The origins of rimmed vacuoles and granulovacuolar degeneration bodies are associated with the Wnt signaling pathway

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Abstract

Inclusion-body myositis (IBM) and Alzheimer's disease (AD) are biochemically characterized by the presence of aggregated β-amyloid protein and tau protein. In addition, both diseases are pathologically characterized by vacuolar changes, including rimmed vacuoles (RVs) in IBM and granulovacuolar degeneration (GVD) in AD. Previously, we demonstrated that RVs and GVD bodies are associated with a set of common molecules, leading us to speculate that both RVs and GVD bodies originate from similar structures on the plasma membrane of muscle cells and neuronal cells, namely, the neuromuscular junction (NMJ) and the postsynaptic spine especially in terms of Wnt signaling pathway. In this study, we investigated the presence of components of NMJ in RVs and/or postsynaptic spine in GVD bodies respectively by immunohistochemistry and immunofluorescence. The antigens probed included the following: (1) dishevelled (Dvl) family proteins (Dvl1, Dvl2 and Dvl3), (2) NMJ-associated proteins (low density lipoprotein-related protein 4 [Lrp4], heat shock protein 70 [Hsp70], β-catenin, phospho-β-catenin, rapsyn, P21-activated kinase 1 [PAK1], adenomatous polyposis coli [APC] and ADP-ribosylation factor 6 [Arf6]), (3) a lipid raft-associated molecule (phosphatidylinositol 4, 5-bisphosphate [PIP2]), and (4)

other proteins [prion, glycogen-synthase kinase 3β (GSK-3β)]. In all cases of sporadic IBM examined, RVs were immunopositive for Dvl3, Hsp70, β-catenin, PIP2, APC, prion and GSK-3β. In all cases of AD examined, GVD bodies were immunopositive for Dvl3, phospho-β-catenin, rapsyn, APC and PIP2. These findings show that RVs and GVD bodies share common molecules associated with the Wnt signaling pathway, indicating that these structures share a common structural and functional origin.

Keywords: Alzheimer's disease, granulovacuolar degeneration, inclusion-body myositis, rimmed vacuole, Wnt

Abbreviations

AD, Alzheimer's disease; APC, adenomatous polyposis coli; Arf6, ADP-ribosylation factor 6; CK1δ, casein kinase 1δ; Dvl, dishevelled; GSK-3β, glycogen-synthase kinase 3β; GVD, granulovacuolar degeneration; Hsp70, heat shock protein 70; Lrp4, low density lipoprotein-related protein 4; PAK1, P21-activated kinase 1; p-β-catenin, phospho-β-catenin; PIP2, phosphatidylinositol 4, 5-bisphosphate; PSD95, postsynaptic density protein 95; RV, rimmed vacuole; s-IBM, sporadic inclusion body myositis.

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1. Introduction

Rimmed vacuoles (RVs) are found in a number of muscular disorders, including inclusion-body myositis (IBM) [1], distal myopathy with RV formation, oculopharyngeal dystrophy [2], and Becker muscular dystrophy [3]. RVs are approximately 3–20 μm in diameter and consist of vacuoles surrounded by filamentous material forming round/oval or cleft-like shapes [1]. Most vacuoles are empty, but are sometimes occupied by granules. Sporadic IBM (s-IBM) is one of the most common muscle diseases, with prominent RVs in patients over the age of 50 [4]. The pathology of s-IBM is similar to that of Alzheimer's disease (AD), in that both are associated with β-amyloid (Aβ) peptide and phosphorylated tau [5].

AD is characterized pathologically by the presence of senile plaques and neurofibrillary tangles. Furthermore, granulovacuolar degeneration (GVD) in the brain is also a pathological hallmark of AD [6]. GVD bodies, which are typically 3–5 μm in diameter, are accumulations of basophilic small granules, each within a clear vacuole, in the perinuclear region of pyramidal neurons [7]. GVD bodies contain many proteins related to tau phosphorylation, including casein kinase 1 (CK1 [CK1δ and CK1ε]) [8] and glycogen-synthase kinase 3β (GSK-3β) [9]. Earlier, we reported that GVD bodies

contain another tau kinase, cyclin dependent kinase 5 (CDK5) [10]. In addition, we demonstrated that the lipid raft-associated molecules, flotillin-1, phosphatidylinositol 4, 5-bisphosphate (PIP2) and annexin 2 are present in GVD bodies [11]. Membrane lipid rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes [12] and those in postsynaptic spines are regarded as major sites of signal transduction, membrane trafficking and molecular sorting [13, 14].

Previously, we demonstrated that GVD bodies in neurons and RVs in muscle cells contain a number of common proteins, including charged multivesicular body protein 2B (CHMP2B), caspase 3, flotillin-1, annexin 2, leucine-rich repeat kinase 2 (LRRK2), CDK5 and CK1δ [15]. CHMP2B, a subunit of the endosomal sorting complex required for transport (ESCRT) complex-III, is important for the formation of multivesicular bodies and subsequent late endosomes [16]. The presence of CHMP2B in both RVs and GVD bodies [15, 17], indicates that plasma membranes might be endocytosed to form these structures. Provided that raft domains on the plasma membrane were endocytosed and formed RVs in muscle cells and GVD bodies in neurons, the origins of these structures might be similar functionally and structurally in both of these cell

types.

In the present study, as such candidate lipid rafts structures we focused on both neuromuscular junctions (NMJ) in the muscle for RVs and postsynaptic spine in the brain for GVD bodies. We aimed to examine the biochemical similarities between RVs and GVD bodies by immunolabeling using antibodies for molecules that are present not only in lipid rafts but also in the NMJ and the postsynaptic spine.

2. Materials and methods

We performed immunohistochemical and immunofluorescent staining procedure as previously described [15]. Ethics statement and detailed processes are described in Supplementary data.

3. Results

3.1. RVs are immunopositive for molecules located in the NMJ

In all cases of s-IBM, RVs were immunopositive for Dvl3, Hsp70, β-catenin, APC, PIP2, prion and GSK-3β (Fig. 1). Rapsyn was detected in RVs of six, but not all, s-IBM cases

(Fig. 1). β-catenin, PIP2 and GSK-3β were also present in some nuclei. Dvl3 and rapsyn were diffusely distributed in the cytoplasm of RV-positive fibers, as well as on RVs. In comparison, Dvl1, Dvl2, Lrp4, phospho-β-catenin, PAK1 and Arf6 were not detected in RVs of any s-IBM cases (data not shown). The cytoplasmic membranes, but not RVs, were positive for Lrp4 in all s-IBM cases (data not shown). A summary of immunostaining results is shown in Table 1.

3.2. GVD bodies are immunopositive for molecules located in the NMJ

In GVD bodies, Dvl3, phospho-β-catenin, rapsyn, APC and PIP2 were detected (Fig. 2). In addition, the signals for these antigens were diffusely detected in the cytoplasm of pyramidal cells. Dvl1, Dvl2, Lrp4, Hsp70, β-catenin, PAK1, Arf6 and prion were not present in GVD bodies (data not shown). Dvl1, Dvl2, Lrp4, Hsp70, PAK1, Arf6 and prion were diffusely detected in the cytoplasm of pyramidal cells, while β-catenin was not detected in these cells (data not shown). We also examined immunoreactivity for synaptophysin, a presynaptic marker, and postsynaptic density protein 95 (PSD95), a postsynaptic marker, to test whether GVD bodies contain presynaptic or postsynaptic components. Neither synaptophysin nor PSD95 were detected in GVD bodies (data not

shown). A summary of immunostaining results is shown in Table 1.

3.3. pTDP43 or CHMP2B colocalizes with all of the antigens present in RVs and CK1δ colocalizes with all of the antigens present in GVD bodies

We performed double immunofluorescence staining using anti-pTDP43 or anti-CHMP2B antibody (markers of RVs) and anti-CK1δ (a marker of GVD bodies) along with antibodies to the antigens detected in RVs and/or GVD bodies. pTDP43 or CHMP2B colocalized with all of the antigens detected in RVs, and CK1δ colocalized with all of the antigens detected in GVD bodies (Fig. 3). Aside from the staining in RVs and GVD bodies, there were no differences in the intensity of cytoplasmic immunostaining for each antigen examined regardless of the presence or absence of RVs or GVD bodies. For optimum visualization of the relationship, images are pseudocolored red (568 nm, for CHMP2B) or green (488 nm, for Hsp70, PIP2 and prion) in (f), (g) and (h), and pseudocolored red (568 nm, for CK1δ) or green (488 nm, for p-β-cat) in (B) in Fig. 3.

4. Discussion

We speculated that RVs in muscle were the counterpart to GVD bodies in the brain [15] because both are considered autophagic vacuoles [1, 18]. Accordingly, we focused on the molecules present in both the NMJ and postsynapse to determine if RVs are derived from the NMJ and GVD bodies from postsynaptic components, particularly because of their structural and functional similarities.

PIP2, a phosphatidylinositol, is a lipid component of rafts [19] and plays an important role in membrane trafficking [20]. In this study, we demonstrated that RVs and GVD bodies are immunopositive for PIP2. We also previously reported that both RVs and GVD bodies contain lipid rafts components, such as flotillin-1, annexin 2 and LRRK2 [11, 15]. These results suggest that RVs and GVD bodies are both derived from raft-associated structures on the plasma membrane.

Numerous studies indicate that lipid rafts are sites of AChR clustering at the NMJ [21-23]. Dvl, an important mediator downstream of Wnt receptors [24], regulates AChR clustering [25]. Hsp70 [26, 27], β-catenin [28], rapsyn [28, 29] and APC [30] are also involved in AChR clustering at the NMJ through the Wnt signaling pathway downstream of Dvl. For example, AChR clustering is regulated by Hsp70 and/or APC, that stabilized by rapsyn through the suppression of calpain activity [28-30]. Recently,

Aittaleb et al. reported that rapsyn plays a role in the localization of lysosomes, independent of AChR clustering [31], suggesting the alternative possibility that the localization of rapsyn in RVs and GVD bodies is related to lysosomes. The presence of these molecules in RVs suggests that NMJ components are involved in the formation of RVs.

Lipid rafts are abundant in the dendritic spines of cultured hippocampal neurons, and the formation of dendritic spines is also regulated by the Wnt signaling pathway [32, 33]. Dvl3, rapsyn, APC and GSK-3β are present not only in RVs, but also in GVD bodies; moreover, we showed that GVD bodies are positive for phospho-β-catenin, consistent with the observation by Ghanevati et al. [34]. Conversely, we previously reported that several molecular markers of GVD bodies, including CDK5, CK1δ and JNK, are also present in RVs [10]. CK1 increases the phosphorylation of Dvl and transduces Wnt signals [35], and JNK regulates dendritic development through the Wnt signaling pathway [36]. The finding that RVs and GVD bodies have common molecular components supports our hypothesis that the generations of these pathologies are both related to the Wnt signaling pathway. Indeed, a recent study suggests that Aβ binds to the Wnt receptor and inhibits Wnt/β-catenin signaling [37]. These findings are in line

with previous studies [38], although clear relationships between Aβ and Wnt signaling were not shown in *Drosophila* models of AD [39].

On the assumption that GVD bodies are derived from the postsynapse, we first examined the presence of synaptophysin, a presynaptic marker, in GVD bodies to exclude the presence of presynaptic components in these structures. The lack of immunoreactivity for synaptophysin suggests that GVD bodies are unlikely to be derived from presynaptic components. Second, we examined GVD bodies for the presence of PSD95, a postsynaptic marker, to demonstrate the presence of postsynaptic components in GVD bodies; however this experiment was inconclusive. Although our results did not show evidence for postsynaptic components in GVD bodies, it is interesting that the synaptic components, nicotinic acetylcholine receptor and rapsyn (previously called protein 43 Kd), are strongly accumulated in RVs [40] and, therefore, indicate a possible origin of RVs. As a control, we also tested for a protein that is absent from the postsynaptic spine and the NMJ, Arf6, a master regulator of PIP2 generation [41]. Arf6 was not detected in RVs or GVD bodies.

Hsp70 and prion were present in RVs, but not in GVD bodies. The Hsp70 family encompasses at least 11 genes and various isoforms [42]. Therefore, the anti-Hsp70

antibody we used might not detect the isoform of Hsp70 present in GVD bodies. We examined for the presence of prion protein in GVD bodies because it is enriched in postsynaptic densities, and because the AD brain harbors prion-binding Aβ species [43]. We showed that the prion protein was present in RVs as previously reported [44], but was undetectable in GVD bodies. In addition, we showed that RVs are positive for β-catenin, and GVD bodies are positive for phospho-β-catenin, as previously reported [34], but phospho-β-catenin was undetectable in RVs and β-catenin was undetectable in GVD bodies. These discrepancies might stem from the difference in the specimens, i.e., frozen section from muscle samples and paraffinized sections from brains. It is noteworthy that phospho- β-catenin, which is designated for proteasomal degradation in the canonical Wnt pathway, escaped degradation and was accumulated in GVD bodies. Recent studies demonstrated that phospho-β-catenin can accumulate in multivesicular bodies in response to Wnt3a by the mechanism termed Wnt-dependent stabilization of proteins (Wnt/STOP) [45-47]. GVD bodies are thought to be related to late-stage autophagic organelles [18]; therefore, the accumulation of phospho-β-catenin in GVD bodies might occur in parallel to that in multivesicular bodies via Wnt/STOP suggesting that Wnt/STOP plays a role in the pathogenesis of AD. The model depicted in

Supplementary figure 1 depicts how phospho-β-catenin and the other molecules associated with Wnt signaling could accumulate in GVD bodies as autophagic vacuoles.

A limitation of this study is that immunohistochemistry was performed for a small number of antigens on only two AD cases. In addition, we investigated RVs only in s-IBM cases and GVD bodies only in the AD brain. Further studies that compare RVs in other muscle diseases with GVD bodies in other tauopathies are required to confirm and expand our current results. The current study supports our previous findings that RVs and GVD bodies share a number of molecules [15]. In conclusion, our findings suggest that RVs and GVD bodies share common pathogenic mechanisms that are associated with the Wnt signaling pathway.

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2. Materials and methods

2.1. Ethics statement

The neuropathological procedures and analyses were performed according to the guidelines of the ethics committee of Hiroshima University, Graduate School of Biomedical and Health Sciences. Samples were donated with written informed consent of patients, except for autopsy cases. Autopsy cases samples were obtained with written informed consent of family members. All data were analyzed anonymously, and neuropathological procedures and analyses were performed according to the principles of the Declaration of Helsinki.

2.2. Samples

Brain tissue samples were obtained post-autopsy from two cases with AD. Both cases fulfilled the National Institute on Aging-Alzheimer's Association guidelines for the neuropathological assessment of AD [1]. After autopsy, the medial temporal lobe, including the hippocampus, was fixed in 10% formalin for 3 weeks. Paraffin-embedded sections (7 μm thick) were then immunostained. Muscle biopsy specimens were obtained from seven cases of s-IBM that fulfilled clinical and histopathological

diagnostic criteria, as previously described [2]. These specimens were immediately frozen in isopentane cooled with liquid nitrogen and sliced into 7-μm-thick sections. The clinical features of all cases are summarized in Supplementary table 1.

2.3. Antibodies

We selected components present in the NMJ, postsynapse and lipid rafts. Antibodies against the following factors were used: (1) dishevelled (Dvl) family proteins (Dvl1, Dvl2 and Dvl3), (2) NMJ-associated proteins (low density lipoprotein-related protein 4 [Lrp4], heat shock protein 70 [Hsp70], β-catenin, phospho-β-catenin, rapsyn, P21-activated kinase 1 [PAK1], adenomatous polyposis coli [APC] and ADP-ribosylation factor 6 [Arf6]), (3) a lipid raft-associated molecule (PIP2), and (4) other proteins (prion and GSK-3β). The subclass of all antibodies was IgG, except for the anti-PIP2 antibody, which was an IgM. The antibodies used for immunohistochemistry are shown in Supplementary table 2.

2.4. Immunohistochemistry

Hippocampal tissue specimens were deparaffinized and dehydrated. For antigen

retrieval, slides were immersed in a staining dish containing citrate buffer heated to 95–100°C and incubated for 40 min. After cooling to room temperature, the slides were washed three times in phosphate-buffered saline (PBS). Slides were then incubated with 3% H_2O_2 in PBS for 30 min to eliminate endogenous peroxidase activity, and washed once in PBS. The tissue sections were incubated with blocking solution (Protein Block Serum-Free; Dako, Glostrup, Denmark) for 15 min to reduce non-specific staining. Each section was incubated with primary antibodies overnight at 4°C. Following three washes in PBS, sections were incubated with HRP-labeled anti-mouse or anti-rabbit secondary antibody (EnVision+ System- HRP Labelled Polymer; Dako) for 30 min. Sections were then washed in PBS three times and incubated with 3, 3'-diaminobenzidine (DAB; Dako). All sections were counterstained with hematoxylin.

Muscle tissue specimens were fixed in 4% formaldehyde in PBS (pH 7.4) for 30 min and washed with PBS. The tissue sections were incubated with blocking solution for 15 min. Each section was then incubated with primary antibodies overnight at 4°C and washed in PBS. Sections were incubated with the HRP-labeled anti-mouse or anti-rabbit secondary antibody for 30 min. After washing in PBS, the sections were incubated with DAB, and then counterstained with hematoxylin. All experiments were

conducted at room temperature, unless indicated otherwise.

2.5. Immunofluorescence staining

Double staining was performed on sections from the hippocampus and muscles. Paraffin-embedded sections were irradiated with UV light overnight at 4°C to reduce autofluorescence. We employed the same primary and secondary antibodies as described above. The primary antibodies were detected using the tyramide signal amplification (TSA®) method with the same HRP conjugated secondary antibodies as described above together with the TSA® KIT #12 with HRP-goat anti-rabbit IgG and Alexa Fluor 488 tyramide, and TSA® KIT #4 with HRP-goat anti-mouse IgG and Alexa Fluor 568 tyramide (Invitrogen, Eugene, OR). Anti-CHMP2B (ab33174, 1:100, Abcam, Cambridge, UK), anti-phosphorylated TAR DNA-binding protein 43 (pTDP43) (CAC-TIP-PTD-M01, 1:100, Cosmo Bio, Carlsbad, CA) and anti-CK1δ (ab85320, 1:100, Abcam) antibodies were detected using Alexa Fluor 488-goat anti-rabbit IgG, Alexa Fluor 488-goat anti-mouse IgG, or Alexa Fluor 568-goat anti-mouse IgG (All 1:1000). Fluorescence images were acquired with a LSM510 confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany).

Case No.	Diagnosis	Sex	Age
sI-1	s-IBM	M	43
sI-2	s-IBM	M	51
sI-3	s-IBM	M	85
sI-4	s-IBM	M	57
sI-5	s-IBM	M	76
sI-6	s-IBM	F	89
sI-7	s-IBM	F	78
AD-1	AD	M	73
AD-2	AD	M	88

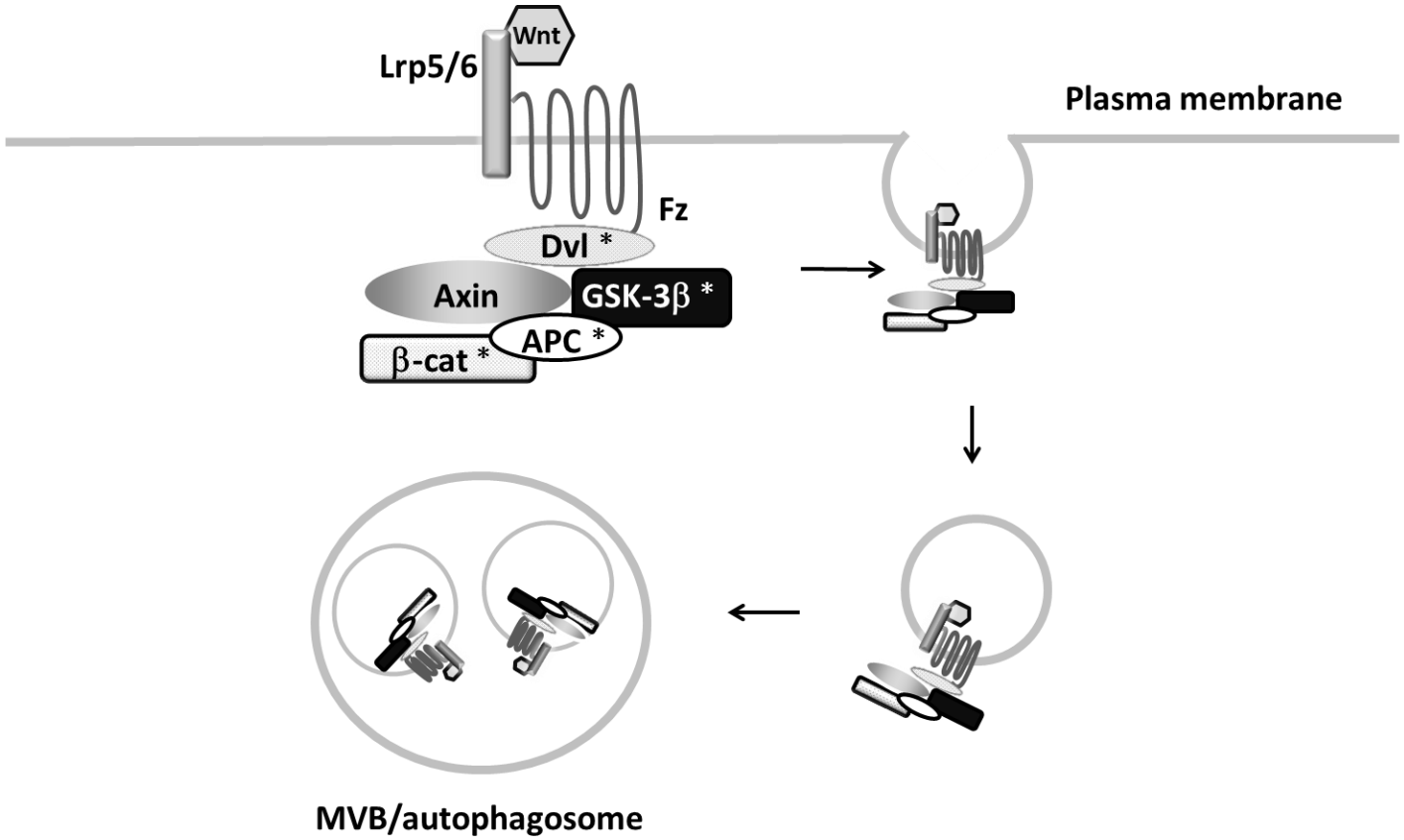
Supplementary table 1. Subject characteristics.

F, female; M, male.

Antigen	Animal	Clone	Source	Dilution		Dilution	
				DAB stain		Immunofluorescence stain	
				Muscle	Brain	Muscle	Brain
Dvl1	mouse	monoclonal	Santa Cruz biotechnology	50	50	N/A	N/A
Dvl2	rabbit	polyclonal	Sigma-Aldrich	100	100	N/A	N/A
Dvl3	rabbit	polyclonal	Sigma-Aldrich	500	100	100	500
Lrp4	rabbit	polyclonal	Merck Millipore	100	100	N/A	N/A
Hsp70	mouse	polyclonal	Transduction Laboratories	100	100	100	N/A
β-catenin	rabbit	monoclonal	Abcam	100	100	100	N/A
p-β-catenin	rabbit	polyclonal	Cell Signaling Technology	100	100	N/A	100
rapsyn	rabbit	polyclonal	Novus Biologicals	100	100	100	100
PAK1	rabbit	polyclonal	Cell Signaling Technology	100	100	N/A	N/A
APC	rabbit	polyclonal	Proteintech	100	100	100	500
Arf6	rabbit	polyclonal	Abcam	100	100	N/A	N/A
PIP2	mouse	monoclonal	Santa Cruz biotechnology	100	100	100	N/A
prion	mouse	monoclonal	Millipore	100	100	100	N/A
GSK3-b	rabbit	monoclonal	Abcam	100	N/A	100	N/A
synaptophysin	rabbit	monoclonal	Epitomics	N/A	100	N/A	N/A
PSD95	rabbit	polyclonal	Abcam	N/A	800	N/A	N/A

Supplementary table 2. List of primary antibodies.

N/A, not assessed.



Supplementary figure 1. Wnt/STOP signaling pathway. In Wnt/STOP pathway, upon Wnt signaling, Dvl and destruction complex components including β-catenin, APC, axin and GSK-3β are sorted into MVB/autophagosome and could be accumulated in GVD bodies as autophagic vacuoles. Wnt/STOP, Wnt-dependent stabilization of proteins; MVB, multivesicular body. Asterisks; the molecules shown to be present in both GVD bodies and RVs in the current study.

Supplementary References

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