

Amphibian $\beta\gamma$ -CAT Defines a Pore-Forming Protein System for Nutrient Acquisition and Metabolic Flexibility

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Summary

Cell membranes form selective barriers for the exchange of materials between cells and their environment. Nutrient uptake in metazoan cells is generally mediated by membrane transporters and endolysosomal pathways. $\beta\gamma$ -CAT, an aerolysin family pore-forming protein (PFP)–trefoil factor complex identified in the toad *Bombina maxima*, has been characterized as a novel regulator of nutrient acquisition. First, the toad cells dispatch $\beta\gamma$ -CAT under starvation to scavenge extracellular proteins for their energy supply and survival. Second, blood $\beta\gamma$ -CAT is an immediate responsive element under fasting, which is able to mediate the transcellular transport of albumin-bound fatty acids for the nutrient requirement of tissue parenchymal cells. Third, $\beta\gamma$ -CAT mediates macropinocytosis in toad osmoregulatory organs for facilitating toad water maintaining. This PFP complex has been found to be tightly regulated at several stages by toad endogenous elements, like AMP-activated kinase signaling, IgG Fc-binding protein, its PFP paralog and ATP. Insights from $\beta\gamma$ -CAT define a previously unrecognized PFP system that drives vesicular delivery and transport. Notably, similar functions and regulatory strategies are now being progressively validated in other species, providing a new paradigm for PFP-driven nutrient acquisition and metabolic flexibility.

Keywords: Pore-forming protein; Nutrient acquisition; Metabolism; Endolysosomal pathways; Immune defense.

Introduction

Metazoan cells take up various substances as their nutrients for energy supply and biomass building, such as glucose and amino acids, macromolecules and micronutrients. Membrane transporters like solute carrier superfamily proteins generally work in small nutrient uptake like glucose and amino acids (Pizzagalli et al., 2021; Saier, 2016). Receptor-mediated endocytosis of nutrient carriers is the main pathway through which cells obtain insoluble nutrients, like cholesterol and iron (Chantranupong et al., 2015; Palm and Thompson, 2017). In addition, metazoan cells can also ingest extracellular macromolecules including proteins through non-selective endocytic pathways like macropinocytosis, which is an ancient feeding behavior conserved from amoebae to mammalian cells (Bloomfield and Kay, 2016; King and Kay, 2019). The question of how a cell's preference for distinct nutrient acquisition

strategies is regulated remains unanswered (Palm and Thompson, 2017).

Pore-forming proteins (PFPs) are featured in existing in a water-soluble monomeric form, and many of them are secreted out of cells (Benton and Bayly-Jones, 2021; Dal Peraro and Van Der Goot, 2016). After undergoing an extensive conformational change under specific conditions, these non-classic membrane proteins can form transmembrane pores (channels) with various sizes (1 to 30 nm). Traditionally, PFPs have long been recognized as either pore-forming toxins (PFTs) for microbial infection (Dal Peraro and Van Der Goot, 2016; Omersa et al., 2019) or host immune executors. Specially, the knowledge on those PFPs derived from animals and plants is mainly focused on their roles in cell death, including Bcl-2 proteins, MACPF/CDC proteins and gasdermins (Banjara et al., 2020; Broz et al., 2020; Lukoyanova et al., 2016). Aerolysin is a β -PFT produced by the bacterium *Aeromonas hydrophila* (Buckley et al., 1981; Parker et al., 1996). The aerolysin domain is defined according to its structural similarity to the transmembrane domain of aerolysin (Szczesny et al., 2011). A diverse array of aerolysin family PFPs (abbreviated af-PFPs, previously referred as aerolysin-like proteins, ALPs) has been identified in various animal and plant species (Dang et al., 2017; Szczesny et al., 2011; Zhang, 2015). Although traditionally viewed principally as cytolytic virulence factors, emerging evidence indicates that certain bacterial pore-forming toxins can also modulate host processes beyond direct membrane disruption, such as influencing extracellular vesicle dynamics and immune signaling (Parveen et al., 2024), a functional versatility conceptually similar to how $\beta\gamma$ -CAT drives regulated vesicle-mediated trafficking in vertebrate cells, as discussed in detail below (Wang et al., 2023; Wei et al., 2022).

Here, we briefly review the discovery of an af-PFP and trefoil factor (TFF) complex, named $\beta\gamma$ -CAT, from the Chinese red belly toad (*Bombina maxima*) as well as its cell biological functions, biological outcomes and molecular regulatory network. $\beta\gamma$ -CAT is a secreted protein complex composed of an aerolysin-like pore-forming protein and a trefoil factor subunit, with tandem N-terminal $\beta\gamma$ -crystallin domains and a C-terminal aerolysin domain that together determine its molecular size and pore-forming capability, and its activity is tightly regulated at multiple levels including subunit assembly, oligomerization state, and physiological concentration (Zhang et al., 2021). Specifically, we focused on our advances on this secretory PFP complex which acts in driving cell vesicular delivery and transport, playing essential roles in toad responses to fasting and toad cell response to starvation. The regulation

of this PFP complex at different levels was summarized, and the similarity and difference among $\beta\gamma$ -CAT and other family PFPs and membrane active peptides (MAPs) were compared. Finally, inherent correlations among nutrient acquisition, immune defense and toxins were discussed.

1 Amphibian *B. maxima* PFP and TFF complex $\beta\gamma$ -CAT

1.1 A “toxic” component of toad *B. maxima* skin secretions to mammals

Toad *B. maxima* lives in the mountainous regions of southwestern China and its skin is very “toxic” to mice. At first, the effort to understand its potential lethal components to mice led to the identification of a protein complex composed of two types of subunits connected non-covalently. As mentioned above, the α -subunit is an af-PFP (two $\beta\gamma$ -crystallin domains fused with an aerolysin domain, termed BmALP1) and the β -subunit is a trefoil factor (TFF) (BmTFF3) with a roughly estimated molecular form of $\alpha\beta_2$, and the PFP complex was named $\beta\gamma$ -CAT to reflect its domain composition (Gao et al., 2011b; Liu et al., 2008). However, accumulated experimental evidence supported a notion that $\beta\gamma$ -CAT should have a non-covalently linked subunit form of $\alpha_2\beta$. Due to the tendency of BmALP oligomerization in its reduced dimer form, a β -subunit (BmTFF3) can stabilize the reduced dimer (Bian et al., 2023; Guo et al., 2019; Wang et al., 2020). In addition, there are BmALP1 homologous in toad *B. maxima*, like BmALP2 in the skin secretions (Wei et al., 2022). Thus, it is reasonably to predict that in addition to BmALP1 homodimer, heterodimer of α -subunit (such as BmALP1 with BmALP2) may occur in $\beta\gamma$ -CAT molecules naturally.

$\beta\gamma$ -CAT can form membrane pores (channels) with a functional diameter about 1.5-2.0 nm (Liu et al., 2021; Liu et al., 2008), which is responsible for the potent hemolytic activity on human erythrocytes and lethal toxicity on mice of *B. maxima* skin secretions (Gao et al., 2011a; He et al., 2008; Qian et al., 2008). Toxicity can be viewed as an uncontrollable biological activity (Zhang, 2015). $\beta\gamma$ -CAT only shows cytotoxicity in digested toad *B. maxima* cells with dosages above 2 μ M (Zhao et al., 2022), which are much more higher than $\beta\gamma$ -CAT physiological concentrations observed (Shi et al., 2022; Xiang et al., 2014). Its cytotoxicity to mammalian cells reflects the absence of regulatory mechanisms for this exogenous PFP protein.

1.2 An immune defense factor in toad *B. maxima*

In the second stage to understand the biological functions and mechanisms involved of $\beta\gamma$ -CAT, the role of the PFP complex in toad immune defense against microbial infection was investigated. Under bacterial challenge, $\beta\gamma$ -CAT was found to oligomerize along cellular endocytic pathways to induce lysosome destabilization, leading to inflammasome activation in toad peritoneal cells and murine skin epithelial cells. This cellular action effectively initiated antimicrobial responses and tissue repairing (Gao et al., 2019; Xiang et al., 2014). Second, it neutralized endocytic organelle acidification to counteract intracellular pathogen invasion (Li et al., 2017). Third, in a murine dendritic cell (DC) model, $\beta\gamma$ -CAT augmented antigen presentation probably via stimulating pinocytosis to enhance antigen (ovalbumin) import, neutralizing endosome acidification and inducing functional exosome release, which led to strong cytotoxic T lymphocyte responses and antigen-specific antibody responses (Deng et al., 2020). Finally, $\beta\gamma$ -CAT directly acted on herpes simplex virus type 1 (HSV-1) envelope to insert into the viral membrane with channel formation. Rapid K^+ and Ca^{2+} efflux from the virus was detected. These actions resulted in the blockade of HSV-1 cytoplasmic entry both via fusion at the plasma membrane as well as along endocytic pathway (Deng et al., 2020).

1.3 The PFP complex $\beta\gamma$ -CAT in toad nutrient acquisition

$\beta\gamma$ -CAT drives extracellular nutrient scavenging under cell starvation: Macromolecules like proteins can supply a range of nutrients when glucose and free amino acids are scarce under starvation (Palm, 2019). The cell nutrient acquisition strategies and metabolic flexibility as well as interior coordination in different cell contexts and nutrient surroundings are incompletely understood (Florey and Overholtzer, 2019; Palm and Thompson, 2017).

Under the deficiency of glucose and glutamine, two basic nutrient components in cell metabolism, *B. maxima* cells $\beta\gamma$ -CAT to scavenge extracellular proteins for their nutrient supply and survival (Liu et al., 2023). Intriguingly, secreted $\beta\gamma$ -CAT interacted with *B. maxima* serum albumin (Bm-SA) or ovalbumin by BmALP1 N-terminal $\beta\gamma$ -crystallin domains (Liu et al., 2023), suggesting that the PFP complex may possess the capacity of sensing and selecting protein cargoes in extracellular

surroundings. Afterwards, $\beta\gamma$ -CAT promoted albumin or ovalbumin uptake via stimulating cell endocytosis. Elevated intracellular amino acids, enhanced ATP production and eventually prolonged cell survival were determined in the presence of $\beta\gamma$ -CAT and the extracellular proteins (Liu et al., 2023).

Mechanistically, the above $\beta\gamma$ -CAT-mediated processes can be separated into steps with different requirements for pore formation. Ligand recognition and binding to protein cargoes such as albumin/ovalbumin (via the N-terminal $\beta\gamma$ -crystallin domains) and initiation of endocytic uptake can occur without necessarily requiring immediate pore opening. In contrast, pore formation on intracellular membranes along endocytic/endolysosomal compartments is critical for downstream events such as ionic flux modulation, changes in vesicular acidification, and intracellular handling/release of internalized cargo. Notably, while evidence supports an essential role of $\beta\gamma$ -CAT channels in endocytic organelle remodeling and vesicle functions, whether pore formation contributes directly to the earliest uptake step remains to be further dissected.

$\beta\gamma$ -CAT promptly responses to toad fasting with capacity to deliver macromolecular nutrients: In response to animal fasting, fatty acids (FAs) have to traverse the endothelium and are taken up by the underlying parenchymal cells, and albumin is the main carrier of FAs in blood plasma (Ko et al., 2020; Mehrotra et al., 2014; Van Der Vusse, 2009; Young and Zechner, 2013). However, the mechanism responsible for transmembrane movement of FAs is incompletely understood (Fung et al., 2018).

$\beta\gamma$ -CAT is a constitutive component in toad *B. maxima* blood serum, which actively response to the animal fasting (Shi et al., 2022). In accordance with above observation (Liu et al., 2023), $\beta\gamma$ -CAT was found to be able to promote cellular albumin and albumin-bound FA uptake in a variety of epithelial and endothelial cells. Furthermore, endothelial cell-derived exosomes (called nutrisomes) containing largely enriched albumin (8 folds) and FAs (3 folds) were released in the presence of $\beta\gamma$ -CAT.

These specific nutrient vesicles were readily taken by starved muscle cells to support their survival. The finding suggested the roles of $\beta\gamma$ -CAT in mediating trans-endothelial cell transport of albumin-bound FAs for tissue nutrient supply (Shi et al., 2022).

$\beta\gamma$ -CAT drives macropinocytosis for toad water maintaining: Amphibians have to face rapid evaporative water loss in dry land surroundings (Haslam et al., 2014; Jorgensen, 1997). Traditionally, aquaporins and ion channels in cell plasma membrane are believed to control this active process (Campbell et al., 2012; Hanukoglu and Hanukoglu, 2016; Suzuki et al., 2015).

$\beta\gamma$ -CAT was constitutively expressed in toad osmoregulatory organs, which was inducible under the variation of osmotic conditions. As a major component of toad skin secretions, deletion of the protein increased animal mortality under hypertonic stress (Zhao et al., 2022). During extracellular hyperosmosis, $\beta\gamma$ -CAT stimulated macropinocytosis in *B. maxima* cells from osmoregulatory organs to facilitate water intake and enhanced exosome release, which simultaneously regulated aquaporin distribution. Channels formed by $\beta\gamma$ -CAT on endocytic organelles enabled Na^+ flux in response to osmotic stress, driving water flow to cytosol. These results uncovered that besides membrane integrated aquaporins, a secretory PFP can facilitate toad water acquiring and maintaining via macropinocytosis induction and exocytosis modulation, especially in responses to osmotic stress (Zhao et al., 2022).

2 $\beta\gamma$ -CAT defines a PFP system working to drive cell vesicular delivery

Thus far, our findings illustrated that the cellular action of $\beta\gamma$ -CAT can facilitate toad *B. maxima* in material acquisition (including nutrients, water and oxygen as well as antigens etc.) and vesicular delivery and transport while maintaining mucosal barrier function as well as fulfilling immune defense (for a review, see Zhang Y et al., 2021 (Zhang et al., 2021)). The presence of $\beta\gamma$ -CAT in toad *B. maxima* can result in such a diverse cellular responses and biological outcomes, indicating that this PFP machine should execute a primary and essential cell biological action. $\beta\gamma$ -CAT is the

first example of an experimentally clarified animal secretory (soluble) endolysosomal channel (SELC) protein. Together with its regulatory network, the PFP machine defines of $\beta\gamma$ -CAT-pathway (a SELC pathway), as proposed before (Zhang et al., 2021). It is reasonable to propose that it is the capacity of $\beta\gamma$ -CAT to drive cell material import and export through cell endolysosomal systems makes the protein a versatile multiple functional protein machine depending on cell contexts and surroundings (Zhang et al., 2021).

$\beta\gamma$ -CAT defines a hitherto unknown animal secretory PFP system working to drive cell vesicular delivery, a basic cellular process relevant to numerous animal biological activities. As summarized in **Figure 1**, toad BmALPs can be converted between the active form (reduced homodimer or even heterodimer) and inactive form (dimer linked by a disulfide bond with high molecular weight polymer formation) by specific redox regulation due to a conserved cysteine residue located in the C-terminal part of BmALP aerolysin domain (Bian et al., 2023; Wang et al., 2020). $\beta\gamma$ -CAT is assembled with the aid of IgG Fc-binding protein (Bian et al., 2023), in which BmTFF3 may act as a BmALP chaperon to stabilize reduced BmALP dimer (also see the next section for a detail). Importantly, it has been shown that assembled $\beta\gamma$ -CAT may have extracellular cargo selectivity via binding of BmALP N-terminal $\beta\gamma$ -crystallin domains to cargo molecules (like Bm-SA, BSA or ovalbumin etc.) (Liu et al., 2023). The PFP complex then targets the acidic glycosphingolipids in the lipid rafts of cell plasma membrane via a double-receptor binding model (Guo et al., 2019) to mediate the internalization of acquired cargoes through stimulating and mediating cell endocytosis (**Figure 1**).

Diverse endocytic pathways are available at the cell surface of metazoan cells including dynamin-dependent pathways and actin filament polymerization mechanisms (Mayor et al., 2014). Macropinocytosis is an ancient endocytic form that mediates non-selective uptake of extracellular fluid and the solutes contained therein (Kerr and Teasdale, 2009; Swanson and Watts, 1995). It has been reported that $\beta\gamma$ -CAT induces macropinocytosis in toad osmoregulatory organs to facilitate toad water acquiring and maintaining (Zhao et al., 2022). However, in $\beta\gamma$ -CAT-mediated nutrient acquisition, it was observed that the PFP binds to extracellular proteins (Bm-SA, BSA or ovalbumin) via BmALP N-terminal $\beta\gamma$ -crystallin domains (Liu et al., 2021). This phenomenon indicated that the intracellular delivery of extracellular proteins by $\beta\gamma$ -CAT and/or its af-PFP homologous can be a specific process with

cargo selectivity (**Figure 1**), which differs from classic pinocytosis/macropinocytosis. Thus, these findings supported a notion that $\beta\gamma$ -CAT is able to employ different cell entry mechanisms to deliver different cargo molecules into cells replying on distinct cell contexts and surroundings.

The large nutrient enrichment (8-folds for albumin and 3-folds for albumin-bound FAs) has been determined in $\beta\gamma$ -CAT-mediated nutrisome (exosome) releasing in nutrient delivery in animal fasting (Shi et al., 2022). Distinct binding capacity of BmALP N-terminal $\beta\gamma$ -crystallin domains to different proteins is at least one of reasons to explain this intriguingly phenomenon. Afterwards, this PFP complex drives the formation of intracellular vesicles (containing enriched nutrient cargoes) with the PFP-channels formed on the organelles to facilitate the uptake and delivery of the nutrients (Zhao et al., 2022) (Liu et al., 2021) (Shi et al., 2022).

Amphibian skin is an important respiration organ (Campbell et al., 2012; Haslam et al., 2014). How oxygen traverses epidermal cells to reach dermis with blood capillary is not clear, and we believe that oxygen free diffusion is not a good explanation. In toad *B. maxima* skin secretions an interesting and confusing biological fact is that $\beta\gamma$ -CAT and its regulatory elements account for the majority of protein components (Wang et al., 2020; Wei et al., 2022; Zhao et al., 2022). The oxygen tension-dependent control of $\beta\gamma$ -CAT by its paralog BmALP3 in the toad skin potentially links the possible protein machinery with skin respiration (Wang et al., 2020). In addition, the haem b cofactor in the toad skin albumin might bind and fix oxygen (Zhang et al., 2005). Furthermore, $\beta\gamma$ -CAT possesses the characteristic cellular action (**Figure 1**). Taken together, the simplest explanation for this spatial expression of haem b-bound albumin and $\beta\gamma$ -CAT in toad epidermis is that the latter may be responsible for mediating the uptake of oxygen-bound toad albumin in outermost layers of toad skin epidermis and delivers it to internal environment, greatly facilitating oxygen uptake in toad skin respiration. However, this remains a working model and will require direct experimental testing *in vivo* and *ex vivo*.

3 The regulation of $\beta\gamma$ -CAT-pathway

Our findings elucidated that $\beta\gamma$ -CAT constitutes a novel PFP system driving cell vesicular delivery and transport. In toad *B. maxima* this newly discovered ingenious PFP-driving system driving cell material delivery works in cell nutrient acquisition

(Liu et al., 2023; Shi et al., 2022), water maintaining (Zhao et al., 2022) and potentially skin oxygen acquiring (as discussed above) as well as in immune defense (Xiang et al., 2014). Reasonably, such a PFP system must be tight regulated. Indeed, emerging experimental evidence revealed that $\beta\gamma$ -CAT is regulated at several levels by distinct endogenous toad proteins and metabolites (**Figure 2**).

3.1 A TFF acts as a cofactor of PFP to regulate $\beta\gamma$ -CAT-pathway

TFFs are well conserved from amphibian to human (Zhang et al., 2011). Though their first-hand actions and mechanisms involved remain elusive, they have been implicated in diverse patho-physiological functions including tumorigenesis, metabolism, mucosal healing, immunity and nervous system diseases (Ghanemi et al., 2021; Hoffmann, 2021; Yang et al., 2022). BmTFF3 is a three domain TFF identified in toad *B. maxima*. BmTFF3 binds non-covalently to BmALP1 crystallin domains to form $\beta\gamma$ -CAT (Gao et al., 2011b). The interaction of BmTFF3 with BmALP1 to form soluble PFP complex $\beta\gamma$ -CAT is a regulatory step in the action of the secreted PFP, in which BmTFF3 acts as a chaperon of reduced BmALP1 dimer which is unstable and tend to oligomerization (Bian et al., 2023; Guo et al., 2019; Wang et al., 2020) (**Figure 1 and Figure 2**). Furthermore, BmTFF3 in BmALP1 oligomers may further serve as a regulatory element of BmALP1-channels formed on membranes. Diverse membrane proteins have been proposed as potential TFF receptor(s), which are believe to mediate TFF biological activities via signaling transduction (Braga Emidio et al., 2019). $\beta\gamma$ -CAT provides an alternative model for our understanding of the first-hand action of vertebrate TFFs and the mechanism involved.

3.2 $\beta\gamma$ -CAT is negatively regulated by an af-PFP paralog

There is a conserved cysteine residue at the C-terminal parts of toad *B. maxima* af-PFPs. An endogenous paralog of BmALPs, named BmALP3, is a negative regulator of $\beta\gamma$ -CAT complex assembly (Wang et al., 2020). BmALP3 is characterized of lacking membrane pore formation capacity. Sensing environmental oxygen tension, BmALP3 was readily oxidized to a disulfide bond-linked homodimer, and this BmALP3 homodimer then oxidized BmALP1 to its homodimer and even water-soluble high molecular weight polymer via disulfide bond exchange, leading to the dissociation of $\beta\gamma$ -CAT complex and the loss of its functions. Thus, BmALPs can be reversibly regulated between active and inactive forms by redox reactions (Wang

et al., 2020) (**Figure 2**). Importantly, the C-terminal cysteine residue is highly conserved in numerous af-PFPs from fishes to reptiles, and has been replaced by a serine residue in birds, highlighting the key role of this site in the regulation of vertebrate af-PFPs.

Interestingly, similar redox- and dimerization-based mechanisms have recently been reported in other pore-forming proteins. In 2024—four years after our report of $\beta\gamma$ -CAT redox regulation—cleavage-independent activation of ancient eukaryotic gasdermins was demonstrated. A basal metazoan gasdermin (TrichoGSDM) was shown to exist as a disulfide-linked autoinhibited dimer that can be activated by reduction, whereas a fungal gasdermin (RCD-1) is activated through heterodimerization (Devant et al., 2023; Li et al., 2024). These findings suggest that reversible, cleavage-independent control may represent a broadly conserved strategy in the regulation of diverse pore-forming protein families.

3.3 IgG Fc-binding protein (FCGBP) mediates the assembly of $\beta\gamma$ -CAT

FCGBP is a mucin-like protein containing repeated units composed of vWF, C8 cysteine rich and trypsin inhibitor-like domains, which were found to be well conserved in vertebrates including human beings (Lang et al., 2016). The protein is widely expressed on mucosal surfaces and in external secretions with unknown functional roles (Ehrencrona et al., 2021; Harada et al., 1997; Kobayashi et al., 2002).

Our newly advance in identifying putative positive regulators for $\beta\gamma$ -CAT assembly demonstrated that toad FCGBP possesses the capacity to reduce BmALP1 oxidized by BmALP3 and meanwhile serve as a scaffold for the interaction of BmALP1 with BmTFF3 to form active $\beta\gamma$ -CAT complex (Bian et al., 2023). BmFCGBP reversed the inhibitory effect of BmALP3 on $\beta\gamma$ -CAT complex. In addition, BmFCGBP reduced BmALP1 polymers and served as scaffold to facilitate $\beta\gamma$ -CAT complex formation in the presence of BmTFF3. Furthermore, recombinant BmALP1 formed biological active $\beta\gamma$ -CAT complex in the presence of BmFCGBP and BmTFF3. Finally, toad peroxiredoxin 6 and thioredoxin were found to be able restore and enhance the effect of BmFCGBP on $\beta\gamma$ -CAT assembly (Bian et al., 2023) (**Figure 2**).

3.4 AMP-activated kinase (AMPK) signaling positively regulates the expression and secretion of $\beta\gamma$ -CAT

The AMPK signaling is involved in sensing availability of nutrients and energy,

which is switched on by lack of energy or nutrients. Once activated by falling energy status, AMPK signaling promotes the catabolism of macromolecules as bioenergetic substrates for ATP production (González et al., 2020; Lin and Hardie, 2018). The assays with pharmacological inhibitors (compound C and SBI-0206965) suggested that AMPK signaling controls the expression and secretion of $\beta\gamma$ -CAT under toad cell starvation (Liu et al., 2023). The findings that $\beta\gamma$ -CAT is positively regulated by AMPK signaling further emphasized and supported a notion that the PFP complex is a necessary protein machine in cell nutrient acquisition and metabolic flexibility (Figure 2).

3.5 High dosages of ATP may negatively regulate $\beta\gamma$ -CAT-channel opening

ATP connects anabolism and catabolism but also fuels biological processes (Bonora et al., 2012). $\beta\gamma$ -CAT did not possess ATP hydrolyzing activity. In liposome assays, the presence of ATP did not obviously affect the oligomerization of $\beta\gamma$ -CAT, but the dying release from $\beta\gamma$ -CAT-channels formed in liposomes was inhibited in a dose-dependent manner by ATP (around 1-5 mM), but not by AMP. The results indicated that the normal intracellular contents of ATP could negatively regulate $\beta\gamma$ -CAT-channel opening (Liu et al., 2023). In another word, $\beta\gamma$ -CAT might sense the abundance of ATP to regulate the switch state of its channels. ATP is an energy situation mark within cells. The negative regulation of $\beta\gamma$ -CAT-channels by high concentrations of ATP (1-5 mM) is in consistent with a fact that this PFP complex is secreted from starved cells to promote the uptake of extracellular nutrients in cell responses to energy falling status. Furthermore, the dosage dependent regulation of $\beta\gamma$ -CAT-channels by ATP could lead to the transcellular delivery of nutrients to tissue internal environments like tissue parenchymal cells by the release of nutrient exosomes (Shi et al., 2022) (Figure 1 and Figure 2).

4 Integrated nutrient acquisition and immune defense

Acquiring environmental materials including nutrients, water and oxygen is essential for a living organism, but it simultaneously brings the risk of suffering potential lethal toxins and antigens as well as pathogens (Florsheim et al., 2021). Consequently, there are inherent correlations in nutrient acquisition and host immune defense, and a common origin for immunity and digestion has been proposed

(Broderick, 2015). A complex interplay between metabolism and immune defense is well-documented (Man et al., 2017; Wang et al., 2019).

4.1 Nutrient scavenging PFP $\beta\gamma$ -CAT in antigen presentation

In accordance with the inherent correlations in nutrient acquisition and immune checking and defending, PFP complex $\beta\gamma$ -CAT has also been shown for its roles in toad *B. maxima* immunity. Consistent with its cellular actions along endolysosomal pathways in nutrient acquisition (**Figure 1**), $\beta\gamma$ -CAT promoted antigen (ovalbumin) internalization via driving pinocytosis in murine DCs (Deng et al., 2020), and the effect was confirmed in toad *B. maxima* peritoneal cells (Shi et al., 2022). Furthermore, the PFP complex neutralized the acidification of endocytic organelles to favor antigen presentation, and the channels formed by $\beta\gamma$ -CAT might serve for the translocation of processed OVA peptides to cytosol. In addition, $\beta\gamma$ -CAT stimulated functional exosome release. Collectively, these effects substantially enhanced antigen presentation and eventually led to robust cellular and humoral immune responses (Deng et al., 2020). $\beta\gamma$ -CAT is the first vertebrate-secreted PFP identified to be able to effectively augment antigen presentation by modulating several crucial steps in DC endocytic and exocytic pathways. Notably, subsequent work in 2023 in mammals identified perforin-2 (Mpeg1) as a pore-forming effector mediating antigen escape from endosomes into the cytosol in cross-presenting dendritic cells, thereby ensuring efficient MHC class I antigen presentation (Rodríguez-Silvestre et al., 2023). This highlights a broader theme whereby PFPs can facilitate vesicular transport steps critical for adaptive immunity.

4.2 $\beta\gamma$ -CAT in inflammasome activation

Nutrient acquisition and metabolism are the core processes underlying all biological phenomena. There is a critical role for metabolism in controlling immunity and that inflammation, in turn, has a profound impact on metabolism (Wang et al., 2019; Ye and Medzhitov, 2019). Both nutrient deficiency and over nutrition influence deeply inflammation, and the metabolic status of a cell is a critical determinant of the type and magnitude of the inflammatory response (Furman et al., 2019; Medzhitov, 2021; O'Neill and Hardie, 2013; Rathinam and Fitzgerald, 2016).

$\beta\gamma$ -CAT-pathway (**Figure 1** and **Figure 2**) is proposed to function primarily in

nutrient acquisition and metabolic flexibility. However, the PFP complex was found to activate inflammasome under pathogen infection and/or facing tissue injuring (Gao et al., 2019; Xiang et al., 2014). In toad *B. maxima* peritoneal cells and murine macrophages, $\beta\gamma$ -CAT was rapidly endocytosed with channels formed on endolysosomes. Lysosomal destabilization and cathepsin B release were detected under bacterial challenge, leading to inflammasome activation and subsequent interleukin-1 β maturation and release. These experimental evidences unveiled a fact that a PFP working in cell nutrient acquisition and vesicular delivery and transport can also act as an effector that initiates tissue inflammation and immune defense under unfavorable environments or noxious stimuli.

4.3 Cell nutrient acquisition mediated by PFP and allergic defense

Potential food antigens can be captured from the gut lumen by passing between (paracytosis) or through (transcytosis) epithelial cells. An estimated 2% of proteins pass through the gut epithelium intact and could be transported directly or indirectly to the liver or to secondary lymphoid tissues (Tordesillas et al., 2017; Yu et al., 2016). Allergic immunity has been proposed to play a role in food quality control by mounting allergic defenses against food antigens associated with noxious substances (Florsheim et al., 2021). $\beta\gamma$ -CAT represents a novel PFP system working to drive cell vesicular delivery, mediating the import and/or transcellular transport of macromolecular nutrients including intact proteins (**Figure 1** and **Figure 2**) (Liu et al., 2023; Shi et al., 2022). Excessive and aberrant transport of food antigens into tissue internal environments including lamina propria by delivery systems like $\beta\gamma$ -CAT may stimulate allergic defenses. The knowledge from $\beta\gamma$ -CAT should be useful in our understanding on the potential roles of a secretory PFP in mediating food immune tolerance and/or in mounting allergic defenses against noxious substances.

4.4 Pathogens hijack PFP-driven cell vesicular delivery systems for infection

Many intracellular pathogens, including bacteria, viruses and fungi, exploit cellular endocytic organelles to invade the host cell (Cossart and Helenius, 2014; Gruenberg and Van Der Goot, 2006). It is reasonable to postulate that at least a part of intracellular pathogens has adapted to hijack and profit $\beta\gamma$ -CAT-like PFP systems driven cell vesicular delivery for their invasion and infection in the long co-evolution

processes. In those cases, the $\beta\gamma$ -CAT-like pathways normally working for nutrient acquisition and transport will be kidnapped by the pathogens to promote their infection.

4.5 Cross-species parallels in PFP-mediated nutrient–immune coupling

Recent evidence in mammals further strengthens the view that pore-forming proteins can act as critical coordinators between nutrient acquisition and immune defense. A study reported recently in 2025 in *Immunity* demonstrated that epithelial gasdermin D (GSDMD) in the small intestine not only promoted brush border assembly and lipid absorption but also rewired enterocyte metabolism to support intraepithelial $\gamma\delta$ T lymphocytes, thereby enhancing mucosal barrier function (Yu et al., 2025). This finding resonates with the amphibian $\beta\gamma$ -CAT paradigm, suggesting that different PFP families across evolution may have convergently evolved to couple nutrient uptake with host immunity.

5 Animal $\beta\gamma$ -CAT-like PFPs

Af-PFPs are widely distributed in invertebrate animals, such as green hydra *Chlorohydra viridissima*, worm *Caenorhabditis elegans*, earthworm *Eisenia foetida* and snail *Biomphalaria glabrata* etc. (Galvin et al., 2008; Pinaud et al., 2021; Sekizawa et al., 1997; Sher et al., 2008). In vertebrates, $\beta\gamma$ -CAT-like af-PFPs are well conserved and readily identified from fishes, amphibians, reptiles, and birds as well as egg laying monotreme mammals like platypus (Lima et al., 2021; Zhang et al., 2021).

Recently, the existence of aerolysin-like domains in proteins of Eutheria mammals has been annotated. Several examples of the pore-forming module of aerolysin-type beta-barrel pore-forming proteins have been found in mammalian species. First, in ankyrin 2 (GenBank: KAF6309469.1) of Kuhl's pipistrelle (*Pipistrellus kuhlii*) an aerolysin domain has been annotated. The ANK2 genes encoding ankyrin 2 are highly conserved in vertebrates including human beings. Second, an aerolysin domain has also been annotated in the cystinosin from various species of camels, including those from *Camelus dromedarius* (Arabian camel) (XP_031325380.1, XP_031325379.1) and *Camelus ferus* (Wild Bactrian camel) (XP_032353886.1). Cystinosins are lysosomal transmembrane proteins which facilitate transport of the disulphide amino acid cystine (CySS) from the lysosomes of cells (Kalatzis et al., 2001). They are

highly conserved in mammals including human beings. We believe that there should be more examples showing the existence of aerolysin-like domains in mammalian proteins, which are waiting for further identification.

It is worthy of pointing out that the $\beta\gamma$ -crystallin domains harboring in BmALP N-terminal part are homologous to mammalian non-lens $\beta\gamma$ -crystallin proteins, such as those of human absent in melanoma 1 (AIM1) and homologous gene products (Liu et al., 2008). Interestingly, proteins encoded by AIM1 gene contain several $\beta\gamma$ -crystallin domains at their N-terminal parts with a C-terminal domain homologous to ricin B chain (Ray et al., 1997). Ricin belongs to the AB-type of protein toxins, similar to bacterial AB toxins (Sowa-Rogozinska et al., 2019). The protein compositions of some human AIM1 gene products are similar to that of $\beta\gamma$ -CAT α -subunit BmALP1 (also see the following section), and knowledge from $\beta\gamma$ -CAT may serve as clues for our understanding on the actions and mechanisms inside of AIM 1 genes and their patho-physiological relevance.

In addition to these vertebrate and mammalian examples, our recent studies have also extended the physiological relevance of af-PFPs to the nematode *Caenorhabditis elegans*. The aerolysin-like pore-forming protein LIN-24, similar to $\beta\gamma$ -CAT in its contribution to antimicrobial defense (Zhang et al., 2023), has also been shown to enhance survival under starvation stress by promoting lipid mobilization, preserving muscle integrity, and inducing mitochondrial remodeling into energy-efficient donut-shaped forms (Lan et al., 2025). These findings provide direct evidence that af-PFPs can regulate metabolic flexibility and tissue homeostasis in invertebrates, highlighting their conserved roles across **evolution** beyond immune defense. And in the jawless vertebrate lamprey, the pore-forming protein Natterin is highly expressed in granulocytes and localizes to lipid droplets, and transgenic expression of Natterin induces browning of white adipose tissue in mice, suggesting an immunometabolic role analogous to that of $\beta\gamma$ -CAT in amphibians and LIN-24 in *C.elegans* (Pang et al., 2025).

6 Comparison of af-PFPs with other family PFPs

6.1 af-PFPs are different from MACPF/CDC and gasdermins

Various families of PFPs documented now can form pores (channels) with diverse

pore sizes, with the largest known pores of MACPF/CDC proteins with diameters of 25–40 nm (Lukoyanova et al., 2016; Tweten et al., 2015). Depending on the size and biochemical property of the pore, different substances pass through, such as ions, small molecules or large molecules including proteins (Benton and Bayly-Jones, 2021; Dal Peraro and Van Der Goot, 2016). Remarkably, the 10–15 nm diameters of the gasdermin pores are large enough to allow the passage of interleukin-1 β and interleukin-18, which have a protein size around 5.0 nm (Evavold et al., 2018; Kovacs and Miao, 2017). Generally speaking, $\beta\gamma$ -CAT differs from these MACPF and gasdermin PFPs in the following several aspects (**Figure 3**):

First, as a member of af-PFPs, $\beta\gamma$ -CAT forms membrane channels with a functional diameter about 1.5–2.0 nm (Liu et al., 2021; Liu et al., 2008), similar to other members of af-PFPs including aerolysin (Degiacomi et al., 2013; Jia et al., 2016). Second, the activation of complements, perforins and gasdermins is mainly depending on proteolytic cleavage by proteases, which is an irreversible process. In contrast, $\beta\gamma$ -CAT is reversibly regulated by redox reactions (Bian et al., 2023; Wang et al., 2020). Although later studies reported that GSDMD can also be influenced by C-terminal cysteine modifications (Devant et al., 2023; Li et al., 2024), this modification does not replace its predominant, irreversible proteolytic activation. Third, though several MACPF proteins have been suggested to play roles in embryonic development and in neural migration with enigmatic mechanisms (Lukoyanova et al., 2016), the cellular action of MACPF and gasdermin PFPs is mainly focus on cell death induction, playing important roles in host inflammation and immunity systems (Broz et al., 2020; Krawczyk et al., 2020). Distinctively, af-PFP member $\beta\gamma$ -CAT defines a novel PFP system driving cell vesicular delivery, playing essential roles in toad *B. maxima* physiology (**Figure 1**).

6.2 af-PFPs are different from short membrane active peptides (MAPs)

A variety of biologically active peptides with low-molecular weights exert their functions through direct interactions with the lipid membrane of cells, either to disrupt it and lead to cell lysis or to translocate through it to deliver cargo into the cell and reach their targets (Avcı et al., 2018; Last et al., 2013). These MAPs, including antimicrobial peptides (AMPs), cell penetrating peptides (CPPs) and amyloid peptides, are either discovered from natural resources including microbes, plants and animals (Chen et al., 2017; Lazzaro et al., 2020) or synthesized artificially under rational

design (Peraro and Kritzer, 2018). CPPs can deliver themselves and attached cargo into cells, which lack cell specificity and have a short duration of action (Liu and Afshar, 2020; Peraro and Kritzer, 2018). To improve the efficacy of endosomal cargo escape, these molecules were designed to switch their structures in endosomes and lyse endosomal membranes (Akishiba et al., 2017; Arafles et al., 2020).

In toad *B. maxima* skin secretions, there are diverse AMPs identified (Lai et al., 2002; Lee et al., 2005a; Lee et al., 2005b). Meanwhile, af-PFPs and TFFs are major protein components (Wang et al., 2020; Zhao et al., 2022). $\beta\gamma$ -CAT differs from MAPs in the following aspects (**Figure 3**): First, $\beta\gamma$ -CAT is a complex protein machine with meticulous regulatory elements. Second, $\beta\gamma$ -CAT has cell selectivity, and it targets sensitive cells with a double receptor-binding model (Guo et al., 2019). Third, $\beta\gamma$ -CAT is capable of driving cell vesicular delivery of extracellular materials and/or plasma membrane components (**Figure 1**). Fourth, in contrast to forming stable and controllable membrane channels by $\beta\gamma$ -CAT for cargo exchange, AMPs and CPPs often make rupture of membrane organelles (Liu et al., 2021). Obviously, $\beta\gamma$ -CAT and similar SELC proteins may have been **evolved** in nature to fulfill cell material (nutrients, water or antigens etc.) vesicular delivery and transport in a selective, efficient and regulatory manner (**Figure 1** and **Figure 2**), which can hardly be achieved by simple peptides (**Figure 3**).

6.3 Comparison with bacterial AB toxins

For their survival and effective infection in hosts, pathogenic bacteria develop a strategy to deliver selected enzymes (effector proteins) into the cytosol of host cells to facilitate pathogen invasion. The delivery of an effector protein often requires specialized machinery to enable the effector protein to cross host membrane barrier. Thus, bacteria produce AB toxins to combine two functional elements (Collier, 2009; Mesquita et al., 2020; Piot et al., 2021). Plants also develop similar protein machines, and ricin is the most well-known example (Sowa-Rogozinska et al., 2019). The B part of bacterial AB toxin is usually a PFP belonging to various protein families, which serves for the translocation of A part (toxin) to cytosol (**Figure 4**). For example, bacterial anthrax toxin, botulinum and tetanus neurotoxins respond to endosome acidification by inserting a channel into the membrane of the early endosome, mediating the direct passage of active A parts cross the endosomal membrane (Abrami et al., 2013; Machen et al., 2021; Pirazzini et al., 2016; Van Der Goot and

Young, 2009). Thus, the B parts of bacterial AB toxins can be viewed as specialized bacterial SELC proteins.

Comparatively, we proposed that $\beta\gamma$ -CAT in toad *B. maxima* can be recognized as a cellular delivering system comprising of X, B and C parts, in which the two subunits of $\beta\gamma$ -CAT complex correspond to B (α -subunit BmALP1) and C (β -subunit BmTFF3) parts of the system, respectively. BmALP1 is a PFP and the additional C part is a chaperon and regulatory accessory of the B part in the toad system (**Figure 4**). Different from bacterial AB toxins, the X part of the toad cellular delivering system can cover a wider range of materials including albumin or albumin-bound FA, water with ions or antigens etc. depending on cell contexts and surroundings (Deng et al., 2020; Liu et al., 2023; Shi et al., 2022; Zhao et al., 2022).

Bacterial AB toxins are exogenous protein components that target host cells as virulence factors, while toad *B. maxima* $\beta\gamma$ -CAT is a vertebrate endogenous physiological system (**Figure 4**). At first glance, these protein systems from prokaryotic and eukaryotic organisms are different from each other in their molecular composition, regulatory and activation patterns, cell targets, cellular acting pathways, and eventually patho-physiological relevance. However, the fundamental similarity shared by these prokaryotic and eukaryotic protein systems is that they work as PFP systems driving cell vesicular delivery, and the common feature of these systems is the presence of a PFP belonging diverse PFP families.

6.4 PFPs from diverse protein families can work in PFP systems driving cell vesicular delivery

Secretory (soluble) PFPs, which act to deliver protein components via endolysosomal pathways, have been determined in bacterial AB toxins (Abrami et al., 2013; Mesquita et al., 2020), a specialized subclass of cellular PFP delivering systems. $\beta\gamma$ -CAT from toad *B. maxima* is a nice example, showing vertebrate af-PFPs may have been evolved to work in the vesicular delivery in cells. The practice and success of these PFPs in delivering cargo molecules into cytosol indicate that PFPs from different protein families (including α -helices and β -barrels PFPs) can work in mediating cell vesicular delivery. These PFP machines possess the capacity to traverse complex cell membrane barrier in vesicular forms along endocytic and exocytic pathways for delivery extracellular components to cytosol and for transcellular transport of environmental cargo molecules to internal tissues, which

should be widely distributed in eukaryotic organisms.

Concluding remarks

Cell membranes with their selective permeability are crucial for the functional compartmentalization and survival of cells, which form barriers for molecule exchange between the cytosol and the extracellular environment. Our findings define a novel amphibian PFP system driving cell vesicular delivery composed of PFP and TFF complex $\beta\gamma$ -CAT and its regulatory network, which functions to drive and mediate the vesicular transport of extracellular materials and/or cell membrane components, playing essential roles in nutrient and water acquisition as well as immune defense in toad *B. maxima* (**Figure 1** and **Figure 2**). $\beta\gamma$ -CAT is a model molecule for af-PFPs in toad *B. maxima*. There should be a group of af-PFPs in the toad. The other af-PFP members or $\beta\gamma$ -CAT homologous may play similar and/or distinct cellular actions with corresponding physiological relevance in the toad.

Nutrient intake is essential for life. During evolution, toxin production has emerged as a competitive strategy to secure nutrients, a process that shares significant similarities with the development of immune defense (Dal Peraro and Van Der Goot, 2016; Zhang, 2015). Accordingly, these complex traits in living organisms are inherently correlated (**Figure 5**). Traditionally, PFPs have long been recognized as either pore-forming toxins for microbial infection or host immune defense effectors. Knowledge from $\beta\gamma$ -CAT provides a new paradigm for PFP-driven cellular transport processes, demonstrating critical roles in nutrient acquisition and metabolic flexibility. Importantly, many of these principles uncovered from amphibian studies have subsequently been echoed in other organisms: cleavage-independent redox regulation of gasdermins in basal metazoans (Li et al., 2024), perforin-2-mediated endosomal escape for antigen cross-presentation in mammals (Rodríguez-Silvestre et al., 2023), epithelial GSDMD coupling nutrient absorption with $\gamma\delta$ T cell-mediated barrier defense in the small intestine (Yu et al., 2025), and the nematode af-PFP LIN-24 enhancing starvation resilience through lipid metabolism and mitochondrial remodeling (Lan et al., 2025). These cross-species findings support the notion that PFP systems are broadly conserved and versatile strategies for coordinating nutrient acquisition with immune defense. From a comparative perspective, $\beta\gamma$ -CAT/SELG provides a framework for considering how secreted pore-forming modules can be repurposed across organisms for regulated vesicular transport in nutrition, immunity,

and environmental adaptation. Furthermore, intelligence (sensing and nerve systems) and body power are essential biological traits evolved for food (nutrients) acquisition along long-term evolution processes (Zhang, 2015), and the roles of PFPs in the origination and development of these important biological traits are perspective and waiting for being investigated (**Figure 5**).

Thus, PFP systems should be regarded as fundamental to living organisms, shaping cell–environment interactions and enabling evolutionary adaptation. The $\beta\gamma$ -CAT pathway proposed here (**Figures 1 and 2**) represents one such framework, illustrating how af-PFPs can mediate nutrient acquisition, water balance, and immune defense. In long-term evolution, PFPs from different protein families may substitute for or complement each other to fulfill these essential roles. These findings also raise the possibility that regulated pore-forming protein systems may have broader relevance beyond amphibians. In mammals, endogenous pore-forming effectors such as gasdermins are central to inflammatory signaling and have been implicated in multiple inflammatory and metabolic disorders. Conceptually, $\beta\gamma$ -CAT/SELC offers a tractable model to explore how pore formation can be coupled to vesicular trafficking and nutrient handling under physiological regulation. A key future challenge is to identify additional analogous regulated PFP systems in mammals, especially humans, and to define their mechanisms and contributions to metabolic regulation, immune homeostasis, and disease pathophysiology.

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Dr. Yun Zhang, the first author of this work, passed away in March 2023. He devoted much of his career to the study of af-PFPs, and this manuscript was largely written by him. Since his passing, related studies from both our group and others have further substantiated the mechanisms he long investigated. This article is presented in memory of his contributions and with the collective respect of all co-authors, his students, and collaborators.

Conflict of interest

We declare that we have no conflicts of interest.

Author contributions

Y.Z., Y.X., W.H.L., S.A.L., Z.H.S., L.Z.L, X.L.B. and QQ Wang conceived and conceptualized the study; Y.Z. and J.R.Z wrote the manuscript. Y.X., W.H.L. and S.A.L. critically revised and edited the manuscript.

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Figures and figure legends

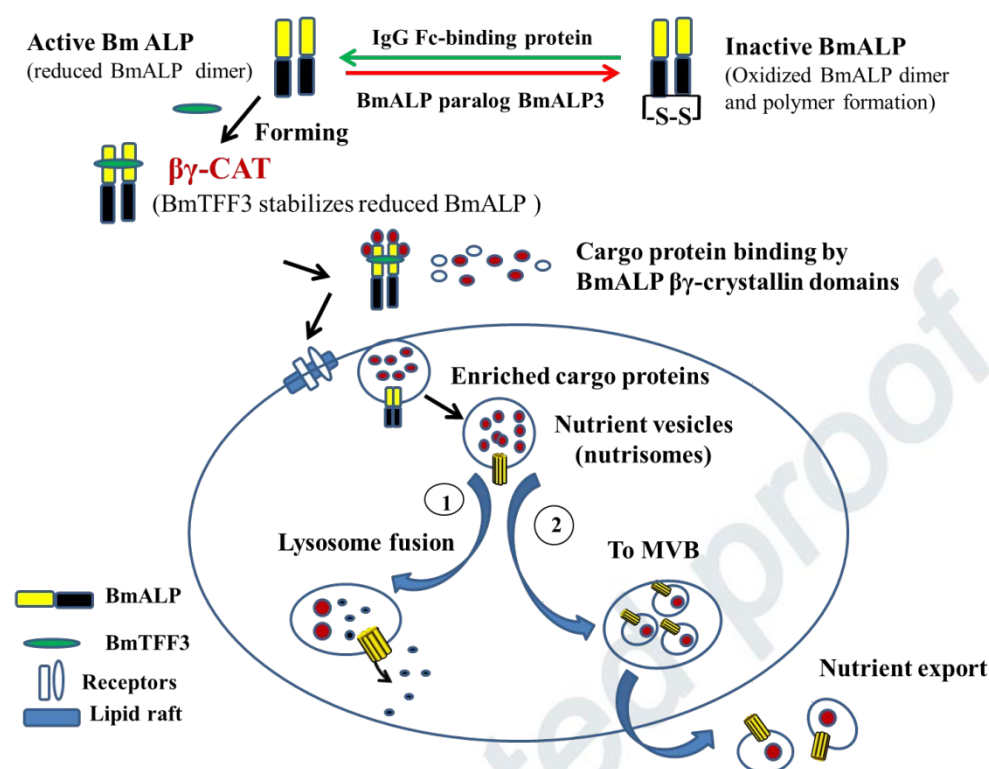


Figure 1 Toad *B. maxima* $\beta\gamma$ -CAT defines a PFP system working to drive cell vesicular delivery and its roles in nutrient acquisition. Under cell starvation or in animal responses to fasting, toad cells secrete $\beta\gamma$ -CAT to drive the uptake, intracellular vesicular delivery and transcellular transport of extracellular macromolecular nutrients (like albumin, ovalbumin and FAs etc.) through endolysosomal pathways (Liu et al., 2023; Shi et al., 2022). Toad BmALPs of af-PFP family can be converted between the active form (either homodimer or heterodimer stabilized by BmTFF3) and inactive form (dimer linked by a disulfide bond) by specific redox regulation (Bian et al., 2023; Wang et al., 2020). $\beta\gamma$ -CAT (BmALP and BmTFF3 complex) possesses nutrient cargo selectivity via selective binding of BmALP N-terminal $\beta\gamma$ -crystallin domains to nutrient molecules (like albumin etc.) (Liu et al., 2023). Afterwards, this PFP complex drives the formation of intracellular vesicles (containing enriched nutrients, called nutrisomes) with the PFP-channels formed on the organelles to facilitate the uptake and delivery of the nutrients (Liu et al., 2023; Shi et al., 2022; Zhao et al., 2022). Depending on cell contexts and surroundings, the nutrisomes can be further developed into two ways. (1) Under cell starvation, they are fused with lysosomes and the cargo molecules are processed

and/or hydrolyzed. The resulted small molecular weight nutrient products can be released through $\beta\gamma$ -CAT-channels to cytosol for cell nutrient supply (Liu et al., 2023). (2) Alternatively, in animal responses to fasting the $\beta\gamma$ -CAT-containing nutrisomes can be matured into multi-vesicular bodies (MVB) by unknown mechanisms, leading to the release of nutrient enriched exosomes and transcellular transport of cargo molecules (including proteins or FAs with a carrier) to support the survival of underlying parenchymal cells via nutrient supply (Liu et al., 2023).

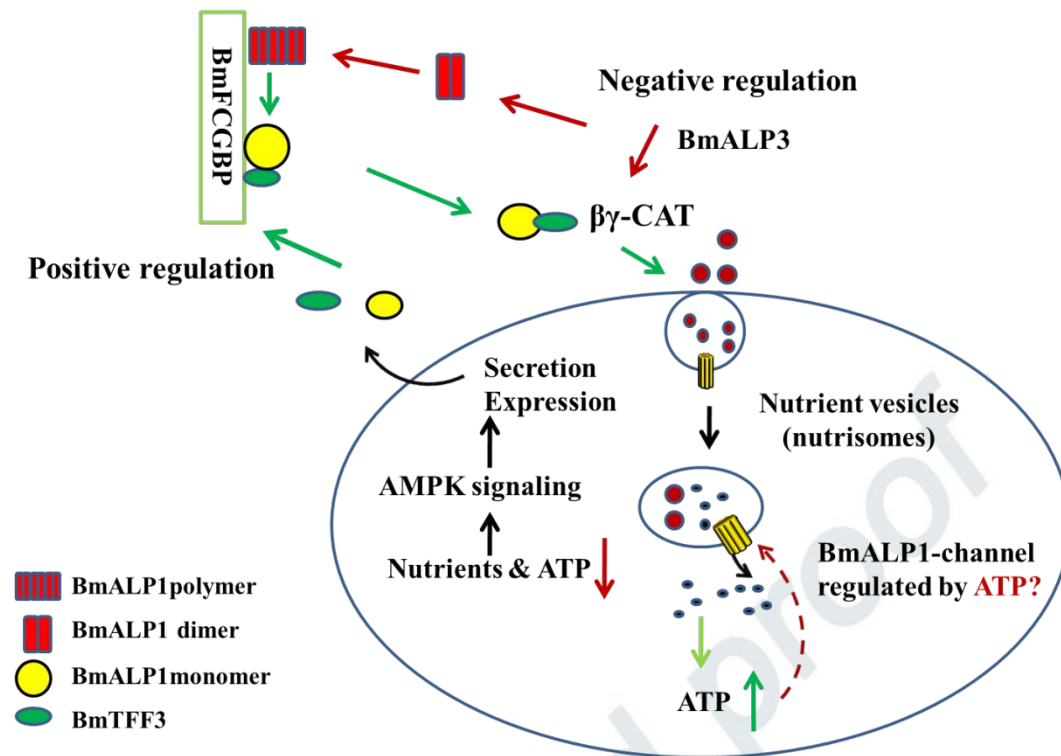


Figure 2 The regulation of $\beta\gamma$ -CAT system. In extracellular surroundings, secreted α -subunit of $\beta\gamma$ -CAT BmALP1 can be reversibly converted between inactive or active forms by specific negative or positive regulators in response to the variation of environmental cues. BmALP3, a paralog of BmALP1, negatively regulates $\beta\gamma$ -CAT assembly by oxidizing BmALP1 (Wang et al., 2020), while BmFCGBP mediates the reduction of BmALP1 homodimer and acts as a scaffold to facilitate the assembly of BmALP1 with BmTFF3 to form $\beta\gamma$ -CAT (Bian et al., 2023). AMPK signaling senses the deficiency of energy and nutrients and positively regulated the expression and secretion of $\beta\gamma$ -CAT subunits. ATP within normal intracellular contents (1-5 mM) may negatively regulate $\beta\gamma$ -CAT-channel opening (Liu et al., 2023).

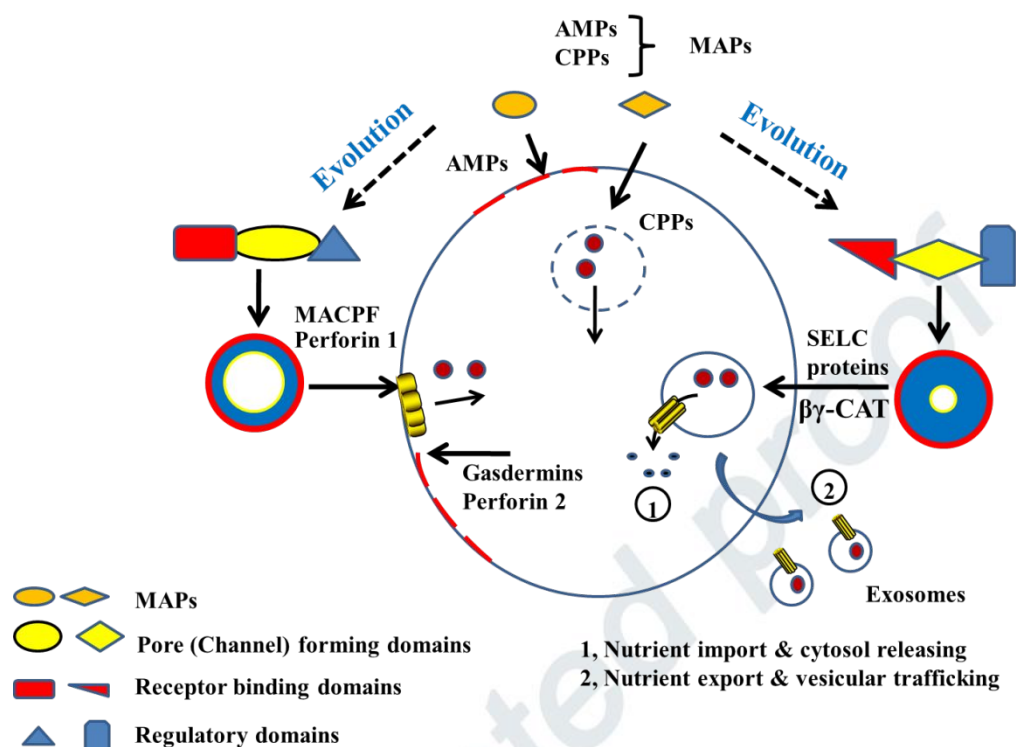
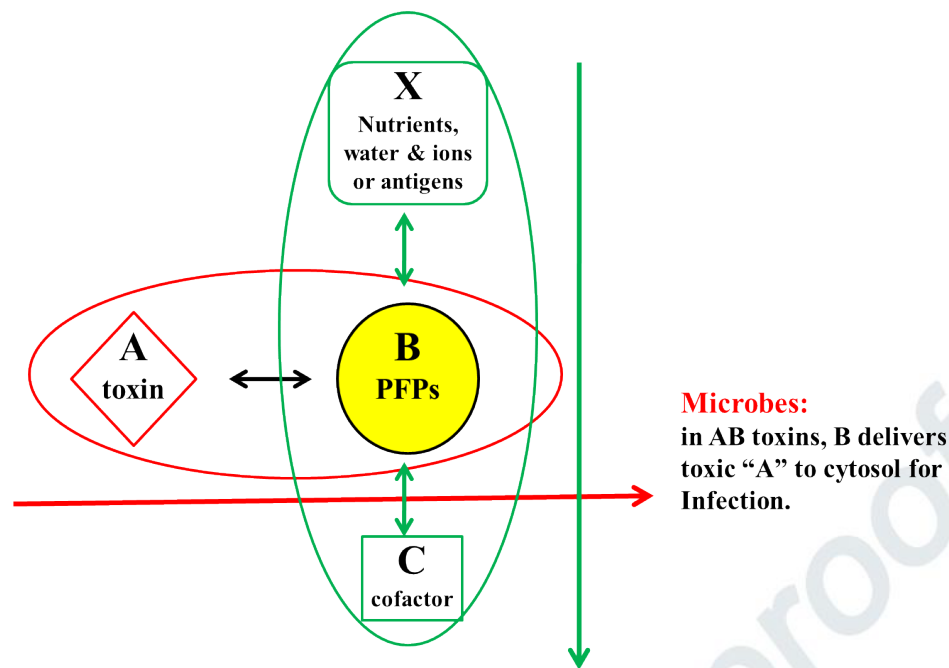


Figure 3 Comparison of PFPs and MAPs. Short MAPs including AMPs and CPPs can be naturally occurred or artificially synthesized (Avci et al., 2018; Last et al., 2013). They lack specific receptor binding and regulatory domains. PFPs normally contain both the receptor binding and regulatory domains (Benton and Bayly-Jones, 2021; Dal Peraro and Van Der Goot, 2016). PFPs of MACPF and gasdermin families form membrane pores with inner diameters (> 10 nm), and these PFPs mainly act on cell plasma membrane as immune effectors via cell death induction either extracellularly (MACPF) or intracellularly (gasdermins) (Broz et al., 2020; Kovacs and Miao, 2017; Lukoyanova et al., 2016). In contrast, af-PFPs form small size channels (1-2 nm) (Degiacomi et al., 2013; Liu et al., 2021; Liu et al., 2008). $\beta\gamma$ -CAT, a member of af-PFPs, forms stable channels on endolysosomes (Xiang et al., 2014; Zhao et al., 2022). $\beta\gamma$ -CAT drives the cell vesicular delivery of materials (like nutrients, water and ions or antigens etc.) (Liu et al., 2023; Shi et al., 2022; Zhang et al., 2021; Zhao et al., 2022). Depending on cell contexts and surroundings, $\beta\gamma$ -CAT can also play roles in immune defense (Deng et al., 2020; Gao et al., 2019; Li et al., 2017; Liu et al., 2021; Xiang et al., 2014). In nature, it is believed that PFPs may be evolved from short MAPs in the long term evolution process. Only animal β -barrel

PFPs are shown here.

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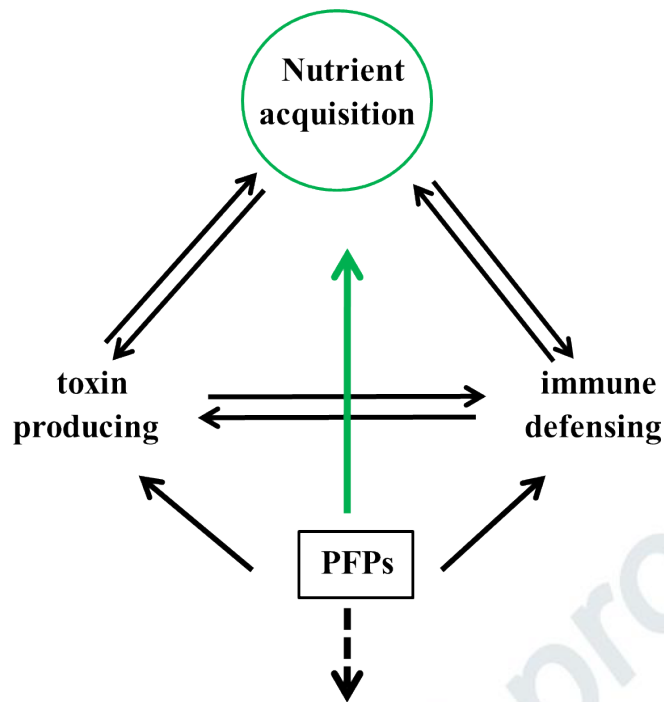




Metazoan: proposed toad *B. maxima* XBC delivering system $\beta\gamma$ -CAT.
B and C drive cell vesicular delivery of materials “X”.

Figure 4 Comparison of bacterial AB toxins and vertebrate toad *B. maxima* $\beta\gamma$ -CAT, two PFP-mediated systems for delivering functional elements into cells.

Bacterial AB toxins are a family of microbial toxins that consist of two components: a toxic part (A) for the catalytic activity of the toxin and a PFP delivery part (B) escorting the A to cytosol (Collier, 2009; Mesquita et al., 2020; Piot et al., 2021). We proposed a vertebrate XBC delivering system, in which toad *B. maxima* PFP complex $\beta\gamma$ -CAT correspond to B (α -subunit BmALP1) and C (β -subunit BmTFF3) parts, respectively. The toad PFP complex (B with C) works to deliver (X) part including macromolecular nutrients, water with ions or antigens etc. (Figure 1 and 2) into cells and/or crossing cells. PFPs (B parts) are the central elements shared by these two cell delivery systems are found in microbes (prokaryotes) and vertebrates (eukaryotes).



Intelligence (sensing & nerve systems) and body power development ?

Figure 5 Inherent correlations among nutrient acquisition, immune defense and toxins in living organisms. Nutrient acquisition is central to a life. This primary biological process shares inherent correlations with immune defense and toxin production in living organisms, including mutual similarity, interaction and coordination. Knowledge from toad *B. maxima* $\beta\gamma$ -CAT uncovered the role of PFPs in cellular nutrient acquisition and vesicular trafficking (green arrow). Thus, PFPs are involved in all these basic biological traits owing to the existence of cellular membrane systems. Furthermore, intelligence (sensing and nerve systems) and body power have been evolved for food (nutrients) acquisition along evolution processes (Zhang, 2015), and the roles of PFPs in the development and differentiation of these complex biological traits are perspective.