Identification of enzymes that are required for Rhodoquinone-dependent metabolism as targets for new species-specific inhibitors

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ABSTRACT

Soil transmitted helminths (STHs) are major human pathogens that infect over a billion people. Resistance to current anthelmintics is rising and new drugs are needed. Here we combine multiple approaches to find druggable targets that are essential for RQ-dependent metabolism, an unusual form of anaerobic metabolism which STHs need to survive in their host. We identified 25 genes predicted to act in RQ-dependent metabolism from sensing hypoxia to RQ synthesis — this includes components of the kynurenine pathway we previously showed to be essential for RQ synthesis (Del Borrello et al., 2019). We found 9 genes to be required — since all have host orthologues, we used comparative genomics and structural modeling to identify those with helminth-specific active sites and found 4 such targets. These 4 high confidence targets open up the possibility of in silico screens to identify STH-specific inhibitors of these enzymes as new anthelmintics.

Introduction

Soil-Transmitted Helminths (STHs) such as *Ascaris*, whipworm and hookworm are parasites that infect over a billion humans and a large proportion of livestock (Bauck et al., 1992; Charlier et al., 2014; Pullan et al., 2014; World Health Organization, 2002). Anthelmintic drugs such as ivermectin (Campbell, 2012; Campbell et al., 1983) and benzimidazoles (Brown and Zweifel, 1961) are listed amongst the WHO Essential Drugs (“WHO model list of essential medicines,” 2019) and have provided frontline treatment for these pathogens for decades. However, resistance is widespread in livestock (Herath et al., 2021; Kaplan and Vidyashankar, 2012; Sangster et al., 2018; Wolstenholme et al., 2004) and in certain regions efficacy for specific drugs has decreased in humans (Geerts and Gryseels, 2001; Kotze et al., 2020; Moser et al., 2019) and few classes of anthelmintics currently exist. There is thus an urgent need for new classes of anthelmintics to combat these major pathogens.

STHs have complex and diverse life cycles but share a key feature that can be exploited for the development of anthelmintic treatments. These parasites must be able to survive in very different environments: the soil environment that they encounter in their free-living stages and the very different host environment they must live in after infection. STHs go through profound changes to adapt to the host environment and some of this involves STH-specific biology and machineries. These STH-specific molecular events are excellent potential targets for new anthelmintics (Kita et al., 2003). One of the best studied is the metabolic switch from standard aerobic energy metabolism that uses ubiquinone (UQ) as a critical electron carrier in the mitochondrial electron transport chain (ETC) to an unusual anaerobic metabolism that requires the unusual electron carrier rhodoquinone (RQ) (Fioravanti and Kim, 1988; Moore and Folkers, 1965; Sato et al., 1972; Sato and Ozawa, 1969) in a rewired ETC (Müller et al., 2012; Tielens,

1994). The key difference is that when UQ is used as an electron carrier, the sole terminal electron acceptor in the ETC is oxygen. When RQ is used, however, it allows electrons to be routed to a number of alternative electron acceptors (Tielens and Van Hellemond, 1998; van Hellemond et al., 2003; Van Hellemond et al., 1995). The RQ-dependent rewired ETC allows STHs to continue using the mitochondrial ETC to power ATP synthesis in the absence of oxygen and lets STHs survive in many highly anaerobic niches in the host such as the intestine. This is reviewed in depth in (Salinas et al., 2020).

RQ-dependent metabolism is essential for parasite survival in the host and it is also a critical difference between parasite and host. Hosts do not have RQ — they only ever make and use UQ. STHs must be able to make and use both UQ and RQ and drugs that block RQ synthesis or its use would thus kill the STHs without affecting their hosts. The need to make and use both RQ and UQ has driven a number of STH-specific molecular changes. The best characterised are the changes surrounding the quinone-binding pocket of succinate dehydrogenase, also known as Complex II (Iwata et al., 2008; Roos and Tielens, 1994; Takamiya et al., 1995). In aerobic conditions, STH Complex II acts as a dehydrogenase, converting succinate to fumarate and transferring two electrons onto UQ as the entry point into the ETC — this is the same basic function as host Complex II. In anaerobic conditions, however, STHs reverse this reaction: Complex II now acts as a fumarate reductase and the two electrons required to convert fumarate to succinate must come from RQ (Fioravanti and Kim, 1988; Tielens and Van Hellemond, 1998). STH Complex II now acts as a point for electrons to exit the ETC and fumarate rather than oxygen is the terminal electron acceptor. Since STH Complex II uses both UQ and RQ, the quinone-binding pocket of STH Complex II must be able to dock both UQ and RQ whereas the host Complex II only ever binds UQ. UQ and RQ are highly related but differ at position 2 of

their benzoquinone rings: UQ has a methoxy group whereas RQ has an amine group (Moore and Folkers, 1965). STH Complex II must be able to bind both ring structures and this difference in quinone-binding requirement has led to a number of STH-specific amino acid changes surrounding the quinone-binding pocket of Complex II (Burns et al., 2015; Kita et al., 2002; Otero et al., 2019). The difference between the quinone-binding pocket of STHs and the hosts has allowed the identification of several STH-specific Complex II inhibitors and we previously showed that these STH-specific inhibitors can efficiently kill *C.elegans*, a helminth, when it requires RQ-dependent metabolism to survive (Del Borrello et al., 2019).

Complex II is not the sole component of quinone-coupled metabolism known to differ between host and STH as a consequence of the difference in quinone utilization. We previously showed that STHs are able to make both RQ and UQ whereas hosts make UQ alone because of differences in the core of the key polyprenyltransferase enzyme COQ-2 (Tan et al., 2020). COQ-2 is highly conserved and almost all eukaryotes share the same COQ-2 core. However, we found that STHs use an alternative splicing event to incorporate two key amino acid changes into the core (Tan et al., 2020). Alternative splicing of two mutually exclusive exons allows STH COQ-2 to be able to use either 4-hydroxy benzoate (4HB) as a substrate for UQ synthesis or 3-hydroxy anthranilate for RQ synthesis — other eukaryotes can only use 4HB. Thus, the requirement to make or use RQ as well as UQ has resulted in STH-specific active sites of several key enzymes. These STH-specific active sites make them ideal targets for STH-specific inhibitors which could result in new classes of anthelmintic drugs. We reasoned that other enzymes that are required for RQ-dependent metabolism may have had similar active site changes and thus could also be targets for STH-specific inhibitors. Our goal in this study is to identify such targets and we take a

two-step approach to identify parasite proteins as high confidence targets for inhibitors of RQ-dependent metabolism for *in vitro* or *in silico* screens.

We initially assembled a set of rationally-chosen candidate genes that are predicted to act in one of four aspects of RQ-dependent metabolism: how the helminth senses the need to switch from UQ to RQ-dependent energy metabolism; how it makes RQ; the quinone-coupled dehydrogenases (QDHs) that directly interact with RQ; and other metabolic enzymes downstream of QDHs that are required for efficient RQ-dependent metabolism (see Figure 1 for schematic). In our two-step approach, we first experimentally test which of these candidate genes is required for RQ-dependent metabolism. We do this using an assay that we previously described that allows us to assess the ability of *C.elegans* to use RQ-dependent metabolism. This step identifies potential anthelmintic targets: if we could specifically block their activity without affecting the host, they would kill the parasite *in vivo*. In our second step, we use computational analyses to determine which of the genes we identify in our experimental testing have helminth-specific changes in druggable regions of the protein — for example, changes surrounding the active site of an enzyme. This second step is critical since RQ-dependent metabolism does not use a dedicated suite of helminth-specific genes but is largely a rewiring of highly conserved genes — indeed almost all of the candidate genes have host orthologues. While most helminth orthologues of human genes show sequence divergence, enzymes often have a high degree of similarity and in particular the active sites tend to be highly conserved. This makes the development of helminth-specific inhibitors very challenging. We therefore combine multi-species sequence alignments with structural modeling to identify which of the potential targets that we identify from our experimental screening have helminth-specific sequence changes at or near the active sites. These would then be the highest priority targets for *in vitro* screens for

inhibitors that can block the unusual RQ-dependent metabolism that parasitic helminths rely on to survive in their host without affecting any host enzymes.

In this paper, we tested 25 candidate genes for their requirement for RQ-dependent metabolism in *C.elegans*. We found 9 genes to be required for *C.elegans* to carry out efficient RQ-dependent metabolism. These include sensors that regulate the switch from UQ-dependent metabolism to RQ-dependent metabolism, enzymes required for RQ synthesis, and a quinone-coupled dehydrogenase. Using sequence alignment and structural modeling we show that 3 of these have active sites that differ between STH and host — these are high confidence targets since the STH-specific active site should allow the development of STH-specific inhibitors that could act as new classes of anthelmintics. We also identify an additional STH-specific target that has no host homologue and that is essential for helminth survival. We believe that this work provides an excellent platform for *in vitro* and *in silico* screens for compounds that block these key enzymes in STHs without affecting their hosts.

A set of candidate genes with potential roles in RQ-dependent metabolism

We previously developed an image-based assay that allows us to test whether any particular gene is required for RQ-dependent metabolism (Del Borrello et al., 2019; Spensley et al., 2018). If so, it is a potential target for anthelmintics since inhibiting its function would kill the STH. In brief outline, we expose *C.elegans* L1 larvae to potassium cyanide (KCN) for 15 hours — KCN prevents oxygen binding to Complex IV of the mitochondrial electron transport chain (ETC) and thus blocks oxidative phosphorylation. As we previously showed (Del Borrello et al., 2019), this forces *C.elegans* to rely on anaerobic RQ-dependent metabolism and wild-type worms readily survive 15 hours of KCN treatment and rapidly recover full movement after removal of KCN.

Worms that cannot make RQ, such as animals with mutations in the gene *kynu-1*, cannot survive and thus show no recovery of movement once KCN is removed (Del Borrello et al., 2019). This assay is powerful but does not have sufficient throughput to carry out genome-scale screens and thus we were forced to take a candidate gene approach. Fortunately, much is known about the pathways that may regulate or be required for RQ-dependent metabolism and thus we manually assembled a set of candidate genes to test for their involvement in RQ-dependent metabolism. These genes are all potential targets for anthelmintics and are predicted from previous literature to act in one of 4 different aspects of RQ-dependent metabolism (shown schematically in Figure 1; genes are listed in Table 1).

First, when helminths move from the aerobic environment outside their host to the anaerobic environment of some host tissues, they must sense the change in environment correctly to switch from UQ to RQ-dependent metabolism (Komuniecki and Harris, 1995). Interfering with this sensor could thus block the ability of parasites to activate RQ-dependent metabolism. Second, in response to the change in environment, parasites alter their quinone synthesis pathways to make more RQ and less UQ. For example, ~14% of the quinone in free-living miracidia-stage *Fasciola hepatica* is RQ, but ~93% is RQ in the adult stage in the host (Van Hellemond et al., 1996). Blocking RQ synthesis will block RQ-dependent metabolism and this is a key potential target for anthelmintics. Third, once RQ has been synthesised, it affects metabolism principally by directly docking to the quinone-coupled dehydrogenases (QDHs) that are linked to the mitochondrial ETC (Nonato et al., 2019; Yamashita et al., 2004). If a compound blocks the docking of RQ to the critical QDHs, this should block RQ-dependent metabolism. There are multiple QDHs that link to the ETC and we must test which are required for RQ-dependent metabolism and thus which are the potential targets. Finally, multiple other metabolic enzymes are required during

RQ-dependent metabolism downstream of the interaction between RQ and QDHs (Müller et al., 2012). By screening 25 candidate genes in our RQ-dependent metabolism assay, we aim to find a set of genes that are essential for *C.elegans* to carry out anaerobic RQ-dependent metabolism. We describe the data from these assays on each of the four classes of genes below.

The switch to RQ-dependent metabolism is not a classic HIF-1-dependent hypoxia response

Parasitic helminths switch from aerobic UQ-dependent metabolism to anaerobic RQ-dependent metabolism when they are exposed to prolonged hypoxia. They must sense this change in environment and blocking the hypoxia response might prevent helminths from switching into anaerobic metabolism. The hypoxia-sensing machinery is thus an excellent potential target for anthelmintics.

The machinery for sensing hypoxia has been well-described in *C.elegans* (Benita et al., 2009; Epstein et al., 2001; Lee et al., 2010; Powell-Coffman, 2010; Shen et al., 2006, 2005) and requires the core hypoxia-regulated transcription factor HIF-1 (Benita et al., 2009; Shen et al., 2005) along with its negative regulators EGL-9 (Epstein et al., 2001; Shao et al., 2009) and VHL-1 (Bishop et al., 2004; Shen et al., 2006), and the atypical splicing regulator IRE-1 (Chen et al., 2009; Mao and Crowder, 2010) and its target XBP-1 (Mao and Crowder, 2010), which respond to an increase in unfolded proteins in the ER due to altered redox levels. We used our assay to examine whether loss-of-function mutations in this core hypoxia-sensing pathway affected the ability of *C.elegans* to carry out RQ-dependent metabolism. If the hypoxia-sensing machinery is required for the switch from UQ-dependent aerobic metabolism to RQ-dependent anaerobic metabolism, mutants lacking the ability to sense hypoxia should be unable to survive 15 hours of KCN exposure.

We examined a set of 4 strains (see Table 1) containing mutations in the hypoxia sensing machinery and found no requirement for the core hypoxia-sensing pathway for *C.elegans* to survive long-term exposure to KCN (Figure 2a). Worms homozygous for loss-of-function mutations in *hif-1* can survive 15 hours of KCN treatment as well as wild-type animals, as can strains with loss of function mutations in *ire-1* or *xbp-1*. This was a surprising result to us as we had expected that KCN treatment would activate a *hif-1*-dependent hypoxia response. To test whether KCN addition caused activation of *hif-1* target genes, and thus invoked a *hif-1*-dependent hypoxia response, we examined induction of expression of *nhr-57*, a well characterized transcriptional target of HIF-1 (Miyabayashi et al., 1999). We find that doses of KCN up to ~50 μ M result in potent induction of *nhr-57* expression as measured using a fluorescent transcriptional reporter (Figure 2b). Wild-type animals initially slow their movement when exposed to these lower doses of KCN, but subsequently recover full movement within ~3 hours; this acute recovery is *hif-1*-dependent (Figure 2c). However, at higher KCN doses (200 μ M), we see no induction of *nhr-57* expression and no difference in movement between wild-type and *hif-1(ia4)* mutant animals — both strains slow their movement and become immobile after ~90 mins (Figure 2d). It thus appears that at lower KCN doses, worms invoke a *hif-1* transcriptional response that allows them to recover from acute KCN treatment, and this recovery is not RQ-dependent. At higher doses of KCN, worms do not activate *hif-1* but instead enter an immobile state where they rely on RQ-dependent metabolism. We suggest that the higher doses of KCN that are needed to drive worms to use RQ-dependent metabolism are mimicking anoxia rather than hypoxia. Consistent with this, *C.elegans* is known to be able to survive long periods of anoxia (LaRue and Padilla, 2011; Mendenhall et al., 2006; Menuz et al., 2009; Peña et al., 2016; Voorhies and Ward, 2000) and *hif-1* is not required for this survival (Padilla et al., 2002).

Our data show that the switch to RQ-dependent metabolism does not require the core hypoxia-sensing pathway. Compounds that block the core hypoxia-sensing pathway are thus unlikely to affect the ability of STHs to activate RQ-dependent metabolism and this is a useful negative result since the hypoxia sensing machinery appeared an obvious candidate target for this. Biologically, it is also important: if helminths do not use the core hypoxia pathway to induce the switch to RQ-dependent metabolism in hypoxic conditions, what is the switch? We address this in the next section.

RQ-dependent metabolism is activated in response to metabolic consequences of anoxia

We found that the core hypoxia-sensing machinery is not involved in the switch to RQ-dependent metabolism. We reasoned that if *C.elegans* is not sensing levels of oxygen directly it might sense the consequences of reduced oxygen instead. Hypoxia has diverse effects on the organism including changes in metabolism (Braeckman, 2009). One immediate consequence of the inability of *C.elegans* to use the aerobic mitochondrial ETC is a reduction in efficiency of ATP generation and thus a likely drop in ATP levels and growth rate. Reduced ATP levels are sensed by AMP-activated kinase (Hardie et al., 2003), a heterotrimeric kinase which in *C.elegans* requires the *aak-1* and *aak-2* encoded catalytic subunits (Apfeld et al., 2004; Narbonne and Roy, 2009, 2006) and we also note that AMPK activity is required for *C.elegans* to survive anoxia (LaRue and Padilla, 2011). We find that *C.elegans* requires AMPK activity to efficiently activate RQ-dependent metabolism (Figure 2a) suggesting that the key switch is not a lack of oxygen per se but the metabolic consequences of reduced oxygen levels. We also examined whether the mTOR pathway is required for the shift to RQ-dependent metabolism. The mTOR complex is required for sensing whether there is sufficient nutrient availability to drive growth and is regulated by AMPK phosphorylation (Blackwell et al., 2019; Howell et al., 1987; Long et

al., 2002). *rikt-1* encodes the *C.elegans* orthologue of RICTOR (Jones et al., 2009; Soukas et al., 2009), a core component of the mTOR complex and loss-of-function mutations in *rikt-1* (Figure 2a) prevent RQ-dependent recovery from KCN. We thus find that *C.elegans* also requires the mTOR complex for efficient activation of RQ-dependent metabolism. We conclude that *C.elegans* activates RQ-dependent metabolism via both AMPK and mTOR in response to dropping ATP levels in anoxic conditions.

Finally, we examined whether *sgk-1* is required for the switch to RQ-dependent metabolism. *sgk-1* encodes the serum and glucocorticoid induced kinase (Hertweck et al., 2004; Jones et al., 2009) and is involved in maintaining mitochondrial homeostasis (Gatsi et al., 2014), in mitochondrial stress responses (Chen et al., 2013), and it affects sensitivity to paraquat which generates Reactive Oxygen Species (Aspernig et al., 2019; Tullet et al., 2008). We found that *sgk-1* is also required for *C.elegans* to use RQ-dependent metabolism (Figure 2a) — worms homozygous for the *sgk-1(ok538)* loss-of-function mutation show reduced recovery after 15 hours of KCN exposure.

Together, our findings here indicate that the switch from UQ-dependent aerobic metabolism to RQ-dependent metabolism is not regulated by the core hypoxia-sensing machinery but instead requires the AMPK/mTOR machinery that senses nutrient levels including ATP levels and the SGK-1 kinase that responds to a variety of cellular stresses. AAK-2, RICT-1 and SGK-1 are all required for *C.elegans* to enter RQ-dependent metabolism and thus these three proteins are potential targets. We next examined each protein to identify whether there were specific residues that are identical across all helminths and that differ from those in their mammalian hosts and whether any of those ‘helminth-specific’ residues reside at or near the active site.

AAK-1 and AAK-2 belong to a complex gene family whose evolutionary relationships were too complex for the method used here (Figure 2 - Figure Supplement 1-3). In contrast, both RICT-1 and SGK-1 have clear evolutionary trees and conserved divergent residues between hosts and parasites. RICT-1 shows divergence between hosts and STHs in 22 residues. However, these are scattered throughout the protein and are not close to any binding interface or active site (Figure 2 - Figure Supplement 4). SGK-1 has 7 residues that differ between STHs and hosts and three are near the active site. Two of these (residues 130 and 140 in *C.elegans*) are close to the active site though not directly at the active site but Phe136 is right at the active site (Hammond et al., 2009; Zhao et al., 2007) suggesting it might be possible to generate an inhibitor that would block the STH SGK-1, disrupting the switch to RQ-dependent metabolism, without affecting the host orthologue. We note however that this active site change is not RQ-specific since it is present in species that do not make or use RQ (Figure 2 - Figure Supplement 7). These residues changes may or may not be sufficient to enable a helminth-specific SGK-1 drug. Taken together, while AMPK, mTOR, and SGK-1 all appear required for the efficient activation of RQ-dependent metabolism, only SGK-1 has STH-specific residues near the active site and these are not uniquely associated with RQ metabolism. We thus conclude that there is no high confidence target for anthelmintics within the ‘sensors’ regulating the switch from UQ to RQ-dependent metabolism, though SGK-1 is perhaps still tractable.

Multiple enzymes required for RQ synthesis have helminth-specific active sites

We and others previously showed that UQ and RQ synthesis share a similar pathway (Del Borrello et al., 2019; Roberts Buceta et al., 2019). The critical difference in UQ and RQ synthesis is the precursor — UQ synthesis in animals uses 4-hydroxybenzoate (4HB) whereas

RQ synthesis starts with 3-hydroxyanthranilate (3HA) as the precursor (Del Borrello et al., 2019; Roberts Buceta et al., 2019). A crucial early step in synthesis of these distinct quinones is the prenylation of either of these two precursors by the polyprenyltransferase COQ-2 — the substrate choice by COQ-2 is the key event that dictates whether UQ or RQ will be synthesised (Tan et al., 2020). We recently found that the choice of substrate for COQ-2 is determined by a single alternative splicing event which changes the enzyme core from a conserved 4HB-utilizing enzyme to a helminth-specific 3HA-preferring enzyme (Tan et al., 2020). The splice variant that uses 3HA as its preferred substrate has two helminth-specific residues deep in its active site making it an attractive drug target since it should be possible to identify inhibitors that specifically inhibit this RQ-synthesising form.

3HA is generated by metabolism of tryptophan via the kynurenine pathway (see Figure 3a) (Mason and Berg, 1952; Takeuchi et al., 1980). In *C.elegans*, the kynurenine pathway requires the genes *tdo-2*, *kmo-1*, *amfd-1* and *kynu-1* and we previously showed that *kmo-1*, *amfd-1* and *kynu-1* are required for RQ synthesis (Del Borrello et al., 2019). These 3 enzymes are thus potential targets for anthelmintics — if we could identify a STH-specific inhibitor of any of these, it should block 3-HA synthesis and thus block RQ synthesis. Comparative genomics and structural modeling showed that the sole potential candidate is KMO-1 where there is a helminth-specific change in a critical region required for binding of kynurenine. This Tyr334Phe is not a major change so we do not believe it to be a high priority target (Figure 3 - Figure Supplement 7-8). Our structural analyses did not find STH-specific residues at the active site of KYNU-1 (Figure 3 - Figure Supplement 9-10) while AMFD-1 protein sequences were too divergent from each other for our analysis pipeline (Figure 3 – Figure Supplement 3). This suggests that it will be hard to identify STH-specific inhibitors to these 3 enzymes. We therefore

turned our attention to Tryptophan Dioxygenase (TDO; TDO-2 in *C.elegans*) which catalyses the initial step in the kynurenine pathway (Hayaishi et al., 1957; Heidelberger and Gullberg, 1948; Kotake and Iwao, 1931), the oxidation of L-tryptophan to N-formyl-L-kynurenine.

We found that like *kmo-1*, *amfd-1* and *kynu-1*, *tdo-2* is also required for survival in conditions where RQ-dependent metabolism is required, confirming that it is also a potential target for anthelmintics (Figure 3b). Intriguingly, TDO requires molecular oxygen which is complexed to a haem molecule in TDO (Basran et al., 2008; Capece et al., 2010). Since RQ synthesis (and thus kynurenine pathway activity) is required under low oxygen conditions, it is possible that helminth TDO has diverged to allow its activity in low oxygen and we note that other oxygen-binding proteins are known to have diverged in helminths. We therefore examined TDO sequence and structure in hosts and helminths. We identified 4 residues that consistently differ between helminths and their hosts (Figure 3c). We note that all 4 are 100% conserved across helminths but also are 100% identical between helminths, molluscs and annelids. These are the three sets of animals that are known to make and use RQ, suggesting that these 4 residues may be critical for TDO activity in low oxygen environments. One is of particular interest since it sits at the active site close to the haem and tryptophan binding site in a small loop (Figs 3d and 3e). This is the same region where characterized TDO inhibitors are known to bind (Dolušić et al., 2011; Lewis-Ballester et al., 2016; Michels et al., 2016) and which is required for the enzymatic activity of TDO-2 in *C.elegans* (Michels et al., 2016). Helminths and hosts thus differ in a single critical residue at the active site of TDO and we suggest that this single amino acid difference may make it possible to develop TDO inhibitors that will be helminth specific and that could block the generation of RQ precursors and thus block the synthesis of RQ itself.

Finally, we also examined COQ-3 and COQ-5, two genes downstream of COQ-2 in the pathway for RQ synthesis. Both these genes were previously shown to be required for RQ synthesis and thus are potential targets (Roberts Buceta et al., 2019). However, RNAi knockdown of *coq-3* and *coq-5* did not greatly affect the ability of the worm to carry out RQ-dependent metabolism (Figure 3 - Figure Supplement 2) and neither COQ-3 nor COQ-5 has helminth-specific residues at the active site (Figure 3 - Figure Supplement 4-6). COQ-5 has no obvious helminth-specific residues that differ consistently between host and helminth. COQ-3 has a single such residue but this is far from the active site (Figure 3 - Figure Supplement 5) and thus is unlikely to result in a helminth-specific inhibitor. We conclude that these are not good anthelmintic targets.

We have thus identified two excellent targets for potential anthelmintics in the RQ synthesis pathway: COQ-2 and TDO-2. Both are required for RQ synthesis and thus for RQ-dependent metabolism and both have helminth-specific residues at the active site.

Two Quinone-coupled dehydrogenases are required for RQ-dependent metabolism

The direct consequence to energy metabolism of switching the main electron-carrying quinone from UQ to RQ is at the level of the quinone-coupled dehydrogenases (QDHs) that are connected to the mitochondrial electron transport chain (ETC) (Müller et al., 2012). In most animals, these QDHs allow electrons to enter the ETC downstream from Complex I and onto the UQ pool to participate in oxidative phosphorylation. In helminths, however, these QDHs can act as either entry points or exit points for electrons. In aerobic conditions, they act as dehydrogenases where they shuttle electrons into the ETC by transferring electrons from substrates onto UQ, just as in other animals; however, in anaerobic conditions, they reverse direction and act as reductases where they transfer electrons from RQ and out of the ETC (Iwata

et al., 2008; Takamiya et al., 2002; Tielens and Van Hellemond, 1998; Van Hellemond et al., 1995) (Figure 4a).

QDHs are excellent candidate targets for anthelmintics since they are the direct site of action of RQ. If we could selectively inhibit the QDHs that are required for RQ-dependent metabolism, we would kill the helminth parasite. Helminth QDHs have different quinone-binding requirements to those of their hosts: the only quinone that interacts with host QDHs is UQ but QDHs in helminths must permit both UQ and RQ to dock at their quinone-binding pocket (Van Hellemond et al., 1995). In Complex II, this difference in quinone binding requirement between host and STH is known to have driven the divergence of the quinone-binding pocket and this has allowed the identification of Complex II inhibitors (Burns et al., 2015; Osanai et al., 2009) that are highly selective for STH Complex II. These inhibitors include flutolanil (Osanai et al., 2009) and wact-11 (Burns et al., 2015) and these inhibitors bind directly at the quinone-binding pocket (Burns et al., 2015; Osanai et al., 2009). We reasoned that if it is possible to generate inhibitors that selectively bind to the quinone binding pocket of Complex II, it may be possible to find inhibitors that selectively bind to the quinone binding pocket of other QDHs that use both UQ and RQ in helminths.

There are 5 QDHs in animals that are linked to the mitochondrial ETC. These comprise Succinate dehydrogenase (also known as Complex II) (Kita et al., 1988; Kmetec and Bueding, 1961; Thunberg, 1909; Van Hellemond et al., 1995) ; Electron-Transferring Flavoprotein (ETF) dehydrogenase (Beckmann and Frerman, 1985b, 1985b; Komuniecki et al., 1989; Ruzicka and Beinert, 1975); Proline dehydrogenase (Abrahamson et al., 1983; Ertel and Isseroff, 1974; McNamer and Stewart, 1974); Glycerol 3-phosphate dehydrogenase (Green, 1936; Mráček et al., 2013; Rogers, 1976); and Dihydroorotic acid dehydrogenase (Miller et al., 1968). We note that

there are other quinone-coupled enzymes in animals including sulphide:quinone oxidoreductase (*sqrd-1* in *C.elegans* (Budde and Roth, 2011)) as well as orthologues of D- and L-hydroxyglutarate dehydrogenase (Achouri et al., 2004; Kranendijk et al., 2012; Lin et al., 2015) (D2HGDH and L2HGDH; *F45D5.12* and *Y45G12B.3* respectively). These are not thought to have any role in energy metabolism, our key focus here, and thus we have not pursued these in this study. We previously showed that Complex II is required for RQ-dependent metabolism in *C.elegans* and showed that helminth-specific residues in the quinone-binding pocket of Complex II in particular are important for RQ utilisation (Del Borrello et al. 2019). However, we do not know which of the other QDHs may also be required and thus we examined the requirement for each QDH in our RQ-dependent metabolism assay.

We used RNAi to examine which of the QDHs are required for RQ-dependent metabolism using our RQ assay. We found that in addition to Complex II, ETFDH is also required for survival for 15 hours in KCN where worms rely on RQ-dependent metabolism (Figure 4b, Figure 4 – Figure Supplement 1). Complex II acts as a fumarate reductase in these conditions, transferring electrons from the RQ pool and generating succinate as a product. ETFDH links BCAA and fatty acid metabolism to the ETC during UQ-dependent aerobic metabolism (Beckmann and Frerman, 1985a, 1985b; Ramsay et al., 1987; Ruzicka and Beinert, 1975) — when acting in reverse during RQ-dependent anaerobic metabolism, it transfers electrons from the RQ pool and generates BCAAs and short chain fatty acids including 2-methyl valerate and 2-methyl butanoate (Komuniecki et al., 1989; Ma et al., 1993). Since Complex II has been extensively studied as an anthelmintic target, we focused our attention on ETFDH.

We used sequence alignment to identify amino acids that consistently differ in ETFDH between hosts and helminths (see Figure 4c) and find 5 such residues i.e. all helminths have one

sequence, all hosts have a different sequence. We modelled *C.elegans* ETFDH (LET-721) on the *Sus scrofa* ETFDH (PDB:2GMH) crystal structure (Zhang et al., 2006) to see where these helminth-specific amino acids sit. While some are dispersed around the structure, one of these host-helminth changes is located in the quinone-binding pocket of ETFDH (Figure 4d). It is a significant change: while hosts have a redox-sensitive cysteine at this position, helminths have the aromatic amino acid phenylalanine that may aid in charge delocalisation. This change right in the quinone-binding pocket suggests that just as it has been possible to identify potent helminth-specific succinate dehydrogenase inhibitors that bind to the quinone-binding pocket of Complex II, it will be possible to identify helminth-specific ETFDH inhibitors that bind to the quinone-binding pocket of ETFDH. ETFDH is thus a high confidence molecular target for anthelmintics.

Metabolic enzymes that change directionality in RQ-dependent metabolism

One of the key features of the switch from UQ-dependent aerobic metabolism to RQ-dependent metabolism is the rewiring of a subset of core metabolic reactions (Butler et al., 2012; Müller et al., 2012; Yilmaz and Walhout, 2016). This often takes the form of a reverse in the direction of a reaction. The canonical example is that of Complex II which uses succinate as a substrate and generates fumarate as a product during aerobic conditions; in anaerobic conditions, it uses fumarate as a substrate and generates succinate as a product (Kmetec and Bueding, 1961; Saz and Vidrine, 1959). There is a similar reverse in reaction direction for ETFDH and as we showed in this paper, ETFDH is also critical for RQ-dependent metabolism. This switch in the direction of core reactions is not unique to quinone-coupled dehydrogenases and other helminth enzymes also switch their direction during RQ-dependent metabolism. The best characterized are those

that involve the TCA cycle and specifically the reactions that lead to the generation of malate and fumarate and this is where we focus.

Malate metabolism is a key decision point between the aerobic and anaerobic TCA cycles in helminths. In aerobic conditions, malate is largely converted to oxaloacetate (OAA) by mitochondrial malate dehydrogenase (MDH-2 in *C.elegans*) as part of a normal animal TCA cycle (Minárik et al., 2002). In anaerobic conditions, however, malate is converted to fumarate by the enzyme fumarase (FUM-1 in *C.elegans*) acting in the reverse direction to the standard aerobic TCA cycle. Generation of fumarate is crucial since it acts as the substrate for RQ-coupled Complex II which is essential for RQ-dependent metabolism. Malate can be generated from a variety of substrates including from fumarate from fumarase (FUM-1), from OAA by reversal of malate dehydrogenases, from pyruvate either directly by the action of malic enzyme (MEN-1 in *C.elegans* (Shaye and Greenwald, 2011)) or via OAA through the action of pyruvate decarboxylase (PYC-1 in *C.elegans* (Liao and Freedman, 2001; Shaye and Greenwald, 2011)).

We used a combination of RNAi and classical genetics to test whether *fum-1*, *mdh-1*, *mdh-2*, *pyc-1* and *men-1* are required for RQ-dependent metabolism. In addition, we tested two genes, *F36A2.3* and *VF13D12L.3* that encode members of a distinct subfamily of malate dehydrogenases (Fuchs et al., 2010; McElwee et al., 2006) that are typically found in archaeal genomes. Importantly for this study, these genes have no host homologues but are present in all STHs making them an attractive target.

As shown in Figure 5a, we find that none of the genes tested appear to be required for RQ-dependent metabolism. There are a number of caveats to this. First, *fum-1* and *mdh-2* are lethal by RNAi so we could not assess their involvement. This is not unexpected — they are required for the TCA cycle which is essential in both aerobic and anaerobic conditions. Second, *F36A2.3*

and *VF13D12L.3* are closely related and the lack of a strong effect of these genes on RQ-dependent metabolism might be due to paralogue redundancy. We therefore used CRISPR genome engineering to make a presumptive null mutation in *VF13D12L.3* and carried out RNAi against *F36A2.3* in a strain homozygous for the *VF13D12L.3* null allele to reduce activity of both paralogues at once — we saw no effect on RQ-dependent metabolism (data not shown). This could reflect the lack of requirement for these genes, but could also be the result of poor knockdown of *F36A2.3* by RNAi. We suspect this to be the case since we used CRISPR engineering to make a presumptive null mutation in *F36A2.3* and found that this is lethal. The fact that the mutant phenotype is lethal but the RNAi phenotype is not suggests that the *F36A2.3* RNAi is only giving a weak knockdown. It is intriguing to note that *F36A2.3* does not show any reproducible strong effect on RQ-dependent metabolism by RNAi but has a variable phenotype — some biological repeats show strong effects, others very little. This is the sole gene for which we saw such variability and variable RNAi phenotypes can often result from weak or incomplete RNAi knockdown. We would thus not feel confident either excluding it as a possible target or including it as a high priority target based on this weak data. Overall, we conclude that we did not find that any of these metabolic enzymes to be definitely required for RQ-dependent metabolism.

More positively, the finding that *F36A2.3* is an essential gene in *C.elegans* suggests that this malate dehydrogenase is a good target for anthelmintics. All animals have both cytoplasmic and mitochondrial malate dehydrogenases (*mdh-1* and *mdh-2* in *C.elegans*). However, a small number of animal species have an additional family of malate dehydrogenases that is closely related to those found in archaea (see Figure 5b). Intriguingly, no STH hosts have this third class of malate dehydrogenase but all species that make RQ (helminths, annelids, and molluscs) have

these. Crucially, since there is no similar gene in any STH host and since *F36A2.3* is an essential gene in *C.elegans*, it is an excellent target for new anthelmintics.

In addition to these changes in reaction direction, helminths can also truncate their TCA cycles to avoid the two steps where carbon atoms are lost in the form of CO₂. This may be beneficial during times of limited carbon source availability (Komuniecki and Tielens, 2003). These steps occur in the serial conversion of isocitrate to alpha-ketoglutarate (α -KG) and α -KG to succinyl-CoA. The truncated TCA cycle that avoids these steps requires the isocitrate lyase ICL-1 to drive the glyoxylate shunt (Liu et al., 1995; Wadsworth and Riddle, 1989). Plants, fungi and helminths can also carry out the glyoxylate shunt (Barrett et al., 1970; Galbraith and Smith, 1969; Kornberg and Beevers, 1957) but hosts have no ICL-1 homologues and thus cannot do this. ICL-1 has thus previously been proposed as a potential anthelmintic target (Butler et al., 2012; Tyagi et al., 2019). We examined whether ICL-1 was required for RQ-dependent metabolism. As shown in Figure 5a, worms homozygous for a loss of function mutation in *icl-1* were no different to wild-type animals in their ability to carry out RQ-dependent metabolism and we conclude ICL-1 is not required.

Together, we conclude that while there are extensive metabolic changes downstream of quinone-coupled dehydrogenases that occur during RQ-dependent metabolism, none of the enzymes involved appear to be individually required for efficient RQ-dependent metabolism. They are thus not worthwhile targets for drugs designed to block RQ-dependent metabolism. We suggest that this may be because there are many parallel routes for the generation of the key metabolite malate including through multiple different malate dehydrogenases and malic enzyme and that inhibiting any single route has little effect. The sole target we identified here is *F36A2.3* which appears to be an essential gene rather than having a dedicated role in RQ-dependent metabolism.

Since there is no host homologue for this unusual malate dehydrogenase, it is an excellent target for anthelmintics.

Functional significance of STH-specific residues

We identified 4 enzymes that are required for RQ-dependent metabolism and that also have helminth-specific residues at their active sites: Complex II, ETFDH, COQ-2e and TDO-2. These are all high confidence targets for anthelmintics since it should be possible to identify compounds that inhibit the helminth and not the host enzyme and thus block RQ-dependent metabolism without affecting the host. We previously showed that mutations that affect the helminth-specific binding pocket of Complex II can block RQ-dependent metabolism and wanted to specifically test the functional significance of the helminth-specific residues in the other three targets we identified. We did this in two ways.

First, we looked at the phylogenetic distribution of the amino acids that differ between STHs and their hosts. These differences at the active sites could have arisen through random genetic drift and have no functional significance. Alternatively, the amino acid changes seen at the active sites of the STH enzymes may be important for their function in RQ-dependent metabolism — if so we would expect to see the same residues in the other animal lineages that use RQ, molluscs and annelids. If they are simply the result of genetic drift, we would not expect this distribution. We see identical amino acids in the active sites of COQ-2e, TDO-2, and ETFDH in STHs, annelids, and molluscs but not in any of the STH hosts. This suggests that these residues may be functionally important for the ability to do RQ-dependent metabolism.

Second, we used genetics to directly test whether the identified residues are critical for the ability to carry out RQ-dependent metabolism. For COQ-2, we had previously shown that all RQ-using

animals make an unusual splice form (COQ-2e in *C.elegans*) that includes the STH-specific residues (Tan et al., 2020), but did not confirm the functional importance of these two amino acids for RQ-synthesis. Here, we used CRISPR genome engineering to change these two residues in the COQ-2a isoform. COQ-2a has the pan-eukaryotic active site and only participates in UQ synthesis; we previously showed that animals that can only make the COQ-2a isoform cannot make RQ and thus cannot survive in conditions where RQ-dependent metabolism is required. However, here we generated a *C.elegans* strain in which animals cannot make the RQ-synthesising COQ-2e isoform but can only make a CRISPR-edited version of COQ-2a where two amino acids in COQ-2a are changed from the pan-eukaryotic consensus to the STH-specific residues in the COQ-2e splice form (see Figure 6a for schematic). If these two residues are indeed sufficient for RQ synthesis, this strain that expresses only the edited COQ-2a form should now be able to make RQ and thus to survive in conditions where RQ is required. We find that mutant worms with this edited COQ-2a form can survive where RQ is required (Figure 6b), suggesting that this mutant can now make RQ. This demonstrates that these two STH-specific residues are indeed critical for RQ synthesis. In the case of TDO-2, we took advantage of an existing mutant, *tdo-2(zg218)* (Michels et al., 2016). The critical STH-specific amino acid in the active site, P133 in *C.elegans* TDO-2, is in a short 3 amino acid loop (PLD, residues 133-135 in *C.elegans*). Crucially, the mutant strain *tdo-2(zg218)* has been engineered to delete only that PLD loop (Michels et al., 2016). We find that *tdo-2(zg218)* animals with the deleted PLD loop cannot carry out RQ-dependent metabolism confirming the functional importance of this STH-specific region (Figure 6c). Finally for ETFDH, we also used CRISPR genome engineering to switch the single STH-specific amino acid in the quinone binding pocket to the host consensus. We find that this single change reduces the ability of *C.elegans* to carry out RQ-dependent

metabolism, confirming its functional importance, though we note the effect is weaker (Figure 6d shows the recovery from three different doses of KCN).

Taken together, both the phylogenetic distribution of the active site amino acid changes and the direct functional testing show that not only do STHs have changes at the active sites of COQ-2e, TDO-2, and ETFDH, compared to their hosts but these active site changes are critical for their ability to use RQ-dependent metabolism. These three enzymes are thus high confidence targets for new anthelmintics.

Discussion

Soil transmitted helminths (STHs) are major human pathogens and anthelmintics like ivermectin (Campbell, 2012; Campbell et al., 1983) benzimidazoles (Brown and Zweifel, 1961), and levamisole (Janssen, 1976) are listed by the WHO as essential medicines. These anthelmintics are powerful but there is a limited number of drug classes that are widely available and resistance is rising to these in both human and animal populations. New classes of anthelmintics are thus needed to combat these pathogens.

There are two fundamental strategies to identify potential new anthelmintics. The first is unbiased screening for compounds that can kill helminths but that do not affect their hosts. This type of unbiased screening has been extremely successful and is the source of all major classes of anthelmintics to date. The screens for these compounds were not directed at any specific helminth target proteins and targets were elucidated afterwards largely through genetic screens. The second, alternative approach would be to search for compounds that inhibit a pre-defined

helminth target protein without targeting any host proteins. Such target-directed approaches allow powerful *in silico* docking methods to identify candidate inhibitors and such strategies have been successful in identifying drugs that specifically inhibit individual kinases that are mutated in cancers, for example (Nath et al., 2017; Sarukhanyan et al., 2020; Ye et al., 2016). However, no such target-directed approaches have been successful thus far for the identification of anthelmintics (Geary et al., 2015). One reason may be the lack of validated targets. Our goal in this study is to provide a set of validated high confidence helminth targets to allow *in silico* methods for the selection of new anthelmintics.

The critical issue for any *in silico* approach to identify potential new anthelmintics is the selection of the target. Helminth genomes typically encode over ~20,000 genes. Which of these is the best target for a potential anthelmintic? We drew insight in target selection from known anthelmintic targets. Unexpectedly, while helminth genomes contain many helminth-specific genes and gene families (“Comparative genomics of the major parasitic worms,” 2019; Gasser et al., 2016), none of these has emerged as a target of major anthelmintics to date. Instead, anthelmintics typically target proteins that are shared between parasites and hosts and their selectivity for helminths is due to subtle differences between the helminth and host protein sequences. For example, both helminths and hosts have nicotinic acetylcholine receptors (nAChRs) that are critical for signalling within the nervous system (Fleming et al., 1997; Hogg et al., 2003; Léna and Changeux, 1998). Levamisole acts as a powerful anthelmintic by binding a specific subclass of nAChR that is only found in helminths (Boulin et al., 2008; Martin et al., 2005), hence its selectivity for paralysing the parasite while leaving the host unaffected. Benzimidazoles target another highly conserved target, beta-tubulin (Barrowman et al., 1984;

Dawson et al., 1984). However, subtle sequence changes between host and helminth beta-tubulin results in high selectivity of benzimidazoles for helminths (Driscoll et al., 1989; Hahnel et al., 2018; Lacey, 1990). These studies indicate that an excellent source of potential anthelmintics targets are proteins that are conserved between host and parasite, but where small sequence differences allow the selection of compounds that only bind the parasite protein with high affinity. These are the types of targets we pursue here.

To identify potential anthelmintic targets, we focused on the difference in energy metabolism between host and parasite and how these have shaped the active sites of shared enzymes. Both hosts and STHs use the mitochondrial electron transport chain (ETC) as the primary way to power the generation of ATP under aerobic conditions. One of the core components of the ETC is the electron carrier ubiquinone (UQ) and both host and STHs use this to carry electrons from Complex I or from quinone-coupled dehydrogenases such as succinate dehydrogenase to Complex III. However, when the STHs live for extended periods in the host, they frequently inhabit niches with extremely low available oxygen and must switch from aerobic to anaerobic metabolism. They must switch from UQ to a highly related and uncommon electron carrier, rhodoquinone (RQ). This allows the STHs to continue to use a truncated ETC in the absence of oxygen as they can use a number of terminal alternative electron acceptors. Because hosts only make and use UQ whereas the parasite must make and use both RQ and UQ, several of these enzymes have undergone critical residue changes that has allowed us to identify key targets for new classes of anthelmintics.

The best characterised molecular change in STHs that is driven by the requirement to be able to use both UQ and RQ is that in the quinone binding pocket of Complex II, succinate dehydrogenase (Kmetec and Bueding, 1961; Osanai et al., 2009; Sato et al., 1972; Saz and Vidrine, 1959; Van Hellemond et al., 1997; Van Hellemond and Tielens, 1994). Amongst the multiple amino acid changes around the quinone-binding pocket of Complex II, we have previously showed that single residue mutations in this pocket can alter the ability of *C.elegans* to use RQ-dependent metabolism suggesting that these helminth-specific residues are likely critical for the docking of RQ (Del Borrello et al., 2019). The helminth-specific Complex II inhibitors flutolanil (Inaoka et al., 2015) and the related compound wact-11 (Burns et al., 2015) rely on the differences in the quinone binding pocket between STH and host Complex II because STH Complex II is required for RQ-dependent metabolism. This requirement of the enzyme for RQ-dependent metabolism and the STH-specific changes to the quinone binding pocket have made it an excellent anthelmintic target. We therefore examined other quinone-couple dehydrogenases and identified ETF dehydrogenase (ETFDH) as also being required for RQ-dependent metabolism. Excitingly, we found that STHs have a single amino acid change in the quinone-binding pocket of ETFDH relative to the host sequence suggesting that like Complex II, ETFDH may also be an excellent target for anthelmintics.

To expand our list of potential targets beyond quinone-coupled enzymes, we tested a wider set of candidate genes that all might have potential roles in RQ-dependent metabolism and thus might be targets for anthelmintics. These included genes that regulate the switching from UQ synthesis to RQ synthesis, the genes required for RQ synthesis, and the metabolic enzymes downstream of quinone-binding enzymes such as those involved in malate metabolism and the TCA cycle. To

identify high quality targets, we followed a two step selection method. We first identified which of the genes were required for RQ-dependent metabolism in an assay we developed in *C.elegans* (Del Borrello et al., 2019; Spensley et al., 2018). Genes that are required for RQ-dependent metabolism are potential targets since inhibiting their activity should kill STHs in the host. Since all the potential targets we identified have a direct host orthologue, we then used multiple species alignments to identify helminth-specific residues and then mapped them onto the known crystal structures of orthologous enzymes. This two step approach allows us to identify proteins that are both required for RQ-dependent metabolism and that also have helminth-specific active sites. Such proteins are high priority targets for *in silico* approaches to identify new anthelmintics.

In total, we identified 4 such high priority targets. Two, TDO-2 and COQ-2e, act in the RQ synthesis pathway and the other two, Complex II and ETFDH, are the direct docking sites for RQ where it can transfer electrons out of the ETC and onto terminal acceptors. All four targets are required for RQ-dependent metabolism and all four also have specific changes between STHs and their hosts in critical regions of the proteins that may allow the development of STH-specific inhibitors. If the specific changes we identify in these proteins are important for the ability to make and use RQ, we would expect similar or identical changes in the other animal lineages that make RQ, molluscs and annelids. We find that molluscs and annelids have the exact same amino acid changes in TDO-2, COQ-2e, and ETFDH as we find for STHs. While this could be the result of identical random drift in all three lineages, it is more likely related to their use of RQ as a key electron carrier. Crucially, to directly test the functional importance of these active site changes, we used genome engineering to explicitly test the importance of each of the identified residues for RQ synthesis. We find that mutating the active sites of COQ-2, TDO-2 or

Complex II from their STH-specific form to the host version abolished the ability of *C.elegans* to do RQ-dependent metabolism and mutating the single STH-specific residue in the quinone-binding pocket of ETFDH to the host form reduced RQ-dependent metabolism. Taken together this shows that the residues that differ between STHs and their hosts in the active sites of COQ-2, TDO-2, ETFDH and Complex II are functionally critical for the ability of STHs to make and use RQ. More importantly, the differences in active sites between STHs and their hosts in these critical enzymes should make it possible to use *in silico* methods to identify STH-specific inhibitors for these key enzymes. These may ultimately lead to new classes of anthelmintics which are urgently needed as resistance to current anthelmintics continues to rise.

Materials and Methods

Caenorhabditis elegans strains and maintenance

The *F36A2.3 (syb769)/hT2, VF13D12L.3.1(syb243), coq-2a(syb2949); coq-2e(syb1721)* and *let-721(syb2817)* mutant strains were generated by SunyBiotech (Fuzhou City, China) using the CRISPR-Cas9 system. The specific mutations were confirmed by DNA sequencing and details of each of these strains are provided in Supplemental Table 1. The *coq-2e(syb1721)* strain used was previously described (Tan et al., 2020) and was a kind gift from Dr. Gustavo Salinas. The *tdo-2(zg216), tdo-2(zg217)* and *tdo-2(zg218)* strains were a kind gift from Dr. Ellen Nollen (Michels *et al.* 2016). The wild-type N2 strain and all other strains listed in Table 1 were obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA), which is

funded by NIH Office of Research Infrastructure Programs (P40 OD010440). All worm stocks were maintained at 20°C on NGM agar plates seeded with the *E. coli* strain OP50 as described elsewhere (Stiernagle, 2006).

Image-based KCN assays

The 15 h KCN recovery assay was performed as previously described (Del Borrello et al., 2019; Spensley et al., 2018). In outline, L1 worms were washed from NGM agar plates and isolated by filtration through an 11 µm nylon mesh filter (Millipore: S5EJ008M04). Approximately 150 L1 worms in M9 were dispensed to each well of a 96 well plate and an equal volume of potassium cyanide (KCN) (Sigma-Aldrich St. Louis, MO) solution was then added to a final concentration of 150-250 µM KCN. Upon KCN addition, plates were immediately sealed using an aluminium seal and incubated at room temperature for 15 h on a rocking platform. After 15 h, the KCN was diluted 6-fold by addition of M9 buffer. Plates were then imaged on a Nikon Ti Eclipse microscope every 10 min for 3 h. After imaging, fractional mobility scores (FMS) were calculated using a custom image analysis pipeline (Spensley et al., 2018). For each strain, FMS scores for the KCN-treated wells were normalized to the M9-only control wells for the WT strain at the first timepoint. 2-3 technical replicates were carried out for each experiment and the final FMS scores taken from the mean of at least 3 biological replicates.

For the acute 3 h KCN assay, L1s were isolated and treated with KCN as described above. After KCN addition, the plates were immediately imaged every 5 min for 3 h. FMS scores of KCN-treated wells were then normalized to the M9-only control wells at each timepoint.

Analysis of the *nhr-57::GFP* reporter

The *nhr-57::GFP* reporter (Miyabayashi et al., 1999) was used to assay HIF-1 activity after KCN treatment. L1 worms were purified by filtration and treated with 40 µl KCN of various concentrations. Worms incubated in M9 alone were used as the control. After 1.5 h and 4.5 h of KCN treatment, worms were imaged using a Leica DMRE fluorescence compound microscope, with levamisole used as an anesthetic. All images were taken using the 40x objective.

RNAi experiments

RNAi-by-feeding was performed as previously described (Kamath et al., 2003). RNAi clones were grown overnight at 37°C in LB media with 1 mM carbenicillin and seeded onto NGM plates supplemented with 1 mM IPTG and 1 mM carbenicillin. For each RNAi knockdown, approximately 350 wild-type L1 worms were grown on NGM plates seeded with dsRNA-expressing bacteria. GFP-targeting dsRNA-expressing bacteria was used as a negative control. After one generation, worms were washed with M9 buffer and filtered to collect L1 progeny. These worms were then transferred to 96-well plates to be used in the KCN recovery assay as described above.

Sequence identification

To identify host, parasite, mollusk/annelid and invertebrate sequences, four search strategies were made using BLASTP (protein databases). The search strategies are detailed below. For each search, the first 100 sequences which met at minimum 30% query cover were selected for

further processing. Sequences were retrieved from NCBI protein and nucleotide databases and from RCSB Protein Data Bank (PDB).

For parasites, each search was made in the non-redundant protein sequences (nr) database against *Fasciola hepatica* (taxid:6192), *Ancylostoma ceylanicum* (taxid:53326), *Strongyloides ratti* (taxid:34506), *Brugia malayi* (taxid:6279), *Trichuris suis* (taxid:68888), *Haemonchus contortus* (taxid:6289) and *Ascaris suum* (taxid:6253). For hosts as well as *C.elegans*, each search was made in the reference proteins (refseq_protein) database against *Caenorhabditis elegans* (taxid:6239), *Ovis aries* (taxid:9940), *Bos taurus* (taxid:9913), *Mus musculus* (taxid:10090), *Sus scrofa* (taxid:9823) and *Homo sapiens* (taxid:9606). For mollusks and annelids, each search was made in the nr database against *Crassostrea gigas* (taxid:29159), *Biomphalaria glabrata* (taxid:6526) and *Capitella telata* (taxid:283909). In order to capture a broad select of other invertebrates, the search strategy used the refseq_protein database included *Insecta* (taxid:50557), *Arachnida* (taxid:6854), *Mollusca* (taxid:6447), *Crustacea* (taxid:6657), *Anthozoa* (taxid:6101), *Onychophora* (taxid:27563), *Xiphosura* (taxid:6845), *Cnidaria* (taxid:6073), *Echinodermata* (taxid:7586) and *Porifera* (taxid:6040) while excluding *Vertebrata* (taxid:7742), *Crassostrea* (taxid:6564), *Biomphalaria* (taxid:6525), *Capitella* (taxid:51293) and *Drosophilidae* (taxid:7214).

The crystal structures were found on PDB by searching for the name of the enzyme and finding the best match, preferentially in a host or parasite and/or one bound with the ligand(s) or an inhibitor.

Sequence analysis

Sequences were aligned using Clustal Omega (Sievers et al., 2011) from the R package msa (Bodenhofer et al., 2015) then ModelTest-NG (Darriba et al., 2020) was used to identify the substitution model and its parameters for BEAST (Suchard et al., 2018). Speciation was based on the Yule Process (Gernhard, 2008; Yule, 1925) and a random local clock was used. BEAST was run using the CIPRES Science Gateway (Miller et al., 2011). Tracer was used to confirm that an effective sample size (ESS) had been reached (Rambaut et al., 2018) and the trees were summarized using TreeAnnotator with a burnin percentage of 10% and a posterior probability limit of 0.5. Trees were visualized using FigTree.

Based on the distribution of sequences in the tree, sequences were selected for each species. Two sets of sequences were selected for each protein; one included only host, mollusk/annelid, *C.elegans* and parasite sequences in addition to any further crystal structure sequences while the second also included the expanded invertebrate sequences. If a sequence was clustered with other sequences from the same species, only one sequence was selected with a preference for first PDB sequences and then XP sequences. If there were multiple clear gene families across species, one sequence per species was selected based on gene families. Gene families which included sequences from other proteins outside of the protein of interest were not included.

Selected sequences were realigned using Clustal Omega (Sievers et al., 2011) and msa (Bodenhofer et al., 2015) then visualized in JDet (Muth et al., 2012). S3det (Rausell et al., 2010) was used with a cut-off of 2 with categories based on host, mollusk/annelid, *C.elegans* and parasite. In cases where there had been a gene family expansion, additional groups were used.

These residues were also checked against the expanded invertebrate list in order to determine whether they were RQ-specific.

Protein analysis

Crystal structures were visualized using Chimera (Pettersen et al., 2004) and the *C. elegans* and *H. sapiens* (where needed) sequences were threaded by homology using Modeller (Sali and Blundell, 1993; Webb and Sali, 2016). Active sites were determined based on the original published crystal structures. Residues of interest were visualized relative to ligands and known active sites.

Figure Legends

Figure 1. Identification of potential anthelmintic targets that act in various aspects of RQ-dependent metabolism. Genes involved in regulating four main areas of RQ-dependent metabolisms are candidate genes for helminth-specific inhibitors. (1) Genes that act to sense and regulate the switch from UQ to RQ-dependent metabolism as STHs migrate to anaerobic environments in the hosts are potential anthelmintic targets ('sensors') (2) Genes involved in RQ synthesis (3) The Quinone-dependent Dehydrogenases (QDHs) that interact with RQ (4) Other metabolic enzymes downstream of QDHs that are important for the rewiring of metabolic pathways in anerobic environments are also potential targets.

Figure 2. The AMPK/mTOR pathway and not the hypoxia pathway is required for RQ-dependent metabolism. (a) Wild-type (N2) and mutant L1 worms were exposed to 200 μ M

KCN for 15 h. KCN was then diluted 6-fold and worm movement was measured over 3 h to track recovery from KCN exposure (see Materials and Methods for details). *kynu-1(e1003)*, a mutant that lacks RQ and cannot survive prolonged KCN treatment was used as a positive control. Data points show biological replicates of movement scores after 3 h recovery, with 2-3 technical replicates for each experiment. (b) Activation of the *nhr-57::GFP* reporter in response to KCN is dose-dependent. L1 worms were treated with 50 or 200 μ M KCN and imaged after 1.5 and 4.5 hours. Worms incubated in M9 buffer alone was used as a negative control. Images are representative of 10-15 worms imaged. (c-d) Wild-type N2 (red curve) and *hif-1(ia4)* (black curve) L1 worms were exposed to 50 or 200 μ M KCN and their movement measured over 3 hours. Curves show the mean of 3 biological replicates.

Figure 3. TDO-2 is required for RQ-dependent metabolism. (a) Schematic of kynurenine pathway. 3-hydroxyanthranilate (3HA), a precursor for RQ synthesis, is derived from tryptophan. Tryptophan Dioxygenase (TDO; TDO-2 in *C. elegans*) is required for the oxidation of L-tryptophan to N-formyl-L-kynurenine while AMFD-1, KMO-1, and KYNU-1 function downstream and were previously shown to be required for RQ synthesis. (b) Wild-type and *tdo-2* loss-of-function mutants were treated with 200 μ M KCN for 15 h followed by a 6-fold dilution with M9. Curves show the recovery from KCN treatment and represent the mean of 4 biological replicates and error bars are standard error. (c) Conserved divergent residues from host (blue), *C.elegans* (yellow), parasite (red) and mollusk/annelids (green) were identified using S3Det (Rausell et al., 2010) with a cutoff of 2 (see Methods). Residues are numbered according to *C.elegans*. One of the residues (arrow) is part of the PLD loop, a previously identified and evolutionarily conserved structural loop required for enzymatic activity (Michels et al., 2016).

(d) Crystal model of human TDO (PDB: 5TI9) with the single monomer highlighted in teal as visualized in Chimera (Pettersen et al., 2004). (e) Single monomer of human TDO (PDB: 5TI9) in complex with Tryptophan and dioxygen with *C.elegans* sequence threaded over by homology (pink residues, circled in red). PLD loop is highlighted in yellow and the A vs P residue is circled in green and is close to tryptophan (orange) and to heme complexed with iron (red).

Figure 4. ETFDH is required for RQ-dependent metabolism. (a) Schematic showing role of QDHs in the electron transport chain in UQ and RQ-dependent metabolism. (b) Requirement for QDHs in RQ-dependent metabolism. RNAi knockdown of QDH genes was performed by feeding dsRNA-expressing bacterial strains to L1 worms until they developed to fertile adults. Their L1 progeny were subjected to 15 h of 200 μ M KCN treatment followed by a 3 h recovery (see Methods). An RNAi clone targeting GFP was used as a negative control. Data show movement scores after 3 h recovery and represent biological replicates, with 2-3 technical replicates in each. (c) Conserved divergent residues from host (blue), *C.elegans* (yellow), parasite (red) and mollusk/annelids (green) were identified using S3Det (Rausell et al., 2010) with a cutoff of 2 (see Methods). Residues are numbered according to *C.elegans*. Phe437 (arrow) is conserved in RQ-containing species but is not present in hosts. (d) Crystal structure of porcine ETFDH (PDB: 2GMH) in complex with UQ5 (orange) as visualized in Chimera (Pettersen et al., 2004) with *C.elegans* sequence (pink residues) and *H.sapiens* sequence (cyan residues) threaded over by homology (residues from (c) circled in red). The Phe437Cys residue (green) is located near the quinone binding site (Zhang et al., 2006) where both RQ and UQ must be able to bind in *C.elegans* and parasites.

Figure 5. Requirement for various metabolic enzymes for RQ-dependent metabolism. (a)

The involvement of various metabolic enzymes in RQ-dependent metabolism was tested using either loss-of-function mutants or RNAi knockdown. Wild-type, mutants, and RNAi-treated L1 worms were exposed to 200 μ M KCN for 15 h followed by a 6-fold dilution, and worm movement was measured after 3 h recovery. For loss-of-function mutants, movement scores were compared to wild-type N2 worms, while for RNAi-treated worms, scores were compared to RNAi against a GFP control. Data show movement scores after 3 h recovery and represent biological replicates, with 2-3 technical replicates in each. (b) Evolutionary tree of the three types of malate dehydrogenase found in helminths as calculated by BEAST (see Methods) and visualized with FigTree. The colour of the circles reflects their posterior probability with the more blue being the most likely. Unlike hosts, helminths possess a third malate dehydrogenase which is more closely related to archaean MDHs (black).

Figure 6. Mutations in STH-specific residues affect ability to survive extended KCN

treatment. (a) Schematic of CRISPR-mediated engineering of a COQ-2 mutant. *coq-2* exons 6a and 6e are alternatively spliced in a mutually exclusive manner in wild-type worms. Mutant worms with exon 6e deleted (*coq-2 Δ 6e*) are unable to synthesise RQ. Exon 6e contains 2 helminth-specific residues and mutations were generated in the *coq-2 Δ 6e* mutant, such that two residues in exon 6a are replaced with their helminth-specific counterparts (F204L, A243S). (b) Helminth-specific residues in COQ-2 are required for survival in KCN. L1 worms were treated with 200 μ M KCN for 15 h followed by a 6-fold dilution, and worm movement was tracked for 3 h. The RQ-deficient *coq-2 Δ 6e* mutant cannot survive extended KCN treatment while the *coq-2 Δ 6e* F204L;A243S mutant (*coq-2a(syb2949);coq-2e(syb1721)*) can now recover from KCN

treatment, suggesting restored ability to synthesise RQ. (c) The TDO-2 PLD loop is required for survival in KCN (Michels et al., 2016). Wild-type (N2, black) or *tdo-2(zg218)* mutant worms (blue) L1 worms were treated with 150-250 μ M KCN for 15 h followed by a 6-fold dilution, and worm movement was tracked for 3 h (d) LET-721 helminth-specific quinone binding pocket is required for normal survival in KCN. Wild-type (N2, black) or *let-721* mutant L1 worms were treated with 150-250 μ M KCN for 15 h followed by a 6-fold dilution, and worm movement was tracked for 3 h. All curves show the mean of 4 biological replicates and error bars represent standard error.

Figure 2 – Figure Supplement 1. Evolutionary tree of AAK-1. Evolutionary tree generated through BEAST and visualized in FigTree of the AAK-1 component on AMPK (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in red. Posterior probability is indicated by the colour of the circles with blue being more strongly supported. Gene families outside of AAK-1 are collapsed.

Figure 2 – Figure Supplement 2. Evolutionary tree of AAKB-1 shows gene family expansion amongst hosts. Evolutionary tree generated through BEAST and visualized in FigTree of the AAKB-1 component on AMPK (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in red. Posterior probability is indicated by the colour of the circles with blue being more strongly supported.

Figure 2 – Figure Supplement 3. Evolutionary tree of AAKG-1 shows large scale gene

family expansion amongst hosts and parasites. Evolutionary tree generated through BEAST and visualized in FigTree of the AAKG-1 component on AMPK (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in red. Posterior probability is indicated by the colour of the circles with blue being more strongly supported.

Figure 2 – Figure Supplement 4. Evolutionary tree of RICT-1. Evolutionary tree generated

through BEAST and visualized in FigTree of RICT-1 (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in magenta and the human sequence used for the crystal structure threading in cyan. Posterior probability is indicated by the colour of the circles with blue being more strongly supported.

Figure 2 – Figure Supplement 5. Conserved divergent residues are dispersed throughout

RICT-1. (a) RICT-1 is a component of the mTOR2 complex which regulates a number of downstream processes including SGK-1. (b) Conserved divergent residues of RICT-1 with a cutoff of 2 using S3Det shows conserved divergence though much of it is not RQ-sepcific. (c) Crystal structure of human mTORC2 (PDB: 5ZCS) as visualized in Chimera (Pettersen et al., 2004) with RICT-1 in green. *C.elegans* sequence (pink residues) are threaded over by homology but are dispersed throughout the protein and seem more likely to affect its dynamics than be druggable.

Figure 2 – Figure Supplement 6. Evolutionary tree of SGK-1 shows the large gene family

expansion amongst hosts. Evolutionary tree generated through BEAST and visualized in FigTree of SGK-1 (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in magenta and the human sequence used for the crystal structure threading in cyan. Posterior probability is indicated by the colour of the circles with blue being more strongly supported. Gene families outside of SGK-1 are collapsed.

Figure 2 – Figure Supplement 7. Conserved divergence in SGK-1 is not RQ-specific. (a)

Conserved divergent residues of SGK-1 with a cutoff of 2 using S3Det shows conserved divergence though much of it is not RQ-sepcific but rather reflects the SGK1/2/3 variation within hosts. The same is true of the previous characterized active site (Hammond et al., 2009; Zhao et al., 2007). (b) Crystal structure of human SGK-1 (PDB: 3HDM/2R5T) as visualized in Chimera (Pettersen et al., 2004). *C.elegans* sequence (pink residues) are threaded over by homology.

Figure 3 – Figure Supplement 1. Evolutionary tree of TDO-2. Evolutionary tree generated

through BEAST and visualized in FigTree of TDO-2 (see Methods). Sections of the tree are highlighted based on whether they below to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in magenta and the human sequence used for the crystal structure threading in cyan. Posterior probability is indicated by the colour of the circles with blue being more strongly supported.

Figure 3 – Figure Supplement 2. Effects of RNAi knockdown of *coq-3* and *coq-5* on worm recovery after extended KCN treatment. RNAi knockdown of *coq-3* and *coq-5* was performed in L1 worms and after one generation their L1 progeny were subjected to 15 h 200 μ M KCN treatment followed by a 3 h recovery (see Methods). An RNAi clone targeting GFP (black curve) was used as a negative control. Curves show the mean of 4 biological replicates and errors bars represent standard error.

Figure 3 – Figure Supplement 3. Evolutionary tree of AFMD-1. Evolutionary tree generated through BEAST and visualized in FigTree of AFMD-1 (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in magenta. Posterior probability is indicated by the colour of the circles with blue being more strongly supported.

Figure 3 – Figure Supplement 4. Evolutionary tree of COQ-3. Evolutionary tree generated through BEAST and visualized in FigTree of COQ-3 (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in magenta and the human sequence used for the crystal structure threading in cyan. Posterior probability is indicated by the colour of the circles with blue being more strongly supported. Gene families outside of COQ-3 are collapsed.

Figure 3 – Figure Supplement 5. Conserved divergent residues of COQ-3 are not close to the active site. (a) COQ-3 catalyzes several reactions in the UQ/RQ biosynthetic pathway. (b) Conserved divergent residues of COQ-3 with a cutoff of 2 using S3Det shows poor conservation

amongst helminths while the known active site (Zhu et al., 2015) is either conserved or very flexible in helminths as with 211. (c) Crystal structure of *E.coli* COQ-3 (PDB: 4KDC) as visualized in Chimera (Pettersen et al., 2004) with *C.elegans* sequence (pink residues) and *H.sapiens* sequence (cyan residues) threaded over by homology. Conserved divergent residues are not near the active site (blue).

Figure 3 – Figure Supplement 6. Evolutionary tree of COQ-5. Evolutionary tree generated through BEAST and visualized in FigTree of COQ-5 (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in magenta and the human sequence used for the crystal structure threading in cyan. Posterior probability is indicated by the colour of the circles with blue being more strongly supported. Gene families outside of COQ-5 are collapsed.

Figure 3 – Figure Supplement 7. Evolutionary tree of KMO-1. Evolutionary tree generated through BEAST and visualized in FigTree of KMO-1 (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in magenta and the human sequence used for the crystal structure threading in cyan. Posterior probability is indicated by the colour of the circles with blue being more strongly supported.

Figure 3 – Figure Supplement 8. A single conserved divergent residue is within the active site of KMO-1. (a) KMO-1 catalyzes a key reaction in the biosynthetic pathway which makes the 3HA, a RQ precursor. (b) Conserved divergent residues of KMO-1 with a cutoff of 2 using

S3Det shows poor conservation amongst helminths while the known active site (Kim *et al.*, 2018) does have a conserved divergent residue (green arrow). (c) Crystal structure of *P.fluorescens* KMO-1 (PDB: 5NAK) and *H.sapiens* KMO-1 (PDB: 5X68) as visualized in Chimera (Pettersen et al., 2004) with *C.elegans* sequence (pink residues) threaded over by homology. A single conserved divergent residues is within the substrate binding active site (blue).

Figure 3 – Figure Supplement 9. Evolutionary tree of KYNU-1. Evolutionary tree generated through BEAST and visualized in FigTree of KYNU-1 (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in magenta and the human sequence used for the crystal structure threading in cyan. Posterior probability is indicated by the colour of the circles with blue being more strongly supported.

Figure 3 – Figure Supplement 10. No conserved divergent residues are near KYNU-1's active site. (a) KYNU-1 catalyzes a key reaction in the biosynthetic pathway which makes the 3HA, a RQ precursor. (b) Conserved divergent residues of KYNU-1 with a cutoff of 2 using S3Det shows strong conserved divergence while the known active site (Lima et al., 2009) does not. (c) Crystal structure of human KYNU-1 (PDB: 3E9K) as visualized in Chimera (Pettersen et al., 2004) with *C.elegans* sequence (pink residues) threaded over by homology.

Figure 4 – Figure Supplement 1. RNAi knockdown of *let-721* results in reduced recovery from extended KCN treatment. RNAi knockdown of various QDH genes was performed in L1

worms and after one generation their L1 progeny were subjected to 15 h 200 μ M KCN treatment followed by a 3 h recovery (see Methods). An RNAi clone targeting GFP (black curve) was used as a negative control. Curves show the mean of at least 3 biological replicates and errors bars represent standard error.

Figure 4 – Figure Supplement 2. Evolutionary tree of LET-721. Evolutionary tree generated through BEAST and visualized in FigTree of LET-721 (see Methods). Sections of the tree are highlighted based on whether they belong to hosts, parasites or other invertebrates and the *C.elegans* sequences are coloured in magenta and the human sequence used for the crystal structure threading in cyan. Posterior probability is indicated by the colour of the circles with blue being more strongly supported.

Figure 2 – Source Data 1. List of fractional mobility scores related to Figure 2a.

Figure 4 – Source Data 1. List of fractional mobility scores related to Figure 4b.

Figure 5 – Source Data 1. List of fractional mobility scores related to Figure 5a.

1004 **Supplemental Table 1. *C. elegans* strains generated using the CRISPR-Cas9 system**

1005

Strain	Allele	Mutation	Sequence change (plus flanking sequences)
PHX76 9	<i>F36A2.3</i> <i>(syb769)/hT2</i>	: 699bp deletion A: 1bp insertion Background: N2	agtggaaaagtaacattcaactttgga taaacg ATGACTATTAAAGACA AAAGAGAATTCAAT GAAACAGATGAAAA- aaaattttgtaaac aagtatacaatatcccaaatgttttagG CCA AAGAGTTTGGGAATCGG ATGGGTAGTATGCC
PHX24 3	<i>VF13D12L.3.1(sy</i> <i>b243)</i>	: 1688bp deletion A: 5bp insertion Background: N2	aaacaatcgaatcaatttcacagagg cag ccgcagcagcaaaatacagtgaaaat aaaa ATGAATT- AAAAAAGAAGCGCCGT CCCCGC

			ACTCGGA ACTAATCCA ATCAGTTTAGCAGC TCCTGGAACTGGTGAT GACTCTTTTGTACT
PHX29 49	<i>coq-2a(syb2949)</i>; A: <i>coq-2e(syb1721)</i>	Substitution (protein change: F204L; A243S) A: Substitution (synonymous mutation) Background: <i>coq-</i> <i>2e(syb1721)</i>	ccacctaaccctccccctggtttctc aa tatctcttacactctctctatcatagtac a gGACTAACGTTAAATT GGGGCGCTCTTCTT GGATGGTGTGCGCTGA AAGGTGATTTGTCG TCTAGTGCACCGTTCT GGATGTATGCAGCT GCACTTCAATGGACAC TGATCTACGACACT ATCTATAGTCATCAGgt ggcctatttgggg attgggactctttgtttgtcatgtttt ccttggtgatgttttgcggttcgt

PHX28	<i>let-721(syb2817)</i>	A:	GACAAGAATATTCGTG
17		Substitution	ATACTTATGTTGTT
		(protein	AAAGAGCTCAAAGCAA
		change:	CCAGAAATATTCGT
		F437C)	CCATCAT TG CAATACTT
			CATTGGGATACATT
		Background:	GGAGGACTGATCTATT
		N2	CTGGAATATTCTAT
			GTTTTTCGGTCGAGGAA
			TTGAACCATGGACT

1006

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1011

1012 Competing Interests

1013 We declare no competing interests.

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Table 1. Candidate genes with potential roles in RQ-dependent metabolism

Category	Gene Name	Perturbation	Genotype	Required for KCN-dependent Metabolism	Active Site Changes
Sensors	<i>aak-2</i>	Mutation	<i>aak-2(rr48)</i> <i>aak-2(ok524)</i>	+	N/A
	<i>hif-1</i>	Mutation	<i>hif-1(ia4)</i>	0	N/A
	<i>ire-1</i>	Mutation	<i>ire-1(zc14);zcls4</i> <i>ire-1(ok799)</i>	0	N/A
	<i>rict-1</i>	Mutation	<i>rict-1(mg360)</i> <i>rict-1(ft7)</i>	++	0
	<i>sgk-1</i>	Mutation	<i>sgk-1(ok538)</i>	+	+
	<i>xbp-1</i>	Mutation	<i>xbp-1(zc12); zcls4</i>	0	N/A
RQ Synthesis	<i>coq-2^a</i>	Mutation	<i>coq-2(syb1721)</i>	++	++
	<i>kynu-1</i>	Mutation	<i>kynu-1(e1003)</i>	++	0
	<i>tdo-2</i>	Mutation	<i>tdo-2(zg216)</i> <i>tdo-2(zg217)</i> <i>tdo-2(zg218)</i>	++	++
	<i>coq-3</i>	RNAi	N/A	0	0
	<i>coq-5</i>	RNAi	N/A	0	N/A
	<i>afmd-1</i>	N/A	N/A	N/A	N/A
	<i>kmo-1</i>	N/A	N/A	N/A	0
RQ Binding	<i>mev-1^b</i>	Mutation	<i>mev-1(tr357)</i>	++	++
	<i>dhod-1</i>	RNAi	N/A	0	N/A
	<i>gpdh-1</i>	RNAi	N/A	0	N/A
	<i>let-721</i>	RNAi	N/A	+	++
	<i>prdh-1</i>	RNAi	N/A	0	N/A
	<i>F27D4.1</i>	RNAi	N/A	+	N/A
Metabolism	<i>F36A2.3</i>	RNAi	N/A	+	N/A
	<i>men-1</i>	RNAi	N/A	0	N/A
	<i>VF13D12L.3.1</i>	Mutation	<i>VF13D12L.3.1(syb243)</i>	0	N/A
	<i>pyc-1</i>	RNAi	N/A	0	N/A
	<i>mdh-1</i>	RNAi	N/A	0	N/A
	<i>icl-1</i>	Mutation	<i>icl-1(ok531)</i>	0	N/A

^a Functional assay and structure analysis described in Tan *et al.*, 2020

^b Functional assay and structure analysis described in Del Borrello *et al.*, 2019

c.

HOST

PARASITE

	49	165	437	504	567
H.sapiens	E	M	C	S	V
O.aries	E	M	C	S	V
S.scrofa	E	M	C	S	V
B.taurus	E	M	C	S	V
M.musculus	E	M	C	S	V
C.elegans	D	L	F	T	I
S.ratti	D	L	F	T	I
A.ceylanicum	-	-	F	T	I
F.hepatica	D	L	F	T	I
T.muris	D	L	F	T	S
H.contortus	D	L	F	T	I
C.telata	D	L	F	T	I
B.glabrata	D	L	F	T	I

▲

a.

b.

b.

c.

d.

AMPK – AAK-1

posterior

1

0

- Hosts
- Helminths
- Mollusks
- Echinodermata
- Arthropods

0.6

Figure 2 - figure supplement 1

AMPK – AAKB-1

0.3

Figure 2 - figure supplement 2

Figure 2 - figure supplement 4

Figure 2 - figure supplement 5

A

S3Det

Active Site

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Protein ID	S3Det	Active Site
NP_001367134.1_C.elegans	ETFFVME	SFKLI NNHLEDKENLTD FGLC
NP_001041299.1_C.elegans	ETFFVME	SFKLI NNHLEDKENLTD FGLC
XP_024498839.1_S.ratti	ETFFVLE	SFKLK ANYLEDKENLTD FGLC
EYC35961.1_A.ceylanicum	ETFFVME	SFKLK ANHLEDKENLTD FGLC
THD23091.1_F.hepatica	ETFFVLE	QFKLV KAYVEDKENLTD FGLC
XP_019924194.1_C.gigas	EVFLIIE	SFKLL KNYVEDKENLTD FGLC
XP_013080258.1_B.glabrata	EVFLIIE	SFKLL KNYVEDKENLTD FGLC
ELU03722.1_C.teleta	EVFLIIE	SFKLV KNYVEDKENLTD FGLC
XP_020934240.1_S.scrofa	SVFLIIE	SFKLL KNYIEDKENLTD FGLC
NP_035491.1_M.musculus	SVFLIIE	SFKLL KNYIEDKENLTD FGLC
3HDM_A_H.sapiens	SVFLIIE	SFKLL KNYIEDKENLTD FGLC
NP_001137149.1_H.sapiens	SVFLIIE	SFKLL KNYIEDKENLTD FGLC
NP_001095503.1_B.taurus	SVFLIIE	SFKLL KNYIEDKENLTD FGLC
XP_004011401.1_O.aries	SVFLIIE	SFKLL KNYIEDKENLTD FGLC
XP_020933825.1_S.scrofa	AVYLIVQ	NYKLL KNYVEDKENLTD FGLC
XP_006499723.1_M.musculus	AVYLIVQ	NYKLL KNYVEDKENLTD FGLC
XP_027833011.1_O.aries	AVYLIVQ	NYKLL KNYVEDKENLTD FGLC
NP_001186193.1_H.sapiens	AVYLIVQ	NYKLL KNYVEDKENLTD FGLC
XP_005214760.1_B.taurus	AVYLIVQ	NYKLL KNYVEDKENLTD FGLC
XP_027833010.1_O.aries	AVYLIVQ	NYKLL KNYVEDKENLTD FGLC
NP_001032848.1_M.musculus	GVFLIIE	SFKLL KNFVEDKENLTD FGLC
NP_001028750.1_H.sapiens	GVFLIIE	SFKLL KNFVEDKENLTD FGLC
XP_020944944.1_S.scrofa	GVFLIIE	SFKLL KNFVEDKENLTD FGLC
XP_004011764.2_O.aries	GVFLIIE	SFKLL KNFVEDKENLTD FGLC
XP_015329947.1_B.taurus	GVFLIIE	SFKLL KNFVEDKENLTD FGLC

B

S3Det

- *C.elegans* residues
- *H.sapiens* residues
- Active site
- ANP
- MMG

Figure 2 - figure supplement 7

TDO-2

0.4

Figure 3 - figure supplement 1

Figure 3 - figure supplement 2

Figure 4 - figure supplement 2

Figure 3 - figure supplement 4

A

B

S3Det		Active Site
Protein ID		
NP_766275.1_M.musculus	ADGHEYL	IAGVKH
NP_059117.3_H.sapiens	ADGHEYL	IASVKH
XP_020945957.1_S.scrofa	ADGHEYL	IAGVKH
XP_004011304.1_O.aries	ADGHEYL	IAGVKH
NP_001069678.1_B.taurus	ADGHEYL	IAGVKH
XP_013062857.1_B.glabrata	SEAHQRI	ILRVPH
ELU05074.1_C.teleta	KNAHQKM	ILRVPH
NP_001041045.1_C.elegans	DRATTTC	VLKVPH
EPB78549.1_A.ceylanicum	KEA-ARI	VLKVQH
KFD49406.1_T.suis	DGAHKQI	VLGVKH
CDJ84378.1_H.contortus	QKADSSV	VLKVQH
THD28425.1_F.hepatica	QRAQQQV	VLGVRH
XP_001893231.1_B.malayi	KRARRH I	VLGVPH
XP_024503724.1_S.ratti	-RAQTDI	IIGVEH
4KDC_E.coli	APALLTV	ILRVKH

C

Figure 3 - figure supplement 5

Figure 3 - figure supplement 6

KMO-1

Figure 3 - figure supplement 7

Figure 3 - figure supplement 9

B **S3Det** **Active Site**

<https://doi.org/10.1101/2021.03.24.436840>; this version posted March 29, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Protein ID		
NP_003475.22.1_M.musculus	162	162
3E9K_H.sapiens	172	172
XP_020931491.1_S.scrofa	FE	FE
NP_001178237.1_B.taurus	FE	FE
XP_004004770.1_O.aries	FE	FE
XP_013090590.1_B.glabrata	YP	YP
ELT96284.1_C.teleta	YP	YP
NP_509023.1_C.elegans	YP	YP
THD19993.1_F.hepatica	YP	YP
XP_001895030.1_B.malay	YP	YP
XP_024501210.1_S.ratti	YP	YP
EPB80867.1_A.ceylanicum	Y-	Y-
CDJ93501.1_H.contortus	YP	YP
KFD57689.1_T.suis	YP	YP

Figure 3 - figure supplement 10

Figure 4 - figure supplement 1

Figure 4 - figure supplement 2