NIH Public Access
Author Manuscript
Immunol Rev. Author manuscript; available in PMC 2016 March 01.

Published in final edited form as:
Immunol Rev. 2015 March ; 264(1): 319–326. doi:10.1111/imr.12256.

Mycobacterial Genes Essential for the Pathogen's Survival in the Host

Sabine Ehrt[1], **Kyu Rhee**[2], and **Dirk Schnappinger**[1]

[1] Department of Microbiology and Immunology, Weill Cornell Medical College, New York, New York, United States of America

[2] Department of Medicine, Weill Cornell Medical College, New York, New York, USA

Summary

Mycobacterium tuberculosis (Mtb) has evolved within the human immune system as both host and reservoir. The study of genes required for its growth and persistence *in vivo* thus offers linked insights into its pathogenicity and host immunity. Studies of Mtb mutants have implicated metabolic adaptation (consisting of carbon, nitrogen, vitamin and cofactor metabolism), intrabacterial pH homeostasis, and defense against reactive oxygen and reactive nitrogen species, as key determinants of its pathogenicity. However, the mechanisms of host immunity are complex and often combinatorial. Growing evidence has thus begun to reveal that the determinants of Mtb's pathogenicity may serve a broader and more complex array of functions than the isolated experimental settings in which they were initially found. Here, we review select examples, which exemplify this complexity, highlighting the distinct phases of Mtb's life cycle and the diverse microenvironments encountered therein.

Keywords

intraphagosomal nutrients; mycobacterial stress resistance; attenuated Mtb mutants

Introduction

The pathogenicity of a microbe is defined by its ability to cause disease in a host organism. For *Mycobacterium tuberculosis* (Mtb), this ability has been shaped by its evolution within humans as both host and reservoir. Its pathogenicity has thus co-evolved with its physiology as a species.

Following infection, Mtb begins its life cycle within the terminal airspace, or alveolus, of the lung. Here, the pathogen is phagocytosed by resident alveolar macrophages and dendritic cells. Mtb is then believed to undergo a period of unrestricted replication, during which it migrates to the local draining lymph nodes, disseminates through the bloodstream, infects more macrophages, and reseeds additional regions of the lung. Infection is usually contained

Correspondence to: Sabine Ehrt, Department of Microbiology and Immunology, Weill Cornell Medical College, 413 East 69th Street, New York, NY 10021 USA, Tel.: +1 646 962 6215, sae2004@med.cornell.edu.

The authors have no conflicts of interest to declare.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

with the onset of cellular immunity and formation of granulomatous lesions, marking the transition to a chronic phase of infection. During this period, Mtb is widely believed to exist in in a clinically asymptomatic state of slowed or arrested replication lasting for decades, if not the lifetime of the host. This period constitutes the longest phase of its life cycle. However, Mtb remains poised to resume replicating throughout.

Mtb ultimately completes its life cycle upon immunologic failure, wherein granuloma rupture is thought to facilitate exposure to fresh nutrients and oxygen, re-entry into cell cycle, release into the extracellular airways, and aerosol transmission to a new host. Mtb's pathogenicity thus derives from the complex requirements of a life cycle in which unrestrained replication would extinguish its host and perfect symbiosis would preclude its spread as a species. Inasmuch as Mtb infects and resides chiefly within the immune compartment, its pathogenicity is likely to have evolved from its specific adaptations to host immunity.

Here we review current knowledge about genes required by Mtb to survive inside the host. Reflecting the weight of existing data, we focus primarily on murine models of pulmonary tuberculosis (TB). While murine models of TB fail to fully recapitulate the pathology of both latent and active human TB (1), experimental infections of other animal models with genetically altered Mtb mutants have been performed only rarely. Moreover, the availability of various inbred strains, reagents, and genetically altered mutants, and the ease with which mice can be infected and analyzed have allowed for the clear identification of genes associated with Mtb's pathogenicity. Nonetheless, the growing use of other animal models such as the guinea pig, rabbit, and non-human primates has begun to yield important insight into those aspects of TB pathogenesis not modeled by the mouse.

While a significant number of genes have been shown to facilitate Mtb's growth and survival within a host, we focus on only few selected examples. We place particular emphasis on primary findings and accompanying limitations associated with their interpretation.

Dietary restrictions

Nutrient acquisition is essential for growth and survival of all organisms. For Mtb, this requirement has been shaped by its evolution with the human immune system. Unlike many pathogens, Mtb infects and resides within immune cells, apart from other microbes. Within immune cells, Mtb can reside within the dynamic and heterogeneous environment of the macrophage phagosome, the cell type and compartment most committed to its eradication.

Knowledge of the genes associated with Mtb metabolism within the host first emerged from transcriptional studies of Mtb recovered from macrophages *in vitro* and from mouse lungs (2-5). These studies implicated catabolism of fatty acids, biosynthesis of lysine and leucine, and acquisition of iron, among others, as required upon entry in host cells. Subsequent work, largely through the use of genetically engineered mutants, has begun to evaluate the function and essentiality of individual genes associated with each of these pathways.

Mtb with a deletion of *icl1*, one of two genes encoding the glyoxylate shunt enzyme isocitrate lyase (ICL) essential for metabolism of even chain fatty acids, was the first

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

metabolic mutant reported to exhibit an *in vivo* survival defect (6). This defect was observed in activated but not in naive macrophages and during the chronic but not during acute phase of infection. These findings suggested an essential role for metabolism of even chain fatty acids during the chronic phase of infection. Subsequent studies with Mtb strains completely lacking ICL activity, due to deletion of both *icl* genes, however showed a complete *in vitro* growth defect on both even and odd chain fatty acids, and profound attenuation during both the acute and chronic phases of mouse infections. These studies thus implicated broader roles for ICL and lipid metabolism in the growth and survival of Mtb during all phases of its life cycle (7, 8).

More specific interpretations of the role of Mtb's ICLs in growth and survival in mice were complicated by the discovery that these enzymes possessed both isocitrate (ICL) and methylisocitrate lyase (MCL) activities and that these activities were encoded by the same active site. ICL and MCL serve separate, though interlinked, pathways associated with the metabolism of even and odd chain fatty acids, respectively (9). Recent metabolomic studies nonetheless showed that the *in vitro* survival defect of ICL-deficient Mtb in media containing even and odd chain fatty acids could be attributed to the specific loss of MCL activity (10). These studies specifically demonstrated that ICL deficiency resulted in the sequestration of tricarboxylic acid (TCA) cycle intermediates into methylcitrate cycle intermediates, depletion of gluconeogenic precursors, inhibition of gluconeogenesis through the accumulation of methylcitrate cycle intermediates, and ultimate loss of membrane potential and intrabacterial pH homeostasis.

These studies further showed that the survival, though not growth, defect associated with ICL-deficiency could be rescued *in vitro* with the addition of exogenous vitamin B_{12}, which activated an alternate pathway for propionate metabolism and mitigated the need for MCL activity. Previous studies had shown that the survival of Mtb mutants lacking the remaining genes of the methylcitrate cycle was similarly impaired in a B_{12}-reversible manner when cultured *in vitro* with fatty acids or in macrophages, yet unaffected in mice, indicating that Mtb has adequate access to B_{12} *in vivo* (11, 12). Thus, while Mtb's ICLs play clear roles in the metabolism of fatty acids, the lack of such activities does not appear sufficient to explain the severe attenuation of ICL-deficient Mtb observed in mice. Interestingly, Mtb's ICLs were recently also reported to serve critical roles in adaptation to hypoxia and antibiotic tolerance through mechanisms independent of fatty acid metabolism (13, 14). It is thus likely that the severe *in vivo* attenuation of ICL-deficient Mtb may reflect the loss of such additional functions.

While the physiologic basis for the attenuation of ICL-deficient Mtb remains unresolved, studies of other Mtb mutants have identified additional genes associated with lipid metabolism required for survival in the host (reviewed in 15). These include mutants lacking three or four phospholipase C enzymes important for lipid degradation, mutants unable to take up or degrade cholesterol and mutants with deficient phosphoenolpyruvate carboxykinase (PEPCK), catalyzing the first committed step of gluconeogenesis (16-21).

Mtb lacking PEPCK failed to replicate in resting macrophages and during acute infections of wildtype and IFNγ-deficient mice (21). This phenotype is consistent with the view that Mtb

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

utilizes gluconeogenic carbon sources to produce biomass during growth *in vivo*. Unexpectedly however, the mutant was not only unable to replicate *vivo* but also died and was rapidly cleared from mouse lungs. Moreover, depletion of PEPCK during the chronic phase of an established infection similarly led to death of the bacilli. Absent any evidence that fatty acids become toxic to Mtb lacking PEPCK, its death *in vivo* remains unexplained. PEPCK has been shown to also operate in the anaplerotic direction assimilating CO_2 and generating oxaloacetate, which is important when the reverse TCA cycle is active (22). The reverse TCA cycle has been demonstrated to operate with glycolytic carbon sources and in a hypoxic environment and Mtb grown in THP1 cells (22-24). It is thus plausible that metabolic cues and environmental conditions inside the host trigger flux though the reverse, reductive TCA cycle.

ICL and PEPCK aside, Mtb has been shown to be capable of utilizing multiple carbon substrates simultaneously (25, 26), suggesting that fatty acids may not be the only carbon source it utilizes on during infections. Many bacteria prefer hexoses such as glucose as carbon and energy source, and glucose serves as dominant carbon source for *Salmonella typhimurium* when replicating in the host cell phagosome (27). Mtb expresses two glucokinases to convert glucose into its phosphorylated form, the entry step into glycolysis. Deletion of the dominant glucokinase PPGK profoundly impaired growth of Mtb with glucose as sole carbon source, but the bacteria were still able to metabolize glucose although far less efficiently than wildtype Mtb (28). Only deletion of both glucokinases, PPGK and GLKA, resulted in a strain that failed to metabolize and grow on glucose. The PPGK/GLKA double mutant replicated normally during the acute phase of a mouse infection but exhibited a persistence defect in the chronic phase. This suggests that Mtb has access to at least some amount of glucose *in vivo* and relies on glucose phosphorylation during persistence in mice. The mechanism by which glucose-phosphate may contribute to Mtb's fitness in chronically infected mice remains to be determined. The most straightforward interpretation of the persistence defect caused by deletion of PPGK and GLKA seems to be that glucose phosphate can serve a metabolic role and enter glycolysis as a source of carbon and energy to maintain viability. Alternatively, but not exclusively, glucose phosphate might serve as source of reducing power (29) and thereby help protect Mtb from oxidative stress. In fact, the glucokinase double mutant was found to be hypersusceptible to killing by H_2O_2, but not other forms of ROS, yet its *in vivo* persistence defect was not rescued in NADPH phagocyte oxidase deficient mice (28). Thus, while the impact of glucokinase deficiency demonstrates the importance of Mtb's metabolic flexibility for its pathogenicity, the glucokinase double mutant highlights the limited ability of *in vitro* phenotypes to inform on *in vivo* activities.

While genes encoding enzymes required for both glycolysis and gluconeogenesis were predicted to be essential for growth of Mtb (30), both fructose-1,6-bisphosphate aldolase (FBA) and triose phosphate isomerase (TPI) were found to be dispensable *in vitro* when Mtb was provided with a combination of a glycolytic and a gluconeogenic carbon source, such as glucose and a fatty acid (31, 32). *In vivo* however, both mutants were unable to establish an infection, and FBA depletion prevented persistence of Mtb during the chronic phase of infection. These studies thus highlighted an *in vivo* form of essentiality whose *in vitro* conditionality proved particularly informative. Moreover, only a specific, balanced ratio of

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

glucose and butyrate allowed growth of the FBA mutant *in vitro*. Hence, its failure to replicate or persist *in vivo*, while consistent with other evidence that Mtb faces an abundance of fatty acids and lipids, leaves open the availability of glycolytic carbon substrates, albeit at non-permissive levels.

Carbon aside, recent work has also begun to implicate essential roles for genes associated with nitrogen assimilation in Mtb (33, 34). Nitrogen is an essential nutrient for all organisms, and bacteria can use amino acids as precursors. Accordingly, deletion of the aspartate transporter AnsP1 in Mtb abolished *in vitro* uptake of aspartate, *in vitro* growth on media containing aspartate as sole nitrogen source, and Mtb replication during the acute phase of infection in mouse lungs (33). Together, these findings implicated a potentially essential role for aspartate as a nitrogen source for Mtb during infection. However somewhat surprisingly, the AnsP1 mutant replicated normally in macrophages. Thus, while it is possible that this finding reflects differences in the nutritional environment provided by macrophages *in vitro* compared to, it may alternatively point to an additional, yet to be identified role of AnsP1 in virulence.

Work from the same group revealed that another transporter AnsP2 is involved in but not essential for uptake of asparagine (34). The AnsP2 mutant replicated normally in macrophages and *in vivo* indicating that Mtb can utilize a redundant asparagine uptake system or that asparagine is replaceable by other nitrogen sources. That asparagine is important for Mtb's pathogenicity is however evident from a mutant lacking the asparaginase AnsA, which Mtb requires to hydrolyze asparagine into aspartate and ammonia. The AnsA mutant failed to replicate normally in macrophages and in mouse lungs. *In vitro*, the mutant not only showed impaired asparagine-derived nitrogen incorporation but also reduced growth at low pH and impaired ammonia secretion implicating asparagine hydrolysis via AnsA as mechanism for pH buffering and acid resistance. The attenuation of the AnsA mutant *in vivo* may thus be the consequence of impaired nitrogen assimilation and/or a deficiency in coping with acidification. Whether Mtb relies on amino acid uptake and nitrogen acquisition for long-term persistence however remains to be investigated.

Looking beyond metabolic enzymes themselves, experimental studies in both mice and guinea pigs have identified an essential role for the Mtb ortholog of the metabolic stress response regulator, RelA (35, 36). The stringent response helps bacteria to adapt to environmental stresses including limited availability of nutrients. This cellular reprogramming involves transcriptional and posttranscriptional responses and is mediated through the accumulation of hyperphosphorylated guanine nucleotides, (p)ppGpp, through the activity of RelA (37). Deletion of *relA* compromised Mtb's ability to survive nutrient starvation in culture (38) and impaired its long-term survival during the chronic phase of infection in mice and acutely infected guinea (35, 36). These phenotypes are consistent with limited nutrient availability *in vivo* but could also be caused by increased susceptibility of Mtb incapable of mounting the stringent response to other forms of stress. Furthermore, deletion of *relA* perturbed the expression of a large number of genes including several dominant mycobacterial antigens(35). The extent to which the attenuation of *relA*-deficient

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Mtb reflects its specific roles in sensing and regulating the metabolic state of Mtb during the acute and chronic phases of infection thus awaits further investigation.

Take your vitamins

Biotin serves as an essential enzyme cofactor and bacteria obtain it either through *de novo* synthesis or active uptake from extracellular sources. Mtb requires biotin–dependent enzymes for the synthesis of fatty acids and lipids that are part of its cell envelope (39). Transposon site hybridization (TraSH) experiments (40) predicted mutants with transposon insertions in genes required to synthesize biotin to be severely attenuated in mice (41). These predictions were confirmed with an Mtb mutant, in which the biotin biosynthetic enzyme 7,8-diaminopelargonic acid synthase (BioA) had been deleted (42). This mutant required external biotin for growth and survival in culture and failed to establish infection in mice. Conditional *bioA*-TetON mutants, in which *bioA* transcription is controlled by tetracycline repressor and dependent on the presence of a tetracycline, were used to demonstrate that BioA activity was not only required to establish infections but also to maintain mycobacterial persistence. Complementation of the *in vitro* growth defect of the *bioA* deletion mutant required a minimum of 50 nM biotin, which is at least 25 times higher than the biotin concentration in human serum. Together these experiments demonstrate that at least in the mouse model Mtb fails to have access to biotin and depends on *de novo* biotin biosynthesis for replication and persistence.

Nicotinamide adenine dinucleotide (NAD) is a central cofactor with chief roles in cellular metabolism and energy production in all living cells. It is important for redox homeostasis and energy metabolism, for the activity of the NAD-dependent DNA ligase, protein ADP-ribosylases, protein deacetylation, in cobalamin biosynthesis and for calcium homeostasis (43, 44). Bacteria can synthesize NAD *de novo* or recycle precursors by a variety of pathways or scavenge it from exogenous sources (43). Mtb has been shown to recycle exogenously provided nicotinamide for NAD synthesis(44). Mtb mutants lacking enzymes of either the *de novo* biosynthesis or the recycling pathway replicated and persisted normally *in vivo* due to the abundance of nicotinamide and nicotinic acid in the host. Chemical inhibition of the NAD synthetase NadE inhibited growth of Mtb *in vitro*, which could not be rescued by the addition of precursors such as nicotinamide, because NadE is required for both the recycling and the *de novo* biosynthesis pathway. Accordingly *nadE* is essential for growth of Mtb and cannot be deleted (30, 45, 46) complicating the analysis whether this enzyme is required for viability of Mtb populations that are nonreplicating or slowly replicating such as those associated with the chronic phase of infection. A recently developed genetic dual control (DUC) switch that simultaneously activates transcriptional repression of a target gene and degradation of the encoded protein was applied to probe the essentiality of NadE in different environments (46). Depletion of NadE in Mtb prevented growth and strong NadE depletion resulted in death of Mtb in culture, during nonreplicating persistence *in vitro*, and during acute and chronic mouse infections. The Mtb kill kinetics in mouse lungs and spleens were fast and similar regardless of the time point at which NadE depletion was initiated, validating NadE as promising target for further antitubercular therapeutic development.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

These and other studies indicate that Mtb has restricted access to vitamins and cofactors during infection. However, studies of Mtb mutants depend on the limitations of the specific host model used. The mouse strains most commonly used for such studies do not form necrotic and caseating granulomas in response to Mtb infection (1, 47, 48). Progression of human gramulomas is accompanied by necrosis, a central caseation that is sometimes surrounded by calcification, and accumulation of extracellular bacilli (49-51). It is plausible that these extracellular populations gain access to nutrients and cofactors that are scarce in intracellular compartments, a hypothesis that remains to be investigated.

Resist to persist

Experimental analyses of infected macrophages from mice or humans have provided biochemical evidence that Mtb is exposed reactive oxygen species (ROS) produced by phagocyte oxidase (Nox2), to reactive nitrogen species (RNS) generated by IFNγ-inducible nitric oxide synthase (iNOS), decreased concentrations of oxygen, and limited acidification (~pH 6.5) in resting macrophages, a maturation block that can be overcome with IFNγ-activation reaching a pH of as low as 4.5 (52-54). That ROS and RNS contribute to control of Mtb during infections is supported by studies with genetically altered mice lacking functional iNOS or Nox2, while immunohistochemistry revealed expression of iNOS and the presence of nitrotyrosine, a marker of nitric oxide, in tuberculous granulomas in humans (55, 56). Reduced oxygen concentrations in TB granulomas have been demonstrated by *in situ* staining with hypoxia-sensitive pimonidazole (51). In addition, studies of pathogen and host transcriptional responses and experimental infection of animals with Mtb mutants that are deficient in response to a given stress *in vitro* have implicated additional host-derived stresses that can vary depending on the host, localization of the pathogen within the host and stage of the infection (57-59).

These studies were soon followed by efforts to identify the genes and specific mechanisms that Mtb employs to resist defined modes of stress. To determine how Mtb resists acid stress, a library of approximately 10,000 transposon mutants was screened for those that exhibited a survival defect at acidic pH 4.5 (60). The majority of isolated mutants showed increased susceptibility to low pH only in the presence of a fatty acid released from BSA or Tween, both constituents of the screening media. Nonetheless, two mutants were hypersusceptible to pH 4.5 in a simple buffer not containing any fatty acid, a condition that wildtype Mtb can withstand for days without losing viability. One of these mutants lacked the periplasmic serine protease encoded by *rv3671c.* This protein was confirmed to function as a protease (61) and to be required to resist acid stress and maintain a near neutral intrabacterial pH *in vitro* and in IFNγ-activated primary macrophages (60). Accordingly, it was named mycobacterial acid resistance protease (MarP) (62). Loss of MarP also attenuated Mtb's growth and long-term persistence in mice consistent with the concept that phagosome acidification is a major host defense mechanism the pathogen has to withstand. However, loss of MarP not only crippled pH homeostasis but also rendered the bacilli hypersusceptible to oxidative stress, certain antibiotics and cell wall damaging agents such as SDS (60, 62). Therefore, while phagosome acidification is clearly one of the host-imposed stresses against which MarP helps defend, the increased susceptibility of the mutant to other stresses likely contributed to its attenuation *in vivo*.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

A similar case can be made for Mtb's proteasome. A screen for RNS hypersusceptible transposon mutants identified a role for the proteasome accessory factor A PafA and *Mycobacterium* proteasomal ATPase Mpa in RNS resistance (63). Depletion and deletion of the proteasome core proteasome PrcBA similarly caused increased susceptibility to nitrosative stress (64, 65). In mice, these mutants were attenuated for growth and persistence, suggesting that the ability to degrade proteins damaged by RNS is crucial for virulence of Mtb (63-66). Yet, genetic deficiency of iNOS did not fully rescue an Mpa mutant, and chemical inhibition of iNOS failed to improve growth or persistence in mice of Mtb lacking the core proteasome suggesting that protection from RNS damage is not the major activity by which the proteasome contributes to Mtb's pathogenicity.

Mtb lacking a fully functional proteasome had defects beyond RNS hypersusceptibility. Mpa and PafA mutants exhibited transcriptional deregulation of two metal-dependent regulons (67), implying that a failure to normally adapt to changes in metal homeostasis in the host could have contributed to the attenuation phenotypes of these mutants (68). The Mtb proteasome core deletion mutant had a growth defect in culture and lost viability in prolonged stationary phase and during nutrient starvation (65). Surprisingly, expression of an active site mutant proteasome complemented the mutant's RNS sensitivity, its slow growth *in vitro*, and growth defect in acute mouse infection, suggesting that the proteasome core may have proteolysis-independent functions in Mtb. In contrast, rescue of the survival defects in stationary phase, during nutrient starvation, and the impaired *in vivo* persistence required a proteoytically active proteasome. These findings linked nutrient limitation with *in vivo* persistence and led to the hypothesis that Mtb might require the proteasome for both amino acid supply and turnover of damaged proteins to enable long term persistence within its host (65). In mammalian cells, proteasomal protein degradation is crucial for supplying amino acids for the synthesis of new proteins during amino acid deprivation (69) and *Legionella pneumophilia* exploits its host's proteasome to generate amino acids as sources of carbon and energy during infection (70). *M. smegmatis* has also been recently found to recycle amino acids via the proteasome in response to nutrient starvation (71).

Like the acid sensitive mutants, mutants with deficiencies in proteasome functions highlighted the complications and complexity associated with interpreting the *in vivo* phenotypes. *In vivo* Mtb does not face any of the stresses that were used to screen for mutants with survival defects in isolation. Instead, the environment and conditions encountered by Mtb *in vivo* are complex and include numerous molecules that impact on each other, for example the pH of the phagosome activates lysosomal hydrolases and potentiates generation of RNS and ROS. Inevitably, interpretation of Mtb mutant phenotypes is often more complicated than one might anticipate.

A striking example highlighting a mycobacterial mechanism of resisting host immunity came from a search for Mtb genes that protect the pathogen from $CD4^+$ T-cell-mediated host immunity (72). A screen expertly designed to identify Mtb's CD4 ‘counteractome’ revealed the importance of tryptophan biosynthesis for Mtb's ability to survive $CD4^+$ T-cell responses. Deletion of *trpE* in Mtb resulted in tryptophan auxotrophy and severe growth attenuation in mice, which was rescued in MHC class II-deficient animals. With a set of elegant experiments the authors demonstrated that IFN-γ-dependent, tryptophan-

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

catabolizing indolamine-2,3-dioxygenase (IDO) mediates tryptophan starvation, which the *trpE* mutant failed to survive. This work not only emphasized how Mtb evolved to evade immune-driven starvation but also provided validation of the tryptophan biosynthesis pathway as potential chemotherapeutic target.

Concluding remarks

The advent of genetic tools and approaches in TB research has rapidly expanded our ability to inventory the determinants of Mtb's pathogenicity, on an organism-wide and gene-specific level. However, our understanding of the functions they serve and the basis for their essentiality have only recently begun to emerge (57, 58, 73, 74). Indeed, growing evidence indicates that many of these factors may serve multiple functions beyond those identified in isolated *in vitro* settings, reflecting the far more complex and heterogeneous *in vivo* settings in which Mtb evolved.

Central carbon metabolism, while not a classical subject of focus when analyzing a pathogen's virulence, has emerged as fundamental to Mtb's pathogenicity (15). Mtb lacks classical carbon catabolite repression and instead co-catabolizes multiple carbon sources simultaneously (25), which likely reflects an adaptation to diverse host niches where the pathogen encounters few microbial competitors. Mutants with defects in central carbon metabolism pathways are among the most strongly attenuated *in vivo*. Work that integrated genetic, biochemical, and metabolomics analyses has taught us that one cannot simply rely on annotation, predicted function, or even demonstrated activity of a given enzyme when interpreting a mutant's phenotype. The studies of Mtb's ICL enzymes serve as an excellent example. While initially thought to reveal a specific metabolic requirement of Mtb inside the host, sophisticated multidisciplinary approaches demonstrated that instead, the mutant's attenuation most likely resulted from the perturbation of a set of complex activities beyond the ones in central carbon metabolism (10, 13, 14). These are not the only studies revealing a connection between metabolism and stress response. The asparaginase AnsA, the metabolic stress response regulator RelA and the proteasome similarly integrate metabolic functions and stress defense activities.

That annotations can be misleading was exemplified by the gene (Rv1248) annotated to encode Mtb's α-ketoglutarate dehydrogenase. Biochemical analysis revealed its activity as thiamine diphosphate-dependent a-ketoglutarate decarboxylase (KGD) (75) followed by metabolomics analysis demonstrating its carboligase activity as hydroxyoxoadipate synthase (76). Further structural and biochemical work revealed that this enzyme is in fact multifunctional and capable of catalyzing α-ketoglutarate decarboxylase, α-ketoglutarate dehydrogenase, and hydroxyoxoadipate synthase activities (77). Accordingly, the understanding of the mutant's *in vivo* phenotype will be challenging.

As has been the case for Mtb's ICL, a better understanding and interpretation of attenuated mutants will require multidisciplinary approaches. Combination of genetics, biochemistry, metabolomics, and structural analysis is crucial to determine gene function and is also required for defining the *in vivo* correlates causing a mutant's attenuation. A look beyond the obvious and the consideration of Mtb's specific host niches and complex pressures

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

encountered therein are equally important and challenging but will provide new knowledge about the genes required by Mtb to survive in the host.

Acknowledgements

Work in the authors' laboratories is supported by grants from the National Institutes of Health (R01 AI63446 to SE, DS, KR; R01 AI092573 to SE, KR; R01 AI09179 to D.S) and grants from the Bill and Melinda Gates Foundation (grant 42848 and OPP1024065 to DS, Burroughs Wellcome Career Award in the Biomedical Sciences.

References

1. Flynn JL. Lessons from experimental *Mycobacterium tuberculosis* infections. Microbes Infect. 2006; 8:1179–1188. [PubMed: 16513383]
2. Sambandamurthy VK, Wang X, Chen B, Russell RG, Derrick S, Collins FM, Morris SL, et al. A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. Nat Med. 2002; 8:1171–1174. [PubMed: 12219086]
3. Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, Dolganov G, et al. Transcriptional Adaptation of *Mycobacterium tuberculosis* within Macrophages: Insights into the Phagosomal Environment. J Exp Med. 2003; 198:693–704. [PubMed: 12953091]
4. Timm J, Post FA, Bekker L-G, Walther GB, Wainwright HC, Manganelli R, Chan W-T, et al. Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. Proc Natl Acad Sci USA. 2003; 100:14321–14326. [PubMed: 14623960]
5. Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, Schoolnik GK. Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. J Exp Med. 2003; 198:705–713. [PubMed: 12953092]
6. McKinney J, zu Bentrup K, Muñoz-Elías E, Miczak A, Chen B, Chan W, Swenson D, et al. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature. 2000; 406:735–738. [PubMed: 10963599]
7. Muñoz-Elías E, McKinney J. *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. Nat Med. 2005; 11:638–644. [PubMed: 15895072]
8. Blumenthal A, Trujillo C, Ehrt S, Schnappinger D. Simultaneous analysis of multiple *Mycobacterium tuberculosis* knockdown mutants in vitro and in vivo. PLoS ONE. 2010; 5:e15667. [PubMed: 21203517]
9. Muñoz Elías E, Upton A, Cherian J, McKinney J. Role of the methylcitrate cycle in *Mycobacterium tuberculosis* metabolism, intracellular growth, and virulence. Mol Microbiol. 2006; 60:1109–1122. [PubMed: 16689789]
10. Eoh H, Rhee KY. Methylcitrate cycle defines the bactericidal essentiality of isocitrate lyase for survival of *Mycobacterium tuberculosis* on fatty acids. Proc Natl Acad Sci USA. 2014; 111:4976–4981. [PubMed: 24639517]
11. Savvi S, Warner DF, Kana BD, Mckinney JD, Mizrahi V, Dawes SS. Functional Characterization of a Vitamin B12-Dependent Methylmalonyl Pathway in *Mycobacterium tuberculosis*: Implications for Propionate Metabolism during Growth on Fatty Acids. J Bacteriol. 2008; 190:3886–3895. [PubMed: 18375549]
12. Griffin JE, Pandey AK, Gilmore SA, Mizrahi V, Mckinney JD, Bertozzi CR, Sassetti CM. Cholesterol Catabolism by *Mycobacterium tuberculosis* Requires Transcriptional and Metabolic Adaptations. Chemistry & Biology. 2012; 19:218–227. [PubMed: 22365605]
13. Eoh H, Rhee KY. Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA. 2013; 110:6554–6559. [PubMed: 23576728]
14. Nandakumar M, Nathan C, Rhee KY. Isocitrate lyase mediates broad antibiotic tolerance in *Mycobacterium tuberculosis*. Nature Communications. 2014; 5:1–10.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

15. Ehrt S, Rhee K. *Mycobacterium tuberculosis* metabolism and host interaction: mysteries and paradoxes. Current Topics in Microbiology and Immunology. 2012; 374:163–188. Chapter 299. [PubMed: 23242856]
16. Raynaud C, Guilhot C, Rauzier J, Bordat Y, Pelicic V, Manganelli R, Smith I, et al. Phospholipases C are involved in the virulence of *Mycobacterium tuberculosis*. Mol Microbiol. 2002; 45:203–217. [PubMed: 12100560]
17. Pandey AK, Sassetti CM. Mycobacterial persistence requires the utilization of host cholesterol. Proc Natl Acad Sci USA. 2008; 105:4376–4380. [PubMed: 18334639]
18. Nesbitt NM, Yang X, Fontan P, Kolesnikova I, Smith I, Sampson NS, Dubnau E. A Thiolase of *Mycobacterium tuberculosis* Is Required for Virulence and Production of Androstenedione and Androstadienedione from Cholesterol. Infect Immun. 2009; 78:275–282. [PubMed: 19822655]
19. Yam KC, D'angelo I, Kalscheuer R, Zhu H, Wang J-X, Snieckus V, Ly LH, et al. Studies of a Ring-Cleaving Dioxygenase Illuminate the Role of Cholesterol Metabolism in the Pathogenesis of *Mycobacterium tuberculosis*. PLoS Pathog. 2009; 5:e1000344. [PubMed: 19300498]
20. Hu Y, van der Geize R, Besra GS, Gurcha SS, Liu A, Rohde M, Singh M, et al. 3-Ketosteroid 9α-hydroxylase is an essential factor in the pathogenesis of *Mycobacterium tuberculosis*. Mol Microbiol. 2010; 75:107–121. [PubMed: 19906176]
21. Marrero J, Rhee KY, Schnappinger D, Pethe K, Ehrt S. Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for *Mycobacterium tuberculosis* to establish and maintain infection. Proc Natl Acad Sci USA. 2010; 107:9819–9824. [PubMed: 20439709]
22. Beste DJV, Nöh K, Niedenführ S, Mendum TA, Hawkins ND, Ward JL, Beale MH, et al. C-Flux Spectral Analysis of Host-Pathogen Metabolism Reveals a Mixed Diet for Intracellular *Mycobacterium tuberculosis*. Chemistry & Biology. 2013:1–10.
23. Watanabe S, Zimmermann M, Goodwin MB, Sauer U, Barry CE, Boshoff HI. Fumarate reductase activity maintains an energized membrane in anaerobic *Mycobacterium tuberculosis*. PLoS Pathog. 2011; 7:e1002287. [PubMed: 21998585]
24. Beste DJV, Bonde B, Hawkins N, Ward JL, Beale MH, Noack S, Nöh K, et al. C Metabolic Flux Analysis Identifies an Unusual Route for Pyruvate Dissimilation in Mycobacteria which Requires Isocitrate Lyase and Carbon Dioxide Fixation. PLoS Pathog. 2011; 7:e1002091. [PubMed: 21814509]
25. de Carvalho LPS, Fischer SM, Marrero J, Nathan C, Ehrt S, Rhee KY. Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. Chemistry & Biology. 2010; 17:1122–1131. [PubMed: 21035735]
26. Rhee KY, de Carvalho LPS, Bryk R, Ehrt S, Marrero J, Park SW, Schnappinger D, et al. Central carbon metabolism in *Mycobacterium tuberculosis*: an unexpected frontier. Trends in Microbiology. 2011; 19:307–314. [PubMed: 21561773]
27. Bowden S, Ramachandran V, Knudsen G, Hinton J, Thompson A. An incomplete TCA cycle increases survival of Salmonella Typhimurium during infection of resting and activated murine macrophages. PLoS ONE. 2010; 5:e13871. [PubMed: 21079785]
28. Marrero J, Trujillo C, Rhee KY, Ehrt S. Glucose Phosphorylation Is Required for *Mycobacterium tuberculosis* Persistence in Mice. PLoS Pathog. 2013; 9:e1003116. [PubMed: 23326232]
29. Hasan MR, Rahman M, Jaques S, Purwantini E, Daniels L. Glucose 6-phosphate accumulation in mycobacteria: implications for a novel F420-dependent anti-oxidant defense system. J Biol Chem. 2010; 285:19135–19144. [PubMed: 20075070]
30. Griffin JE, Gawronski JD, Dejesus MA, Ioerger TR, Akerley BJ, Sassetti CM. High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. PLoS Pathog. 2011; 7:e1002251. [PubMed: 21980284]
31. Trujillo C, Blumenthal A, Marrero J, Rhee KY, Schnappinger D, Ehrt S. Triosephosphate Isomerase Is Dispensable In Vitro yet Essential for *Mycobacterium tuberculosis* To Establish Infection. mBio. 2014; 5:e00085–14–e00085–14. [PubMed: 24757211]
32. Puckett S, Trujillo C, Eoh H, Marrero J, Spencer J, Jackson M, Schnappinger D, et al. Inactivation of Fructose-1,6-Bisphosphate Aldolase Prevents Optimal Co-catabolism of Glycolytic and Gluconeogenic Carbon Substrates in *Mycobacterium tuberculosis*. PLoS Pathog. 2014; 10:e1004144. [PubMed: 24851864]

33. Gouzy A, Larrouy-Maumus GER, Wu T-D, Peixoto A, Levillain F, Lugo-Villarino G, Gerquin-Kern J-L, et al. *Mycobacterium tuberculosis* nitrogen assimilation and host colonization require aspartate. Nat Chem Biol. 2013; 9:674–676. [PubMed: 24077180]
34. Gouzy A, Larrouy-Maumus G, Bottai D, Levillain F, Dumas A, Wallach JB, Caire-Brandli I, et al. *Mycobacterium tuberculosis* Exploits Asparagine to Assimilate Nitrogen and Resist Acid Stress during Infection. PLoS Pathog. 2014; 10:e1003928. [PubMed: 24586151]
35. Dahl JL, Kraus CN, Boshoff HIM, Doan B, Foley K, Avarbock D, Kaplan G, et al. The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. Proc Natl Acad Sci USA. 2003; 100:10026–10031. [PubMed: 12897239]
36. Klinkenberg LG, Lee JH, Bishai WR, Karakousis PC. The Stringent Response Is Required for Full Virulence of *Mycobacterium tuberculosisin* Guinea Pigs. J Infect Dis. 2010; 202:1397–1404. [PubMed: 20863231]
37. Boutte CC, Crosson S. Bacterial lifestyle shapes stringent response activation. Trends in Microbiology. 2013; 21:174–180. [PubMed: 23419217]
38. Primm TP, Andersen SJ, Mizrahi V, Avarbock D, Rubin H, Barry CE. The Stringent Response of *Mycobacterium tuberculosis* Is Required for Long-Term Survival. J Bacteriol. 2000; 182:4889–4898. [PubMed: 10940033]
39. Daniel J, Oh T-J, Lee C-M, Kolattukudy PE. AccD6, a member of the Fas II locus, is a functional carboxyltransferase subunit of the acyl-coenzyme A carboxylase in *Mycobacterium tuberculosis*. J Bacteriol. 2007; 189:911–917. [PubMed: 17114269]
40. Sassetti CM, Boyd DH, Rubin EJ. Comprehensive identification of conditionally essential genes in mycobacteria. Proc Natl Acad Sci USA. 2001; 98:12712–12717. [PubMed: 11606763]
41. Sassetti CM, Rubin EJ. Genetic requirements for mycobacterial survival during infection. Proc Natl Acad Sci USA. 2003; 100:12989–12994. [PubMed: 14569030]
42. Woong Park S, Klotzsche M, Wilson DJ, Boshoff HI, Eoh H, Manjunatha U, Blumenthal A, et al. Evaluating the Sensitivity of *Mycobacterium tuberculosis* to Biotin Deprivation Using Regulated Gene Expression. PLoS Pathog. 2011; 7:e1002264. [PubMed: 21980288]
43. Foster JW, Moat AG. Nicotinamide adenine dinucleotide biosynthesis and pyridine nucleotide cycle metabolism in microbial systems. Microbiol Rev. 1980; 44:83–105. [PubMed: 6997723]
44. Boshoff HIM, Xu X, Tahlan K, Dowd CS, Pethe K, Camacho LR, Park T-H, et al. Biosynthesis and recycling of nicotinamide cofactors in *Mycobacterium tuberculosis*. An essential role for NAD in nonreplicating bacilli. J Biol Chem. 2008; 283:19329–19341. [PubMed: 18490451]
45. Zhang YJ, Ioerger TR, Huttenhower C, Long JE, Sassetti CM, Sacchettini JC, Rubin EJ. Global Assessment of Genomic Regions Required for Growth in *Mycobacterium tuberculosis*. PLoS Pathog. 2012; 8:e1002946. [PubMed: 23028335]
46. Kim J-H, O'Brien KM, Sharma R, Boshoff HIM, Rehren G, Chakraborty S, Wallach JB, et al. A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence. Proc Natl Acad Sci USA. 2013; 110:19095–19100. [PubMed: 24191058]
47. McMurray DN, Collins FM, Dannenberg AMJ, Smith DW. Pathogenesis of experimental tuberculosis in animal models. Curr Top Microbiol Immunol. 1996; 215:157–179. [PubMed: 8791713]
48. Rhoades ER, Frank AA, Orme IM. Progression of chronic pulmonary tuberculosis in mice aerogenically infected with virulent *Mycobacterium tuberculosis*. Tubercle and Lung Disease. 1997; 78:57–66. [PubMed: 9666963]
49. Boshoff H, Barry C. Tuberculosis—metabolism and respiration in the absence of growth. Nat Rev Micro. 2005; 3:70–80.
50. Russell DG, Cardona P-J, Kim M-J, Allain S, Altare F. Foamy macrophages and the progression of the human tuberculosis granuloma. Nat Immunol. 2009; 10:943–948. [PubMed: 19692995]
51. Via LE, Lin PL, Ray SM, Carrillo J, Allen SS, Eum SY, Taylor K, et al. Infect Immun. 2008; Tuberculous Granulomas Are Hypoxic in Guinea Pigs, Rabbits, and Nonhuman Primates. 76:2333–2340. [PubMed: 18347040]
52. Schaible UE, Sturgill-Koszycki S, Schlesinger PH, Russell DG. Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium*-containing phagosomes in murine macrophages. J Immunol. 1998; 160:1290–1296. [PubMed: 9570546]

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

53. Nathan C, Shiloh MU. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci USA. 2000; 97:8841–8848. [PubMed: 10922044]
54. MacMicking JD, Taylor GA, Mckinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science. 2003; 302:654–659. [PubMed: 14576437]
55. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc Natl Acad Sci USA. 1997; 94:5243–5248. [PubMed: 9144222]
56. Choi H-S, Rai PR, Chu HW, Cool C, Chan ED. Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. Am J Respir Crit Care Med. 2002; 166:178–186. [PubMed: 12119230]
57. Stallings CL, Glickman MS. Is *Mycobacterium tuberculosis* stressed out? A critical assessment of the genetic evidence. Microbes Infect. 2010; 12:1091–1101. [PubMed: 20691805]
58. Ehrt S, Schnappinger D. Mycobacterial survival strategies in the phagosome: defence against host stresses. Cell Microbiol. 2009; 11:1170–1178. [PubMed: 19438516]
59. Barry CE, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, Schnappinger D, et al. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. Nat Rev Micro. 2009; 7:845–855.
60. Vandal OH, Pierini LM, Schnappinger D, Nathan CF, Ehrt S. A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*. Nat Med. 2008; 14:849–854. [PubMed: 18641659]
61. Biswas T, Small J, Vandal O, Odaira T, Deng H, Ehrt S, Tsodikov OV. Structural insight into serine protease Rv3671c that Protects *M. tuberculosis* from oxidative and acidic stress. Structure. 2010; 18:1353–1363. [PubMed: 20947023]
62. Small JL, O'Donoghue AJ, Boritsch EC, Tsodikov OV, Knudsen GM, Vandal O, Craik CS, et al. Substrate specificity of MarP, a periplasmic protease required for resistance to acid and oxidative stress in *Mycobacterium tuberculosis*. J Biol Chem. 2013
63. Darwin KH, Ehrt S, Gutierrez-Ramos J-C, Weich N, Nathan CF. The proteasome of *Mycobacterium tuberculosis* is required for resistance to nitric oxide. Science. 2003; 302:1963–1966. [PubMed: 14671303]
64. Gandotra S, Schnappinger D, Monteleone M, Hillen W, Ehrt S. In vivo gene silencing identifies the *Mycobacterium tuberculosis* proteasome as essential for the bacteria to persist in mice. Nat Med. 2007; 13:1515–1520. [PubMed: 18059281]
65. Gandotra S, Lebron MB, Ehrt S. The *Mycobacterium tuberculosis* proteasome active site threonine is essential for persistence yet dispensable for replication and resistance to nitric oxide. PLoS Pathog. 2010; 6:e1001040. [PubMed: 20711362]
66. Cerda-Maira FA, Pearce MJ, Fuortes M, Bishai WR, Hubbard SR, Darwin KH. Molecular analysis of the prokaryotic ubiquitin-like protein (Pup) conjugation pathway in *Mycobacterium tuberculosis*. Mol Microbiol. 2010; 77:1123–1135. [PubMed: 20636328]
67. Festa RA, Jones MB, Butler-Wu S, Sinsimer D, Gerads R, Bishai WR, Peterson SN, et al. A novel copper-responsive regulon in *Mycobacterium tuberculosis*. Mol Microbiol. 2011; 79:133–148. [PubMed: 21166899]
68. Samanovic MI, Li H, Darwin KH. The Pup-Proteasome System of *Mycobacterium tuberculosis*. Subcellular Biochemistry. 2013; 66:267–295. Chapter 10. [PubMed: 23479444]
69. Vabulas RM, Hartl FU. Protein synthesis upon acute nutrient restriction relies on proteasome function. Science. 2005; 310:1960–1963. [PubMed: 16373576]
70. Price CTD, Al-Quadan T, Santic M, Rosenshine I, Abu Kwaik Y. Host Proteasomal Degradation Generates Amino Acids Essential for Intracellular Bacterial Growth. Science. 2011; 334:1553–1557. [PubMed: 22096100]
71. Elharar Y, Roth Z, Hermelin I, Moon A, Peretz G, Shenkerman Y, Vishkautzan M, et al. Survival of mycobacteria depends on proteasome-mediated amino acid recycling under nutrient limitation. EMBO J. 2014; 33:1802–1814. [PubMed: 24986881]

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

72. Zhang YJ, Reddy MC, Ioerger TR, Rothchild AC, Dartois V, Schuster BM, Trauner A, et al. Tryptophan Biosynthesis Protects Mycobacteria from CD4 T-Cell-Mediated Killing. Cell. 2013; 155:1296–1308. [PubMed: 24315099]

73. Russell DG, Vanderven BC, Lee W, Abramovitch RB, Kim M-J, Homolka S, Niemann S, et al. *Mycobacterium tuberculosis* Wears What It Eats. Cell Host Microbe. 2010; 8:68–76. [PubMed: 20638643]

74. Glickman MS, Jacobs WR. Microbial pathogenesis of *Mycobacterium tuberculosis*: dawn of a discipline. Cell. 2001; 104:477–485. [PubMed: 11239406]

75. Tian J, Bryk R, Shi S, Erdjument-Bromage H, Tempst P, Nathan C. *Mycobacterium tuberculosis* appears to lack α-ketoglutarate dehydrogenase and encodes pyruvate dehydrogenase in widely separated genes. Mol Microbiol. 2005; 57:859–868. [PubMed: 16045627]

76. de Carvalho LPS, Zhao H, Dickinson CE, Arango NM, Lima CD, Fischer SM, Ouerfelli O, et al. Activity-Based Metabolomic Profiling of Enzymatic Function: Identification of Rv1248c as a Mycobacterial 2-Hydroxy-3-oxoadipate Synthase. Chemistry & Biology. 2010; 17:323–332. [PubMed: 20416504]

77. Wagner T, Bellinzoni M, Wehenkel A, O'hare HM, Alzari PM. Functional Plasticity and Allosteric Regulation of α-Ketoglutarate Decarboxylase in Central Mycobacterial Metabolism. Chemistry & Biology. 2011; 18:1011–1020. [PubMed: 21867916]