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**CHARACTERIZATION OF THE METABOLIC REGULATORY PROTEIN
5'AMP-ACTIVATED PROTEIN KINASE IN THE HUMAN PARASITE,
SCHISTOSOMA MANSONI:
ANALYSIS OF DEVELOPMENTAL REGULATION, FUNCTION, AND
THERAPEUTIC POTENTIAL**

by

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Dissertation submitted to the Faculty of the
Emerging Infectious Diseases Graduate Program
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In partial fulfillment of the requirements for the degree of
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DEDICATION

To the memory of my grandmother, Virginia Beatrice Blagmon. She was the epitome of a phenomenal, strong, and fearless woman. I share this PhD with her and all other phenomenal women like her, who looked like her, who were denied the opportunity to show the world their talents because of the color of their skin despite the fact that they were good enough, smart enough, talented enough, and more than brilliant enough. Thank you for crawling so I could walk, for making sacrifices that I couldn't even begin to fathom having to make, and for taking the brunt of society's worst so that I might have the opportunity to shine as the best. I am forever indebted. I owe it to you to aim for the highest heights and settle for nothing less- this is just the beginning. I love you Grandma Sissy.

To my mother and father, who never put limits on who or what I could become. You showed me the sky and told me to go farther – for that, I thank you. Though I'll never be able to be able to repay your sacrifices, I hope making you proud comes close.

“Now you understand, just why my head's not bowed. I don't shout or jump about or have to talk real loud. When you see me passing, it ought to make you proud.... 'Cause I'm a woman, phenomenally. Phenomenal woman, that's me.”

- Dr. Maya Angelou

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BOR Meeting: February 4, 2020

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ABSTRACT

Title of Dissertation:

Characterization of the Metabolic Regulatory Protein 5' AMP-Activated Protein Kinase in the Human Parasite, *Schistosoma mansoni*: Analysis of Developmental Regulation, Function, and Therapeutic Potential

Kassandra S. Hunter, Doctor of Philosophy, 2019

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Nearly 300 million people in roughly 70 countries suffer from schistosomiasis, an infectious disease caused by parasites known as schistosomes. Schistosomes are flatworms belonging to the genus *Schistosoma*, of which three species are of particular importance to humans: *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*. These parasitic platyhelminths have the ability to innocuously infect humans and cause chronic inflammation of major organs that often results in serious health complications or death. Despite causing over 200,000 deaths per year and the potential for over 600 million people to contract new infections daily just by living in endemic areas, schistosomiasis remains a neglected tropical disease due to the low-income regions it primarily affects

that tend to be disregarded. In these neglected populations, schistosomiasis often leads to detrimental outcomes for entire communities due to the disease's impact on perpetuating poverty cycles.

Currently there exists no method to prevent schistosomiasis, aside from avoiding infested fresh water- neither prophylactic, nor vaccine. Furthermore, of all the schistosomiasis therapeutics that have been considered in the past, only one has prevailed and is in widespread use to combat disease. Praziquantel, the current gold standard of treatment for schistosomiasis, while effective against all human-relevant *Schistosoma* species, is powerless against juvenile schistosomes and subsequently newly acquired infections. It is paramount that we add to the pool of therapeutics to treat schistosomiasis and, even more importantly, that these novel therapeutics be efficacious against all stages of the parasite life cycle. To achieve this, it is necessary to identify targets that are common amongst all schistosome species and developmental stages.

Regulation of energy metabolism is a process common in and essential to the survival of all organisms. Eukaryotes, in particular, have evolved to express a common protein that is essential to metabolic regulation and maintaining energy homeostasis, known as 5' AMP-Activated Protein Kinase (AMPK). Given the importance of this metabolic regulatory protein in eukaryotic organisms, we hypothesized that schistosomes express a 5' AMP-Activated Protein Kinase that is critical for the regulation of parasite metabolism and, thus, has the potential to serve as a novel therapeutic target. Here, we demonstrate that all stages of the schistosome life cycle possess this integral metabolic regulatory protein, albeit expression of the AMPK α gene is developmentally regulated. We show that the activity of schistosome-expressed AMPK is susceptible to modulation

by external factors, indicating the potential for vulnerability to AMPK-targeted therapeutics. We illustrate that modulation of schistosome AMPK activity has downstream impacts on parasite glycolysis that are potentially mediated through regulation of parasite glycogen stores. Finally, we demonstrate that inhibition of AMPK is detrimental for juvenile schistosomes, but not adult worms, suggesting prophylactic potential of this mechanism. Targeting parasite processes that regulate nutrient metabolism and energy homeostasis has the potential to be critical for disrupting transmission cycles, presenting innovative mechanisms for the development of novel schistosomiasis therapeutics.

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CHAPTER 1: INTRODUCTION

GENERAL OVERVIEW OF SCHISTOSOMIASIS

Schistosome Biology

Schistosomes are digenetic trematodes that cause the disease schistosomiasis. These parasitic platyhelminths, also known as blood flukes, belong to the genus *Schistosoma*, of which more than 20 species exist. Every species of *Schistosoma* is an obligate parasite and has a definitive vertebrate host, whether it be fish, amphibians, or mammals (147). Indirectly, schistosomes can significantly burden humans via their impact on livestock. Directly, schistosomes cause severe, debilitating, and often life-threatening disease in humans. Specifically, three schistosome species are of direct medical importance to humans: *Schistosoma japonicum*, *Schistosoma haematobium*, and *Schistosoma mansoni* (25). The species, *S. mansoni*, is the focus of this present work.

Flukes are generally characterized by their flat bodies, syncytial tegument, incomplete gut, oral sucker, some form of a ventral sucker, and universal requirement for a molluscan intermediate host (46; 148). Schistosomes, while maintaining all of these qualities, remain unique among flukes for numerous biological reasons- the first being evident in the parasite's life cycle. Schistosomes begin their life cycle as an embryonated egg that hatches in fresh water to release a miracidium. A miracidium is the ciliated, juvenile schistosome transmission stage that navigates fresh water to find the intermediate molluscan host (25). Schistosomes are quite selective of their snail hosts as each *Schistosoma* species favors particular snail species, remarkably, without much overlap. In the case of *S. mansoni*, various snail species of the genus *Biomphalaria* are preferred (104).

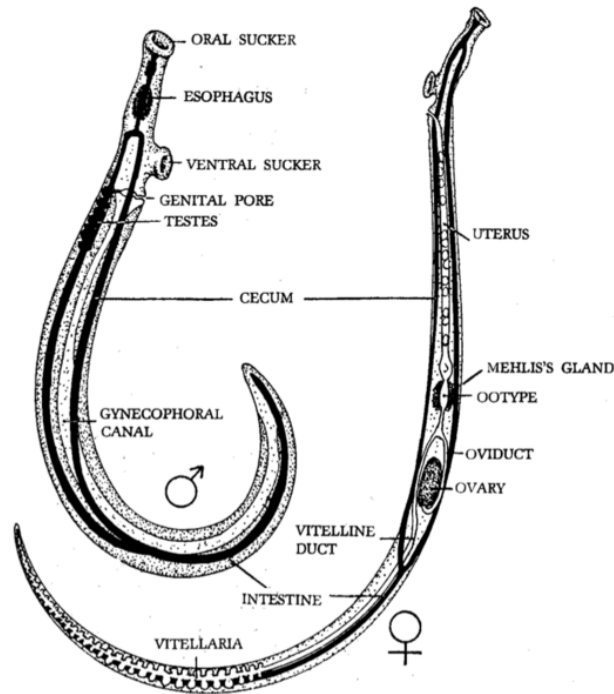
Once inside the snail host, miracidia develop into mother sporocysts which give rise to daughter sporocysts over the course of a few weeks. It is daughter sporocysts that produce the human-infectious juvenile stage, known as cercariae (152). This part of the schistosome life cycle considerably diverges from most trematodes as there is no redial stage (intermediate larval stage that precedes cercariae in some trematode life cycles) that typically follows the sporocyst stage in other flukes. In addition, schistosomes do not produce metacercariae and, therefore, do not require a second intermediate host as is common for trematodes (147; 148). In this sense, the schistosome life cycle, albeit quite complex, is very much truncated compared to most trematodes.

When released from the intermediate snail host, cercariae navigate fresh water in search of their definitive, mammalian host. Cercariae must act quickly, as they are short-lived and only remain viable for up to 3 days (147). When these organisms find a host, they are able to penetrate intact human skin in order to gain entry within. During skin penetration, cercariae lose their tails and transition to the juvenile stage known as schistosomula (147). With the help of the circulatory system, schistosomula migrate throughout the human body over the course of five to six weeks to their final vasculatory resting place of either the mesenteries, in the cases of *S. mansoni* and *S. japonicum*, or bladder wall, in the case of *S. haematobium* (104).

While maneuvering the human body, schistosomes mature into either male or female adult worms. This phenomenon brings about another facet of the life cycle that deviates from typical fluke attributes. The overwhelming majority of flukes are monoecious, with components of both male and female reproductive organs housed within the same organism. Schistosomes are peculiar in that they are one of few digenetic

trematodes to exhibit sexual dimorphism (148). Their dioecious nature is apparent in their distinctive physiology as male schistosomes possess a ventral groove, known as the gynecophoral canal, in which female schistosomes reside to facilitate reproduction. After six weeks post-infection, paired *S. mansoni* females lay as many as hundreds of eggs per pair, per day (104; 152). Newly laid eggs exit the body of the definitive host in order to continue the parasite life cycle. Eggs must traverse the vasculature lining as well as organ tissue to make it to the intestinal lumen where *S. mansoni* eggs can progress towards expulsion. During this difficult journey, eggs become entrapped in host tissue where they are subject to host defenses, such as inflammation and granuloma formation, which often aid in expulsion (25; 152). Eggs that successfully escape host tissue are excreted with feces into the environment to begin the life cycle all over again. Those eggs that are unsuccessful in being expelled from the tissue remain entrapped where they go on to cause a plethora of health complications for the host.

Schistosomes: anatomy of the adults



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Figure 1. Schistosome Physiology

Anatomical features of schistosomes are highlighted in this figure. Note those features common to all digenetic trematodes (e.g. oral and ventral suckers) as well as those unique to schistosomes (i.e. male and female reproductive organs housed in two physiologically distinct organisms). (Image obtained from: <https://quizlet.com/240797061/schistosomiasis-intro-diagram/>)

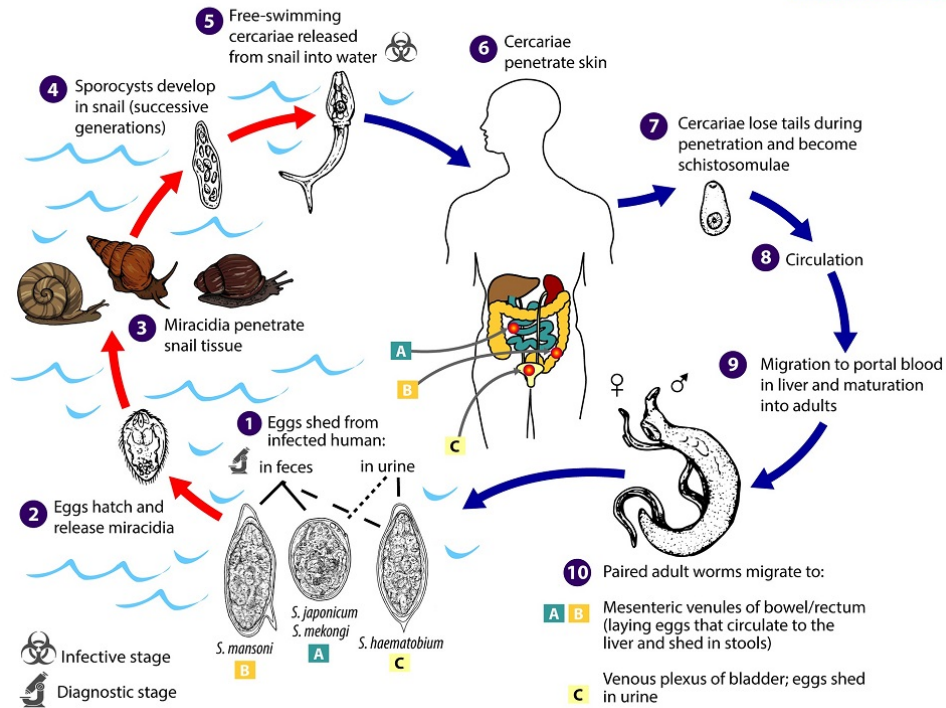


Figure 2. Schistosome Life Cycle

The life cycle of all *Schistosoma* spp. Infected humans shed eggs into the environment that hatch upon contact with fresh water. Miracidia released from hatched eggs infect the intermediate snail host which, in turn, releases the human infectious stage, cercariae. Humans in contact with infested fresh water are infected by cercariae that develop into adult worms inside the human body over the course of a few weeks. Mature, paired adult schistosomes lay eggs to restart the life cycle. (Centers for Disease Control & Prevention [CDC], 2019)

Immune Response to Infection

Schistosomiasis infection can be divided into two stages, either acute or chronic disease. The physical manifestation of both stages is largely driven by the host's immune response to the parasite (143). From the time *S. mansoni* cercariae enter the mammalian host until the time they encounter their final niche of the mesenteric venules as adults, the host is exposed to numerous worm antigens. Secreted worm antigen is highly immunogenic and stimulates an acute inflammatory response in the early stages of infection (26). This acute inflammatory response, profiled as a Th1 response, is characterized by the large production of interferon gamma (IFN- γ), tumor-necrosis factor (TNF), IL-1, and IL-6 (143). As the infection progresses and adult worms begin to lay eggs, the immune response transitions from a pro-inflammatory Th1 reaction to a Th2 response (26). This response is characterized by increased production of the immunoregulatory cytokines IL-10, IL-4, and IL-13 that are elicited by proteins secreted from schistosome eggs (143).

A robust Th2 response is responsible for the classical granulomatous response that forms around schistosome eggs deposited in host tissue (192). CD4⁺ T-helper cells are the orchestrators of this response, primarily through IL-4 production, that leads to the enclosing of tissue-entrapped eggs by CD4⁺ T-cells, macrophages, eosinophils, and collagenous tissue. These granulomas, while helpful to the host, are also useful for promoting the expulsion of schistosome eggs by allowing for them to traverse the altered host tissue architecture into the intestinal lumen (41). Over time, the granulomatous lesions surrounding eggs that persist in the tissue and eventually die, become fibrotic (143). The fibrosis of these lesions, also largely driven by IL-13 production, often leads to health complications for the host (141). In addition to their usefulness in establishing a

granulomatous response to schistosome eggs, CD4⁺ T-cells have also been demonstrated as being essential for the complete development and maturation of adult schistosomes (31).

A delicate balance of Th1 and Th2 responses is necessary for the establishment and long-term control of schistosomiasis. In studies that investigated the effects of unbalanced Th1 and Th2 responses to schistosome infections, outcomes were eventually fatal when either response was left unchecked (192). As schistosome infections progress over time and become chronic, reactions to schistosome eggs are downregulated. Infected individuals, particularly those in endemic areas, often seem to tolerate the infection. This is, in large part, owed to the downregulation of SEA responses which, if left to progress unchecked, would lead to detrimental consequences for the host (25; 26). It is clear that tolerance of schistosome infection is the result of an intricately balanced immune response and is essential for survival of both host and parasite.

Disease Pathology

Early infection with schistosomiasis evokes a range of acute symptoms in humans. Skin penetration by cercariae can cause mild dermatitis at the infection site, often seen in naïve hosts. This acute skin reaction wanes, however, with repeated infections and is often not exhibited in individuals living in endemic areas (104). In the weeks following initial infection, flu-like symptoms are elicited such as fever, malaise, eosinophilia, a non-productive cough, chills and headaches. Collectively, these symptoms are referred to as Katayama fever (25). Similar to cercariae-induced skin reactions, Katayama fever is typically only observed in naïve and non-endemic individuals in the cases of *S. mansoni* (143). In the case of *S. japonicum*, however, these general flu-like

symptoms are commonly seen in both endemic and naïve populations with outcomes generally being more severe than those induced by the other two species. In all cases, the acute pathology of schistosomiasis generally subsides within a month or two. Exceptional cases that result in fatal outcomes during early schistosomiasis infection are typically due to unusually high parasite burden (25; 104).

Much of the chronic symptoms due to schistosomiasis are the direct result of schistosome eggs and the immune responses they beget (25). Eggs trapped in host tissue are subject to a number of defenses that, while often beneficial for egg expulsion, can be quite harmful to the host over long time periods. The canonical granulomatous reactions to schistosome eggs eventually progress fibrotic deposits that replace parenchymal tissues. Over time, as more eggs become embedded in host tissue, granulomatous tissue and fibrotic deposits may become excessive leading to extensive host tissue fibrosis (94). Complications arising from tissue injury and fibrosis due to egg deposition are the underlying etiology of chronic schistosomiasis. In cases caused by *S. mansoni*, these granulomatous reactions primarily occur in the liver and intestinal tissue (25).

Collagenous deposits in the liver due to schistosomiasis notoriously result in the development of white fibrotic tissue in periportal spaces that take the shape of a pipe stem, also known as Symmer's pipestem fibrosis. This characteristic symptom of schistosomiasis gradually leads to broad tissue fibrosis of the liver that occludes portal veins (6). Complications of such extensive liver fibrosis results in decreased portal venous flow that causes increased portal hypertension, hepatomegaly, portosystemic shunting, splenomegaly, and gastrointestinal varices (65; 104). While fibrosis formation can take more than a decade to develop, in the case of *S. mansoni*, the consequences are

severe and can be fatal. Varices that form as a byproduct of liver fibrosis stemming from schistosomiasis often bleed, and in the case of *S. mansoni* infections, do so repeatedly. Repetitive bleeding of varices can result in a number of devastating outcomes including anemia, cachexia, and ascites. In addition, fatal consequences of ruptured varices can occur as a result of massive internal hemorrhaging (65). Although typically thought of as a morbid disease, hepatic fibrosis of chronic and untreated schistosomiasis greatly contributes to mortality rates associated with disease.

Less typical but equally severe consequences of schistosomiasis infection result from the deposition of parasite eggs outside of hepatointestinal tissue or the migration of parasites themselves from the mesenteric vasculature (104). Ectopic schistosomiasis, as this phenomenon is termed, can culminate in numerous manifestations including pulmonary, renal, central nervous system, and genital. Pulmonary involvement can result in heart failure, central nervous system involvement can result in inflammation of the spinal cord and, in some cases, seizures, and genital involvement manifests as lesions that leave those affected vulnerable and significantly more prone to secondary infections (25; 104). In all manifestations, the consequences are undoubtedly severe and typically the result of chronic, advanced *Schistosoma* infection.

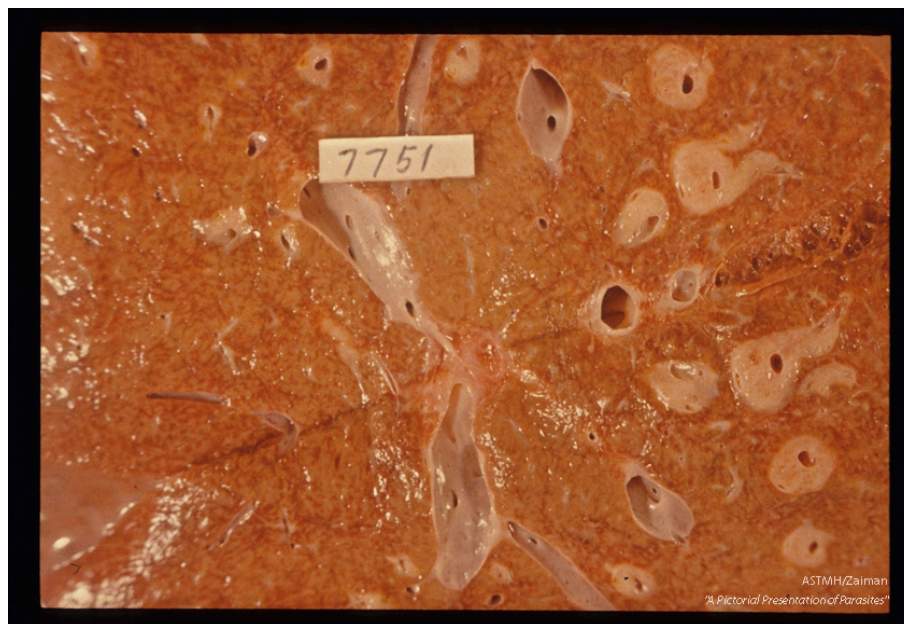
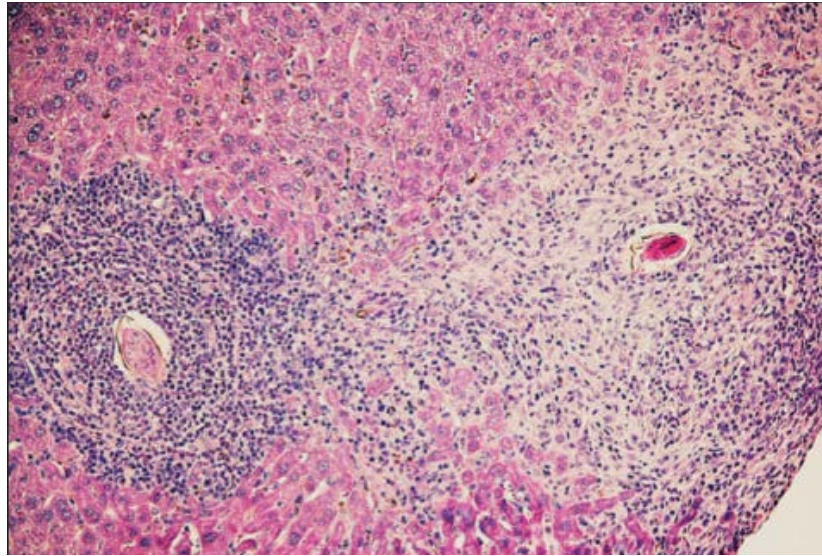


Figure 3. Egg-Induced Pathology

Top: Granuloma formation surrounding schistosome eggs entrapped in the liver of an infected mouse. (Colley *et al*, 2014, *The Lancet*, p. 2256)

Bottom: Characteristic pipe-stem fibrosis that results from schistosome-induced granulomas. (Picture taken by Dr. Herman Zaiman, obtained from ASTMH, 2015)



Figure 4. Gross Pathology of Schistosomiasis

Inflammation due to schistosomiasis often manifests as a distended abdomen in infected individuals. This is mainly due to enlargement of the liver and spleen as well as fluid accumulation in the abdominal cavity (edema). (Senthilingam, 2016, “The Snails Spreading Fever Across Africa”, *Cable News Network [CNN]*)

Global Impact

The global population currently stands at a staggering 7.6 billion humans and is rapidly growing each day (20). A crisis affecting one-sixth of the earth’s population would quickly garner widespread attention and create concern for an international emergency. This is not the case, however, concerning neglected tropical diseases (NTDs) which affect over 1 billion individuals worldwide; one-sixth of the global population (139). To put into perspective this distressing statistic, the number of people who suffer from NTDs is roughly equivalent to the entire population of China or India, the world’s most populous countries (20).

While the causative agents of neglected tropical diseases are numerous and diverse, schistosomiasis is responsible for roughly 25% of all NTD cases (85). With over an estimated 240 million individuals infected worldwide, schistosomiasis remains one of the most important neglected tropical diseases (183). Furthermore, the estimated prevalence is predicted to be substantially greater than originally determined as emerging data reveals the insensitivity of current schistosomiasis diagnostic tools (52). Roughly three times the number of those infected with schistosomiasis live in endemic areas spread across 78 countries and are, therefore, at daily risk of contracting the disease. The overwhelming majority of those affected by and at risk of being affected by schistosomiasis can be found in Sub-Saharan Africa (151; 183). It is estimated that over 90% of all schistosomiasis cases are reported from this geographical region (2; 135).

The impact of schistosomiasis is widespread and far-reaching. Approximately 200,000 deaths due to schistosomiasis occur each year. Those who don't succumb to infection must endure a lifetime of morbidity as a result of the chronic, long-lasting effects of schistosome infection (135). It is estimated that over 4 million disability-adjusted life years (DALYs) can be attributed to schistosomiasis alone (52). This means, that of all the individuals infected with schistosomiasis, a cumulative 4 million years of life are lost due to early death and suffering from poor health and disability. The chronic health impacts of schistosomiasis often leave those infected unable to work or care for their families. Even worse, children with chronic schistosomiasis commonly suffer from severe impacts on and stunting of their physical and cognitive development (103; 105). The burden of schistosomiasis is devastating, not only to individuals but to entire communities. When adults are disproportionately incapacitated and children are

precluded from ever attaining their full intellectual potential, it is easy to see how a cycle of poverty can be perpetuated in schistosomiasis endemic communities that are most often vulnerable and already poverty-stricken (103; 105). It is undeniable, then, that the global burden of schistosomiasis stretches far beyond the millions infected or at risk of being infected. Schistosomiasis is a key factor in the cultivation and maintenance of poverty cycles in the communities it affects, thus, making it an economic and social burden on a global scale (103).

STATUS OF THERAPEUTICS

Anti-schistosome Treatments

Praziquantel is the gold standard of treatment for schistosomiasis. It is currently the only medication that is universally effective and in widespread use for schistosomiasis caused by all three human-relevant species of schistosomes (25). Praziquantel was first acknowledged for its anti-schistosomal properties in the early 1970s after being discarded from studies exploring its usefulness as a tranquilizer (24). After careful toxicological and pharmacological consideration following its acquisition by Bayer, praziquantel began distribution in 1988 under the commercial name, Biltricide, and has been in use ever since (51). Praziquantel has had such a long-standing run as the drug of choice to treat schistosomiasis mainly because it has remained unrivaled by any other compound that can either meet or surpass its impressive qualities. It is safe, effective, economical to produce, and easy to administer.

The current recommended treatment of schistosomiasis caused by *S. mansoni*, is 40 mg/kg praziquantel in pill form, administered as a single, oral dose (25). As of 2002, the cost of one praziquantel tablet decreased to less than a dime, therefore, making it

feasible to treat both children and adults for less than \$.50 each (40). The low cost of praziquantel makes large quantities exceedingly accessible for mass drug administration efforts. In addition, praziquantel is generally well tolerated and doesn't have any negative, long-term health impacts. Treatment does elicit some mild side effects such as abdominal pain, dizziness, headaches, and bloody stool, which are often reported in children (24; 25). The intensity of the mild side effects that are observed usually correlate with the intensity of infection pre-treatment and is thought to be a reaction to dying schistosomes (14; 23; 51). Finally, praziquantel is notable for its broad effectiveness against all human relevant *Schistosoma* species including *S. mansoni*, *S. haematobium*, *S. japonicum*, and the closely related *S. mekongi*. The drug mediates its effect on adult schistosomes by targeting parasite calcium ion channels which results in whole worm paralysis and subsequent death. It is generally thought that this paralysis causes schistosomes to detach from the lining of host vasculature, leaving the parasites substantially more vulnerable to host defenses (40). Post-treatment, it is estimated that praziquantel can yield a cure rate between 60-90%, which is regarded as exceptional in the field. To date, no other drug has become commercially available that has surpassed praziquantel's efficacy while maintaining all of the gold standard's other critical properties, such as potency against all human-relevant schistosome species (23).

While praziquantel has many remarkably positive characteristics, it is not a silver bullet. Not only is it unwise to rely on the use of monotherapy to combat infectious diseases, as decades of mass bacterial drug resistance has demonstrated, but praziquantel also falls short in as many areas as it excels; the need for new anti-schistosome therapeutics of equal and better quality is dire. Praziquantel, although quite effective

against adult schistosomes, is virtually useless against juvenile schistosomes. Individuals recently infected with schistosomes do not benefit from treatment nor is the drug useful for preventing subsequent infections. Praziquantel is also ineffective against schistosome eggs and, therefore, offers no relief for those chronically infected individuals suffering from the effects of tissue-dwelling eggs that remain post-adult parasite killing. In addition to its selective performance, praziquantel has the potential to be rather unappealing to children, who are typically the primary targets of mass drug administration programs, due to the size and bitter taste of the pill (25). The absence of a pediatric praziquantel formulation could have the potential to impact mass drug administration (MDA) programs by influencing the reluctance of children to comply with treatment regimens, although not much research has been conducted to assess if such an impact exists. This could, in part, be indirectly addressed by, first, addressing how praziquantel is manufactured. Praziquantel is chiral; two stereoisomers of the drug exist. Only one of these stereoisomers, the R form, is active against adult schistosomes (24). Currently, the praziquantel pill is manufactured as a racemate mixture, meaning only half of one large pill is actually effective. If production methods were to shift towards manufacturing a pill solely comprised of the anti-schistosome stereoisomer, MDA programs may benefit as smaller doses could be administered while achieving the same result with decreased side effects (23; 24; 53). Finally, and most importantly, evidence indicating the existence of praziquantel resistance is mounting. *In vitro* studies have demonstrated that praziquantel resistance can be artificially induced (97; 115). In addition, field isolates of praziquantel-resistant schistosomes have been reported (40; 48; 96). While the evidence for praziquantel resistance in the field is not overwhelming or widespread, the existence of

some isolates is concerning. Furthermore, recent reports of improved diagnostics have revealed higher schistosomiasis prevalence in endemic areas than previously estimated, indicating that praziquantel may not be as effective a treatment as originally presumed (10). It is pertinent, now more than ever, that improved therapeutics be developed to supplement the use of praziquantel in order to achieve global schistosomiasis eradication.

Other generally effective compounds that are not currently in use to treat schistosomiasis are oxamniquine, metrifonates, and artemisinins. Oxamniquine, a drug used to treat schistosomiasis long before Praziquantel, is not as efficient as the gold standard (24). This anti-schistosomal is only effective against *S. mansoni* and has, therefore, lost popularity in parts of the world outside of South America where multiple schistosome species are prevalent (45). Aside from its ineffectiveness on species outside of *S. mansoni*, oxamniquine is similar to Praziquantel in that high cure rates are achieved, minimal toxic side effects are observed, and it is powerless against juvenile stages. Current work is focused on redesigning oxamniquine to broaden its targeted species repertoire which may help it re-emerge in the future as a praziquantel partner (23). Artemisinins potentially carry the most promise for effective and comprehensive schistosomiasis treatment that, unfortunately, cannot be harnessed. The drug, discovered over 35 years ago, is efficacious against all human-relevant schistosome species with high cure rates. Moreover, artemisinins have been shown to be an effective prophylactic as juvenile schistosomes are the primary drug target, a quality that the current gold standard lacks (23; 40). However, due to its extreme importance to malaria-eradication efforts, there is widespread reluctance to also use these compounds against schistosomiasis (23). Metrifonate is an organophosphorous pesticide that has been

demonstrated to be primarily effective against *S. haematobium*, metrifonate's mechanism of action is similar to Praziquantel in that it causes worm paralysis, although the target is different (45). The drug was removed from widespread use as an anti-schistosomal due to its activity on host proteins causing severe medical outcomes, ineffectiveness against other species of schistosomes, and the requirement to administer multiple, high doses to achieve high cure rates (24; 185). Current strategies to produce new anti-schistosome therapeutics are directed at improving the flaws of current and past treatments.

Prevention Efforts

Decades of research have been devoted to the development of schistosomiasis prophylactics, whether in the form of a vaccine or otherwise, to no avail. The development and implementation of an anti-*Schistosoma* vaccine would be pivotal for turning the tides of the fight to eradicate schistosomiasis. It is well known that prevention is a key component of the schistosomiasis eradication strategy; treatment alone cannot sufficiently address this goal. The idea that a vaccine to prevent schistosomiasis was potentially achievable derived from experiments performed almost 50 years ago that demonstrated protection in mice immunized with irradiated cercariae (23; 128). Since the publication of these results, decades of work has been dedicated to identifying immunogenic parasite antigens and assessing their efficacy as a vaccine (45). Only a handful of schistosome antigens have shown evidence of being effective at preventing schistosomiasis in animal models and only three antigens are currently in clinical trials; one for *S. haematobium* and two for *S. mansoni* (45; 125). None of the candidates currently in clinical trials have yet to yield significant results. While the hope of an

impending anti-schistosome vaccine is promising, the reality is that many more years may pass before the dream of a safe, effective schistosomiasis vaccine is realized.

Efforts to prevent schistosomiasis, then, must heavily rely on existing, non-chemotherapeutic strategies that have been in use alongside, and in some cases before, therapeutics for decades. Current prevention strategies focus on control of the schistosome intermediate molluscan host, community-centered health education, and efforts aimed at strengthening the infrastructure of affected communities via the introduction of clean water and waste management (151). Methods employed to control the intermediate snail host include molluscicides and the introduction of predatory or competing organisms to reduce snail population numbers (23). Although effective at interrupting the parasite life cycle, snail control methods can be difficult to implement as the introduction chemicals or non-native organisms into bodies of water that are essential to communities are often reluctantly accepted by endemic populations. Molluscicides, like niclosamides, are pollutants and often have unintended, off-target effects on other aquatic wildlife. Snail control using competing or predatory organisms, while largely effective, also runs the risk of exerting unintended effects on the balance of aquatic ecosystems (151; 172). Improvements in current snail control methods are aimed at only using molluscicides in focal areas as opposed to widespread treatment, creating environmentally safe molluscicide alternatives, and investigating competing and predatory organisms that pose the least threat to upsetting existing aquatic ecosystems in endemic communities (23). In addition to snail control, health education and improved sanitation are key components of schistosomiasis elimination efforts. Health education programs particularly aimed at endemic communities, both at the local and government

levels, are crucial to drug coverage success. If communities are not familiar with the disease itself as well as drug delivery, patient compliance and drug coverage decrease significantly (151). Education on avoiding behaviors that increase exposure risk is also critical for preventing infection. The profound effect of health education on changed behavior and decreased incidence in schistosomiasis endemic communities has been demonstrated numerous times in the literature; it is a significantly useful prevention tool (104). Equally, if not more, effective are strategies aimed at improving sanitation and access to clean water in endemic communities. This strategy, often referred to as WASH (water, sanitation, and hygiene), is estimated to have the power to reduce incidence by 33% alone (151). Simple improvements on aspects such as water quality, methods of human waste disposal, and protective clothing can have substantial effects on interrupting schistosomiasis transmission and has the potential to be equally as effective as drug therapy (104). Improved sanitation practices have been critical for the eradication of schistosomiasis in multiple countries over the last few decades, highlighting its significance as a major factor contributing to schistosomiasis prevention, control, and elimination efforts (65; 104).

Impediments to Therapeutic Developments

Throughout years of research dedicated to the discovery and development of anti-schistosomiasis therapeutics, the challenges that hinder significant progress remain the same. Factors lending to difficulties that impede advancements typically fall into two categories: (i) inaccessibility of schistosome targets and (ii) the lack of shared targets across schistosome species and across life cycle stages among the same species. Many schistosome proteins that would make excellent targets against which to direct anti-

schistosomal efforts are intracellularly located. This presents a hurdle to designing therapeutics as these intracellular proteins are largely inaccessible to human immune effectors, specifically those of humoral origin (45). As a result, vaccine design and therapeutic efforts have mainly focused on proteins found in the schistosome tegument because they are believed to be more visible to the immune system than an intracellular target, however this has posed an issue with inaccessibility as well. Studies have revealed that only a few proteins displayed on the parasite tegument are actually accessible to the human immune response (86). To further complicate the matter, mimicry of host molecules and rapid parasite membrane turnover make it that much more difficult for the immune system to hone in on and access schistosome antigens (29; 54; 156). From the moment that schistosomes enter the definitive host, their tegument is undergoing constant changes that make it less susceptible to defenses mounted by the human immune system. Furthermore, it has been reported that schistosomes employ the use of proteins that bear striking similarities to host proteins as an immune evasion mechanism (54; 156). In addition, there is also evidence that schistosomes stifle immunogenic parasite proteins by hiding them in lipid-rich areas of their tegument, allowing them to go virtually undetected by the human immune system (44; 54; 60). The various mechanisms that schistosomes employ to keep parasite proteins out of reach not only makes it hard for the host immune system to keep up but presents a significant barrier to anti-schistosome therapeutic and prophylactic developments.

Lack of shared targets across schistosome species and among stages of the parasite life cycle within schistosome species is particularly evident when evaluating the status of schistosomiasis therapeutics, both present and past. Most of therapeutics that

have been discarded were due to their ineffectiveness at treating all human-relevant schistosome species and, therefore, impracticality for use in mass drug administration efforts. Although the current and only schistosomiasis therapeutic in widespread use, Praziquantel, is effective against all species of schistosomes that plague humans, it too is a testament to this common flaw as it is ineffective against juvenile *Schistosoma* parasites. The lack of common targets among schistosomes is a critical issue that has impeded the development of a pan-species, pan-life cycle stage therapeutic for quite some time. Of the targets that are shared between species and amongst life cycle stages, the overwhelming majority are cytosolic, falling under the hurdle category of being inaccessible to the immune system (193). Efforts should be focused on the development of therapeutics that target these common cytosolic proteins without relying on assistance from the immune system if hopes to achieve a universally effective anti-schistosomal are to be realized.

Status of Current Approach for Future Therapeutics

The science of “-omics” is leading the way in the discovery of novel schistosome antigens and the development of novel anti-schistosome therapeutics (125). Genomics data has made possible huge strides in schistosomiasis research within the last decade. The sequencing of *Schistosoma* genomes has not only allowed for the identification of unique intraspecies targets but has made room for comparative studies between schistosome species, permitting the identification of shared, interspecies targets (185). Proteomics, transcriptomics, and other recently developed high throughput technologies, have also been instrumental in identifying novel antigens against which to direct anti-schistosome efforts (38; 174). Furthermore, immunomics has been helpful in recognizing

immunogenic schistosome proteins and predicting the host immune responses they will elicit (59; 102; 144). These tools are complemented by compound libraries that have advanced drug discovery progress against schistosomiasis (125). Current efforts are focused on combining these techniques to create novel therapeutic strategies via developing Praziquantel derivatives or completely novel compounds, exploring natural alternatives, revisiting pre-existing drugs to assess their usefulness against schistosomiasis, and assessing previously unexplored antigens as novel vaccination targets (23; 45; 62; 185).

MOUSE MODEL OF SCHISTOSOMIASIS

The mouse model of schistosomiasis is unique in that it nearly identically replicates the clinical signs and pathology of disease using a human-relevant pathogen. Mice are naturally permissive to infection with *Schistosoma mansoni* (49). This is important because it removes confounding factors that must be otherwise considered when collecting animal model data using related but non-human-relevant pathogens. Mice are able to be infected with *Schistosoma* parasites via skin contact, mimicking the natural route of infection that occurs between humans and schistosomes. In addition, the course of infection from parasite skin-penetration to egg deposition observed in humans is recapitulated in the murine model of schistosomiasis (21).

Mice infected with schistosomiasis generate an initial mixed Th1, Th2, and Th17 response to schistosome infection that shifts to a Th2 response, correlating with egg deposition (26). Some controversy exists regarding whether certain mouse strains are more amenable for schistosomiasis studies than others given differences in the range of responses that some genotypes can display post-infection. Generally, C57BL/6 mice are

known for the Th1 immune responses that dominate during the course of an infection while BALB/c mice are known for their dominant Th2 immune responses (191). In the context of schistosomiasis, differences in the immunologic profiles of these two mouse genotypes typically manifests as minor differences in disease pathology, such as egg burden and granuloma size, which are heavily influenced by the immune response (5). Regardless, both genotypes have been consistently used in research studies with much success.

SCHISTOSOME ENERGY METABOLISM

Schistosomes largely rely on glycolysis to meet their metabolic demands. This reliance is initially established upon skin-penetration of the definitive mammalian host by schistosome cercariae. Cercariae, as well as the other free-living schistosome stages, depend on oxidative phosphorylation to fuel their short-lived lifespan. Upon transformation into schistosomula and entry into the human host, however, the metabolic switch is made to nearly exclusive dependence on glycolysis (47; 189). Adult schistosomes are unable to use oxygen as the final electron acceptor and, as a result, do not oxidize carbohydrates completely to CO₂. These parasites, therefore, rely on anaerobic methods to extract energy from glucose, primarily being glycolysis (179). To supply this high glycolytic demand, schistosomes must consume large amounts of glucose. It is estimated that within 5 hours, adult schistosomes consume their dry weight in glucose, emphasizing the importance of and the parasite's reliance on this energy source (18). For some time, it was believed that it was possible for schistosomes to use lipids to meet their metabolic requirements. However, this misconception has been put to rest. Recent evidence has thoroughly debunked any lingering belief that schistosomes

oxidize lipids as a means of energy production, as they are genetically unable to do so (16). Glucose uptake and metabolism via anaerobic methods, then, are critical for parasite survival.

Glucose Uptake and Regulation

Because of the extreme importance of glucose, schistosomes have developed mechanisms to ensure the consistent uptake and availability of this energy source. Schistosomes acquire glucose through their outer membrane, or tegument (171). Decades of research investigating the mechanisms by which they do so have revealed the presence of multiple glucose transporters that function in the uptake and transport of glucose across the schistosome tegumental membrane. Although four schistosome glucose transporters have been identified in the *S. mansoni* genome (SGTPs1-4), only two have been documented to actively function in transporting glucose from the host bloodstream into parasite cells (SGTP1 and SGTP4) (168; 198). The tegument of schistosomes is composed to two lipid bilayers that nutrients must cross (168). Each functioning glucose transporter operates to accomplish this task. SGTP4 is located on the apical side of the tegument membrane, at the host-parasite interface and transports glucose from the extracellular milieu into the cytoplasm of the tegumental syncytium. To move glucose further into the body of the worm, beyond the lipid bilayer, SGTP1, located on the tegumental basal membrane, transports glucose from the tegument into the interstitial fluid to nourish internal parasite tissues (198). Glucose transporters are always expressed throughout the schistosome life cycle, further emphasizing the necessity of glucose access to schistosome survival. SGTP1 can be found in both juvenile and adult stages while SGTP4 is only expressed in intra-mammalian forms (171). Furthermore,

suppression of these transporters in adult worms impedes their ability to uptake glucose, ultimately impacting viability, and transporter suppression in schistosomula results in impaired ability to establish infection (108; 168).

Schistosomes have evolved an additional layer of regulation of glucose transporters via insulin to maximize glucose uptake. While schistosomes are unable to synthesize insulin themselves, they do express insulin receptors and possess insulin-degrading proteases, presumably to interact with host-derived insulin (198). Host insulin has been demonstrated to affect the growth, metabolism, development, and fecundity of adult schistosomes. In addition, it has been shown to influence parasite glucose uptake (3; 198). One of the two identified schistosome insulin receptors are located in the same anatomical positions as schistosome glucose transporters and vaccination against schistosome insulin receptors results in detrimental parasite outcomes, highlighting its significance in supporting overall parasite health (197; 198). Given the importance of insulin in aiding glucose uptake in host cells, it is not surprising that schistosomes have developed similar mechanisms coinciding with their definitive host to facilitate the uptake of this vital nutrient source.

While the vast majority of glucose transported into schistosome cells is funneled into glycolysis to fuel the metabolic demands of functions like reproductive activity, a significant amount of glucose is stored as glycogen (180). Adult schistosomes contain large glycogen stores with males containing up to 10.5% and females containing up to 2.5% of their dry weight in glycogen (168). These stores are metabolically active and continuously cycling between synthesis and degradation. It is speculated that the purpose of schistosome glycogen stores in a typically glucose rich environment, is to serve as

energy reserves in instances where nutrient restriction may occur in tight anatomical spaces (181; 182). Evidence suggests that the regulation of glycogen store maintenance is also heavily influenced by insulin and the activity of schistosome insulin receptors (198). Much work remains to be done to fully elucidate the importance and function of glycogen in schistosomes.

5'-AMP ACTIVATED PROTEIN KINASE (AMPK)

All living organisms face the challenge of acquiring sources of energy and maintaining energy homeostasis, both at the organismal and cellular level. At the cellular level, methods of regulating energy and maintaining balance have been preserved amongst all eukaryotes. Specifically, the metabolic regulatory protein, 5' AMP-activated Protein Kinase (AMPK), is a feature well conserved in virtually all eukaryotes. This key protein, believed to have evolved very early in the eukaryotic lineage, is integral for sensing cellular energy levels and subsequently modulating cellular functions to meet energy demands. As more complex eukaryotes have evolved (i.e. multicellular organisms), AMPK has likewise evolved to reflect the complexity of the expanded demands it must meet (77).

Mammalian AMPK Structure, Function, and Regulation

Structure

5' AMPK-activated Protein Kinase is a heterotrimeric protein comprised of three structurally and functionally distinct α , β , and γ - subunits. All eukaryotes harbor at least one gene for each subunit (78). The catalytic α subunit contains the kinase domain located at the N-terminus of the protein. Within the kinase domain is a conserved threonine residue that is phosphorylated in order to activate the kinase; in mammals this

is typically T172 (63). The remainder of the α -subunit consists of an autoinhibitory domain (AID) that conformationally regulates kinase domain activity, followed by a linker, and a C-terminal domain that functions in binding to the β -subunit. The β -subunit is the scaffold that holds all three subunits together as it contains a C-terminal domain that allows for simultaneous binding to α and γ -subunits. The β subunit also contains a carbohydrate binding motif (CBM) that allows for the close association of AMPK with cellular glycogen stores (78). The γ -subunit is the energy sensing domain of AMPK. It contains four cystathionine β -synthase (CBS) repeats that function as four ligand binding sites for adenosine-containing compounds. Each site possesses its own unique binding capabilities. Sites 1 and 3 serve regulatory functions as ATP, ADP, and AMP compete for binding which ultimately affects AMPK activation. Cellular energy levels determine which adenosine molecule is able to outcompete the others for binding. The other two CBS repeat sites have less important, non-regulatory functions. Site 4 appears to constitutively have AMPK bound and site 2 remains empty (34; 78).

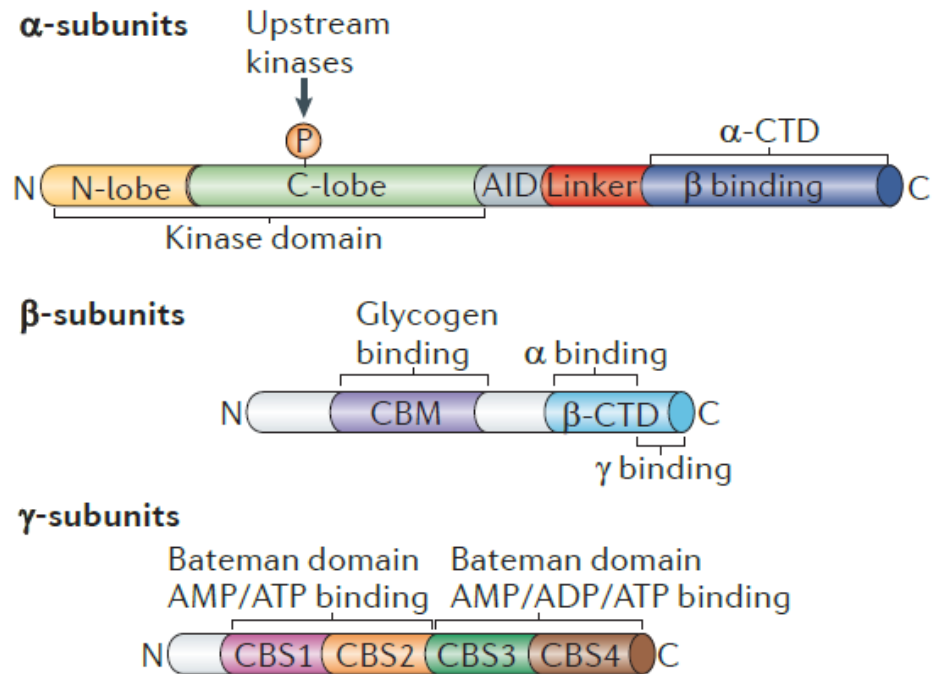


Figure 5. AMPK Structure

5' AMP-activated Protein Kinase (AMPK) is a heterotrimeric protein comprised of α , β , and γ subunits. The α -subunit is the catalytic portion of the protein, containing the kinase domain that is phosphorylated and, subsequently, activates the protein. The β -subunit binds to α and γ -subunits, holding the trimer together. This subunit also contains a glycogen binding domain that allows for close association of AMPK with cellular glycogen stores. The γ -subunit senses energy levels within the cell by binding ATP, ADP, and AMP according to their cellular concentrations. (Hardie *et al*, 2012, *Nature Reviews: Molecular Cell Biology*, p. 253)

Regulation

Activation of mammalian AMPK is regulated through multiple mechanisms. The most common method of activation is elicited through phosphorylation of threonine 172 (T172) located in the kinase domain of the catalytic α -subunit. The kinase primarily responsible for phosphorylation of AMPK is the tumor suppressor LKB1. LKB1 is constitutively active and typically found in complex with two other proteins, STRAD and MO25, which are a pseudokinase and scaffold protein, respectively. This complex is often referred to as LKB1-STRAD-MO25. Alternatively, calmodulin dependent kinase kinases (CaMKKs), which are located upstream of AMPK, have also been demonstrated to phosphorylate AMPK (76). The activity of CaMKKs are largely driven by cellular Ca^{2+} concentrations (78). In instances where cellular Ca^{2+} rise above physiologically normal levels, CaMKKs become activated, resulting in downstream CaMKK phosphorylation of AMPK (78). The ability of these upstream kinases to phosphorylate T172 in the α -subunit is tightly controlled by cellular ratios of AMP, ATP, and ADP (63). As cellular AMP and ADP levels rise to outnumber ATP, AMP and ADP outcompete ATP for binding of the γ -subunit leading to AMPK conformational changes that either promote phosphorylation of the α -subunit by the aforementioned upstream kinases or protect the catalytic subunit from dephosphorylation. Studies have identified AMP as the major effector of these conformational changes, although there is much debate regarding how significantly ADP contributes as well (63; 66; 75). In addition to α -subunit phosphorylation regulation, AMPK can also be alternatively regulated by AMP binding that results in allosteric activation. Allosteric activation can be attributed to AMP binding alone and occurs distinctly from activation caused by T172 phosphorylation (64; 76).

In addition to the aforementioned canonical AMPK regulatory pathways, recent evidence has shed light on the role of the β -subunit in regulating AMPK activity. The glycogen binding region of the β -subunit allows for close association of the kinase with cellular glycogen stores as a means of sensing glycogen levels (122). When glycogen levels are fully synthesized, and as result highly branched, AMPK is activated. As glycogen is depleted, however, the branching structure is affected leading to inhibition of AMPK (123).

Function

Regardless of the method of AMPK activation, once activated, the functions of AMPK are generally conserved throughout mammalian systems. AMPK functions to counteract the metabolic stresses that are responsible for increased ratios of AMP and ADP to ATP via the activation of catabolic, ATP generating processes that restore energy balance. While most of the targeted pathways are metabolic, they are not limited to this role as typically most cellular process are energy consuming (79). In mammals, pathways that AMPK typically activates to quickly restore cellular energy levels include glucose and fatty acid uptake, fatty acid oxidation, and glycolysis. AMPK has been known to also promote mitochondrial biosynthesis, autophagy, and mitophagy, the effects of which take a bit longer to manifest but equally contribute to the restoration of energy homeostasis. In addition, AMPK inactivates a host of other pathways, mainly biosynthetic, that consume considerable quantities of energy including fatty acid, triglyceride, nucleic acid, and protein synthesis pathways, among others (77).

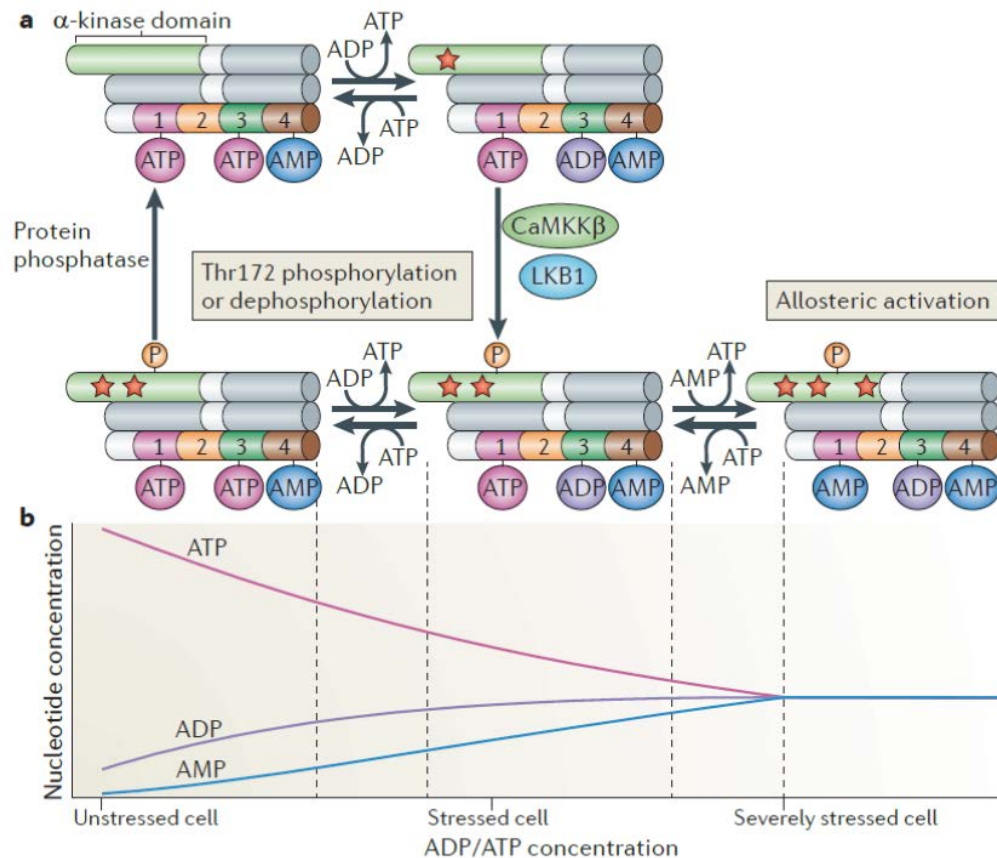


Figure 6. Regulation of AMPK Activation

Activation of AMPK is regulated by phosphorylation via upstream kinases as well as allosterically. As cellular ATP levels fall, and conversely ADP and AMP levels rise, ADP and AMP outcompete ATP for binding of the γ -subunit. This allows for conformational changes that make AMPK more amenable to phosphorylation by upstream kinases. In addition, AMP binding of the γ -subunit can allosterically activate AMPK as well. (Hardie *et al*, 2012, *Nature Reviews: Molecular Cell Biology*, p. 252)

AMPK Regulation of Metabolic Processes

In efforts to restore the cellular energy balance, AMPK regulates intracellular metabolic processes by inhibiting anabolic, ATP-consuming pathways and stimulating catabolic, ATP-generating pathways (84). Various pathways that contribute to the metabolism of protein, lipids, and glucose are regulated by AMPK via the phosphorylation of key proteins that are important for the coordination of each of these metabolic systems (78). AMPK directly controls lipid metabolism by phosphorylating the major lipid regulatory enzymes acetyl-coA carboxylase (ACC) 1 and 2. Phosphorylation of ACC1 results in inhibition of fatty acid synthesis while phosphorylation of ACC2 results in an upregulation of fatty acid oxidation (58). Aside from lipid metabolism concerning fatty acids, AMPK also manipulates cholesterol metabolism via the phosphorylation of HMG-CoA reductase resulting in abrogation of cholesterol synthesis (78; 84). AMPK has been implicated in promoting lipid uptake via upregulation of fatty acid transporters and lipid utilization by phosphorylating and, subsequently, activating lipases that aid in lipid release from triglyceride stores as well as absorption (58; 73; 84). Furthermore, AMPK has been demonstrated to exert control over lipid metabolism at the transcriptional level as well by phosphorylating and inhibiting transcriptional factors that promote lipogenesis (e.g. sterol regulatory binding protein 1, a master transcriptional regulator of lipid synthesis) (58).

AMPK's effect on cellular protein metabolism is inhibitory. These effects are primarily mediated through AMPK's relationship with another key metabolic regulatory protein known as mechanistic target of rapamycin, mTOR (58). The mTOR complex is noteworthy for its central role in regulating cellular growth in response to nutrient and growth factor signals, much of which is accomplished through its effect on protein

translation. AMPK negatively regulates mTOR both directly, via phosphorylation and inhibition of a subunit of the complex, and indirectly, via activation of mTOR's upstream negative regulators (58; 116). Inhibition of mTOR's effects greatly reduces protein synthesis and, subsequently, overall cellular growth, ultimately resulting in reduced energy usage (84). Aside from effects mediated through mTOR, AMPK additionally limits protein synthesis via inhibition of transcription factors responsible for the production of ribosomal RNA as well as factors responsible for elongation of proteins during the synthesis process (58; 78).

On a larger scale, AMPK is equally as important for whole-body metabolism as it is for cellular metabolism. The activity of AMPK in the hypothalamus of the brain is largely important for regulating metabolism and nutrient uptake via the control of appetite. AMPK responds to the feeding-associated enzymes leptin and ghrelin. Leptin, the satiation-stimulating hormone, and ghrelin, the hunger-stimulating hormone, inhibit and activate AMPK, respectively. These effects on AMPK have downstream implications on particular sets of neurons in the hypothalamus which manifests as either increased or decreased appetite (78).

Role of AMPK in Glycolysis Regulation

Glucose is one of, if not the most, important energy source within the cell. Because glycolysis can significantly contribute to cellular energy stores in a relatively short amount of time, the management of this process can be pivotal for restoring energy homeostasis. As a result, AMPK enacts multiple different mechanisms to regulate glycolysis. AMPK can affect glycolysis indirectly by regulating glucose uptake or directly via interactions with enzymes essential for performing glycolysis and managing

glucose storage. AMPK is well known for its stimulatory effects on the enzyme 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3 via phosphorylation, which produces fructose-2, 6-bisphosphate. Increased production of this compound, in turn, stimulates the glycolytic rate-limiting enzyme, phospho-fructokinase 1, leading to increased rates of glycolysis (99). In addition, AMPK is known to phosphorylate proteins involved in increasing the expression of the glucose transporters, GLUT1 and GLUT4, at the cell surface which facilitates increased rates of glucose transport into the cell (58). AMPK phosphorylation of phospholipases has also been implicated in increased cellular glucose uptake (84). AMPK has also been demonstrated to phosphorylate the enzymes glycogen synthase (GS) and glycogen phosphorylase (GP) which results in inhibition of glycogen synthesis and activation of glycogen breakdown (78; 99). AMPK's effect on glycogen synthase and phosphorylase are mediated primarily through its close proximity to glycogen via the β -subunit. In periods of high glycogen availability, glycogen-bound AMPK is active and, thus, inhibits GS and activates GP via phosphorylation (123). The influx of intracellular glucose from increased glucose uptake and glycogen breakdown aid in stimulating increased rates of glycolysis as well.

Similar to its effects on protein metabolism, AMPK also controls glucose metabolism at the transcriptional level. Gluconeogenesis is inhibited by AMPK via the phosphorylation and exclusion of cofactors required to transcribe gluconeogenic genes. AMPK also directly inhibits transcription factors important for transcribing other genes involved in glucose synthesis (58; 126).

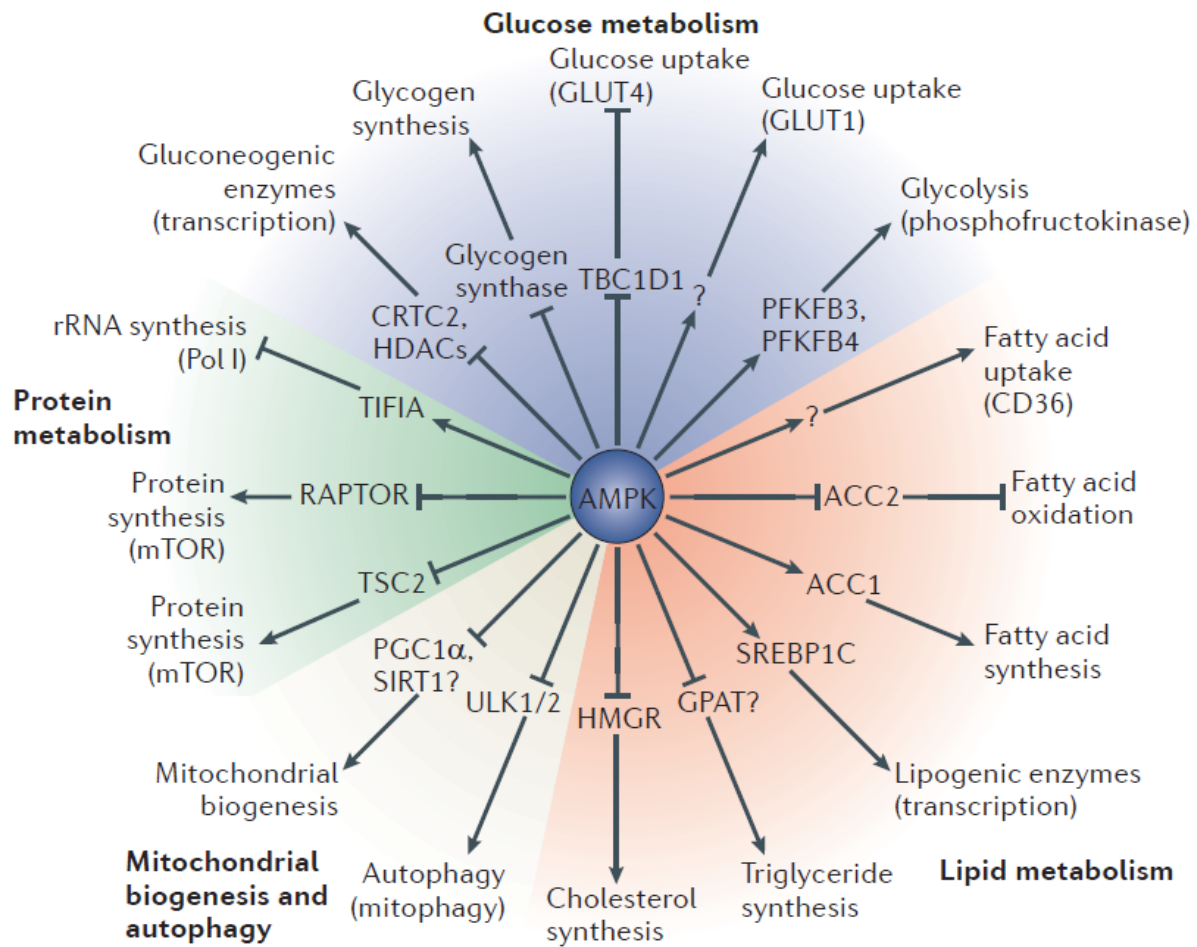


Figure 7. Impact of AMPK Activation on Cellular Metabolic Processes

Metabolic proteins directly phosphorylated by activated AMPK as well as the final outcomes are summarized here. AMPK activation can lead to a host of metabolic outcomes within the cell involving glucose, lipid, and protein metabolism to fulfill the aim of persevering energy usage and stimulating energy production. Autophagy is another method enacted by the cell to replenish energy levels. Question marks in the inner wheel denotes an activation pathway that has yet to be fully elucidated. (Hardie *et al*, 2012, *Nature Reviews: Molecular Cell Biology*, p. 256)

Non-Mammalian AMPK

As previously mentioned, AMPK is well conserved and can be found as a heterotrimeric complex in the vast majority of eukaryotic organisms. In all AMPK-possessing organisms, the function remains the same: to maintain cellular energy balances and restore energy homeostasis when said balances are disrupted. However, as energy demands and intracellular processes of eukaryotes vary with organismal complexity, the activity of AMPK in these organisms varies as well (75; 77). In yeast, the AMPK ortholog, while maintaining its heterotrimeric structure, is a bit less sophisticated than its mammalian counterpart. The gene encoding an AMPK ortholog, known as Snf1, was first identified over 40 years ago after sequencing of the *Saccharomyces cerevisiae* genome. The connection to mammalian AMPK, however, was not made until almost 20 years after its discovery (116). Snf1 regulates the usage of alternative energy sources in yeast when glucose is either limited or unavailable by the derepression of genes necessary for alternative carbohydrate metabolism (83; 116). Other cellular processes that are controlled by Snf1 in response to glucose limitation include cellular developmental processes like meiosis and sporulation as well as aging. In addition to nutrient stress, Snf1 is also involved in the regulation of other cellular processes in response to environmental stressors like heat shock and oxidative stress. Similar to mammalian AMPK, the Snf1-activating effects of cellular stress are mediated through upstream kinases that regulate the kinase's activity (83).

Likewise, in other non-mammalian organisms, AMPK behaves similarly with either less or more intricate behaviors depending on the organism. In plants, the AMPK ortholog is known as Snf1-related kinases (SnRK1) (77). SnRK1 regulates the growth response to nutrient stress in the form of darkness. In both simpler plants like

Physcomitrella patens (moss) and more complex plants like *Arabidopsis thaliana*, SnRK1 deficiencies result in the dysregulation of growth and failure to thrive in darkness due to the inability to upregulate genes necessary to compensate for light starvation (75; 77; 116). Similarly, *Drosophila*'s AMPK ortholog has been implicated in regulating nutrient intake as well as cell growth (17) and in *C. elegans* the AMPK ortholog, AAK-2, is critical for regulation of aging and energy homeostasis in response to starvation stress (187). The conservation of AMPK's role and function throughout various species emphasizes its importance to the survival of all eukaryotic organisms.

PURPOSE OF RESEARCH AND PROPOSED SPECIFIC AIMS

Schistosomiasis is a devastating disease that wreaks havoc on the individuals, families, and communities it affects. The chronic impact of this disease on infected populations is only surmounted by its global impact and burden. Despite decades of research and field efforts dedicated to eradicating schistosomiasis, we have yet to accomplish this goal. With the World Health Organization's (WHO) objective to eliminate schistosomiasis on the horizon in 2025, there are doubts that this hefty task can be achieved in time. Studies evaluating the feasibility of achieving this objective reported that in areas with moderate to high schistosomiasis prevalence, WHO's goal is unattainable on our current trajectory (183). Something must change. One of the most significant challenges that interferes with the complete realization of global eradication of schistosomiasis is the current state of affairs surrounding treatment options. Our current and only schistosomiasis therapeutic, Praziquantel, stands alone and has done so for decades now. The drug is waning in efficacy and does not prevent infection or protect against re-infection.

One of the biggest flaws of current and past schistosomiasis therapeutics is their inability to target parasite features common to all schistosomes as well as every stage of the parasite life cycle. This is why Praziquantel fails to protect against the establishment of novel infections. This is why past treatments failed to protect against all human-relevant schistosome species. We desperately need anti-schistosome treatments that are effective across schistosome species and across life cycle stages. Therapeutic targets must be re-examined to focus on features present in every schistosome, at every developmental stage.

Energy metabolism and management are features that must be present in every schistosome and at every developmental stage as they are essential to all living organisms. While there are various metabolic pathways that are unique to different organisms, commonalities and redundancies exist in the ways metabolism is regulated across all life forms. Specifically in eukaryotic organisms, AMPK is a metabolic regulatory protein that is of extreme importance to the management of metabolic processes and energy homeostasis. AMPK is present in and essential to all extracellular eukaryotes. The use of metabolism as a target for therapeutics has become the focus of efforts to mount defenses against diseases caused by bacteria (132), viruses (140; 188), parasites (68; 178), and cancer (69; 119). Specifically, AMPK has gained attention as an attractive metabolic target for and has significantly advanced the treatment of diabetes, related metabolic disorders, and cancer (27; 74; 114; 204). **We, therefore, hypothesized that schistosomes express a 5'-AMP-activated Protein Kinase that is central to regulating parasite metabolism and present in all stages of the life cycle, thus, presenting a novel potential drug target.** The identification of a drug target common to

all schistosome species and developmental stages would be critical for the advancement of schistosome eradication efforts. To test this hypothesis, the following aims were proposed:

Specific aim 1: Identify and characterize the *S. mansoni* ortholog of AMPK

Sub aim 1.1: Clone *S. mansoni* AMPK

Sub aim 1.2: Detect expression of schistosome AMPK at both the transcript and protein levels

Sub aim 1.3: Determine whether schistosome AMPK expression is developmentally regulated throughout the parasite life cycle

Specific aim 2: Assess the activity and function of schistosome AMPK

Sub aim 2.1: Determine factors that influence the activity and regulation of schistosome AMPK

Sub aim 2.2: Determine the role of AMPK in regulating schistosome metabolism

Specific aim 3: Evaluate the potential of *S. mansoni* AMPK as a therapeutic target

Sub aim 3.1: Test whether inhibition of AMPK is effective against adult schistosomes

Sub aim 3.2: Test whether AMPK inhibition is an effective prophylactic mechanism

CHAPTER 2: Host Adaptive Immune Status Regulates Expression of the Schistosome AMP-Activated Protein Kinase

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*Reference: Hunter KS, Davies SJ. 2018. Host Adaptive Immune Status Regulates Expression of the Schistosome AMP-Activated Protein Kinase. *Front Immunol* 9:2699

ABSTRACT

Schistosomes exhibit profound developmental adaptations in response to the immune status of their mammalian host, including significant attenuation of parasite growth, development and reproduction in response to deficits in host adaptive immunity. These observations led us to hypothesize that schistosomes regulate the utilization of energy resources in response to immunological conditions within the host. To test this hypothesis, we identified and characterized the *Schistosoma mansoni* AMP-activated protein kinase (AMPK), a heterotrimeric enzyme complex that is central to regulating energy metabolism at the cellular and organismal level in eukaryotes. We show that expression of the catalytic α subunit is developmentally regulated during the parasite life cycle. While there is evidence of this critical protein in all developmental stages, transcription of the AMPK α gene is down-regulated in cercariae and re-initiated once inside the mammalian host. We also show that schistosome AMPK activity is sensitive to changes in the parasite's environment, suggesting a mechanism by which schistosome metabolism may be responsive to host immune factors. Finally, we show that AMPK α expression is significantly down-regulated in parasites isolated from immunodeficient

mice, suggesting that modulation of parasite energy metabolism may contribute to the attenuation of schistosome growth and reproduction in immunodeficient hosts. These findings provide insights into the molecular interactions between schistosome and vertebrate host and suggest that parasite energy metabolism may represent a novel target for anti-schistosome interventions.

Author Summary

Schistosoma mansoni is a parasitic pathogen that significantly impacts morbidity and mortality of millions worldwide. With only Praziquantel available to treat disease and no vaccine, developing methods to disrupt transmission are paramount to the success of global eradication efforts. The recent focus on exploiting metabolism and energy usage regulation as alternative therapeutic options have proven lucrative for the treatment of cancer as well as bacterial and parasitic infections. Similar approaches could be beneficial for combatting *S. mansoni*. Here, we present evidence of a protein expressed in *S. mansoni* that is centrally involved in regulating energy usage and metabolism in other eukaryotes: 5'-AMP Activated Protein Kinase (AMPK). We demonstrate that expression of the AMPK catalytic α subunit gene varies at different stages in the parasite's life cycle. Lack of the AMPK catalytic α subunit gene expression in the human transmission stage reveals a vulnerable point in the life cycle that could potentially be targeted. Finally, we show that the activity of this critical metabolic protein can be influenced by artificial changes in the parasite's environment and, potentially, the host environment as well. Understanding schistosome energy metabolism regulation and what role host factors play may be key to identifying avenues for targeting parasite metabolic mechanisms for transmission disruption.

INTRODUCTION

Parasitic platyhelminths of the genus *Schistosoma* are the causative agents of schistosomiasis. It is estimated that at least 230 million individuals worldwide suffer from schistosomiasis (25; 52; 53), while approximately 600 million more are at risk of infection (2; 35). Most human schistosomiasis is attributed to just three parasite species - *S. haematobium*, *S. japonicum*, and *S. mansoni*. In the case of *S. japonicum* and *S. mansoni*, adult male and female worms reside in the mesenteric vasculature and produce hundreds or thousands of eggs per pair each day (142). Eggs either pass into host feces and exit into the environment to continue the parasite life cycle, or are trapped in host tissues where they cause inflammation and potentially life threatening complications. Virtually all the immunopathology associated with schistosomiasis, such as granulomas, fibrosis and subsequent portal hypertension, are solely due to the entrapment of parasite eggs in host tissue (42; 65). Furthermore, mouse models of schistosome infection have revealed that host factors can have profound effects on parasite development and reproduction. For example, in certain immunodeficient mice, schistosome growth and development is delayed and reproductive fitness is dramatically impaired (32; 81). We have shown that components of the host's adaptive immune system, particularly CD4⁺ T cells and T cell-derived cytokines, facilitate schistosome development and promote parasite egg production (32; 33; 112). Because of the immunopathological significance of schistosome eggs, targeting pathways that regulate egg production would be useful in decreasing global mortality and morbidity due to schistosomiasis.

While it is clear that schistosomes are auxotrophic for energy-producing substrates, such as glucose and fatty acids, and must acquire them from the host to sustain schistosome life and reproduction, it is unclear what intracellular processes control

schistosome utilization of these resources, especially as it relates to egg production. Male and female schistosomes mainly engage in glycolysis to meet their bioenergetic needs (19; 164; 168; 198), but catabolism of fatty acids via β -oxidation was also shown to be necessary for egg production by female schistosomes (91; 142). How environmental signals, such as those emanating from the host immune system, are integrated with metabolic regulation to control parasite bioprocesses such as egg production is unknown. Our previous work showed that pathways involved in cellular metabolism are altered in schistosomes isolated from immunodeficient mice (177). We found that expression and activity of schistosome protein kinase A (PKA), a key enzyme in eukaryotic cells with a central role in modulating cellular metabolic activity, is attenuated in schistosomes isolated from immunodeficient mice, correlating with reduced growth and reproductive fitness. Expression and activity of parasite PKA could be restored in immunodeficient mice by stimulating the host's immune system, suggesting a link between schistosome metabolic activity and the immune system of the mammalian host. Furthermore, restoration of PKA activity and expression correlated with restored parasite reproductive fitness, suggesting that control of cellular metabolism is not only important for schistosome egg production, but may also play a causal role in the attenuated developmental and reproductive phenotype of schistosomes in immunodeficient mice (177).

Evidence that PKA is involved in regulating schistosome reproduction prompted us to examine whether enzymes that are directly involved in specifically regulating energy metabolism are also implicated in regulating parasite reproductive activity. 5'-adenosine monophosphate-activated protein kinase (AMPK) is a

heterotrimeric protein complex that plays a key role in cellular metabolic regulation and energy homeostasis. The trimeric AMPK complex is comprised of a catalytic α subunit, and β and γ subunits that perform regulatory functions (111). Within the protein kinase domain of the α subunit, a highly conserved threonine residue in the activation loop is characteristically phosphorylated when the protein is catalytically activated (76; 78; 79). The β subunit mainly acts as the scaffold holding the trimer together, and may also play a role in determining the subcellular localization of AMPK (95). The γ subunit contains four repeats of a cystathionine- β -synthase (CBS) domain that act to bind ATP, ADP, and AMP. When cellular ATP levels are low, AMP outcompetes ATP and ADP for binding the γ subunit, promoting the phosphorylation of the activation loop threonine in the α subunit kinase domain and leading to α subunit activation (4; 78; 79). Upon activation, AMPK activates pathways that produce ATP, and inhibits the activity of energy-consuming processes, in order to restore cellular equilibrium. Thus, these three subunits act together as a crucial nutrient sensor within the cell, integrating signals from the extracellular environment, transmitted via PKA and other upstream components, with cellular energy levels, to engage downstream effectors and processes that ensure energy homeostasis (76; 126).

In recent years, AMPK has received increasing attention, for its role in overall growth and reproduction in many organisms, and for its potential as a therapeutic target (27; 37; 74; 117; 195; 204). In both yeast and *Drosophila*, AMPK is essential for regulating nutrient uptake and proper organismal growth, as the absence of AMPK abrogates nutrient uptake and storage and results in abnormal or delayed growth, and is sometimes lethal (17; 57; 87). In *C. elegans*, AMPK plays a fundamental role in

formation of dauer larvae, a developmental stage triggered by limited environmental nutrient availability that exhibits decreased metabolic activity and rationing of energy stores - a state that is similar to the phenotype of schistosomes isolated from immunodeficient mice (7; 133; 187). Furthermore, AMPK is the target of new therapeutics for cell proliferative and metabolic diseases like cancer and diabetes, because exploiting the unique metabolic conditions in these diseases has proven a successful strategy for discriminating between diseased and normal tissue (27; 37; 74; 129). Because mounting evidence emphasizes the importance of AMPK to the survival of organisms of many species, elucidating the role of AMPK in schistosome survival, growth, and reproduction may reveal new opportunities to disrupt the parasite life cycle. Here, we present an initial characterization of the AMPK α subunit of *S. mansoni*. In addition to an analysis of *S. mansoni* AMPK α subunit expression and activity during the parasite life cycle, we provide evidence that AMPK α activity can be modulated in response to extrinsic factors, including those emanating from the host immune system.

MATERIALS & METHODS

Ethics statement

All animal procedures were performed according to the current edition of the National Research Council's *Guide for the Care and Use of Laboratory Animals* (The National Academies Press) and pre-approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences, permit number A3448-01.

Parasite materials

The *Schistosoma mansoni* (NMRI strain) life cycle was maintained using *Biomphalaria glabrata* (NMRI strain) snails and C57BL/6 mice as intermediate and definitive hosts, respectively. Mice were purchased from Jackson Laboratories. Snails infected with *S. mansoni* miracidia were provided by the Biomedical Research Institute. Infected snails were incubated under light for one hour to promote the release of cercariae. To prepare schistosomula, cercariae were mechanically transformed by multiple passages through an emulsification needle. Cercarial heads were separated from the tails by swirling in deep petri dishes. To obtain adult schistosomes, mice were infected with 150 cercariae each by tail skin exposure. At 8 weeks post-infection, adult worms were recovered from infected mice by portal vein perfusion. Eggs were isolated by homogenizing livers of infected mice and passing homogenates through stacked sieves of decreasing pore size (425µm, 180µm, 106µm, 45µm). Eggs restricted by the smallest pore size were collected and cleaned for use. Miracidia were collected by hatching viable eggs in distilled water.

Cloning and sequencing of *S. mansoni* AMPK α

Adult *S. mansoni* cDNA was used as the template for amplifying the full-length AMPK α cDNA sequence. To prepare cDNA, RNA was extracted from adult worm homogenates using RNeasy RLT RNA Isolation Reagent. Crude RNA extracts were further purified using the RNeasy MinElute Cleanup Kit with DNase I digestion (Qiagen). 1µg RNA was used as the template for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A 533 bp fragment of the putative *S. mansoni* AMPK α , corresponding to nucleotides 668 to 1201 of the

predicted reference sequence Smp_142990 (mRNA XM_018799915.1) was amplified by conventional PCR using Accuprime Pfx Supermix (Thermo Fisher Scientific). The remaining full length cDNA sequence was then obtained using the RNA-ligase mediated rapid amplification of 5' and 3' cDNA ends (RACE) kit (Invitrogen). Products obtained by PCR and RACE were analyzed via 1.0 % agarose gel, excised, and purified from the gel using the QIAquick Gel Extraction Kit (Qiagen). Purified fragments were then cloned into pCR-BluntII-TOPO vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and used to transform One Shot Top10 Chemically Competent *E. coli* cells (Invitrogen). Transformants were plated on LB agar containing 50 µg/mL kanamycin and incubated at 37 °C overnight. Single colonies were isolated and grown in LB overnight at 37 °C with shaking. Cells were lysed and plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen). Sequencing of plasmids was performed using the BigDye Terminator cycle sequencing kit (Thermo Fisher) and T7 primers. Sequence data was analyzed and contigs assembled using Geneious software, version 11.0.5 (Biomatters Ltd.).

Preparation of parasite protein extracts

Parasite tissue was homogenized using an electric homogenizer in either RIPA lysis buffer or boiling 1X LDS sample buffer (NuPAGE LDS Sample Buffer, Invitrogen, NP0007) containing 10 % 2-mercaptoethanol. Buffers were supplemented with phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail, Thermo Scientific) and protease inhibitors (Halt Protease Inhibitor Single-Use Cocktail, Thermo Scientific) to a final concentration of 1X. To obtain a homogenous solution, samples were further subjected to sonication. Samples homogenized in RIPA buffer were centrifuged at 13,000

rpm for 10 minutes to remove insoluble material. Samples were, then, boiled at 95°C for 10 minutes and stored at -80°C for later use.

Western blotting

Protein concentrations of parasite lysates were determined using Pierce 660 nm Protein Assay (Thermo Scientific) according to manufacturer's instructions. 10 % Bis-Tris gels (NuPage) were loaded with 7 µg or 10 µg of protein per sample. Proteins were separated by SDS-PAGE in MES Buffer at 200 V for 1 h and transferred onto Invitrolon polyvinyl difluoride membranes (PVDF) (Novex) immediately after for 90 min at 25 V, 160 mA. Membranes were blocked for 30 min in TBS StartingBlock Blocking Buffer (Thermo Scientific) with 0.05 % Tween-20. After blocking, membranes were incubated for 1 h with either anti-AMPK α -1, 2 rabbit polyclonal antibody (PA5-36045, Invitrogen; Research Resource Identifier (RRID) AB_2553341) diluted 1:500, anti-phospho-AMPK α (Thr172) (40H9) rabbit monoclonal antibody (2535S, Cell Signaling Technology) diluted 1:1000, or anti-beta tubulin rabbit polyclonal antibody (Abcam) diluted 1:5000. After primary antibody incubation, membranes were washed with 1X TBS Tween-20 buffer (TBST) (Thermo Scientific) and incubated for 1 h in goat anti-rabbit IgG secondary antibody, HRP (G21234, Invitrogen) diluted 1:2000. All antibodies were diluted in blocking buffer with 0.05 % Tween-20. To detect bound antibody, membranes were incubated in SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific). Membranes were imaged using an ImageQuant LAS 4000 system (GE Healthcare Life Sciences). Densitometry measurements were performed using ImageJ software (160).

Immunoprecipitation of AMPK α

AMPK α was immunoprecipitated from adult and schistosomula lysates using nProtein A Sepharose Fast Flow beads (GE Healthcare) according to the manufacturer's instructions. Before immunoprecipitation (IP), beads were washed in 1 % NP-40 buffer (150 mM NaCl, 1 % NP-40, 50 mM Tris-HCl pH 7.4, 1 mM EDTA). Upon final wash, a 50 % slurry was prepared by mixing equal volumes of beads and NP-40 buffer. Adult worms were homogenized in NP-40 buffer. Schistosomula were lysed in boiling 1X LDS sample buffer. Lysates were precleared with nProtein A Sepharose Fast Flow 50 % slurry by gently mixing for 1 h at 4° C. Precleared lysates were then centrifuged at 12000 x g for 20 s and immunoprecipitation was performed using 50 μ g parasite protein in a final volume of 250 μ l NP-40 buffer. Either 1 μ g anti-AMPK α or anti-phospho-AMPK α was added and the mixtures were incubated for 1 h at 4° C with gentle mixing. Lysate/antibody mixture was then incubated for 1 h at 4° C with 50 μ l nProtein A Sepharose Fast Flow to precipitate immune complexes. Immunoprecipitated proteins were eluted by incubating immune complexed Sepharose beads in 30 μ l 1X LDS sample buffer at 95°C for 3 minutes. Supernatants were analyzed by SDS-PAGE as described above.

λ Phosphatase sensitivity anti-phospho AMPK α antibody

The specificity of the anti-phospho AMPK α for phosphorylated *S. mansoni* AMPK α was tested using Lambda Protein Phosphatase (Lambda PP) (New England BioLabs, P0753S). A 5 ml 1X solution of 10X NEBuffer was prepared by diluting in 1X TBST supplemented with 500 μ l MnCl₂. 25 μ l of Lambda PP was added to each 5 ml preparation of 1X NEBuffer. PVDF membranes containing 10 μ g of adult schistosome lysate per lane were incubated in 5 ml of the Lambda PP preparation either before or after

incubation in anti-phospho-AMPK α diluted 1:1000. Bound antibody was detected as described above. Phosphatase treated membranes were also incubated in anti-AMPK α 1, 2 and anti-beta tubulin as controls for protein loading.

***In vitro* treatment of adult schistosomes with AMPK agonists**

Immediately post -perfusion, fresh adult worms (5 pairs per well) were placed in the wells of 12 well tissue culture plates in 2 ml of modified Basch medium (Basal Medium Eagle, 5.55 M glucose, 50 μ g/ml hypoxanthine, 1X MEM vitamin solution, 1X MEM non-essential amino acids, 1X L-glutamine, 10 mM HEPES, 1X Pen/Strep, 10 % FBS) containing either 100 μ M or 200 μ M forskolin or 0.1 mM, 0.3 mM, or 0.5 mM metformin. To determine the effects of no glucose, modified Basch medium was prepared without 500 mg glucose. Schistosomes were incubated in their respective culture conditions for 2 h at 37 °C. After incubation, worms were collected, lysates were prepared and analyzed by SDS-PAGE/Western blotting with anti-phospho-AMPK α , anti-AMPK α -1, 2, and anti-beta tubulin antibodies as described above.

Detection of AMPK α gene expression in *S. mansoni* life cycle stages

To determine expression levels of AMPK α , quantitative real-time RT-PCR was performed on *S. mansoni* cDNA from every developmental stage. Total RNA from *S. mansoni* developmental stages was obtained from BEI Resources. 500 ng of RNA was used as template for cDNA synthesis reactions performed as previously described. A 64 bp fragment (nucleotide positions 751-815 of the *S. mansoni* AMPK α cDNA) was produced by PCR (TaqMan Fast Universal PCR Master Mix, ThermoFisher Scientific) from 1 μ l cDNA reaction using the following primers: forward 5'-AGAATGATTACTGTGGACCCGATT-3' and reverse 5'-

AACCACGGATGTCGTCTGATTT-3'. A TaqMan MGB probe conjugated to a primer sequence situated at nucleotide position 776 (5'-AACGTGCAACCATAGAA-3') was used to detect amplification of this fragment. A 557 bp fragment of Sm AMPK α (nt 388-944, of the *S. mansoni* AMPK α cDNA) was ligated into pCR-BluntII-TOPO vector (Invitrogen) as a control plasmid. Dilutions of this construct were used to generate a standard curve, from which AMPK α transcript copy number could be determined. All amplification reactions were performed and quantified using a 7500 Fast Real-Time PCR System (Applied Biosystems).

Statistical analyses

For densitometry data derived from Western blots, the data in each experiment were normalized to that of one of the conditions tested, so that data from repeat experiments could be compared. Data from repeat experiments (at least two independent biological replicates were performed in every case) were considered to be paired repeated measures and were analyzed using paired t tests. For quantitative PCR data, standard curves were used to calculate absolute transcript number in each sample. For each source of RNA, at least three independent biological replicates were tested. The data for each RNA source were first tested for evidence of significant differences in variance, using F tests. Because no significant differences in variance were found, the differences in transcript number between RNA sources were tested using parametric unpaired t tests. All statistical analyses were performed using GraphPad Prism software, version 7.01 (GraphPad Software, Inc.)

RESULTS

Identification of the *Schistosoma mansoni* 5'-AMP-activated Protein Kinase α subunit

Annotation of the *S. mansoni* genome identified a putative single gene encoding for an AMPK α subunit, consisting of 14 exons, at locus Smp_142990 (Gene ID 8351843) on the complementary strand of the W chromosome, with predicted mRNA and protein products identified by accession numbers XM_018799915.1 and XP_018653781.1 respectively. While exhibiting high overall identity and similarity to AMPK α subunits of other species, this putative *S. mansoni* α subunit contained 830 amino acids, almost 300 residues more than AMPK α subunits of other organisms (Supplementary Figure 1A). Approximately 175 of the additional residues, encoded by the first six exons, are present in an N-terminal extension found in no other species (Supplementary Figure 1A). Comparison with the Conserved Domains Database (CCD) at the National Center for Biotechnology Information (NCBI) (120) revealed that the N-terminal extension contains a metal-dependent phosphohydrolase (HD_3) domain (CCD accession number pfam13023) (8), a feature not found in the AMPK α subunit of any other species we have examined. Using the putative mRNA sequence (XM_018799915.1) in a BLAST query of the expressed sequence tag (EST) database at NCBI (101), we identified many matching *S. mansoni* ESTs, with coverage across the entire length of the putative mRNA (Supplementary Figure 1B), suggesting the sequence is present in the *S. mansoni* transcriptome. However, no ESTs spanned the junction between the regions encoding the phosphohydrolase domain and the AMPK α subunit proper (Supplementary Figure 1B), suggesting the sequences may be derived from separate transcripts. To address this discrepancy, we used PCR and RACE to clone the *S.*

mansoni AMPK α subunit cDNA from adult parasite cDNA. We succeeded in isolating a cDNA that corresponded to the eight 3' exons of the putative XM_018799915.1 mRNA (beginning at nucleotide position 538 of XM_018799915.1), but could find no evidence of the six 5' exons that encode the HD_3 domain, despite exhaustive 5' RACE and targeted PCR with specific primers. Together, these findings indicate that the HD_3-encoding exons do not contribute to the *S. mansoni* AMPK α subunit transcript, but rather represent a separate gene, located upstream on the complementary strand of the W chromosome and separated from the AMPK α gene by 5 kb of intergenic sequence (Figure 8A). We conclude that the cDNA we isolated (Genbank accession number MH445971) represents the complete *S. mansoni* AMPK α transcript.

The MH445971 cDNA contained a short 29-nucleotide 5' untranslated region (UTR) followed by an open reading frame (ORF), beginning with a start methionine at nucleotide position 30, that encodes for a protein of 651 amino acids and predicted mass of 72 kDa. The predicted protein exhibited high identity and similarity to the AMPK α subunits of other organisms (Figure 8B). Analysis of the amino acid sequence using the Conserved Domains Database (120) revealed that the predicted protein contained all the canonical features of a typical AMPK α subunit (194): an AMPK α subunit serine/threonine kinase catalytic domain (CCD accession number cd14079) at amino acids 17-272 (E-value = 0e+00), an AMPK α UBA-like autoinhibitory domain (CCD accession number cd14336) at amino acids 289-353 (E-value = 1.08e-28), and an AMPK α C-terminal regulatory domain (CCD accession number cd12122) at amino acids 474-649 (E-value = 7.53e-36) (Figure 8B). Alignment of the *S. mansoni* AMPK α sequence with AMPK α subunits of other species revealed that the amino acid sequence was highly

conserved throughout the kinase domain (Figure 8B), and particularly in the region of the activation loop (Figure 8C), where the conserved phosphorylation site of the human AMPK α subunits (Thr183 and Thr172 of human AMPK α -1 and AMPK α -2, respectively) was conserved at Thr176 of the schistosome protein (Figure 8C).

In addition to considerable lengths of conserved sequence, the *S. mansoni* AMPK α subunit also contained roughly 100 additional non-conserved amino acids, mostly clustered in the C-terminal half of the protein in two large insertions between the autoinhibitory and C-terminal regulatory domains (Figure 8B). These additional amino acids account for an appreciable difference in the predicted mass of the *S. mansoni* protein (72 kDa), as compared to human AMPK α (62 kDa).

Detection of native *S. mansoni* AMPK α protein

The high level of identity between the schistosome and human AMPK α subunits in the region of the activation loop (Figure 8C) suggested that a rabbit polyclonal antibody raised against residues 150-200 of human AMPK α -1 (RRID AB_2553341) may cross-react with the schistosome AMPK α , as there are only five amino acid differences between the two proteins in this region (Figure 8C). Furthermore, the conservation of the phosphorylation site threonine (Thr176) and surrounding amino acids in the schistosome protein (Figure 8C) suggested that a monoclonal antibody that specifically binds human AMPK α -1/2 only when phosphorylated at Thr183/Thr172 (clone 40H9) may also bind specifically to phosphorylated schistosome AMPK α . When used to probe adult *S. mansoni* protein extracts by Western blot, both the anti-AMPK α polyclonal antibody and anti-phospho-AMPK α monoclonal antibody bound to a single band migrating slightly above the 70 kDa molecular mass marker (Figure 9A), consistent

with the predicted molecular mass of the protein encoded by the cDNA we isolated (72 kDa). Western blots of adult schistosome and human embryonic kidney cell extracts probed with the two antibodies confirmed the expected difference in molecular mass between the AMPK α subunits of the two species (Figure 9B), the schistosome protein being approximately 10 kDa heavier due to the C-terminal insertions between the autoinhibitory and C-terminal regulatory domains (Figure 8B).

To confirm the specificity of the anti-phospho-AMPK α antibody for phosphorylated *S. mansoni* AMPK α , we tested whether λ phosphatase treatment of the protein was able to diminish antibody binding. When membranes containing *S. mansoni* adult worm lysate were treated with a λ protein phosphatase solution, anti-phospho-AMPK α antibody binding to the expected 72 kDa band was ablated or diminished (Figure 9C), while binding of the anti-AMPK α polyclonal antibody was not, providing evidence that the antibody binds specifically to a phosphorylated form of schistosome AMPK α .

To test whether the two antibodies were indeed binding to the same protein species, we performed reciprocal immunoprecipitation experiments, where we tested whether each antibody was able to bind to the protein immunoprecipitated by the other antibody. When immunoprecipitations of adult worm lysate performed with the anti-AMPK α polyclonal antibody were probed with the anti-AMPK α and anti-phospho-AMPK α antibodies, both antibodies bound to a 72 kDa band in the immunoprecipitates (Figure 9D). This band was not present when parasite lysate was omitted from the immunoprecipitate, confirming that the protein originated in the parasite lysate and was not derived from the preparation of immunoprecipitating antibody (Figure 9D). Likewise,

when the anti-phospho-AMPK α antibody was used to immunoprecipitate, both the anti-AMPK α and anti-phospho-AMPK α antibodies identified a 72 kDa band in the immunoprecipitations that was absent when the parasite lysate was omitted. These data support the conclusion that both antibodies bind the same protein species in schistosome protein extracts.

AMPK α expression and activation in *S. mansoni* larval stages

To determine if AMPK α protein is expressed and phosphorylated in larval stages of the *S. mansoni* life cycle, we used Western blotting to probe extracts of larval stages with the anti-AMPK α and anti-phospho-AMPK α antibodies. In preliminary experiments, we compared the suitability of different extraction methods to prepare lysates for Western blot analysis. Using cercariae as test material, we found that reactivity with both antibodies was lost altogether when parasites were lysed in RIPA buffer, and that a ladder of lower molecular mass bands was observed when the cercariae were lysed directly in 1 x sample buffer (data not shown), suggesting that the AMPK α was subject to rapid proteolytic degradation when extracted under these conditions, even though protease inhibitors were included in the extraction buffer. We found that the 72 kDa species was only extracted without significant degradation when the cercariae were lysed in boiling 1 x sample buffer, underscoring both the apparently labile nature of the schistosome AMPK α and the high protease content of cercariae. All larval stages were subsequently extracted using the boiling sample buffer method.

Lysates of *S. mansoni* eggs, miracidia, cercariae, and schistosomula (30 minutes post-transformation) were prepared using boiling sample buffer, equal amounts of protein were subjected to SDS-PAGE, and analyzed by Western blot. In blots of *S. mansoni* eggs,

both antibodies produced only a weak signal at 72 kDa, with multiple lower molecular mass species now also detected, especially with the anti-AMPK α antibody, suggesting the protein may be subject to proteolytic degradation in this life cycle stage (Figure 10A). Blots of miracidia produced a clearer and more intense signal at 72 kDa, but also a pattern of lower molecular mass bands similar to that observed in eggs, most notably a doublet between the 55 and 40 kDa markers (Figure 10B). As miracidia only hatch from viable eggs, the miracidium extract used in Figure 10B is essentially enriched for the contents of viable eggs. The reactivity of the antibodies to egg extracts observed in Figure 3A may therefore be explained, at least in part, by the heterogeneous nature of egg preparations obtained from infected mouse livers, which contain dying, dead and non-viable eggs, in addition to viable eggs, and could therefore contain AMPK α in various states of degradation.

Western blotting of cercarial extracts produced a single 72 kDa band with both the anti-AMPK α and anti-phospho-AMPK α antibodies (Figure 10C), similar to that observed in adult worms (Figure 9). Immunoblotting of schistosomula extracts also revealed a single band (Figure 10D). However, in schistosomula the band appeared at a lower molecular mass (approximately 60 kDa), apparently displaced by a large quantity of another unknown species running at approximately the same position as the 70 kDa mass marker (Figure 10D). To test whether this single reactive band in schistosomula was indeed the same species as that observed at 72 kDa in other life cycle stages (Figure 9, Figure 10A-C), we immunoprecipitated the protein from schistosomula extracts using the anti-AMPK α antibody, to isolate the protein from the abundant species at 70 kDa. When the immunoprecipitates were probed with the anti-AMPK α and anti-phospho-AMPK α

antibodies, we found that migration of the reactive band was restored to 72 kDa (Figure 10E), suggesting that its migration on direct blots was indeed distorted by the abundant unidentified species at 70 kDa.

As a loading control, equal amounts of the extracts of all life cycle stages were probed with an anti- β -tubulin antibody by Western blot. A band of approximately the expected mass (55 kDa) was detected in all life cycle stages, although in cercariae and schistosomula, the signal was weaker, with evidence of lower molecular mass fragments, again emphasizing the difficulty of completely neutralizing the protease content of these life cycle stages when making protein extracts (Figure 10F).

To further investigate the expression of the AMPK α gene throughout the life cycle of *S. mansoni*, we used quantitative real-time RT-PCR to quantify AMPK α transcripts in each life cycle stage. Total RNA was extracted from every developmental stage, 500 ng of which was used as template for RT-PCR with primers and probe specific for the *S. mansoni* AMPK α cDNA. AMPK α message was detected in eggs, miracidia, sporocysts, and adult schistosomes, with the highest concentrations detected in adults (Figure 10G). In contrast, AMPK α transcript was nearly undetectable in cercariae and schistosomula.

Schistosome AMPK α expression in adult female and male worms

Next, we compared the expression and phosphorylation of AMPK α in adult female and male schistosomes (Figure 11). Lysates were prepared from separated (i.e. previously paired) adult female and male *S. mansoni* and equal amounts of protein were analyzed by Western blot. Overall, female schistosomes appeared to contain more total AMPK α protein than male schistosomes, relative to the β -tubulin content of the extracts,

although this difference was not significant when statistical analysis was performed on results from two biological replicates (Figure 11A). When AMPK α phosphorylation was assessed by Western blotting using the anti-phospho-AMPK α antibody, female and male schistosomes appeared to contain comparable amounts of phosphorylated AMPK α (Figure 11B). Interestingly, when phosphorylated AMPK α was normalized relative to total AMPK α protein, we found that AMPK α was phosphorylated to a marginally greater extent in males (Figure 11B). Data from two biological replicates revealed almost identical small increases in relative AMPK α phosphorylation in males, and statistical analysis of these data found the small difference was significant (Figure 11B). Finally, AMPK α gene expression in female and male worms was compared using PCR to measure transcript levels in RNA extracted from separated female and male worms. Adult male RNA contained significantly more AMPK α transcripts than adult female RNA (Figure 11C), but the difference was less than twofold. From these limited data, we conclude that while some differences in AMPK α protein content and phosphorylation and AMPK α gene expression may exist between female and male schistosomes, the protein is clearly expressed and active in both sexes.

Regulation of schistosome AMPK α phosphorylation in response to external stimuli

The data presented in Figures 9 and 11 suggest that AMPK α is constitutively phosphorylated in freshly isolated adult worms. We therefore sought to test whether further phosphorylation of schistosome AMPK α could be induced in adult worms immediately *ex vivo*, as evidence that the AMPK pathway in adult schistosomes is responsive to changes in the worm's environment. Adult worms were incubated in control medium, in medium lacking glucose, or in control medium supplemented with

increasing concentrations of forskolin (an adenylate cyclase agonist that indirectly activates AMPK α by raising cellular cAMP concentrations (43; 196)) or metformin (a direct AMPK α agonist (55; 74; 134; 205)). After 2 h of incubation under these conditions, lysates were prepared and equal amounts of total protein were analyzed by Western blot. The signal obtained with the anti-phospho-AMPK α antibody was normalized to that obtained with the anti-AMPK α antibody, which in turn was normalized to the signal obtained with the anti- β -tubulin antibody. Glucose deprivation consistently resulted in increased relative phosphorylation of AMPK α (Figure 12A, 12B). Likewise, exposure to forskolin and metformin resulted in increased phosphorylation of AMPK α , in a dose dependent manner (Figure 12A, 12B).

***S. mansoni* AMPK α expression is attenuated in RAG^{-/-} mice**

Data presented in Figure 12 suggest that schistosome AMPK α activity can be modulated by extrinsic factors (e.g. environmental glucose availability, AMPK α agonists) in the parasites' environment. We therefore tested whether *S. mansoni* AMPK α phosphorylation was modulated in immunodeficient RAG-1^{-/-} mice, where schistosomes exhibit attenuated growth and reproductive fitness (32). Worms recovered from wild type and RAG-1^{-/-} mice were assessed for AMPK α expression and phosphorylation by Western blot. Probing with the anti-AMPK α antibody, we found that worms isolated from wild type mice contained approximately twice as much total AMPK α protein as worms from RAG-1^{-/-} mice, relative to the β -tubulin content of the lysates (Figure 13A). This decrease in total AMPK α protein content in worms from RAG-1^{-/-} mice was found to be reproducible and statistically significant when data from four biological replicates were analyzed (Figure 13A). When the lysates were probed with the anti-phospho-AMPK

α antibody, worms from wild type mice again contained more phosphorylated AMPK α than worms from RAG-1^{-/-} mice (Figure 13B), mirroring the difference in total AMPK α content of the worms. However, when the levels of phosphorylated AMPK α were normalized relative to the total AMPK α protein content of the lysates, we found that relative phosphorylation of schistosome AMPK α was similar in both host genotypes, with no significant difference evident when data from four biological replicates were analyzed (Figure 13B). When the AMPK α mRNA content of the worms was compared by PCR, we found that worms from RAG-1^{-/-} mice contained less than half the concentration of AMPK α transcripts as worms from wild type mice (Figure 13C). Thus, expression of AMPK α in worms from RAG-1^{-/-} mice is down-regulated at the level of both mRNA and protein (Figure 13A, 13C), but levels of AMPK α activity are similar to those in wild type mice (Figure 13B).

DISCUSSION

Overall, schistosome AMPK α exhibits high homology to AMPK catalytic subunits of other organisms, with the exception of a 100 amino acid insertion in the C-terminal half of the schistosome sequence that is not found in the AMPK α of any other organism. The impact of this insertion on the tertiary structure of *S. mansoni* AMPK α will require specific structural and functional analyses, but we hypothesize that the greater distance between the protein's C-terminal regulatory and N-terminal kinase/autoinhibitory domains may introduce greater flexibility into the molecule, and increase the potential for these domains to engage in intermolecular interactions with other proteins. If this unique sequence does have functional significance, it may have the

potential to serve as a unique target to interrupt AMPK activity in schistosomes, without detrimentally affecting the human orthologue.

Our Western blotting experiments show that AMPK α protein is present and active in all life cycle stages we examined, likely reflecting a need for active energy homeostasis throughout the schistosome life cycle. In contrast, AMPK α transcripts were detectable in all life cycle stages examined, with the notable exception of cercariae and schistosomula. Previous studies have shown that cercariae generally do not actively engage in transcription, with the exception of a subset of genes that are mostly expressed in cercarial tails (157; 170), and that transcription of most genes is reinitiated once the parasite enters the mammalian host (149). Our data suggest that the AMPK α gene is also regulated in this manner, such that AMPK proteins are prepackaged into cercariae before leaving the molluscan host, and the AMPK α gene is not actively expressed again until the parasite enters the mammalian host. We found that AMPK α transcripts were most abundant in adult worms, so at some point between cercarial skin penetration and adulthood, dramatic up-regulation of AMPK α transcription must occur. Studies are underway to identify the exact time point at which this occurs. The presence of AMPK α protein, but not transcripts, during the cercariae/schistosomulum stage of the life cycle suggests this may be an opportune point to target schistosome AMPK α , when the parasite is unable to synthesize new protein.

The results of our in vitro experiments showed that AMPK α activity is responsive to changes in the parasites' environment, suggesting AMPK α could potentially play a role in sensing host factors. The impaired growth and reduced reproductive fitness of schistosomes infecting immunodeficient mice is well documented

and is an obvious developmental response to a change in host factors (32; 81; 112; 113). We were therefore curious to explore whether any changes in AMPK α expression or activity correlated with this developmental adaptation. Here we found that this attenuated developmental phenotype coincided with a reduction in overall AMPK α protein levels, while the AMPK α that is expressed is phosphorylated to a similar extent to AMPK α in parasites from immunocompetent hosts. One possible explanation for why schistosome growth and reproduction are attenuated in immunodeficient hosts is that the parasites are unable to secure sufficient essential host resources to sustain normal growth and development in an immunocompromised environment. However, we have been unable to show that the availability of fatty acids or glucose are reduced in immunodeficient hosts (Hunter and Davies, unpublished), and the consistent level of AMPK α phosphorylation in worms from immunocompetent and immunodeficient mice argues that the parasites are not struggling to meet their bioenergetic needs in an immunodeficient environment. If schistosomes growing in immunodeficient mice were experiencing metabolic stress, we would expect AMPK α phosphorylation to be increased in these worms as they engage mechanisms that serve to restore cellular ATP levels, but this is not the case. While we cannot rule out the possibility that a reduction in the availability of some other essential host resource accounts for the impaired development of schistosomes in immunodeficient mice, our data argue that impeded acquisition of energy substrates is not the cause of the attenuated parasite development in this context.

An alternative hypothesis for the attenuated developmental phenotype of schistosomes in immunodeficient mice postulates that (i) the parasites require a key but as yet unidentified developmental signal from the host to develop and reproduce at

normally rates, and (ii) that this developmental signal either originates from the host's immune system or is otherwise produced as a result of the host immune response. Hence, in immunodeficient mice the putative developmental signal is not expressed and the parasites revert to an alternative developmental program, where development and reproduction proceeds but at a slower pace than normal (32; 113). Our data showing that schistosome AMPK α transcripts and protein are expressed at a lower level in immunodeficient hosts could be consistent with an alternative developmental state, where bioenergetic demands are different and AMPK α expression is regulated accordingly. Indeed, AMPK α transcript levels in worms from RAG-1^{-/-} mice are more similar to those found in miracidia than in adult worms from wild type mice. These results closely resemble the findings we previously published concerning schistosome PKA, which is also expressed at significantly reduced levels in immunodeficient hosts (177). Together these results suggest that schistosome metabolism is subject to significant modification in response to host immune status. While it is unlikely that schistosomes, under natural conditions, encounter hosts as profoundly immunodeficient as the RAG-1^{-/-} mice used in our studies, causes of secondary or acquired immunodeficiency, such as malnutrition and coinfection with human immunodeficiency virus and other pathogens, are prevalent within the geographic range of schistosomes (28; 121; 127; 166; 175). Hence, the ability to modify parasite metabolic activity in response to alterations in host immune function may confer a selective advantage.

The downstream effects of AMPK activation are numerous. In mammals and other eukaryotes, phosphorylation of AMPK α ultimately leads to the upregulation of catabolic processes, including fatty acid oxidation and glycolysis, and mitochondrial

biogenesis and autophagy (4; 78; 79). Further metabolic analyses will be required to determine whether the activation of schistosome AMPK α results in the same consequences. Given that glycolysis and fatty acid oxidation have both been shown to be essential for parasite survival and reproduction (19; 91; 142; 164; 168; 198), elucidating the connection between host immune signals, AMPK, and energy generating processes in schistosomes is of considerable interest. The influence of schistosome AMPK on parasite glucose transporter expression is also being explored. In mammals, the mechanism by which AMPK activation upregulates glycolysis is in part fueled by an increase in glucose uptake via enhanced expression of glucose transporters (1; 78; 136). Identifying schistosome metabolic processes regulated by AMPK and mechanisms by which this occurs may reveal new opportunities for disrupting parasite growth, development and reproduction.

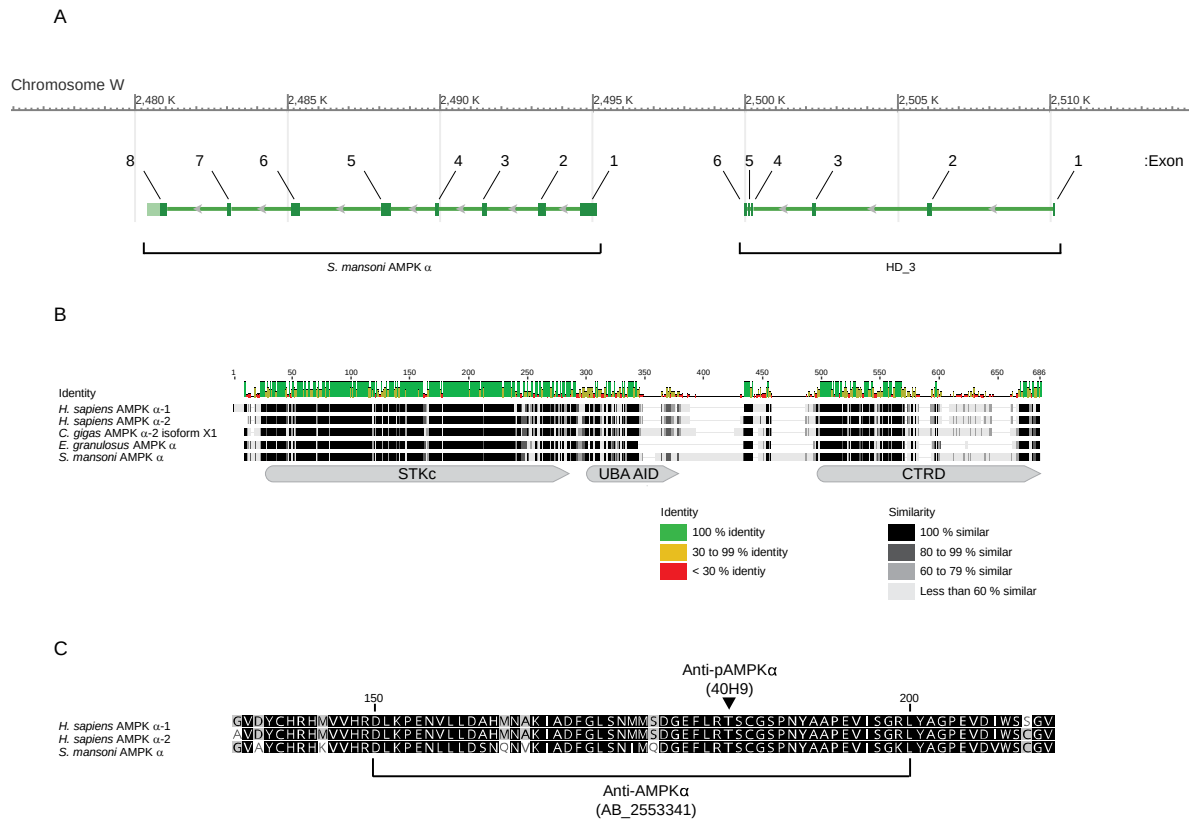


Figure 8. Sequence and structure of *S. mansoni* AMPK α

A, Arrangement of the eight exons that contribute to the *S. mansoni* AMPK α mRNA MH445971, on the complementary strand of the W chromosome. Six exons that encode for a metal-dependent phosphohydrolase (HD_3) domain are located upstream, separated by 5 kb of intergenic sequence. B, The amino acid sequence of *S. mansoni* AMPK α (MH445971) aligned with sequences of *Homo sapiens* AMPK α -1 (NP_006242) and AMPK α -2 (NP_006243), *Crassostrea gigas* AMPK α -2 (XP_011446066), and *Echinococcus granulosus* AMPK α (AER10553), showing sequence identity and similarity, and the approximate locations of the AMPK α

serine/threonine kinase catalytic domain (STKc, CCD accession number cd14079), AMPK α UBA-like autoinhibitory domain (UBA AID, CCD accession number cd14336), and AMPK α C-terminal regulatory domain (CTRD, CCD accession number cd12122) .

C, Alignment of *H. sapiens* AMPK α -1 (NP_006242) and AMPK α -2 (NP_006243), and *S. mansoni* AMPK α (MH445971), in the region recognized by a rabbit polyclonal antibody (RRID AB_2553341) raised against aa 150-200 of human AMPK α -1 (NP_006242). The square brackets denote the 50 aa sequence of NP_006242 used as the immunogen. The arrowhead indicates the position of the activation loop threonine that is phosphorylated upon activation, corresponding to Thr183 and Thr172 of NP_006242 and NP_006243, respectively, and Thr176 of the schistosome sequence (MH445971). When phosphorylated, this residue is recognized by the phospho-specific monoclonal antibody 40H9. Amino acid numbering corresponds to the sequence of NP_006242.

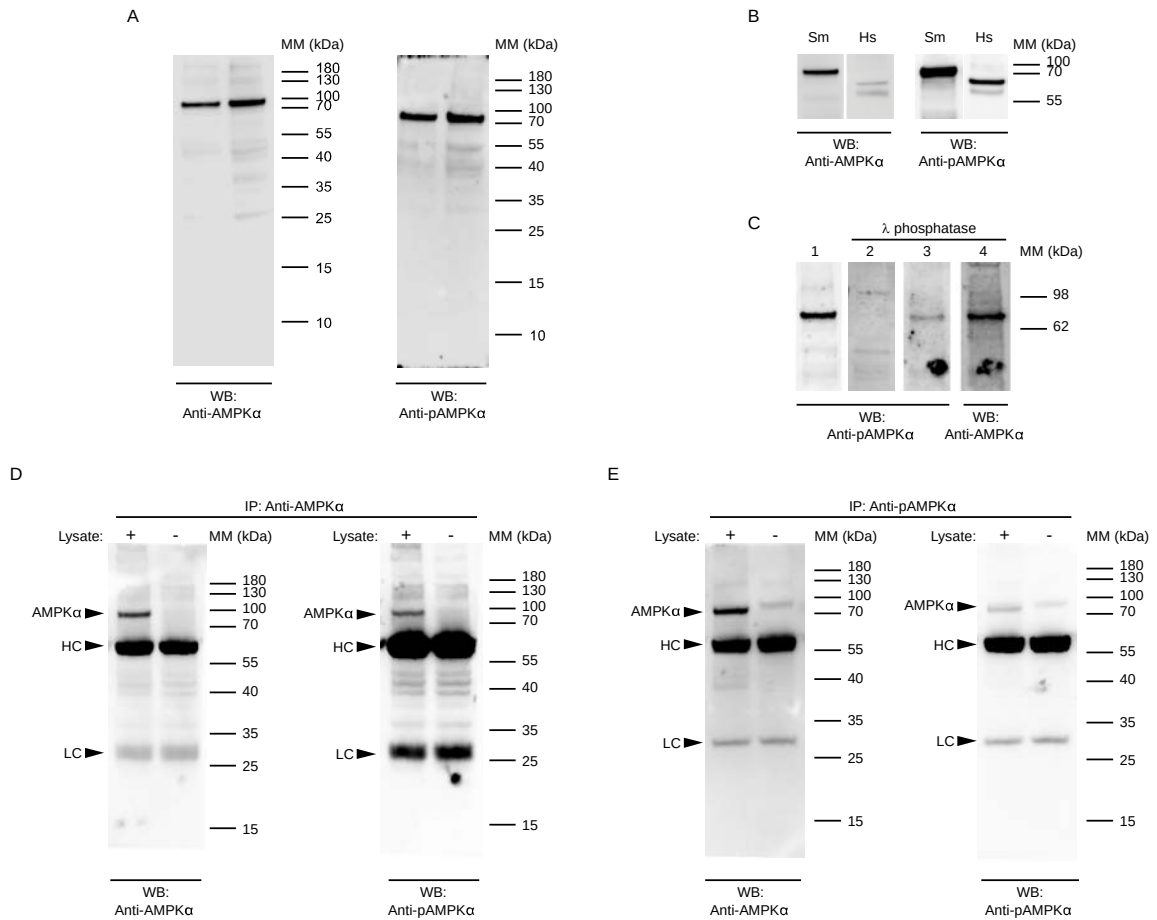


Figure 9. Detection of native *S. mansoni* AMPK α

A, Duplicate samples of adult *S. mansoni* worm lysate were probed by Western blot with a polyclonal antibody raised against the catalytic domain of human AMPK α -1/2 (anti-AMPK α , RRID AB_2553341, left panel) and a phospho-specific monoclonal antibody that recognizes human AMPK α -1/2 when phosphorylated at Thr183/Thr172 (anti-pAMPK α , clone 40H9, right panel). B, Lysates of adult *S. mansoni* worms (Sm) and human embryonic kidney cells (Hs), probed with anti-AMPK α (AB_2553341, left panel) and anti-pAMPK α (clone 40H9, right panel). (C) PVDF membranes bearing 10 μ g adult *S. mansoni* worm lysate were probed with anti-pAMPK α (clone 40H9) either before (lane 1) or after treatment with λ protein phosphatase (lane 2). Lanes 3 and 4 are the same

membrane as in lane 1, reprobed with anti-pAMPK α (lane 3) or anti-AMPK α (lane 4) after treatment with λ phosphatase. D, E, AMPK α protein was immunoprecipitated from adult schistosome lysates using either anti-AMPK α (D) or anti-pAMPK α (E). The immunoprecipitates were then probed by Western blot with anti-AMPK α (left panel) and anti-pAMPK α (right panel). Control immunoprecipitates where the lysate was omitted were also included. Arrowheads indicate the position of the schistosome AMPK α (AMPK α) and the immunoglobulin heavy chain (HC) and light chain (LC) of the immunoprecipitating antibody. WB: indicates the antibody used for Western blotting. IP: indicates the antibody used for immunoprecipitation. Positions of molecular mass (MM) markers, in kilodaltons (kDa), are indicated to the right of each panel.

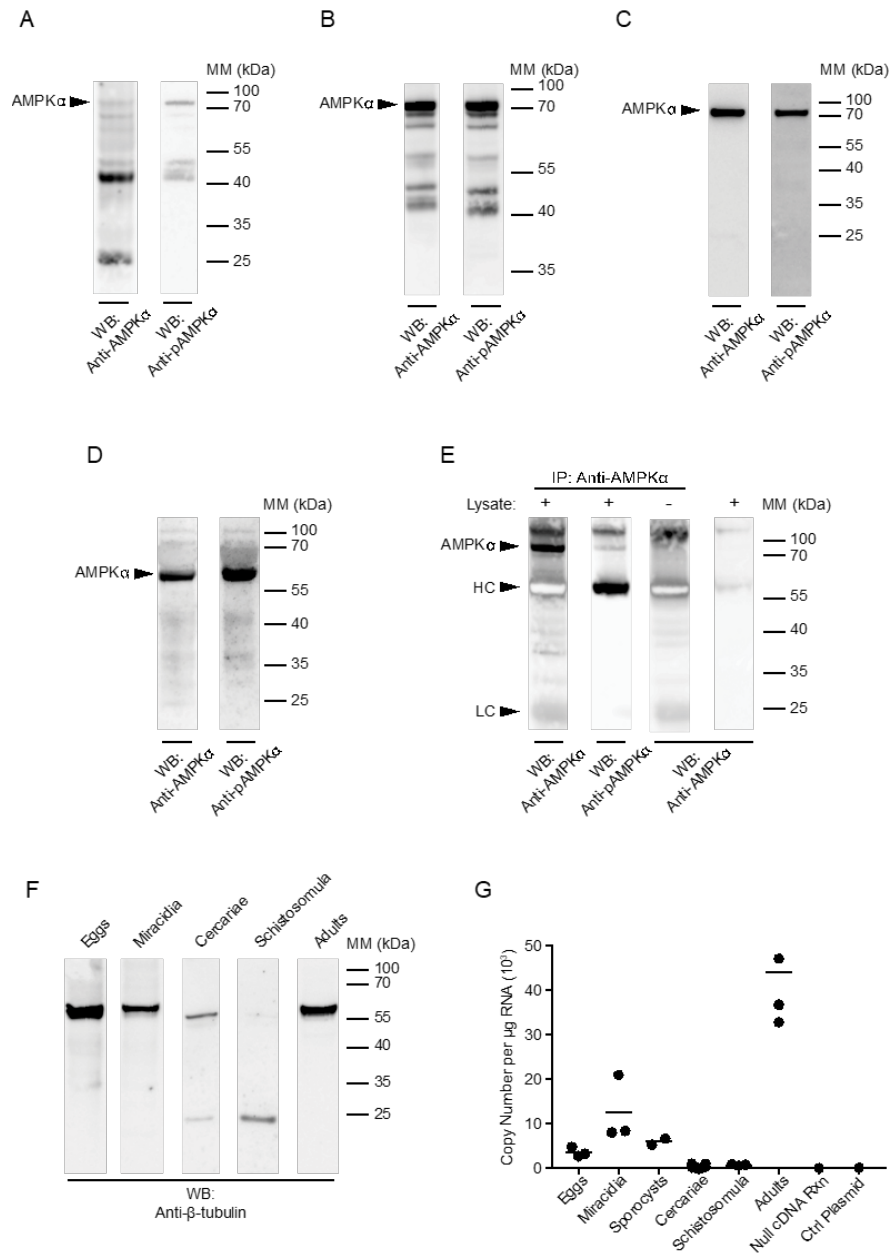


Figure 10. Detection of AMPK α in *S. mansoni* larval stages

A-D, Lysates of *S. mansoni* eggs (A), miracidia (B), cercariae (C), and schistosomula (D, 30 min post-transformation) were probed by Western blot using anti-AMPK α (left lane) and anti-pAMPK α (right lane) antibodies. E, AMPK α was immunoprecipitated from schistosomula lysate using anti-AMPK α , then probed by Western blot using the anti-AMPK α and anti-pAMPK α antibodies. Control

immunoprecipitations, where either the lysate or the immunoprecipitating anti-AMPK α were omitted, were also probed with anti-AMPK α . Arrowheads indicate the position of the schistosome AMPK α (AMPK α) and the immunoglobulin heavy chain (HC) and light chain (LC) of the immunoprecipitating antibody. F, Lysates of each larval stage and adult worms were probed by Western blot with an anti- β -tubulin antibody. G, AMPK α transcripts in each *S. mansoni* developmental stage were quantified using quantitative real-time RT-PCR. Control reactions containing no cDNA template and a control plasmid lacking the AMPK α target sequence were also included. WB: indicates the antibody used for Western blotting. IP: indicates the antibody used for immunoprecipitation. Positions of molecular mass (MM) markers, in kilodaltons (kDa), are indicated to the right of each blot.

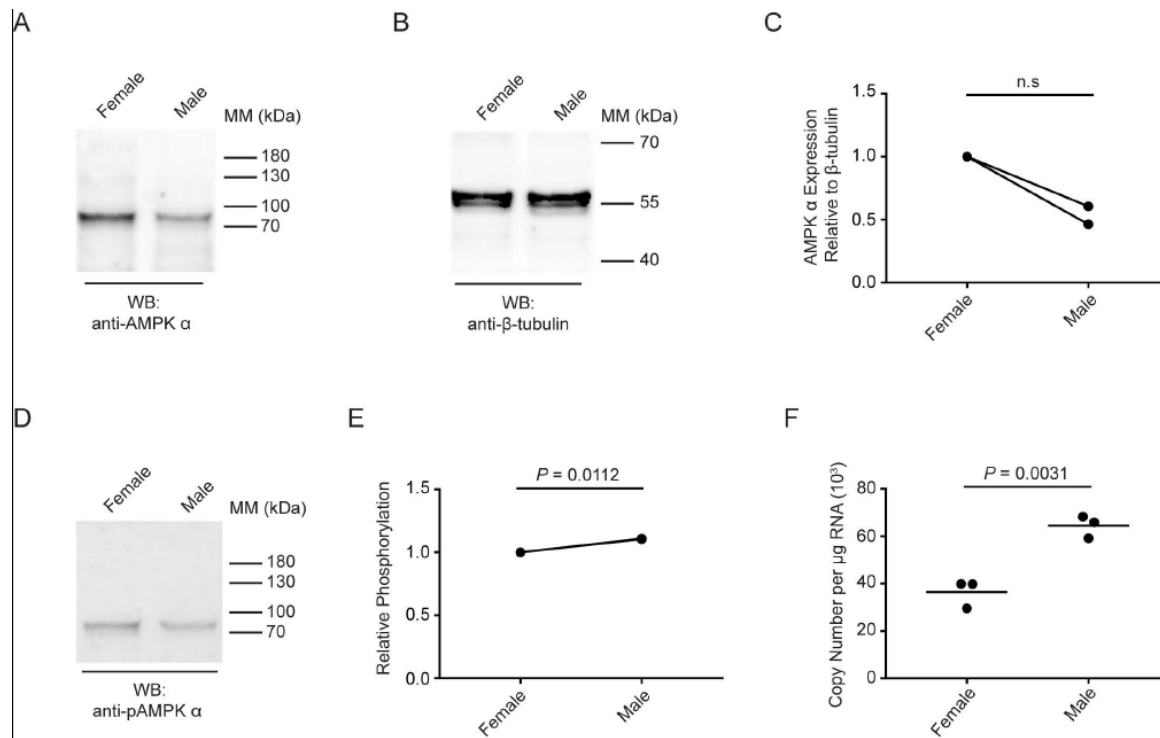


Figure 11. Expression and phosphorylation of AMPK α in female and male schistosomes

A, Lysates of adult female and male schistosomes were probed by Western blot with anti-AMPK α (left panel) and anti- β -tubulin (center panel) antibodies. The intensity of the anti-AMPK α signal relative the anti- β -tubulin signal for each sample was determined, and the signals normalized to that obtained with female worms (right panel, displaying results for two independent biological replicates). B, Lysates of adult female and male schistosomes were probed by Western blot with anti-pAMPK α antibody (left panel). The intensity of the anti-pAMPK α signal relative the anti-AMPK α signal (from A) for each sample was determined, and the signals normalized to that obtained with female worms (left panel, displaying results for two independent biological replicates). C,

AMPK α transcripts in adult female and male *S. mansoni* were quantified using quantitative real-time RT-PCR. WB: indicates the antibody used for Western blotting. Positions of molecular mass (MM) markers, in kilodaltons (kDa), are indicated to the right of each blot.

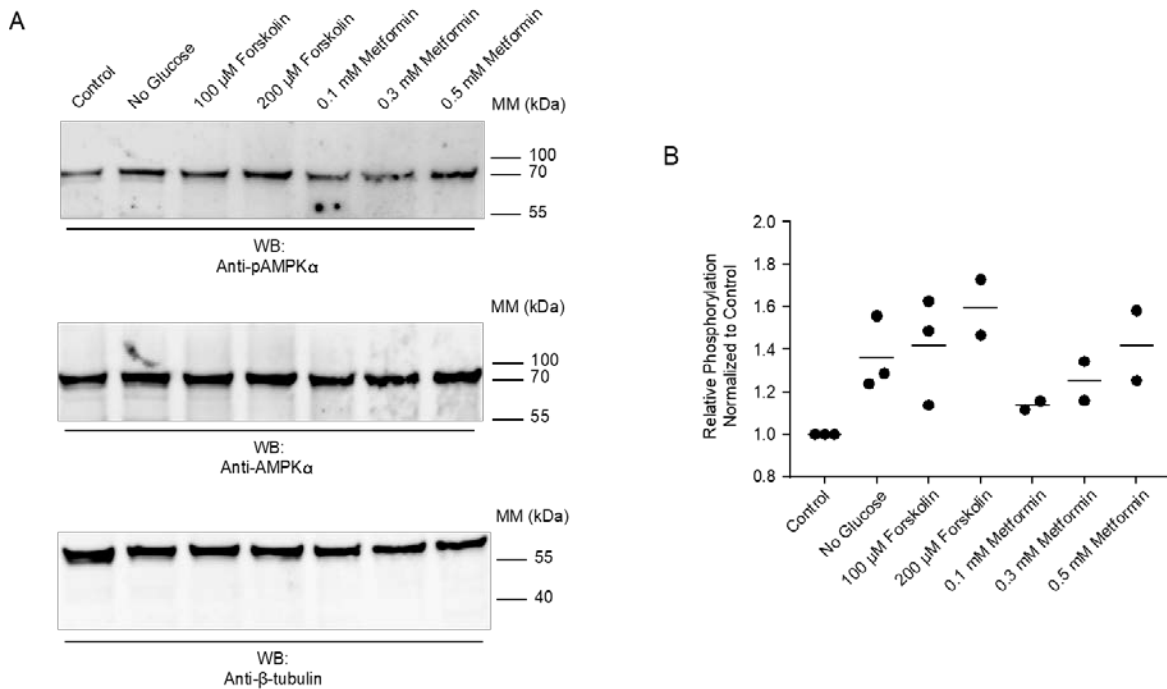


Figure 12. Responsiveness of schistosome AMPK α to external factors

A, Freshly isolated adult schistosomes were incubated in control medium, medium lacking glucose, or medium supplemented with various concentrations of forskolin or metformin. After two hours, lysates were prepared and equal amounts of protein were probed by Western blot, using anti-AMPK α antibody (top panel), anti-pAMPK α antibody (center panel) and anti- β -tubulin antibody (bottom panel). WB: indicates the antibody used for Western blotting. Positions of molecular mass (MM) markers, in kilodaltons (kDa), are indicated to the right of each blot. B, Relative AMPK α phosphorylation under each condition was calculated by determining the ratio of the anti-pAMPK α signal to that of the anti-AMPK α signal, and then correcting for the intensity of the anti- β -tubulin signal. Relative AMPK α phosphorylation values were normalized relative those obtained with worms incubated in control medium. Data points from 2-3 independent biological replicates are shown.

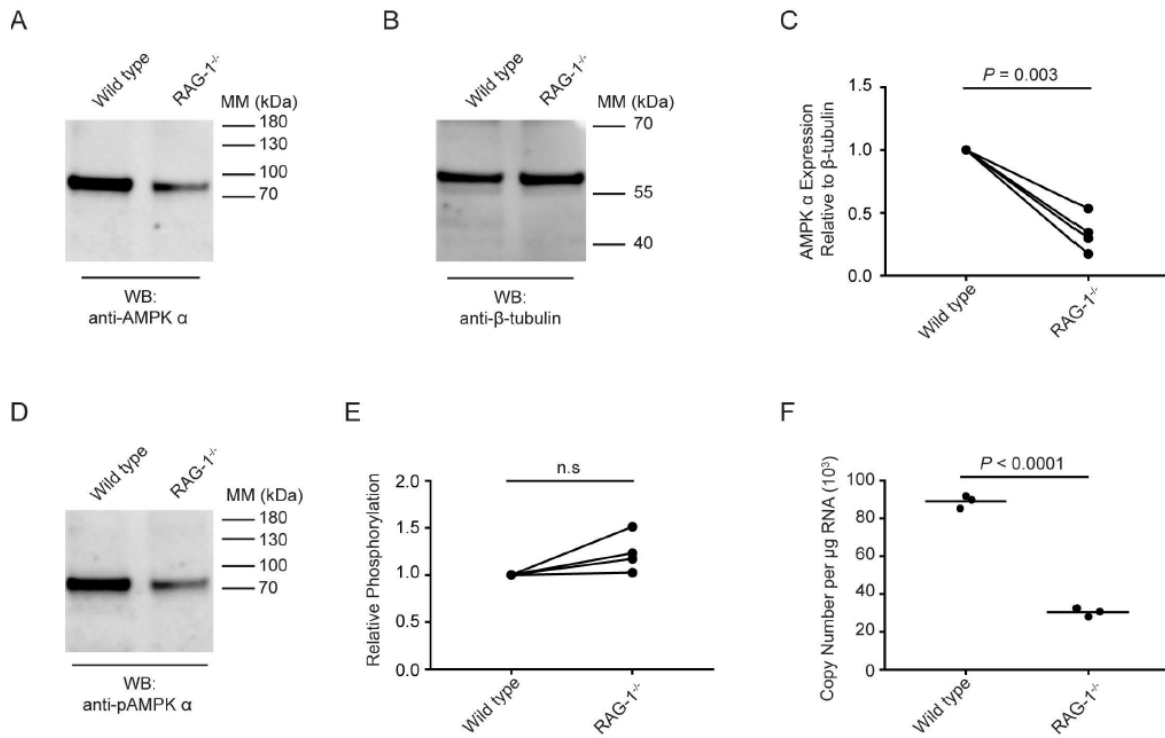


Figure 13. Expression and phosphorylation of schistosome AMPK α in RAG-1^{-/-} mice

A, Lysates were prepared from schistosomes recovered from wild type (C57BL/6) and RAG-1^{-/-} mice and probed by Western blot with anti-AMPK α (left panel) and anti- β -tubulin (center panel) antibodies. The intensity of the anti-AMPK α signal relative the anti- β -tubulin signal for each sample was determined, and the signals normalized to that obtained with worms from wild type mice (right panel, displaying results from four independent biological replicates). B, Lysates of schistosomes from wild type and RAG-1^{-/-} mice were probed by Western blot with anti-pAMPK α antibody (left panel). The intensity of the anti-pAMPK α signal relative the anti-AMPK α signal (from A) for each sample was determined, and the signals normalized to that obtained with worms from wild type mice (left panel, displaying results from four independent biological replicates). C, AMPK α transcripts in *S. mansoni* worms isolated from wild type and RAG-1^{-/-} mice

were quantified using quantitative real-time RT-PCR. WB: indicates the antibody used for Western blotting. Positions of molecular mass (MM) markers, in kilodaltons (kDa), are indicated to the right of each blot.

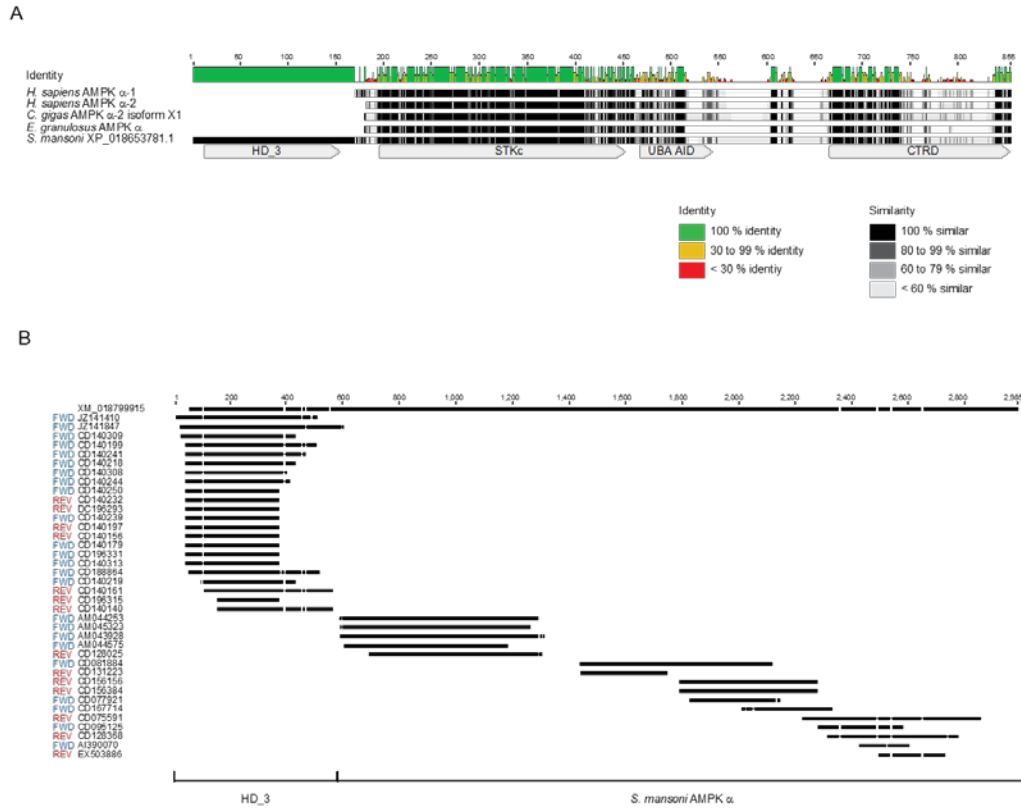


Figure 14. Sequence predictions of *S. mansoni* AMPK α incorrectly annotate N-terminal phosphohydrolase domain

A, The amino acid sequence of *S. mansoni* AMPK α originally predicted by the genome consortium (XP_018653781.1) aligned with AMPK α -subunits of *Homo sapiens* AMPK α -1 (NP_006242) and AMPK α -2 (NP_006243), *Crassostrea gigas* (XP_011446066), and *Echinococcus granulosus* (AER10553), showing an N-terminal extension present in schistosome AMPK α that is absent from all other organisms. The extension is annotated as a phosphohydrolase domain (HD_3). B, Alignment of expressed sequence tags (ESTs) spanning the nucleotide sequence of predicted *S. mansoni* AMPK α (XM_018799915). Both the annotated phosphohydrolase domain and our confirmed schistosome AMPK α -subunit are well represented by ESTS, however, without overlap.

CHAPTER 3: Schistosome AMPK is required for larval schistosome viability and regulates glycogen metabolism in adult parasites

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ABSTRACT

On entering the mammalian host, schistosomes transition from a freshwater environment where resources are scarce, to an environment where there is an unlimited supply of glucose, their preferred energy substrate. Adult schistosome glycolytic activity consumes almost five times the parasite's dry weight in glucose per day to meet the parasite's energy demands, and the parasite glycolytic enzymes and mechanisms for glucose uptake that sustain this metabolic activity have previously been identified. However, little is known of the parasite processes that regulate schistosome glucose metabolism. We previously characterized the *Schistosoma mansoni* ortholog of 5'-AMP-Activated Protein Kinase (AMPK), which is a central regulator of energy metabolism in eukaryotes, and demonstrated the modulation of its activity by external factors. Here we sought to explore the function of AMPK in schistosomes and test whether it represents a potential target for interventions intended to disrupt the parasite life cycle. Adult schistosomes mounted a compensatory response to chemical inhibition of AMPK α , resulting in increased AMPK α protein abundance and activity. RNAi inhibition of AMPK α expression, however, suggests that AMPK α is not required for adult

schistosoma viability. Schistosomula, on the other hand, are sensitive to chemical AMPK α inhibition, and this correlates with inactivity of the AMPK α gene in this life cycle stage that precludes a compensatory response to AMPK inhibition. While our data indicate that AMPK is not essential in adult schistosomes, our data suggest that AMPK regulates adult worm glycogen stores, influencing both glycogen utilization and synthesis. AMPK may therefore play a role in the ability of adult schistosomes to survive in vivo stressors such as transient glucose deprivation and oxidative stress. Our data suggest that AMPK warrants further investigation as a potential drug target, especially for interventions aimed at preventing establishment of a schistosome infection.

Author Summary

More than 250 million individuals across the globe suffer from schistosomiasis. This chronic inflammatory disease is caused by parasitic flatworms known as schistosomes. There is no vaccine or prophylactic to prevent schistosomiasis and only one therapeutic is in widespread use to treat established infections. The need to identify novel schistosome targets for the development of additional therapeutics or prophylactics is paramount. Recently, we identified an ortholog of the metabolic regulatory protein, 5'AMP-Activated Protein Kinase (AMPK), in the human parasite, *Schistosoma mansoni*. Mammalian AMPK is a therapeutic target for treating diabetes and similar metabolic diseases, and its potential as a target for novel cancer interventions has also been explored. In this paper, we assessed AMPK's function in regulating schistosome metabolism and evaluated its potential as a therapeutic target. We show that AMPK is essential for schistosomula, with inhibition of the protein being toxic for the juvenile

parasites. While we found that AMPK is not essential for adult schistosomes, it is involved in the storage and breakdown of schistosome glycogen stores, suggesting it may play a role in allowing the parasite to overcome stressful situations inside the body. The findings argue that schistosome AMPK deserves further exploration as a potential target for new drugs, especially those aimed at preventing the establishment of infection.

INTRODUCTION

Schistosomiasis is a chronic inflammatory disease caused by infection with parasitic platyhelminths of the genus *Schistosoma*. The species *S. japonicum*, *S. haematobium*, and *S. mansoni* are of primary concern in human medicine as they cause the majority of human schistosomiasis (104). It is estimated that over 250 million individuals are infected with schistosomes (25; 85; 138), resulting in the loss of over 10 million disability-adjusted life years (DALYs). Illness is hallmarked by severe inflammation of critical organs, ultimately resulting in the accumulation of irreparable and life-threatening tissue damage if left untreated (106).

Humans contract schistosomiasis via contact with bodies of fresh water where the infectious schistosome larval stages known as cercariae are found. Cercariae penetrate intact human skin (124) and, once inside the body, the juvenile schistosomes migrate through the vasculature for several weeks until the vessels that are the preferred final destination are reached. Once mature, adult male and female schistosomes pair and female worms begin to produce hundreds to thousands of eggs per day. Some eggs successfully exit the human body to restart the schistosome life cycle in the outside environment (104). Many eggs, however, are unable to exit the host and remain in the

tissue where they incite immune responses, resulting in inflammation and granuloma formation. Over time, granulomatous tissue surrounding schistosome eggs becomes fibrotic, leading to tissue scarring and complications such as hepatosplenomegaly, periportal fibrosis, portal hypertension, and esophageal varices (25). Currently, there is only one therapeutic, praziquantel, available to treat schistosomiasis, and this drug is only effective against adult schistosomes. There is no prophylactic available to prevent infection (185).

Schistosomes are obligate parasites that rely on their hosts to supply essential factors that the helminths are unable to manufacture themselves. One of the most important nutrients that schistosomes must acquire from the external host environment is glucose, as it is the parasite's principle energy substrate (198). Previous studies have demonstrated that when glucose access is interrupted, schistosome survival is detrimentally impacted (108). Adult schistosomes consume as much as 20 % of their dry weight in glucose per hour, the vast majority of which is funneled into glycolysis (18). Schistosomes almost exclusively depend on glycolysis to metabolically support their growth and reproduction (179; 198). Previous efforts to elucidate the systems that schistosomes employ to meet their high metabolic demand revealed that these parasites are unable to metabolize lipids, leaving glucose metabolism as their sole source of energy production (16).

Because of the importance of glucose metabolism in schistosomes, previous research has focused on elucidating the mechanisms by which schistosomes acquire glucose, as well as factors that might influence glucose uptake, in order to uncover potential opportunities for intervention. Previous studies have identified schistosome

insulin receptors (3; 199), parasite glucose transporters that facilitate glucose uptake (169; 171), and schistosome glycolytic enzymes (198). However, little is known about the regulatory mechanisms that control schistosome energy metabolism. Analysis of schistosome genomic sequences have predicted that the regulatory networks that control energy metabolism in other eukaryotes are conserved in schistosomes (15; 159; 200), but little has been done to characterize these networks in schistosomes or test their potential as targets for novel interventions. Targeting the regulation of intracellular energy metabolism, specifically those controlling glucose metabolism, have proven to be lucrative interventions to diabetes and other related metabolic diseases (27; 74; 204). Similar methods are also being explored for further therapeutic development given their established efficacy against various cancer types *in vitro* (69; 114; 202). This approach could prove to be useful against schistosomes as well.

In mammals and other eukaryotes, numerous pathways are involved in the regulation of glucose uptake and metabolism, including the insulin signaling pathway (155), protein kinase A (PKA) (100; 109), Akt (162; 176), and 5' AMP-activated Protein Kinase (AMPK) (73; 107). AMPK in particular is conserved in all eukaryotes, and is the intracellular master regulator of cellular energy metabolism and energy homeostasis (75; 84). AMPK is a heterotrimeric protein, comprised of α , β , and γ subunits that each serve a unique purpose in regulating the cellular energy balance. The γ subunit senses the cellular energy status via direct binding of ATP, ADP and AMP. In instances where cellular ATP levels are low, AMP and ADP out compete ATP for binding to the AMPK γ subunit, leading to phosphorylation and activation of the catalytic α subunit kinase domain (63; 73; 78). Activation of AMPK leads to multiple metabolic outcomes that achieve the

common goal of dampening anabolic processes to decrease ATP consumption (e.g. gluconeogenesis, lipid synthesis, and glycogen synthesis), while simultaneously stimulating catabolic processes to promote ATP generation (e.g. glycolysis, fatty acid oxidation, and glycogen breakdown) (111). Mammalian AMPK activation results in increased glucose uptake, indirect stimulation of the key glycolytic enzyme phosphofructokinase-1, inhibition of glycogen synthesis via phosphorylation of glycogen synthase, and activation of glycogen breakdown via phosphorylation of glycogen phosphorylase (99). While these effects are mainly mediated by the kinase activity of the AMPK α subunit, the β subunit also plays a significant role in regulating glycolysis by interacting with glycogen. A glycogen binding domain in the AMPK β subunit allows for the direct association of AMPK with glycogen, allowing AMPK to gauge cellular glycogen stores (98; 122; 123; 145).

Previously, we reported the presence of an AMPK ortholog in the human parasite *S. mansoni* (92). While the schistosome AMPK α subunit exhibits high levels of identity and similarity to the AMPK α of humans and other species, the schistosome sequence diverges considerably in the C-terminal half of the protein due to the presence of approximately 100 additional, non-conserved amino acids. Furthermore, we showed that while the *S. mansoni* AMPK α protein is present in all developmental stages of the parasite, the gene is not actively expressed in the infectious cercaria and early intra-mammalian stages. Lastly, we demonstrated that schistosome AMPK activity is vulnerable to modulation by host factors and chemical agents (92).

Because AMPK is a central regulator of energy metabolism in other eukaryotes, we hypothesized that schistosome AMPK is critical for the regulation of parasite

glycolysis and could therefore serve as a novel therapeutic target for disrupting schistosome energy metabolism. To test this hypothesis, we sought to characterize the relationship between schistosome AMPK activity and parasite glycolysis, and ascertain the metabolic and phenotypic consequences of targeting parasite AMPK activity. Our data suggest that the schistosome AMPK fulfills different roles in larval and adult parasites, being required for viability in the former and regulating cellular glycogen stores in the latter.

MATERIALS & METHODS

Ethics Statement

All animal procedures were performed in accordance with guidelines outlined in the National Research Council's *Guide for the Care and Use of Laboratory Animals* (The National Academies Press) and were pre-approved by the Institutional Animal Care and Use Committee (IACUC) of Uniformed Services University (permit number A3448-01).

Parasite Materials

Albino *Biomphalaria glabrata* infected with *Schistosoma mansoni* miracidia (NMRI strain) were provided by Biomedical Research Institute (BRI; Shady Grove, MD) within a week post-miracidial exposure. At 6 weeks post-infection, *B. glabrata* snails were exposed to light for 1-1.5 hours to promote the emergence of cercariae. Cercariae were collected in a conical tube and either used to infect mice or were transformed into schistosomula. Mice were infected with *S. mansoni* via tail skin exposure to 135 cercariae for 30 minutes, after which they were returned to their cages. After 8 weeks, adult schistosomes were recovered from infected mice via portal vein perfusion. Cercariae

were mechanically transformed into schistosomula via 20-30 passages through an emulsification needle using 30-ml syringes (Luer-Lok Tip, BD). Cercarial heads (newly transformed schistosomula) were separated from tails via multiple rounds of swirling in RPMI + 1 % HEPES buffer + 2 % antibiotic/antimycotic (Invitrogen) using deep petri dishes.

Chemical Compounds

Metformin hydrochloride (Tocris Bioscience, catalog no. 2864) was dissolved in ultrapure distilled water (Invitrogen) to a stock concentration of 100 mM and stored at -20 °C. 2-Deoxy-D-glucose (2-DG) (Sigma Aldrich, catalog no. D8374) was dissolved in ultrapure distilled water (Invitrogen) to a stock concentration of 500 mM and stored at -20 °C. Stock solutions of 2-DG were discarded after one freeze-thaw cycle. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) (Sigma Aldrich, catalog no. A9978) was solubilized in ultrapure distilled water (Invitrogen) to a stock concentration of 33 mM and stored at -20 °C. Dorsomorphin was supplied in either dimethyl sulfoxide (DMSO) soluble (Abcam, catalog no. ab120843) or water-soluble forms (Dorsomorphin dihydrochloride, Tocris Bioscience, catalog no. 3093). Either DMSO (Fisher Scientific) or ultrapure distilled water (Invitrogen) were used as appropriate to produce stock solutions of 2.5 mM. Aliquots of DMSO-soluble Dorsomorphin were stored at -20 °C. Dorsomorphin dihydrochloride stock solutions were prepared fresh when possible or stored at -80 °C and thawed after one freeze-thaw cycle. Akt Inhibitor X (10-(4'-(N,N-Diethylamino)butyl)-2-chlorophenoxazine, HCl, 4-(2-Chloro-10H-phenoxazin-10-yl)-N,N-diethylbutan-1-amine, HCl) (Calbiochem, catalog no. 124039) was supplied as a 20 mM solution in water and stored at -80 °C. Glycogen Phosphorylase Inhibitor (2-chloro-

4,5-difluoro-N-[[[2-methoxy-5-
[[[(methylamino)carbonyl]amino]phenyl]amino]carbonyl]-benzamide) (EMD Millipore Corp, catalog no. 361515) was supplied as 1 mg and dissolved in 1 mL DMSO to make a 2.4 mM stock solution that was stored at 4 °C.

Agilent Seahorse XFe96 Glycolysis Assay

An Agilent Seahorse XFe96 Analyzer was used to assess the metabolic output of adult and juvenile schistosomes in response to AMPK modulating factors. To determine the rate of glycolysis, the extracellular acidification rate was measured over time using the Agilent Seahorse XFe96 Extracellular Flux Assay Kit cartridge (Agilent Technologies, catalog no. 102905-100) and Agilent Seahorse XFe96 Spheroid Microplates (Agilent Technologies, catalog no. 102978-100). Twenty-four hours prior to the start of an experiment, cartridge probes were soaked in 200 µl Ultrapure Distilled Water (Invitrogen) at 37 °C. The water was replaced with 200 µl Agilent Seahorse XF Calibrant (Agilent Technologies, catalog no. 100840-000) an hour prior to starting the experiment. Adult schistosomes were recovered by perfusion from infected mice immediately prior to the start of the experiment and washed with 1 X PBS to remove perfusion fluid. Either a single schistosome pair, containing one male and one female adult schistosome, or 500 newly transformed schistosomula were placed in each well of an Agilent Seahorse XFe96 Spheroid Microplate with 180 µl of Seahorse XF DMEM medium (Agilent Technologies, catalog no. 103575-100) supplemented with 1 mM Seahorse XF pyruvate solution (Agilent Technologies, catalog no. 103578-100), 10 mM Seahorse XF glucose solution (Agilent Technologies, catalog no. 103577-100), 2 mM Seahorse XF L-glutamine solution (Agilent Technologies, catalog no. 103579-100), and 1 X antibiotic/antimycotic.

Cartridge compound-administration chambers were loaded with 25 µl of either media or AMPK modulating compounds at pre-calculated concentrations to achieve desired final concentrations in a final volume of 205 µl of media before beginning Seahorse XFe96 Analyzer experiment.

Agilent Fluorescent Glycolysis Assay

Glycolytic rates, both basal and post-exposure to AMPK-modulating compounds, of adult schistosomes and schistosomula immediately-post transformation were measured using the pH-Xtra Glycolysis Assay (Agilent Technologies, catalog no. PH-200-4).

Manufacturer's instructions described in the pH Xtra Glycolysis User Manual were modified to adapt the standard protocol to use with parasites. Briefly, either 2 adult worm pairs or 1000 schistosomula were distributed per well in a black, clear bottom 96-well plate (Corning). PH-Xtra Reagent was solubilized in 1 ml ultrapure distilled water (Invitrogen). One pH-Xtra respiration buffer tablet was fully dissolved in 50 ml Ultrapure Distilled Water (Invitrogen) and warmed to 37 °C. The pH was adjusted to 7.4 using KOH and NaOH. After pH-adjustment, the respiration buffer solution was sterilized via filtration through a 0.22 µm filter. Parasites were washed twice in warmed respiration buffer and resuspended in 60 µl buffer + 10 µl pH-Xtra reagent per well, to maximize fluorescent signal. Compounds were added to respective wells at volumes calculated to achieve desired final concentrations. Plates were kept at 37 °C and fluorescence was measured every 15 minutes using a Spectramax M2 spectrophotometer (Molecular Devices) at an excitation/emission wavelength of 350/615 nm.

***S. mansoni* AMPK Kinase Activity Assay**

AMPK was immunoprecipitated from adult *S. mansoni* lysate using anti-AMPK α antibodies according to the immunoprecipitation protocol we described previously (92). Briefly, 1 μ g anti-AMPK α (Invitrogen, catalog no. PA5-36045) was incubated with 50 μ g of pre-cleared adult schistosome lysate prepared in 1 % NP-40 lysis buffer for 1 h at 4 °C. The lysate/antibody was then incubated with a 50 % nProtein A Sepharose Fast Flow (GE Healthcare) bead slurry for 1 h at 4 °C with gently mixing to precipitate immune complexes. After incubation, the mixture was centrifuged at 12,000 x g for 30 seconds to isolate the bead-bound *S. mansoni* AMPK α -antibody complexes. After washing five times in 1 % NP-40 buffer, bead-bound immune complexes were resuspended in 1 X kinase buffer (prepared from 10 X kinase buffer, Cell Signaling Technologies, catalog no. 9802S) supplemented with adenosine triphosphate (ATP) (Sigma Aldrich, catalog no. A1852) and adenosine monophosphate (AMP) (Sigma Aldrich, cat no. 01930, each to a final concentration of 200 μ M). To determine the effects of dorsomorphin on Sm AMPK activity, dorsomorphin was added to the kinase buffer/immune complex mixture to a final concentration of 10 μ M followed by the addition of .2 μ g acetyl-CoA carboxylase (ACC). The mixture was incubated at 37 °C for 10 minutes after which the reaction was terminated by incubation in 1 X LDS sample buffer (25 % 4 X LDS sample buffer, 65 % ddH₂O, 10 % beta-mercaptoethanol) at 95 °C for 5 minutes. The supernatant was separated from nProtein A Sepharose beads by centrifugation at 12,000 x g for 30 seconds, transferred to a new tube, and analyzed via Western blot to assess ACC phosphorylation status. The kinase assay outlined above was adapted from a protocol previously described by Hardie *et al* (80).

Preparation of Parasite Protein Extracts

Immediately post-perfusion, adult schistosomes were either directly used to extract protein or flash frozen in liquid nitrogen or stored at -80 °C for protein preparation at a later date. Whole protein extracts were prepared by homogenizing parasites in 1 X LDS sample buffer (25 % 4 X LDS sample buffer, 65 % ddH₂O, 10 % beta-mercaptoethanol) supplemented with 100 X Halt protease inhibitor cocktail (Invitrogen) and 100 X phosphatase inhibitor to a final concentration of 1 X using an electric homogenizer. To further homogenize remaining particulate matter, samples were briefly subjected to one round of sonication at intervals of 10 seconds for 4 minutes. Samples were incubated for 10 minutes at 95 °C. Insoluble material was removed from the lysate via centrifugation for 5 minutes at 1800 x *g*. Schistosomula protein was similarly prepared, however, samples were homogenized in boiling 1 X LDS sample buffer to reduce the activity of cercarial proteases.

Western Blotting

Protein concentrations of parasite lysates were determined using the Pierce 660 nm Protein Assay Kit (Thermo Scientific). Protein separation by SDS-PAGE was performed by loading 5-10 µg of sample into 10 % Bis-Tris gels (NuPAGE) in 1X MES Buffer (Novex) and run at 200 V for 1 h. Immediately following separation, proteins were transferred onto Invitrolon polyvinyl difluoride membranes (PVDF) (Novex) for approximately 90 min at 25 V, 160 mA. After transfer, PVDF membranes were blocked in Startingblock TBS Blocking Buffer (Thermo Scientific) with 0.05 % Tween-20 for 30 min. Blocked membranes were probed for 1 h or overnight with either AMPK alpha- 1,2 rabbit polyclonal antibody (Invitrogen, catalog no. PA5-36045) diluted 1:500, phospho-AMPK alpha (T172) rabbit monoclonal antibody (Cell Signaling Technology, catalog no.

2535) diluted 1:1000, Acetyl CoA carboxylase rabbit monoclonal antibody (Cell Signaling Technology, catalog no. 3676) diluted 1:1000, phospho-Acetyl CoA (Ser179) rabbit monoclonal antibody (Cell Signaling Technology, catalog no. 11818) diluted 1:1000, or beta tubulin rabbit polyclonal antibody (Abcam, catalog no. ab15568) diluted 1:5000. After primary antibody incubation, membranes were washed five times in 1 X TBST Buffer (Thermo Scientific) then probed with Goat anti-rabbit IgG (H+L) cross-adsorbed HRP secondary antibody (Thermo Scientific, catalog no. G21234) diluted 1:2000 for 1 h. All antibodies were diluted in Startingblock TBS Blocking Buffer with 0.05 % Tween-20. Finally, membranes were incubated in SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) for 5 min to detect bound antibody. Membranes were imaged using an Amersham Imager 680 blot and gel imager (GE Healthcare Life Sciences). Densitometry measurements were calculated using ImageJ Software (161).

***In vitro* Dorsomorphin Parasite Treatment**

Adult schistosomes were transferred to 12-well tissue culture treated plates immediately post-perfusion in 2 ml DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic. Newly transformed schistosomula were placed in 12- or 24-well tissue culture treated plates in 2 ml Modified Basch Medium (11) immediately following transformation. Dorsomorphin was added directly to media in various concentrations. Plates were swirled to evenly distribute dorsomorphin throughout media and incubated at 37 °C, 5 % CO₂. Schistosomes were collected at 2 and 24 hours post-exposure to dorsomorphin and either phenotypically scored or flash frozen in liquid nitrogen. Frozen parasites were stored at -80 °C for later use.

Parasite RNA Extraction

Total RNA was extracted from frozen parasite samples according to the manufacturer's technical bulletin (Sigma Aldrich). Briefly, two or more adult schistosome pairs per sample were homogenized in 1 ml RNazol RT (Sigma Aldrich, catalog no. R4533) using an electric homogenizer. The resulting homogenate was mixed vigorously with 400 μ l water for 15 seconds then stored at room temperature for 15 min before centrifugation at 12,000 x *g* for 15 min. The supernatant was transferred to a new tube and mixed with 1 mL isopropanol to precipitate RNA. After incubation for 10 min at room temperature followed by centrifugation at 12,000 x *g* for 10 min, the RNA pellet was washed three times with 75 % ethanol. After the final wash, the RNA pellet was dissolved in ultrapure distilled water (Invitrogen). To further purify and concentrate extracted RNA, the RNeasy MinElute Cleanup Kit (Qiagen) was used according to the supplied "RNA Cleanup" protocol. The optional DNase digestion step was performed prior to starting the "RNA Cleanup" protocol using the RNase-Free DNase Set (Qiagen) according to the supplied protocol. The resulting, cleaned up RNA was stored at -80 °C for later use.

RT-PCR Detection of AMPK α Transcripts

Quantification of AMPK α transcripts was performed using RT-PCR according to our previously described protocol (92). Briefly, 500 ng parasite RNA was used as template for cDNA synthesis reactions using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). PCR reaction was performed using TaqMan Fast Universal PCR Master Mix (ThermoFisher Scientific) with 1 μ l cDNA as template and primers targeting nucleotide positions 751-815 of *S. mansoni* AMPK α (forward 5'-AGAATGATTACTGTGGACCCGATT-3' and reverse 5'-

AACCACGGATGTCGTCTGATTT-3') to produce a 64 bp fragment. To detect amplification of the fragment, a TaqMan MGB probe conjugated to an oligo sequence positioned at nucleotide 776 (5'-AACGTGCAACCATAGAA-3') was used. AMPK α copy numbers were calculated using a standard curve. All reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). Subsequent quantification of transcripts was performed using GraphPad Prism software, version 7.05 (GraphPad Software, Inc).

Parasite Phenotypic Scoring

Phenotypic screening of parasites following dorsomorphin exposure and RNAi was performed according to a protocol adapted from Kyere-Davies *et al* (110). Briefly, single word descriptors for parasite phenotypic characteristics such as movement, shape, translucence, and orientation were used to characterize treatment effects that could be observed visually. Table 1 contains each single word descriptor for which parasites were assessed and assigned a number ranging from 1- unaffected, healthy to 4- severely affected or damaged. The average was calculated in order to determine an overall severity score. Parasites were assessed using the described phenotypic scoring system at 2 and 24 h post *in vitro* exposure to dorsomorphin. Following RNAi, the phenotypic scoring system was used to assess the impact of AMPK α transcript reduction at 24, 48, and 72 h post-RNAi. For example, an adult schistosome pair on its side and exhibiting uncoordinated movement would be assigned a higher severity score than a pair that remained suctioned to the tissue culture plate, the typical orientation of a healthy schistosome pair.

Preparation of dsRNA for RNAi

DsiRNA oligos targeting *S. mansoni* AMPK α at three different sequence positions, 26 nucleotides in length, were prepared by Integrated DNA Technologies (IDT): DsiRNA #1- CD.Ri.195363.13.5 (forward 5'-rGrUrCrArArArGrUrUrGrGrArArUrUrCrArCrArArArUrCTA-3' and reverse 3'-rUrArGrArUrUrUrGrUrGrArArUrUrCrCrArArCrUrUrUrGrArCrUrU-5') targeting nucleotide positions 120-145, DsiRNA #2- CD.Ri.195363.13.4 (forward 5'-rCrArCrUrGrGrArUrCrUrGrCrUrArGrUrCrCrArArCrCrAAT-3' and reverse 3'-rArUrUrGrGrUrUrGrGrArCrUrArGrCrArGrArUrCrCrArGrUrGrCrU-5') targeting nucleotide positions 1805-1830, DsiRNA #3- CD.Ri.195363.13.2 (forward 5'-rArGrUrArUrUrUrArArArGrCrArArUrGrArArUrUrCrArCTT-3' and reverse 3'-rArArGrUrGrArArUrUrCrArUrUrGrCrUrUrUrArArArUrArCrUrUrC-5') targeting nucleotide positions 1499-1524. Scrambled Negative Control DsiRNAs were also supplied by IDT. DsiRNAs were reconstituted in Duplex Buffer (Integrated DNA Technologies) to a final concentration of 100 μ M then heated at 95°C for 2-3 minutes and allowed to cool at room temperature for 1 h before use.

Adult Schistosome RNAi

Immediately post-perfusion, freshly isolated adult schistosomes were collected and separated into microcentrifuge tubes of 5-7 pairs each. Parasites were washed three times with Opti-MEM to ensure serum removal. After washing, parasites were transferred to 4 mm electrode gap cuvettes and resuspended in 100 μ l Opti-MEM supplemented with 6 μ l of 100 μ M DsiRNA stock solution per sample. To test the effect of all three oligos combined, 2 μ l of each oligo's 100 μ M stock were used. Parasites in Opti-MEM were gently mixed to evenly distribute dsiRNA then electroporated at room temperature with a

single square wave pulse for 20 ms at 125 V using the GenePulser Xcell Electroporation System (BioRad). Parasites were immediately removed from cuvette, transferred to a 12 well tissue-culture treated plate in 2 ml complete DMEM medium, and incubated at 37 °C, 5 % CO₂. At 24, 48, and 72 h post-electroporation, parasites were collected to assess effects of RNAi on AMPK α transcript abundance. At 7 days post-electroporation, parasites were collected to assess AMPK α protein levels or flash frozen in liquid nitrogen and stored at – 80 °C for later use.

Measuring Parasite Glycogen Content

Adult schistosomes in culture or newly transformed schistosomula were collected in microcentrifuge tubes. Parasites were washed twice with 1 X PBS to remove trace amounts of media then flash frozen in liquid nitrogen to prevent breakdown of glycogen. Frozen parasites were resuspended in 100 μ l ultrapure distilled water (Invitrogen) and homogenized using an electric homogenizer. Upon complete homogenization of parasite tissue, samples were boiled for 5 min then centrifuged at 4 °C at 13,000 x *g* for 5 minutes to remove insoluble material. Glycogen concentration of supernatants were determined using the Sigma-Aldrich Glycogen Assay Kit (catalog no. MAK016) according to the manufacturer's instructions for colorimetric readout. For normalization purposes, sample protein concentrations were determined using the Pierce BCA Protein Assay (Thermo Scientific). The colorimetric signal produced, which is proportional to the glycogen present, was read at 570 nm using a Spectramax M2 spectrophotometer (Molecular Devices).

Cloning and Sequencing of *S. mansoni* AMPK β

Cloning, sequencing, and analysis of *S. mansoni* AMPK β were performed similarly to our previously described methods used for AMPK α (92). Briefly, adult *S. mansoni* cDNA was used as the template for amplifying the full-length AMPK β cDNA sequence. To prepare cDNA, parasite RNA was extracted from adult schistosomes as previously detailed. Crude RNA extracts were further purified using the RNeasy MinElute Cleanup Kit with DNase I digestion (Qiagen). 1 μ g RNA was used as the template for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Using the putative *S. mansoni* AMPK β mRNA (Smp_170160) predicted by the genome project (15) and ESTs, a consensus sequence was formed that extended the prediction by 77 bp at the 5' end and 43 bp at the 3' end. This consensus sequence was used as template to design primers that amplified AMPK β in its entirety. Two overlapping fragments, one 573 bp fragment (forward primer: 5'-GGACCCGTGGCTAATTTCCA-3', targeting the 5' UTR, and reverse primer: 5'-TTTTGAACACCGGTCGGACT-3') and one 608 bp fragment (forward primer: 5'-AGTCCGACCGGTGTTCAAAA-3' and reverse primer: 5'-ACTCTCTCCTCTTCCTCCTCT-3', targeting the 3' UTR), were amplified by conventional PCR using Accuprime Pfx Supermix (Thermo Fisher Scientific). Purified fragments were then cloned into pCR-BluntII-TOPO vector and sequenced using a BigDye Terminator cycle sequencing kit (Thermo Fisher) and T7 primers. Sequence data was analyzed and contigs assembled using Geneious software, version 11.0.5 (Biomatters Ltd.). Sequence alignment data comparing AMPK β sequences between *S. mansoni* and other organisms was also created using Geneious software.

Statistical Analysis

For both Agilent Seahorse XFe96 and fluorescent glycolysis assay graphs, area under the curve (AUC) analyses were performed. For Seahorse XFe96 data, the AUC from the time point immediately preceding the addition of drug until experiment's completion was calculated. For fluorescent assay data, the AUC for the entire experiment beginning at time point "0" until experiment's completion was calculated. A one-way ANOVA was performed on the calculated AUCs using the untreated group as the reference for comparison. For Western blot data derived from the kinase assay, dorsomorphin treatment, and RNAi experiments, densitometry was performed to quantify band intensity. Densitometry data for each experimental condition was normalized to one of the control conditions tested, so that data from repeat experiments could be compared. Data from repeat experiments (at least three independent biological replicates were performed in every case) were considered to be paired repeated measures and were analyzed using paired t tests. For quantitative PCR data, standard curves were used to calculate absolute transcript number in each sample. For each source of RNA, at least three independent biological replicates were tested. The data for each RNA source were first tested for evidence of significant differences in variance, using F tests. Because no significant differences in variance were found, the differences in transcript number between RNA sources were tested using parametric unpaired t tests. For glycogen assay data, three or more biological replicates were tested. The data for each parasite glycogen point were first tested for significant differences in variance, then, tested for significant differences between glycogen content using parametric unpaired t tests. All statistical analyses were performed using GraphPad Prism software, version 7.05 (GraphPad Software, Inc.).

RESULTS

Inhibition of schistosome AMPK results in increased parasite glycolysis

Previously, we demonstrated that phosphorylation of *S. mansoni* AMPK α was increased *in vitro* when adult schistosomes were exposed to chemical AMPK agonists or deprived of glucose (92). If schistosome AMPK regulates parasite metabolic activity in a manner similar to the AMPK of other organisms, we predicted that increased schistosome AMPK activity would be associated with increased parasite glycolytic activity, while reduced AMPK activity would lead to diminished glycolysis (89). To test this hypothesis, we monitored the glycolytic activity of adult schistosomes exposed to the following AMPK modulatory factors: (1) metformin, a biguanide with antihyperglycemic activity that is thought to directly activate AMPK in mammals; (2) 5-aminoimidazole-4-carboxamide (AICAR), an inosine monophosphate intermediate that stimulates mammalian AMPK by mimicking increased cellular AMP concentrations; and (3) dorsomorphin, a pyrazolopyrimidine that potently and reversibly inhibits AMPK by binding directly to the activation site of the α subunit kinase domain (70). To monitor schistosome glycolytic activity, we used the Agilent Seahorse platform to detect changes in the extracellular acidification rate (ECAR) of schistosomes exposed to these compounds *in vitro*. While we had previously shown that metformin stimulated schistosome AMPK α activity (92), metformin exposure had no significant effect on schistosome glycolytic activity (Figure 15A). Similarly, AICAR, another AMPK agonist, also had no significant effect on parasite glycolytic activity (Figure 15B). In contrast, higher concentrations of the AMPK inhibitor dorsomorphin stimulated a paradoxical increase in schistosome glycolytic activity (Figure 15C). Because these findings were contrary to our expectations, we confirmed that our experimental system was capable of

detecting reduced rates of schistosome glycolysis by monitoring the ECAR of schistosomes exposed to 50 mM 2-DG. As expected, 2-DG exposure induced a significant decrease in glycolytic activity (Figure 15D).

Dorsomorphin inhibits *S. mansoni* AMPK kinase activity

Previous studies have demonstrated that dorsomorphin inhibits AMPK in mammalian cells, leading to decreased AMPK α phosphorylation and inhibition of downstream processes regulated by AMPK (e.g. glycolysis) (117; 165; 190). The paradoxical increase in glycolytic activity induced by dorsomorphin in schistosomes therefore led us to question whether dorsomorphin was an effective inhibitor of schistosome AMPK. To address this question, we immunoprecipitated *S. mansoni* AMPK from adult schistosomes and assessed its kinase activity either in the presence or absence of dorsomorphin, using human acetyl-coA carboxylase-1 (ACC) as substrate (13; 79). The extent of ACC phosphorylation was then assessed by Western blot using anti-ACC and anti-phospho-ACC antibodies (Figure 16A). Phosphorylation of ACC by immunoprecipitated schistosome AMPK was significantly reduced in the presence of 10 μ M dorsomorphin (Figure 16B). These data suggest dorsomorphin is an effective inhibitor of *S. mansoni* AMPK and that the paradoxical effect of dorsomorphin on schistosome glycolytic activity is not explained by a failure of dorsomorphin to inhibit schistosome AMPK.

Dorsomorphin increases phosphorylation and expression of AMPK α in *S. mansoni* adults

Previous studies have shown that dorsomorphin exposure inhibits mammalian AMPK phosphorylation and kinase activity (22; 117). However, when adult schistosomes

were exposed to 10 μ M dorsomorphin for 24 h, a significant increase in phosphorylation of AMPK α was detected via Western blot (Figure 17A, Figure 17B). In addition, a significant increase in overall schistosome AMPK α protein abundance was also detected via Western blot in response to dorsomorphin treatment (Figure 17C). Similar results were noted at a shorter incubation time of 2 h and at lower concentrations of 2.5 μ M and 5 μ M dorsomorphin (data not shown). To determine if the increase in total AMPK α protein post-dorsomorphin exposure was due to an upregulation of AMPK α gene expression, we used quantitative RT-PCR to assess AMPK α mRNA abundance in adult schistosomes after 24 h of dorsomorphin exposure. No significant differences in AMPK α transcript content were found in untreated and dorsomorphin-treated schistosomes (Figure 17D). These data suggest that both post-transcriptional and post-translational mechanisms contribute to a compensatory increase in schistosome AMPK activity in response to dorsomorphin inhibition. Consistent with the finding that dorsomorphin failed to induce a loss of function of AMPK in adult schistosomes, we observed that incubation in 10 μ M dorsomorphin for 24 hours resulted in minimal fitness consequences for these organisms. Other than for an increased tendency for paired males and females to separate, no obvious phenotypic changes were induced by dorsomorphin exposure (Figure 17E). Quantification of phenotypic observations using a phenotypic scoring system (110) further emphasized the absence of any significant phenotypic differences between dorsomorphin-treated and untreated or vehicle control-treated worms (Figure 17F).

Inhibition of AMPK is toxic for schistosomula

We previously showed that while cercariae and newly transformed schistosomula contain AMPK α protein, AMPK α transcripts are not detectable in these life cycle stages, suggesting the AMPK α gene is not actively transcribed at this stage of the life cycle (92). Because adult schistosomes appear to avoid the toxic effects of dorsomorphin by upregulating AMPK α activity and protein abundance (Figure 17), we hypothesized that cercariae and schistosomula, which do not contain AMPK α transcript, might be more susceptible to dorsomorphin-induced toxicity. First, we evaluated whether AMPK inhibition induced any metabolic changes in schistosomula. Exposure of schistosomula to increasing concentrations of dorsomorphin did not significantly alter glycolytic activity when compared to control parasites (Figure 18A). This was in contrast to the dorsomorphin-induced increase in glycolytic activity we observed in adult worms (Figure 15). Furthermore, we found that prolonged exposure of schistosomula to dorsomorphin for 48 h resulted in significant dose-dependent loss of glycolytic activity (Figure 18B). When we examined the phosphorylation status and abundance of AMPK α protein in dorsomorphin-exposed schistosomula by immunoblot, we found no evidence of significant increases in AMPK α phosphorylation (Figure 18C, 18D) or overall protein levels (Figure 18C, 18E) in response to 10 μ M dorsomorphin, consistent with our prediction that schistosomula would not be able to synthesize additional AMPK α due to the absence of AMPK α transcripts in this life cycle stage. Lastly, we noted that incubation of schistosomula in 10 μ M dorsomorphin resulted in notable phenotypic changes compared to untreated and vehicle control-treated parasites. Schistosomula exposed to dorsomorphin appeared rounded, granular, and dark in appearance, while control schistosomula were translucent and maintained a rod-like shape (Figure 18F).

Application of phenotypic scoring to dorsomorphin-exposed and control schistosomula produced largely similar scores after 2 h of exposure, but after 24 h of exposure, schistosomula treated with dorsomorphin exhibited severity scores approaching 4 (the highest severity score in the phenotypic scoring system; Figure 18G). This high score reflected the phenotypic changes that were evident in the dorsomorphin-exposed parasites, such as dark color, granular appearance and rounded shape (Figure 18F), as well as a loss of motility.

Knockdown of *S. mansoni* AMPK α via RNAi is effective yet causes no significant impact on adult worm fitness and survival

To further assess the effects of reduced AMPK function in adult schistosomes, we attempted to reduce the abundance of AMPK α protein by RNAi-mediated targeting of the AMPK α transcript. We tested three different DsiRNAs designed to target the schistosome AMPK α mRNA at separate nucleotide positions. Adult schistosomes treated with 6 μ M of either each DsiRNA alone or the combination of all three DsiRNAs exhibited at least 50% reductions in AMPK α transcripts within 24 - 48 h following electroporation (Figure 19A). By 72 h post-DsiRNA electroporation, AMPK α transcript levels rebounded in schistosomes that received each of the three DsiRNAs alone, returning to about 70 % of transcript levels in control worms. Schistosomes that received the combination of all three DsiRNAs exhibited a more durable reduction in AMPK α transcripts, with mRNA abundance reduced to approximately 30 % over the 72 h following electroporation. At 7 days post-DsiRNA electroporation, adult worms were collected to evaluate the effect of RNAi-mediated AMPK α transcript reduction on AMPK α protein abundance. All three DsiRNAs, individually and in combination, were successful in inducing a reduction in

overall AMPK α protein, with 12 – 56 % of protein remaining when compared to control worms (Figure 19B).

At 24, 48, and 72 h post-RNAi, adult worms were carefully examined for phenotypic changes (Figure 19C) and subjected to phenotypic scoring (Figure 19D). The most noticeable effects of AMPK α knockdown were observed 24 h post-DsiRNA electroporation, after which the phenotypic effects of RNAi gradually became less apparent. Phenotypic effects of AMPK α knockdown included incoordination, with worms temporarily losing their ability to remain suctioned to the tissue culture plate, decreased male/female pairing, and decreased parasite activity/mobility. However, by 72 h post-DsiRNA treatment these effects had diminished and, phenotypically, worms appeared to return to their pre-RNAi state, as evidenced by the restoration of coordination/suctioning, and pairing, among other factors (Figure 19D). AMPK α targeting did not result in any apparent phenotypic changes at 7 days post-RNAi, when AMPK α -targeted worms were indistinguishable from control worms (data not shown).

Inhibition of schistosome AMPK leads to increased glycogen breakdown

Given the considerable amount of evidence supporting a role for AMPK in regulating glycogen synthase in mammalian cells (78; 99), we hypothesized that the rapid increase in glycolysis observed in dorsomorphin-treated adult schistosomes might be fueled by rapid breakdown of parasite glycogen stores. Consistent with this hypothesis, we found that adult schistosomes incubated in 10 μ M dorsomorphin for 24 h contained significantly lower glycogen content than worms maintained in media alone (Figure 20A). Next, we tested whether the dorsomorphin-induced depletion of worm glycogen content could be blocked by glycogen phosphorylase inhibitor (GPI) (173; 186). Fresh

adult worms were maintained *in vitro* in medium containing dorsomorphin, GPI, or a combination of both for 24 h. The decrease in glycogen content induced by dorsomorphin was blocked by GPI (Figure 20B). To test whether breakdown of glycogen was required for the dorsomorphin-induced increase in glycolytic activity, worms treated with dorsomorphin, GPI, or a combination of both were subjected to metabolic analysis using the Agilent Seahorse platform. The increase in schistosome glycolytic activity in response to dorsomorphin was abolished by GPI (Figure 20C), consistent with the conclusion that the dorsomorphin-induced increase in adult worm glycolysis is fueled by glucose derived from the breakdown of glycogen. Last, we tested whether AMPK was necessary for glycogen depletion by testing whether RNAi-mediated AMPK α knockdown preserved glycogen content in adult worms. However, adult schistosomes electroporated with a combination of all three AMPK α -specific DsiRNAs contained significantly less glycogen than control worms electroporated with scrambled DsiRNA (Figure 20D), suggesting that AMPK may be required for the establishment and/or maintenance of parasite glycogen stores, in addition to regulating glycogen utilization.

In other organisms, AMPK may regulate cellular energy homeostasis in part by controlling the activity of glycogen synthase, thereby determining whether glucose is diverted toward the synthesis of glycogen or made available for the production of ATP via glycolysis. Central to interaction with cellular glycogen stores is the ability of AMPK to physically interact with glycogen, via a carbohydrate binding motif located within the AMPK β subunit. Because our data suggest AMPK plays a central role in the regulation of schistosome glycogen stores, we explored whether the *S. mansoni* AMPK β protein retains the glycogen binding domain found in the β subunits of other organisms.

Searching the National Center for Biotechnology Information (NCBI) Nucleotide database identified a putative mRNA sequence for the *S. mansoni* AMPK β subunit (accession number XM_018790591), which encodes for a putative AMPK β protein of 287 amino acids (accession XP_018646108). The database entry for XM_018790591 referenced locus genomic Smp_170160, located at position 223300 – 234844 on supercontig Sc0049 of the *S. mansoni* genomic sequence (https://www.genedb.org/#/chromosome/Schisto_mansoni.SC_0049), as the source of the mRNA. However, comparison of the putative XM_018790591 mRNA sequence with the proposed exon-intron structure of Smp_170160 revealed that 177 nucleotides from the 3' end of exon 4 of Smp_170160 had been omitted from the XM_018790591 sequence. Thus the predicted mRNA derived from Smp_170160 would encode for a considerably larger protein of 346 amino acids. To address this discrepancy, we independently cloned and sequenced the AMPK β cDNA from adult *S. mansoni*. First, we used the XM_018790591 and Smp_170160-derived mRNA sequences to identify expressed sequence tags (ESTs) in the NCBI EST database that provided 5' and 3' untranslated sequence upstream and downstream of the putative open reading frame. Then primers designed to hybridize either side of the ORF were used to amplify the coding region in its entirety. The sequence of the resulting PCR product exactly matched that of the mRNA predicted from Smp_170160, confirming the inclusion of the 177 nucleotides from exon 4 in the mRNA. This sequence is available in the NCBI Nucleotide database (accession number MN480434). Analysis of the predicted 346 amino acid sequence revealed that the *S. mansoni* AMPK β protein is considerably bigger than the β subunits of vertebrates (e.g. human, Norway rat, fish), and the AMPK β of the cestode *Echinococcus granulosus*

(Figure 20E). However, similarly sized AMPK β subunits have been predicted by the genome projects of several other trematodes, including five other species of schistosome, suggesting that an extended AMPK β subunit may be a conserved feature of trematode AMPKs, with the additional sequence concentrated in the C-terminal half of the polypeptide (Figure 20E). Despite these differences, considerable sequence identity and similarity exist between all the trematode sequences and those of vertebrates, and analysis of the *S. mansoni* sequence using the NCBI Conserved Domain Database (CDD) readily identified an AMPK glycogen recognition site (AMPK1_CBM, accession number pfam16561) at amino acids 94-175 (E-value 4.37e-41), in addition to a characteristic AMPK β interaction domain (AMPKBI, accession number smart01010) at the C terminus (E-value 1.24e-25) (Figure 20E).

DISCUSSION

Because maintenance of blood glucose concentrations within narrow limits is essential for life of the vertebrate host, adult schistosomes likely never face situations similar to those encountered by free-living organisms, where the supply of energy substrates is limited. Hence, it could be argued that adult schistosomes have no requirement to control their consumption of glucose, and the prodigious rate by which schistosomes metabolize glucose by glycolysis is consistent with this argument (18). We previously found constitutively high levels of AMPK α phosphorylation in freshly isolated adult schistosomes, indicating that schistosome AMPK is constitutively active under steady state conditions within the mammalian host. Indeed, we estimate schistosome AMPK in freshly isolated adult worms is approximately four times more active than human AMPK in HEK cells grown under glucose-replete in vitro conditions

(92). While comparisons between species and between whole organisms and cell lines should be interpreted with caution, these observations do suggest that, like glycolysis, schistosome AMPK activity is constitutively high, and perhaps close to maximal even under steady state conditions. Thus, while we previously demonstrated that phosphorylation of schistosome AMPK α could be modestly enhanced when parasites are exposed to AMPK agonists (92), any downstream effects on energy metabolism could be difficult to detect if both AMPK activation and glycolysis are already occurring at high levels. Consistent with this model, neither of the AMPK agonists we tested, metformin and AICAR, caused any measurable increase in parasite glycolysis. There are other possible explanations for these observations. For example, while metformin and AICAR are well characterized AMPK agonists in other species, perhaps neither are especially potent in activating schistosome AMPK, and perhaps there are as yet undiscovered agonists of schistosome AMPK that would cause substantial increases in schistosome AMPK α phosphorylation and parasite glycolysis. Furthermore, we did not explore other potential effects of AMPK agonists on schistosomes, such as increased rates of autophagy. These possibilities will be explored in future studies.

Paradoxically, chemical inhibition of AMPK by dorsomorphin treatment resulted in an increase in adult schistosome glycolysis. This result was unexpected, as dorsomorphin treatment of live cells consistently reduces AMPK α phosphorylation and interrupts downstream metabolic processes (88; 190). These data led us to question whether dorsomorphin is actually capable of inhibiting schistosome AMPK, as there are significant differences in the sequence and size of *S. mansoni* AMPK α compared to those of other species (92). However, results from an in vitro kinase assay confirmed that

dorsomorphin does indeed impair the catalytic activity of the schistosome AMPK α subunit. Further analysis to understand the mechanism behind this unexpected result revealed that dorsomorphin exposure also induced increases in AMPK α phosphorylation and overall AMPK α protein levels as well. We hypothesize that these increases represent a compensatory response by the parasite to surmount the inhibition of this critically important enzyme. Though increased abundance of AMPK α protein post-dorsomorphin treatment could be detected via Western blot, no significant difference in transcript abundance was detected between untreated and dorsomorphin-treated adult schistosomes when assessed by RT-PCR. However, transcript levels may not directly correlate with protein levels for several reasons (12; 118). First, it is possible that a sudden loss of AMPK α activity due to dorsomorphin exposure could induce post-transcriptional regulatory mechanisms that result in a rapid increase in AMPK α protein synthesis from existing AMPK α mRNA (36; 184), which is abundant at this stage of the life cycle (92). Second, studies in mammalian systems have identified microRNAs (miRNAs) that can directly target AMPK α mRNA and affect rates of protein synthesis (82). As there is already evidence that schistosomes utilize miRNAs for post-transcriptional regulation (39; 61; 72), it is possible that schistosome AMPK α protein abundance is also regulated by this mechanism. There are alternative explanations for the paradoxical increases in glycolysis and AMPK α phosphorylation induced by dorsomorphin in adult schistosomes. For example, it is possible that increases in AMPK α protein abundance and phosphorylation in response to dorsomorphin are unrelated to a direct effect of dorsomorphin on AMPK, as dorsomorphin is known to have activity against other signaling pathways, e.g. the bone morphogenetic protein (BMP) pathway (30; 71; 154;

190; 201). While this pathway is conserved in schistosomes (56), it is unclear how interruption of BMP signaling would result in increased glycolysis and AMPK α protein abundance. It is also possible that schistosome AMPK is indirectly activated by dorsomorphin. For example, some catalytic-site inhibitors of other kinases, like PKB/Akt (137), exert an activating effect on AMPK by indirectly raising cellular AMP/ADP concentrations (9; 163; 203), though why this would also result in an increase in AMPK α protein abundance is unclear.

In contrast to adult schistosomes, newly transformed schistosomula do not exhibit any increase in glycolytic activity in response to dorsomorphin, nor is there any detectable increase in AMPK α protein abundance or phosphorylation. This latter finding is predictable, as we had previously shown that AMPK α mRNA is virtually undetectable in schistosomula (92), suggesting the gene is not actively transcribed in this developmental stage, and leaving the parasites without a means to compensate for the inhibition of AMPK α protein by dorsomorphin. Furthermore, newly transformed schistosomula exhibited significant phenotypic deterioration after exposure to dorsomorphin for 24 hours, and diminished metabolic activity after 48 hours, indicating parasite death. We therefore conclude that AMPK is essential to schistosomulum viability, and that an inability to synthesize de novo AMPK α protein underlies the sensitivity of schistosomula to dorsomorphin. While we cannot exclude the possibility that dorsomorphin mediates toxic effects against schistosomula via some other target, the correlation of the compensatory response of adult worms with their resistance to dorsomorphin, and the absence of this compensatory response in dorsomorphin-susceptible schistosomula, argues that a direct effect on dorsomorphin on schistosome

AMPK α is the most parsimonious explanation for the anti-schistosomular effects of dorsomorphin.

The rapid upregulation of AMPK α protein abundance and phosphorylation by adult worms in response to dorsomorphin-mediated inhibition of AMPK suggests that AMPK fulfills an important role at this stage of the life cycle. We therefore sought to disrupt this compensatory mechanism by targeting AMPK α mRNA by RNAi. Using a combination of DsiRNAs, we were able to significantly deplete AMPK α mRNA and protein levels in adult schistosomes. However, loss of AMPK α protein did not result in any obvious phenotypic changes in the parasites, nor did it reduce schistosome glycolytic activity (data not shown). These results suggest that schistosome glycolysis is not controlled by AMPK and proceeds regardless of AMPK expression and activation. We therefore explored other explanations for the rapid increase in glycolysis and phosphorylated AMPK α observed in dorsomorphin-treated adult schistosomes. Interestingly, we noted that adult worm exposure to dorsomorphin resulted in rapid depletion of parasite glycogen stores, suggesting that glycogen breakdown might fuel the increase in parasite glycolytic activity. Consistent with this hypothesis, treatment of adult worms with a glycogen phosphorylase inhibitor to prevent glycogen breakdown also blocked the dorsomorphin-induced increase in glycolysis. These data suggest that schistosome AMPK does not directly stimulate glycolysis in adult worms, but can drive an increase in glycolytic activity by increasing the supply of substrate for glycolysis through increased glycogen breakdown. To test whether dorsomorphin-induced glycogen depletion was mediated by AMPK, we examined glycogen levels in adult worms where AMPK α protein was depleted by RNAi. However, we found that even in the absence of

dorsomorphin exposure, AMPK α -depleted worms exhibited significantly reduced glycogen stores. This result is perhaps to be expected, as AMPK is suspected to play a role in maintaining glycogen stores in other organisms by maintaining the intracellular glucose concentrations required for glycogen synthase activity (98). For example, AMPK may contribute to maintenance of the intracellular glucose concentration by driving glucose transporter expression, activating the enzyme 6-phosphofructo-2-kinase/fructose-2, 6bisphosphatase 3 (PFKFB3) which stimulates rates of glycolysis, inhibiting glycogen synthesis through phosphorylation of glycogen synthase, or inhibiting gluconeogenesis (73; 78). In addition, direct association of AMPK with cellular glycogen via the β -subunit also contributes to the maintenance of glycogen stores and, ultimately, intracellular glucose concentrations. A carbohydrate binding motif located towards the N-terminal of AMPK allows for binding to and sensing of cellular glycogen levels in order to ensure that they are maintained (84; 123). We have shown that this significant glycogen binding motif is also conserved in *S. mansoni* AMPK β . This suggests that similar interactions that occur between mammalian AMPK and cellular glycogen may also take place in schistosomes, and supports our data showing a relationship between AMPK inhibition and glycogen depletion.

Taken together, our data suggest that in adult schistosomes, AMPK does not directly regulate energy production by glycolysis, which appears to proceed regardless of AMPK. Rather, our data suggest that one of the functions of AMPK at this stage of the life cycle is to regulate the breakdown and synthesis of glycogen. Given the stability and consistency of blood glucose concentrations maintained by the vertebrate host, it is unclear what the function of adult schistosome glycogen stores might be. Previous work

has shown that adult schistosome glycogen stores are highly dynamic, undergoing constant breakdown and re-synthesis (181; 182). It has been speculated that perhaps adult schistosome pairs switch to glycogen as a glucose source during oviposition, when the parasites squeeze into narrow venules where blood flow might be reduced or occluded altogether for brief periods. However, reports from other organisms suggest glycogen may fulfill roles beyond serving as merely a backup energy source. For example, in mammalian cells there is evidence that glycogen contributes to protection against oxidative stress, due to its preferential use as a substrate for the pentose phosphate pathway that produces NADPH, which neutralizes reactive oxygen species (50; 150; 167). Similar observations regarding the connection between glycogen and oxidative stress have been noted in the free-living nematode *C. elegans* (67). As schistosomes are presumably subject to constant oxidative pressure from the host's immune system (130; 158), these proposed alternative functions of glycogen could be important. If a primary function of AMPK in adult schistosomes is to regulate the turnover of glycogen stores, targeting AMPK in vitro might not result in any obvious detrimental effects due to the absence of significant oxidative stress. However, targeting AMPK in vivo might render the parasites more susceptible to oxidative damage.

In conclusion, our data suggest that schistosome AMPK fulfills different roles in larval and adult parasites. In the schistosomulum, when the parasite is transitioning to life inside the definitive host, AMPK α is required for viability, and as the AMPK α gene is not actively transcribed at this point, schistosomula might be especially vulnerable to AMPK targeting. These observations suggest that AMPK α may be a useful target for interventions aimed at preventing the establishment of a schistosome infection. While the

precise function of AMPK in schistosomula is unclear, the absence of significant glycogen stores at this stage in the life cycle (data not shown) raise the possibility that perhaps AMPK fulfills other roles in schistosomula. In adult worms, our initial hypothesis that AMPK regulates the production of energy by glycolysis is not supported by our data, which instead suggest that glycolysis and AMPK activation are uncoupled in this developmental stage. However, AMPK appears to still play an important role in adults, as AMPK inhibition induces a compensatory upregulation in AMPK α abundance and phosphorylation. While this compensatory mechanism makes it difficult to test the function of AMPK α in adult parasites, our data point to a significant role in the regulation of parasite glycogen stores, the function of which has been the subject of much speculation. Our data provide fresh insights into the regulation of energy substrate utilization in a parasite that capitalizes on host glucose homeostasis and its anatomic location in the host bloodstream, and suggest that parasite energy metabolism may yet exhibit unique features that can be targeted to the detriment of the parasite.

ACKNOWLEDGMENTS

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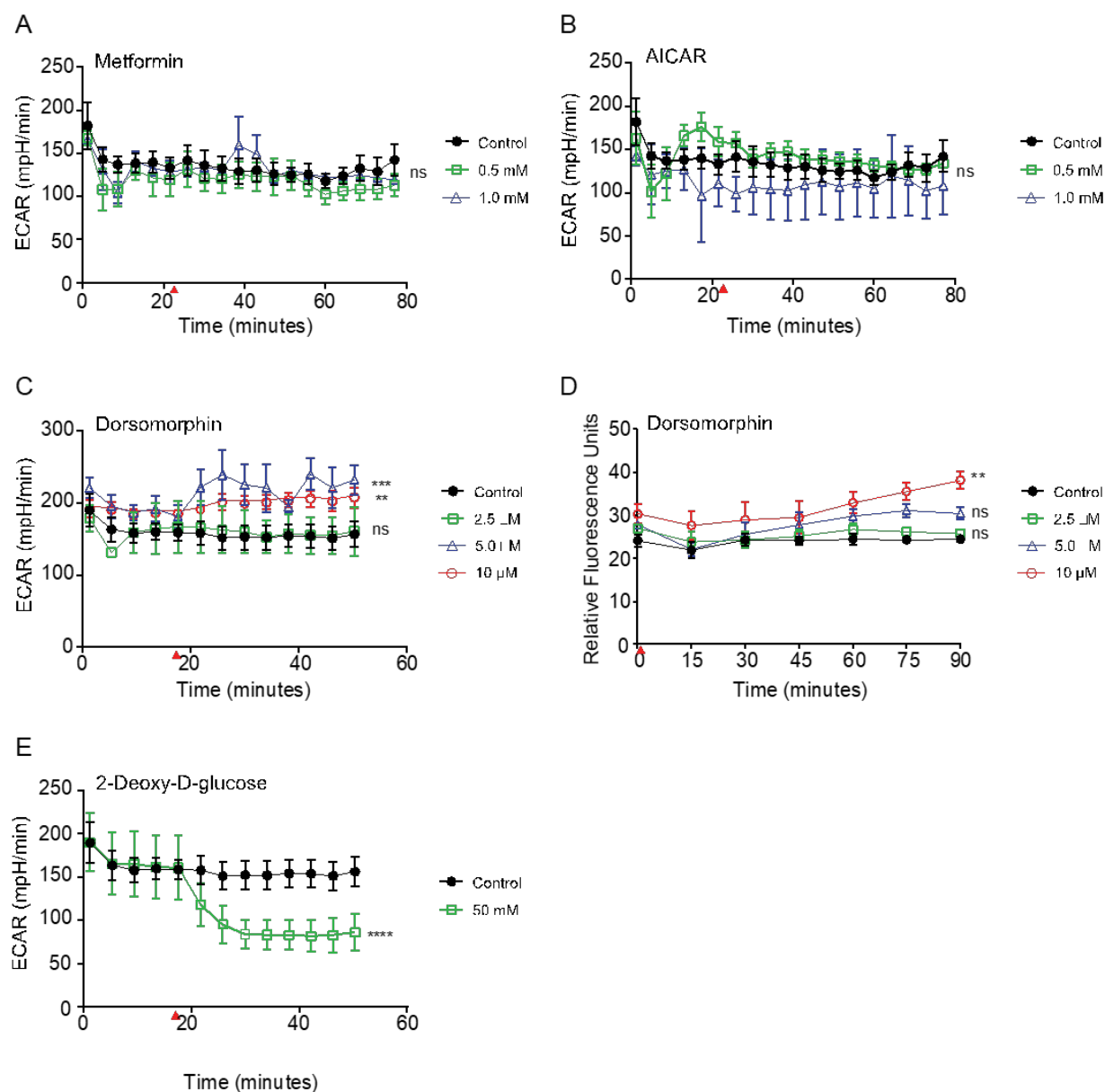


Figure 15. Effects of AMPK agonists and inhibitor on *S. mansoni* glycolytic activity

A-C, The extracellular acidification rate (ECAR) of freshly isolated adult *S.*

mansoni pairs before and after exposure to metformin (A), AICAR (B), and

dorsomorphin (C), were measured using Agilent Seahorse XFe96 system. D,

Extracellular acidification, measured in relative fluorescence units (rfu), of freshly

isolated adult schistosome pairs after exposure to dorsomorphin, measured using Agilent pH-Xtra Glycolysis Assay. E, ECAR of freshly isolated adult *S. mansoni* pairs before and after exposure to 2-Deoxy-D-glucose. Red arrows in A-C, E indicate introduction of compound into parasite culture media. Each graph is representative of at least 2-3 independent experiments. Data points shown are the average of at least 3 biological replicates. (ns = $P > 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$)

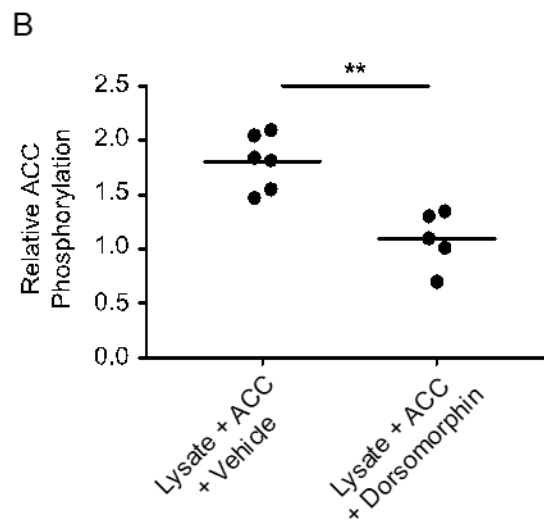
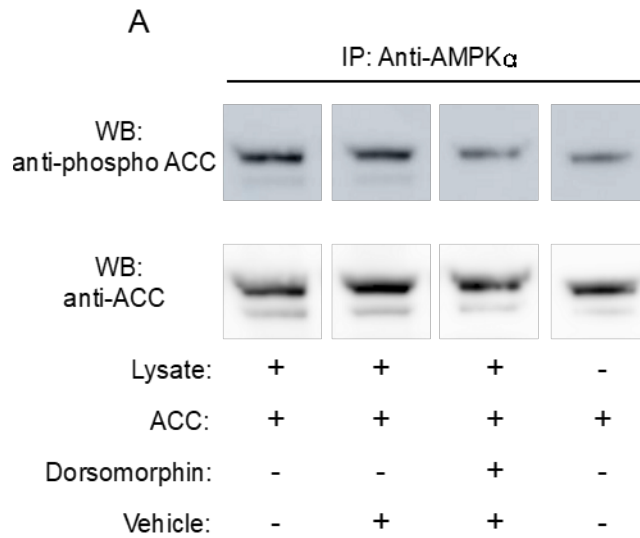


Figure 16. Dorsomorphin inhibits *S. mansoni* AMPK α kinase activity

A, AMPK α was immunoprecipitated from lysates of freshly isolated adult schistosomes using anti-AMPK α , then resuspended in 1 X kinase buffer with 0.2 μ g acetyl-coA-carboxylase (ACC) in either the presence or absence of 10 μ M Dorsomorphin. Control kinase assays were performed where the lysate (source of the kinase) was omitted. Kinase assay supernatants were probed by Western blot using anti-

phospho ACC and anti-ACC antibodies. WB: indicates the antibody used for Western blotting. B, The intensity of the anti-phospho ACC signal relative to the anti-ACC signal for each sample was determined. Each data point represents an independent kinase reaction. (** = $P \leq 0.01$)

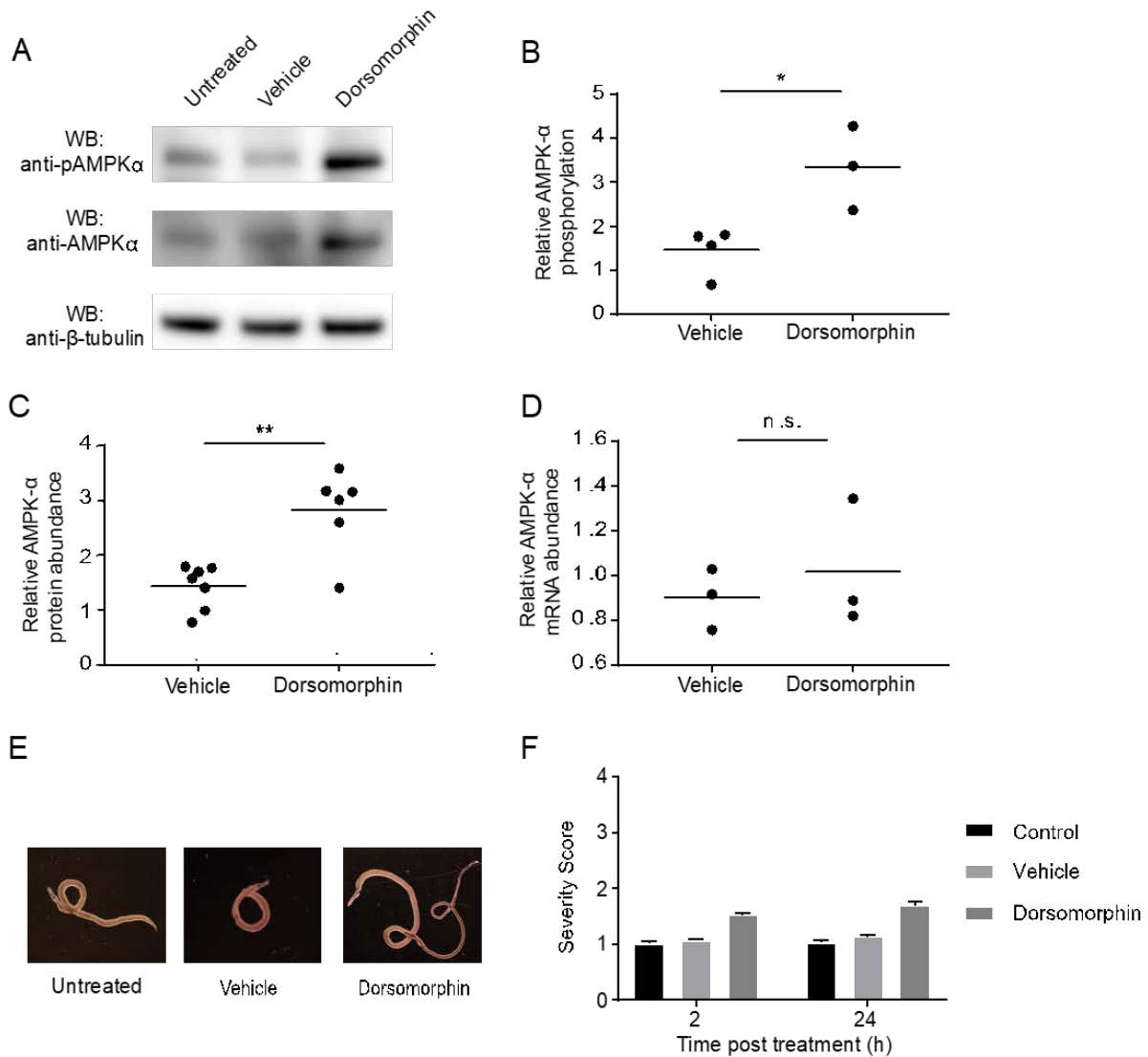


Figure 17. Chemical inhibition of AMPK induces compensatory increases in AMPK α protein and phosphorylation

A, Freshly isolated adult schistosomes were incubated in either media alone (untreated) or media supplemented with 10 μ M dorsomorphin or vehicle. After 24 h, lysates were prepared and equal amounts of protein were probed by Western blot using anti-pAMPK α antibody (top panel), anti-AMPK α antibody (center panel), and anti- β -tubulin antibody (bottom panel). WB: indicates the antibodies used for Western blotting.

B, Relative AMPK α phosphorylation under each condition was calculated by determining the ratio of anti-pAMPK α signal intensity to that of the anti-AMPK α signal intensity, and then normalizing to the intensity of the anti- β -tubulin signal to control for protein loading. Vehicle and dorsomorphin values shown are relative to untreated ratios (which were normalized to a value of 1). C, Relative AMPK α protein abundance under each condition was calculated by determining the ratio of the intensity of the anti-AMPK α signal to that of anti- β -tubulin. Vehicle and dorsomorphin values shown are relative to untreated. D, AMPK α transcripts of adult schistosomes exposed to the described conditions were quantified using quantitative real-time RT-PCR. AMPK α transcript abundance in vehicle and dorsomorphin samples relative to that of untreated samples are shown. Data points shown are biological replicates. E, Freshly isolated adult schistosome pairs photographed after 24 h incubation in either medium alone (untreated) or medium supplemented with 10 μ M dorsomorphin or vehicle. F, Worms incubated in either medium alone (control) or medium supplemented with 10 μ M dorsomorphin or vehicle were phenotypically scored after 2 and 24 h incubation in the aforementioned conditions. Data shown are averages of at least 3 biological replicates. (ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$)

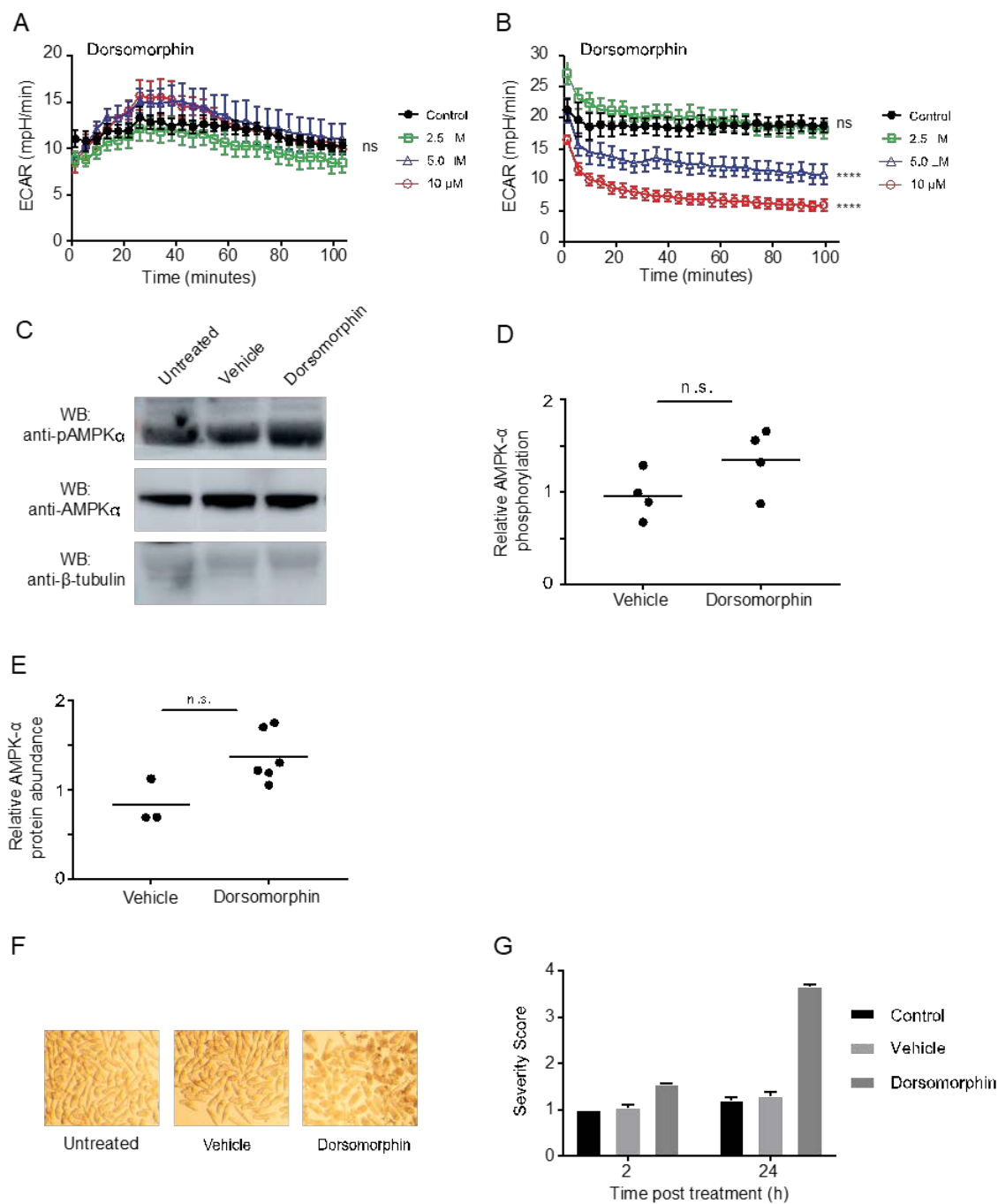


Figure 18. Chemical inhibition of AMPK is toxic for juvenile schistosomes

A-B, The extracellular acidification rate (ECAR) of schistosomula (30 minutes post-transformation) before and immediately after exposure to dorsomorphin (A), and after 48 h of exposure to dorsomorphin (B) was measured using the Agilent Seahorse

XFe96. C, Schistosomula (30 minutes post-transformation) were incubated in either medium alone (untreated) or media supplemented with 10 μ M dorsomorphin or vehicle. After 24 h, lysates were prepared and equal amounts of protein were probed by Western blot using anti-pAMPK α antibody (top panel), anti-AMPK α antibody (center panel), and anti- β -tubulin antibody (bottom panel). WB: indicates the antibodies used for Western blotting. D, Relative AMPK α phosphorylation under each condition was calculated by determining the ratio of anti-pAMPK α signal intensity to that of anti-AMPK α signal intensity, which was then normalized to the intensity of the anti- β -tubulin signal to control for differences in protein loading. Vehicle and dorsomorphin values shown are relative to untreated control. E, Relative AMPK α protein abundance under each condition was calculated by determining the ratio of the intensity of the anti-AMPK α signal to that of anti- β -tubulin. Vehicle and dorsomorphin values shown are relative to untreated. Each data point shown represents a biological replicate. F, Schistosomula (30 minutes post-transformation) were immediately placed in culture, in either medium alone (untreated) or medium supplemented with 10 μ M dorsomorphin or vehicle, and photographed 24 h post-incubation. G, Schistosomula incubated in either medium alone (control), or medium supplemented with 10 μ M dorsomorphin or vehicle were phenotypically scored after 2 and 24 h incubation. Data shown are averages of at least 3 biological replicates. (ns = $P > 0.05$, **** = $P \leq 0.0001$)

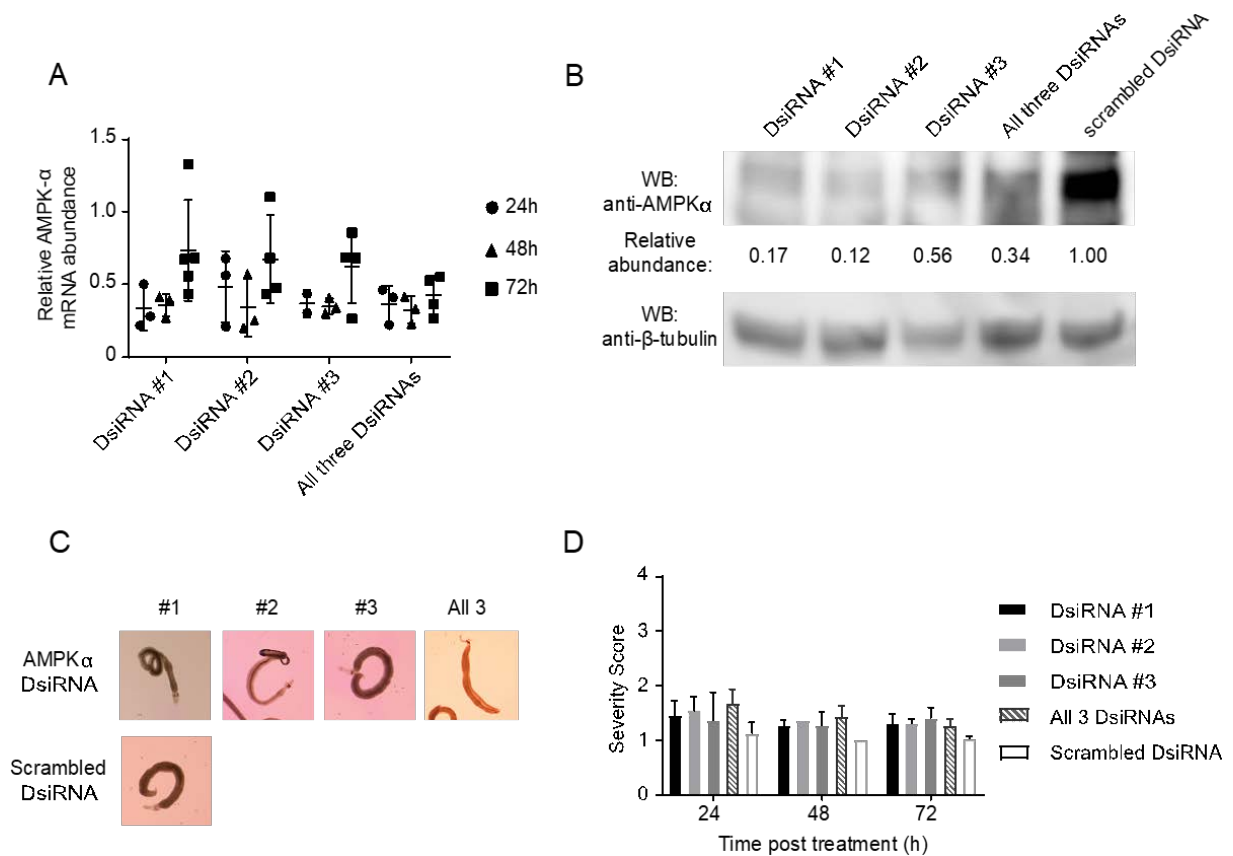


Figure 19. Transcriptional reduction of AMPK α in adult schistosomes

A, RNAi was performed on freshly isolated adult schistosomes by electroporation in the presence of three *S. mansoni* AMPK α -targeting DsiRNA constructs, either separately (DsiRNA #1, DsiRNA #2, DsiRNA #3), or in combination (all three DsiRNAs). Control parasites were electroporated with a scrambled DsiRNA control construct. AMPK α transcripts were quantified at 24, 48, and 72 h post-RNAi using quantitative real-time RT-PCR. AMPK α transcript abundance relative to the scrambled DsiRNA control were calculated. Data points represent independent biological replicates.

B, Lysates of adult schistosomes 72 h post-RNAi were prepared and equal amounts were

probed by Western blot using anti-AMPK α and anti- β -tubulin antibodies. Protein abundance was determined by normalizing the anti-AMPK α signal to the anti- β -tubulin signal for each sample. Relative protein abundance compared to the scrambled DsiRNA samples was then calculated. WB: indicates the antibodies used for Western blotting. Western blot pictured is representative of at least 3 biological replicates. C, Adult schistosomes electroporated in the presence of either AMPK α -targeting DsiRNA #1, #2, #3, all 3 constructs combined, or scrambled DsiRNA were photographed 72 h post-RNAi. D, DsiRNA-treated adult worms were phenotypically scored at 24, 48, and 72 h post-electroporation. Data shown are averages of at least 3 biological replicates.

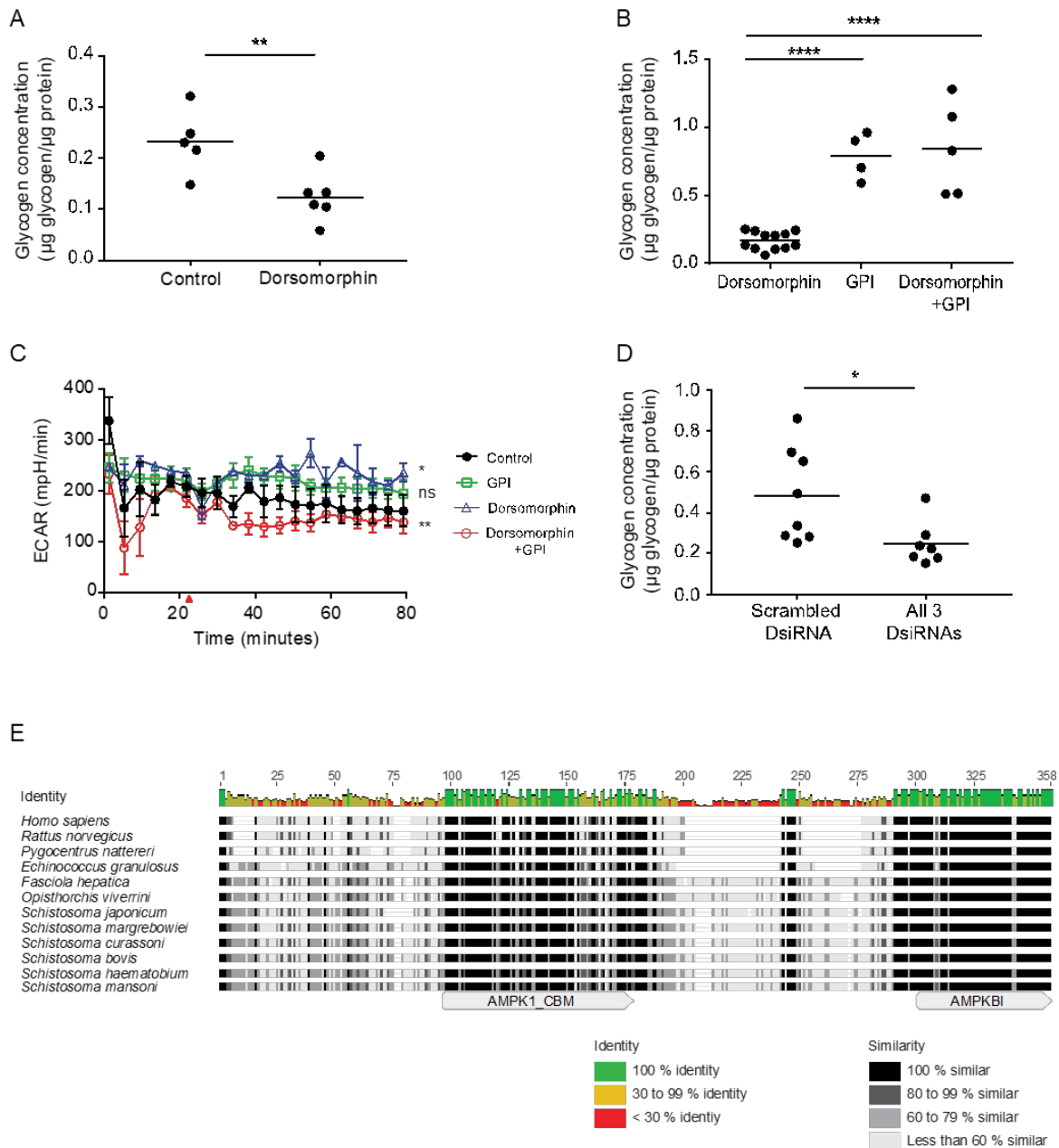


Figure 20. AMPK regulates glycogen metabolism in adult schistosomes

A, Freshly isolated adult schistosomes were incubated in either medium alone (control) or medium supplemented with 10 µM dorsomorphin. After 24 h, lysates were prepared and glycogen content was measured. B, Freshly isolated adult schistosomes were incubated in medium supplemented with 10 µM dorsomorphin, 75 nM glycogen

phosphorylase inhibitor (GPI) or 10 μ M dorsomorphin combined with 75 nM GPI. After 24 h, lysates were prepared and glycogen content was measured. In A and B, each data point indicates one schistosome pair, and graphs are representative of at least 3 independent experiments. C, ECAR of adult worms before and after exposure to 10 μ M dorsomorphin, 75 nM GPI, or a both combined, was measured using Agilent Seahorse XFe96. Red arrow indicates the time point at which compounds were introduced into parasite culture media. Each data point is the average of at least 3 biological replicates. D, Freshly isolated adult schistosomes were electroporated with either control scrambled DsiRNA or a combination of all 3 AMPK α -targeting DsiRNAs. 72 h post-electroporation, lysates were prepared and glycogen content was measured. Each data point represents a biological replicate. E, The amino acid sequence of *S. mansoni* AMPK β (MN480434) aligned with AMPK β sequences of *Homo sapiens* (NP_006244), *Rattus norvegicus* (NP_114182.1), *Pygocentrus nattereri* (XP_017539403.1), *Echinococcus granulosus* (AER10555), *Fasciola hepatica* (THD24738.1), *Opisthorchis viverrini* (XP_009174364.1), *Schistosoma japonicum* (TNN15813.1), *Schistosoma margrebowiei* (VDP35519.1), *Schistosoma curassoni* (VDO62757.1), *Schistosoma bovis* (RTG86190.1), and *Schistosoma haematobium* (XP_012797764.1). The alignment shows sequence identity and similarity as well as the approximate locations of the glycogen binding domain (AMPK1_CBD) and C-terminal domain (AMPKB1) that associates with AMPK α and γ subunits. (ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, **** = $P \leq 0.0001$)

CHAPTER 4: Discussion

SUMMARY OF FINDINGS

In this present work, we have demonstrated that the human parasite *Schistosoma mansoni* expresses a 5'AMP-Activated Protein Kinase (AMPK), like most eukaryotes. However, unlike most documented examples of AMPK, *S. mansoni* AMPK (Sm AMPK) does not appear to be overtly necessary for the regulation of overall intracellular metabolism in adult schistosomes. Evidence suggests that Sm AMPK contributes to glycogen regulation in this stage, although it is not required for adult parasite survival. In schistosomula, however, AMPK is indispensable as it is essential for juvenile schistosome viability. In our pursuit to characterize Sm AMPK, describe its developmental regulation, and define the metabolic implications of its activity, we have established the following:

1. The catalytic, α -subunit of *S. mansoni* AMPK is roughly 100 amino acids larger than human AMPK α due to insertions in the C-terminal portion of the protein that are absent from AMPK α subunits of human and other closely related organisms. Despite these areas of divergence, schistosome AMPK α shares moderate homology with AMPK α -subunits from human and other related species, with residues important for kinase activation being highly conserved.
2. *S. mansoni* AMPK α is differentially regulated throughout the parasite's life cycle. All developmental stages of the *S. mansoni* life cycle harbor AMPK α protein but the human transmission (cercariae) and early intra-mammalian

(schistosomula) stages do not actively express the transcripts necessary for protein production.

3. AMPK activity in adult schistosomes is vulnerable to modulation by factors emanating from sources of artificial and host origin.
4. Modulation of *S. mansoni* AMPK activation correlates with changes in schistosome glycolytic activity. Specifically, inhibition of schistosome AMPK stimulated an increase in glycolytic rates of adult schistosomes. The opposite occurred in schistosomula, with glycolytic activity significantly decreased after 48 hours post-AMPK inhibition.
5. Adult and juvenile schistosomes respond differently to chemical inhibition of AMPK. AMPK inhibition is tolerated by adult schistosomes but toxic for schistosomula.
6. Increased AMPK α phosphorylation and total protein correlates with AMPK inhibition in adult schistosomes. Schistosomula exhibit increased AMPK α phosphorylation in the absence of increased total AMPK α protein following inhibition.
7. Inhibition of AMPK in adult schistosomes coincides with a reduction in parasite glycogen stores. This reduction in the wake of AMPK inhibition is abrogated when parasite glycogen phosphorylase is inhibited.
8. Regulation of cellular glycogen stores by *S. mansoni* AMPK is most likely mediated via the β -subunit as we have demonstrated that this portion of the schistosome protein bears homology to human AMPK β , with a conserved domain significant for glycogen binding.

INTERPRETATION & SIGNIFICANCE OF FINDINGS

We initiated our characterization of schistosome AMPK by, first, describing the α -subunit. This was incredibly important since AMPK α is the subunit that is phosphorylated and responsible for kinase function (58). Through examination of schistosome AMPK α , we revealed that the subunit shares overall high homology with AMPK α -subunits from human and other closely related organisms, as evidenced through extensive stretches of conserved sequences, especially those required for kinase activation. In addition, we uncovered insertions of about 100 amino acids in length in the C-terminal portion of schistosome AMPK α protein situated in the “linker region” (58) between the autoinhibitory and regulatory domains. These insertions are absent from the α -subunits of human and other closely related organisms and contribute to the appreciable size difference between schistosome AMPK α and most other documented α -subunits. The implications of this considerably-sized sequence divergence have yet to be fully investigated. Initial speculations regarding the significance of the large insertion were that it increases the range of possible interactions between schistosome AMPK α and other proteins, although this remains to be experimentally confirmed. We hypothesize that the placement the insertion situated between the autoinhibitory and C-terminal regulatory domains, both responsible for α -subunit regulation, may be significant for augmenting schistosome kinase activity. The autoinhibitory domain and linker region of AMPK α have been previously demonstrated as essential for the regulation of enzyme activity through interaction with the γ -subunit (58). Through these interactions, AMPK remains in an inactivated state when cellular energy levels are balanced, however, binding of AMP to the γ -subunit alters this association, allowing for AMPK α to be phosphorylated for activation (58; 153). Increased separation between these two domains

may disrupt their regulatory functions, potentially causing AMPK enzyme activity to deviate from expected norms due to changes in autoregulation. Furthermore, these differences between schistosome and human AMPK α -subunits highlight a potential advantage in targeting parasite AMPK. It is important to thoroughly investigate how we can take advantage of sequence dissimilarities between schistosome and human AMPK that are typically not afforded in the α -subunits of most other organisms in order to unveil novel opportunities for intervention.

Following sequencing of schistosome AMPK α , we investigated how the parasite may regulate this protein at different stages throughout the life cycle and revealed a lack of transcripts in cercariae and early schistosomula, despite AMPK α protein being present. We hypothesized that the absence of AMPK α transcripts in cercariae and early schistosomula can be attributed to the notorious lack of transcriptional activity in the *S. mansoni* human transmission stage (149). Cercariae are most likely pre-packaged with all essential proteins necessary to sustain their short lifespans before leaving the intermediate molluscan host and resume transcriptional activity as well as protein production at some point once inside the definitive mammalian host. The lack of AMPK α transcripts in these developmental stages is potentially responsible for their vulnerability to AMPK inhibition and we, therefore, predict that these stages would make favorable targets against which to direct therapeutic efforts via AMPK inhibitory mechanisms.

After assessing the differential regulation of AMPK expression across the parasite life cycle, we turned our focus to the enzyme's activity and investigated whether it was amenable to augmentation. Indeed, we demonstrated that through the introduction of chemical AMPK agonists to parasite culture media, AMPK activity could be modulated.

The ability of schistosomes to respond to changes in their external environment via increased AMPK α activity, as illustrated by increased phosphorylation, indicated to us that this protein was potentially important for the parasite's homeostatic response to environmental fluctuations, possibly fluctuations in nutrient availability or factors emanating from the host. Additionally, this remarkable feature suggested the potential susceptibility of schistosome AMPK to targeting via therapeutic compounds. In an effort to further investigate the role that AMPK plays in regulating schistosome energy homeostasis, we assessed the metabolic implications of modulating schistosome AMPK α . We demonstrated in adult schistosomes that glycolytic activity is not significantly affected (or increased as expected) when exposed to AMPK α agonistic factors, however, when exposed to AMPK α inhibitory conditions, the glycolytic response increases. AMPK inhibition is not fatal for adult schistosomes; they are able to live under these conditions for prolonged periods of time and exhibit only mild physical effects. RNAi-mediated AMPK reduction had similar effects on adult schistosomes. No effect on AMPK α transcript reduction was noted and glycolysis proceeded as normal. This observation suggested that AMPK is not essential in adult schistosomes and is potentially not needed for metabolic processes to proceed, contrary to what we initially hypothesized.

Juvenile schistosomes (i.e. immediate intra-mammalian stages), however, are detrimentally impacted by AMPK inhibition and exhibit severe physical consequences to which they succumb, *in vitro*. We examined the underlying cause for this disparity and established that adult schistosomes respond to AMPK inhibition with increased α -subunit phosphorylation and overall protein nearly four times the levels existing under non-

inhibitory conditions. Juvenile schistosomes exposed to identical AMPK inhibitory conditions are unable to respond similarly as insignificant changes in AMPK α expression and phosphorylation are observed. While a significant increase of overall AMPK α protein was apparent in adult schistosomes in response to AMPK inhibition, no significant increase was observed in AMPK α transcripts. It is possible that the stark increase in adult schistosome AMPK α protein will not directly correspond to apparent and significant differences in AMPK α transcripts. It is, also, possible that AMPK inhibition impacts the stability of schistosome AMPK or results in the upregulation of post-transcriptional mechanisms that leads to an accumulation of AMPK α protein (36; 184).

Finally, after examination of some compensatory mechanisms potentially underlying the increased glycolytic activity elicited post-AMPK inhibition, we uncovered that schistosome AMPK inhibition coincides with depleted parasite glycogen stores. The depletion of glycogen as well as the increase in glycolytic activity following AMPK inhibition were both interrupted when glycogen phosphorylase, the enzyme responsible for breaking down glycogen into its glucose precursor, was inhibited. This evidence suggests a link between *S. mansoni* AMPK and glycogen, potentially via glycogen phosphorylase. In mammals, a functional connection between AMPK and glycogen has been well established, although the details are complex and remain incompletely understood. AMPK's β -subunit has been demonstrated to be important for binding to glycogen (as indicated by its carbohydrate binding domain) as a potential gauge of cellular energy stores (122). Previous data has demonstrated that the activity of mammalian AMPK can be impacted by cellular glycogen availability. Studies have also

shown that the dynamics of cellular glycogen storage and usage are regulated by AMPK (98). The structure of glycogen impacts AMPK's activation which, in turn, dictates whether glycogen is formed or utilized through its activity on glycogen-associated enzymes. High levels of cellular glycogen levels can be detected by the presence of highly branched glycogen molecules. Normally, glycogen-bound AMPK is kept in its active state by this highly branched structure causing glycogen synthase, the enzyme responsible for glycogen formation, to remain inactive via phosphorylation by active AMPK. As glycogen stores are depleted and branching diminishes, AMPK is inhibited, causing activation of glycogen synthase due to interruption in phosphorylation by AMPK (98). We have demonstrated that the β -subunit of schistosome AMPK shares this conserved glycogen binding feature, therefore, supporting the potential role of AMPK in regulating parasite glucose metabolism via glycogen as our data suggests. While the observed effects of AMPK inhibition on parasite glycogen stores appears to be counterintuitive to the AMPK-glycogen relationship described in other eukaryotic systems in the literature (particularly mammalian), there are a few mechanisms that could potentially explain this phenomenon. On one hand, it is possible that the stimulation of glycogen breakdown is the result of increased total AMPK levels (and subsequently increased phosphorylation of these increased AMPK levels) induced by the inhibition of existing AMPK pools in adult schistosomes. Additionally, when AMPK levels were reduced via RNAi, a decrease in the amount of AMPK available to regulate glycogen stores could have contributed to increased glycogen breakdown. Finally, the potential for Sm AMPK to behave in a manner contrary to what has been previously documented in other organisms cannot be ruled out. It is possible that in adult schistosomes, AMPK is a

negative regulator of glycogen breakdown, thus, when AMPK is inhibited, glycogen breakdown proceeds unrestricted. Further experimentation would be required to parse out the precise mechanism underlying glycogen breakdown following AMPK inhibition in order to fully describe the relationship between AMPK and glycogen in this parasite.

While the connection between schistosome AMPK and glycogen is supported by a wealth of data demonstrating similar connections in mammals and other non-mammalian organisms, the effects of AMPK inhibition on glycolysis and glycogen depletion were somewhat unexpected. Given the well documented downstream effects of AMPK activation on increased glycolytic activity and decreased glycogen formation, we predicted, then, to observe the opposite effects when inhibiting schistosome AMPK (58; 74). Similar, non-conventional effects of AMPK activity have been noted in mammalian muscle cells. In mammalian muscle, chronic activation of AMPK has been shown to unconventionally activate glycogen synthase due to increased muscle glycogen accumulation resulting in allosteric glycogen synthase activation (93). Long-term AMPK activation causes increased glucose uptake and, subsequently, pools of glucose-6-phosphate increase. As glucose-6-phosphate levels rise within the cell, glycogen synthase inactivation mediated by activated AMPK is overcome allosterically, resulting in activated glycogen synthase and subsequent glycogen formation (93). In our work with *S. mansoni* AMPK, we have consistently observed persistent activation and high phosphorylation ratios of overall AMPK α . It is possible that schistosome AMPK may behave in an unconventional manner similar to mammalian muscle cells due to chronic AMPK activation. In this case, AMPK inhibition may not manifest in the ways we would expect which may explain the effect on glycogen depletion.

It has been well established that schistosomes possess extensive glycogen stores that are actively (and often simultaneously) formed and broken down despite inhabiting presumably glucose-rich host environments (181; 182). While stored forms of excess glucose is nearly universal to all living organisms, one can't help but question the significance of this process in a parasitic organism like *S. mansoni* that typically has uninterrupted access to glucose. Previous studies investigating the dynamics of schistosome glycogen storage have speculated that these stores may serve as sources of glucose for parasites that have navigated into tight spaces within the host where access to external glucose may be restricted (182). An examination of glycogen usage in *Caenorhabditis elegans* and some mammalian cells, however, may provide an alternative hypothesis for the significance of glycogen in schistosomes. Glucose derived from glycogen metabolism has been demonstrated to be useful for protection against oxidative stress in an AMPK-dependent manner in *C. elegans*. This protection contributes to prolonged lifespan in the nematode as well. Similar conclusions have been drawn in human hepatocytes (67). Human cancer cells have, also, been shown to benefit from glycogen metabolism as it contributes to tumor growth and free radical protection (50). The protective effects of glycogen metabolism demonstrated in these examples are mediated through the pentose phosphate pathway (PPP). Glucose produced via glycogen breakdown is preferentially funneled into the PPP which generates NADPH, a critically important component for combating the effects of oxidative stress (131). Schistosomes spend the entirety of their adult lives under constant oxidative attack by the host immune system. Much work has been done to examine the ways in which schistosomes counteract reactive oxygen species, however, no studies have investigated the potential for glycogen

metabolism to contribute to these counteractive mechanisms (90; 158). Genetically, schistosomes possess all components necessary to participate in the oxidative arm of the pentose phosphate pathway (15). Further investigation is necessary to determine whether products of glycogen metabolism in schistosomes are purposed for the PPP and what role, if any, schistosome AMPK has in directing this pathway.

GENERAL CONCLUSIONS

In general, we can conclude that *S. mansoni* AMPK is not essential for the regulation of parasite energy metabolism, specifically glycolysis, as we originally predicted. Given that parasite glycolytic activity carried on as normal and could not be stimulated beyond basal levels when AMPK was activated by chemical stimulants, indicated that AMPK does not play as significant a role in regulating glycolysis as it does in other organisms. It is quite possible that AMPK is already functioning at peak operational levels, thus, further stimulation through increased phosphorylation may not directly translate to obvious increases in glycolysis. Glucose is the primary energy source for schistosomes and these parasites rely, almost entirely, on glycolysis to fulfill their bioenergetics needs. In their definitive mammalian host, these parasites are constantly bathed in glucose that is strictly maintained at consistent concentrations at all times. It is possible that glycolytic regulation is not necessary in schistosomes and the most efficient approach would be to have all systems involved in glucose metabolism invariably activated to maximal efficiency.

The most important conclusions of this work are that we have identified an *S. mansoni* AMPK ortholog, of which we have isolated sequences of the α and β subunits, that is amenable to being targeted by external compounds, is important for regulation of

glycogen stores in adults, and is essential for schistosomula viability. Regulation of schistosome glycogen stores by AMPK suggests that glycogen is a critical substrate and its maintenance needs to be tightly controlled. Whether glycogen metabolism is important for supplying an additional energy source or serves alternative roles for schistosomes remains to be determined. However, this is an interesting revelation in schistosome biology and should be further investigated to fully appreciate the significance of glycogen in *S. mansoni* parasites. In addition, the toxicity of AMPK inhibition for schistosomula indicates the potential for this mechanism to inform the design of novel intervention approaches. Future work would greatly benefit from examining how this vulnerability in juvenile schistosomes can be exploited.

FUTURE EXPERIMENTS

There is much to be done to fully understand the function of *S. mansoni* AMPK and how it compares those of other organisms. It will be necessary to explore interactions between Sm AMPK and other cellular proteins as well as identify substrates in order to elucidate intracellular networks in *S. mansoni*. Furthermore, we have previously demonstrated that Sm AMPK α is significantly more phosphorylated than human AMPK α , indicating the potential for increased activity compared to its human counterpart (92). This requires a more in-depth investigation to assess whether increased phosphorylation of Sm AMPK α indeed translates to increased activity and what this means for parasite metabolic processes compared to other organisms. This can only be done as more substrates of schistosome AMPK are identified. While we have assessed the impact of AMPK activity on schistosome glycolysis, we did not look at other processes such as protein metabolism, autophagy, and transcriptional downstream effects.

These processes are also worth examining to better understand the significance of AMPK in schistosomes and how its activity impacts other cellular functions.

To determine the implications of schistosome AMPK α C-terminal insertions on kinase behavior, future studies would benefit from constructing schistosome AMPK α mutants with insertions of varying lengths and compare their activity with wild-type schistosome and human AMPK α . This would assist determining contributions of schistosome AMPK α C-terminal insertions to overall enzyme activity, function, and regulation. Moreover, because the C-terminal insertions in schistosome AMPK α creates a significant portion of divergence from an otherwise homologous human AMPK α , this may be a lucrative point to target in the development of novel anti-schistosome therapeutics. AMPK inhibitory compounds would be the most advantageous approach to targeting schistosome AMPK α , as the divergent region of the parasite's catalytic subunit would be unreachable by antibodies. Potential differences in schistosome AMPK α structure and overall kinase function due to the insertions may reveal disparate responses to AMPK inhibitors rendered useless against the human enzyme. Libraries of pre-existing and novel AMPK inhibitors as well as other kinase inhibiting compounds should be screened against both schistosome and human AMPK to identify those that are effective against parasite protein while simultaneously ineffective against the human protein in order to uncover compounds for further development as potential schistosomiasis therapeutics.

If parasite AMPK is to be pursued as a therapeutic target, our data suggests that directing efforts against early schistosomula would be most productive as AMPK inhibition is toxic in this developmental stage. We hypothesized that the inability of early

intra-mammalian stage schistosomes to respond with increased AMPK protein, due to lack of AMPK α transcripts (149), prevents them from inducing homeostatic responses necessary to survive environmental AMPK inhibitory conditions. It would be important to resolve the kinetics of AMPK expression in juvenile schistosome stages in order to determine a timeline for which targeting juvenile schistosomes via AMPK inhibition would be most effective. To do so, future experiments would need to determine at what time point AMPK α transcriptional activity in juvenile schistosomes resumes within the mammalian host. Additionally, in elucidating the kinetics of AMPK α gene expression in juvenile schistosomes, we would be able to determine whether the toxic effects of AMPK inhibition in early schistosomula are truly due to a lack of transcripts or if this effect is mediated through other processes that are either impacted by or coincide with dorsomorphin treatment.

Likewise, further investigation is necessary to determine if increased AMPK α protein in adult schistosomes is solely responsible for adult parasite survival post-AMPK inhibition. It would be interesting to evaluate how temporary inhibition of transcription or protein production while inhibiting AMPK in adult schistosomes would impact parasite survival. In doing so, we would, in part, be able to pinpoint which mechanisms are responsible for the increase in total AMPK α as well as determine if this increase is responsible for or just coincides with parasite survival following AMPK inhibition. It should be acknowledged that this would not be a particularly focused approach as other cellular functions would be affected by the loss of transcriptional and translational activity. However, it may provide insight into whether the generation of additional AMPK protein in the wake of AMPK inhibition is necessary for the survival of adult

schistosomes. Furthermore, it will be important to assess whether other metabolic pathways such as glucose uptake are enlisted in response to AMPK inhibition and contribute to adult parasite survival outcomes. Inhibition of glucose uptake and autophagy in adults while inhibiting AMPK would be useful for identifying what compensatory mechanisms, if any, are upregulated by the parasite to ensure survival. This experiment will also be useful for determining whether glucose uptake also contributes to increased glycolysis following AMPK inhibition.

We have shown that Sm AMPK is important for the regulation of cellular glycogen stores and when enzyme activity is dampened, either through chemical inhibition or transcript reduction, glycogen stores are depleted. It would be beneficial for future studies to assess the significance of schistosome glycogen beyond the purpose of additional fuel sources in glucose restricted spaces. We speculated the potential for glycogen breakdown to be important for oxidative protection via the pentose phosphate pathway in *S. mansoni*, as has been demonstrated in *C. elegans*. Experiments designed to investigate the contribution of glycogen breakdown products to the PPP in schistosomes and test whether this influences protection from reactive oxygen species would be necessary. This could be achieved by assessing adult schistosome viability in culture when exposed to exogenous hydrogen peroxide either in the presence or absence of AMPK inhibiting conditions. Furthermore, compounding these conditions with an inhibitor of the oxidative arm of the PPP would help to further tease out the contributions of AMPK inhibition-induced glycogen breakdown to oxidative PPP and ultimately protection from oxidative stress. In addition, another useful experiment would be to examine how AMPK inhibition affects adult schistosome responses to oxidative pressure

in vivo. To achieve this goal, *S. mansoni* adults infecting wild-type, ROS-overexpressing, and ROS-deficient mice under control and AMPK-inhibitory conditions would be evaluated for overall worm burden and phenotypic effects upon 6-8 weeks post-infection. As the pressure of reactive oxygen species is typically absent under normal culture conditions, it would be interesting to evaluate potential differential parasite responses to AMPK inhibition when inside a mammalian host.

We demonstrated that Sm AMPK regulation of parasite glycogen is most likely mediated via the β -subunit given its shared homology to human AMPK β and features significant for glycogen binding. While we have provided sequence-based evidence of glycogen association, direct binding of schistosome AMPK β with glycogen should be confirmed experimentally. Future studies focused on furthering this work would benefit from establishing the binding kinetics between *S. mansoni* AMPK β and glycogen to determine the contribution of this relationship to overall kinase activity as well as determine the direct interactions between schistosome AMPK and glycogen-associated enzymes. Additionally, future experiments should also focus on determining the significance of the insertion in the C-terminal portion of *S. mansoni* AMPK β that contributes to a size difference between the parasite protein and AMPK β -subunits of most other organisms. Experiments designed to create schistosome AMPK β mutants lacking this insertion and comparing activity of AMPK containing the mutant β -subunit to wild-type AMPK would inform how, if at all, the insertions contribute to parasite AMPK activity and function.

Earlier in this discussion, I alluded to the potential for schistosome AMPK to behave similarly to mammalian muscle cell AMPK that also behaves paradoxically due

to chronic activation of the enzyme. Given that schistosome AMPK appears to remain in a chronically and highly activated state (92), future experiments should assess the effects of chronic AMPK activation in schistosomes on glucose uptake and parasite glycogen synthase activity. This could potentially be done by evaluating the amount of glucose absorbed and the activity of parasite glycogen synthase when schistosome AMPK is reduced by RNAi and comparing to that of schistosome AMPK at wild-type levels.

Last, given that we have demonstrated *in vitro* the toxicity of chemical inhibition of AMPK for juvenile schistosomes, future work should extrapolate these findings to an *in vivo* model. Determining whether *in vitro* findings are upheld in an animal model would be a critical first step for assessing whether AMPK inhibition is a viable intervention mechanism that could one day be used in the field. Future experiments would greatly benefit from the *in vivo* testing of dorsomorphin analogs and other kinase-inhibiting compounds shown to be effective against AMPK in order to determine their efficacy in killing juvenile schistosomes and preventing schistosomiasis infections.

Extrapolation of the findings presented in this work via further experimentation will ultimately yield better understanding of schistosome biology and the mechanisms involved in energy metabolism, energy homeostasis, and parasite survival which may one day culminate in the production of novel therapeutics targeting these elucidated mechanisms to help eradicate schistosomiasis.

REAL WORLD IMPLICATIONS

Prior to the report of these findings, *S. mansoni* AMPK had only been predicted by the genome consortium. To our knowledge, this is the first time that an expressed AMPK has been described and characterized in schistosomes. This is significant for a

number of reasons. This work adds to the body of knowledge surrounding schistosome biology as it sheds light on the intracellular mechanisms that these parasites employ to respond to external factors in order to survive within the host. It is critical that future work is dedicated to further elucidating intracellular networks involved in maintaining energy homeostasis in order to better understand schistosome energy requirements, metabolism, and regulation, potentially unveiling novel therapeutic targets. Additionally, the evidence presented in this work establishes a target that is present in every schistosome life cycle stage and vulnerable to exploitation. Chemical inhibition of this protein in *S. mansoni* early intra-mammalian stages was demonstrated to be lethal, a finding that should be expanded upon and investigated further for its potential as an anti-schistosome therapeutic target. Currently, no prophylactic to prevent schistosome infection is available and the current gold standard of treatment is ineffective against these early intra-mammalian schistosome stages (25). A therapeutic that would prevent the establishment of schistosome infection would be pivotal for the reduction of morbidity due to disease as well as interruption of the transmission cycle. While we acknowledge that dorsomorphin would not be ideal given its documented toxicity and effects on human AMPK, potentially less-toxic analogs of this compound could be useful to combat early stages of schistosomes, against which current treatment options are useless. As previously mentioned, screening of AMPK inhibitory compound libraries for activity better suited against schistosome AMPK as opposed to human AMPK (given their α -subunit sequence and structural differences) may be key in identifying compounds most effective against early schistosome stages while maintaining minimal toxicity for the host to be used as potential prophylactics or early-infection therapeutics. Although

this method of therapeutic could have schistosome life cycle-interrupting potential, it is important to note that this mechanism would be more fitting to treat emergency exposures in travelers or military populations stationed in schistosomiasis-endemic areas. Given that endemic populations are often in habitual contact with fresh water that facilitates schistosome transmission, it would be impractical to use this method as it would require constitutive drug exposure to be effective for these populations.

In closing, targeting AMPK in schistosomes, particularly in juveniles, is a mechanism that demonstrates substantial therapeutic promise. Our data shows that schistosomula are incapable of surmounting AMPK inhibitory conditions, rendering fatal outcomes for the parasite. This vulnerability should absolutely be exploited. Additionally, revelations about schistosomula vulnerability have the potential to aid in the development of other therapeutics focused on non-AMPK targets as a result of the mechanisms described in this work.

APPENDIX A. Miscellaneous Glycolytic Rate Data

INTRODUCTION

Additional data on the glycolytic response of adult schistosomes post-exposure to various compounds were collected using both Agilent Seahorse XFe96 and pH-Xtra Glycolysis plate reader-based technologies. The metabolic response to Metformin and AICAR were additionally assessed using Agilent's plate reader-based assay as supplementation to data collected using Seahorse technology. In addition, the metabolic response in adult schistosomes exposed to Akt inhibitor was also evaluated. Akt is a serine/threonine kinase that has been implicated in impacting the upregulation of cellular glucose metabolism via pathways independent of AMPK. Given the increase in glycolytic activity following AMPK inhibition observed in adult schistosomes, we hypothesized that this unexpected response could be mediated via other pathways also involved in glucose metabolism. To evaluate this hypothesis, we chemically inhibited both Akt and AMPK simultaneously to determine if the increase in glycolytic activity following AMPK inhibition alone could be abrogated. These experiments were performed using both methods previously described for glycolytic rate data collection using Agilent technologies.

RESULTS

Stimulating AMPK activity in adult schistosomes with either Metformin or AICAR did not stimulate glycolytic activity. Adult schistosomes exposed to Metformin exhibited no significant differences in the acidification of worm culture media, as indicated by relative fluorescent unit values (rfus) mirroring those of control worms (Figure 21A). Parasites exposed to AICAR maintained rfus over time that were

significantly lower than their control counterparts (Figure 21B). Additionally, exposure to Akt inhibitor alone induced glycolytic rates that were lower than those of adult worms under control conditions. When Akt inhibitor was paired with dorsomorphin exposure, the significant increase in glycolytic activity typically observed following dorsomorphin treatment alone, although dampened, was not abrogated (Figure 22).

CONCLUSION

Glycolytic rate data of adult schistosomes post-exposure to Metformin and AICAR collected using Agilent's plate reader-based assay corroborated data collected under similar circumstances using Agilent Seahorse XFe96 technology. Stimulating schistosome AMPK activity beyond basal levels with AMPK agonistic compounds does not cause increased glycolytic activity, most likely due to the pre-existing highly active nature of AMPK in adult schistosomes. Furthermore, given Akt's documented direct contributions to glucose metabolism, it is not surprising that treatment of adult parasites with Akt inhibitor alone reduces glycolytic activity. Despite this, inhibition of Akt is not sufficient to counteract the glycolytic effects of AMPK inhibition. Dampening but not full interruption of increased glycolysis in adult schistosomes when exposed to both Akt inhibitor and dorsomorphin simultaneously indicates that the effects of AMPK inhibition on parasite glycolytic activity are not due to potential off-target or compensatory effects on Akt.

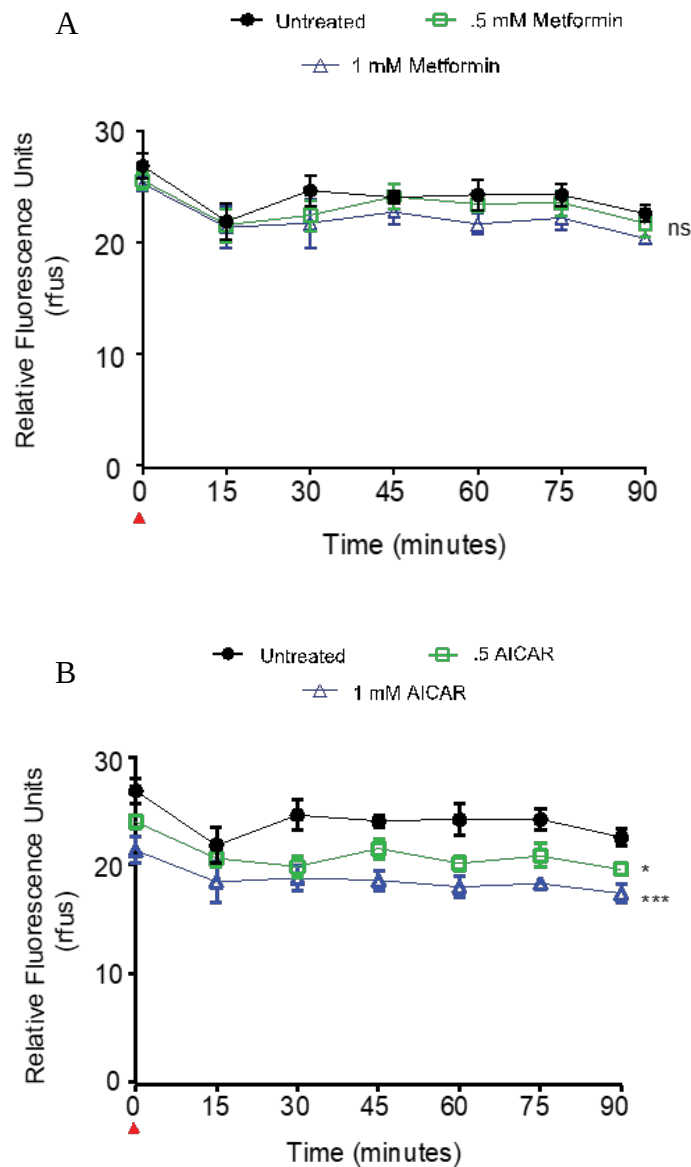


Figure 21. Effects of metformin and AICAR treatment on glycolytic activity in adult schistosomes (supporting data)

Extracellular acidification of media, correlating with changes in relative fluorescence units (rfus), in wells containing two pairs each of freshly isolated adult schistosomes post-exposure to metformin (A) and AICAR (B) was measured using Agilent pH-Xtra Glycolysis Assay. Red arrows indicate introduction of compound into media.

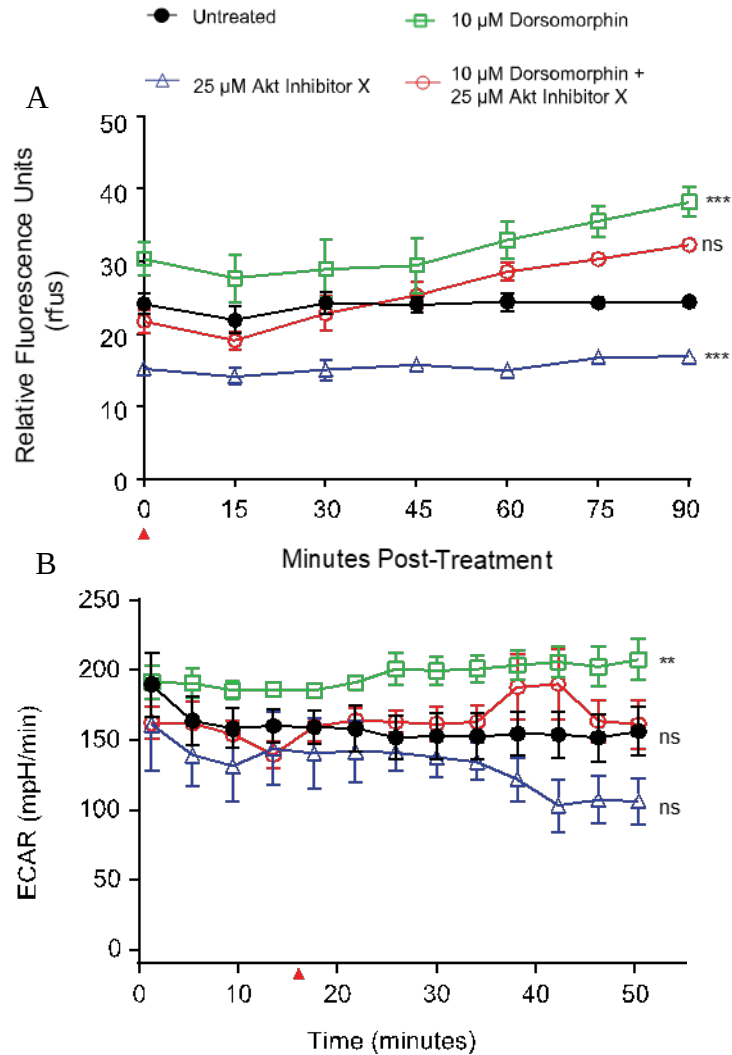


Figure 22. Akt inhibition does not prevent dorsomorphin-mediated glycolysis increase

A, Extracellular acidification of media, correlating with changes in relative fluorescence units (rfus), in wells containing two pairs each of freshly isolated adult schistosomes post-exposure to dorsomorphin, Akt inhibitor, and a combination of both was measured using Agilent pH-Xtra Glycolysis Assay. B, Extracellular acidification rate (ECAR) in wells containing one pair of adult worms post exposure to the aforementioned compounds was measured using Agilent Seahorse XFe96 technology. Red arrows indicate introduction of compound into media.

APPENDIX B. Effects of Dorsomorphin on the Establishment of Schistosome Infection *in vivo*

INTRODUCTION

In vitro treatment of schistosomula with dorsomorphin immediately post-transformation detrimentally impacted fitness and survival. Early schistosomula exposed to 10 μ M dorsomorphin in culture for 24 h exhibited severe phenotypic impacts marked by immobility, loss of translucence, and granular appearance (all indications of extremely unhealthy or dead juvenile parasites). While the physical manifestations of dorsomorphin's effects on early schistosomula became apparent by 24 h, these parasites retained some metabolic activity up until 48 h exposure to 10 μ M, after which their metabolic activity correlated with their dismal physical appearance. The adverse effects of dorsomorphin administration on early intra-mammalian stage schistosomula *in vitro* indicated the potential for AMPK inhibition to be used as a prophylactic mechanism. We, thus, hypothesized that dorsomorphin could prevent the establishment of schistosome infection via killing of juvenile parasites if administered early in the infection time course. Previous studies have examined the feasibility of *in vivo* dorsomorphin administration to achieve diverse outcomes in the field of oncology. Specifically dorsomorphin was demonstrated to effectively reduce the size of artificially induced tumors in mice when one 10 mg/kg dose was administered intraperitoneally (146). Given the impact of dorsomorphin on juvenile schistosomes *in vitro* and the feasibility of *in vivo* administration in murine models, we were prompted to assess the potential efficacy of dorsomorphin as a schistosomiasis prophylactic in preliminary animal studies.

METHODS

Female, 10-week C57BL/6 mice (Jackson Laboratories) were administered dorsomorphin (10 mg/kg) via intraperitoneal (i.p.) injection immediately before infection with *S. mansoni* cercariae. Mice were returned to their cages promptly after infection. One hour post-infection, mice were administered a second injection of dorsomorphin (10 mg/kg) via i.p. injection. Control mice were administered the volume equivalent of vehicle via i.p. injection. Mice were closely monitored for 4 hours following dorsomorphin administration before returning to animal facility. At 6 weeks post-infection, mice were euthanized and perfused to assess worm burden.

RESULTS

In vivo administration of 10 mg/kg dorsomorphin immediately before and shortly after infection by *S. mansoni* cercariae did not have a significant effect on preventing the establishment of schistosome infection in mice. Mice that received dorsomorphin according to the described regimen had worm burdens comparable to mice that received vehicle alone (Figure 23A). When the ratio of paired to overall collected schistosomes was compared between dorsomorphin-treated and vehicle-treated mice, no significant differences were observed (Figure 23B).

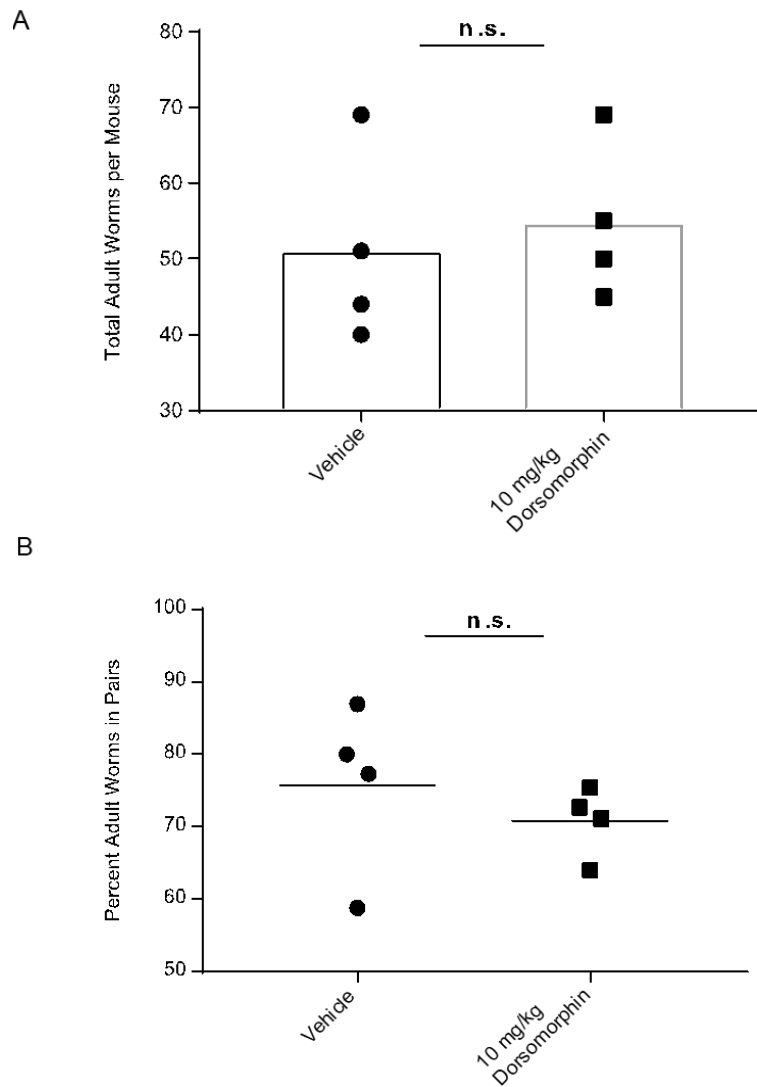


Figure 23. Effects of *in vivo* dorsomorphin administration on adult schistosome burden and pairing

S. mansoni infected C57BL/6 mice treated with either dorsomorphin or vehicle were euthanized and perfused at 6 weeks post-infection to collect adult schistosomes. A, Total number of adult parasites retrieved per mouse were counted and expressed as worm burden. B, The ratio of adult schistosomes in pairs at time of perfusion relative to the total number of worms collected was calculated per mouse and expressed as a percentage.

CONCLUSIONS

Given the effect of 10 μ M dorsomorphin on schistosomula *in vitro*, we expected to observe either a reduction or complete abrogation of adult schistosome burden in infected mice. However, 10 mg/kg dorsomorphin administered via the described regimen was not sufficient to impede schistosome infection as evidenced by comparable worm burdens between control and dorsomorphin-treated mice. There are multiple ways this could be interpreted. First, *in vitro* experiments cannot account for how dorsomorphin may be metabolized once administered. As a result, we cannot fully anticipate the potential varied effects of dorsomorphin on schistosomula once inside the host. Additionally, it is possible that a dose of 10 mg/kg is not adequate to achieve an evident effect on schistosome infection. Finally, dorsomorphin is a reversible inhibitor of AMPK. Schistosomula that succumbed to 10 μ M dorsomorphin treatment *in vitro* were under constant drug pressure for 24 h. It is possible that it is necessary to administer dorsomorphin more frequently *in vivo* to maintain drug pressure conditions similar to those tested *in vitro* in order to observe an impact on worm burden. Additional experiments that test a range of drug concentrations and administration frequencies are required to fully elucidate the impact of dorsomorphin on schistosome infection *in vivo* and its potential as a schistosomiasis prophylactic. In addition, it will be fundamental to assess dorsomorphin pharmacokinetics in mice to assess the concentration that ultimately reaches juvenile parasites in the bloodstream and tissues.

APPENDIX C. Determining AMPK α Protein Presence in Cercariae and Special Considerations for Preparing Juvenile Schistosome Protein

INTRODUCTION

In our early efforts to describe AMPK α throughout the life cycle of *S. mansoni*, we were initially unable to detect AMPK α protein via Western blot of cercariae. Given the generally accepted importance of AMPK to all extracellular eukaryotes, we were skeptical to accept this finding. Although cercariae are notorious for their transcriptional inactivity, we found it unlikely that this transmission stage did not at least house AMPK protein as it is presumed necessary for the regulation of energy stores. Here, we enlisted additional techniques (i.e. confocal microscopy and preparation of parasite lysate via harsher methods) to thoroughly assess the status of AMPK α in juvenile schistosomes. All subsequent work concerning the detection and characterization of AMPK α in cercariae and early schistosomula can be attributed to methods worked out via the troubleshooting described herein.

METHODS

Staining of AMPK α in cercariae for confocal microscopy

Isolated cercariae were fixed and permeabilized in 4% formaldehyde diluted in 1 X PBS supplemented with 0.3% w/v Triton X-100 (PBSTx) for 30 minutes at room temperature. Fixed and permeabilized cercariae were washed with PBSTx three times then resuspended in PBSTx. Cercariae were stained with Hoescht 34580 (ThermoFisher Scientific, catalog no. H21486) at a concentration of 2 ug/ml, Alexa Fluor 488 Phalloidin (ThermoFisher Scientific, catalog no. A12379) diluted 1:40, and anti-AMPK α -1, 2 rabbit polyclonal antibody (Invitrogen, catalog no. PA5-36045) diluted 1:500 for 30 minutes at

room temperature. Stained cercariae were washed twice with PBSTx then incubated with Alexa Fluor 647goat anti-rabbit IgG (H + L) highly cross-absorbed secondary antibody (Thermo Fisher Scientific, catalog no. A21245) at a concentration of 2 ug/ml for 30 minutes at room temperature. Cercariae were washed twice with PBSTx then transferred to a glass slide with 20 µl ultrapure glycerol (Invitrogen, catalog no. 15514-011) and covered with a glass coverslip. Slides were visualized the same day using a Zeiss LSM700 confocal microscope.

Preparation of cercariae lysate for western blotting to detect AMPK α

Freshly isolated cercariae were prepared for Western blot by homogenizing in either RIPA buffer or 1 X LDS sample buffer to assess each method's effectiveness at preventing protein degradation. Using an electric homogenizer, cercariae were lysed in RIPA buffer supplemented with phosphatase inhibitors (Halt Phosphatase Inhibitor Cocktail, Thermo Scientific) and protease inhibitors (Halt Protease Inhibitor Single-Use Cocktail, Thermo Scientific) to a final concentration of either 1 X, 20 X, or 50 X then incubated for 10 minutes at 95 °C and sonicated. 4 X LDS sample buffer was diluted to a final concentration of 1 X in distilled water with 10 % 2-mercaptoethanol and supplemented with the same inhibitors to a final concentration of 1 X. Using an electric homogenizer, cercariae were lysed in 1 X LDS sample buffer prepared at either room temperature or at 95 °C, then incubated at 95 °C and sonicated. After sonication, protein concentration was quantified and lysates were analyzed via Western blot as previously described by Hunter and Davies, 2018 (92).

RESULTS

Although AMPK α protein was absent from Western blots of *S. mansoni* cercariae lysate, we were able to detect a strong AMPK α signal in cercariae when examined via confocal microscopy (Figure 24). Because AMPK α was detectable in fixed, intact cercariae but undetectable in parasite lysate, we hypothesized that strong cercarial proteases degraded AMPK α upon cercariae lysis before we were able to detect protein by Western blot. This prompted us to explore alternative lysate preparation methods to determine how best to prevent protein degradation before Western blot analysis. Detection of AMPK α protein via Western blot was restored when cercariae lysate was prepared in RIPA buffer supplemented with protease and phosphatase inhibitors to final concentrations of 20 X and 50 X (Figure 25, lanes 1-3). AMPK α band intensity at ~ 72 kDa correlated with increasing inhibitor strength (Figure 25, lanes 1-3). Despite restoring cercariae protein detection, it was evident that degradation was still occurring as evidenced by the presence of low molecular weight proteins detected by anti-total AMPK α antibody. As a result, we used even harsher methods to attempt to resolve these issues. Room temperature 1 X LDS sample buffer supplemented with inhibitors to a final concentration of 1 X produced an even more intense AMPK α band at ~ 72 kDa in cercariae indicating its efficacy at preserving some full length protein, however, still in the face of significant degradation (Figure 25, lane 4). Only similarly prepared 1 X LDS sample buffer pre-heated to 95 °C prior to cercariae lysis was adequate for complete abrogation of protein degradation, as evidenced by a single AMPK α band at ~ 72 kDa in the absence of any low molecular weight degradation products (Figure 25, lane 5).

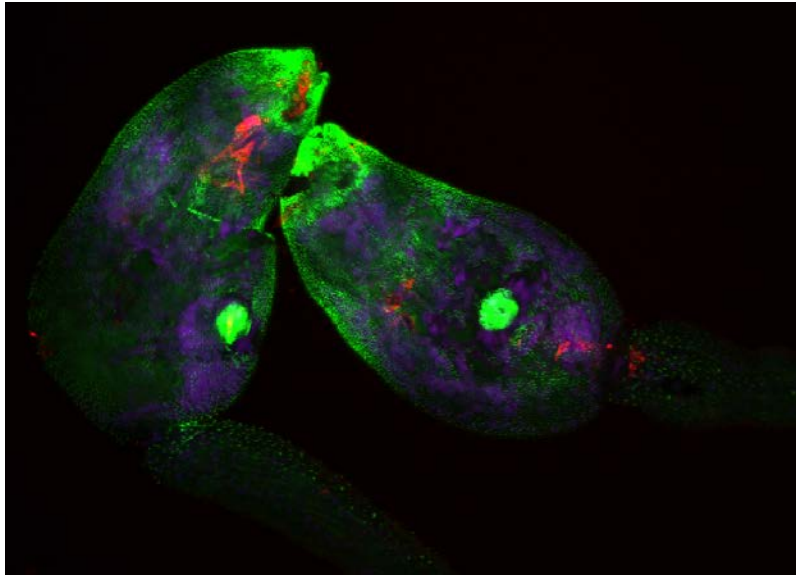


Figure 24. Visualization of AMPK α in *S. mansoni* cercariae via confocal microscopy

Fixed and stained *S. mansoni* cercariae were analyzed via confocal microscopy for the presence of AMPK α (red). Cercariae nuclei (blue) and actin (green) were also stained to provide structural context. Areas of purple staining indicate where AMPK α and nuclei signals overlap.

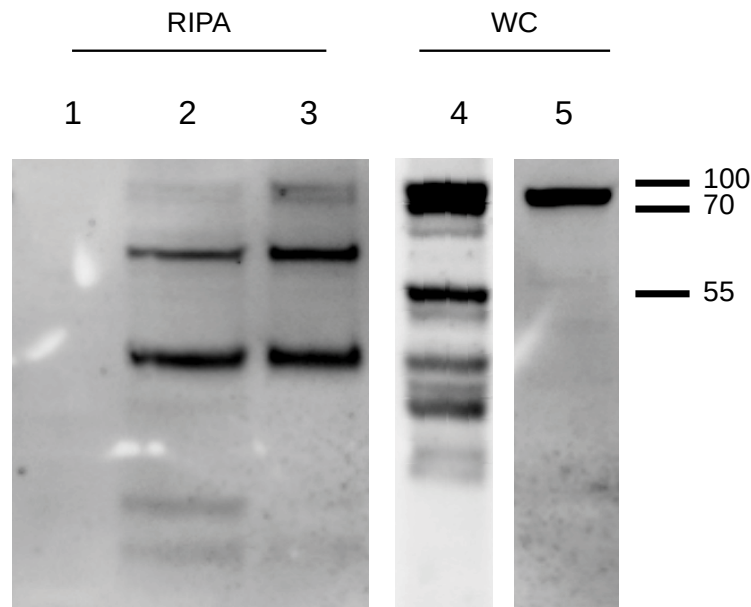


Figure 25. Comparison of cercariae protein preparation methods for detection of AMPK α via Western blot

Cercariae lysates were prepared either in RIPA buffer supplemented with 1 X protease inhibitors (1), 20 X protease inhibitors (2), and 50 X protease inhibitors (3) or 1 X LDS sample buffer supplemented with 1 X protease inhibitors at room temperature (4) or 95 °C (5), separated using SDS-PAGE, then analyzed via Western blot probed with anti-AMPK α -1, 2 to evaluate the impact of cercarial proteases on perturbing detection of AMPK α in cercariae.

CONCLUSIONS

Ensuring that cercarial proteases are completely disabled is integral for thorough investigation of protein expression of any kind in *S. mansoni* cercariae. This was particularly important for determining the status of AMPK in cercariae. Had we not done so, our presumptions based on the initial absence of AMPK α protein from Western blots of cercariae lysate would have been incorrectly accepted and reported. As our data

demonstrates, standard protein preparation practices for Western blot of cercariae lysate using RIPA buffer supplemented with 1 X protease inhibitors is insufficient for inhibiting protein degradation. We recommend always preparing *S. mansoni* cercariae lysate with pre-boiled 1 X sample buffer to ensure optimal reduction in protein degradation or else risk misinterpretation of protein data.

APPENDIX D. Disruption of Glycolysis via 2-Deoxy-D-Glucose Stimulates Increased Total AMPK α Expression in *S. mansoni* Adults

INTRODUCTION

AMPK exerts its regulatory effects over intracellular energy metabolism in various ways, one of them being through glycolysis. In instances of low cellular energy states, AMPK is activated via phosphorylation and employs various mechanisms to stimulate glycolytic activity in order to re-stabilize cellular energy levels. Our previously published data illustrated the ability of *S. mansoni* AMPK α activity, as indicated by phosphorylation status, to be modulated post-exposure to external AMPK agonists. These findings suggested the capacity of schistosome AMPK to respond to changes that affect cellular homeostasis. Based on this data, we aimed to investigate the impact of interrupting ATP generation from glycolysis on schistosome AMPK by exposing *S. mansoni* adults to 2-deoxy-D-glucose (2-DG), a glucose analog and glycolysis inhibitor.

METHODS

Immediately post-perfusion, fresh *S. mansoni* adults were placed in DMEM supplemented with 10 % FBS, 1 X antibiotic/antimycotic, and either 2-deoxy-D-glucose (2-DG) to a final concentration of 5 mM or the volume equivalent of vehicle as a control. Worms were incubated under these conditions for 24 h at 37 °C. After incubation, adult schistosomes were collected and flash frozen in liquid nitrogen. Frozen parasites were homogenized and prepared for analysis via Western blot as previously described by Hunter and Davies, 2018 (92). Densitometry was performed on Western blots using ImageJ software.

RESULTS

Adult schistosomes exposed to 5 mM 2-DG *in vitro* for 24 h exhibited increased phosphorylated and total AMPK α protein as compared to equal amounts of protein from parasites that received vehicle alone (Figure 26). When quantified, total AMPK α is increased in response to 5 mM 2-DG nearly twice as much as control conditions (Figure 27).

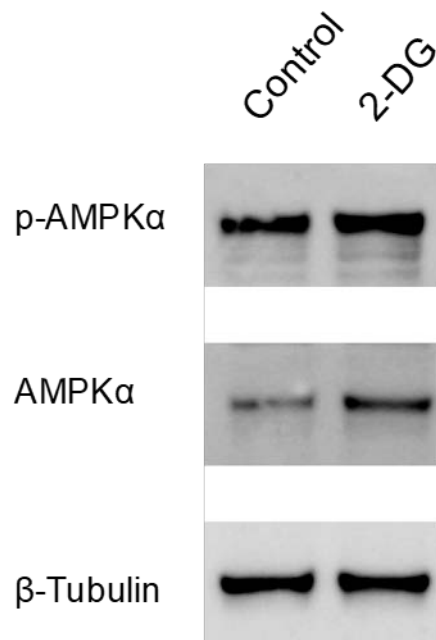


Figure 26. *In vitro* 2-DG exposure increases phosphorylation and expression of AMPK α in *S. mansoni* adults

Lysates of adult schistosomes maintained *in vitro* exposed to either vehicle or 5 mM 2-DG were analyzed via Western blot for AMPK α phosphorylation (p-AMPK α) and total AMPK α levels (AMPK α). As a loading control and for normalization purposes, blots were also probed for beta-tubulin (β -tubulin).

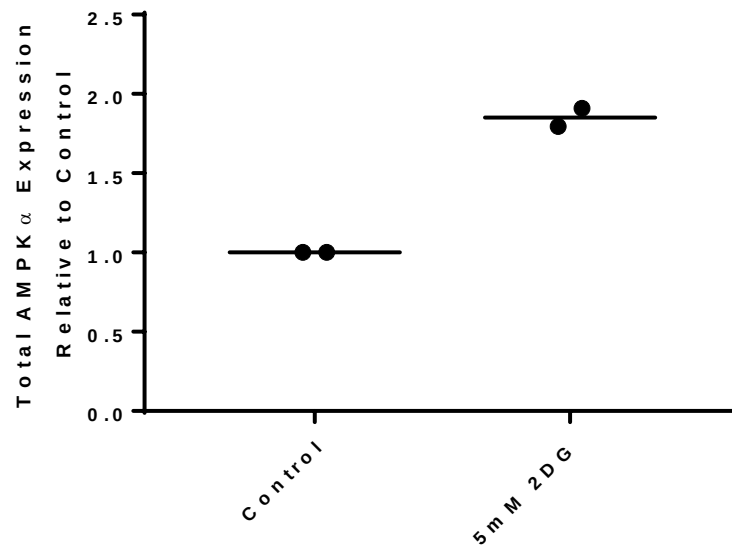


Figure 27. Quantification of AMPK α expression from Western blots of adult schistosomes maintained *in vitro* either in the presence or absence of 2-DG

Using ImageJ software, densitometry was performed on Western blots of adult

schistosome lysate probed with anti-total AMPK α and anti- β -tubulin from parasites

incubated in the presence or absence of 5 mM 2-DG for 24 h. Total AMPK α

densitometry values for each condition were, first, normalized to β -tubulin densitometry

values. Normalized total AMPK α values are shown as relative to control values which

are expressed as “1.0”.

CONCLUSION

Previously, we demonstrated that phosphorylation of schistosome AMPK α can be increased when adult schistosomes are exposed to AMPK agonistic factors *in vitro*. Here, we demonstrate that not only can adult schistosomes respond to environmental changes with increased phosphorylation of AMPK α but also with increased expression of the protein as well. These findings shed light on the regulation of schistosome AMPK and its

potential importance in maintaining cellular energy homeostasis. Furthermore, this data reveals mechanisms that adult schistosomes employ to cope with unfavorable environmental conditions.

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