ARTICLE IN PRESS

Biochemical and Biophysical Research Communications xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The Daple-CK1ε complex regulates Dvl2 phosphorylation and canonical Wnt signaling

Nobutoshi Esaki [a], Atsushi Enomoto [a], Maki Takagishi [a], Yasuyuki Mizutani [a, b], Tadashi Iida [a, b], Kaori Ushida [a], Yukihiro Shiraki [a], Shinji Mii [a], Masahide Takahashi [a, c, *]

[a] Department of Pathology, Nagoya University Graduate School of Medicine, Nagoya, 466-8550, Japan
[b] Department of Gastroenterology and Hepatology, Nagoya University Graduate School of Medicine, Nagoya, 466-8550, Japan
[c] International Center for Cell and Gene Therapy, Fujita Health University, Toyoake, 470-1192, Japan

ARTICLE INFO

Article history:
Received 17 August 2020
Accepted 20 August 2020
Available online xxx

Keywords:
Daple
Dvl2
Casein kinase
Wnt

ABSTRACT

The canonical Wnt signaling pathway plays a crucial role in embryonic development, tissue homeostasis and cancer progression. The binding of Wnt ligands to their cognate receptors, the Frizzled (Fzd) family of proteins, recruits Dishevelled segment polarity protein (Dvl) to the plasma membrane and induces its phosphorylation via casein kinase 1 (CK1), which leads to the activation of β-catenin. Previous studies showed that Dishevelled-associating protein with a high frequency of leucine residues (Daple) is an important component of the Wnt signaling pathway and essential for Dvl phosphorylation. However, the mechanism by which Daple promotes CK1-mediated phosphorylation of Dvl is not fully understood. In this study, we found that Daple overexpression induced CK1ε-mediated Dvl2 phosphorylation at threonine 224 (Thr224). A Daple mutant (Daple ΔGCV) that lacks a carboxyl-terminal motif to associate with Dvl, retained the ability to interact with CK1ε, but did not induce Dvl phosphorylation, suggesting the importance of the Daple/Dvl/CK1ε trimeric protein complex. We further found that Thr224 phosphorylation of Dvl was required for full activation of β-catenin transcriptional activity. Consistent with this, wild-type Daple promoted β-catenin transcriptional activity, following dissociation of β-catenin and axin. Finally, Wnt3a stimulation increased the membrane localization of Daple and its association with Dvl, and Daple knockdown attenuated Wnt3a-mediated β-catenin transcriptional activity. Collectively, these data suggested a essential role of spatial Daple localization in CK1ε-mediated activation of Dvl in the canonical Wnt signaling pathway.

1. Introduction

The canonical (β-catenin dependent) and non-canonical (β-catenin independent) Wnt signaling pathways are crucial for an array of cellular functions, intercellular communication, embryonic development and tissue homeostasis [1,2]. Both pathways are highly conserved among species. Many studies have shown that dysfunction and deregulation of these pathways cause the initiation and progression of cancer, suggesting the significance of tight control of the Wnt signaling pathways [3]. Wnt ligands are secreted lipoglycoproteins that bind to their receptor complex, which is composed of the Frizzled (Fzd) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) to initiate the canonical Wnt signaling pathway [4]. Dishevelled segment polarity protein (Dvl in mammals) is a major component of the canonical Wnt signaling pathway. It becomes polymerized by interacting with Wnt-bound Fzd and LRP5/6 receptors [5,6]. Dvl proteins undergo phosphorylation by different kinases, including casein kinase 1 (CK1) [7,8]. Phosphorylation of Dvl inhibits the β-catenin destruction complex composed of Axin, glycogen synthase kinase 3-β (GSK-3β) and adenomatous polyposis coli (APC). As a result, β-catenin is stabilized and translocated to the nucleus. Nuclear β-catenin then interacts with T cell factor (TCF)/lymphoid enhancer factor (LEF) and initiates transcription of its target genes [9].

The Dvl proteins are multifunctional scaffold phosphoproteins. Dvl phosphorylation is indispensable for signal transduction in both the canonical and non-canonical Wnt signaling pathways

Abbreviations: CK, casein kinase; LRP, low-density lipoprotein receptor-related protein; APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase 3-β.

* Corresponding author. Department of Pathology, Nagoya University Graduate School of Medicine, Nagoya, 466, Japan.
E-mail address: mtakaha@med.nagoya-u.ac.jp (M. Takahashi).

https://doi.org/10.1016/j.bbrc.2020.08.066
0006-291X/© 2020 Elsevier Inc. All rights reserved.

[5,6]. Previous studies identified many phosphorylation sites in Dvl2, a Dvl isoform, that positively regulate the canonical Wnt pathways. For example, the phosphorylation of serine residues at 298 and 480 of Dvl2 via receptor-interacting protein kinase 4 promotes β-catenin stability in the canonical Wnt signaling pathway, which is crucial for the growth of cancer and pluripotent human embryonal carcinoma cells [10]. In the non-canonical Wnt signaling pathway, CK1δ- and ε-dependent phosphorylation of Ser143 and Thr224 in Dvl2 promotes primary cilia disassembly, followed by cell cycle progression that depends on polo-like kinase 1 activity [11]. Although the role of Ser143 phosphorylation in the canonical Wnt signaling pathway was revealed [12], the significance of Thr224 phosphorylation has not been determined. Thus, a complete picture of Dvl2's role and the biological significance of its intricate phosphorylation functions have not yet been established.

Dishevelled-associating protein with a high frequency of leucine residues (Daple) was initially identified as a Dvl-interacting protein [13]. Daple interacts with the PDZ (Postsynaptic density 95, Discs Large, Zonula occludens-1) domain of Dvl through its carboxyl (C)-terminal 3 amino acid residues, glycine-cysteine-valine (GCV) [14]. Recent studies have revealed that Daple is a scaffold protein essential for diverse cellular processes, including the regulation of the non-canonical Wnt signaling pathway that involves the small guanosine triphosphatase (GTPase) Rac and signaling driven by trimeric G proteins [14–16]. Moreover, Daple binds microtubules and regulates their dynamics, which is essential for the orientation of motile cilia to the anterior side in ependymal cells [16]. Thus, the role of Daple in the non-canonical Wnt signaling pathway has been well appreciated. An unresolved issue, however, is that it remains unclear how Daple works in the context of the canonical Wnt signaling pathway. A previous study showed that Daple overexpression induced a mobility shift of Dvl in Western blot analysis [13]. However, the detailed mechanisms by which Daple controls the phosphorylation and function of Dvl in the canonical Wnt signaling pathway are unknown.

In the present study, we further characterized the role of Daple in Dvl phosphorylation and its downstream β-catenin activation in the canonical Wnt signaling pathway. We identified a phosphorylation site in Dvl2 induced by CK1ε in cells overexpressing Daple. We also found that Dvl2 interacts with CK1ε in a Daple-dependent fashion, suggesting the importance of a Daple/Dvl/CK1ε protein complex. Dvl2 phosphorylation and possibly its interaction with Daple were crucial for downstream β-catenin activation. Finally, Wnt3a stimulation increased Daple/Dvl2 interaction, which was accompanied by the membrane localization of Daple. These data collectively improve our understanding of the important roles of Daple in the canonical Wnt signaling pathway.

2. Materials and methods

2.1. Cell culture

HEK293 and L cells stably expressing Wnt3a (L/Wnt3a) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.2. Construction and transfection of expression vectors

A cDNA carrying mouse *Ccdc88c*, the gene for Daple, encoded a 2009 amino acid protein that was isolated as described previously and was inserted into a pCAG-myc plasmid and a pEF-myc plasmid [13,14]. A Daple cDNA deficient for the C-terminal GCV domain (Daple ΔGCV) was also generated [14]. Human Dvl2 cDNA was previously subcloned into the pCGN-HA plasmid [14]. The pCGN-HA-Dvl2 plasmid was used to introduce mutations (T224A) into the Dvl2 cDNA. Human *CSNK1E* cDNA in a pCEP-4HA plasmid was purchased from Addgene (Watertown, MA, USA). Daple fragments, NT (1–259), CT (1380–2009), ΔGCV (1–2006), ΔNT (671–2009), ΔCT (1–1750) and CT ΔGCV (1380–2006), were inserted into a pEF-BOS-GST plasmid to generate fusion proteins. β-catenin-firefly luciferase reporter plasmid TOP-FLASH was purchased from Millipore (Bedford, MA, USA). A *Renilla* luciferase cDNA inserted into pRL vector was purchased by Promega (Madison, WI, USA).

2.3. Antibodies and reagents

α-Daple antibody (ABS515) were purchased from Merck (Billerica, MA, USA). α-Daple antibody (#28147) was purchased from IBL (Gunma, Japan). α-Dvl2 monoclonal antibody (sc-8026), α-c-myc monoclonal antibody (sc-40), α-GFP monoclonal antibody (sc-390,394), α-HA monoclonal antibody (sc-7932) and α-α-tubulin antibody (sc-5286) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). α-GST monoclonal antibody (#2624), α-Dvl2 monoclonal antibody (#3224), α-β-actin monoclonal antibody (#4970) and α-Axin-1 monoclonal antibody (#2074) were purchased from Cell Signaling Technology (Danvers, MA, USA). α-Dishvelled2 (phospho Thr224) monoclonal antibody (ab124941) was purchased from Abcam (Cambridge, GB, United Kingdom). α-β-catenin monoclonal antibody (AF1329), α-Casein kinase 1ε monoclonal antibody (AF4567) and recombinant human Wnt3a (5036-WN-500) were purchased from R&D Systems (Minneapolis, MN, USA). α-HA-tag antibody (561) was purchased from MBL (Nagoya, Japan). α-Transferrin receptor antibody (136,800) was purchased from Invitrogen (Waltham, MA, USA). Casein kinase 1δ, ε inhibitor, IC261, was purchased from Sigma Aldrich (Saint Louis, MO, USA).

2.4. Analysis of Wnt-stimulated cells

Wnt3a conditioned medium (CM), which was harvested from L/Wnt3a cells cultured for 2 days, was concentrated 5-fold using Amicon Ultra 15 (Millipore, Bedford, MA, USA) at 2000×*g* for 10 min. HEK293 cells were serum starved for 24 h, and the cells treated with the concentrated CM for each analysis. Control CM was obtained from control L cells.

2.5. Isolation of cell surface proteins by biotin labeling

Biotin labeling and isolation of cell surface proteins were carried out with a Cell Surface Protein Isolation Kit (Thermo Fisher Scientific) following the manufacturer's protocol. Briefly, cells (1.0×10^7) were washed 3 times with ice-cold PBS, and surface proteins were labeled with sulfo–NHS–SS-biotin for 30 min. Subsequently, cells were treated with cell lysis buffer supplemented with complete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail and centrifuged at 12,000×*g* at 4 °C to remove insoluble materials. Biotin-labeled surface proteins were captured on NeutrAvidin agarose resin at room temperature for 1 h. After the resin was washed, bound proteins were eluted with 1 × SDS-PAGE sample buffer, and analyzed by Western blot analysis.

2.6. Knockdown of daple in cells

The target sequences for shRNA-mediated Daple knockdown were as follows (only the sense sequence is shown): Daple shRNA#1, 5′-GGTGCAAGCTCGATGTGTA-3'; Daple sh RNA#2, 5′-GCACCAAAGGCTATAACTC-3'; and Daple shRNA#3, 5′-GCCTGGAGCGTGACAACAA-3'. The oligonucleotide pairs were inserted into the pSIREN-RetroQ retroviral shRNA expression vector (Clontech Laboratories Inc., Palo Alto, CA, USA) to generate

Please cite this article as: N. Esaki et al., The Daple-CK1ε complex regulates Dvl2 phosphorylation and canonical Wnt signaling, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.08.066

recombinant retroviruses as previously described [14], followed by infection of HEK293 cells and puromycin selection to generate stable Daple knockdown cells.

2.7. β-catenin activity assay

To examine β-catenin activity, Firefly and *Renilla* luciferase activities were determined using the Dual Luciferase Stop & Glo Reporter Assay System (Promega). HEK293 cells (1.0×10^5) were transiently transfected with the TOP-FLASH Firefly luciferase reporter vector and *Renilla* luciferase vector by Lipofectamine 2000. Following transfection (24 h), the cells was lysed with 1 × passive lysis buffer (PLB), and light emission was measured by addition of either Firefly or *Renilla* luciferase substrate. Firefly and *Renilla* luciferase activities were determined by luminometer (Lumat3, Berthold Technology, Bad Wildbad, Germany). Experiments were performed in triplicate for at least 3 different samples. Results are shown as the fold-induction of the Firefly/*Renilla* activity ratio relative to the control.

2.8. Biochemical analysis

Methods for Western blot analysis, immunoprecipitation and GST-pull down assay are described as Supplemental Material.

2.9. Data analysis

Data are presented as means ± standard deviations of the means. P values of less than 0.05 were considered statistically significant using unpaired t-tests. Statistical analyses were conducted using GraphPad Prism 7.00 (GraphPad Software Inc.).

3. Results

3.1. Daple overexpression induces the phosphorylation of Dvl2 at Thr224 via CK1ε

A previous study showed that Daple interacts with Dvl and that Daple overexpression induces a mobility shift of Dvl in Western blot analysis [13]. The same study showed that Daple overexpression inhibited β-catenin transcriptional activity in canonical Wnt signaling. The effect, however, was marginal, and the significance of Daple in canonical Wnt signaling has not been conclusively determined. Therefore, we aimed to reveal whether Daple overexpression induced the phosphorylation of Dvl, a mediator of β-catenin transcriptional activity. We first confirmed that the Daple C-terminal domain (CT, 1380–2009 aa), but not its mutant CTΔGCV (1380–2006 aa) which lacked the C-terminal 3 amino acids (GCV motif), interacts with endogenous Dvl2 (Sup Fig. 1A–C). The data reproduced the previous studies that found that the C-terminal GCV motif is responsible for the binding with Dvl [13,14]. Another study has showed the significance of Dvl2 phosphorylation at Ser143 in canonical Wnt signaling [12], but the association between phosphorylated Dvl2 at Thr224 and canonical Wnt signaling pathway has been unknown. Therefore, we examined the effect of Daple overexpression on phosphorylated Dvl2 at Thr224 (Fig. 1A). The overexpression of Daple, but not its ΔGCV mutant, induced a Dvl2 mobility shift as well as the phosphorylation site, suggesting that Daple interaction with Dvl2 is essential for Dvl2 phosphorylation at Thr224 (Fig. 1A). The data showed that the mobility shift of Dvl that was observed in Western blot analysis in a previous study [13] reflected the phosphorylation of at least Thr224 in Dvl2.

It has been reported that CK1δ and CK1ε are Serine/Threonine kinases responsible for the phosphorylation of Dvl2 at Thr224 [11]. Treatment of HEK293 cells with IC261, which is a specific inhibitor of CK1δ and ε, suppressed the phosphorylation of Dvl2 at Thr224 induced by Daple-expressing cells (Fig. 1B). Next, we examined whether exogenous Daple is co-immunoprecipitated with Dvl2, CK1ε and CK1δ in IP experiment. We found that Daple is more co-immunoprecipitated with CK1ε than CK1δ, which indicated that Daple, Dvl2 and CK1ε form a tertiary protein complex (Fig. 1C). Therefore, we focused on CK1ε for the functional analysis of Dvl2 phosphorylation induced by Daple. Consistent with previous study [11], phosphorylation of Dvl2 at Thr224 was increased by exogenous CK1ε (Fig. 1D). These data showed that CK1ε mediated the phosphorylation of Dvl2 at Thr224 induced by overexpression of Daple.

Interestingly, a Daple ΔGCV mutant that lacks the ability to bind Dvl2 retained interaction with CK1ε, suggesting that Daple interaction with CK1ε was not mediated by Dvl2 (Fig. 1E). Next, we generated Daple Knockdown (KD) HEK293 cells by pSIREN-RetroQ retroviral shRNA expression vector using three types of the target sequences, and we decided target sequence #1 because of the efficacy for Daple KD (Fig. 1F). We found that Daple KD resulted in a decrease in Dvl2/CK1ε interaction, suggesting the possibility that CK1ε/Dvl2 interaction was mediated by Daple (Fig. 1G).

3.2. Effects of Thr224 phosphorylation of Dvl2 and daple overexpression on β–catenin transcriptional activity

Several phosphorylation sites of Dvl2 promote β-catenin transcriptional activity [9,10]. A study indicated that Dvl2 phosphorylation at Ser143 was associated with β-catenin transcriptional activation [12], but it remains unknown whether Dvl2 phosphorylation at Thr224 promotes β-catenin transcriptional activation. Therefore, we addressed the role of Dvl2 phosphorylation at Thr224 in β-catenin transcriptional activity. To that end, we generated a Dvl2 mutant T224A, in which Thr224 residue was substituted by an alanine, followed by its overexpression in HEK293 cells. Successful generation of the mutant was confirmed by Western blot analysis (Fig. 2A). To examine whether Thr224 phosphorylation of Dvl2 mediated β-catenin transcriptional activity, TOP-FLASH was co-transfected with a wild-type (WT) Dvl2 or its T224A mutant into HEK293 cells (Fig. 2B and C). Transduction of WT Dvl2 significantly upregulated the TOP-FLASH reporter by almost 5-fold relative to the negative control, a process that is transcriptionally activated by nuclear β-catenin, (Fig. 2B). On the other hand, the non-phosphorylated mutant (T224A) of Dvl2 activated the reporter to a smaller extent than did WT Dvl2 (Fig. 2C). These data indicated that CK1ε-mediated phosphorylation of Thr224 residue of Dvl2 is required for full activation of β-catenin.

The next important question was whether the phosphorylation of Dvl2 induced by Daple mediates β-catenin transcriptional activity. Previous studies showed that phosphorylated Dvl enhanced the dissociation of β-catenin from the destruction complex, including Axin, GSK3-β, and APC, which resulted in the nuclear translocation of β-catenin in the canonical Wnt signaling pathway [9,17]. We found that the overexpression of Daple induced the dissociation of β-catenin and Axin (Fig. 2D). A TOP-FLASH reporter assay showed that WT Daple activated the reporter more than the negative control (Fig. 2E). Consistent with this, the treatment of cells with the CK1ε and δ inhibitor IC261 reduced β-catenin transcriptional activity induced by Daple overexpression (Fig. 2F).

3.3. The role of Daple in Wnt3a-dependent β-catenin transcriptional activity

A previous study in which Daple was first identified did not address its role in Wnt-dependent β-catenin transcriptional activity [13]. We therefore examined the effect of Daple KD on Dvl2

Please cite this article as: N. Esaki et al., The Daple-CK1ε complex regulates Dvl2 phosphorylation and canonical Wnt signaling, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.08.066

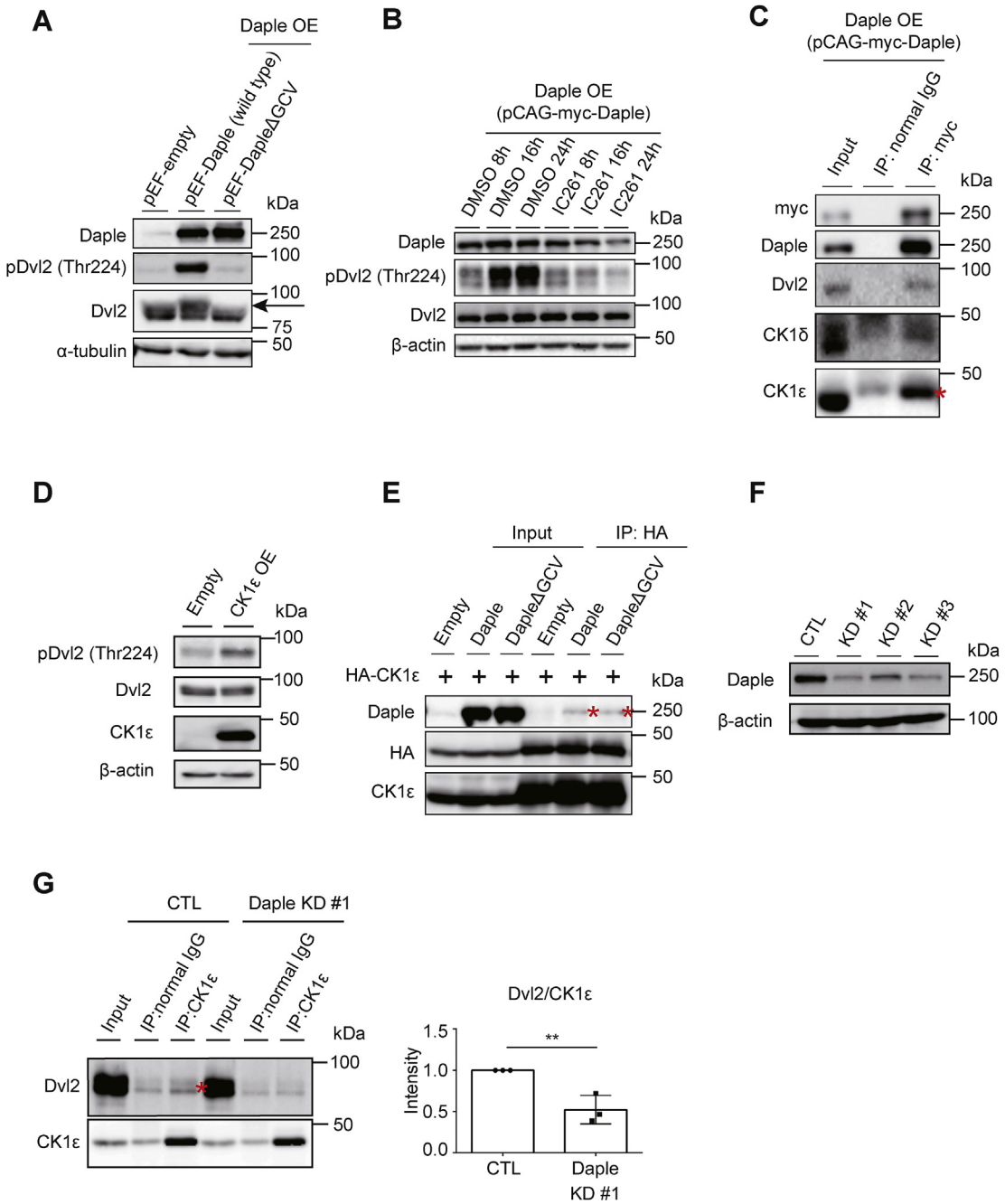

Fig. 1. Daple overexpression promotes the phosphorylation of Dvl2 via CK1ε

(A) HEK293 cells were transiently transfected with either pEF-myc empty, Daple or Daple ΔGCV plasmid, followed by the analysis of total cell lysate with the indicated antibodies. An arrow denotes the mobility shift of endogenous Dvl2 that is found in cells overexpressing wild-type Daple. OE, overexpression.

(B) HEK293 cells were transiently transfected with the pCAG-myc-Daple plasmid, followed by treatment with IC261 (40 μM) and Western blot analysis using the indicated antibodies.

(C) HEK293 cells were transiently transfected with the pCAG-myc-Daple plasmid, followed by immunoprecipitation with α-myc antibody and Western blot analysis. An asterisk indicates Daple proteins bound to CK1ε.

(D) HEK293 cells was transfected with pCEP 4HA-CK1ε plasmid were examined by Western blot analysis using the indicated antibodies.

(E) pCEP 4HA-CK1ε plasmid was co-transfected with either a pEF-myc empty, Daple, or Daple ΔGCV plasmid into HEK293 cells, followed by immunoprecipitation with α-HA antibody. Asterisks indicate Daple proteins bound to CK1ε.

(F) HEK293 cells were transfected with pSIREN-RetroQ retroviral shRNA expression vectors, followed by Western blot analysis

(G) Control and Daple KD (#1) HEK293 cells were immunoprecipitated with α-CK1ε antibody, followed by Western blot analysis. An asterisk indicates Dvl2 protein bound to CK1ε. Quantification of the intensity of Dvl2 bands normalized with that of the CK1ε is shown in right panel. $^{**}P < 0.01$.

phosphorylation in cells stimulated by Wnt3a. Wnt3a specifically promotes β-catenin transcriptional activity in the canonical Wnt signaling pathway. We found that Wnt3a stimulation increased the phosphorylation of Dvl2 at the Thr224 residue, a process that was attenuated by Daple KD (Fig. 3A). After 6 h of Wnt3a stimulation, TOP-FLASH reporter assays showed that β-catenin transcriptional activity decreased by 70% less in Daple KD cells than in WT cells, further supporting the role of Daple in the canonical Wnt signaling

Please cite this article as: N. Esaki et al., The Daple-CK1ε complex regulates Dvl2 phosphorylation and canonical Wnt signaling, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.08.066

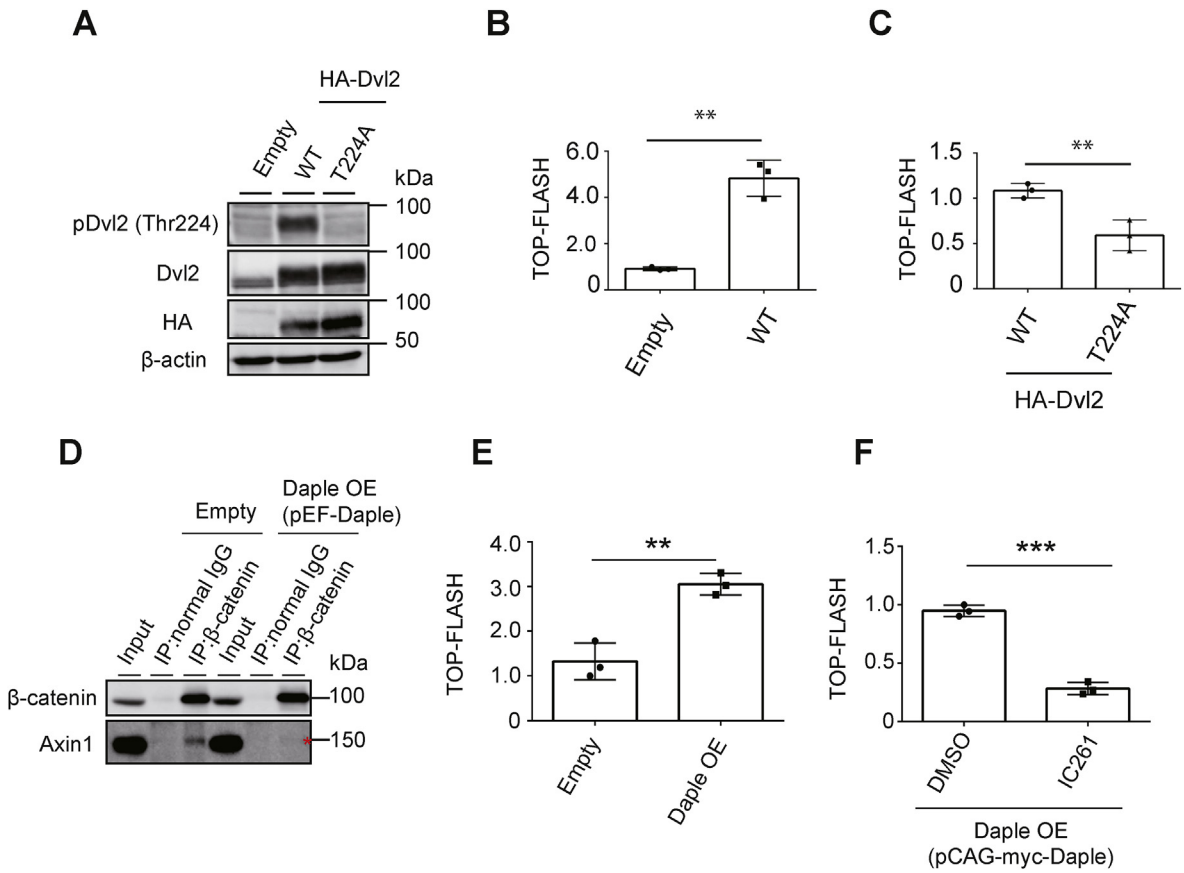

Fig. 2. Effects of Thr224 phosphorylation of Dvl2 and Daple overexpression on β−catenin transcriptional activity

(A) HEK293 cells were transiently transfected with the indicated plasmids encoding wild-type (WT) and non-phosphorylated mutants of Dvl2 tagged with the HA epitope, followed by Western blot analysis with the indicated antibodies.

(B, C) HEK293 cells were transiently co-transfected with the TOP-FLASH reporter plasmid and the indicated plasmids encoding wild-type or non-phosphorylated mutant of the Dvl2 plasmid, followed by the analysis of total cell lysates for luciferase activity. **P < 0.01.

(D) HEK293 cells were transiently transfected with either pEF-myc empty or Daple plasmid, followed by IP with α-β-catenin antibody.

(E) HEK293 cells were transiently co-transfected with the TOP-FLASH reporter plasmid and either pEF-myc empty, or Daple plasmid, followed by the analysis of total cell lysates for luciferase activity. **P < 0.01.

(F) HEK293 cells transiently transfected with the TOP-FLASH reporter plasmid and pCAG-myc-Daple plasmid were treated with IC261 (40 μM) for 12 h, followed by an analysis of total cell lysates for luciferase activity. ***P < 0.001.

pathway (Fig. 3B).

Finally, we examined Wnt3a-dependent changes in the formation of the Daple/Dvl2/CK1ε protein complex. Wnt3a stimulation increased exogenous Daple co-immunoprecipitation with Dvl2 (Fig. 3C). Another interesting finding was that the amount of Daple found in the membrane fraction was increased in Wnt3a-stimulated cells (Fig. 3D). Taken together, these data implied that Wnt3a stimulation enhanced the recruitment of Daple to the plasma membrane, where Daple formed a stable complex with Dvl2 to stimulate its CK1ε-dependent phosphorylation, which led to β-catenin transcriptional activity (Fig. 4).

4. Discussion

Daple was previously identified as a Dvl-binding protein with multiple roles in both the canonical and non-canonical Wnt signaling pathways [13−16]. In the present study, we further characterized aspects of Daple in the context of the canonical Wnt signaling pathway. We identified Daple-induced phosphorylation sites of Dvl2 and interaction of Daple with CK1ε. We also investigated roles of Daple in the regulation of Dvl2/CK1ε interaction and β-catenin transcriptional activity. Our studies suggested that Wnt3a stimulation increased the membrane localization of Daple and its association with Dvl2, which may be vital for Dvl2 phosphorylation at Thr224 and its downstream β-catenin transcriptional activity (Fig. 4).

Our findings contradict the conclusions of previous study with regard to the role of Daple in β-catenin transcriptional activity. Our data showed that Daple overexpression promoted β-catenin transcriptional activity in HEK293 epithelial cells, whereas Oshita et al. reported the opposite effect using L cells [13]. The reason for this discrepancy is not understood at present. Considering that β-catenin is an essential component of E-cadherin-mediated adherent junctions at cell-cell contacts in epithelial cells and L cells lack E-cadherin expression [18,19], the distribution and functions of β-catenin may be different among cell types [20]. Further studies of Daple using different types of cells will be needed to address this issue.

A possible shortcoming of the present study is that we did not address the involvement of Daple-mediated Dvl2 phosphorylation and β-catenin transcriptional activity in the pathology of human disease. Recent studies have revealed that mutations in the human *CCDC88C* gene, which encodes Daple, are the cause of severe congenital hydrocephalus. That finding is consistent with the appearance of hydrocephalus in Daple-deficient mice [16]. Other studies have shown that Daple is overexpressed in colorectal and

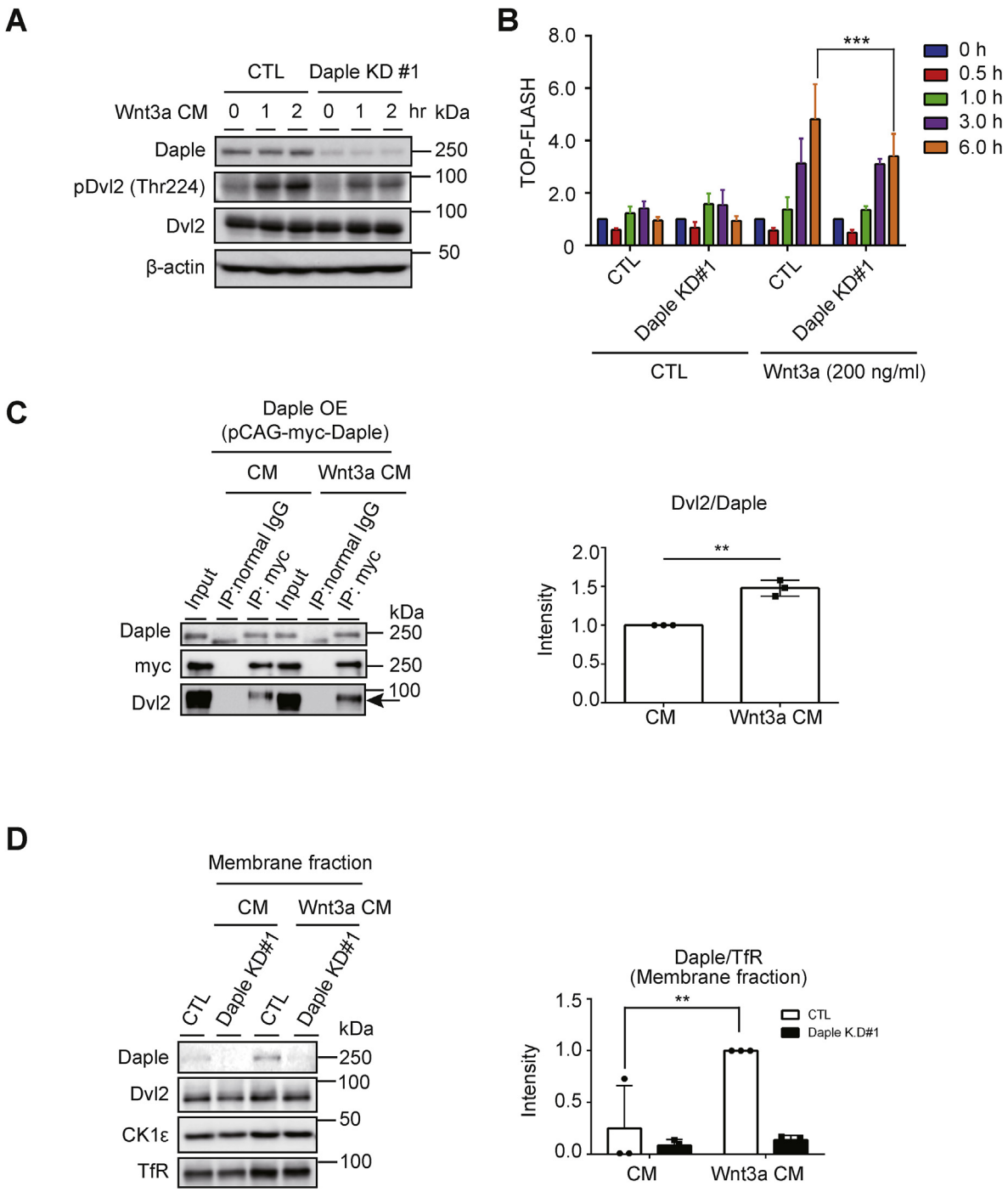

Fig. 3. The role of Daple in Wnt3a-dependent β-catenin transcriptional activity

(A) Control and Daple KD (#1) HEK293 cells were treated with conditioned medium (CM) from L cells stably expressing Wnt3a for the indicated time, followed by Western blot analysis.

(B) Control and Daple KD (#1) HEK293 cells transiently transfected with the TOP-FLASH reporter plasmid were treated with recombinant Wnt3a (200 ng/mL) for 0, 0.5, 1, 3 or 6 h, followed by the analysis of total cell lysates for luciferase activity. ***$P < 0.001$.

(C) HEK293 cells transiently transfected with pCAG-myc-Daple plasmid were treated CM from L cells stably expressing Wnt3a for 1 h, followed by IP with α-myc antibody and Western blot analysis. The arrow indicates Dvl2 co-immunoprecipitated with Daple. Quantification of the intensity of Dvl2 bands normalized with that of the Daple is shown in right panel. **$P < 0.01$.

(D) Control and Daple KD (#1) HEK293 cells treated with conditioned medium from L cells stably expressing Wnt3a for 1 h, followed by isolation of the membrane fraction and Western blot analysis. Quantification of the intensity of Daple bands normalized with that of the Transferrin receptor (TfR), a plasma membrane marker, is shown in right panel. **$P < 0.01$.

Please cite this article as: N. Esaki et al., The Daple-CK1ε complex regulates Dvl2 phosphorylation and canonical Wnt signaling, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.08.066

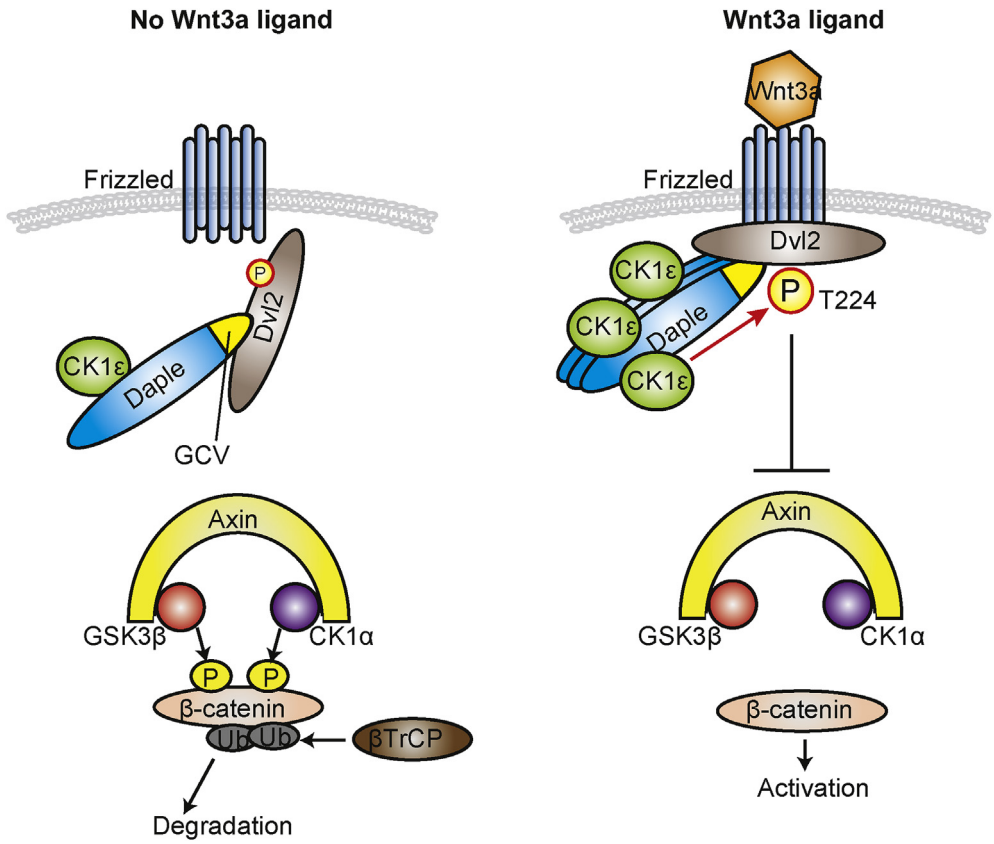

Fig. 4. The role of Daple in Wnt3a-dependent β-catenin transcriptional activity that is accompanied by changes in Daple/Dvl2 interaction and localizationSchematic illustration of the working hypothesis on the role of Daple in the canonical Wnt signaling pathway. In cells lacking the Wnt3a ligand, the interaction of CK1ε and Dvl2 is weak (Left panel). In contrast, Daple is recruited to the membrane and robustly interacts with Dvl2 in cells stimulated with Wnt3a, where Dvl2 is highly phosphorylated by CK1ε (right panel). Daple-mediated Dvl2 phosphorylation is required for suppressing the β-catenin destruction complex and its transcriptional activity in the nucleus.

gastric cancer cells, and its expression levels correlated with the prognosis of the cancer patients [15,21]. However, it remains unknown how Daple-mediated Dvl2 phosphorylation is involved in the etiology of these human diseases [15,21,22]. Our preliminary experiments on a mouse pancreatic cancer cell line showed that Daple is essential for the proliferation of the pancreatic cancer cells and is indispensable for Dvl2 phosphorylation (data not shown). Further work will be necessary to delineate context- and disease type-dependent roles of Daple and its downstream signaling, findings that may lead to the identification of novel therapeutic targets for these human diseases.

Declaration of competing interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Kozo Uchiyama for technical support in the present study. This work was supported by a Grant-in-Aid for Scientific Research (S) (26221304 to M.Takahashi.) and a Grant-in-Aid for Young Scientists (B) (17K15593 to M.Takagishi.) commissioned by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.08.066.

References

[1] C.Y. Logan, R. Nusse, The WNT signaling pathway IN development and disease, Annu. Rev. Cell Dev. Biol. 20 (2004) 781–810, https://doi.org/10.1146/annurev.cellbio.20.010403.113126.
[2] R. Nusse, H.E. Varmus, Wnt genes, Cell. 69 (7) (1992) 1073–1087, https://doi.org/10.1016/0092-8674(92)90630-U.
[3] T. Zhan, N. Rindtorff, M. Boutros, Wnt signaling in cancer, Oncogene 36 (11) (2017) 1461–1473, https://doi.org/10.1038/onc.2016.304.
[4] F. Cong, L. Schweizer, H. Varmus, Wnt signals across the plasma membrane to activate the β-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP, Development. 131 (20) (2004) 5103–5115, https://doi.org/10.1242/dev.01318.
[5] C. Gao, Y.G. Chen, Dishevelled: the hub of Wnt signaling, Cell. Signal. 22 (5) (2010) 717–727. https://doi:10.1016/j.cellsig.2009.11.021.
[6] M. Sharma, I. Castro-Piedras, G.E. Simmons Jr., K. Pruitt, Dishevelled: a masterful conductor of complex Wnt signals, Cell. Signal. 47 (2018) 52–64, https://doi.org/10.1016/j.cellsig.2018.03.004.
[7] J.M. Peters, R.M. McKay, J.P. McKay, J.M. Graff, Casein kinase I transduces Wnt signals, Nature 401 (6751) (1999) 345–350, https://doi.org/10.1038/43830.
[8] C. Sakanaka, P. Leong, L. Xu, S.D. Harrison, L.T. Williams, Casein kinase iepsilon in the wnt pathway: regulation of beta-catenin function, Proc. Natl. Acad. Sci. U. S. A. 96 (22) (1999) 12548–12552, https://doi.org/10.1073/pnas.96.22.12548.
[9] W.J. Jeong, E.J. Ro, K.Y. Choi, Interaction between Wnt/β-catenin and RAS-ERK pathways and an anti-cancer strategy via degradations of β-catenin and RAS by targeting the Wnt/β-catenin pathway, NPJ Precis Oncol 2 (1) (2018) 5, https://doi.org/10.1038/s41698-018-0049-y.
[10] X. Huang, J.C. McGann, L.Y. Bob, et al., Phosphorylation of dishevelled by protein kinase RIPK4 regulates wnt signaling, Science 339 (6126) (2013) 1441–1445, https://doi.org/10.1126/science.1232253.
[11] K.H. Lee, Y. Johmura, L.R. Yu, et al., Identification of a novel Wnt5a-CK1ε-Dvl2-Plk1-mediated primary cilia disassembly pathway, EMBO J. 31 (14) (2012) 3104–3117, https://doi.org/10.1038/emboj.2012.144.
[12] T. He, D. Wu, P. Lin, et al., DDX3 promotes tumor invasion in colorectal cancer via the CK1ε/Dvl2 axis, Sci. Rep. 6 (2016) 21483, https://doi.org/10.1038/srep21483.

[13] A. Oshita, S. Kishida, H. Kobayashi, et al., Identification and characterization of a novel Dvl-binding protein that suppresses Wnt signalling pathway, Gene Cell. 8 (12) (2003) 1005–1017, https://doi.org/10.1111/j.1365-2443.2003.00692.x.
[14] M. Ishida-Takagishi, A. Enomoto, N. Asai, et al., The Dishevelled-associating protein Daple controls the non-canonical Wnt/Rac pathway and cell motility, Nat. Commun. 3 (2012) 859, https://doi.org/10.1038/ncomms1861.
[15] N. Aznar, K. Midde, Y. Dunkel, et al., Daple is a novel non-receptor GEF required for trimeric G protein activation in Wnt signaling, ELife 4 (2015), e07091, https://doi.org/10.7554/eLife.07091.
[16] M. Takagishi, M. Sawada, S. Ohata, et al., Daple coordinates planar polarized microtubule dynamics in ependymal cells and contributes to hydrocephalus, Cell Rep. 20 (4) (2017) 960–972, https://doi.org/10.1016/j.celrep.2017.06.089.
[17] S. Kim, H. Huang, M. Zhao, et al., Wnt stabilization of β-catenin reveals principles for morphogen receptor-scaffold assemblies, Science 340 (6134) (2013) 867–870, https://doi.org/10.1126/science.1232389.
[18] W.C. Chen, B. Obrink, Cell-Cell contact mediated by E-Cadherin Restrict Invasive behavior of L-cells, J. Cell Biol. 114 (2) (1991) 319–327, https://doi.org/10.1083/jcb.114.2.319.
[19] M. Gloerich, J. Bianchini, K. Siemers, et al., Cell division orientation is coupled to cell–cell adhesion by the E-cadherin/LGN complex, Nat. Commun. 8 (1) (2017) 13996, https://doi.org/10.1038/ncomms13996.
[20] R.N. Hannoush, Kinetics of wnt-driven β-catenin stabilization revealed by quantitative and temporal imaging, PloS One 3 (10) (2008) e3498, https://doi.org/10.1371/journal.pone.0003498.
[21] H. Ara, M. Takagishi, A. Enomoto, et al., Role for Daple in non-canonical Wnt signaling during gastric cancer invasion and metastasis, Canc. Sci. 107 (2) (2016) 133–139, https://doi.org/10.1111/cas.12848.
[22] M. Takagishi, N. Esaki, K. Takahashi, et al., Cytoplasmic dynein functions in planer polarization of basal bodies with ciliated cells, iScience 23 (6) (2020) 101213, https://doi.org/10.1016/j.isci.2020.101213.

Please cite this article as: N. Esaki et al., The Daple-CK1ε complex regulates Dvl2 phosphorylation and canonical Wnt signaling, Biochemical and Biophysical Research Communications, https://doi.org/10.1016/j.bbrc.2020.08.066