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Snapshots from within the cell: Novel trafficking and non trafficking functions of Snap29 during tissue morphogenesis

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ABSTRACT

Membrane trafficking is a core cellular process that supports diversification of cell shapes and behaviors relevant to morphogenesis during development and in adult organisms. However, how precisely trafficking components regulate specific differentiation programs is incompletely understood. Snap29 is a multifaceted Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor, involved in a wide range of trafficking and non-trafficking processes in most cells. A body of knowledge, accrued over more than two decades since its discovery, reveals that Snap29 is essential for establishing and maintaining the operation of a number of cellular events that support cell polarity and signaling. In this review, we first summarize established functions of Snap29 and then we focus on novel ones in the context of autophagy, Golgi trafficking and vesicle fusion at the plasma membrane, as well as on non-trafficking activities of Snap29. We further describe emerging evidence regarding the compartmentalisation and regulation of Snap29. Finally, we explore how the loss of distinct functions of human Snap29 may lead to the clinical manifestations of congenital disorders such as CEDNIK syndrome and how altered SNAP29 activity may contribute to the pathogenesis of cancer, viral infection and neurodegenerative diseases.

1. Introduction

Morphogenesis, which shapes tissues and organs throughout development, relies on both cell polarity and signaling. Cell polarity refers to the structural and functional asymmetry within cells while cell signaling to the constant communication between cells. Together, these processes enable the coordination of cell proliferation, differentiation and migration during development.

A major cellular process contributing to the support of polarity and signaling is membrane trafficking. In fact, both require spatially-regulated endocytosis (vesicle internalization from the plasma membrane) and exocytosis (vesicle delivery to the plasma membrane). Common to endocytosis and exocytosis are 3 key steps: vesicle budding from donor membranes, vesicle transport and fusion of the vesicles to target membranes. Tight regulation of these steps contributes to the multi-layered specificity of membrane trafficking and consequently enables highly asymmetric distribution of macromolecules – a requirement to establish and maintain cell polarity and to enable correct signaling [1].

Regulation of trafficking is broadly imparted by the concerted actions of several regulatory molecules acting at membranes, including motor protein adapters, coat proteins, Rab GTPases, phosphoinositides, tethering factors and fusion proteins, such as Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptors (SNAREs). SNAREs are small (100–300 amino acids), elongated proteins, composed of at least one alpha-helical SNARE domain. The presence of a glutamine (Q) or arginine (R) residue at the 'zero-ionic layer' within the otherwise hydrophobic SNARE domains, defines their classification as either Q- or R-SNARE [2]. Generally, functional SNARE complexes observed *in vivo* are composed of three Q-SNARE domains (Qa, Qb and Qc) that function as a receptor for a single R-SNARE domain. These Q- and R-SNARE domains self-assemble into a stable four helix bundle [3]. As Q-SNAREs are

Abbreviations: AC, anchor cell; CEDNIK, Cerebral Dysgenesis, Neuropathy, Ichthyosis, and Keratoderma; COG, conserved oligomeric Golgi; CTPsyn, cytidine triphosphate synthetase; ER, Endoplasmic Reticulum; HOPS complex, homotypic fusion and protein sorting complex; MEDNIK, mental retardation, enteropathy, deafness, neuropathy, ichthyosis, and keratodermia; NEK3, NIMA-never in mitosis gene A-related kinase 3; NEM, N-ethylmaleimide; NF-κB, Nuclear Factor κB; NSF, N-ethylmaleimide-sensitive fusion protein; O-GlcNAc, O-linked β-N-acetylglucosamine; PMLD, Pelizaeus-Merzbacher-like disease; PrPC, cellular prion protein; SNARE, Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor; TNFα, tumour necrosis factor α.

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generally associated to the target membrane and R-SNAREs to the vesicle primed for fusion, they are also commonly referred to as t-SNARE (target) and v-SNARE (vesicle). Following the tethering of a vesicle to a target membrane, distinct sets of SNAREs assemble into a trans-SNARE complex across opposing membranes, bringing them into close proximity for fusion. The energy released by the formation of the trans-SNARE complex overcomes the energy barrier required to fuse the vesicle to the target membrane [4,5]. Once fused, the now cis-SNARE complex on the target membrane is disassembled by the concerted action of the ATPase N-ethylmaleimide-sensitive fusion protein (NSF), and of the attachment factor α-SNAP. This step allows regeneration of t-SNAREs and budding of v-SNAREs, in essence allowing recycling of the fusion machinery [6].

As part of the t-SNARE complex, Qb- and Qc- SNARE domains are peculiar in that they can be separated into two proteins or be part of a single protein, referred to as Qbc-SNAREs. In mammals, Qbc-SNAREs include Snap25 [7], Snap23 [8], Snap29 [9,10] and Snap47 [11]. While other SNAREs are bound to certain vesicles or target membranes by means of transmembrane domains or protein modifications, Snap29 and Snap47 are not stably associated with membranes, making it more challenging to determine their involvement in specific cellular pathways [9–11]. Differently from its paralogs, Snap29 contains an N-terminal acidic Asn-Pro-Phe (NPF) motif, involved in endocytic trafficking, and a linker between the Qb and Qc SNARE domains that diverges from that of Snap25, Snap23 and Snap47 [9,12]. While Snap47 appeared only in metazoans, Snap29 is present in all plant and animal cells [13]. To date, Snap47 functions have not been extensively investigated, as is the case for Snap29 activity in plant cells. In contrast, a number of studies have revealed a multitude of Snap29 functions in animal cells that impact on cell polarity, proliferation and differentiation. It is now clear that Snap29 supports cellular health by modulating a number of trafficking pathways, including macroautophagy (autophagy hereafter), endocytic trafficking and secretion. Surprisingly, Snap29 appears to also mediate trafficking-independent cellular functions, sometimes referred to as moonlighting functions, that are only partly understood. We have recently extensively discussed trafficking and non trafficking functions of Snap29 [14]. However, in the last 3 years new evidence has emerged regarding the functions and the regulation of Snap29 activity that is relevant to the regulation of animal morphogenesis. In this review, we first summarize established knowledge about Snap29. We then focus on selected, recently discovered aspects of Snap29 function and finally we discuss their impact on morphogenesis in the context of human disease.

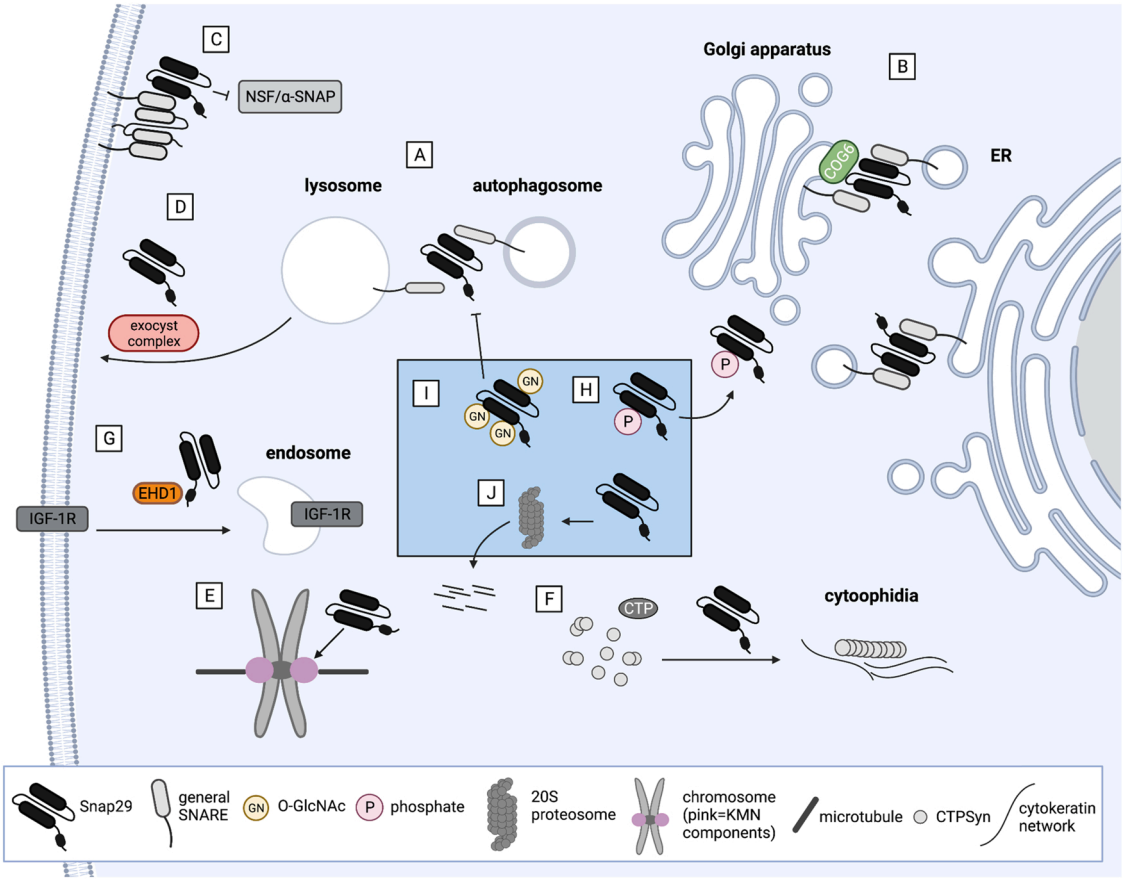

Fig. 1. Function and regulation of Snap29 in trafficking and non-trafficking processes. (A) Snap29 acts in association with Syntaxin17 (Syx17) and Vamp8 or Syntaxin7 and Ykt6 to mediate autophagosome-lysosome fusion. (B) At the Golgi, Snap29 interacts with the SNAREs Syntaxin 5 (Syx5) and Sec22b and with the conserved oligomeric Golgi 6 (COG6) complex to support anterograde transport. At the ER, Snap29 interacts with Syntaxin18 (Syx18) and Sec22b to promote retrograde transport. (C) Snap29 interacts with the quaternary cis-SNARE complex Syx1A-Snap25-Vamp2 at the plasma membrane to inhibit NSF/α-SNAP-mediated dissociation. (D) In cooperation with the exocyst complex, Snap29 mediates the fusion of lysosomes to the plasma membrane to mediate lipid delivery for membrane expansion. (E) Snap29 is recruited to the outer portion of the kinetochore during cell division to ensure correct kinetochore formation. (F) SNAP29 interacts with KRT8 to support the formation of CTPsyn filaments (cytoophidia). (G) The NPF motif of Snap29 interacts with EH domain-containing proteins to mediate endocytic trafficking, such as by interacting with EHD1 to mediate the recycling of IGF-1 receptor (IGF-1R). (H) Snap29 phosphorylation is required for its localisation at the Golgi. (I) Snap29 is inhibited by O-GlcNAcylation (GN) leading to inhibited autophagosome-lysosome fusion. (J) Snap29 is targeted by ubiquitin-independent 20 S proteasome degradation.

2. Functions and regulation of Snap29

2.1. Autophagic degradation

The trafficking functions of Snap29 are well characterized in the context of autophagy, a homeostatic process initiated by various stimuli, whereby organelles, long-lived cytoplasmic proteins and other cellular catabolites are sequestered in autophagosomes and are eventually degraded in lysosomes, so that the molecular components can be recycled and made available for anabolic processes (Fig. 1A). It is now well known by studies in human and *Drosophila melanogaster* cells that Snap29 can be recruited to the mature autophagosome by the Qa SNAREs syntaxin 17 (human STX17 or *Drosophila* Syx17) to promote fusion with lysosomes exposing the V-SNARE VAMP8 (Vamp7 in *Drosophila*) [15–17]. Indeed, a loss of functional Snap29 *in vivo* results in an autophagy block with accumulation of autophagosomes in *Drosophila* [17], *Danio rerio* [18] and *Mus musculus* [19]. Snap29-mediated autophagosome-lysosome fusion additionally involves the homotypic fusion and protein sorting (HOPs) complex [20,21], Rab7 [22] and EPG5 [23]. Recently, it was further discovered that in human cells SNAP29 can also act with the R-SNARE YKT6 [24]. STX17-SNAP29 and YKT6-SNAP29 can alternatively interact with the lysosome-bound R-SNAREs VAMP8 or with the Qa-SNARE STX7, respectively, to form trans-SNARE complexes that mediate fusion of autophagosomes with lysosomes [24]. A parallel study in *Drosophila* indicates that Ykt6-carrying vesicles might interact with Syx17 and Snap29 as part of a stabilization/tethering regulatory step involving the HOPS complex. Indeed, a form of Ykt6 that should not be able to form a correct four helix bundle because it carries a Q instead of R in the zero-ionic layer is able to support fusion [25]. Future work is required to determine whether *Drosophila* and human cells differ in the involvement of Ykt6 in autophagosome to lysosome fusion.

2.2. ER and Golgi trafficking

Snap29 also appears to act directly during endoplasmic reticulum (ER) and Golgi trafficking (Fig. 1B). Early evidence in human fibroblasts indicated that, in absence of SNAP29, the architecture of the Golgi apparatus complex is altered, in that cisternae are often fragmented and disorganized [26]. Golgi apparatus alterations have been observed in *Caenorhabditis elegans* [27], *Drosophila* [17] and Zebrafish [18] mutants for Snap29. Building on these findings, Morelli and coworkers recently confirmed previous observation in HeLa cells, and showed that sub-stoichiometric amounts of SNAP29 can be immunoprecipitated with the ER Qa-SNAREs syntaxin 18 (STX18) and the Golgi apparatus Qa-SNARE syntaxin5 (STX5), as well as with the ER R-SNARE SEC22B. Interaction with syntaxin18 and Sec22 appears conserved as it is also observed in *Drosophila* cells. Authors also reported that ectopic expression of SNAP29 mutants with Q to A substitutions at the Qb and Qc residues, predicted to block dissociation from partner SNAREs, recapitulates the loss of Golgi apparatus integrity. Interestingly, it also decreases the association of SNAP29 with SEC22B but not with STX18. In contrast, SNAP29 depletion strongly reduces interaction of SEC22B with STX18 [28]. Together, these data indicate that SNAP29 is part of t-SNARE complexes at the ER and Golgi apparatus. Specifically, the loss of interaction with SEC22B in Q to A mutant forms suggests the existence of a regulatory, rather than fusogenic, role of SNAP29 in trafficking at the ER and Golgi apparatus.

2.3. Secretion at the plasma membrane

In neurons, Snap29 is likely to play a regulatory role at the plasma membrane (Fig. 1C). Fusion of synaptic vesicles to the plasma membrane is mediated by the well-established SNARE complex composed of the Qa-SNARE Syntaxin1A (Syx1A), the membrane associated Qb-Qc-SNARE Snap25 and the R-SNARE Vamp2. To recycle SNAREs and enable fast and efficient fusion cycles, the cis-SNARE complex on the plasma membrane must be dissociated rapidly by the concerted action of α-SNAP and NSF. In this context, Snap29 was found to reduce binding of α-SNAP to the Syx1A-Snap25-Vamp2 complex, without affecting its assembly, ultimately inhibiting synaptic transmission between superior cervical ganglion neurons and between rat hippocampal neurons in culture. Thus, it appeares likely that Snap29 negatively regulates synaptic transmission by competing with α-SNAP for the cis-SNARE complex at the plasma membrane [29,30]. Such findings hinted for the first time at the possibility that Snap29 might regulate, rather than promote, membrane fusion.

A further role of Snap29 at the plasma membrane was identified during vulval development in *Caenorhabditis elegans* (Fig. 1D). A previous study demonstrated that uterine anchor cells (ACs) initially invade the basement membrane, which separates the uterine and vulva tissues, by forming F-actin-rich, invadopodia-like structures [31]. This is followed by the formation of a single invasive protrusion that pushes the basement membrane aside to generate the uterine-vulval connection [32]. Implicating Snap29 in this process, the same group later demonstrated that Snap29 mediates the fusion of lysosomes to the AC plasma membrane, thereby delivering new membrane material to support the growth of the invasive protrusion. Indeed, a uterine-specific knockdown of Snap29 prevents increases in AC size and reduces the invasion protrusion growth rate. The fusion of lysosomes to the plasma membrane, and subsequent protrusion growth, was further shown to require components of the exocyst complex [33]. How the exocyst complex and Snap29 cooperate to mediate membrane fusion at the plasma membrane, and whether other tethering factors or SNAREs are involved, requires further investigation.

2.4. Non-trafficking functions

Our group unexpectedly reported in 2016 that SNAP29 in human cells and in *Drosophila* can also act independently of membranes. In fact, we found the presence of Snap29 on *Drosophila* chromosomes during cell division. Loss of function analyses indicated that Snap29 supports the formation of the outer part of the kinetochore, the multi protein structure that tethers mitotic chromosomes to spindle microtubules [34] (Fig. 1E).

Surprisingly, a new trafficking-independent, moonlighting function of Snap29 emerged recently in human HEp-2 cells in culture: recent data from Chakraborty et al. demonstrates that histidine-induced formation of cytoophidia along the cytokeratin network is reduced in SNAP29 knockdown human HEp-2 cells [35]. Cytoophidia are cytoplasmic polymers of the cytidine triphosphate synthetase (CTPsyn) enzyme that are found in bacteria, flies, yeast and mammalian cells. CTPsyn is a homotetrameric enzyme that catalyzes the rate limiting step in *de novo* synthesis of CTP, which is essential for DNA, RNA, and phospholipid synthesis [36–38]. Proximity and co-immunoprecipitation assays in human HEp-2 cells suggested that both CTPsyn1 and SNAP29 associate directly with the intermediate filament protein Keratin Type II, Cytoskeletal 8 (KRT8) at the cytokeratin network. While initial reports from bacteria suggested that cytoophidia self-assemble to inhibit CTPsyn catalytic activity [38,39], their regulation and function in eukaryotes now appears more nuanced. Inhibition of the mTOR pathway, for instance, appears to reduce the formation of cytoophidia in yeast, *Drosophila* and mammalian cells [40,41]. Additionally, nutrient starvation induces cytoophia formation in yeast, *Drosophila* and human cell lines [37,42–44]. While the mechanisms underlying starvation-induced cytoophidia formation remain unclear, histidine-mediated methylation of human CTPsyn1 appears to be required for cytoophidia polymerization in glutamine-depleted HEp-2 cells. Treatment of histidine-induced HEp-2 cells with the SNARE complex disassembly inhibitor, NEM, results in increased SNAP29-KRT8 association and concomitant fragmentation of CTPsyn1 filaments. It is important to note that CTPsyn1 localisation at the cytokeratin, and the structure of the cytokeratin

network, remain unchanged by SNAP29 knockdown or N-ethylmaleimide (NEM) treatment [44]. Taken together, these data suggest that reduced cytoplasmic SNAP29 – either due to genetic knockdown or impaired recycling – impairs CTPsyn1 filament formation during nutrient starvation, though not its polarized localisation to the cytokeratin network (Fig. 1F).

2.5. Regulation of localization

Given the multitude of trafficking and non trafficking activities of Snap29, how Snap29 might gain association from the cytoplasm to membranes of specific compartments or to non membrane-associated partners is still mostly unclear. Several lines of evidence suggest that Snap29 may interact with a number of non-SNARE partners to localize to specific cellular compartments. For instance, initial evidence highlighting the activity of Snap29 in endocytic recycling of transferrin receptors and integrin indicated that one mode of association with partners is the interaction of the acidic NPF motif of Snap29 with EH domain-containing proteins [12,26,45]. Such mode of recruitment appears at play also for IGF-1 receptor trafficking and during ciliogenesis [46,47] (Fig. 1G). Consistent with the fact that only part of the function of Snap29 might be regulated by NPF-dependent localization, a form of Snap29 lacking the NPF motif can partially rescue organ formation in *Drosophila* [17].

A second modality of recruitment is likely to occur at the Golgi apparatus, mediated by conserved oligomeric Golgi (COG) tethering complexes. In fact, in human cells SNAP29 has been found to interact with COG6 by yeast two hybrid and immunoprecipitation [48,49] (Fig. 1B). Which portion of SNAP29 interacts with COG6 has not yet been determined, however the interaction is likely to be direct at least in the artificial setup of a two hybrid experiment. Because the SNARE domains of SNAP29 are engaged in the four helix bundle during fusion events during ER and Golgi trafficking, it is likely that either the external surface of the SNARE domains, or the linker domains might be responsible for interaction with COG complexes. Indicating the former may be the case, *in vitro* binding assays with the yeast COG complex suggest that it preferentially interacts with the complete, quaternary SNARE complex, rather than with isolated SNARE domains or binary or ternary SNARE complexes [50].

Finally, Snap29 recruitment to the outer portion of the kinetochore during cell division is supported by interaction with components of the KMN network (formed by the Knl1, Mis12 and Ndc80 complexes) and depends on the first SNARE domain in the wing disc of developing *Drosophila* larvae (Fig. 1E). In human cells, either SNARE domain of SNAP29 appears to stabilize association of the Knl1 complex with the more internal Mis12 complex. In addition, SNAP29 forms with Q to A substitutions at the Qb and Qc residues, predicted to block dissociation from partner SNAREs, ectopically recruit KNL1 [34]. Whether these interactions are direct remains to be tested, however, it is known that portions of the Mis12 as well as of the Knl1 complexes contain coiled-coil repeats that could interact directly with Snap29 [51–54]. The ability of KNL1 to interact with a SNAP29 that cannot be released from SNARE complexes, suggest that the interaction of KNL1 with SNAP29 might occur on the side of the SNARE domain that is not engaged in the SNARE bundle and/or parts of the linker domain.

2.6. Regulation of activity

Beyond its interaction with several non-SNARE partners, Snap29 also appears to be regulated by post-translational modifications. Recently, membrane association of SNAP29 at the Golgi apparatus has been demonstrated to depend on phosphorylation of S105 in the first SNARE domain by the kinase NEK3 (NIMA-never in mitosis gene A-related kinase 3) (Fig. 1H). SNAP29-defective cells rescued with a phosphomutant SNAP29 present altered Golgi apparatus architecture as well as integrin-mediated focal adhesion defects suggesting that such mode of regulation might affect not only ER-Golgi transport but also endocytic recycling [55].

In the context of autophagy, Snap29 appears to be modified by the addition of an O-linked β-N-acetylglucosamine (O-GlcNAc), in both human and *Caenorhabditis elegans* cells [56–58] (Fig. 1I). Such modification, occurring at 3 Ser and 1 Thr residues (not conserved among human and *Caenorhabditis elegans* Snap29), reduces interaction with Syx17 and subsequent formation of the Syx17-Snap29-Vamp7/8 SNARE complex. High levels of O-GlcNAc Snap29 are observed in high nutrient conditions in which mTOR is active, while low levels are found in starving cells that need to extract nutrients from autophagic degradation [57]. In addition, treatment of primary neonatal rat cardiomyocytes with high glucose results in Snap29 O-GlcNAcylation and reduces autophagic flux [58]. Similarly, autophagic flux in *Caenorhabditis elegans* is increased by expression of an O-GlcNAc-defective Snap29 mutant. Strikingly, in SARS-CoV2 infected HeLa cells, wherein STX17 expression is reduced, inhibition of SNAP29 O-GlcNAcylation rescues the autophagic flux [59].

Finally, both SNAP29 and STX17 appear to be targets for ubiquitin-independent 20 S proteasome degradation: stimulation of the 20 S proteasome by TCH-165 treatment in HeLa cells reduces both SNAP29 and STX17 protein levels and consequently reduces autophagosome-lysosome fusion [60] (Fig. 1J). At present, whether inhibition of SNAP29 activity by O-GlcNAcylation or proteasomal degradation may regulate SNAP29 activity beyond autophagy has not yet been determined.

3. Human SNAP29 and disease

How do the emerging functions and regulations of Snap29 affect morphogenetic processes? A clue comes from the study of human diseases associated with alteration of SNAP29 activity. Interestingly, SNAP29 is the only Qbc-SNARE paralog whose loss is known to cause multisystemic disorders in humans. In fact, SNAP29 causes or contributes to different congenital disorders with partly overlapping clinical manifestations (Table 1). These can be classified into two groups: those caused by single nucleotide substitutions in SNAP29 and those associated to copy number variants of chromosome region 22q11.2 onto which SNAP29 maps in humans. However, how the alteration of the many cellular functions of SNAP29 are associated with the morphogenetic defects observed in humans remain largely unclear.

3.1. CEDNIK

In 2005, Sprecher et al. described a novel neurocutaneous syndrome caused by homozygous nonsense mutations in SNAP29 and characterized predominately by Cerebral Dysgenesis, Neuropathy, Ichthyosis, and Keratoderma (CEDNIK) [61]. Since its initial description, the description of the clinical traits of CEDNIK syndrome have broadened, with the disorder having been diagnosed in 25 patients to date [61–68]. As clearly evaluated in a comprehensive literature review by Mah-Som et al., the clinical manifestations of CEDNIK appear to fall on a spectrum: while a global developmental delay and abnormalities of the corpus callosum of the brain have been observed in 100% and 95% of patients respectively, other traits relating to dermatologic, muscle skeletal, eye and ear, neurologic abnormalities as well as various dysmorphic features, including palmar crease, epicanthal folds and synophrys show varying expressivity [64,68]. Taken together however, these clinical manifestations of CEDNIK syndrome point towards a broad involvement of SNAP29 in tissue development and morphogenesis. Interestingly, Vici Syndrome, a rare congenital disorder caused by loss of function mutations in EPG5, a protein involved in supporting the stabilization of the SNARE complex during autophagosome-lysosome fusion, results in similar clinical phenotypes as those observed in CEDNIK, including cataracts, developmental delay and microcephaly [69]. Such partial overlap of clinical traits between CEDNIK and Vici

Table 1
Congenital disorders associated with SNAP29 mutations.

Disease	Main clinical manifestations	Predicted genetic alteration	Most investigated processes likely affected	Reference
CEDNIK	Cerebral dysgenesis, neuropathy, ichthyosis, and keratoderma	Homozygous mutations in *SNAP29*/ loss of function	Autophagy, Golgi trafficking	[61–68]
PMLD	Cerebral dysgenesis and hypomyelination	Compound heterozygous *SNAP29* mutations/ partial loss of function	Secretion	[100]
DiGeorge/Velocardiofacial syndrome with CEDNIK-like syndrome	Congenital heart disease, palatal abnormalities, immune deficiency and learning difficulties + CEDNIK-associated dermatological abnormalities	22q11.2 deletion with hemizygous mutations in *SNAP29*/Loss of function	Autophagy, golgi trafficking	[89]
Dup22q11 syndrome with ocular manifestations	Marcus Gunn jaw wink phenomenon, tortuous retinal vasculature and macrocephaly	~1.4–2 Mb duplication of chromosome 22q11.2/increase *SNAP29* gene dosage?	Endocytosis	[98]
Schizophrenia	Delusions and hallucinations	Mutation in *SNAP29* promoter region/ unknown	Synaptic signalling	[93–95]

Syndrome, suggest that at least the step of fusion of autophagosomes with lysosomes plays a critical role in morphogenesis during human development.

3.2. SNAP29 and skin development

Despite the heterogeneity of CEDNIK, the most distinctive and best characterized traits of the disease are the ichthyosis and keratoderma - the scaling and thickening of the skin immediately apparent in newborns [61,62]. The human epidermis, comprising 5 morphologically distinct layers, is a paradigm of morphogenesis in that it undergoes continuous differentiation during human development and throughout adulthood [70]. The primary cell type of the epidermis are the keratinocytes, which originate from the skin's deepest layer - the stratum basale - and undergo terminal differentiation and programmed cell death as they migrate up through the layers of the epidermis. This process, referred to as cornification, allows the rapidly lost keratinocytes of the stratum corneum to be continually replaced and forces the skin to undergo constant morphogenesis [71]. Electron microscopy and immunohistochemistry analysis of skin biopsies of CEDNIK patients with dermatological abnormalities, as well as of mouse models of CEDNIK [61,72], showed clear alterations in epidermal morphology, particularly including the thickening of the stratum corneum, referred as hyperkeratosis (Fig. 2A).

To begin to understand how a loss of SNAP29 leads to the skin manifestations of CEDNIK syndrome, the role of autophagy in keratinocyte differentiation should be considered. Several studies have demonstrated that autophagic proteins are required during epidermal differentiation [73–78]. Indeed, the autophagosome marker LC3 is expressed in all layers of the epidermis in healthy individuals, with the densest distribution in the stratum granulosum where keratinocyte terminal differentiation initiates [78]. Corroborating these results, skin grafts of Atg7-deficient mice, which are characterized by acanthosis and hyperkeratosis, show reduced levels of the keratinization-associated proteins loricrin [79]. The specific function of autophagy in relation to keratinocyte differentiation, however, remains elusive. One possibility is that terminally differentiating keratinocytes undergo autophagy induced cell death, whereby cytoplasmic organelles are degraded [78,79] (Fig. 2B). This raises the possibility that a loss of SNAP29 could contribute to impaired autophagic degradation of organelles in keratinocytes (Fig. 2B). Suggesting that this autophagosome-lysosome fusion may be independent of SNAP29, it is important to note that no accumulation of autophagosomes is observed in the skin of CEDNIK patients.

Further consistent with the evidence that autophagy is required for keratinocyte differentiation, calcium chloride-induced differentiation of human primary keratinocytes is accompanied by significant increases in lysosomal biogenesis compared to control, non-treated keratinocytes *in vitro*. The lysosomal vesicles appear to originate from the Golgi. Indeed, brefeldin A treatment – which impairs Golgi function and induces Golgi fragmentation by inhibiting ARF-1 activity – leads to impaired differentiation and lysosomal biogenesis in primary human keratinocytes [80]. Consequently, it is interesting to consider a possible role of Snap29 in Golgi-dependent lysosomal biogenesis in the context of CEDNIK syndrome skin abnormalities (Fig. 2B). Indeed, the possibility that the effects of loss of SNAP29-dependent Golgi trafficking, rather than SNAP29-dependent autophagosome-lysosome fusion, may contribute to the skin defects of CEDNIK patients is supported by the fact that Vici syndrome patients with defective EPG5 show no further dermatologic abnormalities beyond oculocutaneous hypopigmentation [68].

Keratinocyte differentiation is accompanied by maturation and secretion of lamellar bodies [71]. Lamellar bodies are secretory

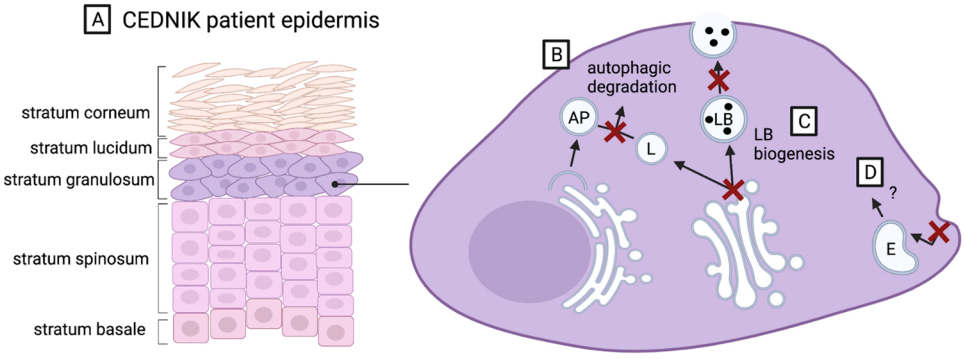

Fig. 2. Possible pathology underlying a loss of SNAP29 in the epidermis of CEDNIK patients. (A) The human epidermis is composed of 5 morphologically distinct layers. CEDNIK patients show increased thickness of the stratum corneum (hyperkeratosis). (B) autophagic degradation of cytoplasmic organelles likely contributes to autophagy induced cell death and is required for terminal differentiation of keratinocytes. In CEDNIK patients, impaired autophagosome (AP)-lysosome (L) fusion and/ or reduced lysosome biogenesis may result in impaired autophagic degradation of organelles. (C) Lamellar bodies (LB) appear to be generated from the Golgi apparatus and are required to secrete essential lipids that make up the lipid matrix of the stratum corneum. The reduced presence of lipids in the stratum corneum of CEDNIK patients may stem from disrupted Golgi trafficking leading to impaired lamellar body biogenesis. (D) Endosomes (E) may play a role in epidermal morphogenesis and thus impaired endocytic trafficking may further contribute to the pathogenesis of CEDNIK.

organelles found predominantly in the stratum granulosum and are responsible for maintaining the skin barrier of the stratum corneum by secreting lipids and proteins that make up the lipid matrix of the stratum corneum. Implicating Snap29 also in this essential step of epidermal morphogenesis, Snap29 mutant mice show a reduced epidermal glucosylceramide distribution [19]. Consistently, the stratum corneum from skin with keratoderma and ichthyosis show an accumulation of glucosylceramide-positive lamellar bodies in granular cells, indicating that polarized trafficking and secretion, and perhaps other trafficking processes involving lamellar bodies, could too be impaired [61]. Sprecher et al. propose that the reduced presence of lipids in the extracellular space between granules cells and cornified cells may cause impaired barrier formation, contributing to the skin traits of CEDNIK patients. As the biogenesis and trafficking of lamellar bodies remains elusive, it is difficult to deduce how a loss of SNAP29 may contribute to impaired lamellar body function. Suggesting that the Golgi may be the source of lamellar bodies, nascent lamellar bodies appear bud off the trans-Golgi network cisternae [81]. Further, keratinocyte-specific knockout of the Golgi pH regulator (GPHR) in mice results in reduced presence of lamellar bodies in the skin 5 days after birth [82]. Taken together, a loss of Snap29 at the Golgi may contribute to impaired lamellar body biogenesis (Fig. 2C).

Considering that COG6 mutations in humans cause a specific congenital disorder of glycosylation (CDGs) with traits that include microcephaly and hyperkeratosis of the skin [83], it is possible that the COG6-SNAP29 interaction at the Golgi apparatus could be relevant to the nervous system and skin phenotypes of CEDNIK. In this context, it would be interesting to test whether CEDNIK cells display glycosylation defects and could be classified as a form of CDG.

Finally, an interesting parallel may be drawn between CEDNIK syndrome and MEDNIK syndrome, a disorder caused by mutations in *AP1S1* and characterized by mental retardation, enteropathy, deafness, neuropathy, ichthyosis, and keratoderma [84]. The overlap in functions of AP1 and SNAP29 in endocytosis and the apparent overlap in clinical manifestations of the distinct disorders, inherently suggests an additional possible role of endocytosis in keratinocyte differentiation (Fig. 2D).

3.3. SNAP29 and cerebral dysgenesis

In contrast to the skin manifestations, the cerebral dysmorphogenesis and developmental delays, including deficits in gross and fine motor skills as well as sensory and visual tracking abnormalities observed in individuals with CEDNIK, have not been investigated beyond descriptions of clinical findings. The cerebral dysmorphogenesis include microcephaly, corpus callosum dysgenesis and cortical dysplasia with pachygyria and polymicrogyria, hypoplastic optic discs, sensorineural deafness [61–68]. It remains unclear how loss of SNAP29 activity could contribute to neurodevelopmental abnormalities observed in CEDNIK.

Some of the hypoplastic defects described could stem from cell death induced by loss of SNAP29. In fact, in the zebrafish *Danio rerio* it has been demonstrated that loss of Snap29 leads to cell death during head development [18]. This may be in part attributed to defective kinetochore formation, leading to impaired cell division and eventually cell lethality [34]. Additionally, disrupted autophagy due to a loss of SNAP29 may be a contributing factor to cell death. An explicit link between altered SNAP29 activity, reduced autophagy and increased cell death was recently reported in a rat model of perinatal fluoride-induced toxicity [85]. Specifically, rats with perinatal exposure to sodium-fluoride show reduced levels of Atg14, Stx17, Snap29 and Vamp8, coupled with an expected block in autophagy and impairments in learning and memory at 2 months old. Critically, the fluoride-induced block in autophagy appears to induce cell apoptosis *in vitro* and *in vivo*. Overexpression of Atg14 in sodium-fluoride treated cells increases Snap29, Vamp8 and Stx17 protein levels, thereby rescuing the block in autophagy and concomitant cell apoptosis. This is in alignment with previous studies which show that Atg14 is required for SNARE complex formation [86]. Taken together, cell death during neurodevelopment, possibly due to impaired cell division or autophagy, could contribute to the cerebral dysgenesis observed upon loss of *SNAP29* in CEDNIK patients.

Given the emerging evidence regarding the tight regulation of cytoophidia formation and CTPsyn catalytic activity throughout development, the functional consequences of reduced SNAP29 on cytoophidia formation at the cytokeratin network poses interesting implications for the neurodevelopmental trait of CEDNIK syndrome. In fact, cytoophidia have been found to be abundant in the fast-growing neuroepithelial stem cells of the developing optic lobe of *Drosophila* larvae. Intriguingly, Tastan and Liu demonstrate that CTPsyn mutants exhibit altered neuroepithelial morphology and have smaller larval brains compared to the wildtype, a key characteristic of microcephaly. Conversely, overexpression of CTPsyn inhibits the transition from neuroepithelium to neuroblast [87]. Consistent with this, Li et al. demonstrate that the formation of cytoophidia in developing cortical neurons impairs neuronal migration and accelerated differentiation [88]. As altered neurogenesis, and in particular microcephaly, are characteristic of CEDNIK, it is tempting to propose that cytoophidia dysregulation due to loss of *SNAP29* could represent a novel disease mechanism. Whether SNAP29 facilitates the formation of cytoophidia in neuroepithelial cells during development, and the functional consequences thereof, requires further investigation.

Pathogenic *SNAP29* variants have been associated with other congenital disorders that present prominent neurodevelopmental traits overlapping with CEDNIK clinical symptoms. This is the case for a number of DiGeorge syndrome/Velocardial Facial syndrome patients with CEDNIK-like phenotypes [89]. DiGeorge syndrome/Velocardial Facial syndrome affects around 1 in 4000 live births and is characterized by developmental delay, cleft palate and cardiac malformations [90]. The heterozygous ~1.5–3 Mb deletion at chromosome 22q11.2, causing DiGeorge/Velocardial Facial syndrome, encompasses 46 protein coding genes, including *SNAP29*. In patients with additional CEDNIK-like symptoms, unnmasking of hemizygous *SNAP29* mutations combined with heterozygous loss of the 22q11.2, causes emergence of CEDNIK syndrome alongside the DiGeorge syndrome/Velocardial Facial syndrome [89].

Interestingly, individuals with DiGeorge syndrome/Velocardial Facial syndrome show a significant increase in the risk of developing psychiatric disorders, including Schizophrenia [91,92]. Three independent studies have identified an association between SNAP29 and schizophrenia: two identified polymorphisms in the *SNAP29* promoter region while the latter further identified copy number variations impacting *SNAP29* [93–95]. Thus, *SNAP29* may be a susceptibility gene in Schizophrenia, a disorder characterized predominantly by impaired synaptic signaling [96]. Perhaps consistent with this, excess neuronal branching is observed in *SNAP29* mutant and morphant zebrafish [18]. The opposite phenotype is observed upon depletion of SNAP25. Together, these results appear to be in line with the role of SNAP29 in negatively regulating SNAP25-mediated functions at the plasma membrane [29,30]. Whether changes in this regulatory function of SNAP29 are involved in schizophrenia or in the development of the cerebral dysgenesis of CEDNIK remains to be explored.

A second SNAP29-mediated process that might contribute is Golgi trafficking to the synapse. Indeed, both SNAP29 and COG6 components have been found to physically interact with dysbindin, encoded by one of the genes most frequently associated with schizophrenia [97].

Another pathogenic alteration of *SNAP29* associated to copy number variants of the chromosome region 22q11.2 has been described in an individual with a microduplication of the 22q11.2 region that includes *SNAP29* and *MAPK1*, with ocular manifestations including Marcus Gunn jaw wink phenomenon (involuntary movements of upper eyelid upon lower jaw movements) and tortuous retinal vasculature [98]. In relation to the observed abnormal retinal vasculature, Cordovez et al. suggest a

specific involvement of impaired vascular endothelial growth factor (VEGF) signaling as a consequence of altered *SNAP29* dosage. Indeed, increased VEGF signaling has been directly linked to increased tortuosity of retinal vessels humans [99]. As SNAP29 interacts with EHD1 to likely mediate EGF-1 receptor endocytosis, this represents a plausible mechanism underlying the observed ocular manifestations. Further, conversely to the microcephaly observed in CEDNIK patients, it is also interesting to note that the individual showed pronounced macrocephaly. Whether the altered dosage of *SNAP29* could alternatively lead to micro- or macrocephaly remains unclear.

Two compound heterozygous loss of function mutations in *SNAP29* have been identified in a 12-year-old patient with symptoms of Pelizaeus-Merzbacher-like disease (PMLD), a hypomyelinating leukodystrophy disorder. The clinical phenotypes of the patient – including cerebral dysgenesis and hypomyelination – reflect those of CEDNIK but lack the typical dermatological abnormalities initially described in the characterisation of the disease [100]. Genetic analysis revealed that one of the two mutations of the patient is nonsense and predicted loss of function and the second is a missense, in-frame change predicted to generate a SNAP29 form lacking the NPF motif. Interestingly, the N-terminus of SNAP29 containing the NPF motif has been shown to bind to RAB3A to promote the secretion of myelin proteolipid in glial cells [101]. While authors show that SNAP29 expression in patient samples is strongly reduced, the lack of skin manifestations but PMLD features might arise from a partial loss of *SNAP29* activity but a complete absence of the functions associated with the NPF motif. Taken together, it is also fair to speculate that different degrees of loss of function of *SNAP29* might contribute to pathogenesis of PMLD and CEDNIK.

Finally, a heterozygous nonsense mutation in *SNAP29*, predicted to generate a truncated protein, has also been linked to Autosomal Dominant Nocturnal Frontal Lobe Epilepsy in one 32-year-old patient, without dermatological or developmental retardation phenotypes [102]. Similarly, seizures have been observed in 36% of CEDNIK patients to date, suggesting that epilepsy might be a frequent consequence of reduced SNAP29 activity. A heterozygous 1 Mb microdeletion in 22q11.2, involving *SNAP29*, *LZTR1* and *P2RXL1*, has also been reported in a patient with obesity, hyperphagia, aggressive behavior, major depressive disorder and immune deficiency [103,104]. While it is interesting to note again the occurrence of psychiatric disorders in association with a loss of SNAP29, it is not clear whether heterozygous loss of function of *SNAP29* might cause the emergence of any trait.

3.4. Dysregulation of SNAP29 beyond congenital disorders

Beyond the aforementioned congenital disorders associated with mutations in *SNAP29*, limited lines of evidence are beginning to highlight how the inhibition of SNAP29 activity, specifically in the context of autophagy, may contribute to the pathogenesis of a wide spectrum of diseases that impact homeostasis of adult tissues, including cancer, viral infection and neurodegenerative diseases.

In cancer cells, altered SNAP29 activity has been demonstrated in response to tumor necrosis factor (TNF)α signaling. Despite being extensively investigated as a potential cancer therapeutic, TNFα may also act as an endogenous carcinogen in certain contexts [105]. Indeed, among its wide range of downstream targets, TNFα can activate both the anti-apoptotic nuclear factor κB (NF-κB) signaling pathways or induce cell death by apoptosis or necrosis. TNFα-mediated induction of the NF-kB signaling cascade has been shown to be enhanced by the cellular prion protein (PrPC) [106]. Concurrently, overexpression of the cellular prion protein (PrPC) in cancers has been associated with increased resistance to cell death induced by antitumor drugs, such as TNFα [107]. Recent evidence from a breast cancer cell line suggests that TNFα can negatively regulate the transcriptional activation of SNAP29 via inhibition of the FOXP3 transcription factor, leading to reduced autophagic degradation of PrPC [108]. The TNFα-induced downregulation of SNAP29 may thus shift the response to TNFα towards the anti-apoptotic, and thus carcinogenic, NF-kB signaling cascade. A further link between TNFα signaling and autophagy-dependent SNARE activity has recently been reported. Specifically, TNFα-induced necroptosis, by concomitant treatment with a caspase inhibitor, weakens the interaction of SNAP29 with VAMP8, STX17 and STX7. While the observed cleavage of STX17 upon TNFα-induced necroptosis may account for the reduced interaction of SNAP29 with VAMP8 and STX17, the cause of impaired SNAP29-STX7 association remains to be clarified [109].

In the context of autophagy, it is further interesting to note that induced overexpression of the mitochondrial surface protein BNIP3 by berbamine, a natural compound that might have anticancer properties, reduces SNAP29 availability for SNARE complex formation and consequently leads to inhibited autophagosome-lysosome fusion [110]. Taken together with the emerging evidence on regulation by TNFα, these data highlight a possible role of autophagy-related SNAP29 activity in tumorigenesis which warrants further investigation.

Ras proteins are a family of ubiquitously expressed and highly conserved GTPases that act upstream in the MAPK signaling pathway to mediate diverse cellular processes including cell growth, proliferation and differentiation. A wide range of mutations in Ras genes have been associated with many types of cancer [111]. K-Ras activity is critically dependent on its polarized trafficking to the plasma membrane, where it relays extracellular signals from growth factors into intracellular signals, such as by activating Raf, a MAPK kinase kinase [112]. Unlike other Ras proteins, the K-Ras isoform KRAS4B is not palmitoylated and thus relies on a Golgi-independent pathway to reach the plasma membrane [111–113]. Interestingly, a triple knockout of *SNAP29, VAMP3* and *SNAP23* results in the mislocalization of KRAS4B to recycling endosomes. While its interaction with VAMP3 appears to be indirect as evidenced from co-immunoprecipitation assays, KRAS4B was found to directly interact with SNAP29 and SNAP23 via its C-terminal hypervariable domain. It is important to note that SNAP29 may have a redundant role in K-Ras trafficking: while independent knockouts of SNAP23 and VAMP3 resulted in reduced tumor growth of a K-Ras-dependent human tumor cell line *in vivo*, *SNAP29* knockout did not affect tumorigenesis [114].

A vulnerability of Snap29 in the context of autophagosome-lysosome fusion has also emerged in response to pathogen infection. As a well-established mechanism, autophagosomes can provide viruses with a compartment for evading host cell detection. Autophagosomes also support the virus replication machinery and act as a vessel for unconventional secretion. Surprisingly, inactivation of Snap29 appears to be a common mechanism among a number of viruses to enable these functions and to escape lysosomal degradation. In particular, viral proteins of the human parainfluenza virus 3 (HPIV3) [115], of the Hantaan virus (HTNV) [116] as well as of three picornaviruses (CVB3, EVD68 and EV-A71) [117–119] have been demonstrated to directly inhibit SNAP29 function, either by competitive binding to SNAP29 or by catalyzing SNAP29 cleavage. In addition, SNAP29 appears to be specifically degraded during pathogenic *Escherichia coli* infection [120].

Finally, α-synuclein has been shown to bind to and inhibit SNAP29 to disrupt autophagosome-lysosome fusion and subsequent autophagic clearance in a human dopamine neuron cell line. Interestingly, impaired autophagic degradation of α-synuclein was observed in combination with an apparent compensatory increase in the release of extracellular vesicles [121]. Thus, inactivation of Snap29 could also play a role in forms of Parkinson's disease and altered polarized trafficking upon Snap29 inhibition may represent a new mechanism by which α-synuclein is transmitted between neurons.

4. Conclusions

In this review, we have discussed emerging evidence suggesting how Snap29 - a multipurpose SNARE with a number of canonical as well as non canonical functions - might contribute to tissue morphogenesis. Considering the pleiotropy of Snap29 activity in most cells, it is

currently difficult to attribute a distinct developmental defect observed in patients lacking *SNAP29* to alteration of a particular SNAP29 function. Taking this into account, we foresee a few key areas of research that might help pinpoint functions to disease traits, as well as some strategies that might lead to their future amelioration. These strategies might also guide future clarification of whether and how SNAP29 prevents pathogenesis associated with cancer, infectious disease and neurodegeneration.

4.1. How is Snap29 localized and activated at specific cellular locales?

Considering that all functions of Snap29 are mediated by a common cytoplasmic pool, regulation by posttranslational modifications or by specific protein-protein interactions are likely to be crucial to direct Snap29 to certain cellular locales at specific moments of the life of a cell. It is therefore first advisable to extend the search for mechanisms that regulate Snap29 activity as well as its localization. O-GlcNACylation and Ubiquitin-independent proteasomal degradation have been studied only in the context of autophagy and phosphorylation only in the context of its localization at the Golgi apparatus. This highlights a need to study these modes of regulation, as well as any novel mechanisms, in the context of the diverse roles of Snap29. Could O-GlcNAcylation of Snap29, for example, also regulate its endocytic and secretory functions? Are the enzymes involved in these reactions restricted to certain compartments? If not, one could propose that Snap29 could act as a nexus to coordinate diverse cellular pathways. A compelling question is also whether cell cycle specific regulation might alternatively regulate the trafficking versus cell division functions of Snap29. To address these outstanding questions, genetic reconstitution experiments with forms of Snap29 unable to sustain a certain regulation may prove valuable.

4.2. What are the structures that Snap29 can entertain? with which interactors?

In line with a need to further elucidate the mechanisms underlying Snap29 regulation, the way Snap29 interacts with other proteins warrants further investigation. Indeed, protein-protein interactions of Snap29 have so far only been described for the SNARE domains - which interact with coordinate SNAREs and with KMN network components - and the NPF motif - which interacts with EH-domain containing proteins and RAB3A. The contribution of the extended domain linking the SNARE domains, which is highly divergent with that of paralogs but is loosely conserved in orthologs, to protein-protein interactions has not been experimentally investigated. Also, whether the linker can entertain direct electrostatic interactions with membranes is unknown. Finally, while the structural properties of the SNARE domains are well understood, it is unclear whether the linker is flexible enough to allow even the incorporation of a single molecule of Snap29 in 4-helix bundles. Answering these questions would help distinguish between fusion and regulatory/tethering properties of Snap29.

4.3. Do we know of all the ways in which SNAP29 dysregulation contributes to pathogenesis?

First, while Snap29 is likely to be expressed ubiquitously, a better understanding of transcriptional regulation of Snap29 in the context of disease models is needed. Evidence so far hints at the possibility that some traits might be associated with complete loss of SNAP29 activity (e.g. skin manifestations), while some might be common and present also in condition of partial loss of function. In addition, considering the described mutations in the *SNAP29* promoter region, it is currently not clear whether Schizophrenia might be associated with reduced or increased SNAP29 activity. In line with this, an important area for future investigation is to understand how increased or reduced activity of SNAP29 can impact neurodevelopment.

Mutations in Snap29 have not been identified to be significantly associated with cancer. However, the many processes regulated by SNAP29, especially at the plasma membrane, during kinetochore formation and, importantly, in autophagy, suggest that SNAP29 might be a convenient cellular player to be disrupted by cancer cells. We predict that SNAP29 might represent a convenient target to counteract pathogenic processes involved in cancer, such as genetic instability, oncogenic signaling and escape from cell death.

A final area of future study revolves around the role of Snap29 in neurodegeneration. Inhibition of Snap29 appears to be a common and potent mechanism to block autophagic degradation, which is vital for the long-term health of neurons. This raises the question whether SNAP29 variants with different efficiency exist or whether changes in neuronal proteostasis might depend on SNAP29. The limited evidence emerged so far suggests a contribution of Snap29 inhibition to both reduced α-synuclein degradation and its subsequent propagation via extracellular vesicles, highlighting an intriguing mechanism that may be common across different neurodegenerative diseases. Whether alterations of Snap29 could impact the pathogenesis of this group of non-developmental diseases, thus represents an interesting direction for future research. We look forward to witnessing the emergence of more scientific research connecting Snap29 to healthy aging.

4.4. Strategies for disease amelioration

Remarkably, the few examples of physiologic and pathogen-dependent regulation of SNAP29 activity represent a convenient entry point into manipulation of SNAP29 activity to counteract disease. This might become highly relevant if future studies reveal further involvement in tumorigenesis and neurodegeneration. However, the initial findings that inhibition of SNAP29 O-GlcNAcylation rescues SARS-CoV2, already set the stage for further investigations into how genetic or pharmacologic manipulations of Snap29 regulation could modulate activity in the context of congenital diseases that might still have minimal SNAP29 activity, such as the case of selected CEDNIK/DiGeorge/PMLD patients. Additionally, it is intriguing to hypothesize that inhibiting SNAP29 O-GlcNAcylation may at least partially overcome the autophagy defects seen in Vici Syndrome, resulting from *EPG5* loss of function mutations, or indeed in neurodegenerative diseases. With increased knowledge regarding the regulation of Snap29, its interactions with protein partners and its dysregulation in diseases, further and more precise strategies to ameliorate the specific traits of patients with defective SNAP29 activity are likely to emerge.

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