Regulation of V-ATPase Assembly in Nutrient Sensing and Function of V-ATPases in Breast Cancer Metastasis

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V-ATPases are proton pumps that function to acidify intracellular compartments in all eukaryotic cells, and to transport protons across the plasma membrane of certain specialized cells. V-ATPases function in many normal and disease processes, including membrane traffic, protein degradation, pathogen entry, and cancer cell invasion. An important mechanism of regulating V-ATPase activity in vivo is regulated assembly, which is the reversible dissociation of the ATP-hydrolytic V₁ domain from the proton-conducting V₀ domain. Regulated assembly is highly conserved and occurs in response to various nutrient cues, suggesting that it plays an important role in cellular homeostasis. We have recently found that starvation of mammalian cells for either amino acids or glucose increases V-ATPase assembly on lysosomes, possibly to increase protein degradation (for amino acid homeostasis) or for the utilization of alternative energy sources (during glucose starvation). While regulation of assembly in response to amino acid starvation does not involve PI3K or mTORC1, glucose-regulated assembly involves both PI3K and AMPK. Another important form of V-ATPase regulation is the targeting of the enzyme to different cellular membranes, which is controlled by isoforms of subunit a. We have shown that V-ATPases are localized to the plasma membrane of highly invasive breast cancer cells, where they promote cell migration and invasion. Furthermore, overexpression of the a³ isoform is responsible for plasma membrane targeting of V-ATPases in breast tumor cells leading to their increased invasiveness.

Keywords: V-ATPase, proton transport, acidification, regulated assembly, nutrient sensing, cancer, metastasis

INTRODUCTION

V-ATPase Function

Vacuolar H⁺-ATPases (V-ATPases) are ATP-dependent proton pumps present in intracellular membranes in all eukaryotes and at the plasma membrane of certain specialized cells (Forgac, 2007; Kane, 2012; Breton and Brown, 2013; Marshansky et al., 2014; Cotter et al., 2015b; McGuire et al., 2017). V-ATPase-dependent proton transport acidifies endocytic, secretory, autophagic and extracellular compartments, and is therefore essential for a variety of important processes (Forgac, 2007; Casey et al., 2010). Acidification of endosomes induces dissociation of internalized ligands...
from their cognate receptors, enabling receptor recycling and ligand targeting for degradation (Forgac, 2007; Maxfield, 2014). A similar process occurs in late endosomes, where acidification is required for dissociation of proteases from the mannose 6-phosphate receptor, facilitating enzyme delivery to the lysosome and receptor retrieval to the trans-Golgi network (Ghosh et al., 2003; Forgac, 2007). Furthermore, formation of endosomal carrier vesicles, which transport cargo between early and late endosomes, is also low pH-dependent (Gu and Gruenberg, 2000; Forgac, 2007). In synaptic vesicles, V-ATPases generate both a proton gradient and a membrane potential that drive neurotransmitter uptake (Farsi et al., 2016). V-ATPases also acidify secretory granules to facilitate the proteolytic processing of prohormones (Rhodes et al., 1987). Such processing also takes place in dendritic cell lysosomes, where internalized antigens are packaged for presentation on MHCII molecules (ten Broeke et al., 2013). Many pathogens co-opt endosomal acidification to gain entry into the cytoplasm, including diphtheria and anthrax toxin, as well as viruses such as influenza and Ebola (Gruenberg and van der Goot, 2006; Forgac, 2007; Grove and Marsh, 2011). Investigation into the role of V-ATPases in viral entry has provided valuable insight into the signaling pathways regulating V-ATPase activity in mammalian cells.

Acidification plays several essential roles in cellular nutrient and energy homeostasis. Amino acid homeostasis – in which cells balance amino acid generating and consuming processes – is dependent on V-ATPases, because lysosomal protein degradation is a major way that cells generate free amino acids (Xu and Ren, 2015). While the low pH of the lysosome is essential for proteolysis, the proton gradient imposed by the V-ATPase also drives the coupled export of amino acids into the cytoplasm (Xu and Ren, 2015). Another important energy-generating process is macroautophagy. During macroautophagy, cellular components are engulfed within double-membranated autophagosomes, which fuse with lysosomes to become autolysosomes (Feng et al., 2014). During this process, acidification is essential both autophagosome/lysosome fusion and for subsequent breakdown of intraluminal contents (Yamamoto et al., 1998; Kawai et al., 2007). In addition to maintaining the low pH of lysosomes, the V-ATPase associates with nutrient sensing machinery on the lysosomal surface and is required for recruitment of the metabolic regulators mTORC1 and AMPK (Zoncu et al., 2011; Zhang et al., 2014).

Finally, V-ATPases are localized to the plasma membrane of certain specialized mammalian cells where they function to transport protons from the cytosol to the extracellular space. Osteoclasts, epididymal clear cells, and renal alpha intercalated cells localize V-ATPases to the plasma membrane where they function in bone resorption, sperm maturation, and urine acidification, respectively (Breton et al., 1996; Frattini et al., 2000; Smith et al., 2000). Plasma membrane V-ATPases have also been observed in several cancer cell lines and human cancer samples. Our lab and others have obtained evidence that plasma membrane V-ATPases aid in cancer cell invasiveness and therefore represent an attractive therapeutic target to prevent metastatic spread in patients. This review will focus on recent contributions of our lab on regulated assembly of V-ATPases and their role in cancer cell invasion.

**V-ATPase Structure and Mechanism**

V-ATPases are large, multi-subunit complexes composed of a peripheral V₁ domain that hydrolyzes ATP, and an integral V₉ domain that translocates protons (Forgac, 2007; Zhao et al., 2015; Roh et al., 2018; [Figure 1]). V₁ comprises eight distinct subunits designated A through H in a stoichiometry of A₃B₃CDE₃FG₃H, while V₉ comprises five distinct subunits designated a, c, c′, d, and e, in a stoichiometry of ac⁵c′de (yeast have an additional subunit, c’′) (Cotter et al., 2015b). Subunits A and B are arranged in a hexameric configuration containing three catalytic ATP binding pockets (Forgac, 2007). ATP hydrolysis causes a conformational change in the hexamer which drives rotation of a central stalk composed of subunits D, F, and d, and a proteolipid ring composed of subunits c and c′ (Noji et al., 1997; Hirata et al., 2003; Forgac, 2007; Cotter et al., 2015b; Zhao et al., 2015; Roh et al., 2018). Each proteolipid subunit contains a buried glutamate residue that becomes protonated as the subunit rotates past the membrane-embedded C-terminal domain of subunit a (Forgac, 2007). Protons access the buried glutamate residues by entering from the cytoplasm through an aqueous channel in subunit a (Forgac, 2007; Toei et al., 2011). These glutamate residues remain protonated as they are forced to rotate through the hydrophobic bilayer, and then deprotonate upon stabilization by a critical arginine residue in subunit a (Kawasaki-Nishi et al., 2001b). Protons then exit through a second hexameric subunit a, which faces either the lumen or extracellular space (Forgac, 2007; Toei et al., 2011). The AB hexamer resists the torque of rotation and is held stationary relative to subunit a by three EG heterodimers, which link the top of V₁ to subunits C, H and the N-terminal domain of subunit a (Cotter et al., 2015b; Roh et al., 2018).

**V-ATPase Regulation**

Because so many essential processes are pH-dependent, regulation of V-ATPase activity is tightly controlled. While several modes of V-ATPase regulation have been observed, among the most important is regulated assembly, in which the V₁ and V₉ domains undergo reversible dissociation from each other. In the dissociated state, both the catalytic function of V₁ and the proton transport function of V₉ are inactive (Forgac, 2007; Kane, 2012). Regulated assembly occurs in response to various nutrient cues, and the next section of this review will focus on the role of regulated assembly in nutrient sensing.

Another important form of V-ATPase regulation is the targeting of V-ATPases to different cellular membranes, which is controlled by isoforms of subunit a. In yeast, subunit a is encoded by two genes, VPH1 and STV1, with Vph1p-containing V-ATPases being targeted to the vacuole, while Stv1p-containing V-ATPases are targeted to the Golgi (Manolson et al., 1994; Kawasaki-Nishi et al., 2001a). Mammals have four isoforms of subunit a, with isoforms a1 and a2 being localized primarily intracellularly, whereas a3 and a4 are targeted to the plasma membrane of osteoclasts and renal intercalated cells, respectively (Frattini et al., 2000; Smith et al., 2000). Importantly, V-ATPases...
Regulated Assembly in Yeast

The first demonstrations of regulated assembly of the V-ATPase were in yeast and in tobacco hornworm midgut cells (Kane, 1995; Sumner et al., 1995). In insects, assembly decreases during times of reduced feeding (such as molting), while in yeast, assembly decreases during glucose starvation. Since disassembled V-ATPases are catalytically inactive, decreased assembly during times of nutrient depletion is thought to help preserve cellular ATP stores. In yeast, V-ATPase disassembly upon glucose withdrawal is rapid, reversible and does not require new protein synthesis (Kane, 1995). Starvation-induced disassembly requires a catalytically active V-ATPase, which suggests that a particular conformation may be required for dissociation, and also requires an intact microtubule network (Parra and Kane, 1998; MacLeod et al., 1999; Xu and Forgac, 2001). Reassembly requires the glycolytic enzymes aldolase and phosphofructokinase, and the heterotrimeric RAVE complex, which directly binds the V-ATPase in a glucose-dependent manner (Smardon et al., 2002; Lu et al., 2004; Chan and Parra, 2014). Interestingly, RAVE only promotes assembly of Vph1p-containing V-ATPases (Smardon et al., 2014), and only V-ATPases localized to the vacuole undergo disassembly in response to glucose withdrawal, while V-ATPases localized to the Golgi do not (Kawasaki-Nishi et al., 2001c). Our laboratory has shown that regulated assembly of the yeast V-ATPase in response to changes in glucose availability is controlled by the Ras/cAMP/PKA pathway (Bond and Forgac, 2008). We identified Ira2p, a Ras GAP which is inhibited by elevated glucose, as a protein whose disruption leads to V-ATPase

THE ROLE OF REGULATED ASSEMBLY IN NUTRIENT SENSING

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activates mTORC1, which inhibits catabolic processes such as autophagy and promotes anabolic processes such as translation (Dibble and Manning, 2013). When V-ATPase function was disrupted either genetically or pharmacologically, mTORC1 remained inactive, even when amino acids were abundant (Zoncu et al., 2011). Because of this finding, and since reversible assembly is a major way that cells regulate V-ATPase activity, we wished to see if V-ATPase activity and assembly were modulated by amino acids. Indeed, we found that amino acids regulate the V-ATPase in mammalian cells, with amino acid starvation leading to a rapid and reversible increase in V-ATPase assembly and activity in lysosomes (Stransky and Forgac, 2015). Unlike other examples of regulated assembly in mammalian cells, this effect is PI3K and mTORC1-independent (Stransky and Forgac, 2015), indicating a novel signaling pathway regulates the V-ATPase in response to changes in amino acid availability. Although changes in V-ATPase assembly are not required for amino acid dependent changes in mTORC1 activity (Stransky and Forgac, 2015), we hypothesize that cells increase lysosomal V-ATPase assembly during amino acid starvation to enhance protein degradation, thereby increasing free amino acids. Thus, we propose that regulated assembly of the V-ATPase is a novel mechanism contributing to amino acid homeostasis.

In addition to being essential for mTORC1 activation by amino acids, the V-ATPase is also required for AMPK activation during glucose starvation (Zhang et al., 2014). AMPK directly senses and responds to cytosolic AMP/ATP ratios, such that during energy-poor conditions, AMPK inhibits anabolic processes (in part by inhibiting mTORC1), and promotes catabolic processes (Hardie, 2014). It was recently reported that AMPK, like mTORC1, is recruited to the lysosomal surface where it is activated, and that the V-ATPase is required for this process (Zhang et al., 2014). We have now shown that glucose starvation increases V-ATPase assembly and activity on lysosomes in mammalian cells (McGuire and Forgac, 2018). These effects are preceded by AMPK activation, indicating they are not required for the increase in AMPK activity upon glucose starvation (McGuire and Forgac, 2018). Furthermore, treatment of cells with the AMPK inhibitor dorsomorphin or the PI3K inhibitor LY294002 blocked the changes in V-ATPase assembly and activity, suggesting that these kinases regulate the V-ATPase in response to glucose starvation in mammalian cells (McGuire and Forgac, 2018). Future studies will focus on understanding the mechanism by which these signaling pathways modulate V-ATPase activity and testing the hypothesis that cells increase V-ATPase assembly during glucose starvation to promote the utilization of energy sources derived from autophagy.

**THE ROLE OF V-ATPASES IN CANCER CELL INVASIVENESS**

V-ATPases are thought to aid in cancer cell survival through several mechanisms, including reducing cytosolic acid generated by aerobic glycolysis, conferring drug resistance through sequestration and efflux, and participating in oncogenic signaling pathways (for an extensive review see, Stransky et al., 2016). A number of studies have also proposed a role for plasma
membrane V-ATPases in promoting cancer cell invasiveness, and this phenomenon has been best characterized in models of human breast cancer. Highly invasive MDA-MB-231 cells were found to possess greater plasma membrane V-ATPase activity than non-invasive MCF7 cells, and pharmacological V-ATPase inhibition reduced MDA-MB-231 cell migration and invasion in vitro, while having no effect on MCF7 cells (Sennoune, 2004). SKBR3 breast cancer cells also localize V-ATPases to the plasma membrane, and treatment with the V-ATPase inhibitor archazolid reduced their in vitro migration (Wiedmann et al., 2012).

Because cellular targeting of V-ATPases in normal cells is controlled by isoforms of subunit a, we hypothesized that a isoforms may be important for plasma membrane localization in breast cancer cells. We found that highly invasive MDA-MB-231 cells express 70-fold higher levels of a3 mRNA than non-invasive MCF7 cells (Hinton et al., 2009). Moreover, siRNA-mediated knockdown of either a3 or a4 reduced invasion to levels comparable to that observed with pan-V-ATPase inhibition by Concanamycin A (Hinton et al., 2009). This is a key result, because it suggests that targeted inhibition of specific isoform-containing V-ATPases can be as efficacious at blocking invasion as inhibiting all the V-ATPases in the cell. To compare more closely related cell lines, we next examined metastatic, Ras-transformed MCF10CA1a cells and the non-cancerous MCF10a breast epithelial line from which they were derived (Santner et al., 2001). MCF10CA1a cells express higher levels of a3 mRNA than MCF10a cells and localize more V-ATPases to the plasma membrane (Cappeci and Forgac, 2013). siRNA-mediated knockdown of a3 or a3 plus a4 (but not a1 or a2) significantly inhibits invasiveness of MCF10CA1a cells (Cappeci and Forgac, 2013). Furthermore, transient overexpression of a3 (but not the other a subunit isoforms) in MCF10a cells leads to increased plasma membrane V-ATPase localization and in vitro invasiveness (Cappeci and Forgac, 2013). These results suggest that a3 specifically promotes invasiveness of breast cancer cells, in part by targeting V-ATPases to the plasma membrane. We next wished to test whether specific inhibition of cell surface V-ATPases could inhibit in vitro migration and invasion. We generated an MDA-MB-231 line stably expressing the c subunit with a C-terminal V5 epitope tag that is accessible to extracellular antibody binding when incorporated into plasma membrane V-ATPases. Treatment of cells with an anti-V5 antibody selectively inhibited plasma membrane V-ATPase activity in c-V5 expressing cells and inhibited both cell migration and invasion (Cotter et al., 2015a). The same effect was observed using a membrane impermeant form of the V-ATPase inhibitor bafilomycin (Cotter et al., 2015a). Both treatments reduced invasion to the same degree as pan-V-ATPase inhibition with a cell-permeant small molecule, suggesting that
plasma membrane V-ATPases are essential to breast cancer cell invasiveness. Utilizing isoform-specific antibodies, we have now confirmed that the invasive lines MDA-MB-231, SUM149 and MCF10CA1a localize more a3-containing V-ATPases to the plasma membrane than non-invasive MCF10a cells (Cotter et al., 2016). Moreover, when examining a cDNA array containing all stages of human breast cancer, we found a3 to be upregulated anywhere from 2.5 to nearly 50-fold in all 42 samples, relative to normal breast epithelial tissue (Cotter et al., 2016). a3 is localized to the leading edge of migrating breast tumor cells and is broadly upregulated in regions of invasive breast carcinoma relative to adjacent non-invasive tumors and normal tissue (Cotter et al., 2016). It should be noted that a3 has also been found to be important for metastasis of the B16 melanoma model (Nishishio et al., 2011). Taken together, these observations suggest that a3 containing V-ATPases at the plasma membrane of tumor cells may play an important role in cancer metastasis.

How do plasma membrane V-ATPases function in tumor cell invasion? One possibility is through the activation of secreted cathepsins, which can directly degrade components of the basement membrane and extracellular matrix, and also promote activation of other secreted proteases, such as matrix metalloproteases (Chung et al., 2014; Webb et al., 2011; Damaghi et al., 2013; Kubisch et al., 2014; Kulshrestha et al., 2015; Figure 2). Plasma membrane V-ATPases may create localized zones of low pH within partially sealed extracellular spaces that promote the activity of secreted proteases. Conversely, intracellular V-ATPases may also be important for invasion by activating proteases prior to their exocytosis. Nevertheless, it is highly encouraging that specific inhibition of plasma membrane V-ATPases is as efficacious as pan-V-ATPase inhibition at blocking invasion in vitro (Cotter et al., 2015a). This suggests it may be possible to specifically target cancer cells that overexpress plasma membrane and/or a3-containing V-ATPases to inhibit cancer cell invasion, while sparing most normal tissues. Targeted inhibition of a3-containing V-ATPases would have the added benefit of reducing osteoclast function, thereby inhibiting the ability of breast cancer cells to recruit osteoclasts for metastasis to bone (Steeg, 2016; Lambert et al., 2017). Future studies will be needed to determine whether these promising in vitro findings are confirmed using in vivo models of metastasis.

AUTHOR CONTRIBUTIONS
MC drafted the manuscript. MF revised the manuscript and approved the final version.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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