Biochimica et Biophysica Acta 1837 (2014) 857–879

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Review

Eukaryotic V-ATPase: Novel structural findings and functional insights

Vladimir Marshansky [a,b,c,*], John L. Rubinstein [d,e,f], Gerhard Grüber [g,h]

[a] Center for Systems Biology, Program in Membrane Biology, Division of Nephrology, Simches Research Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA
[b] Department of Medicine, Harvard Medical School, Boston, MA 02114, USA
[c] Kadmon Pharmaceuticals Corporation, Alexandria Center for Life Science, 450 East 29th Street, New York, NY 10016, USA
[d] Molecular Structure and Function Program, The Hospital for Sick Children Research Institute, University of Toronto, Toronto, ON M5G 1X8, Canada
[e] Department of Biochemistry, University of Toronto, Toronto, ON M5G 1X8, Canada
[f] Department of Medical Biophysics, University of Toronto, Toronto, ON M5G 1X8, Canada
[g] Nanyang Technological University, Division of Structural Biology and Biochemistry, School of Biological Sciences, Singapore 637551, Republic of Singapore
[h] Bioinformatics Institute, A*STAR, 60 Nanyang Drive, Singapore 637551, Republic of Singapore

ARTICLE INFO

Article history:
Received 20 July 2013
Received in revised form 25 December 2013
Accepted 27 January 2014
Available online 4 February 2014

Keywords:
Eukaryotic V-ATPase
Rotary proton-pumping nano-motor
Trafficking via endocytotic and exocytotic pathways
Regulation of cellular receptors function
Cytohesin-2/Arf's signaling
pH sensor

ABSTRACT

The eukaryotic V-type adenosine triphosphatase (V-ATPase) is a multi-subunit membrane protein complex that is evolutionarily related to F-type adenosine triphosphate (ATP) synthases and A-ATP synthases. These ATPases/ATP synthases are functionally conserved and operate as rotary proton-pumping nano-motors, invented by Nature billions of years ago. In the first part of this review we will focus on recent structural findings of eukaryotic V-ATPases and discuss the role of different subunits in the function of the V-ATPase holocomplex. Despite structural and functional similarities between rotary ATPases, the eukaryotic V-ATPases are the most complex enzymes that have acquired some unconventional cellular functions during evolution. In particular, the novel roles of V-ATPases in the regulation of cellular receptors and their trafficking via endocytotic and exocytotic pathways were recently uncovered. In the second part of this review we will discuss these unique roles of V-ATPases in modulation of function of cellular receptors, involved in the development and progression of diseases such as cancer and diabetes as well as neurodegenerative and kidney disorders. Moreover, it was recently revealed that the V-ATPase itself functions as an evolutionarily conserved pH sensor and receptor for cytohesin-2/Arf-family GTP-binding proteins. Thus, in the third part of the review we will evaluate the structural basis for and functional insights into this novel concept, followed by the analysis of the potentially essential role of V-ATPase in the regulation of this signaling pathway in health and disease. Finally, future prospects for structural and functional studies of the eukaryotic V-ATPase will be discussed.

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1. Introduction

The eukaryotic V-type ATPase (V-ATPase) is a multi-subunit membrane protein complex that functions as a rotary proton-pumping nano-motor. The structure of the V-ATPase is similar to that of the F-ATP synthase found in the inner mitochondrial membranes of eukaryotes and the plasma membranes of eubacteria. It is also closely related to the A-ATP synthase found in the plasma membranes of archaea and some eubacteria [1–7]. They all have in common a membrane-bound sector $V_O/A_O/F_O$, with the "$_O$" indicating the oligomycin sensitive sector, which has been described for eukaryotic F-ATP synthases [8]. This membrane-integrated sector consists of the motor and ion channel. The $V_1/A_1/F_1$-sectors, which historically are named according to the soluble factor (F_1) of the beef heart mitochondrial F-ATP synthase, contain the ATPase/ATP synthase catalytic sites [9]. As indicated by the bipartite names, these ATPases/ATP synthases are evolutionarily related, functionally conserved and operate as rotary proton-pumping nano-motors, invented by Nature billions of years ago [1,10–18].

The eukaryotic V-ATPase is a 900 kDa membrane-intrinsic protein complex consisting of multiple subunits called: A, B, C, D, E, F, G, H, *a*, *c*, *c′*, *c″*, *d* and *e* that are distributed in two sectors called V_1 and V_O (Table 1). A fundamental feature exclusive to V-ATPases is the reversible assembly/disassembly of the V_1 and V_O sectors. Originally, this important mechanism was observed in response to ceased feeding in *Manduca sexta* (*M. sexta*) [19,20] and in response to glucose depletion in *Saccharomyces cerevisiae* (*S. cerevisiae*) [21–23]. Notably, neither disassembly nor reassembly of V-ATPase requires new protein synthesis. The relative scarcity of the sample and its tendency to disassemble into V_1 and V_O sectors has so far prevented crystallization of the intact enzyme. Instead, a hybrid approach to the structural analysis of the

* Corresponding author at: Center for Systems Biology, Program in Membrane Biology, Division of Nephrology, Simches Research Center, Massachusetts General Hospital and Department of Medicine, Harvard Medical School, CPZN No. 8212, 185 Cambridge Street, Boston, MA 02114, USA. Tel.: +1 617 724 9815, Kadmon Pharmaceuticals Corporation, Alexandria Center for Life Science, 450 East 29th Street, New York, NY, 10016, USA, Tel.: +1 646 666 7960.
E-mail addresses: Vladimir_Marshansky@hms.harvard.edu, Vladimir.Marshansky@kadmon.com (V. Marshansky).

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http://dx.doi.org/10.1016/j.bbabio.2014.01.018

Table 1
Homology of subunit isoforms and the corresponding genes of V-ATPase, A-ATP synthase and F-ATP synthase. The table shows the list of the V_1-sector subunits, followed by the subunits of V_O sector and, finally, by the accessory subunits. The subunits that form the "stators" of these nano-motors are shown in black, while "rotor" forming subunits are indicated in red. The F-subunits of V-ATPase and A-ATP synthase are homologues in their 3D structure and different to the ε—subunit of F-ATP synthase. However, all of these subunits are proposed to be involved in the coupling mechanisms between the catalytic headpieces and the membrane-embedded parts of these enzymes. The homology of subunit G of V-ATPases and A-ATP synthases to the bacterial *b* subunit of F-ATP synthases is based on the sequence similarity of their soluble domain. The homology of a-subunits of the V-ATPases and A-ATP synthases to the a-subunit of F-ATP synthases is related to their functional role in ion-pumping activity. However, a-subunits of V-ATPases and A-ATP synthases are ~100 kDa proteins compared to the ~25 kDa membrane-embedded a-subunit of F-ATP synthases.

Eukaryotic V-ATPase V1 Vo				***A-ATP synthase A1 Ao***	***F-ATP synthase F1 Fo***
Human		***Yeast***			
Subunit isoform	Gene	Subunit isoform	Gene	Subunit isoform	Subunit isoform
V1 sector					
A	ATP6V1A	**A**	VMA1	**A**	β
B1	ATP6V1B1	**B**	VMA2	**B**	α
B2	ATP6V1B2	–	–	–	–
C1	ATP6V1C1	**C**	VMA5	–	–
C2	ATP6V1C2	–	–	–	–
D	ATP6V1D	**D**	VMA8	**D**	γ
E1	ATP6V1E1	**E**	VMA4	**E**	–
E2	ATP6V1E2	–	–	–	–
–	–	–	–	–	δ
F	ATP6V1F	**F**	VMA7	**F**	ε
G1	ATP6V1G1	**G**	VMA10	**G**	**b**
G2	ATP6V1G2	–	–	–	–
G3	ATP6V1G3	–	–	–	–
H	ATP6V1H	**H**	VMA13	–	–
Vo sector					
a1	ATP6V0A1	**a**	VPH1/STV1	**a**	**a**
a2	ATP6V0A2	–	–	–	–
a3	ATP6V0A3	–	–	–	–
a4	ATP6V0A4	–	–	–	–
d1	ATP6V0D1	**d**	VMA6	**C**	–
d2	ATP6V0D2	–	–	–	–
c	ATP6V0C	**c**	VMA3	**c**	**c**
–		**c′**	VMA11	**c′**	–
c″	ATP6V0B	**c″**	VMA16	**c″**	–
e	ATP6V0E	**e**	VMA9	–	–
Accessory subunits					
Ac45	ATP6VAP1	–	–	–	–
M8-9	ATP6VAP2	–	–	–	–

V-ATPase has proven most successful. Single particle electron cryo-microscopy (cryo-EM) provides low-resolution maps of the intact enzyme, while X-ray crystallography and NMR spectroscopy provide atomic models of individual subunits and subcomplexes. Thus, in the first part of this review we will focus on the novel structural findings from cryo-EM of holocomplexes and X-ray crystallography/NMR spectroscopy of subunits recently determined for eukaryotic V-ATPases from *M. sexta* and *S. cerevisiae*. Based on this hybrid approach, we will also analyze the general principles and functional insights into the regulation of V-ATPase's primary function as a proton-pumping rotary nano-motor.

In spite of the structural and functional similarities between rotary ATPases, the eukaryotic V-ATPases are the most complex nano-motors that have acquired some unconventional cellular functions during evolution. In particular, during the last decade unconventional roles of the V-ATPases in the regulation of cellular receptors and their trafficking via endocytotic and exocytotic pathways were uncovered. Thus, in the second part of this review we will discuss these novel roles of V-ATPases in modulation of function of cellular receptors that are involved in the development and progression of diseases such as cancer and diabetes as well as neurodegenerative and kidney disorders. Moreover, we have recently described that the V-ATPase also functions as an evolutionarily conserved pH sensor and receptor for cytohesin-2/Arf-family GTP-binding proteins. Thus, in the final part of this review we will evaluate the structural basis for and functional insights into this novel concept, followed by the analysis of the potentially essential role of V-ATPase in the regulation of this signaling pathway in health and disease.

2. Structural insights into the eukaryotic V-ATPase: from moderate to high-resolution structures

2.1. The structure of the eukaryotic V_1V_O ATPase

2.1.1. Overall structure of the V_1V_O holocomplex

Eukaryotic V-ATPases are multiprotein complexes that consist of 14 different subunits $A_3B_3CDE_3FG_3Hac_Xc'_Yc''_Zde$, where the stoichiometry (X,Y,Z) of the c, c′, and c″-subunits are not known. Many of these subunits are present in multiple isoforms [1,3,4]. The V-ATPase has a bipartite structure consisting of a soluble cytoplasmic V_1-sector (subunits $A_3B_3CDE_3FG_3H$) and a membrane-integrated V_O sector (subunits *a*, *c*, *c*′, *c*″, *d*, and *e*), which together form the V_1V_O holocomplex (Table 1) (Fig. 1A–D). Electron microscopy (EM) image analysis [5,24–30] and small-angle X-ray scattering (SAXS) [28,31,32] have provided a general outline for the structural organization of the V_1 and V_O sectors as well as the assembled V_1V_O holocomplex (Fig. 1A–D). Both sectors are linked by connecting regions that are important for coupling proton translocation in V_O with ATP hydrolysis in V_1, and are involved in regulating the activity of the enzyme by reversible disassembly. These

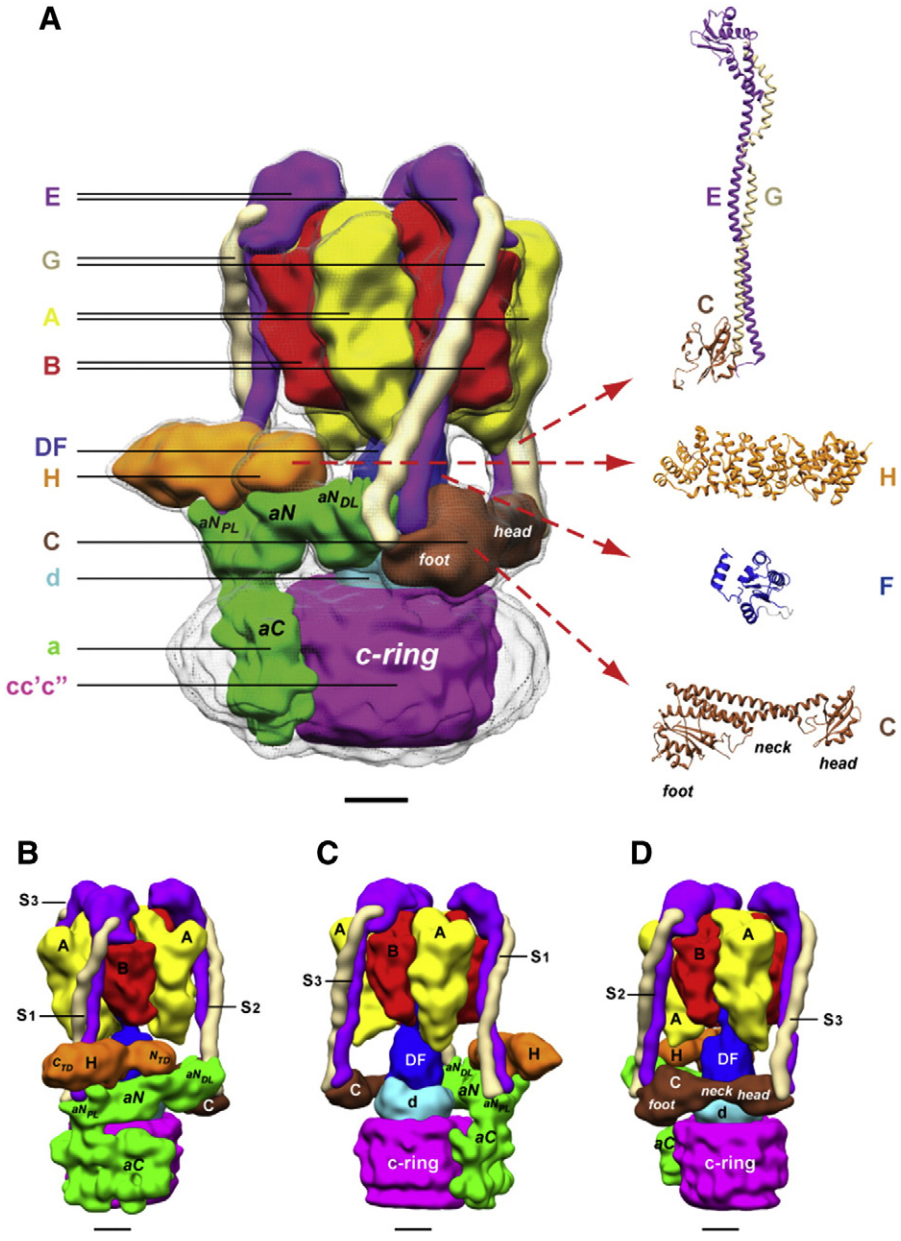

Fig. 1. Summary of current structural information for the eukaryotic V-ATPase. A) The structure of the *S. cerevisiae* V-ATPase was determined from single particle cryo-EM at ~11 Å resolution. Most of the subunits of the complex could be identified in this map, including subunits A (yellow), B (red), D and F (blue), d (light blue), E (purple), G (beige), H (orange), C (brown), a (green), and the cc′c″ ring (pink). Crystal structures are known for the H-subunit (orange) [61], C-subunit (brown) [58], the EG&C-head subcomplex (purple, beige and brown) [57] and F-subunit (blue) [63]. B) The structure of the *S. cerevisiae* V-ATPase after ~90° of counter clockwise rotation of the image shown in Panel A. The positions of three peripheral stalks are indicated as S1, S2 and S3, respectively. However, while the peripheral stalks S1 and S2 are clearly seen, the peripheral stalk S3 is hidden behind A_3B_3 hexamer. C) The structure of the V-ATPase after a ~180° of counter clockwise rotation of the image is shown in Panel A. The positions of peripheral stalks S1 and S3 are indicated. D) The structure of the V-ATPase after ~270° of counter clockwise rotation of the image shown in Panel A. The positions of peripheral stalks S2 and S3 are indicated. The cytosolic N-terminal part of a-subunit is indicated as (aN), while the transmembrane C-terminal part is indicated as (aC). The proximal lobe of the a2N is marked as (aN_{PL}) and distal lobe is marked as (aN_{DL}). The C-terminal domain and N-terminal domain of H-subunit are shown as (C_{TD}) and (N_{TD}), respectively. Foot, neck and head domains of the C-subunit are also indicated. (C-subunit, PDB ID: 1U7L) (H-subunit, PDB ID: 1HO8) (F-subunit, PDB ID: 4IX9) (E-subunit, PDB IDs: 4dl0 and 4efa) (G-subunit, PDB IDs: 4dl0 and 4efa) The scale bar represents 25 Å. Adapted with permission from [30].

connecting regions consist of the central stalk subunits (D, F, and d), central ring (*c*, *c*′, and *c*″) and peripheral stalk subunits (E, G, C, H, e and a) (Fig. 1A–D).

2.1.2. Structural features of the V_1 sector: catalytic hexamer A_3B_3

The alternating arrangement of A and B subunits in the V_1 region of the V-ATPase was expected from the crystal structure of the F_1 region from the related F-ATP synthase [33]. Indeed, 3D maps of the V_1 part [24,34] and V_1V_O ATPase of *M. sexta* and *S. cerevisiae*, derived from single particle analysis of electron micrographs also showed a similar arrangement of the V_1 complex [24,30]. The most notable structural difference between the A and B subunits is due to the protuberances at the top of the catalytic subunits A, that is formed by an insert of 80–90 amino acids and called "non-homologous region" [35,36]. These structural differences were further confirmed by the crystallographic structures of homologous subunits A [37] and B [38] of the related A-ATP synthase. The asymmetric crystal structures of the entire A_3B_3 and A_3B_3DF complexes of the A-ATP synthase from *Enterococcus hirae* (*E. hirae*) were recently solved at high resolution, giving insights into the rotational mechanism inside the catalytic hexamer A_3B_3 [39].

2.1.3. Structural features of the V_1 sector: peripheral stalks

The most striking structural difference between evolutionarily related F-ATP synthases, A-ATP synthases and eukaryotic V-ATPases is the number of peripheral stalks of these nano-motors. Importantly, these features may also reflect some fundamental differences in their function and regulation. Using a combination of EM approaches, including 2D and 3D negative stain EM as well as 3D cryo-EM and mass-spectrometry analysis it was demonstrated that unlike the F-ATP synthase, which has one peripheral stalk [40–44], A-ATP synthases have two [45–50] and V-ATPases have three peripheral stalks [5,28,29,51–56]. In particular, the evidence for two peripheral stalks in A-ATP synthases came from negative stain EM of holocomplexes from *Methanococcus jannaschii* (*M. jannaschii*) [45], *Caloramator fervidus* (*C. fervidus*) [46] and *Thermus thermophilus* (*T. thermophilus*) [47] enzymes. This model of subunit arrangement in A-ATP synthases was further supported by mass spectrometry [48] and 3D maps from cryo-EM [49,50]. In particular, cryo-EM of the A-ATP synthase from *T. thermophilus*, initially reached a resolution of ~16 Å [49], which was sufficient to discern the arrangement of subunits within the complex. Subsequent cryo-EM analysis yielded a map at ~10 Å resolution which allowed tracing many of the alpha helices of the enzyme [50]. The first evidence for three peripheral stalks in the eukaryotic V-ATPase was obtained from mass spectrometry [29,55] and negative stain EM [28,29] analysis. These structures were then seen clearly in cryo-EM of the *M. sexta* [5] and *S. cerevisiae* enzymes [30] (Fig. 1). The shape of these peripheral stalks fits well with SAXS-derived structures of subunits EG [28] and EGC [28,56] as well as the crystallographic structure of the subunit assembly EGC_{head} of the *S. cerevisiae* V-ATPase [57], including the head domain of subunit C. Thus, these data further confirmed the EM, mass spectrometry and biochemical evidence demonstrating that three E and G-subunits are found in the eukaryotic V-ATPase complex, forming three EG-heterodimers in the three peripheral stalks (Fig. 1A–D) [5,28,29,55].

2.1.4. Structural features of the V_1 sector: collar-like structure

The combination of EM analysis [28–30] and the SAXS-derived structure of the EGC-domain [28,56] as well as electron microscopy with antibody labeling [54] demonstrated that the boot-shaped 103 Å long subunit C [56,58] is positioned parallel to the membrane-embedded V_O sector, and links two of the peripheral stalks, referred to as S2 and S3, by interacting with the N-termini of these EG-heterodimers (Fig. 1B–D) [59,60]. The 3D maps of *M. sexta* and *S. cerevisiae* V-ATPases revealed that beside the domain filled by subunit C, two additional subunits form the collar-like structure. The crystal structure of subunit H of the *S. cerevisiae* V-ATPase [61] produced a volume that fits in the density of the upper domain (Fig. 1A–C). In this arrangement, the C-terminal part of subunit H has a large surface interaction with the middle section of the N-terminal part of the V_O subunit *a*, which connects two peripheral stalks, including the one (S2) that is in close proximity to the foot domain of subunit C (Fig. 1A, B, and D). In comparison, the N-terminal part of subunit H is well exposed and accessible from all sides for binding to proteins or to act as an adaptor between Nef and the adaptor protein complex 2 (AP-2) [62].

2.1.5. Structural features of the V_1 sector: central stalk

Low-resolution structures of the eukaryotic V_1 sector and V_1V_O holocomplex of ATPases had shown that the catalytic A_3B_3 hexamer and the V_O sector are separated by an approximately 100 Å long central stalk, consisting of the subunits D, F and *d*, with subunit *d* forming the bottom of the central stalk (Fig. 1C, D) [5,28–31]. The N and C termini of subunit D penetrate into the cavity of the A_3B_3 headpiece (Fig. 1C, D). The NMR solution structure and crystal structure of the *S. cerevisiae* subunit F [63] fits well into the 11 Å cryo-EM map of the *S. cerevisiae* V-ATPase [30,63]. The N terminus of subunit F and the bottom segment of D fit into a cavity in subunit *d* [63], which, according to the structure of the related A-ATP synthase subunit C, has a funnel shaped structure [64,65] with a central cavity. The bottom region of the A-ATP synthase subunit C has a diameter of about 30 Å, which is suitable for binding to the central cavity of the *c*-ring of the V_O sector. The A-ATP synthase subunit C and eukaryotic V-ATPase subunit *d* have been described to be a spacer that plays a role in coupling rotation between the *c*-ring and V_1 regions of the enzyme (Fig. 1C, D) [6,64,65].

2.1.6. Structural features of the V_O sector: a-subunit and c-ring

In eukaryotic V-ATPases six subunits and two accessory proteins have been identified as a part of the V_O sector. In *S. cerevisiae*, V_O consists of subunits *a* (two isoforms in yeast are coded by the VPH1 and STV1 genes), *c*, *c*′, *c*″, *d* and *e* (Table 1) [4,66]. In comparison, the mammalian V_O sector is composed of five different subunits, since no gene has been found for the *c*′ subunit (Table 1). Thus, the mouse and human V_O sectors are formed by the following subunits: *a* (*a*1, *a*2, *a*3 and *a*4 isoforms), *c*, *c*″, *d* (*d*1 and *d*2 isoforms) and *e* (*e*1 and *e*2 isoforms), respectively (Fig. 1). Moreover, in contrast to *S. cerevisiae*, the mammalian V_O contains the two additional subunits Ac45 and M8-9 [4,66]. According to current structural models, the V_O sector is composed of a ring of *c*-subunits and the adjacent single copy of the *a*-, *e*-, Ac45 and M8-9 subunits [4,66,67]. However, the stoichiometry of the *e*-, Ac45 and M8-9 subunits remains to be clarified and they are not shown in the structural V-ATPase model (Fig. 1). As suggested by the 3D map of the V_O sector from bovine brain clathrin-coated vesicles V-ATPase, accessory subunit Ac45 contacts the *c*-ring from the lumen side [27,68]. On the other hand, the N-terminal cytosolic tail of the *a*-subunit is oriented parallel to the cytoplasmic surface of the membrane and in close proximity to the N terminus of the H-subunit [28–30] (Fig. 1A–C). A recent SAXS-derived structure of the N-terminal tail $a_{104-363}$ of the *S. cerevisiae* V-ATPase, suggested the organization of the connection between the cytoplasmic N-terminal and the transmembrane C-terminal domains of *a*-subunit [32]. This arrangement makes the N-terminal part of subunit *a* accessible for cytohesin-2 and Arf-family GTP-binding proteins, described to be essential for various signaling pathways [1,69,70] (see details below). The C-terminal part of subunit *a* is membrane-embedded and its transmembrane topology remains controversial [70]. Previously, six [71,72], eight [73,74], and nine [75,76] transmembrane helix topology models of the C terminus of *a*-subunit of the V-ATPase have been proposed. However, recent studies strongly indicate an eight transmembrane topology for the yeast a-isoforms (Vph1p and Stv1p) [77,78]. According to the topology model with eight transmembrane helices, both N and the C termini of these isoforms are located in the cytosol, which is supported by experimental data showing interaction of phosphofructokinase-1 with the C-terminal tail of the human *a*4- and *a*1-isoforms [79]. The recent cryo-EM map of the *S. cerevisiae* V-ATPase showed that the contact between the

C-terminal and ion-translocating domain of *a*-subunit and the *c*-ring is small and occurs near the middle of the membrane region [30]. In this 3D map the *c*-ring has an average outer diameter of about 85 Å and an average inner diameter of about 40 Å, enabling the fit of the crystallographic structure of the *c*-ring of the related *E. hirae* A-ATP synthase, which is composed of ten *c* subunits, each with four transmembrane α-helices [80]. The *c*- and *c*′-subunits are 16 kDa proteins, proposed to contain four transmembrane helices with two cytosolic loops exposed to the cytosol, while the *c*″ subunit is a 23 kDa polypeptide with five putative transmembrane helices, two loops and a C-terminal tail exposed to the cytosol [81,82]. At the moment, the subunit composition and number of transmembrane helices in the *c*-rings of eukaryotic V-ATPases are an open questions.

2.2. High-resolution structures of key eukaryotic V-ATPase subunits and their implication to function

To date, as with many other proteins, homologues of the eukaryotic V-ATPase from thermophilic eubacteria or archaea A-ATP synthase have led the way for structural analysis. Crystal structures have been determined for the A_1 regions (A_3B_3DF) from *T. thermophilus* [83] and *E. hiraea* [39], the EG-peripheral stalk subcomplex from *T. thermophilus* [84], and the isolated DF subcomplex from *E. hiriae* [85]. From the A_O region of enzymes from thermophilic archaea and eubacteria, crystal structures have been determined for the equivalent of the *d* subunit from *T. thermophilus* [64,65], the N-terminal domain of the equivalent of the a-subunit from *Meiothermus ruber (M. ruber)* [86], and the c_{10}-ring from *E. hirae* [80]. Importantly, all these crystal structures were useful for fitting into cryo-EM maps of both eukaryotic *M. sexta* and *S. cerevisiae* V-ATPases (see discussion above). For the eukaryotic V-ATPase, only a few crystal structures are available and all from the *S. cerevisiae* enzyme. In particular, during the last decade the individual subunits C [58], E [57,87,88], F [63], G [57,89,90] and H [61] of the *S. cerevisiae* V-ATPase have been solved at high resolution, Most recently, the crystal structure of an EGC_{head} subcomplex was determined [57]. These structures fit into a cryo-EM map of the V-ATPase as shown in Fig. 1A.

2.2.1. C-subunit: collar-like structure

As shown for its hydrated [56] and crystallized form [58], subunit C is a boot-shaped protein with an upper head domain, composed of both α-helices and β-strands (residues 166–263) and a globular foot domain (residues 1–55 and 320–392). Both domains are connected by an elongated helical neck domain (Fig. 1A, D) [58]. During physiological stress, such as deprivation of glucose from the media or a drop in the ATP/ADP ratio [78], eukaryotic V-ATPases undergo a reversible disassembly of their V_1 and V_O sectors and regulatory dissociation of C-subunit from V-ATPase [91]. The critical role of the C-subunit in V-ATPase function was also supported by mutational analysis studies [59]. The location and orientation of the C-subunit in the V-ATPase complex enables its binding to actin [58], ADP/ATP [92] and WNT-kinase [93]. These interactions take place via its C-terminal foot domain, which is in proximity to the N-termini of an EG-heterodimer, forming peripheral stalk S2, as well as in the neighborhood of the N-terminal region of subunit *a* (Fig. 1A, D). It is noteworthy that interaction affinity of the EG-peripheral stalk (S2) and the foot domain of subunit C is lower compared to the interaction affinity between subunit C and the EG-peripheral stalk (S3 in Fig. 1A, D) [94,95]. Moreover, it was suggested that ATP/ADP-binding [92] and/or phosphorylation [93] of subunit C could alter the stability of a-subunit EGC-assembly by affecting its binding properties with either the EG-heterodimer or with actin. Thus it is tempting to propose that modulation of these interactions is critical in reversible disassembly of V_1 and V_O sectors and regulatory dissociation of C-subunit from V-ATPase [19,21,22,95].

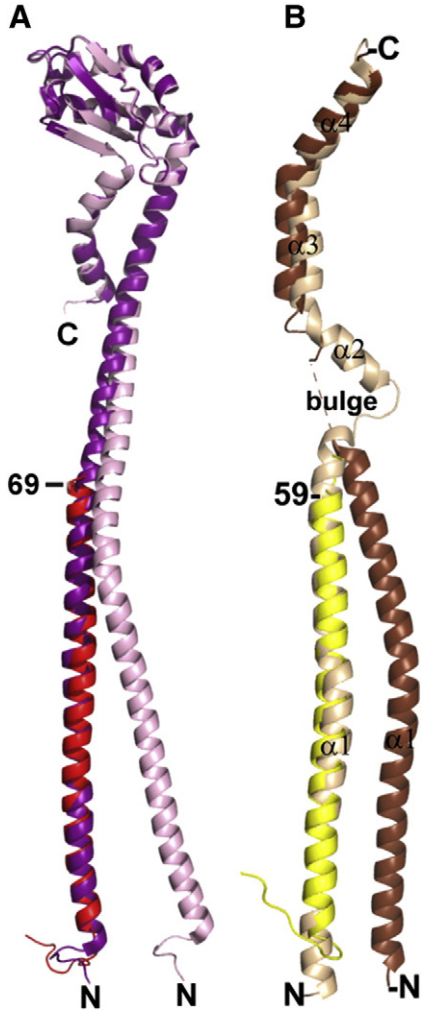

Fig. 2. High-resolution structures of subunits E and G of eukaryotic V-ATPase. A) Superposition of subunit E of the *S. cerevisiae* V-ATPase solved at 2.91 Å (purple; PDB ID: 4dl0) and 2.82 Å resolution (pink; PDB ID: 4efa) [57] and the NMR solution structure of $E_{1\text{-}69}$ (red, PDB ID: 2KZ9) [87]. B) Comparison of the *S. cerevisiae* G subunit determined at 2.91 Å (wheat; PDB ID: 4dl0) and 2.82 Å resolution (brown; PDB ID: 4efa) [57] and the NMR structure of $G_{1\text{-}59}$ (yellow, PDB ID: 2K88) [90]. The short random coil "bulge" includes the residues 63GGVG66.

2.2.2. Subunits E and G: peripheral stalks

Subunit E of the *S. cerevisiae* V-ATPase contains a 110 amino acids long N-terminal α helix and a globular C terminus, consisting of a mixture of α-helices and β-sheets, arranged as β1:α1:β2:β3:β4:α2, and connected by flexible loop regions (Fig. 2A) [57,87,88]. Subunits E and G form a ~150 Å long complex (Figs. 1A, 2A, B and 3E) with both N-termini folded into a noncanonical, right-handed coiled coil. The N-terminal helix of subunit G (α1) (Fig. 2B) contains a deformity starting around N61, characterized by a short random coil "bulge", which includes the residues $_{63}$GGVG$_{66}$. These amino acids are flanked by residues predicted to be disordered [57], a phenomenon also proposed for the equivalent region in subunit E. These areas in EG are proposed to be important during the association of the EG-heterodimer and subunit C [57]. A short helix between E67 to Q77, called the "rectifying" helix (α2) (Fig. 2B), follows the random coiled structure in subunit G and allows G to cross subunit E at an angle of about 45° and to bring the E and G-subunits back into a parallel orientation (Figs. 1 and 3E). Subsequently, two α helices, made up by the residues 78G to K90 (α3) and K91 to K104 (α4), follow the "rectifying" helix at an angle of 120° and 103°, respectively (Fig. 2B). Due to the interruption and orientations of these α-helices in the C terminus of subunit G, a

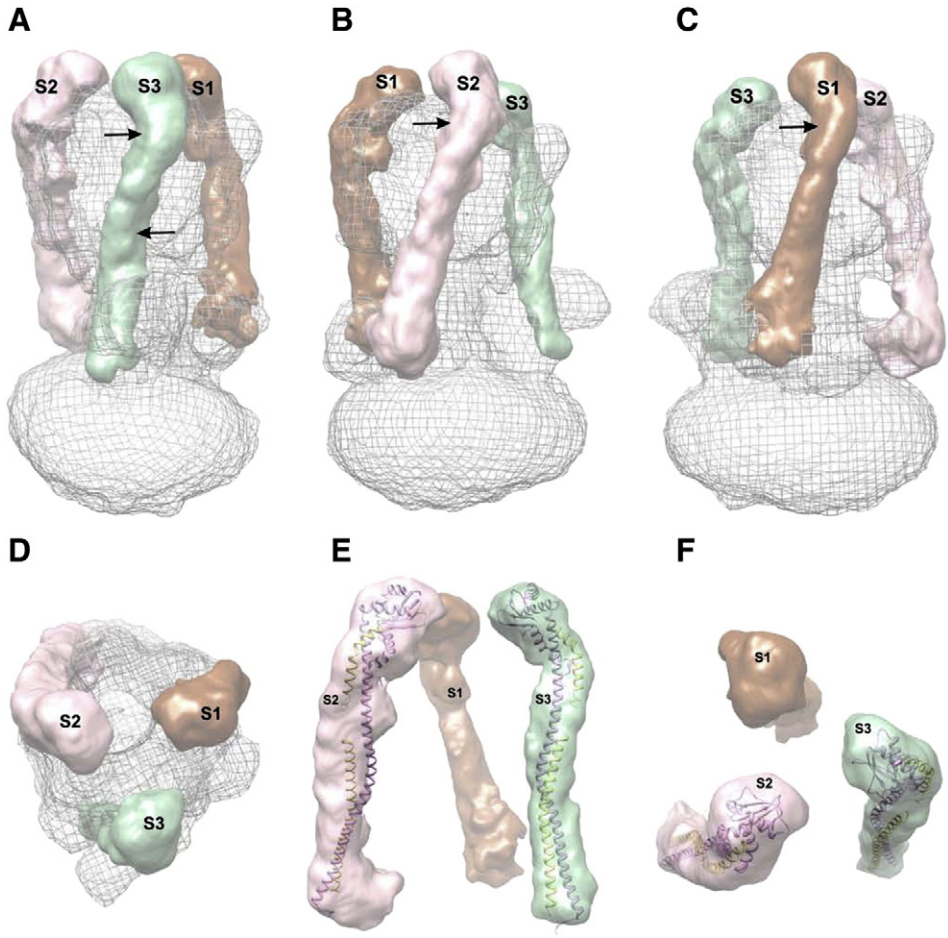

Fig. 3. Structural insights of the three EG-heterodimers and their implication to V-ATPase function. A–D) The 3D reconstruction EM map of the 11 Å resolution EM density of the *S. cerevisiae* V-ATPase [30] in three different side views (A–C) and one top view (D). A–C) The three peripheral stalks, formed by the three EG-heterodimers, show different kinked features in the upper C-terminal- and/or middle domain of the peripheral stalk (indicated by arrows). The three differently shaped peripheral stalks are indicated as S1 (brown), S2 (light pink) and S3 (pale green). Side view (E) and top view (F) of the three peripheral stalks of the EM map fitted with the atomic structures of curved 2.82 Å resolution structure of the *S. cerevisiae* EG-heterodimer. E, F). The crystallographic structure of the subunit E (pink) and G (light orange) assembly fits into the peripheral stalk S2 and S3 of the EM density. In contrast, a fit could not be achieved with peripheral stalk S1.

three-helix bundle with an extensive hydrophobic core can be arranged with the C-terminal helix of subunit E, revealing relative the stability in the C-terminal domain of the EG-heterodimer (Figs. 1A and 3E). In parallel, a second conformation of the EG-heterodimer, including the head domain of subunit C, has been determined at 2.82 Å resolution [57]. Whereas the N-terminal α-helix (residues Q3 to A62) of the 2.91 Å resolution structure described above [57] fits well with the NMR solution structure of the N-terminal helix of G_{1-59} of the *S. cerevisiae* V-ATPase [89], the N-terminal helix of G in the 2.82 Å resolution is turned by around 47° (Fig. 2B). A similar phenomenon can also be seen in the comparison of the N-terminal helix of subunit E (Fig. 2A). A second hinge region rearrangement between both structures in the C-terminal region of E and G are in proximity to the "rectifying" helix of subunit G and the region close to residue M84 in subunit E [57]. The two structures of the EGC_{head} of the *S. cerevisiae* V-ATPase indicate that concerted rearrangements in the individual subunits E and G will allow the incorporation of the peripheral stalk(s) into the enzyme during assembly of V_1 and V_O and *vice versa* during the process of disassembly.

As seen in the 3D maps of the *S. cerevisiae* enzyme [29,30], the three peripheral stalks are not identical, with a different degree of twisting in the C-terminal and/or middle part of the shape and called S1, S2 and S3 (Fig. 3). The 2.82 Å resolution crystal structure of the curved *S. cerevisiae* V-ATPase EG-heterodimer fits into peripheral stalks S2 and S3 of the 11 Å resolution EM density of the *S. cerevisiae* V-ATPase with relative correlation coefficients of 0.916 and 0.972, respectively (Fig. 3E, F). In comparison, no proper fitting could be achieved with peripheral stalk S1, indicating the differences of the three peripheral stalks in the V-ATPase complex (Fig. 3E, F). In all arrangements the C termini of the EG-heterodimers are located at the very top of the A_3B_3-hexamer and enable the connection(s) of the catalytic headpiece with the subunit C, H and *a*, which, as discussed above, form the collar domain of the enzyme (Figs. 1A–D, 3A–C).

Most recently, the peripheral stalks of V-ATPases have been proposed to provide transient elastic energy during the rotary catalytic cycle, with subunit E tethering the peripheral stalk to the V_1-headpiece sector [57,84,96] (Fig. 3A–C). This suggestion has been made based on the first determined heterodimeric right-handed coiled-coil (RHCC) structure of the *T. thermophilus* EG-subunits of the related A-ATP synthase, where the RHCC contains a hendecad- and a quindecad repeat [84]. As shown in Fig. 2B, subunit G of the *S. cerevisiae* V-ATPase is entirely α-helical with a long N-terminal helix and a shorter C-terminal helix, which are linked by a sharp kink. These, together with the two observations of the more straight E and curved-shaped E structure, support the proposed flexibility in the stator subunits, which alters the conformation of the extended N-terminal helix of subunit E from a straight to a curved structure after a rearrangement of the two flexible helices and *vice versa*. These alterations move the N-terminal helix of subunit E away or closer to the N-terminal helix of subunit G (Fig. 3E). Combined with the

rotation of the left-handed coiled-coil structure of the central subunit D, the movements of subunit E relative to G would facilitate the storage of transient elastic energy during rotary motion. Whether the arrangements of the random coiled bulge region, the "rectifying" helix α2 and the C-terminal helices α3–α4 in subunit G (Fig. 2B) contribute to the storage of elastic energy due to ATP hydrolysis and/or whether they store during the processes of reversible disassembly, have to be investigated in the future. Importantly, direct evidence of flexibility within the V-ATPase during its function as rotary nano-motor has been recently obtained using electron microscopy [97,98].

2.2.3. Subunit F and H: regulatory subunits

During catalysis the central stalk subunit F is proposed to undergo structural alterations by interacting with subunits A, B, D and *d* in a nucleotide dependent manner (Fig. 1C, D) [63,99,100]. The N-terminal 94 amino acids of *S. cerevisiae* subunit F has an elliptical shape with a size of 30 × 16 × 38 Å (Fig. 1A). It contains four-parallel β-strands, which are intermittently surrounded by four α-helices forming an egg-shaped structure (Figs. 1A and 4A). This elliptical N-terminal domain is connected via a linker segment to the C-terminal α5-helix with the residues 103 to 113 [63,101]. The surface electrostatic potential of F shows that one side of the protein is hydrophobic and proposed to face the rotating subunit D, whereas the opposite side is composed of both positive and negative charge [63]. Two loops, which are conserved in eukaryotic F subunits, have been determined in the crystal structure, located between the α1–β2 ($_{26}$GQITPETQEK$_{35}$) (Fig. 4A) and α2–β3 ($_{60}$ERDDI$_{64}$). In the V-ATPase complex the $_{26}$GQITPETQEK$_{35}$-loop (Fig. 4A) faces the C-terminal serine residue S_{381} of subunit H (Fig. 4B), revealed to be involved in cross-linking subunit F of the disassembled V_1 sector [102,103]. This arrangement of the $_{26}$GQITPETQEK$_{35}$-loop relative to the C terminus of H led to the proposal, that in the process of V_1 and V_O disassembly the flexible C-terminal domain of subunit H moves slightly closer to its nearest neighbor, the exposed $_{26}$GQITPETQEK$_{35}$-loop of subunit F, where it causes conformational changes, leading to an inhibitory effect of ATPase activity in the V_1 ATPase [30,63,103]. ^{15}N-[^{1}H] heteronuclear NOE studies on the *S. cerevisiae* subunit F revealed a rigid core formed by ß-strands, ß1 to ß4, and α2 to α4. In comparison, the N- and C-terminal helices α1 and α5 with their adjacent loops $_{26}$GQITPETQEK$_{35}$ and $_{94}$IPSKDHPYD$_{102}$, respectively, are more flexible in solution [63]. The N-terminal helix α1 of subunit F and the bottom segment of subunit D form the neighborhood with subunit *d* [63]. It has been proposed that this area undergoes alterations during the process of disassembly and reassembly of the V_1 and V_O sectors. In this scenario the higher flexibility of α1 in subunit F would allow it to transmit the alteration of subunit *d* during dissociation from the DF-heterodimer and also allow the movement of subunit H closer to F, via the neighboring $_{26}$GQITPETQEK$_{35}$-loop [63].

The crystal structure of the *S. cerevisiae* subunit H (54 kDa) is an α-helical subunit, whose N- and C-terminal domain form a shallow groove, which are connected by a flexible four-residue loop (Figs. 1A, B and 4B) [61]. The structural rearrangement of both the N- and C-terminal domain, which is needed to bring both subunit H and F in close proximity, is mediated through the flexible four-residue linker segment regulating the ATPase activity of the enzyme [30]. The 11 Å resolution cryo-EM map of the V-ATPase from *S. cerevisiae* [30] allowed for accurate docking of the yeast V-ATPase crystal structure of subunit H. This docking suggested a mechanism by which the H-subunit of the enzyme inhibits ATP hydrolysis upon separation of the V_1 and V_O sectors as shown by biochemical experiments [103,104]. This model suggests that upon V_1V_O disassembly the inhibitory C-terminal domain of the H-subunit loses its binding site on the N-terminal domain of subunit a (Fig. 1B). Due to flexibility in the linker that connects the N- and C-terminal domains of the H-subunit, the C-terminal domain is free to sample different orientations, so that it can bind to an inhibitory position on V_1 [30,102–104]. The molecular mechanism of this inhibition was discussed above in detail (Fig. 4) [63].

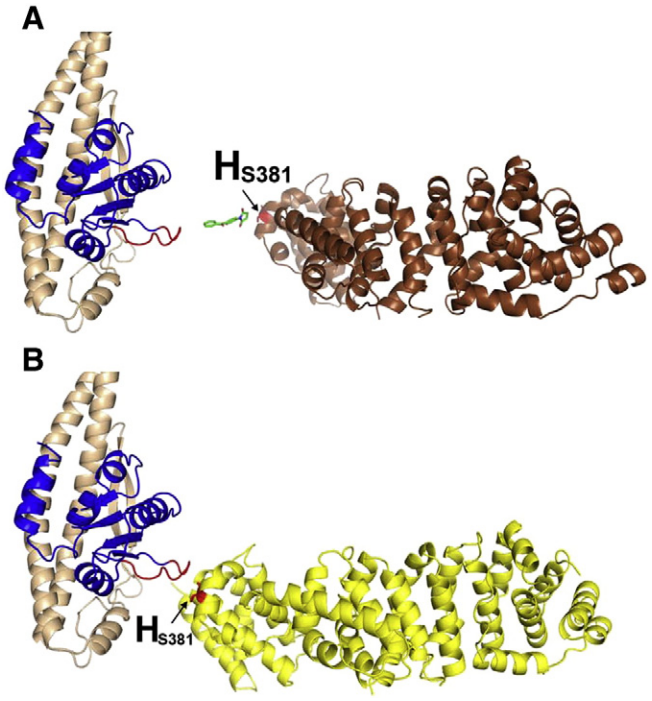

Fig. 4. High-resolution structures of the eukaryotic V-ATPase subunit F and H. A, B) Structure of the *S. cerevisiae* subunit F (blue; PDB ID: 4IX9) [63] with its characteristic $_{26}$GQITPETQEK$_{35}$-loop (red). Subunit D is shown in beige. A) The sulfhydryl cross-linker 4-(N-maleimido)benzophenone (MBP) (stick; green) is described to link the H-subunit (brown) (PDB ID: 1HO8) [61] and F subunit via the H-subunit residue S381 (red) only in the free *S. cerevisiae* V_1 domain [103]. B) It has been proposed that the disassembly of the V_1 from the V_O part causes alterations in the C-terminal domain of subunit H (yellow), bringing the epitope around residue S381 in close proximity to the exposed loop $_{26}$GQITPETQEK$_{35}$ (red) of subunit F [63].

3. V-ATPase is the nano-motor invented by Nature billions of years ago

3.1. Function of V-ATPase as a rotary proton-pumping machine

As discussed above, eukaryotic V-ATPase consists of a soluble catalytic V_1 ($A_3B_3DE_3FG_3H$) and a membrane-bound V_O sectors (*acc′c″de*) (Table 1) (Figs. 1 and 5). However, within these two sectors there are other functionally identifiable subcomplexes. The DF$c_Xc'_Yc''_Zd$ subcomplex comprises the rotor of the nano-motor which consists of central stalk (DF*d*) subunits and proteolipid *c*-ring ($c_Xc'_Yc''_Z$) subunits (Fig. 5, rotor is shown in gray and outlined by the dashed-lines). The rotor of the V-ATPase nano-motor is surrounded by its stator which consists of: i) a catalytic hexamer (A_3B_3) subcomplex and ii) collar-like structure formed by the subunits C, H and a. These two subcomplexes are connected by three peripheral stalk (EG) subcomplexes, while subunits *a* and *e* form the membrane-embedded part of the stator (Table 1) (Figs. 1 and 5). During its principal activity of ATP-driven proton pumping, ATP hydrolysis in the interfaces of the A and B subunits induces conformational changes that drive rotation of the rotor subcomplex (Fig. 5, rotor). Clockwise rotation of the *c*-ring of the rotor against the *a*-subunit of V_O sector drives the translocation of protons across the membrane. The peripheral stalk subcomplexes serve to restrain the *a*-subunit to the A_3B_3-subcomplex, allowing the relative rotation of the rotor against the *a*-subunit. It is very likely that the *c*-ring of the rotor also rotates against the *e*-subunit of V_O sector, however, its exact location in the V_1V_O V-ATPase holocomplex is currently unknown.

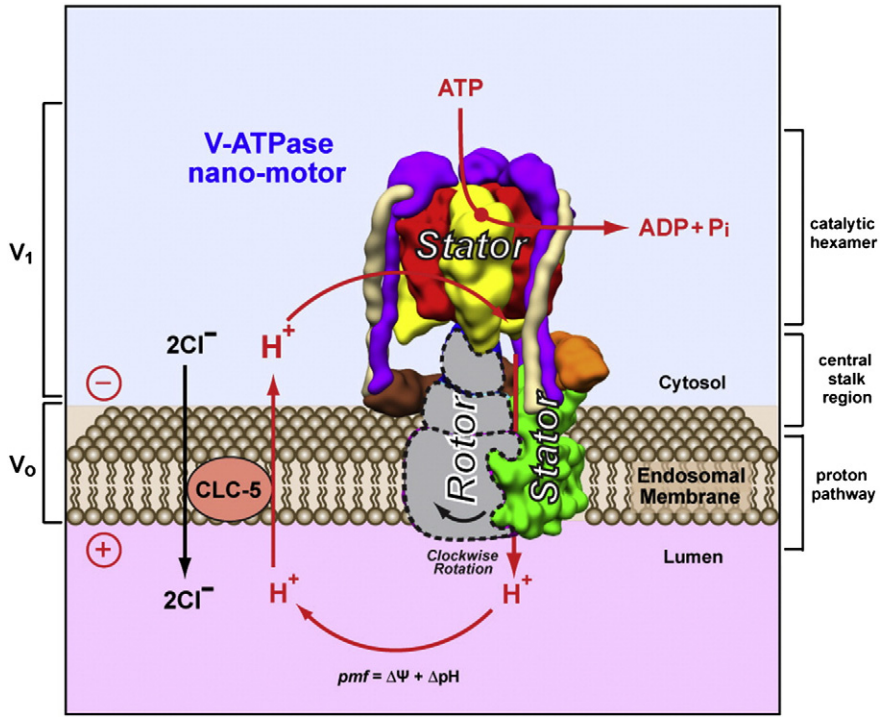

Fig. 5. V-ATPase is a primary proton-pumping nano-motor invented by Nature billions of years ago. During function of V-ATPase as a proton-pumping nano-motor, the hydrolysis of ATP induces conformational changes that promote clockwise rotation of the rotor subcomplex against the stator subcomplex (viewed from the V_1 region toward the V_O region). The colors of the stator subunits of V-ATPase are indicated as in Fig. 1, while the *c*-ring and DF*d* subunits forming rotor are shown in gray. This rotation in turn drives the translocation of protons (indicated by red arrows) from the cytosol to lumen of endomembrane organelles of endocytic (endosomes, lysosomes, and phagosomes) and exocytic pathways (Golgi, secretory vesicles) and, thus, gives rise to the generation of an electrochemical proton gradient or a proton-motive force (*pmf*) across the membrane. The *pmf* consists of membrane potential ($\Delta\Psi$) and a proton gradient (ΔpH). The values of $\Delta\Psi = 27$ mV and ΔpH $= 2.2$ units were previously determined in early phagosomes using direct a FRET approach [118]. In endosomes, the V-ATPase driven current of protons is neutralized by electrogenic CLC-5 ($2Cl^-/H^+$-exchanger), which promotes an additional acidification and accumulation of Cl^- ions (indicated by black arrows). Importantly, the coupled function of V-ATPase with $2Cl^-/H^+$-exchangers is a universal mechanism, which provides an additional control of differential acidification generated by V-ATPases located either on plasma membrane or in endomembranes of other intracellular organelles [1]. The localization of the cytosolic V1-sector and transmembrane V_O sector is shown on the left, while the location of catalytic hexamer, central stalk region and proton pathway is shown on the right. The movies showing the rotation of yeast V-ATPase and bacterial F-ATP synthase nano-motors were previously published [1].

The hydrolysis of ATP by the catalytic hexamer (A_3B_3) of V-ATPase induces conformational changes that drive clockwise rotation (viewed from the V_1 region toward the V_O region) of the rotor against the stator (Fig. 5). In contrast, the ATP synthase nano-motors rotate in a counter clockwise direction (viewed from the V_1 region toward the V_O region), when driven by proton gradients. Thus, this structural design of ATPases/ATP synthases allows the relative rotation of the membrane-embedded *c*-ring rotor against the stator's transmembrane subunits, which in turn drives the translocation of protons across the membrane. The basis of rotary catalysis in the V_1 regions of ATPases/ATP synthases is currently emerging from high-resolution crystal structures from A-ATPases [39,63,83] and reconstitution studies [105]. In particular, a recent study has reported the asymmetric structures of the nucleotide-free and nucleotide-bound A_3B_3 hexamer of the *E. hirae* A-ATPsynthase, and thus, has revealed the conformational changes of A_3B_3 hexamer during the cooperative binding of nucleotides in this rotary nano-motor [39]. On the other hand, the question of how rotation of the central rotor is coupled to proton translocation through the membrane is still enigmatic. The dominant model for this mechanism proposes two offset half-channels: one channel that exchanges protons from one side of the membrane and the middle of the lipid bilayer, and a second that exchanges protons from the middle of the lipid bilayer and the other side of the membrane [16,17]. Once delivered to the middle of the lipid bilayer, protons may bind conserved glutamate residues on the ring-forming c-subunits of the ATPase. The ring is driven to rotate by the power provided by ATP hydrolysis in the V_1 region. The recent cryo-EM map of the A-ATP synthase from *T. thermophilus* [50] provided some experimental evidence for this model of proton translocation by showing offset contacts between the a-subunit and two different ring-forming c-subunits in that enzyme. However, the proton path and chemistry of this interaction are not known. The eukaryotic V-ATPase has rings composed of different subunits (subunits *c*, *c*′, and *c*″ in the yeast and *c*, *c*″ in mammalian cells) and it is not clear how these subunits function together to allow proton translocation by the V-ATPase. Finally, another apparently unique attribute of the eukaryotic V-ATPase is that during dissociation of the V_1 and V_O sectors the proton translocation by the V_O region also ceases [106]. However, the nature of the conformational or chemical change in the V_O region that stops proton translocation after dissociation of V_1V_O holocomplex has not been established.

3.2. Regulation of V-ATPase nano-motor and modulation of V-ATPase driven acidification

As a result of ATP hydrolysis and rotary-driven proton pumping, V-ATPases generate an electrochemical proton gradient or proton-motive force (*pmf*) across the membranes (Fig. 5), which gives rise to acidification of intracellular compartments. All intracellular compartments of eukaryotic cells require maintaining V-ATPase dependent acidic luminal pH [1]. The V-ATPase is also targeted to the plasma membrane and is involved in extracellular acidification of some specialized cells in kidney [107,108], epididymis [109,110] and bone [72,111–114] tissues. It is also acidifies extracellular environment in metastatic cancer cells [115–117]. The pH becomes more acidic as the exocytic and endocytic vesicular trafficking pathways reach their destination [1]. The regulation

of V-ATPase function and V-ATPase driven differential acidification is achieved by the following mechanisms: i) modulation of V-ATPase dependent acidification via chemiosmotic mechanism; ii) regulation of coupling of the V-ATPase nano-motor; iii) subunit-specific targeting of V-ATPase and iv) regulation of V-ATPase activity via reversible association/dissociation of V_1 and V_O sectors.

3.2.1. Chemiosmotic mechanism

The proton-motive force (*pmf*) generated by V-ATPases consists of a proton gradient (ΔpH) and membrane potential ($\Delta\Psi$) components. In phagosomes, the values $\Delta pH = 2.2$ units and $\Delta\Psi = 27$ mV were experimentally determined using a FRET approach [118]. It is noteworthy that according to the Nernst equation the value of $\Delta pH = 2.2$ units corresponds to the value of $\Delta\Psi = 129.8$ mV, and thus, it is the predominant component of the V-ATPase driven *pmf*. However, this component of *pmf* and the corresponding levels of acidification could vary depending on the origin of intracellular organelles. In particular, the differential level of acidification in endosomes and lysosomes of endocytic pathways depend on the coupled function of V-ATPase in these organelles with the function of electrogenic $2Cl^-/H^+$-exchangers called CLC-3, CLC-5 and CLC-7 (Fig. 5) [119–121]. These exchangers shunt electrogenic proton currents and promote accumulation of chloride anions in endocytotic compartments [122]. A similar chemiosmotic mechanism is also involved in the regulation of acidification of the compartments along the exocytic pathway, since CLC-3 is present on synaptic vesicles and CLC-4, CLC-5 can reach the plasma membrane [123]. Thus, a crucial role of the Cl^-/H^+- and also Na^+/H^+-exchangers is generally accepted as an important chemiosmotic mechanism of regulation of V-ATPase driven acidification of intracellular organelles during cellular homeostasis [119,123–125].

3.2.2. ATP hydrolysis/proton-pumping coupling mechanism

The modulation of V-ATPase activity and differential acidification of intracellular compartments is also achieved by the regulation of the coupling between ATP hydrolysis and proton pumping during its function as a rotary nano-motor. In yeast, the a-subunit isoforms and non-homologous region of the a-subunit V-ATPase have been implicated in this type of regulation [126–128]. Moreover, a "mouse/yeast" hybrid V-ATPase approach has uncovered an essential role of the E- and C-subunit isoforms in regulating the coupling efficiency of *S. cerevisiae* V-ATPase [129,130].

3.2.3. Subunit specific V-ATPase targeting mechanism

In mammalian cells various subunit isoforms have been identified: i) four isoforms for a-subunit (a1, a2, a3 and a4); ii) three isoforms for the C and G-subunits; iii) and two isoforms for the B, E, H and d-subunits (Table 1 and Fig. 1) [1,4,130–132]. The levels of expression of these multiple isoforms of V-ATPase are tissue and cell specific and their gene regulation is largely unknown. On the other hand, the targeting of V-ATPase subunit isoforms is also cell and intracellular compartment specific and the mechanism of this phenomenon is currently emerging [1,114,132].

In yeast, V-ATPase is targeted by the two a-subunit isoforms Vph1p and Stv1p to the vacuole and Golgi/endosomes, respectively [127,133]. Studies with chimeric Vph1p and Stv1p proteins revealed that the targeting information is located in the cytosolic N-terminal domain of a-subunit (Fig. 1) [127]. Recently, random mutagenesis studies of Stv1p N terminus have identified the W(83)KY sequence as a novel signal that is both necessary and sufficient for targeting of V-ATPase to the Golgi/endosomes in yeast [134]. Similarly, in mammalian cells, localization of V-ATPase in endocytic and exocytic compartments and targeting to the plasma membrane depend on a-subunit isoforms [1,4,114,132]. However, the specific targeting signal of mammalian a-subunits has not been yet determined.

The a1-subunit isoform is specifically targeted to presynaptic membranes and exocytic synaptic vesicles in mammalian neurons [135,136]. Recent studies with neurosecretory PC12 cells revealed that a1-subunit functions cooperatively with a2-subunit in order to regulate the acidification and neurotransmitter uptake, storage and release by exocytic vesicles [137]. On the other hand, in microglial cells of brain the a1-subunit was implicated in modulation of the endocytic pathway via its role in the fusion between phagosome and lysosomes during phagocytosis, an important process of microglial-mediated neuronal degeneration [138]. Moreover, recent immunocytochemistry and cell fractionation experiments demonstrated that in presenilin-1 (PS1) null blastocysts neurons the a1-isoform containing V-ATPases is also targeted to lysosomes of endocytic pathway [139]. In this study the authors proposed that physical interaction of the unglycosylated a1-isoform with PS1 is required for its targeting and delivery from ER to lysosome. Thus, according to this study the PS1 is essential for V-ATPase targeting to the lysosomes, lysosomal acidification and proteolysis during autophagy associated with Alzheimer's disease (AD) [139]. However, this concept and the role of PS1 in the V-ATPase dependent lysosomal acidification and protein degradation during AD were recently challenged [140–143].

The a2-subunit isoform targets V-ATPases to early endosomes of the endocytic pathway both in MTC cells *in vitro* and in kidney proximal tubule epithelial cells *in situ* [69,70,144]. Overexpression of recombinant a2-isoform (a2-EGFP) in these cells targets V-ATPase to endosomal compartments [145]. In contrast, in cultured osteoclast cells and B16 cells both endogenous a2- and a1-isoforms are targeted to the Golgi complex of secretory vesicles in the exocytic pathway [114,137]. Similarly, overexpression of recombinant a2-isoform (a2-EmGFP) in neuroendocrine PC12 cells targets V-ATPase to the Golgi apparatus [114,137].

The a3-subunit of V-ATPase is a lysosomal specific isoform in osteoclasts, which is relocated to plasma membrane during osteoclast differentiation [111,114]. Recent studies revealed, that the V-ATPase a3-subunit mutation (R444L), which causes infantile malignant osteopetrosis in humans, in a mouse model gives rise to its defective glycosylation, retention in endoplasmic reticulum and defective trafficking to the plasma membrane [146]. Subsequently, the specific sites of the a3-subunit glycolylation were also identified and characterized [78]. During bacterial infection, the nascent phagosomes of macrophages also acquire a3-subunit containing V-ATPase from lysosomes [147]. In contrast, in neuroendocrine PC12 cells the recombinant a3-isoform (a3-EmGFP) is targeted to early endosomes of the endocytic pathway, while in pancreatic β-cells the endogenous a3-isoform is specifically targeted to insulin containing secretory granules of the exocytic pathway [1,114]. Finally, V-ATPase containing the a3-isoform is also targeted to the plasma membrane of osteoclasts [72,111–114] and breast cancer cells [115–117], in which it is involved in bone reabsorption and metastasis, respectively.

The a4-isoform is highly specific for kidney and epididymis, in contrast to the tissue ubiquitous a1-, a2- and a3-isoforms. In these tissues the a4-isoform is specifically targeted to the apical plasma membrane of collecting duct intercalated cells and epididymal clear cells [148,149]. In these specialized cells, V-ATPase containing the a4-isoform is involved in maintenance of acid-balance [107,108] and sperm-maturation [109,110], respectively. Thus, depending on the cell type and tissue specificity the V-ATPase is differentially targeted by the a-subunit isoforms to the various intracellular compartment and plasma membrane. In turn, this cell and tissue specific targeting and assembly of V-ATPase may modulate its function, giving rise to the differential levels of acidification of intracellular endocytic/exocytic organelles and extracellular milieu [1,3,110,132].

3.2.4. Reversible assembly/disassembly of V_1V_O sectors

The regulation of V-ATPases by reversible assembly/disassembly of the V_1V_O sectors was first described in response to glucose depletion in *S. cerevisiae* [21–23] and in response to ceased feeding in *M. sexta* [19,20]. In yeast, assembly/disassembly of the V_1V_O sectors is regulated by the a-subunits (Vph1 and Stv1 isoforms). It is also dependent on the

micro-environment, where these isoforms are located and on levels of vacuolar luminal acidification [4,126,150]. Importantly, in yeast the reversible assembly/disassembly of V_1V_O sectors is tightly controlled by the following two distinct mechanisms: i) disassembly involves the cytosolic microtubular network, while ii) the assembly requires the cytosolic RAVE/rabconnectins complex (Rav-1, Rav-2 and Skp1) [1,4,23,151]. Moreover, the yeast RAVE complex is an a-isoform specific factor. Recent study revealed that RAVE complex is necessary for assembly of Vph1p-containing but not for Stv1p-containing V-ATPase in yeast [152]. However, the molecular details of this regulation remain obscure. In yeast, the reversible assembly/disassembly of the V_1V_O sectors is also controlled by the direct interaction of V-ATPase with cytosolic aldolase (a central enzyme of the glycolytic pathway), which was suggested to act as a cytosolic glucose-sensor [153–155]. It is noteworthy that the glucose-dependent assembly/disassembly of V-ATPase and its interaction with aldolase is modulated by the Ras/cAMP/PKA pathway [156]. However, in kidney proximal tubule epithelial cells the effect of glucose on V-ATPase function is mediated by the phosphatidylinositol 3-kinase (PI3K) pathway [157]. Thus, it has become generally accepted that multiple regulatory pathways are involved in and intersect with each other to control the reversible assembly/disassembly of V-ATPase along the endocytic pathway [158]. In mammalian cells, the regulation of V-ATPase by this mechanism was also previously shown in lysosomes during the maturation of dendritic cells, in which it is critical for lysosomal acidification, protein degradation and antigen presentation [159]. Recently, it was demonstrated that assembly of V-ATPase in dendritic cells is also regulated via a PI3K and mTOR-dependent pathways [160]. Finally, the reversible assembly/disassembly of V_1V_O sectors was also recently identified as an important regulatory mechanism of signaling, trafficking and degradation of EGFR/ErbB-receptors within endosomal/lysosomal protein degradation pathway [161].

4. Unconventional functions of V-ATPase in eukaryotic cells: role in health and disease

During the last decade unconventional roles of V-ATPases in the regulation of signaling, trafficking and degradation of variety cellular receptors were described. Here we will discuss these novel emerging roles of V-ATPase in the modulation of function of cellular receptors and their regulatory complexes. These findings will be also analyzed taking into consideration the emerging crucial role of V-ATPase in the development and progression of diseases like cancer and diabetes as well as neurodegenerative and kidney disorders.

4.1. Function of V-ATPases in the endocytotic pathway and its role in disease states

Endocytosis is a fundamental cellular process that is used by eukaryotic cells to communicate between the intracellular environment and external milieu. This mechanism is used by cells to internalize an enormous variety of macromolecules, nutrients and hormones, as well as

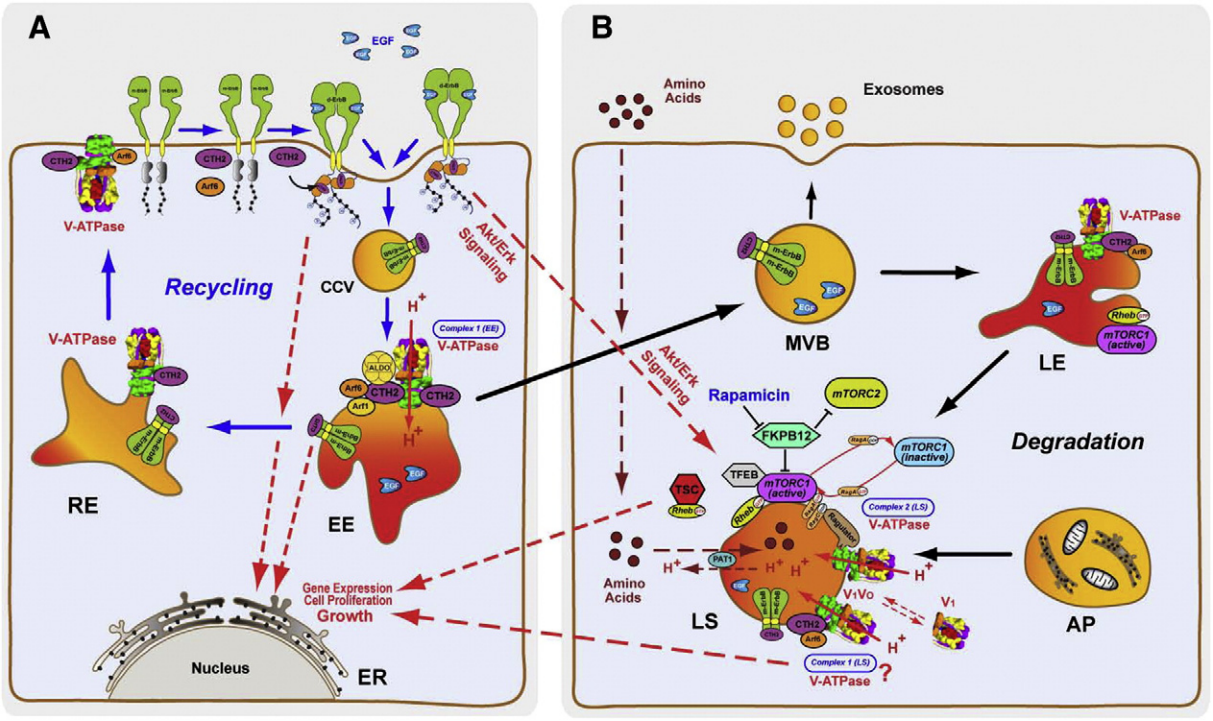

Fig. 6. Role of V-ATPase in signaling and trafficking of EGFR/ErbB-receptors. Signaling of epidermal growth factor (EGF) and trafficking of the EGFR/ErbB-receptors via clathrin-dependent endosoma/lysosomal protein degradative pathway. The shown image of single cell is divided in two parts depicting two major branches of endocytic vesicular trafficking pathway: A) the recycling branch (blue arrows) and B) the degradation branch (black arrows). The endocytic compartments of this pathway are shown in yellow/red and labeled as follows: i) clathrin-coated vesicles (CCV); ii) early endosomes (EE); iii) recycling endosomes (RE); iv) multivesicular bodies (MVB); v) late endosomes (LE); vi) lysosomes (LS) and vii) autophagosomes (AP). A) The super-complex containing V-ATPase/CTH2/ALDO/Arf proteins (*Complex 1, EE*) was previously identified in early endosomes [265]. The V-ATPase of this super-complex functions as pH-sensing and cytohesin-2 signaling receptor, which is involved in: i) formation of MVB's; ii) modulation trafficking between early and late endosomes and iii) potentially, in the regulation of trafficking of EGFR/ErbB-receptors and their signaling to control genes expression and cell proliferation. B) A novel super-complex containing V-ATPase/Ragulator/RagA/C/mTORC1/Rheb proteins *(Complex 2, LS)* was recently identified in lysosomes [190,192,194]. The V-ATPase of this super-complex is involved in sensing of levels of amino acids and modulation of mTORC1-dependent downstream cellular programs and cell growth. Importantly, recent studies demonstrated, that the reversible assembly/disassembly of V_1V_O sectors of lysosomal V-ATPase is regulated by signaling of EGFR/ErbB-receptors via Akt/Erk pathway (dashed red arrows) [161]. The V-ATPase and its subunits are shown as in Fig. 1. Cytohesin-2 (CTH2); ADP-ribosylation factor 1 (Arf1); ADP-ribosylation factor 6 (Arf6); aldolase B (ALDO); epidermal growth factor (EGF); monomeric EGFR/ErbB-receptor (mErbB); dimeric EGFR/ErbB-receptor (dErbB); mammalian target of rapamycin complex 1 (mTORC1); mammalian target of rapamycin complex 2 (mTORC2); Ras homolog enriched in brain (Rheb GTPase); tuberous sclerosis complex (TSC complex); Ragulator complex (Ragulator); Rag A/C GTPases (RagA/C); FK506/rapamycin binding protein (FKPB12); transcription factor EB (TFEB) and proton-coupled aminoacid transporter 1 (PAT1).

microorganisms, viruses and DNA. It is also crucial for the internalization of a variety of cellular receptors localized at the plasma membrane. In particular, the constitutively operated clathrin-dependent endocytosis (CDE) pathway mediates internalization of such receptors as: EGFR/ErbB, Fz/LRP6, PRR, Notch, transferrin and megalin/cubilin among many others (Fig. 6) [145]. After binding to corresponding ligands, the receptor/ligand complexes initiate signaling and are then retrieved from the plasma membrane, followed by their trafficking via the endosomal/lysosomal pathway. In this pathway, the receptor/ligand complexes are delivered via clathrin-coated vesicles (CCV) to early endosomes (EE), multivesicular bodies (MVB), late endosomes (LE) and then to lysosomes (LS) for acidification-dependent protein degradation (Fig. 6 A,B, degradation branch). Alternatively, after V-ATPase-driven pH-dependent dissociation from ligands in early endosomes, these receptors are returned back to the plasma membrane via recycling endosomes (RE) (Fig. 6A, recycling branch). However, as discussed below, mounting evidence indicates that establishing the acidic environment of intracellular organelles is not the only function of V-ATPase. This nano-motor complex is also involved in variety of direct protein–protein interactions and could directly modulate the function of various receptors and their regulatory proteins along the endocytic pathway.

4.1.1. Frizzled (Fz) and low-density receptor-related protein (LRP6) receptors

The Wnt/β-catenin, Wnt/PCP (planar cell polarity) and Wnt/Ca^{2+} signaling pathways are fundamental mechanisms that control embryonic tissue development, homeostasis, cell proliferation, polarity and apoptosis [162,163]. They are strongly linked to the development of a variety of human diseases including metastatic cancers [162–164]. Recently, an unexpected direct role of the V-ATPase in the regulation of Wnt/β-catenin and Wnt/PCP signaling pathways was uncovered [165,166]. It was shown that signal transmission after association of Wnt ligands with Fz/LRP6 co-receptors requires direct interaction of LRP6 with an accessory M8-9 subunit of V-ATPase, also called V-ATPase lysosomal accessory protein-2 (ATP6AP2). This interaction takes place in early endosomes and the ATP6AP2 subunit acts as an adaptor that brings together V-ATPase and the Wnt/Fz/LRP6 receptor complex. Thus, this work revealed that both direct and electrochemical regulation by V-ATPase are involved in signaling of Wnt/Fz/LRP6 in the early endosomal compartment of the protein degradation pathway.

4.1.2. (Pro)renin receptor (PRR)

The (pro)renin receptor (PRR), a single transmembrane domain cell surface receptor, plays a central role in the activation of the local renin-angiotensin system (RAS). Binding of prorenin to PRR induces a conformational change, allowing conversion of angiotensinogen to angiotensin-I, which is subsequently converted to angiotensin-II by an angiotensin-converting enzyme (ACE) [167,168]. However, two angiotensin-independent functions of PRR were also recently discovered. Firstly, it was demonstrated that binding of prorenin to PRR induces its own intracellular signaling via activation of the p38 MAP kinase pathway [168–170]. Secondly, PRR was also identified as an accessory ATP6AP2 subunit of V-ATPase (see above) [165,166,168,171]. Tissue-specific conditional knockout experiments confirmed an essential role of ATP6AP2 subunit in assembly of the V_1V_O holocomplex of V-ATPase [172]. However, further studies are needed in order to reveal the interplay between these novel functions of PRR. Importantly, the level of prorenin is elevated during diabetes and over-activation of PRR is strongly associated with development of hypertension and diabetic kidney disease [173]. The role of PRR in kidney function and its association with diabetes and hypertension was recently reviewed [168,171,174]. Thus, future studies in this area could lead to the development of novel therapeutic approaches for the treatment of hypertension, diabetes and its complications.

4.1.3. Notch receptor

The cell-to-cell signaling by the Notch receptor pathway is critical during development and tissue renewal for controlling the balance between cell proliferation and apoptosis. Pathological deregulation of Notch receptor signaling is also a hallmark of different cancers [175,176]. Activation of the Notch receptor by ligands gives rise to its cleavage by γ-secretase-mediated intra-membrane proteolysis followed by activation of specific target genes. Surprisingly, recent studies revealed that in *Drosophila* V-ATPase driven acidification may control two opposite processes in Notch signaling: i) lysosomal degradation and deactivation of Notch receptors; and ii) γ-secretase-mediated Notch receptor activation in early endosomes [177]. Moreover, both in *Drosophila* and mammalian cells Notch receptor signaling is also controlled by Rabconnectin-3A/B (Rbcn-3) via its regulation of V-ATPase function [178,179]. It is important to underline that mammalian Rbcn-3 protein is a homolog of yeast Rav-1, which forms a part of the RAVE (Rav-1, Rav-2 and Skp1) complex, and which directly interacts with the V-ATPase and is essential for assembly of the V-ATPase V_1V_O holocomplex (see also above) [23,151,180]. Thus, similar to Wnt-signaling these studies also revealed two novel mechanisms of Notch-signaling modulation by V-ATPase in mammalian cells: i) via subsequent Rbcn-3, V-ATPase, Notch regulation and ii) via V-ATPase-driven acidification-dependent/electrochemical γ-secretase activation of Notch signaling [166,177–179].

4.1.4. Insulin-like growth factor (IGF-I) receptor and heme-binding protein (HRG-1)

Both the growth hormone (GH) and insulin-like growth factor 1 (IGF-I) exert powerful control over lipid, protein and glucose metabolism. The function of GH/IGF-I axis is associated with longevity, and thus, aging related morbidities including diabetes and cancer [181,182]. It also plays an important role in muscle maintenance and repair [183]. Signaling by insulin-like growth factor receptor (IGF-IR) controls expression of heme-binding protein (HRG-1) among others proteins. A recent study revealed specific targeting of this protein to early endosomes and its direct interaction with the c-subunit of the V-ATPase [184]. Moreover, the HGR-1 expression correlates with function of V-ATPase, levels of endosomal acidification and endocytic trafficking of receptors, which facilitate tumor growth and cancer progression [184].

4.1.5. mTOR complex 1 (mTORC1) and complex 2 (mTORC2)

The mammalian target of rapamycin (mTOR) is a large cytosolic serine–threonine kinase that controls cellular growth and metabolism. Under physiological conditions it is involved in neonatal autophagy and survival as well as development of obesity and aging processes in adulthood [185,186]. Abnormal function of mTOR is implicated in the pathogenesis of many diseases including cancer, diabetes, and neurodegenerative and kidney disorders [185,187–189]. The mTOR belongs to the superfamily of phosphatidylinositol-3 kinase related-kinases (PI3KK) that forms the core of two functionally distinct complexes: mTORC1 and mTORC2. In particular, mTOR complex 1 (mTORC1) responds to the levels of growth factors, amino acids, oxygen, energy and stress and thus integrates/communicates signaling of a variety of cellular stimuli (Fig. 6B) [185,187,190]. On the other hand, mTORC2 plays a central role in the growth factor and insulin signaling cascades. It also regulates cytoskeleton function, metabolism and cell survival [187,191]. Recently, V-ATPase was identified as an important component of the mTORC1 regulatory super-complex and signaling pathway [192]. This novel V-ATPase containing super-complex consists of V-ATPase/Ragulator/Rag/mTORC1/Rheb proteins and is associated with late endosomes (LE) and lysosomes (LS) of the protein degradation pathway (Fig. 6B) [192,193]. Localization of mTORC1 on the lysosomal membrane is critical for its activation as a multifunctional serine–threonine kinase and is regulated by two types of small GTPases: i) Rheb GTPase (Ras homolog enriched in brain); and ii) Rag GTPases.

It is well recognized, that Rheb is a potent activator of mTORC1, which funnels signaling of growth factors, oxygen, energy supply and stress via the tuberous sclerosis complex (TSC), acting as a GTPase activating protein (GAP) for Rheb small GTPase. On the other hand, while the central role of amino acids as an important nutrient supply in the modulation of cell growth and homeostasis is generally accepted, the molecular aspects of their regulation of mTORC1 function remained elusive. However, recently the V-ATPase was uncovered as a major player in the amino acids dependent recruitment, activation and signaling to mTORC1 [192]. It was demonstrated that V-ATPase is involved in sensing of the levels of intra-lysosomal amino acids via its direct interaction with the Ragulator complex, that acts as a GTP/GDP-exchange factor (GEF) for Rag small GTPases [194]. This cell biological event results in V-ATPase/Ragulator "inside-out" signaling from the lysosomal lumen, that leads to activation and recruitment of cytosolic RagA/B small GTPase. Interestingly, the V-ATPase is not required to be active for this signaling to occur (Fig. 6B) [190,192,194]. The authors proposed that the primary function of amino acid-dependent V-ATPase/Ragulator/Rag-signaling is to promote the recruitment of mTORC1 to lysosomal membrane, and in this way, to trigger the TSC/Rheb-driven "ignition key" for the activation of the kinase activity of lysosomal mTORC1 complex. Thus, in this scenario the V-ATPase plays a direct role in intra-lysosomal sensing of amino acids and transmembrane signaling to mTORC1. It is noteworthy that this mechanism requires physical interaction of V-ATPase with Rag-GEF Ragulator and RagA/B small GTPases (Fig. 6B).

4.1.6. Epidermal growth factor receptors (EGFR/ErbB's)

The epidermal growth factor receptor (EGFR) was among the first discovered growth receptors that regulate crucial cell biological processes including cell proliferation [195,196]. The EGFR/ErbB-receptors (EGFR/ErbB's) family comprises four members: i) EGFR; ii) ErbB-2; iii) ErbB-3 and ErbB-4 and are involved in the development of a variety of cancers [197,198]. These receptors are composed of five domains including: i) extracellular domain; ii) transmembrane domain; iii) juxtamembrane domain; iv) tyrosine kinase (TK) domain and v) C-terminal tail [199]. Activation of EGFR/ErbB-receptors by extracellular EGF ligand promotes their hetero-dimerization with subsequent activation of TK-domains and tyrosine trans-phosphorylation of the cytoplasmic tail. However, the cytoplasmic proteins that are able to directly modulate EGF-induced activation and signaling of EGFR/ErbB-receptors were largely unknown. Recently cytohesin-2 (CTH2) was identified as a cytoplasmic activator of EGFR/ErbB-receptors (Fig. 6A) [200,201]. Cytohesin-2 enhances trans-dimerization and activation of EGFR/ErbB's by direct binding with TK-domains of dimerized receptors and by facilitating conformational changes and trans-phosphorylation of these domains. Fig. 6A illustrates the signaling of the epidermal growth factor (EGF) via EGFR/ErbB's localized at the plasma membrane and early endosomes and the crucial role of CTH2 in hetero-dimerization of these receptors. It also depicts trafficking and signaling of EGFR/ErbB-receptors via the clathrin-dependent endocytosis (CDE) endosoma/lysosomal protein degradative pathway. After ligand binding and CTH2-dependent activation at the plasma membrane, the EGFR/ErbB's are rapidly internalized into early endosomes (EE) from where they either: i) undergo dephosphorylation/deactivation and recycled back via recycling endosomes (RE) to the plasma membrane (Fig. 6A), or ii) are delivered via multivesicular bodies (MVB) and late endosomes (LE) to lysosomes (LS) for degradation (Fig. 6B). It is noteworthy that while initiated at the plasma membrane, signaling of EGFR/ErbB-receptors is sustained, enhanced and in some conditions actually originates from early endosomes [202,203]. Thus, V-ATPase-dependent acidification may play a key role in the modulation of EGFR/ErbB-receptors function in EE and is pivotal for their fate in sustained signaling, recycling or degradation (Fig. 6A). Moreover, it is reasonable to suggest, that similarly to the Wnt- and Notch pathways (see above), the cytohesin-2 may act as adaptor between EGFR/ErbB-receptors and V-ATPase at the plasma membrane, early endosomes and even lysosomes (Fig. 6A and B). However, this potentially important role of cytohesin-2 must be explored experimentally.

On the other hand, it is important to underline, that a novel important role of the V_1V_O assembly/disassembly mechanism and V-ATPase-dependent acidification of LE and LS in EGFR/ErbB-receptors function was recently revealed [161]. Previous studies clearly demonstrated that EGF/EGFR-dependent signaling contributes to proliferation of liver cells and is an important regulator of hepatic regeneration *in vivo* [204]. This signaling involves EGF-induced activation of mTORC1 on late endosomes/lysosomal compartments of hepatocytes [205,206]. It was shown that EGF-induced activation of mTORC1 involves the Akt/Erk activation, TSC complex inhibition and Rheb(GTP) formation. However, in contrast to amino acid-induction studies [192], this signaling does not accompany mTORC1 recruitment from the cytosol and its translocation to the lysosomal membrane [161,192]. Surprisingly, it was demonstrated that EGF/EGFR-dependent signaling promotes the rapid recruitment of cytosolic V_1-sectors of the V-ATPase and gives rise to its increased assembly as V_1V_O-holocomplexes on late endosomal/lysosomal compartments. This assembly in turn gives rise to increased V-ATPase driven lysosomal acidification, increased protein degradation and release of amino acids from lysosomal compartments needed for Rheb(GTP) and mTORC1 activation (Fig. 6B). In this scenario, signaling of EGFR/ErbB-receptors results in Erk/Akt activation and consequent assembly of V_1V_O-holocomplexes of LE/LS V-ATPase. Erk/Akt activation promotes an increased V-ATPase driven acidification of these compartments, increased protein degradation and amino acid production followed by their release and "cytosolic sensing" by Rheb and activation of the mTORC1 complex. According to this study, the V-ATPase is playing an indirect role in EGF-dependent activation of mTORC1 signaling pathway and it occurs by modulating the V_1V_O-holocomplexes assembly/disassembly mechanism of V-ATPases [192]. Therefore, this study provides the first evidence showing the functional assembly of V-ATPase V_1V_O-holocomplexes in response to the signaling of EGFR/ErbB-receptors. It also revealed an unexpected role of V-ATPase in the regulation of mTORC1 signaling and trafficking EGFR/ErbB-receptors within the endosomal/lysosomal protein degradation pathway (Fig. 6 A and B) [161,192]. However, in this scenario, the V-ATPase is indirectly involved in the regulation of amino acid levels and their sensing in the lysosomal lumen and cytosol. According to this electrochemical mechanism, the V-ATPase driven lysosomal acidification may modulate the function of mammalian pH-dependent amino acid transporters (PATs), which equilibrate the amino acid pools between cytosol and lysosomal lumen [185,207–209]. Thus, additional studies are needed to clarify the differential direct and indirect roles of V-ATPase in mTORC1 function promoted either by: i) modulation of levels of amino acids [192] or ii) activation of EGFR/ErbB's signaling pathway [161] (Fig. 6 A and B).

In summary, the lysosomal organelles play a central regulatory role in cellular protein degradation and energy production using V-ATPase/mTORC1 "sensing machinery" to monitor both lysosomal and cytosolic amino acid content as indicator of nutritional status of the cell. This important physiological information is further communicated to the nucleus to activate the feedback gene expression programs allowing lysosomes to regulate their own function. This two-step mechanism involves the transcription factor EB (TFEB) that acts as: i) a sensor of nutritional status directly binding to mTORC1 on lysosomal membrane; and ii) an effector of lysosomal function after its translocation to the nucleus [210]. In addition, other functions of the lysosomes-dependent mTOR signaling are the cellular clearance and removal of organelles by autophagy. This quality control process declines over lifespan, contributing to aging and age-associated diseases, and thus, is considered as an important therapeutic drug target. During recent years extensive studies in animal models and clinical trials have uncovered the beneficial action of rapamycin, an FDA-approved mTOR inhibitor, for treatment

of variety age-associated diseases including cancers and neurodegenerative disorders [185,187] (Fig. 6). Inhibition of mTOR by rapamycin is also beneficial for treatment of kidney diseases including: i) renal cell carcinoma, ii) diabetic nephropathy and ii) polycystic kidney disease [188,189]. However, due to its side effects caused by the differential action of rapamycin on both mTORC1 and mTORC2, there is growing necessity for more specific and efficient targeting of these pathways [185,187,191]. Therefore, specific targeting of amino acid-sensing V-ATPase/Ragulator/Rag protein-protein interactions by small molecules that will give rise to differential targeting of the mTORC1 and mTORC2 super-complexes offers an attractive therapeutic approach to control aberrant localization and function of mTORC1 and mTORC2 in variety of age-related disease states [185,187].

4.1.7. V-ATPase, cytohesin-2/Arf's and EGFR/ErbB-receptors in modulation of the macropinocytosis pathway

The clathrin-dependent endocytosis (CDE) pathway is the best studied pathway of endocytic uptake and trafficking receptors including EGFR/ErbB's (Fig. 6A, B). However, various clathrin-independent endocytosis (CIE) pathways have been also identified and include: i) Arf6-dependent endocytosis and ii) macropinocytosis pathways [211,212]. The Arf6-dependent CIE pathway operates constitutively and intersects in early endosomes with the CDE pathway [213,214]. This pathway is involved in constitutive trafficking of a variety of proteins such as: MHC1, β-integrins and GPI-anchored proteins in the "resting" state of HeLa and COS cells. However, the modulation of Arf6-dependent signaling quickly promotes the switch from a constitutive to a stimulated uptake involving an elaborate interplay between cytohesin-2/EFA6 GEFs and Arf6/Arf1 small GTPases [213,214]. The cell specific macropinocytosis also co-exists and cross-talks with the CDE endosomal/lysosomal protein degradative pathway (Fig. 6). Its regulation depends on particular cellular requirements and macropinocytosis is transiently stimulated by signaling of c-Src or EGFR/ErbB-receptors [215,216]. For example, while at low levels of EGF, the internalization of EGFR/ErbB's takes place via endosomal/lysosomal dependent CDE pathway, at high levels of EGF the internalization of EGFR/ErbB-receptors occurs via a vigorous macropinocytosis pathway [217–219]. Thus, these studies uncovered the EGF-ligand dependent activation of macropinocytosis and its cross-talk between endocytic endosomal/lysosomal pathway in trafficking of EGFR/ErbB-receptors [217–219]. Additional studies of this phenomenon have shown that plasma membrane NHE-exchanger driven cytosolic acidification and Rac1/Cdc42 small GTPase dependent actin cytoskeleton remodeling are downstream effectors of EGF-ligand dependent activation of the macropinocytosis pathway [220]. Importantly, the recent study reported that in Ras-transformed cancer cells macropinocytosis represents an important route of nutrient uptake in tumors, pointing to the possible exploitation of this process in the development of anticancer therapies [221].

However, the involvement and cell biological role of V-ATPase in the regulation of this an important endocytic pathway was largely unknown. Thus, recently we addressed the potential implication of V-ATPase and cytohesin-2/Arf small GTPase in modulation of the macropinocytosis pathway [222]. We studied the cell biological action of the novel V-ATPase derived cell permeable peptides that are potent inhibitors of the cytohesin-2 GEF activity and modulators of Arf6/Arf1 signaling. It was shown that V-ATPase derived cell permeable peptides could dramatically activate an uptake of albumin-Alexa555 and RITC-dextran endocytic markers. Analysis of the early cell biological events revealed that appearance of the V-ATPase derived anti-cytohesin-2 peptides promotes: i) cell shape remodeling, ii) formation of the large vacuoles and iii) accelerated movement of early endosomal compartments. These cell biological events are consistent with activation of the cytohesin-2/Arf small GTPases dependent macropinocytosis pathway, which was previously described in HeLa and COS cells [211–214]. It was proposed that signaling between V-ATPase and cytohesin-2/Arf small GTPases may serve as a regulatory switch in cross-talk between macropinocytosis and receptor-mediated endosomal/lysosomal protein degradative pathways in the regulation of trafficking variety receptors including EGFR/ErbB's (Fig. 6) [222]. However, the exact cell biological and molecular events and downstream effectors underlying this novel regulatory mechanism need to be clarified.

4.2. Functions of V-ATPase in the exocytotic pathway and its role in disease states

Vesicular trafficking within the exocytotic pathway is involved in communication between intracellular organelles (endoplasmic reticulum, Golgi, lysosomes) and in delivery of newly synthesized receptors and secretory proteins to the plasma membrane [223]. In the following chapter we will focus on the emerging role of V-ATPase in the fission/fusion process and organelle biogenesis as well as its role in protein glycosylation, exocytosis from ER/Golgi, and trafficking to lysosomes and plasma membrane.

4.2.1. Role of V-ATPase in modulation of fission/fusion process, organelle biogenesis and exocytosis

Budding/fission of vesicles from exocytotic donor organelles and their fusion with an acceptor compartment/membrane is a tightly controlled process that is regulated by V-ATPase [1,224]. In particular, a direct role of the *c*-subunits of the V_O sector was implicated in membrane fusion along this pathway and the molecular details of the fusion-dependent conformational changes of the *c*-subunits were recently analyzed [225]. In *S. cerevisiae* the fusion process is regulated by Vph1 (yeast homolog of a-subunit of V-ATPase) Rab-GTPase Ypt7 and calmodulin [224,226,227]. The regulatory role of a-subunit isoforms of V-ATPase was also uncovered in the exocytotic pathway of other organisms. The direct interaction of the a1-soform with calmodulin is involved in fusion of synaptic vesicle in *Drosophila melanogaster* synapses [136,228], while secretion of Hedgehog-related proteins from exosomes to the apical membrane depends upon the a-subunit in *Caenorhabditis elegans* [229]. In mammals, the V-ATPase controls exocytotic trafficking and secretion of hormones. The a3-subunit isoform of V-ATPase is highly expressed in endocrine tissues including pituitary, adrenal, parathyroid and thyroid glands. Thus, it was suggested that the a3-isoform is commonly involved in the regulation of the exocytotic pathway and secretion [1,230]. In particular, the a3-isoform is specifically targeted to the V-ATPase in secretory vesicles containing insulin of mouse pancreatic β-cells [1]. Moreover, oc/oc-mice (a null mutant of the a3-isoform gene) are defective in insulin exocytosis. This study suggests that insulin secretion by mouse pancreatic β-cells specifically requires intact a3-subunit isoform containing V-ATPase, that could not be replaced by the a2-subunit isoform. The a3-subunit isoform is also specifically targeted to the plasma membrane of vertebrate osteoclasts [1,111]. In these cells, the V-ATPase is involved in the bone resorption process by acidifying the "bone resorption lacuna", an extracellular space formed between the plasma membrane and the bone surface. The V-ATPase dependent acidic environment of this space is necessary for matrix protein degradation and for mineral dissolution. The a3-isoform is also specifically targeted to lysosomes but upon differentiation is exocytosed to the plasma membrane of the osteoclast-like RAW264.7 cells [1,111]. Thus, the localization of the V-ATPase a3-isoform is a dynamic trafficking and targeting process. In accordance to the pivotal role of V-ATPase in bone homeostasis, multiple mutations of the a3-isoform give rise to diseases of bone resorption and are associated with osteopetrosis in both mice models [231] and in humans [232,233]. In human osteoclasts the a3-subunit is specifically assembled and functions with the d2-isoform of the V-ATPase holocomplex [234]. Accordingly, in the studies of d2-isoform knockout mice the direct role of the d2-isoform in the fusion of osteoclast progenitors was suggested [235].

Numerous eukaryotic organelles, including mitochondria, endosomes, lysosomes and Golgi apparatus among others, change their copy number, size and morphology in response to the cellular environmental conditions and their nutrient status. This homeostasis of organelles is controlled by the coordinated balance between fission and fusion processes. As discussed above, whereas the fusion of membranes was relatively well studied, the membrane fission and organelle fragmentation remains poorly understood. However, their differential regulatory mechanisms were recently identified using purified yeast vacuoles in cell-free reconstitution, fission/fragmentation and fusion experiments [224,236]. These studies uncovered the dual function of V-ATPase in the control of the vacuolar/lysosomal fusion and fission. While vacuolar/lysosomal fusion requires physical interactions with the V_O sector of V-ATPase, the vacuolar/lysosomal fission process depends upon proton pumping and acidification capacity of the intact V-ATPase nano-motor. Importantly, these studies also revealed an unexpected role of vacuole-associated TORC1 (the yeast analog of mammalian mTORC1 complex) in vacuolar/lysosomal membrane dynamics in *S. cerevisiae*. Under nutrient restriction conditions, yeast TORC1 is inactivated and promotes specific fusion but not fission of the vacuoles [224,236]. Thus, these studies have uncovered a complex interplay between V-ATPase and TORC1 complex in the regulation of vacuolar/lysosomal dynamics via both direct involvement of V-ATPase and its role as proton-pumping nano-motor.

4.2.2. Role of V-ATPase in protein glycosylation and exocytosis from ER/Golgi, and trafficking to lysosomes and the plasma membrane

The importance of the structural and functional integrity of the a-subunit isoforms in V-ATPase function is strongly supported by a variety of human genetic diseases associated with mutations in these proteins [232,233,237]. Genetic defects in a-subunit V-ATPase genes include mutations in the a3-subunit (ATP6V0A3), causative of infantile malignant autosomal recessive osteopetrosis (ARO) [232], and a4-subunit (ATP6V0A4), causative of recessive distal renal tubular acidosis (dRTA) [237]. The pathophysiology of these human diseases was intensively studied during the last decade and is reviewed elsewhere [1,4]. However, recently studied loss-of-function mutations in the V-ATPase a2-subunit isoform (ATP6V0A2) were also identified as cause of an autosomal recessive cutis laxa type II (ARCL II) or wrinkly skin syndrome (WSS) [238]. To date, a total of 200 patients and 41 different mutations were identified, of which 18 frame-shift and missense mutations were located on the N-terminal cytosolic tail of the a2-subunit (a2N)(Fig. 1) [239–242]. Importantly, one of the frame-shift mutations described in these studies is involved in modification of integrity of the $a2N_{1\text{-}17}$ epitope, the structure and function of which was determined in our recent study (see below). Cutis laxa is a rare inherited disease accompanied with decreased elasticity of the skin and appearance of wrinkles. Formation of laxed and wrinkled skin is a physiological process of normal human aging caused by a degeneration of elastic fibers of the extracellular matrix. Homeostasis of the extracellular matrix of the connective tissue of the skin depends on very active post-translational glycosylation of proteins (e.g. collagen, fibronectin, and fibulins) in Golgi complex and their secretion via exocytotic pathway. Thus, since ARCL II patients frequently present with an aged appearance, this syndrome at least in part may be related to human aging processes of the skin. Indeed, at the molecular and cellular level, loss-of-function mutations in the ATP6V0A2 gene give rise to loss of V-ATPase function and are accompanied with: i) defects in Golgi acidification; ii) impaired N- and O-glycosylation of proteins in Golgi complex; iii) impaired tropoelastin secretion and aggregation in the Golgi and iv) increased apoptosis of elastogenic cell [239–242]. Thus, the discovery of the cutis laxa related mutations in the human gene encoding a2-subunit V-ATPase underlined the crucial role of V-ATPase in the regulation of protein glycosylation within the Golgi complex and their trafficking via exocytotic pathway. However, further studies are necessary to elucidate the exact direct and/or indirect/electrochemical role of V-ATPase in modulation of Golgi function, defects of proteins glycosylation, pathogenesis of cutis laxa disease and the potential role of these cellular events in normal human aging processes.

It is generally accepted that the V-ATPase dependent acidification of Golgi complex is essential for synthesis and delivery of the lysosomal hydrolases from ER/Golgi to lysosomes via the mannose-6-phosphate receptor pathway [243,244]. However, the molecular mechanism involved in the assembly of the V-ATPase complex itself in the ER/Golgi followed by its trafficking and specific targeting to lysosomal compartments remains elusive. Recent studies have suggested PS1 as a potential chaperon for a1-subunit V-ATPase and the oligosaccharyl-transferase (OST) during its N-glycosylation needed for proper folding and trafficking to lysosomes [139]. However, this concept was recently disputed by three laboratories (also see above) [140–143].

5. V-ATPase is a novel evolutionarily conserved pH-sensing and cytohesin-2 signaling receptor

The primary physiological function of the V-ATPase nano-motor is acidification of intracellular compartments and extracellular milieu. However, recent studies uncovered that V-ATPase itself also operates as a pH-sensing and cytohesin-2 signaling receptor (Fig. 7) [145]. This function of the V-ATPase is evolutionarily conserved, and thus, may be a universal attribute of eukaryotic cells from yeast to humans. The chapter below describes the structural basis and functional insights of this novel concept of V-ATPase function and will briefly discuss its potential role in health and disease.

5.1. Function of V-ATPase as a putative pH-sensing receptor

Original studies from our laboratory and others have proposed the presence of a hypothetical “pH-sensing protein” (PSP) that modulates the recruitment of cytosolic proteins to external membrane outer-leaflet of intracellular organelles in response to pH-levels of organelle lumen [1,69,70]. In particular, work from the Schulz laboratory demonstrated that the interaction of Arf GTP-binding proteins with purified pancreatic microsomal vesicles depends upon their acidification [245,246]. However, the specific members of the Arf-family and specific compartments involved in this interaction remain obscure. Subsequently, Gruenberg and coworkers have proposed an involvement of a hypothetical PSP in endosomes of baby hamster kidney (BHK) cells, based on the finding of acidification-dependent recruitment of β-COP, ε-COP and Arf1 proteins [247–249]. However, in these studies the pH-sensing mechanism and importantly the origin of PSP were not determined. On the other hand, in a search for unknown PSP, our laboratory focused on studies of the functional interplay between V-ATPase and cytohesin-2/Arf's in function of early endosomes of megalin/cubilin-receptor protein degradative pathway of the kidney proximal tubules (PT) epithelial cells [250,251]. The Arf-family GTP-binding proteins belong to the Ras-superfamily of small GTPases that are involved in the regulation of a great variety of cellular pathways [252]. These regulatory proteins function as “molecular switches” and the transition between “on” and “off” states of this molecular device is mediated by a GDP/GTP cycle. In particular, activation of Arf's is accomplished by the cytohesin-subfamily of guanine nucleotide exchange factors (GEFs). Cytohesin-family GEFs includes cytohesin-1, cytohesin-2 (ARNO), cytohesin-3 (or GRP1) and cytohesin-4. It is important to underline that cytohesin-2 (CTH2) is also generally known and was referred in our previous publications as ARNO (ADP-ribosylation nucleotide-side opener) [69,251,253,254]. These are highly conserved proteins composed of four domains: i) an N-terminal coiled-coil (CC); ii) a central Sec7 domain (Sec7); iii) a pleckstrin homology (PH) domain; and iv) a C-terminal polybasic (PB) domain (Fig. 8A, B) [255–257]. In particular, the Sec7 domain binds and activates Arf's via catalysis of the GDP/GTP-exchange reaction. The generally accepted functions of cytohesin/Arf proteins are regulation of organelle biogenesis, modulation of vesicular trafficking and actin

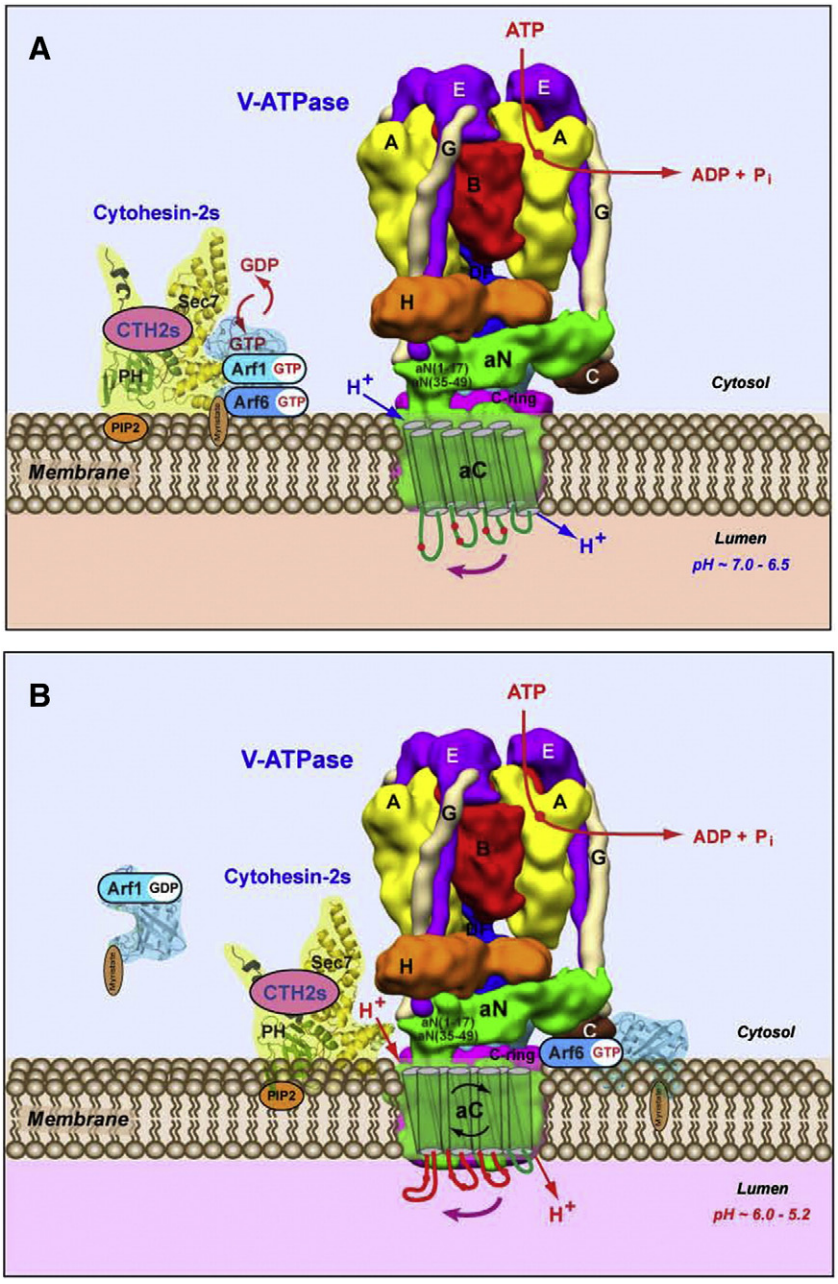

Fig. 7. The V-ATPase nano-motor is a novel evolutionarily conserved pH-sensing and cytohesin-2 signaling receptor. Model of the novel role of V-ATPase nano-motor as a pH-sensing and cytohesin-2 signaling receptor. The molecular details of the interactions of V-ATPase with cytohesin-2 and Arf GTP-binding proteins at high (A) and low (B) levels of luminal acidification. A) Schematic representation of the rotary proton-pumping eukaryotic V-ATPase, which does not interact with cytohesin-2 and myristoylated Arf1/Arf6 GTP-binding proteins anchored on the PIP_2 containing membrane. The structure of V-ATPase and subunit composition are shown as in Fig. 1. The position of epitope a-subunit isoforms formed by aN(1–17) and aN(35–49) peptides that is involved in the interaction with the Sec-7 domain of cytohesin-2 is also indicated. B) The V-ATPase is sensing low luminal pH with its transmembrane aC part of a-subunit isoform. This results in recruitment and interaction of cytohesin-2 with the cytosolic aN part of a-subunit of V-ATPase. The molecular details uncovered in our recent studies revealed that this binding involves N-terminal epitope of V-ATPase a-subunits formed by aN(1–17) and aN(35–49) peptides and Sec7 domain of cytohesin-2 [145,266]. This interaction of the V-ATPase with cytohesin-2 could modulate its signaling with Arf6 and Arf1 GTP-binding proteins and also may be involved in modulation of the function of V-ATPase itself. Structure of Sec7/Ar1 complex pdb code 1S9D. Adapted with permission from [145].

cytoskeleton remodeling [255–257]. However, cytohesins have also recently emerged as central modulators of signaling and trafficking of variety plasma membrane receptors including: i) integrin-; ii) EGFR/ErbB-, iii) VEGFR2- and iv) insulin-receptors [200,201,258–264]. In particular, cytohesin-1/2 have been identified as activators of EGFR/ErbB's that are involved in oncogenesis [200,201]. Cytohesins are also crucial downstream effectors for the insulin-receptor signaling cascade involved in development of insulin resistance and metabolic syndrome during diabetes [261–264].

Studies from our laboratory have also revealed a central role of cytohesin-2/Arf6 in function of the V-ATPase involved in the regulation of megalin/cubilin-receptors mediated endosomal/lysosomal protein degradative pathway. It was shown that cytohesin-2 and Arf6 are specifically targeted to early endosomes of this pathway and colocalized with the endosomal V-ATPase in kidney PT *in situ* (Fig. 7) [251,253]. Moreover, *in vitro* reconstitution experiments with purified early endosomes demonstrated that specific recruitment of both cytohesin-2 and Arf6 (but not Arf1) from the cytosol to endosomal membranes depends upon V-ATPase driven intra-endosomal acidification. Whereas the existence of the unknown "pH-sensing protein" (PSP) and its direct interaction with both cytohesoin-2 and Arf6 were suggested in these studies, its nature and the mechanism of its pH-dependent interaction

with cytohesin-2/Arf6 remained elusive [251]. However, subsequent work revealed that V-ATPase itself is a long-sought after PSP or pH-sensing receptor. In particular, it was shown that V-ATPase containing transmembrane a2-subunit isoform is specifically targeting to early endosomes of the kidney PT cells (Fig. 7). Importantly, the direct interaction of a2-subunit with cytohesin-2 and c-subunit with Arf6 GTP-binding proteins was shown. These interactions are specific and dependent upon the V-ATPase-driven acidification of the endosomal lumen (Fig. 7). Thus, the a2-isoform containing endosomal V-ATPase was identified as a putative pH-sensing receptor [69]. A model of this novel V-ATPase function was also proposed, in which the histidine amino acids residues in the a2-subunit of V-ATPase are involved in the pH-sensing mechanism of the V-ATPase function [70]. These histidine residues form a part of a transmembrane/luminal domain of a-subunit (aC) that senses the intra-endosomal pH and transmits this information through its conformational changes to the cytoplasmic domain (aN) of the protein (Fig. 7) [70,145]. Therefore, according to this novel concept, the eukaryotic V-ATPase nano-motor is not only responsible for proton pumping and the generation of a pH gradient across the membranes. It is also involved in sensing levels of acidification or measuring pH and transmitting this information from one membrane side to another. In summary, the function of V-ATPases as a putative pH-sensing receptor would, therefore, add acidic pH to the growing list of signals that can be transmitted via a still uncharacterized transmembrane signaling mechanism (Fig. 7).

5.2. Function of V-ATPase as a cytohesin-2 signaling receptor

Original work from our laboratory uncovered V-ATPase as pH-sensing receptor that recruits the cytosolic cytohesin-2 and Arf6 in an acidification-dependent manner (Fig. 7) [69,70,251]. Moreover, it was also shown that interaction with cytohesin-2 is not only restricted to the a2-subunit isoform but also occurs with the three other a-subunit isoforms (a1, a3 and a4) of the V-ATPase [265]. Taken together, these data indicate that pH-sensing by V-ATPase and interaction with cytohesin-2/Arf's is a general cell biological phenomenon, which may take place in other acidic organelles of both the exocytotic and the endocytic pathways (Fig. 6). However, the following questions remain to be addressed: i) what are the molecular mechanism and cell biological significance of the V-ATPase/cytohesin-2 interaction and ii) what are other downstream effectors and pathways intertwined with this cell biological event. Our recent studies have addressed these issues and uncovered the molecular details of another unexpected function of V-ATPase, as an evolutionarily conserved cytohesin-2 signaling receptor (Fig. 8).

First, we focused on the molecular mechanism of interaction between cytosolic tail of a2-subunit V-ATPase (a2N) and cytohesin-2 (CTH2) [254]. The interaction sites between these two proteins were mapped using the combination of recombinant proteins/synthetic peptides pull-down experiments. In particular, recombinant protein pull-down experiments demonstrated that a2N recombinant protein interacts strongly with the Sec7-domain of one molecule of cytohesin-2 (CTH2s). In these experiments the weak interaction of another molecule of cytohesin-2 (CTH2p) via its PH-domain was also detected (Fig. 8A, B, and C). On the other hand, using a synthetic peptide pull-down approach, we identified the N-terminal epitope of the a2N as a major interacting site with CTH2s. This epitope corresponds to the peptide $a2N_{1-17}$, which is formed by the first seventeen amino acids (MGSLFRSESMCLAQLFL) of the a2-subunit isoform of V-ATPase. Moreover, additional surface plasmon resonance (SPR) experiments also

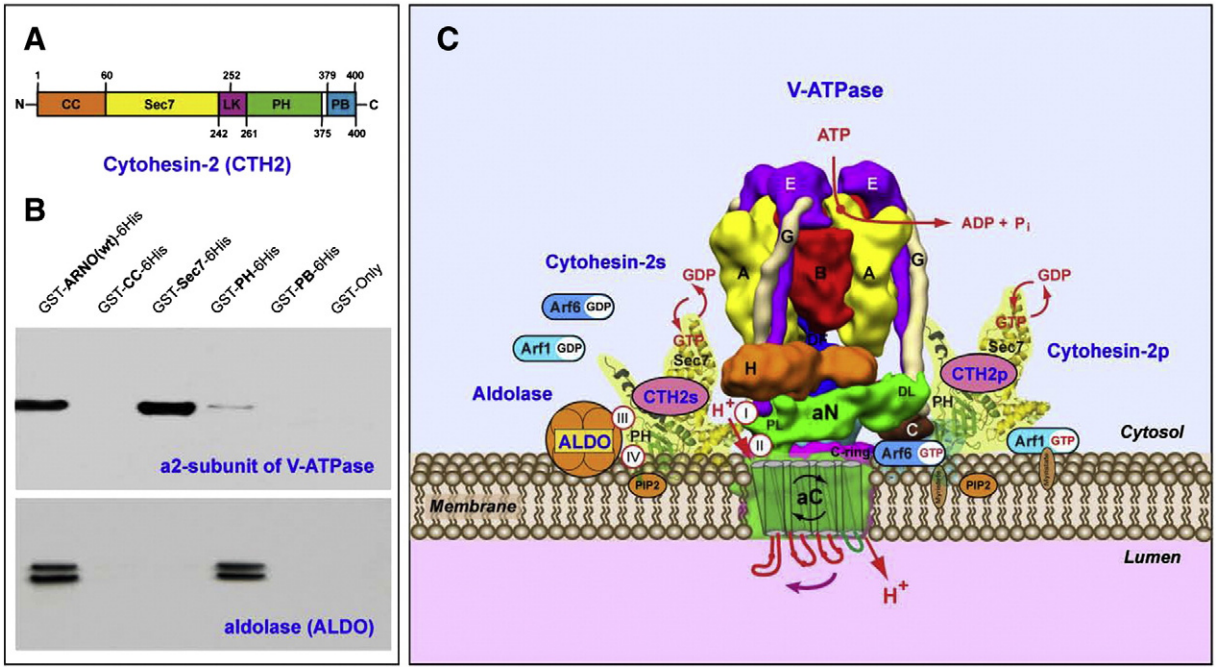

Fig. 8. Identification of V-ATPase as a novel cytohesin-2 signaling receptor and characterization of V-ATPase/cytohesin-2/aldolase/Arf's super-complex. A) Schematic representation of domains and regulatory elements of cytohesin-2 (CTH2). These structures are indicated as follows: i) CC-domain (1–60aa) in orange; ii) Sec7-domain (61–242aa) in yellow; iii) Sec-7-PH-linker (LK) (242–261aa) in magenta; iv) PH-domain (262–375aa) in green; v) PB-domain (376–400aa) in blue. Boundaries of the domains are indicated as amino acid numbers. B) Western blot analysis showing the interaction of cytohesin-2 with a2-subunit of V-ATPase via its Sec7 and PH-domains. Western blot analysis showing the interaction of cytohesin-2 with aldolase via its PH-domain. C) The diagram shows the structure of the novel V-ATPase/CTH2/ALDO/ Arf's super-complex (*Complex 1*, *EE*) localized on endosomal membrane (Fig. 6). It illustrates two binding sites for two cytohesin-2 (CTH2, in yellow) molecules with an N-terminal tail of a2-subunit (a2N, in green) of V-ATPase. The first cytohesin-2 molecule (CTH2s) is shown on the left, which, via its Sec7-domain, is binding to the proximal lobe (PL) to the epitope formed by the peptides a2N(1–17) and a2N(35–49) of a2N. The second cytohesin-2 molecule (CTH2p) is shown on the right, which, via its PH-domain, is binding to the distal lobe (DL) of epitope formed by the peptides a2N(198–214) and a2N(215–230) of a2N [145,266]. While binding of CTH2s to V-ATPase inhibits its GEF activity with Arf1 and Arf6, binding of CTH2p might have stimulatory action on its GEF activity with Arf1 and Arf6. The binding of cytohesin-2 with aldolase (ALDO) as well as Arf6 with *c*-subunit of V-ATPase is also shown. Roman numbers indicate interfaces and affinities of interaction: I) CTH2 with a2N(1–402) and II) Sec7 domain with a2N(1–17) and III,IV) CTH2 with ALDO. As suggested in our original study, two aldolase bands represent the full length and a partially translated but interaction-competent version of recombinant human aldolase [265].

confirmed that these structural elements are major binding sites between a2N of V-ATPase and CTH2s. In particular, this analysis revealed a strong binding affinity between this $a2N_{1-17}$ peptide and the Sec7-domain of CTH2s, with a dissociation constant of $K_D = 3.44 \times 10^{-7}$ M, similar to the binding affinity $K_D = 3.13 \times 10^{-7}$ M between full-length $a2N_{(1-402)}$ and CTH2 proteins (Fig. 8C, interfaces I and II). Based on these real-time kinetic experiments, we suggested that this V-ATPase epitope-forming $a2N_{1-17}$ peptide is crucial for V-ATPase/CTH2s signaling, and could be involved in the regulation of cytohesin-2 enzymatic Arf-GEF activity by V-ATPase. To test this hypothesis a combination of enzymatic and structural approaches were applied [145]. Indeed, these studies revealed that $a2N_{1-17}$ peptide is a potent inhibitor of the enzymatic GDP/GTP-exchange activity of wild-type CTH2s, that is acting via its direct interaction with the catalytic Sec7 domain. The α-helical structure of $a2N_{1-17}$ and its residues F_5, M_{10}, Q_{14} binding with the Sec7 domain were also identified by NMR spectroscopy analysis (Fig. 9A, B). *In silico* docking studies have shown that $a2N_{1-17}$ epitope of V-ATPase competes with the switch-2 region of Arf's for binding to the Sec7 domain of CTH2s (Fig. 9C, D). Together, these experiments revealed the structural basis and molecular details of a novel mechanism of signaling between the V-ATPase V_1V_O-holocomplex and CTH2/Arf's GTP-binding proteins (Fig. 9). Sequence alignment of the N-terminal epitope of all four a1-, a2-, a3- and a4-subunit isoforms has shown that the V-ATPase amino acids involved in the interaction with Sec7 domain are highly conserved in all eukaryotes from yeast to humans, and these peptides are also efficient inhibitors of CTH2s [145]. Finally, the conserved character of this signaling event was also confirmed in experiments showing binding of human CTH2 to the purified intact yeast V-ATPase V_1V_O-holocomplex (Fig. 1)[145]. In summary, these studies have uncovered an unexpected function of the V-ATPase as a novel cytohesin-2 signaling receptor (Fig. 8C). However, the downstream effectors and pathways that are regulated by this signaling event remain obscure.

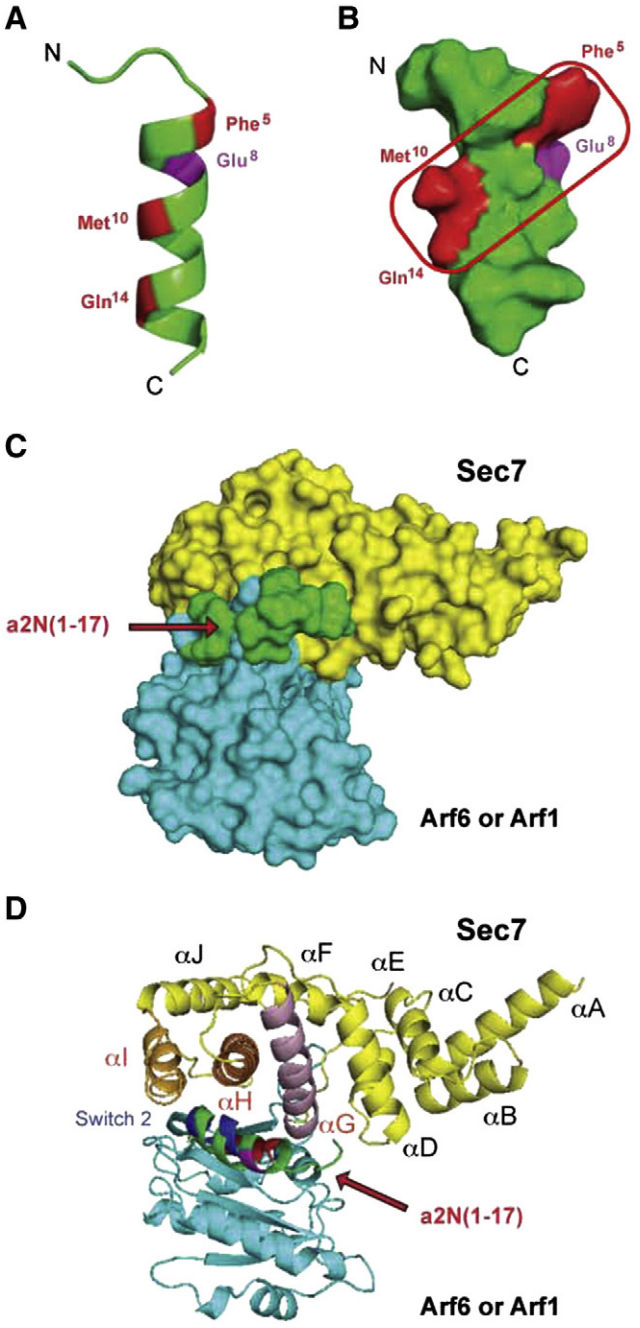

Fig. 9. Structural basis and molecular mechanism of signaling between V-ATPase and cytohesin-2/Arf's. A) Determination of interaction-competent amino acids and the interaction surface plane of a2N(1–17) peptide involved in binding with Sec7 domain. Location of the four amino acids F5, M10, Q14 (in red) and E8 (in magenta) that were determined by NMR to be involved in the interaction of a2N(1–17) peptide with Sec7 domain. B) Identification of the interaction surface plane formed by amino acids F5, M10, Q14 (shown in red square) that is involved in binding of a2N(1–17) with Sec7 domain. C) *In silico* docking experiments revealed the binding site of a2N(1–17) peptide near the catalytic site of the Sec7 domain with Arf1 and Arf6 GTP-binding proteins. D) The binding of the a2N(1–17) peptide on the Sec7 domain involves the αG, αH and αI helixes, which are crucial for its catalytic activity with Arf1 and Arf6 GTP-binding proteins. Spatial structure of Sec7 domain and Arf1 was taken from the crystal structure of the complex of Sec7/Arf1 with brefeldin A as a stabilization agent (pdb code 1S9D). The atomic coordinates and structure factors of peptide a2N(1–17) (pdb code 2LX4) have been deposited in the Protein Data Bank (http://wwpdb.org/). Adapted with permission from [145].

Second, to address these questions the structural model of the complete N-terminal cytosolic tail of a2-subunit ($a2N_{1-402}$) was generated using a combination of homology modeling and NMR structural analysis [266]. The complete molecular model of $a2N_{1-402}$ revealed that all six a2N-derive and CTH2-interacting peptides are clustered into just two binding sites, in the proximal lobe (PL) and distal lobe (DL) subdomains of $a2N_{1-402}$ (Fig. 8C). These data suggest that while the PL subdomain is the major interacting site with the Sec7 domain of the first molecule of cytohesin-2 (CTH2s), the DL sub-domain most likely interacts with the PH-domain of the second molecule of cytohesin-2 (CTH2p) (Fig. 8C). Moreover, further analysis of this model revealed, that binding sites of both cytohesin-2 molecules (CTH2s and CTH2p) are located in a close proximity to binding sites of S1 and S2 EG-heterodimers with a-subunit, which form the peripheral stalks in the V_1V_O-holocomplex of V-ATPases (Figs. 1 and 8C). These data indicate that pH-dependent binding and signaling between V-ATPase and CTH2 may modulate the interaction of a-subunit isoforms with the S1 and S2 EG-heterodimer peripheral stalks (Figs. 1B and 3), and consequently modulate the reversible association/dissociation of the V_1 and V_O sectors of the V-ATPase holocomplex (Fig. 8C). In conclusion, it is tempting to hypothesize that an evolutionarily acquired by eukaryotes function of V-ATPase, as a pH-sensing and cytohesin-2/Arf6-signaling receptor, might be an integral part of the self-regulation mechanism of the primary V-ATPase function as a proton-pumping rotary nano-motor.

Third, in search for downstream effectors of the signaling between V-ATPase and cytohesin-2/Arf the potential role of aldolase in this function was recently explored [265]. The role of fructose bisphosphate aldolase (ALDO) as a central regulatory enzyme of glycolysis is well studied and generally accepted. However, recent identification of additional aldolase protein-protein interactions has pointed out its alternative function as a scaffolder protein. Aldolase was implicated in the regulation of: i) cytoskeleton rearrangement and cell motility; ii) trafficking and recycling of membrane proteins; iii) signal transduction during endocytosis and iv) modulation of V-ATPase function [265]. Indeed, in *S. cerevisiae* the reversible association/dissociation of V_1V_O sectors of the V-ATPase is modulated by levels of glucose [21–23]. It was proposed that in yeast this glucose-dependent mechanism is

controlled by the direct interaction of V-ATPase with aldolase that plays a role as a cytosolic glucose-sensor [153–155]. Three different subunits of the V-ATPase are involved in direct interaction of aldolase with yeast V-ATPase including a-subunit of the V_O sector and E- and B-subunits of the V_1-sector (Fig. 1) [1]. Thus, it was suggested that since V-ATPase interacts with both cytohesin-2 and aldolase, these two proteins could in turn interact with each other and coordinate the regulation of V-ATPase function. Indeed, using recombinant protein pull-down assay the direct interaction of aldolase with cytohesin-2 was shown and its binding via the PH-domain was mapped (Fig. 8A, B) [265]. The kinetics of aldolase-binding with CTH2 was further studied in surface plasmon resonance (SPR) experiments, showing a two-step binding ($K_{D1} = 1.1 \times 10^{-4}$ M and $K_{D2} = 2.7 \times 10^{-6}$ M) between these two proteins (Fig. 8C, interfaces III, IV). Moreover, cell fractionation experiments have confirmed the formation of V-ATPase/CTH2/ALDO/Arf's super-complex located on the early endosomes of the endosoma/lysosomal protein degradative pathway (Figs. 6 and 8) [265]. In conclusion, recent studies have uncovered V-ATPase as a novel pH-sensing and cytohesin-2 signaling receptor. This V-ATPase function takes place as an integral part of V-ATPase/CTH2/ALDO/Arf's super-complex that may be involved in: i) formation of multivesicular bodies (MVB) and control trafficking between early and late endosomes, and therefore, ii) regulation of trafficking and signaling of insulin- and EGFR/ErbB-receptors among others (Fig. 6). These findings have also clearly shown that signaling between V-ATPase a-subunit isoforms and cytohesin-2/Arf's is a general and evolutionarily conserved cell biological phenomenon. Thus, taking into consideration that a-isoforms target the V-ATPase to different cellular compartments, this signaling phenomenon may also play an important role in function of other organelles (lysosomes, Golgi, secretory vesicles and plasma membrane among others) along both endocytic and exocytic pathways of eukaryotic cell.

6. Future prospects in studies of eukaryotic V-ATPase

6.1. Future prospects for structural studies

The hybrid approach of combining cryo-EM with X-ray crystallography is likely to yield significant insight into the structure and function of the eukaryotic V-ATPase. Continued improvements in cryo-EM methods promise to deliver substantially improved resolutions for cryo-EM maps of the V-ATPase. On the other hand, crystallographic studies of the A-ATP synthase from thermophilic sources suggest that crystallography of the V_1 region of the eukaryotic enzyme should be feasible, once a sufficiently abundant source for this region of the complex can be established. While crystallization of the intact V_1V_O-holocomplex of V-ATPase may be also possible, inherent instabilities in the complex, heterogeneity due to subunit isoforms, and scarcity of the enzyme from natural sources offer significant barriers to this goal. To overcome these experimental limitations, *S. cerevisiae* offers a valuable source for cryo-EM and X-ray studies of the V-ATPase V_1V_O-holocomplex because, unlike most higher organisms, it only posses two isoforms for the a-subunit, which are encoded by the VPH1 and STV1 genes. Thus, as was recently performed, the heterogeneity of the *S. cerevisiae* enzyme could be eliminated by creating a yeast strain that lacks the less abundant subunit isoforms [30].

6.2. Future prospects for functional studies

The primary role of eukaryotic V-ATPase as a rotary proton-pumping nano-motor remains unchanged from the time of invention of this design by Nature billions of years ago and its implementation in archaea A-ATP synthase and mitochondrial F-ATP synthase. However, in eukaryotic cells this function of the V-ATPase requires a tight and efficient regulation in response to changes of cellular homeostasis, signaling of multiple specific receptors and micro-environment of variety intracellular compartments of both endocytic and exocytic pathways. Therefore, during an evolution the eukaryotic V-ATPases have acquired a unique regulatory mechanism of regulation of proton-pumping activity by reversible assembly/disassembly of V_1 and V_O sectors of V-ATPase V_1V_O-holocomplex. Moreover, the eukaryotic V-ATPases has also acquired the following alternative roles: i) as a receptor, capable of sensing and transmembrane signaling as well as, ii) direct and indirect modulator of trafficking and signaling of other cellular receptors. Importantly, since these alternative roles of V-ATPase are functionally related, it is tempting to hypothesize that these evolutionarily acquired secondary functions of V-ATPase are also intertwined with the cell biological mechanisms of their regulation. Undoubtedly, future functional studies will shed light on the molecular mechanisms and cell biological significance of these functions of eukaryotic V-ATPases. Finally, eukaryotic V-ATPase are also emerging as an important drug targets, useful to control signaling and trafficking of EGFR/ErbB, Fz/LRP6, Notch and insulin-receptors among others. Moreover, modulation of V-ATPase function by small molecules may be also useful in the regulation of cross-talks between these receptors with endosomal V-ATPase/CTH2/ALDO/Arf's and lysosomal V-ATPase/Ragulator/Rag/mTORC1/Rheb super-complexes. Therefore, the successful combination of structural and functional studies reviewed here, will very likely open an avenue to identify novel targets, which will lead to a generation of new drugs useful for the treatment of a variety of human diseases including cancer and diabetes as well as neurodegenerative and kidney disorders.

Acknowledgements

Original work in the authors' laboratories is supported by *NIH DK038452*, *BADERC DK057521-08* (Marshansky) as well as by *A*STAR BMRC*, 09/1/22/19/609, Ministry of Education Tier 2 (MOE2011-T2-2-156; ARC 18/12), Singapore (Grüber) and by *CIHR MOP81294* (Rubinstein). We thank Dr. S. S. M. Malathy (School of Biological Sciences, NTU) for the art work of the Figs. 2–4.

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