Histol Histopathol (2013) 28: 805-815
DOI: 10.14670/HH-28.805

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Review

Vacuolar-type proton pump ATPases: Acidification and pathological relationships

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Summary. Vacuolar H^+-translocating ATPase (V-ATPase) is a universal proton pump, and its activity is required for a variety of cell biological processes, such as membrane trafficking, receptor-mediated endocytosis, lysosomal degradation of macromolecules, osteoclastic bone resorption, and the maintenance of acid-base homeostasis by renal intercalated cells. V-ATPase is targeted to various membranes and has different compositions depending on its cellular location. Here, we focus on recent knowledge concerning the targeting mechanism of V-ATPase, a process associated with a wide spectrum of diseases. We also discuss the functions of this enzyme in macrophages and cancer cells-2 characteristic cell types with clinical importance.

Key words: V-ATPase, Acidification, Macrophage, Cancer

General introduction of V-ATPase

Vacuolar H^+-translocating ATPase (V-ATPase) is a ubiquitous enzyme found in various eukaryotes of the Animalia, Plantae, Fungi, and Protista kingdoms. Its function is to transport protons across membranes at the expense of ATP. As in the case of its sibling, the F-type ATP synthase, chemiosmotic energy conversion involves mechanical rotation within the enzyme complex (Hirata et al., 2003). In this respect, the F- and V-type proton translocating ATPases are highly unique enzymes that carry out chemical, electroosmotic, and mechanical energy transductions (Futai et al., 2012). These complicated reactions are performed via the interaction between the multiple subunits. However, the 2 enzymes do not share any components with each other. Another distinctive nature of the 2 proton pumps is their physiological function: V-ATPase participates in proton transport across membranes to acidify 1 side of the membrane, even though it can perform the reverse reaction, whereas the F-type ATPase mainly functions in the production of ATP from ADP and phosphate using the chemiosmotic gradient of protons generated by the respiratory chain or photosynthesis (Hirata et al., 2000; Wada et al., 2000). However, bacterial F-ATPase participates in the regulation of proton concentration by pumping out protons from the cytosol to the cell exterior (Shibata et al., 1992), and in archaebacteria, ATP synthesis is executed by the A-type ATPase, which closely resembles V-ATPase (Gogarten et al., 1989).

V-ATPase is a complex enzyme composed of at least 14 different subunits. The enzyme is organized into 2 main parts (Fig. 1). The V_1 sector is a complex that is located on the cytoplasmic side of the membrane and contains the catalytic center for ATP hydrolysis, whereas the V_O sector is embedded in the membrane and is responsible for the translocation of protons from the cytoplasm to the lumen or extracellular environment. The V_1 sector is composed of 3 copies of *A* and *B* subunits, and a single copy of *C, D, E, F, G,* and *H* subunits. V_O contains 6 different subunits. Yeast VO is composed of *a, d, e, c, c'* and *c"*, whereas higher eukaryotes contain the accessory subunits Ac45 (Jansen et al., 2010) and Ac8-9 (Kinouchi et al., 2010), but lack subunit *c'*. The proteolipid subunits (*c, c'* and *c"*) are highly hydrophobic and form a ring-like structure with a

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stoichiometry of 4 copies of c and single copies of the c' and c'' subunits (Powell et al., 2000; Wang et al., 2007). In mammals, the structural gene for c' is not found in the genome, therefore mammalian enzymes are highly likely to be composed of the c (16 kDa) and c'' (23 kDa) subunits (Sun-Wada et al., 2001). Mammalian genes and their corresponding yeast genes are summarized in Table 1. Overall, the V_OV_1 complex is a very large supramolecule with an apparent molecular mass of c.a. 900 kDa. Its atomic structure has not yet been resolved, although it is the subject of active research. V-ATPase is present in relatively low abundance; therefore, its purification and crystallization appear difficult compared to the F_OF_1 ATPase, which is abundantly expressed in the mitochondria of bovine hearts. Further, as we will discuss below, molecular heterogeneity, due to the presence of multiple subunit isoforms, negatively contributes to structural studies on the enzyme complex.

V-ATPase carries out rotational catalysis like the F-ATPase, whose mechanism has been extensively studied. It has been shown that the V_1 subunits, other than *A* and *B*, form 2 types of stalks, peripheral and central, which connect the V_1 and V_O domains. These stalks have distinct functions in the rotary mechanism by which V-ATPases couple ATP hydrolysis to proton transport. The central stalk, comprising subunits *F* and *D*, serves as a rotor that couples the energy released from ATP hydrolysis to the rotation of a ring of proteolipid subunits in V_O. The peripheral stalks, comprising subunits *C, E, G, H*, and the N-terminal domain of subunit *a*, serve to prevent the rotation of the *A*3*B*3 head during ATP hydrolysis, and therefore, serve a stator function (Forgac, 2007) (Fig. 1).

Subcellular localization of V-ATPase: targeting signal

V-ATPase is distributed in a wide array of endomembrane systems constituting the secretory and endocytic pathways, and at the cell surface of highly differentiated cells. This subcellular distribution appears uncommon for membrane proteins; many membrane-associated proteins, in general, show rather restricted localization in subcellular organelles. We postulated that the localization of V-ATPase is, at least in part, determined by the structural variations of the enzyme complex itself (Futai et al., 2000). The yeast genome contains a single structural gene for each V-ATPase subunit, except the largest membrane spanning 100-kDa *a* subunit. The yeast *a* subunit is encoded by 2 genes,

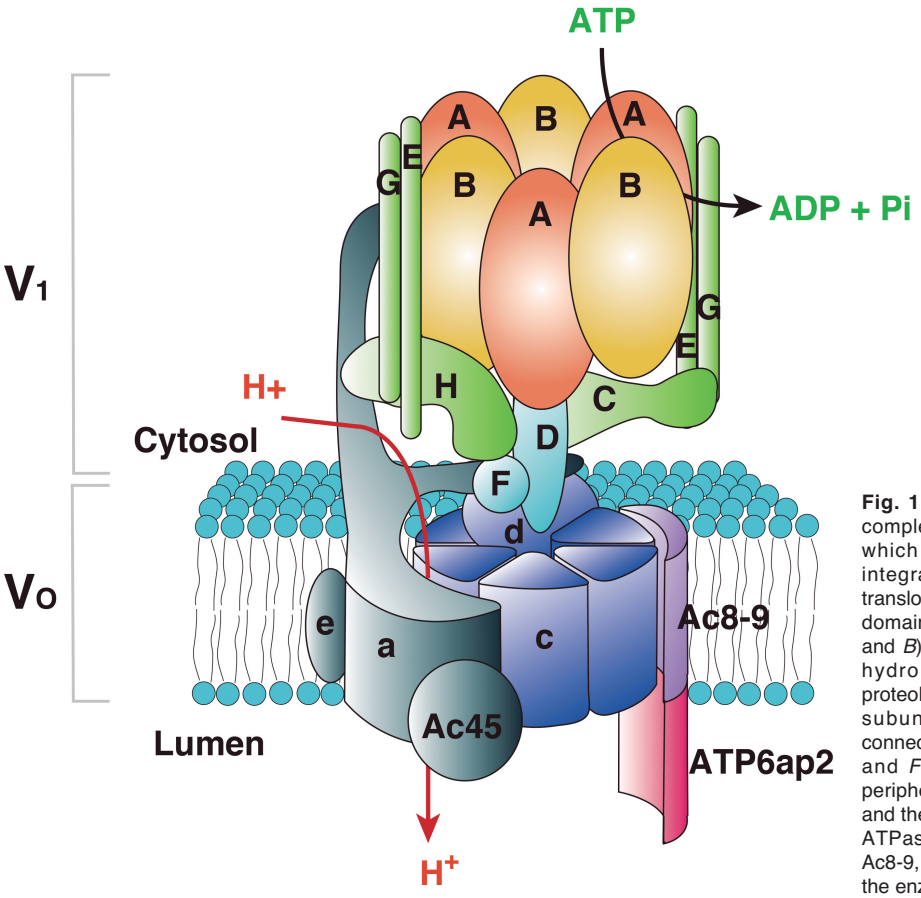

Fig. 1. Structure of the V-ATPase. The V-ATPase complex is composed of a peripheral domain (V_1), which is responsible for ATP hydrolysis, and an integral domain (V_O), which is involved in proton translocation across the membrane. The core of the V_1 domain is composed of a hexamer of alternating (*A* and *B*) subunits, which participate in ATP binding and hydrolysis. The V_O domain includes a ring of proteolipid subunits (*c, c', and c''*) that are adjacent to subunits a and e. The V_1 and V_O domains are connected by a central stalk, composed of subunits (*D* and *F*) of V_1 and subunit d of V_O, and multiple peripheral stalks, composed of subunits *C, E, G, H*, and the N-terminal domain of subunit a. Mammalian V-ATPase contains the accessory proteins Ac45 and Ac8-9, both of which are essential for the function of the enzyme.

VPH1 and *STV1*, exhibiting 54% identity in amino acid sequence, and Vph1p (Manolson et al., 1992) and Stv1p (Manolson et al., 1994) are localized to vacuoles and prevacuolar compartments (possibly the Golgi or endosomes), respectively.

In higher eukaryotes, including vertebrates and invertebrates, V-ATPases acquire more complex variations via a combination of various subunit isoforms. For instance, the mammalian V_O sector is composed of 1 of 4 isoforms of the 100-kDa *a* subunit and common *c* and *c"* subunits. In the metazoan *Paramecium* at least 17 different isoforms for the V_O *a* subunit have been identified (Wassmer et al., 2006). Like yeast proton pumps, each *a* subunit isoform exhibits rather restricted subcellular localization along the endocytic and exocytic pathways (Toyomura et al., 2000, 2003; Wassmer et al., 2006), suggesting that the V_O *a* subunit isoforms, at least in part, carry localization signals.

Most recently, Stevens and colleagues identified the localization signal of Stv1, which resides within a fairly large hydrophilic domain located at the N-terminal end of the protein. This is the first demonstration of a subcellular localization signal in the V-ATPases (Finnigan et al., 2012). This observation is an important step forward in terms of the pathogenic role of V-ATPase, given that some microorganisms alter their subcellular localization of V-ATPase so that they can escape from the cytotoxic environment created by the proton pump and successfully adapt to the host cells. We will discuss this in the later part of this article. In mammals, 4 isoforms of the V-ATPase *a* subunit have been identified (Sun-Wada et al., 2003b). The *a*1, *a*2, and *a*3 isoforms are ubiquitously expressed, although their cellular localizations are different (Toyomura et al., 2003). The *a*4 isoform is specifically expressed in the kidney and other epithelial tissues (Oka et al., 2001; Smith et al., 2001; Kawamura et al., 2010). These *a* subunit isoforms exhibit ~50% amino acid sequence identity with each other. However, the N-terminal regions (amino acid residues 60–300) exhibit lower identity of ~40% (Toyomura et al., 2000), suggesting that the N-termini of these proteins is responsible for the diverse cellular localization of the enzyme in mammalian cells.

Catalysis and enzymatic reaction

In addition to the 100-kDa *a* subunit of the V_O sector, the *B, C, D, E, G,* and *H* subunits constituting the V_1 catalytic sector contain multiple isoforms, which exhibit tissue-specific expression profiles [for reviews, see (Sun-Wada and Wada, 2010) and (Smith et al.,

Table 1. Characteristics of V-ATPase subunit.

Domain		Subunit	Yeast gene	Mouse isoforms (expression)	Mouse gene
V_1	Catalytic hexamer (*A3B3*)	*A*	*VMA1*		*Atp6A1*
		B	*VMA2*	*B*1 (renal , epididymis, otic) *B*2 (ubiquitous)	*Atp6V1B1* *Atp6V1B2*
	Stalks	*C*	*VMA5*	*C*1 (ubiquitous) *C*2-a (lung) *C*2-b (lung, kidney)	*Atp6V1C1* *Atp6V1C2a* *AtpV1C2b*
		D	*VMA8*	–	*Atp6V1D1*
		E	*VMA4*	*E*1 (testis) *E*2 (ubiquitous)	*Atp6V1E1* *Atp6V1E2*
		F	*VMA7*		*Atp6V1F1*
		G	*VMA10*	*G*1 (ubiquitous) *G*2 (neural) *G*3 (renal, epididymis)	*Atp6V1G1* *Atp6V1G2* *Atp6V1G3*
		H	*VMA13*	*H*1 (2 alternatively spliced isoforms found in humans)	*Atp6V1H1*
V_0	Proton pathway	*d*	*VMA6*	*d*1 (ubiquitous) *d*2 (renal, epididymis)	*Atp6Vod1* *Atp6Vod2*
		e	*VMA9*		*Atp6v0e*
		a	*STV1* *VPH1*	*a*1(ubiquitous, synaptic vesicle localization) *a*2 (ubiquitous, Golgi localization) *a*3 (ubiquitous, lysosomal localization) *a*4 (renal, epididymis, optic)	*Atp6Voa1* *Atp6Voa2* *Atp6Voa3* *Atp6Voa4*
		c	*VMA3*		*Atlp6Voc*
		c'	*VMA11*	No mammalian gene	
		c"	*VMA16*		*Atp6Vof*
		Ac45	No yeast gene		*ATP6AP1*
		Ac8-9	No yeast gene	Identical to the (pro)rennin receptor	*ATP6AP2*

2003)]. It has been shown that the V-ATPases in a particular membrane of a given cell type contain specific combinations of subunit isoforms (Sun-Wada et al., 2003a,c, 2005). These isoforms may also contribute to the subcellular localization of V-ATPases; however, only a few lines of experimental evidence have been reported to date. The isoforms confer different enzyme kinetic properties on the proton pumps. For example, the mouse *E* subunit has *E1* and *E2* isoforms, and *E2* is ubiquitously expressed in all the tissues, whereas *E1* is restricted to the testis. Interestingly, V-ATPase with E1 decoupled the proton transport and ATP hydrolysis at 37°C, the physiological temperature in mammals, but higher than the normal temperature range for the tissue where *E*1 is expressed (Sun-Wada et al., 2002). Although the physiological significance of the biochemical characteristics of V-ATPases with the *E*1 isoform remains to be examined, the domain between Lys26 and Val83 of *E*1, which includes 7 residues not conserved between *E*1 and *E*2, is responsible for the unique thermo-labile energy coupling properties (Hayashi et al., 2008).

Membrane orientation

Although the tertiary structure of V-ATPase remains to be solved, the topologies of the membrane-intrinsic V_O subunits have been extensively analyzed and discussed for decades. Forgac and colleagues expressed a series of yeast *a* subunits with Cys substitutions at various positions, and treated the vacuoles with membrane-permeable and membrane-impermeable maleimide, which reacts with Cys residues. Covalent chemical modification occurs at the Cys residues accessible to the maleimide reagents. Using this biochemical strategy, they determined the membrane topology of the 100-kDa subunit of the yeast proton pump, and showed that the N-terminal part is localized to the cytosol (Toei et al., 2011). This orientation is consistent with the model that this part contains the signals for subcellular localization, and interacts with the retention/localization machinery present in the cytosol. The N-terminal region is also known to interact with cytosolic proteins such as ARF nucleotide-binding site opener (ARNO) and Arf6, underscoring the cytosolic orientation of the N-terminal domain (Hurtado-Lorenzo et al., 2006).

In contrast to the N-terminal domain, orientation of the C-terminal domain remains rather ambiguous. The hydrophobicity profiles of the primary structure for the 100-kDa subunits predict 8 or 9 possible transmembrane segments, and a rather short C-terminal domain of approximately 40 amino acid residues. The differential modification study using membrane-permeable and membrane-impermeable reagents suggests 8 transmembrane segments, thus predicting a model with the C-terminal domain facing the cytosol (Toei et al., 2011). An *a*3-GFP fusion protein was expressed in mouse cells and tissues, and the GFP fluorescence completely matched the immunofluorescence of the *a*3 subunit, suggesting that the *a*3-GFP fusion protein in the cells retained the GFP moiety. Further, most fusion proteins existed as full-length products, and essentially no degradation products or truncated proteins were found in cell extracts, despite the highly degradative nature of the lysosomal lumen (Sun-Wada et al., 2009). We therefore postulate that the C-terminus of the V-ATPase *a* subunit is highly likely to be localized to the cytosol, rather than to the lysosome/phagosome lumen where digestive enzymes are accumulated. A similar topological model has been proposed for the 100-kDa *a* subunit in other organisms, including humans and *Dictyostelium* (Clarke et al., 2002; Su et al., 2003).

By contrast, a recent study, which identified a role for the C-terminal peptide of the *a*3 subunit, suggested an alternative orientation (Mouline et al., 2012). The V-ATPase *a*3 subunit is also known as Tirc7, which was discovered by its involvement in T-cell activation (Utku et al., 1998). Both products, i.e., *a*3 and Tirc7, are encoded by the same gene, *Tcirg1*, and the 2 products, with distinct translation initiation points, are generated by alternative splicing of the messenger RNA. Therefore, they share the same primary structures in the C-terminal half. The 40-amino acid C-terminal peptide of the *a*3 subunit promotes the differentiation of macrophage-derived RAW264.7 cells into osteoclasts, when added to the culture medium (Mouline et al., 2012). This observation suggests that the C-terminal region of the *a*3 subunit perceives extracellular signals for lymphocyte differentiation, therefore, it should orient toward the luminal/extracellular spaces rather than the cytosol.

Loss of the *a*3 subunit produces a severe bone phenotype known as osteosclerosis, in which the bone becomes denser and fragile. Indeed, the mouse *oc* mutation, a causative factor in severe osteosclerosis, was identified as a deletion of exons 1–3 of the structural gene for the V-ATPase *a*3 subunit (Scimeca et al., 2000). Li and colleagues created a targeted mutation (*Atp6i*$^-$) of exons 2–5 (Li et al., 1999). Both mutations have been reported to produce almost identical phenotypes. The part of the gene that encodes the C-terminal portion of the *a*3 subunit is unaffected in these mutant alleles. Thus, the Tirc7 protein is presumably translated and sustains the differentiation of osteoclasts carrying the *oc* or *Atp6i* mutations (Mouline et al., 2012). We have reported that mice homozygous for an allele lacking the exons encoding the C-terminal region exhibit bony phenotypes identical to the *oc*- and *Atp6i*- mutant mice (Sun-Wada et al., 2009; Kawamura et al., 2010). This observation suggests that the Tirc7 C-terminal peptide is not an absolute requirement for the differentiation and maintenance of osteoclasts.

Macrophages and V-ATPase

A unicellular organism like cellular slime mold, *Dictyostelium*, eats bacterium by phagocytosis, a specific

form of endocytosis. The phagocytosed bacterium is then digested in the acidic environment created by the function of V-ATPases (Clarke et al., 2010). In mammals, a professional phagocytic cell, macrophage, performs surveillance and clearing tasks to protect against pathogens. Macrophages defend against pathogens and other unfavorable materials present in tissues. When these target materials are encountered, the macrophages attach to them and internalize them by phagocytosis. After internalization, the pathogens are degraded in the endocytic apparatus called phagosomes, which finally form compartments known as phagolysosomes, in which various hydrolases are enriched. The conversion of the nascent phagosomes into mature phagolysosomes is a complex process involving highly dynamic material exchange between the plasma membrane and the early and late endosomes as well as lysosomes. The endoplasmic reticulum (ER) may also participate in the assembly of phagosomes (Gagnon et al., 2002; Hatsuzawa et al., 2006), although this view has been under debate (Touret et al., 2005a,b).

One of the characteristics of this phagosome maturation is the gradual decrease in the luminal pH due to the active transport of protons from the cytosol to the lumen. The proton-rich environment in the maturing phagosome supplies the substrate for reactive oxygen species generation by NADPH oxidase. Further, the acidic interior provides a suitable chemical environment for multiple hydrolases. Like in *Dictyostelium*, this acidification of mammalian phagosomes is mediated by V-ATPase.

From the bacterial perspective, the maturation of phagosomes, including the recruitment of V-ATPase, is life threatening. It has been shown that pathogenic microorganisms prevent the recruitment of V-ATPase to phagosomes, where they reside. *Legionella pneumophila* establishes intracellular infection in macrophages (Xu et al., 2010). The bacterium secretes a specific subset of proteins to establish its intracellular habitat. SidK, a protein recently identified by screening yeast cells for mutations that phenocopied V-ATPase deficiency, has been shown to be a direct inhibitor of the V-ATPase complex. SidK binds to the A subunit of V-ATPase, a key component of the V1 sector, and prevents phagosomal acidification by inhibiting ATP hydrolysis. In addition to this inhibition of V-ATPase enzymatic activity, the host subcellular architecture is modulated at various levels by other proteins secreted by the internalized bacterium. For example, SidM affects the GTP cycles of rab1, a small GTP-binding protein that is normally a resident of the ER, and recruits it to the phagosome membrane (Muller et al., 2010, 2012). Similarly, other bacterial factors are known to modify the rab1 small GTPase by adenylation or phosphocholination and alter its GDP/GTP cycle (Neunuebel et al., 2011; Tan and Luo, 2011). These alterations in the function and localization of the rab protein result in a loss of phagosomal signatures, including acidification, as well as proteolytic activity from the bacterium-containing phagosomes.

Mycobacterium, which is a clinically highly important pathogenic microorganism, prevents the recruitment of V-ATPase to the phagosomes, where they have settled. Although this interesting strategy was discovered decades ago, the underlying molecular mechanism remains largely unknown. The mechanism may involve the inhibition of the phagosome/lysosome interaction, which inevitably prevents the bacterium-containing phagosomes from acquiring V-ATPases from lysosomes or other sources, or the selective exclusion of V-ATPase from the phagosomes. Either way, the phagosomes are devoid of the proton pump for luminal acidification.

The defective acidification of phagosomes allows the bacteria to avoid being killed, and to even proliferate in the "host" cells. Therefore, the understanding of V-ATPase dynamics in the immune system is essential from a clinical perspective. However, our knowledge of how V-ATPase is specifically targeted in the cells, and how the invaders modify this host cell mechanism, is still limited.

Even for non-pathogenic bacteria and other substances, the mechanisms by which phagosomes acquire V-ATPases from other subcellular compartments are not fully understood. This is partly due to the lack of appropriate experimental tools to detect the V-ATPase complex. Phagocytosis, like endocytosis and exocytosis, is composed of multiple steps involving membrane budding, translocation, and fusion. Furthermore, the interactions between the different membranes are mediated by multiple mechanisms, including vesicle-mediated transport, formation of membrane tubules, and engulfment of different compartments. These processes are quite dynamic, and the transitional structures may be highly sensitive to physicochemical fixation. Therefore, conventional microscopic observations are difficult. In addition, fixed specimens provide only static images, and the time sequences of the events have to be reconstructed from the acquired stacks of images. The recent advances using fluorescent proteins and synthetic fluorophore techniques have entirely changed this situation and have provided critical insights into the fundamental nature of the endocytic and exocytic pathways. However, studies on phagocytosis have not immediately received such benefits. Expression of fluorescently tagged molecules requires the introduction of genetic material into cells, and most phagocytic cells are highly resistant to such genetic manipulation. Even if this intrinsic problem is solved by strategies such as the use of macrophage-like cell lines and lentivirus-mediated transfection, ectopic expression of the intrinsic membrane components of V-ATPase may cause cell death. We have attempted to introduce various expression vectors into several cell lines, including the most popular HEK293 and HeLa cells; however, these efforts were unsuccessful, most likely due to potential

toxicity of the ectopically expressed V-ATPase subunits, particularly those of the membrane-embedded V_O sector, including the *a* isoforms, *c* (16-kDa components), and *c*'' (23-kDa components) subunits (our unpublished observations).

To circumvent these problems, we attempted to express a GFP-tagged ATPase under the most physiological condition, namely, from its authentic locus. We modified the mouse *Tcirg1* locus encoding the *a*3 subunit, which is the major *a* subunit found on phagolysosomal membranes, to produce an *a*3-GFP fusion protein via ES-cell mediated gene targeting (Sun-Wada et al., 2009; Sakurai et al., 2012).

Using this method, we were able to selectively detect the V-ATPase with the *a*3 subunit isoform, and perform live-cell imaging of the dynamics of the *a*3 subunit (Fig. 2, movies 1 and 2). It is noteworthy that *a*3 GFP-mice are a source of a variety of differentiated cells and tissues for live imaging of *a*3-expressing cells, thus providing a valuable tool to dissect the physiology and dynamics of the vacuolar pump in highly specialized functions (Sun-Wada et al., 2007, Kikuta et al., 2013). However, although current technical improvements provide highly efficient and labor-saving means for creating knockin and knockout mice, such reverse genetic approaches are practically time-consuming. It is noteworthy that Saw et al. reported the successful expression of GFP-tagged *a*1 and *a*2 in pheochromocytoma-derived PC12 cells using a lentiviral expression vector (Saw et al., 2011), showing that expression of the V_O subunits is possible at least in some cell lines, which will provide a good experimental system to explore the mechanisms underlying the localization as well as the function of the enzyme.

Interestingly, accumulating evidence indicates that V-ATPase is, at least transiently, in proximity to membrane trafficking machinery, such as SNAREs, rab GTP-binding proteins, etc. An interaction between V-ATPase and SNARE proteins has been reported in *Drosophila* neurons and kidney epithelium (Banerjee et al., 1999; Hiesinger et al., 2005). Furthermore, we have recently shown that SNARE, the well-known fusion machinery in membrane trafficking, directly interacts with V-ATPase in macrophages. It is noteworthy that V-ATPase is required for the efficient secretion of insulin-containing secretory granules from pancreatic ß-cells, indicating that V-ATPase has a definite role in membrane fusion (Sun-Wada et al., 2006). Similar observations have been made in other tissues and species (Hiesinger et al., 2005). Microglia are residents of the central nervous system with active phagocytic activity. They are responsible for protecting the central nervous system (CNS) from infectious pathogens, and further, they participate in the maintenance/development of the CNS via clearance of apoptotic neurons. Of course, the latter function is tightly regulated in order to prevent neurodegenerative disorders. Peri and Nusslein undertook live imaging of the V-ATPase *a* subunit in the zebrafish CNS to reveal the function of V-ATPase-containing microglia. They found that inhibition of the V-ATPase *a*1 subunit by a morpholino-based approach severely affected phagocytosis. However, the acidification of the microglial phagosomes remained nearly normal (Peri and Nusslein-Volhard, 2008). This interesting observation suggests that the *a*1 subunit is not a prerequisite for phagosomal acidification, probably due to compensation by other *a* subunit isoforms or another acidification system present on the plasma membrane, although phagocytic membrane dynamics is highly dependent on the presence of the *a*1 subunit. Thus, it has once again been shown that V-ATPase function is intimately involved in the fusion of different membranes during phagocytosis. Most recently, Wong et al. reported that the V-ATPase H subunit and the Vps33B protein are targets of the Mycobacterium protein phosphatase A (PtpA), whose function is indispensable for avoiding phagosomal killing. Importantly, Vps33B and the V-ATPase subunit were co-immunoprecipitated from macrophage cell lysates, indicating that both proteins are components of a large complex (Wong et al., 2011).

The osteoclast is also a macrophage-derived cell specialized for bone resorption. It attaches to the bone surface to form a microenvironment called lacuna, actively secretes protons into the lacuna to resolve the bone matrix, and phagocytoses the resolved materials and proteins. This extracellular acidification is dependent on the V-ATPase with the *a*3 isoform (Kikuta et al., 2013). Genetic loss of *TCIRG1* results in a complication known as autosomal recessive osteoporosis (ARO) (also known as stone bone or marble bone disease) due to severe impairment in osteoclast function. Interestingly, the differentiation of osteoclasts itself is not affected by the loss of the *a*3 subunit. In addition to

Movie 1. Macrophages homozygous for *Tcirg1*^{floxGFP} were cultured in a glass-bottom dish and stained with LysoTracker, as described in Figure 2. The time sequence of the live images was recorded under a wide-field fluorescence microscope, Leica-ASMDW. The Nomarski, *a*3-GFP signal (green), LysoTracker signal (red), and the merged images for GFP and LysoTracker are shown. See the movie in http://www.hh.um.es/pdf/Movies/11_302/movie_1.mov

Movie 2. Splenocytes were isolated from mice homozygous for *Tcirg1*^{floxGFP} and cultured in a glass-bottom dish. After pulse labeling with Texas-Red transferrin (25 ng/mL for 15 min), the cells were observed with Leica-ASMDW. In live splenocytes, *a*3-GFP (green) is associated with tubular structures extending from the perinuclear region toward the cell periphery. Texas-Red transferrin (red), a marker for early and/or recycling endosomes, moves along tubular structures. See the movie in http://www.hh.um.es/pdf/Movies/11_302/movie_2.mov

the bone pathology, loss of *a*3 causes multiple complications in immune function and the sense organs, like blindness and deafness; however, the mechanism is not fully understood (Li et al., 1999; Kornak et al., 2000; Kawamura et al., 2010).

Many of the ARO mutations in *TCIRG1* occur in regulatory motifs or splice sites, or result in large deletions or truncations; however, the recessive missense mutation R444L results in a particularly malignant form of infantile osteopetrosis, which is lethal in infancy or

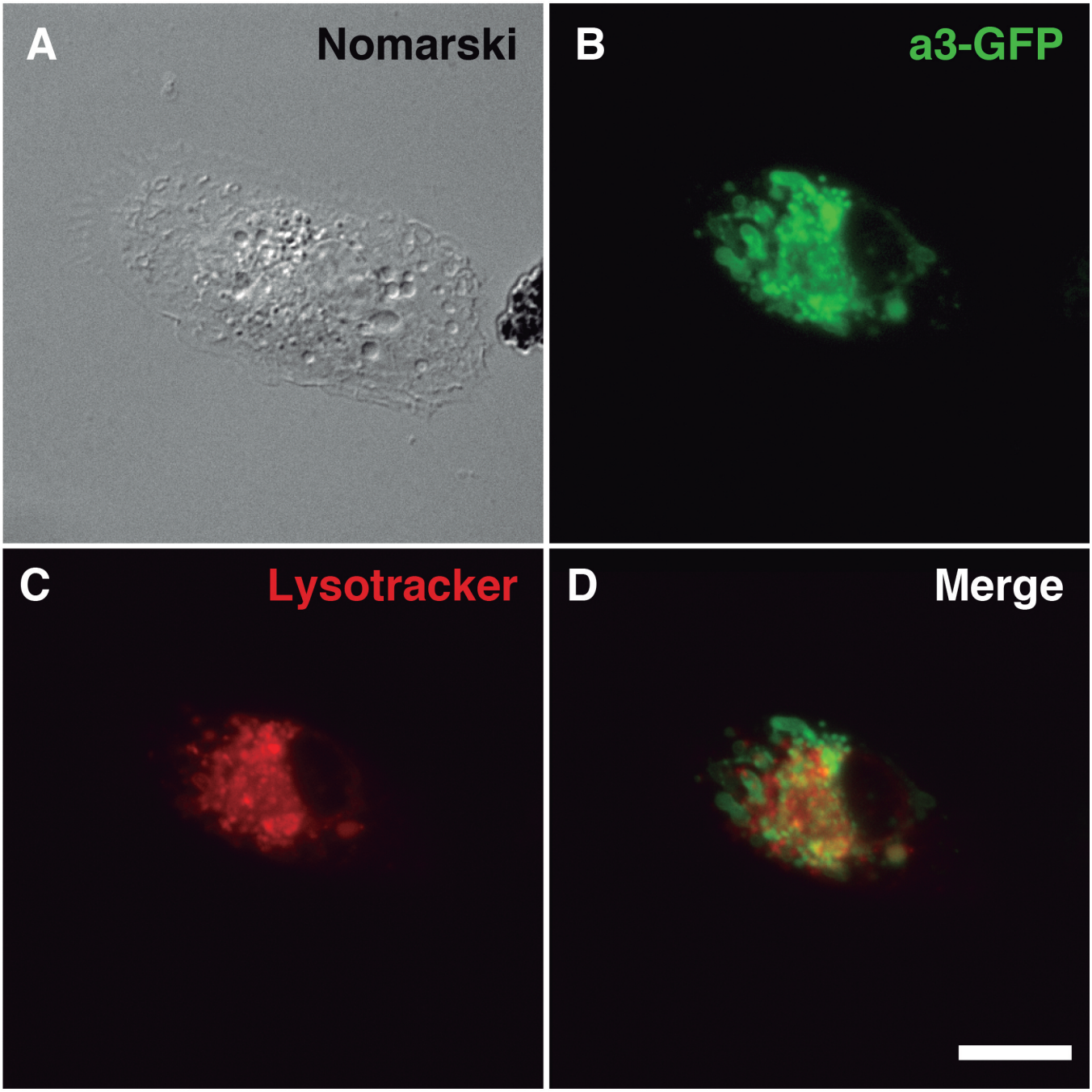

Fig. 2. Live imaging of macrophages expressing the *a*3-GFP fusion protein. Macrophages homozygous for *Tcirg1*^{floxGFP} were isolated and cultured in a glass-bottom dish placed on a microscope stage in a climate chamber (5% CO_2 atmosphere, 37°C). The medium was replaced with one containing 1 μM LysoTracker, a fluorescent dye that accumulates in the lumen of acidic compartments. *a*3-GFP signals are detected on the membranes of tubular structures **(B)**, and the LysoTracker signal **(C)** accumulates in *a*3-GFP–positive compartments. The merged image is shown in D. Bar: 10 μm.

early childhood. Manolson and colleagues have examined the effect of a homologous mutation (R445L) in the mouse and found that the mutant *a*3 localized to the ER instead of lysosomes, and its oligosaccharide moiety was misprocessed. In addition, the mutant *a*3 is degraded at an increased rate (Bhargava et al., 2012).

Recently, Aubin and colleagues performed an N-ethyl-N-nitrosourea (ENU) screen and identified a dominant missense mutation (R740S) in *Tcirg1* that causes high bone density (Ochotny et al., 2011). R740S, which is dominant negative for proton pumping and bone resorption, also uncouples proton pumping from ATP hydrolysis but has no effect on ruffled border formation or polarization of osteoclasts, suggesting again that the V_O complex has proton-pumping-independent functions in mammalian cells.

V-ATPase and cancer cells

The function of V-ATPase in extracellular acidification is also implicated in cancer metastasis. In a pioneering study in this context, Sennoue et al. found that tumor cells exhibit upregulated proton extrusion, which is mediated by V-ATPase localized at the plasma membrane. This extracellular acidification enables the cells to survive in a hypoxic environment by maintaining the cytosolic pH neutral, and simultaneously creates an acidic extracellular microenvironment (Sennoune et al., 2004). They found that the metastatic potential of cancer cells correlated with the activity of the cell surface V-ATPase. During cancer development, the carcinoma detaches from the original site of the tumor by degrading the extracellular matrix (ECM) and cell adhesion molecules, and then moves to remote sites. During this process, epithelial-mesenchymal transition (EMT) is a major morphological cue. Although experimental models remain to be developed, the extracellular acidification during EMT is critical for releasing the carcinoma cells from the ECM and the neighboring cells, the first step in migration via the lymph and/or blood circulation. We have recently shown a positive correlation between metastatic nature and the expression levels of V-ATPase in mouse B16 melanoma sub-lines (Nishisho et al., 2011). The highly metastatic nature of melanoma, the malignant transformants of melanocytes, causes serious complications after the onset of migration from the origin, and results in poor prognosis. The high-metastatic B16-F10 melanoma cells strongly expressed the *a*3 isoform V-ATPase compared to the low-metastatic B16 parental cells. Knockdown of the *a*3 isoform or administration of a specific V-ATPase *a*3 inhibitor reduced the bone metastasis of B16-F10. These results suggest that *a*3 V-ATPase promotes distant metastasis of B16-F10 cells by creating acidic environments via proton secretion. Our results also raise the possibility that inhibition of the development of cancer-associated acidic environments by suppressing *a*3 V-ATPase may be a novel therapeutic approach for the treatment of cancer metastasis.

The mechanisms underlying the expression, regulation, and plasma membrane targeting of V-ATPase in metastatic tumor cells remain unclear. It is highly likely that V-ATPase localized to melanosomes may be translocated to the plasma membrane, where they serve a proton-secreting function and thus confer the melanoma with a highly invasive nature. However, this may be too simple a speculation. We previously attempted to localize the V-ATPase in melanoma cells, and discovered that the most mature melanosomes lose the V-ATPase during the maturation process (Tabata et al., 2008). This observation does not rule out the possibility that even a small amount of V-ATPase in the melanosomes may be sufficient to provide the malignant melanomas with migration capability.

Gliomas, which are malignant neoplasms in the brain, are often associated with the ectopic expression of the *a*4 isoform of the V-ATPase, which is normally restricted to certain types of absorbing epithelial cells in renal, cochlear, and ocular ciliary bodies. The expression of *a*4 in gliomas is likely to be correlated with their invasiveness and drug resistance; however, in tissue culture cells derived from gliomas, suppression of *a*4 by siRNA does not negate these parameters highly related to malignancy (Gleize et al., 2012). Further investigation is necessary to evaluate the functional importance of *a*4 expression in glioma cells.

In addition to the V_O subunit, the V_1 subunit may also contribute to tumor malignancy. It is reported that patients with oral squamous cell carcinoma, the most common malignancy of the oral cavity, exhibited significantly high *ATP6V1C1* expression in their jugal mucosa. A statistically significant relationship, characterized by an increasing gradient, was also observed between *ATP6V1C1* levels and tumor stage (Perez-Sayans et al., 2010).

It is reported that patients with tuberous sclerosis or tuberous sclerosis complex (TSC), a syndrome that causes the growth of non-malignant tumors in the brain and in other vital organs, such as the kidneys, heart, eyes, lungs, and skin, express melanocytic markers such as HMB45 and MelanA in non-melanoma tumors (Martignoni et al., 2008). TSC arises from mutations in either the *TSC1* or *TSC2* gene, resulting in constitutive activation of mTORC1 (mammalian target of rapamycin complex 1), a pivotal regulator of cell growth, cell proliferation, nutrient status, and differentiation. This protein is well conserved from yeast to humans. Therefore, its dysfunction is directly associated with cancer (Efeyan and Sabatini, 2010). Burgarolas and colleagues showed that mTORC1 induces the expression of V-ATPase and other lysosomal genes via activation of an oncogenic transcription factor, TFEB (Pena-Llopis et al., 2011). On the other hand, knockdown of mTORC1 and mTORC2 induced mesenchymal-epithelial transition, and inhibition of mTOR signaling attenuated the migration and invasion of colorectal cancer (Gulhati et al., 2011). This observation suggests that pharmacological and molecular biological inhibition of

mTORC1 activity is a promising strategy for controlling cancer metastasis at multiple levels, including V-ATPase activity.

The identification of V-ATPase as an essential component of signal transduction cascades regulating cell proliferation/differentiation has highlighted its importance in cancer biology. ATP6ap2, an essential component of V-ATPase, is important for Wnt-dependent signaling, which has important regulatory circuits in multiple organs, especially in endoderm-derived tissues (Cruciat et al., 2010). mTOR signaling, which is the key regulator of the switch between cell differentiation, cell proliferation, and cell death, is mechanistically linked to the endosomal acidic environments established by V-ATPase function (Zoncu et al., 2011a,b). The observation that V-ATPase function is involved in cancer metastasis suggests that this proton pump can serve as a diagnostic and prognostic target. Furthermore, selective inhibition of V-ATPase in a spatial and/or temporal fashion may provide a new strategy to treat cancer metastasis.

Acknowledgements. The original work from our laboratories described in this article was carried out in collaboration with H. Tabata, N. Kawamura, M. Aoyama, and other co-workers, whose names appear in the references. This study was supported by grants-in-aids from the Ministry of Education, Culture, Science and Technology Japan, Japan Science and Technology Agency, Hayashi Foundation, and Noda Foundation (to YW and GHSW), and was supported in part by Individual Research Grants in Doshisha Women's College of Liberal Arts to GHSW.

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Accepted February 6, 2012