnt signaling is through the autophagy pathway, we used 3-MA, a specific inhibitor of autophagy, to examine the status of Wnt signaling. Firstly, we compared the effect of 3-MA on the luciferase activity corresponding to Wnt signaling. The results showed that, while HA-GABARAPL1 repressed the luciferase activity stimulated by Wnt1, Dvl2 and β -catenin, addition of 3-MA significantly recovered the luciferase activity (Fig. 4 A, B, C, comparing the open and black columns), suggesting that the inhibitory role of GABARAPL1 on the Wnt signaling is dependent on autophagy. Secondly, we examined the protein level of Flag-Dvl2 in the presence of HA-GABARAPL1 with or without addition of 3-MA. A Western blot result showed that an increasing amount of expressed HA-GABARAPL1 resulted in a

Fig. 5. Over-expression of GABARAPL1 represses MCF7 proliferation and tumor growth. (A) Generation of stable cell lines with over-expression of GABARAPL1. Western blotting demonstrates the expression of HA-GABARAPL1 (HA-GRAPL1) in the two selected cell lines. Cells were selected with G418 after transfection with HA-GABARAPL1 (HA-GRAPL1). (B) Over-expression of GABARAPL1 represses MCF7 proliferation. A ^3H -TdR incorporation assay was performed in wild type, mock and GABARAPL1 stably over-expressed MCF7 cell lines. (C-D) Over-expression of GABARAPL1 represses MCF7 tumor growth. Nude mice (6 in each group) were injected with wild type, mock MCF7 and MCF7/GABARAPL1 cells. Photographs of representative nude mice are shown in (C) and the curves of tumour growth are shown in (D).



decreased level of Flag-Dvl2 proteins in cells in the absence of 3-MA, however, the Flag-Dvl2 protein level was recovered when 3-MA was added to the cells (Fig. 4D). These results suggested that the decreased protein level of Flag-Dvl2 is due to a degradation process mediated by GABARAPL1 through the autophagy pathway.

Since autophagic degradation is depended on hydrolases in lysosome at the last stage of autophagy [21], we questioned whether lysosome inhibitors, leupeptin and chloroquine, could recover the effect of GABARAPL1 on the protein degradation of Dvl2. A Western blot analysis indicated that both leupeptin and chloroquine recovered the Dvl2 protein level decreased by GABARAPL1 (Fig. 4E).

p62 has been reported to be required for the autophagy-mediated protein degradation after ubiquitination of the aggregated protein [27]. We hypothesized that both ubiquitin and p62 would enhance Dvl2 degradation mediated by GABARAPL1. To examine this hypothesis, HEK293T cells were co-transfected with Dvl2, ubiquitin (or p62) and GABARAPL1. The results demonstrated that either ubiquitin (Fig. 4F, lane 2) or p62 (Fig. 4G, lane 2) promotes Dvl2 degradation. Intriguingly, the degradation of Flag-Dvl2 by HA-GABARAPL1 was further accelerated by either ubiquitin (Fig. 4F, lane 4) or p62 (Fig. 4G, lane 4). As is expected, addition of 3-MA recovered Flag-Dvl2

protein levels significantly (Fig. 4F and 4G, lanes 5, 6, 7 and 8). Taken together, we concluded that GABARAPL1 mediates degradation of Dvl2 via the autophagy pathway.

GABARAPL1 represses MCF7 cells proliferation and tumor growth

Based on the inhibitory role of GABARAPL1 on Wnt signaling, we speculated that GABARAPL1 could lead to an inhibition of tumor cell growth. To examine this hypothesis, we stably over-expressed HA-GABARAPL1 in MCF7 cells (Fig. 5A). A ^3H -TdR incorporation assay indicated that MCF7/GABARAPL1 cells grow significantly less slowly than the wild type and mock cells (Fig. 5B), suggesting that GABARAPL1 represses MCF7 cell proliferation. Furthermore, a tumorigenicity assay demonstrated that mice implanted with MCF7/GABARAPL1 cells had significantly reduced tumors, compared with mice implanted with wild type and mock MCF7 cells (Fig. 5C, D). Taken together, we concluded that GABARAPL1 inhibits cell proliferation and represses tumor growth.

Discussion

Wnt signaling is critical for development, stem cell and tumor formation. As a major component, Dvl proteins

function as a hub for the Wnt signaling pathways. In this study, we reported that GABARAPL1 interacts with Dvl2. We provided lines of evidence that the interaction of GABARAPL1 with Dvl2 leads to inhibition of Wnt signaling and degradation of the Dvl2 protein through the autophagy pathway. Importantly, we observed that over-expression of GABARAPL1 in Wnt activated cells results in retarded cell growth both *in vitro* and *in vivo*. Our data suggest that the GABARAPL1 induced autophagy plays an important role in Wnt signaling.

Recently, our colleagues reported that Wnt signaling is negatively regulated by autophagy through the interaction of Dvl2 with LC3. Their results also indicated that Dvl2 interacts with GABARAP [25]. In this study, we demonstrated that Dvl2 interacts with GABARAPL1, a protein belonging to GABARAP family. Similarly, we found that p62 adapts the interaction of GABARAPL1 and Dvl2. Two previous studies revealed that GABARAPL1 is involved in autophagy [19, 36]. Therefore, our study provides a solid base for the role of autophagy in the regulation of Wnt signaling through degradation of Dvl2. Our study also provides information that GABARAP family proteins are involved in the regulation of Wnt signaling through autophagy pathway.

Another interesting observation in this study is that GABARAPL1 also regulates the activity of β -catenin. In our study, we found that Wnt signaling activated by over-expression of β -catenin is also impaired by GABARAPL1 (Fig. 3C). This result indicated that GABARAPL1 repressed Wnt signaling not only at the Dvl level but also at the β -catenin level. Our preliminary data showed that GABARAPL1 also mediates degradation of β -catenin (data not shown). We speculated that β -catenin might be degraded together with Dvl2 in the cytoplasm by the autophagic complexes. Whether β -catenin is aggregated and degraded by autophagy is of interest for further study.

Ubiquitination has been identified that involved in selective autophagy [18]. In general, ubiquitinated proteins at K48 site are thought to be degraded by the ubiquitin proteasome system (UPS), while proteins ubiquitinated at K63 site or just be mono-ubiquitinated is degraded by autophagy [37]. Our result showed that ubiquitin accelerates Dvl2 degradation under over-expression of GABARAPL1 (Fig. 4F). The question is which E3 ligase is involved in Dvl2 ubiquitination for autophagy degradation. Recently, Gao et al reported that pVHL, an E3 ligase, was required for Dvl ubiquitination in response to starvation [25]. pVHL is found to promote Dvl2 ubiquitination and degradation by autophagy. Although we

have a consistent result that GABARAPL1, similarly to GABARAP, mediates the degradation of Dvl2, it remains unclear whether other E3 ligases are involved in the ubiquitination of Dvl2 during autophagy mediated degradation. It may be possible that both GABARAP and GABARAPL1 associated with Dvl2 being ubiquitinated by the same E3 ligase.

Although autophagy has a double-sword function in health and diseases [38], it plays a suppressive role in breast cancer [39]. Our results demonstrated that GABARAPL1 inhibits Wnt/ β -catenin signaling pathway (Fig. 4A, B, C) and represses MCF7 cell proliferation and tumorigenesis (Fig. 5). Consistently, Klebig et al reported GABARAP expression was decreased in breast cancer and over-expression of GABARAP repressed the tumorigenicity of cells in nude mice [40]. Meanwhile, Berthier et al reported that over-expression of GABARAPL1 inhibited MCF7 proliferation and the mRNA level of GABARAPL1 was down-regulated in breast cancer. These authors demonstrated that high expression of GABARAPL1 is correlated with a better outcome for patients with lymph node-positive breast cancer [41]. In our study, we found that GABARAPL1 significantly inhibits the tumor growth from breast cancer cell line. Taken together with all the results, we proposed that GABARAPL1 is a tumor suppressor and negatively regulates Wnt signaling by mediating Dvl2 degradation through the autophagy pathway.

Conclusion

In this study, we found that GABARAPL1 promotes ubiquitinated Dvl2 degradation via autophagy pathway and inhibits Wnt/ β -Catenin signaling. Our results also demonstrated that GABARAPL1 is a tumor repressor, which shed new light on cancer diagnosis and therapy.

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