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Loss of vitamin C biosynthesis protects from the pathology of a parasitic infection

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The ability to synthesize essential molecules is sometimes lost in evolution. A classic example is ascorbate (vitamin C), which is synthesized in most animals by L-gulonolactone oxidase (GULO), an enzyme lost multiple independent times in animal evolution. This event is thought to be evolutionarily neutral; however, *GULO*-deficient animals including humans need to obtain ascorbate from their diet and are prone to ascorbate deficiency and scurvy. We therefore hypothesized that this disadvantage of GULO loss is offset by physiological benefits. Here, we show that ascorbate deficiency benefits mice infected with schistosome parasites, which cause schistosomiasis, a debilitating parasitic disease that afflicts 250 million people. *Schistosoma mansoni* worms required host ascorbate to produce eggs in vivo. Consequently, ascorbate-deficient mice were protected from schistosomiasis pathologies and transmission. Intermittent ascorbate deficiency protected *Gulo*-deficient mice from both scurvy and schistosomiasis mortality. The effects of ascorbate on schistosome reproduction were mediated by ascorbate-dependent histone demethylation which promoted vitellocyte development in female schistosomes. We propose that vitamin deficiencies are not always detrimental but can protect animals from pathogens which need to obtain vitamins from their host.

ascorbate | parasitology | host-microbe interactions | vitamins | schistosomes

Significance

Ascorbate synthesis was lost in the evolution of several species, turning this molecule into an essential micronutrient, vitamin C. Are there any benefits to losing ascorbate synthesis and becoming prone to ascorbate deficiency? We show that loss of ascorbate synthesis and consequent transient ascorbate deficiency protects mice infected with schistosomes, parasites which cause the major parasitic disease schistosomiasis. This is because schistosomes require ascorbate from the host to lay eggs and cause pathology. Our work shows that a vitamin deficiency can have physiological benefits by protecting animals from the pathology of a major parasitic disease. We propose that resistance against the pathology of a parasitic infection may explain why some animals lost the ability to synthesize vitamin C.

Ascorbate is a small molecule synthesized in most animals by an evolutionarily ancient metabolic pathway terminating in the enzyme L-gulonolactone oxidase (GULO) (1). GULO, whose only known function is ascorbate synthesis, was lost repeatedly and independently in the evolution of many species, including haplorrhine primates, cavy rodents, fruit bats, teleost fishes, and passeriform birds (1) (Fig. 1*A*). This loss transformed ascorbate into a vitamin, whose consumption is essential for health and survival in GULO-deficient species, including humans. It is assumed that loss of ascorbate synthesis incurs no fitness costs because it can be fully replaced by dietary intake, and *GULO* loss is a paradigmatic evolutionarily neutral gene loss (2). However, unlike ascorbate-synthesizing organisms such as mice which maintain uniformly high systemic ascorbate levels, *GULO* loss allows for large variations in plasma ascorbate levels (3, 4). Low ascorbate levels in humans are common and are associated with increased mortality from noncommunicable disease (4, 5). Ascorbate is an antioxidant (6) and stimulates the activity of many oxygenases (7). Ascorbate deficiency impairs hematopoiesis (8), germline development (9), bone development (10), norepinephrine synthesis (11), collagen production, vascular integrity (12), and other cellular processes (13). Persistent deficiency causes scurvy, a fatal disease (14, 15). Why would some species lose the capacity for ascorbate synthesis if ascorbate has many important functions, and its deficiency causes disease? We hypothesized that ascorbate deficiency must confer some robust benefit to the organism in particular physiological settings that may outweigh its harms. However, no such benefits have been identified. Because GULO evolved in the earliest eukaryotes (Fig. 1*A*), any eukaryote which does not have it must have lost it during evolution and therefore may require exogenous ascorbate. This also applies to pathogens, some of which contain GULO or related genes and can synthesize their own ascorbate, and some of which cannot (Fig. 1*B*).

Schistosome parasitic flatworms infect 250 million people every year and cause significant mortality and morbidity in the world's poorest regions (16, 17). Schistosomes live in the circulation for decades and lay hundreds or thousands of fertilized eggs every day (18, 19). This massive fecundity causes schistosomiasis pathology and transmission (18, 19). Eggs lodge in organs, causing inflammation and the chronic disabling symptoms of schistosomiasis. Eggs trigger granuloma formation, facilitating egg shedding from the

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body (20) and parasite transmission to the freshwater snails which serve as intermediate hosts (18). Praziquantel, the only available treatment, is partially effective, is ineffective against immature worms (21), and its intensive use may cause resistance (22). It is essential to understand the molecular mechanisms of schistosome reproduction in order to prevent pathology and transmission.

Schistosome reproduction is governed by a coordinated developmental program in which contact with a male in the host stimulates female maturation and production of oocytes and vitellocytes (23). Vitellocytes provide nutrients to fertilized eggs and express proteins which are cross-linked to form the eggshell (24). Eighty years ago it was reported that schistosome eggshell formation is disrupted in infected guinea pigs with scurvy, but whether this confers benefits to the host has been unclear (25). Addition of ascorbate to culture media promotes production of viable eggs from *Schistosoma mansoni* and *Schistosoma japonicum* (26, 27). Therefore, we hypothesized that vitamin C deficiency protects animals from schistosomiasis by depriving schistosomes of vitamin C, preventing parasite reproduction, host pathology, and disease transmission.

Results

Ascorbate Deficiency Prevents Schistosomiasis Liver Pathology. To test whether vitamin C deficiency protects from schistosomiasis, we infected wild-type mice, which can synthesize ascorbate endogenously, or ascorbate-deficient *Gulo*$^{-/-}$ mice with the human pathogen *S. mansoni*. *Gulo*$^{-/-}$ mice were fed an ascorbate-deficient diet starting from 1 wk before infection to induce ascorbate deficiency throughout the experiment (Fig. 1*C*). Worms isolated from wild-type mice contained ascorbate, but worms isolated from ascorbate-deficient *Gulo*$^{-/-}$ mice did not (Fig. 1*D*). Therefore, schistosomes obtain ascorbate from their host. The number and size of worms were not significantly different between wild-type and *Gulo*$^{-/-}$ mice, suggesting that ascorbate was not required for schistosome infection, survival, or growth in vivo (Fig. 1 *E* and *F*). Infected wild-type mice had hepatomegaly and splenomegaly typical of schistosomiasis, (28) but infected *Gulo*$^{-/-}$ mice had no hepatomegaly and their splenomegaly was attenuated (Fig. 1 *G–I*). Extensive granulomas were present on the surface of infected wild-type but not *Gulo*$^{-/-}$ livers (Fig. 1*J*). In this depletion schedule, ascorbate-deficient *Gulo*$^{-/-}$ mice were weak and had weight loss consistent with development of scurvy (Fig. 1*K*). To test whether ascorbate deficiency can block schistosomiasis pathology without causing scurvy, in parallel we provided *S. mansoni*–infected *Gulo*$^{-/-}$ mice with 1% ascorbate in the diet – which normalizes plasma ascorbate levels (8)—before and for up to 3 wk after infection and then transferred to an ascorbate deficient diet until analysis at 8 to 9 wk after infection (Fig. 1*C*). This time-restricted depletion schedule removed ascorbate specifically during the egg-laying period whose onset is 4 to 5 wk after infection (19). *Gulo*$^{-/-}$ mice in this treatment group did not show signs of scurvy, including weight loss, lethargy, or weakness (Fig. 1*K*). Similar to the complete depletion group, time-restricted ascorbate depletion prevented hepatomegaly and reduced splenomegaly (Fig. 1 *G–I*). *Gulo*$^{-/-}$ mice on a time-restricted ascorbate depletion had no viable eggs deposited in their livers and no or minimal granuloma formation and liver fibrosis, in contrast to wild-type mice which had abundant egg deposition, granuloma formation, and liver fibrosis (Fig. 1 *L–P*). Histological analysis of the spleen showed that infection in wild-type mice induced white pulp expansion with follicular hyperplasia and red pulp expansion with extramedullary hematopoiesis (EMH), effects that were reduced in ascorbate-depleted *Gulo*$^{-/-}$ mice (Fig. 1*Q*).

Provision of ascorbate to *Gulo*$^{-/-}$ mice throughout the infection period in the chow or water restored hepatomegaly (*SI Appendix*, Fig. S1*A*) consistent with ascorbate deficiency being responsible for the phenotype of infected *Gulo*$^{-/-}$ mice. Therefore, ascorbate depletion can protect *S. mansoni*–infected mice from egg deposition, granuloma formation, inflammation, liver fibrosis, hepatomegaly, and splenomegaly in the absence of scurvy.

Ascorbate Deficiency Prevents Hematopoietic and Immune Aberrations Caused by Schistosomiasis. *S. mansoni* infection causes hematopoietic and immune aberrations, including eosinophilia (19, 28). Complete or time-restricted ascorbate depletion blunted the infection-induced increase in blood, bone marrow, spleen, and liver eosinophils (Fig. 2 *A–F*) and eosinophil precursors (Fig. 2 *G–I*). Ascorbate depletion prevented increased blood monocytes in infected mice (Fig. 2*J*). Consistent with our prior results (28), infection impaired the development of the erythroid and platelet lineages, as suggested by a decreased frequency of megakaryocyte-erythroid progenitors (MEPs) and an increased frequency of platelet/erythroid-biased hematopoietic progenitors (HPC-2) (29, 30). These effects were rescued by ascorbate depletion (Fig. 2 *K–L*). Schistosomiasis severely disrupted bone marrow erythropoiesis (28), and this was also rescued by ascorbate depletion (Fig. 2 *M–O*).

Granulomas form around schistosome eggs a few days after deposition and continue to grow for several weeks (20, 31). To test whether granuloma maintenance is affected by ascorbate, we supplemented *Gulo*$^{-/-}$ mice with ascorbate in the drinking water for 1 wk during the egg-laying period of the infection, followed by a 2-wk ascorbate depletion and analysis (*SI Appendix*, Fig. S1*B*). In this setting, many granulomas at analysis were formed around eggs laid during the supplementation period. Ascorbate supplementation restored the effects of schistosomiasis on hepatosplenomegaly, granuloma formation, and hematopoiesis (*SI Appendix*, Fig. S1 *C–N*). This suggests that ascorbate is not required for the maintenance of granulomas after they have formed. The number of schistosome eggs in the liver was reduced (*SI Appendix*, Fig. S1*O*), and eosinophilia was blunted (*SI Appendix*, Fig. S1 *P–U*) after a 2-wk ascorbate depletion, consistent with a reduction in ongoing egg deposition during the infection.

Ascorbate Deficiency Protects from Mortality and Disease Transmission. For ascorbate depletion to be protective against schistosomiasis, benefits to the host must outweigh the adverse effects of scurvy. Development of scurvy requires ascorbate depletion for several months in humans (32, 33), and for around 6 wk in mice, (12) but schistosomes lay eggs daily. We hypothesized that the different timescales of the harms compared to the benefits of ascorbate deficiency might provide a survival advantage during infection. To assess this, we tested whether intermittent ascorbate depletion for 3 wk followed by repletion for 1 wk may benefit the host by decreasing the intensity of egg laying and pathology while preventing scurvy. Infected *Gulo*$^{-/-}$ mice with *S. mansoni* were placed on this long-term intermittent ascorbate depletion schedule (Fig. 3*A*). 10/16 infected wild-type mice died after infection, but only 1/19 of intermittently depleted *Gulo*$^{-/-}$ mice died (Fig. 3*A*). Serial bleeds revealed a reduction in eosinophilia and monocytosis in infected *Gulo*$^{-/-}$ as compared to wild-type mice, suggesting reduced inflammation (Fig. 3 *B* and *C*). Infected wild-type mice had thrombocytopenia which was rescued in intermittently depleted *Gulo*$^{-/-}$ mice (Fig. 3*D*). Therefore, intermittent ascorbate depletion provides a robust survival advantage to *Gulo*$^{-/-}$ mice infected with *S. mansoni*.

Ascorbate Deficiency Prevents In Vivo Egg Laying and Disease Transmission. Ascorbate is necessary for viable schistosome egg production in vitro (26, 27). We tested whether host ascorbate is necessary for viable egg production 8 to 9 wk postinfection in vivo. Eggs were often seen in the center of liver granulomas in wild-type mice but not in the liver of *Gulo*$^{-/-}$ mice depleted from ascorbate during the egg-laying period (Fig. 3*E*). *S. mansoni* egg shedding in the feces transmits schistosomiasis (20). Feces of wild-type mice contained many normal eggs, but feces of ascorbate-depleted *Gulo*$^{-/-}$ mice contained no eggs (Fig. 3*F*), suggesting that host ascorbate deficiency blocks disease transmission. Worms isolated from wild-type mice produced viable eggs ex vivo for at least 2 d, (26, 27) but worms isolated from *Gulo*$^{-/-}$ mice produced only malformed, dead eggs (Fig. 3 *G* and *H* and *SI Appendix*, Fig. S2). To confirm the direct effect of ascorbate on egg formation in this context, paired worms isolated at 7 wk postinfection from ascorbate-depleted *Gulo*$^{-/-}$ mice were cultured in ascorbate-supplemented media. By day 4, ascorbate-supplemented worms began producing normal eggs, and the proportion of normal eggs increased progressively with time in ascorbate-supplemented culture (*SI Appendix*, Fig. S3). By day 8, the vitelline glands of ascorbate-supplemented worms matured, whereas those of the control group remained undeveloped (*SI Appendix*, Fig. S3). Therefore, ascorbate acts directly on worms to rescue the egg production defects observed in worms isolated from ascorbate-depleted *Gulo*$^{-/-}$ mice.

Inflammatory activity and granuloma formation in schistosomiasis is primarily triggered by antigens from mature eggs (20), suggesting that protection from inflammation and granulomas in infected ascorbate-depleted *Gulo*$^{-/-}$ mice is imparted by impaired egg production. However, ascorbate also affects hematopoietic and immune cells (8, 34). To test whether the protective effects of ascorbate deficiency on inflammation and granuloma formation were mediated by the action of ascorbate on egg production rather than on the immune system, we administered *S. mansoni* eggs intravenously in wild-type or ascorbate-depleted *Gulo*$^{-/-}$ mice. In this system, eggs mainly lodge and trigger an immune response in the lung (35). Both wild-type and ascorbate-depleted *Gulo*$^{-/-}$ mice exhibited a vigorous type 2 lung inflammatory response to the eggs marked by elevated eosinophils and CD4^{+} T cells, including those that produced the type 2 cytokines IL-4 and IL-13 (*SI Appendix*, Fig. S4 *A–L*). The frequency of CD8^{+} T cells or other immune cells in the lungs did not change or decreased after egg administration in both wild-type and *Gulo*$^{-/-}$ mice (*SI Appendix*, Fig. S4 *A–L*). Wild-type and *Gulo*$^{-/-}$ mice showed a mild systemic response to this treatment marked by elevation of CD4^{+} T cells and eosinophils in the blood and liver (*SI Appendix*, Fig. S4 *M–P*). Granulomas surrounding injected eggs were formed in the lungs of both wild-type and ascorbate-depleted *Gulo*$^{-/-}$ mice (*SI Appendix*, Fig. S4*Q*). Therefore, intact eggs rescue the effects of ascorbate deficiency on inflammation and granuloma formation in schistosomiasis. This suggests that the effects of ascorbate are mediated by its action on worms rather than the host.

To test whether a different schistosome species also requires host-derived ascorbate for egg production and pathology, we analyzed *Gulo*$^{-/-}$ mice infected with *S. japonicum*. Adult *S. japonicum* females isolated from ascorbate-depleted *Gulo*$^{-/-}$ mice produced malformed eggs which adhered to each other, in contrast to females isolated from ascorbate-supplemented *Gulo*$^{-/-}$ mice which produced eggs with a normal morphology (*SI Appendix*, Fig. S5*A*). Ascorbate-depleted *Gulo*$^{-/-}$ mice were protected from *S. japonicum*-induced hepatomegaly, liver granulomas and fibrosis as compared to ascorbate-supplemented *Gulo*$^{-/-}$ mice (*SI Appendix*, Fig. S5 *B–D*). Therefore, ascorbate deficiency protects against egg production and pathology of infection from multiple schistosome species.

Ascorbate Is Required for Female Schistosome Vitellocyte Development. Our results suggest that host-derived ascorbate is essential for schistosome egg production, disease development, and transmission. Ascorbate depletion did not affect parasite survival or growth in vivo (Fig. 1 *E* and *F*), suggesting that ascorbate specifically regulates reproduction rather than general growth processes. To understand how ascorbate regulates egg production, we first assessed the effects of ascorbate on female schistosome reproductive organ development. Maturation of the ovaries and vitellaria of virgin female schistosomes is triggered by pairing with a male schistosome (36). No changes were observed in the gross structure of ovaries of paired mature female *S. mansoni* isolated from ascorbate-depleted *Gulo*$^{-/-}$ as compared to wild-type mice (Fig. 4*A*), suggesting that ovary maturation does not require ascorbate. In contrast, vitellaria maturation was inhibited in female *S. mansoni* isolated from ascorbate-depleted *Gulo*$^{-/-}$ as compared to wild-type mice (Fig. 4*A*). Female *S. japonicum* vitellaria development and egg production but not ovarian development was impaired in paired infections of ascorbate-depleted as compared to ascorbate-supplemented *Gulo*$^{-/-}$ mice (*SI Appendix*, Fig. S6). These results suggest that ascorbate is required for female schistosome vitellaria maturation in the infected host.

We previously showed that ascorbate is crucial for egg formation in cultured worms (26). To assess the mechanisms by which ascorbate promotes egg laying, we set up an in vitro system in which we could control the timing of female sexual development using the male peptide pheromone β-alanyl-tryptamine (BATT). BATT can trigger female sexual maturation in the absence of males (36). Virgin females isolated from unisexual mouse infections were stimulated with BATT and cultured with or without ascorbate. After 10 d in culture, females cultured with ascorbate laid normal eggs, but those cultured without ascorbate produced smaller eggs with malformed or missing structures typical of normal eggs, such as a smooth external shell, a prominent lateral spine, and a viable oocyte (Fig. 4*B*). Vitellaria from ascorbate-treated virgin females were strongly stained with Fast Blue BB, indicative of vitellaria maturation, but untreated females had reduced staining indicative of impaired maturation (Fig. 4*B*). We did not observe differences in ovary maturation between the two groups (Fig. 4*B*). Therefore, the effects of ascorbate on female sexual maturation in the host could be recapitulated in this controlled developmental in vitro system.

To explore the molecular changes induced by ascorbate, we performed RNA-seq analysis on ascorbate-treated or untreated females stimulated with BATT. Ascorbate treatment changed the expression of many genes (Fig. 4*C* and *SI Appendix*, Table S1) (37, 38). To pinpoint the tissues affected by ascorbate at the transcriptional level, we mapped the gene expression changes induced by ascorbate onto a single-cell gene expression atlas we previously generated (39). Most of the top 50 transcripts upregulated by ascorbate were specifically expressed in cells of the late vitelline lineage (Fig. 4*D*). Genes whose expression was upregulated by ascorbate included several involved in eggshell synthesis (*SI Appendix*, Fig. S7). These data suggest that female schistosomes depend on host-derived ascorbate to support vitellaria development.

To determine processes influenced by ascorbate treatment in an alternative way, we performed metabolomics in ascorbate-treated or untreated female schistosomes. The top metabolite enriched in ascorbate-treated schistosomes was L-DOPA, a product of tyrosinase activity, which crosslinks vitellocyte proteins to form the schistosome eggshell (*SI Appendix*, Fig. S8 and Table S2) (40), consistent with a role for ascorbate in vitellocyte development.

Molecular Mechanisms of Ascorbate Action in Female Schistosomes. We investigated the molecular basis for the ascorbate requirement in egg production. Ascorbate is a major

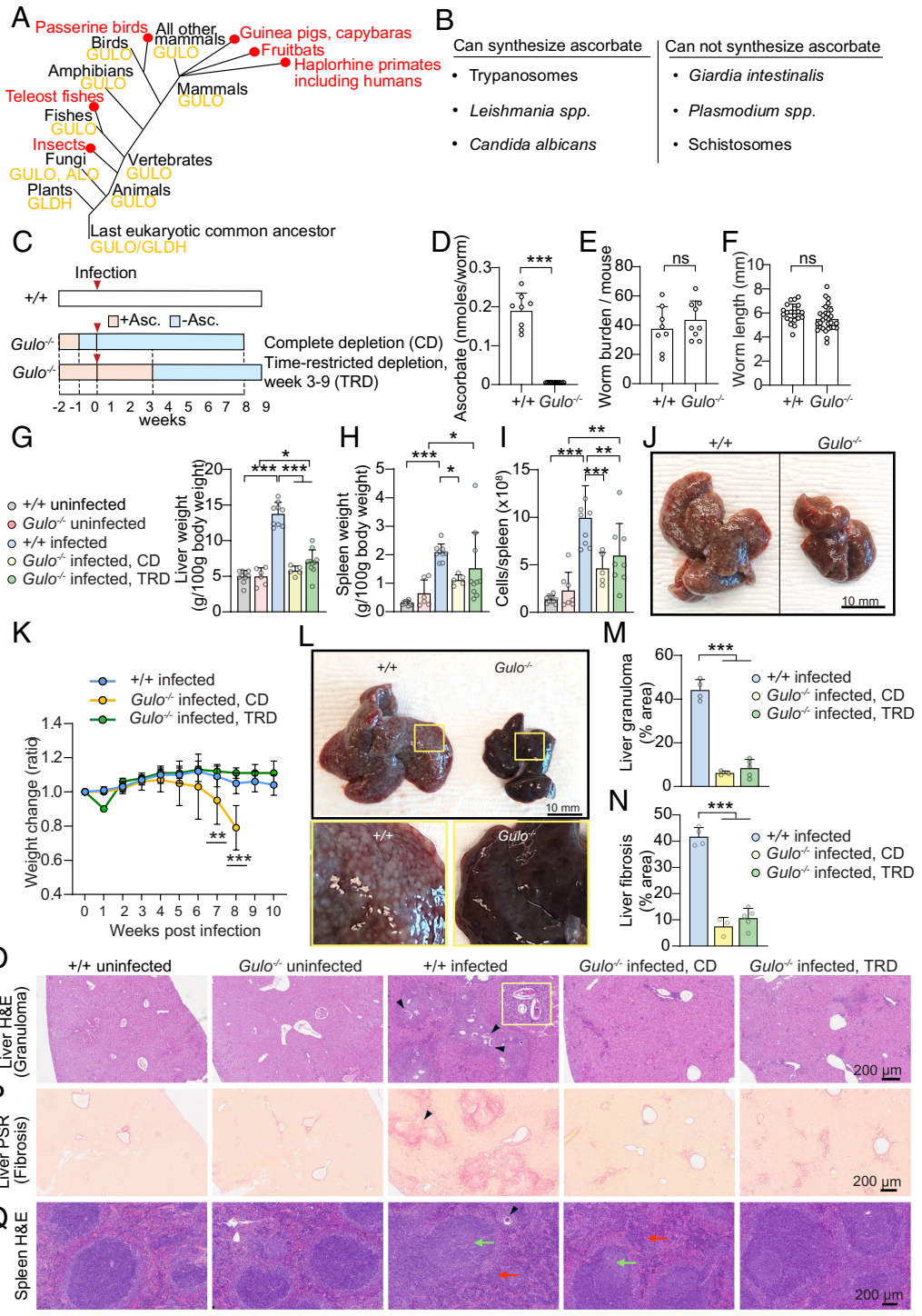

Fig. 1. Ascorbate deficiency prevents schistosomiasis pathology. (*A*) Ascorbate synthesis through L-gulonolactone oxidase (GULO) or its paralogues GLDH/ALO evolved in the last eukaryotic common ancestor and was lost multiple independent times during animal evolution. The presence of a functional GULO or paralogue gene is indicated with an orange gene name, and the loss of GULO is indicated with a red circle. (*B*) Examples of human pathogens which can or cannot synthesize their own ascorbate (or ascorbate analogues such as erythroascorbate). (*C*) *S. mansoni* infection of *Gulo*$^{-/-}$ mice. *Gulo*$^{-/-}$ mice were placed on complete ascorbate depletion (CD) starting from 1 wk before infection or on time-restricted ascorbate depletion (TRD) starting from 3 wk after infection. Wild-type control mice synthesize their own ascorbate and were not provided with exogenous ascorbate. (*D*) Ascorbate levels in worms isolated from infected wild-type and *Gulo*$^{-/-}$ mice (n = 8 wild-type and 10 *Gulo*$^{-/-}$ mice, two worms/mouse). (*E* and *F*) Worm number (n = 8 to 9 mice/treatment) and length (n = 21 to 29 worms/treatment) isolated from wild-type or ascorbate-depleted *Gulo*$^{-/-}$ infected mice at 6 to 7 wk postinfection. (*G–I*) Liver and spleen weights and spleen cellularity at 8 wk or 9 wk postinfection (n = 5 to 10 mice/treatment) (*J*) Granulomas at 7 wk postinfection in the liver of infected wild-type but not *Gulo*$^{-/-}$ mice with complete ascorbate depletion. (*K*) Effect of complete (CD) or time-restricted (TRD) ascorbate depletion on body weight after infection. n = 5 to 7 mice/treatment. (*L*) Granulomas at 9 wk postinfection in the liver of infected wild-type but not *Gulo*$^{-/-}$ mice with time-restricted ascorbate depletion. *Insets* show a magnification of the boxed area. (*M*) Area of H&E-stained liver sections occupied by granulomas. n = 3 to 5 mice/treatment. (*N*) Area of Picrosirius Red (PSR)-stained liver sections which shows fibrosis. n = 3 to 5 mice/treatment. (*O*) Representative images of H&E-stained liver sections. Infected wild-type but not *Gulo*$^{-/-}$ mice show severe granuloma formation associated with heavy egg lodging (black arrowhead). The *Inset* shows a magnified region with egg deposition. (*P*) Representative images of PSR-stained liver sections. Infected wild-type but not *Gulo*$^{-/-}$ mice show severe hepatic fibrosis marked by collagen deposition. (*Q*) Representative images of H&E-stained spleen sections. Infected wild-type mice show proliferation of white pulp with follicular hyperplasia/germinal centers (green arrow) and expanded red pulp (red arrow) with associated extramedullary hematopoiesis (EMH). These features are attenuated in infected *Gulo*$^{-/-}$ mice. All graphs show means ± SD. Statistical significance was assessed with Welch's *t* test (*D*), unpaired *t* test (*E* and *F*), and one-way ANOVA (*G–N*). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

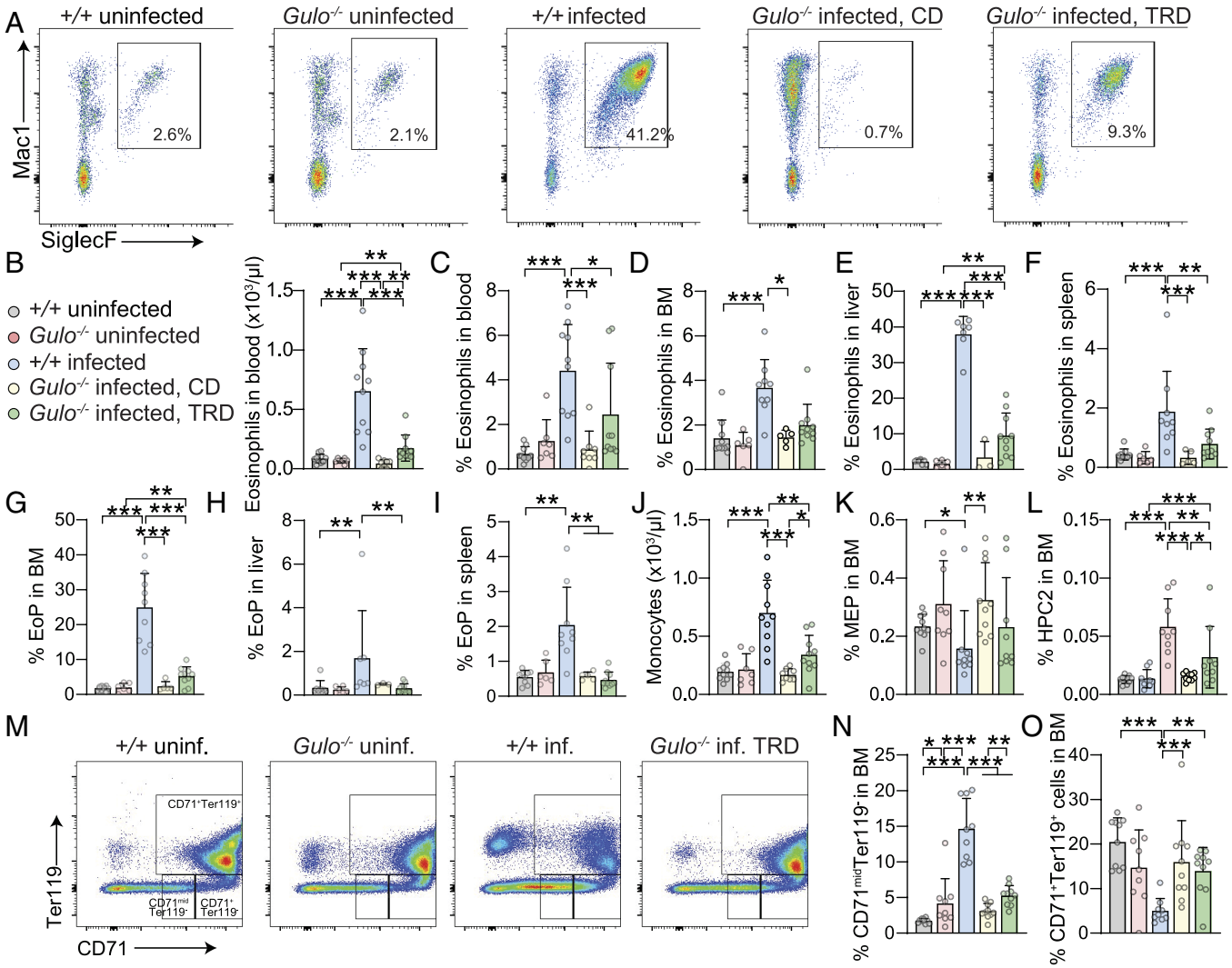

Fig. 2. Ascorbate deficiency prevents hematopoietic aberrations in *S. mansoni*–infected mice. (*A*) Representative flow plots of liver eosinophils from uninfected wild-type and *Gulo*$^{-/-}$ mice, or infected wild-type and *Gulo*$^{-/-}$ mice on a time-restricted (TRD, weeks 3 to 9) or complete (CD) ascorbate depletion. Plots are gated out of CD45^{+}CD115^{-}Ly6C^{+} cells. (*B* and *C*) Number and frequency of eosinophils in the blood. n = 7 to 10 mice/treatment. (*D*–*F*) The frequency of eosinophils in bone marrow (BM), liver, and spleen. n = 3 to 10 mice/treatment. (*G*–*I*) The frequency of SiglecF^{+}Mac1^{-} eosinophil precursors (EoP) cells in bone marrow (BM), liver, and spleen. n = 5 to 10 mice/treatment. (*J*) The number of monocytes in the blood. n = 5 to 10 mice/treatment. (*K* and *L*) The frequency of MEP and HPC-2 in bone marrow. n = 7 to 10 mice/treatment. (*M*) Representative flow plots of erythrocyte progenitors quantified in *N* and *O*, gated out of Mac1^{-}B220^{-}CD3^{-} cells. (*N* and *O*) The frequency of CD71^{mid}Ter119^{-} and CD71^{+}Ter119^{+} erythroid progenitor cells. n = 9 to 10 mice/treatment. All graphs show means ± SD. Statistical significance was assessed with one-way ANOVA of log-transformed values (*B*, *C*, *G*, and *L*), Kruskal–Wallis test (*D*, *H*, and *K*), one-way Brown–Forsythe ANOVA (*E*, *I*, and *J*), one-way ANOVA (*F* and *O*), and one-way Brown–Forsythe ANOVA of log-transformed values (*N*). *P < 0.05, **P < 0.01, and ***P < 0.001.

antioxidant, (6) but other antioxidants including vitamin E or glutathione could not replace ascorbate in promoting vitellaria development and egg production in vitro, suggesting that ascorbate does not act as an antioxidant (Fig. 5*A*). The action of ascorbate on egg production was stereospecific (Fig. 5*A*) (26). Transport of the stereoisomer D-ascorbate into schistosomes was significantly lower than transport of the physiological L-ascorbate form (Fig. 5*B*). This suggested that the basis for stereospecificity in egg-induction was transport rather than enzymatic action, consistent with work in mammalian systems suggesting stereospecific transport as the basis for stereospecific ascorbate activity (41). Ascorbate promotes the activity of many iron-and-α-ketoglutarate-dependent dioxygenases (α-KGDDs) in enzymatic assays, cell culture, and animals (7). We therefore searched for dioxygenases which may be ascorbate targets in *S. mansoni*. Ascorbate activates TET enzymes which oxidize methylated cytosine (5mC) to produce 5-hydroxymethylcytosine (5hmC) on DNA (8, 42–44). Analysis with a sensitive mass spectrometry method (8) did not detect 5mC or 5hmC in the schistosome genome (*SI Appendix*, Fig. S9 *A* and *B*). AlkB-family α-KGDDs can demethylate 6-methyladenosine (m6A) on mRNA. Schistosome mRNA contained m6A (*SI Appendix*, Fig. S9*C*); however ascorbate supplementation did not significantly change its levels (*SI Appendix*, Fig. S9*D*). In mammals, ascorbate is a cofactor for the activity of collagen prolyl-4-hydroxylase (45). However, neither total hydroxyproline levels nor newly synthesized hydroxyproline (*SI Appendix*, Fig. S9 *E* and *F*) increased in ascorbate-treated schistosomes. Carnitine synthesis also relies on α-KGDD enzymes, but carnitine levels were not affected by ascorbate treatment (*SI Appendix*, Fig. S9*G*). Knockdown of *S. mansoni* orthologs of enzymes for nucleic acid modification or proline or lysine hydroxylation in paired female *S. mansoni* did not impair egg production (*SI Appendix*, Fig. S10).

Jumonji C-domain histone demethylases are α-KGDDs whose activity is ascorbate-dependent in several systems, including purified enzyme assays, pluripotent stem cells in vitro, and animals (46). Pharmacological inhibition of the KDM6a/UTX H3K27me2/me3

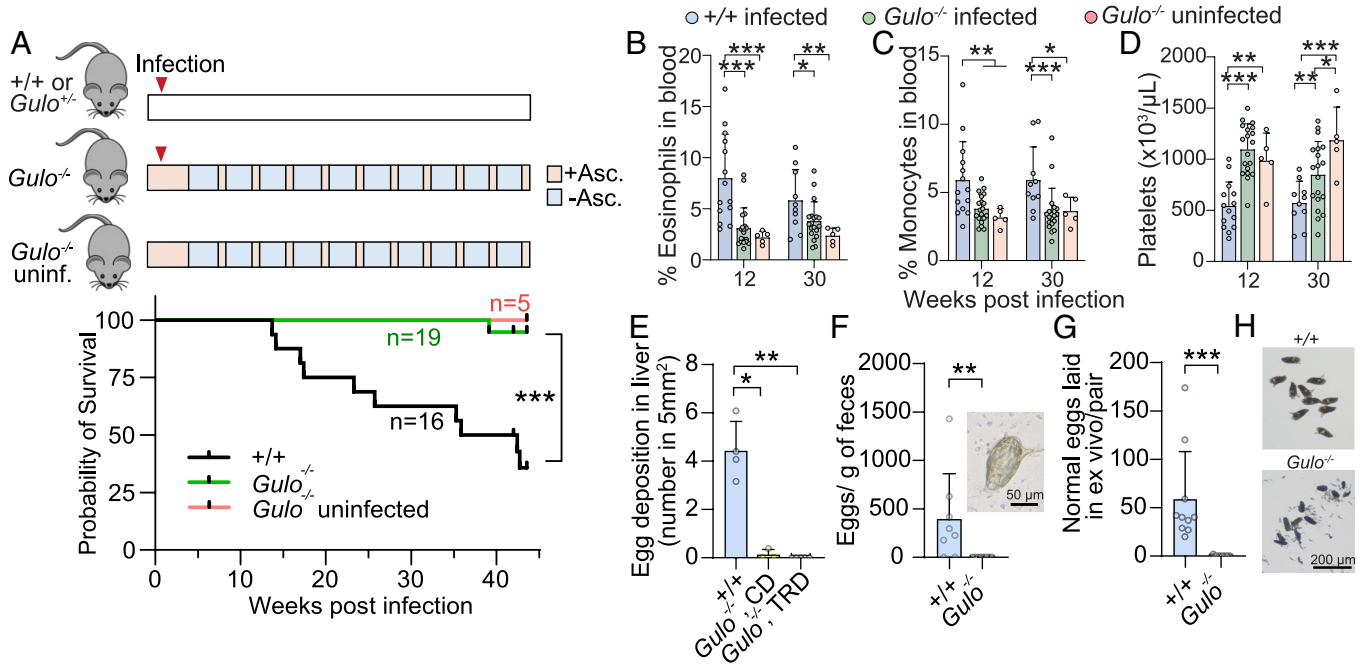

Fig. 3. Ascorbate deficiency blocks schistosomiasis mortality, worm reproduction, and transmission. (*A*) Schedule of *S. mansoni* infection with long-term alternating ascorbate depletion/repletion and Kaplan–Meier survival curve of wild-type or *Gulo*$^{-/-}$ infected mice. *Gulo*$^{-/-}$ mice were supplied with ascorbate until 2.5 wk after infection and then treated on a 3 wk-depletion/1 wk-repletion cycle. n = 16 to 19 mice/treatment. 5 of 5 uninfected *Gulo*$^{-/-}$ control mice survived throughout the experiment. (*B–D*) The frequency of eosinophils and monocytes in blood and platelet counts of infected wild-type or *Gulo*$^{-/-}$ mice on the alternating depletion/repletion schedule. n = 5 to 19 mice/treatment. (*E*) Egg deposition in the liver of wild-type or *Gulo*$^{-/-}$ mice that were either on a time-restricted (TRD, weeks 3 to 9) or a complete (CD) ascorbate depletion, analyzed at 8 to 9 wk after infection. Egg number was counted from H&E-stained liver sections. n = 3 to 5 mice/treatment. (*F*) Fecal egg load of infected wild-type and *Gulo*$^{-/-}$ TRD mice, analyzed 9 wk after infection. n = 8 mice/genotype. The image shows a miracidium recovered from feces of wild-type mice. (*G*) Rate of normal egg production per worm pair from worms isolated from infected wild-type or ascorbate-depleted *Gulo*$^{-/-}$ mice. Egg number was counted at days 1 and 2 after isolating worms from mice and culturing in BM169 media without ascorbate. (*H*) Example of normal or malformed eggs laid by worms isolated from, respectively, infected wild-type or ascorbate-depleted *Gulo*$^{-/-}$ mice. All graphs show means ± SD. Statistical significance was assessed with the Mantel–Cox log-rank test (*A*), one-way ANOVA of log-transformed values (*B* and *C*), one-way ANOVA (*D*), and Mann–Whitney test (*E–G*). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

demethylase homologue *Smp_034000* inhibits schistosome egg production (47, 48). We thus tested whether the effects of ascorbate in schistosome reproduction were mediated by histone demethylation. L-ascorbate-treated BATT-stimulated female schistosomes had reduced levels of H3K27me3 as compared to untreated controls (Fig. 5*C*). The sequence of schistosome KDM6 JmjC domain has high homology to that of KDM6 in humans and other organisms (*SI Appendix*, Fig. S11), suggesting that inhibitors designed for human KDM6 could also be effective in schistosomes. Consistent with prior results (47, 48), pharmacological inhibition of H3K27me2/me3 demethylase activity with GSK-J4, an ester prodrug of the KDM6 inhibitor GSK-J1 (49) inhibited normal egg production (*SI Appendix*, Fig. S12*A*). Ester-conjugated prodrugs may have spurious effects (50). We therefore validated these results with treatment with GSK-J1, which can enter cells, albeit less efficiently than GSK-J4, and may be more selective for KDM6 (51). To avoid effects on male worms, we evaluated the action of GSK-J1 in BATT-stimulated virgin female *S. mansoni*. GSK-J1 did not cause gross morphological abnormalities in treated females but impaired vitellaria development at a dose of 6 µM (*SI Appendix*, Fig. S12*B*). GSK-J1-treated females produced fewer and abnormal eggs, as compared to females treated with the inactive control isomer GSK-J2 (Fig. 5*D*) and had impaired vitellaria but not ovary development (Fig. 5*E*). Similar to BATT-stimulated females, paired adult females treated with GSK-J1 also laid abnormal eggs compared to those treated with GSK-J2 (*SI Appendix*, Fig. S12*C*). To test whether KDM6 genetically mediates the effects of ascorbate, we knocked down *Smp_034000* in females in the presence of ascorbate. Similar to the effects observed with GSK-J1 treatment, knockdown of *Smp_034000* inhibited ascorbate-induced vitellaria maturation and egg production, without impacting ovarian maturation (Fig. 5 *F* and *G* and *SI Appendix*, Fig. S12*D*). GSK-J1 treatment or *Smp_034000* knockdown also inhibited ascorbate-induced vitellaria maturation and normal egg production in virgin females paired with males (*SI Appendix*, Fig. S13). These results suggest that KDM6 inhibition phenocopies ascorbate deprivation in impairing egg production.

Discussion

Our work suggests that the enormous fecundity of the female schistosome and resulting morbidity, mortality, and disease transmission depend on ascorbate supply from the host. The effect of ascorbate is specific to reproduction rather than general schistosome survival. Ascorbate regulates mammalian stem cell function, cell differentiation, and reproduction via DNA or histone methylation (8, 10, 43, 44). Our finding that ascorbate regulates female *S. mansoni* reproduction via histone demethylation suggests that regulation of the epigenome by ascorbate is an evolutionarily generalizable mechanism for the control of cell differentiation and reproduction. Although our search did not reveal ascorbate targets aside from histone demethylation, we do not exclude the possibility that ascorbate may act via additional molecular and cellular mechanisms to control schistosome egg production. In mammals, ascorbate is imported into cells mainly via SLC23A1/A2; however, no homologues of these transporters are annotated in *S. mansoni*. Schistosomes may uptake ascorbate via a different, unknown transporter, or in its oxidized form via glucose transporters (52, 53). Our results support the idea that the abundance of a dietary micronutrient in the host

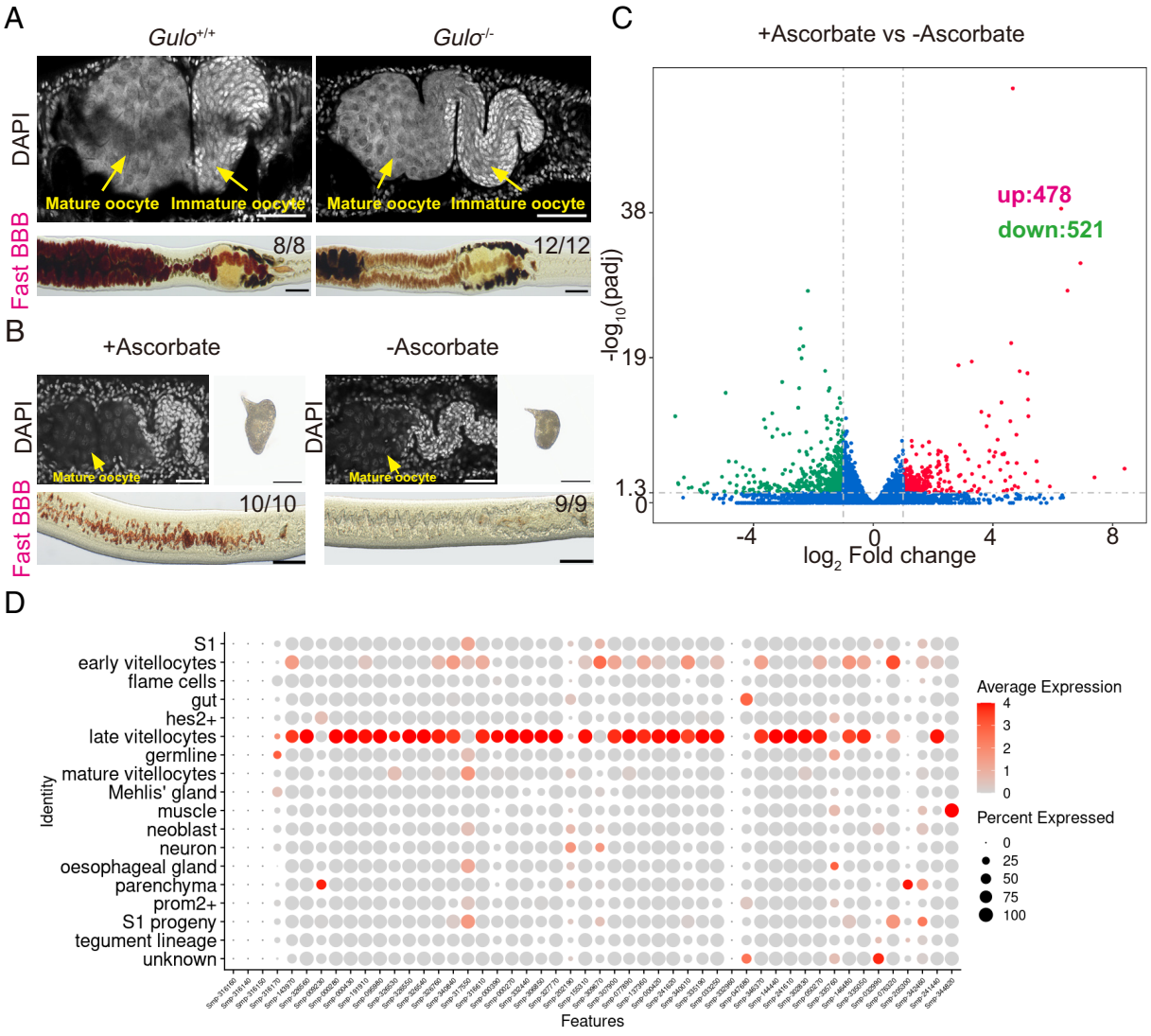

Fig. 4. Host-derived ascorbate is essential for schistosome vitellaria development. (*A*) DAPI and Fast BBB staining showing the ovary (*Top*) and vitellaria (*Bottom*) of adult female parasites from wild-type mice or *Gulo*$^{-/-}$ mice. (*B*) Reproductive organs and eggs laid by virgin female parasites cultured with BATT in the presence or absence of ascorbate. Vitellaria were labeled by Fast BBB staining, and nuclei were stained with DAPI. (*C*) Volcano plot of the differentially expressed genes in virgin females treated with or without ascorbate. Genes were considered differentially expressed at Log_2Fold change>1 and *padj* <0.05. (*D*) Dot plot displaying the cell types which express the 50 most upregulated genes between ascorbate-supplemented and deprived conditions at the single-cell level. (Scale bar, 50 μm for the ovary and egg or 100 μm for the vitellaria.)

can affect the pathogenesis and transmission of parasitic disease by modulating parasite reproduction.

Ascorbate in the blood is depleted after several challenges including inflammation and infection (54–60). Our results suggest that this depletion may be a mechanism to protect against pathogens which themselves require ascorbate. Ascorbate levels in the blood vary 10-fold in the United States and United Kingdom (4, 5). Ascorbate levels are low in schistosomiasis-endemic regions, suggesting that ascorbate deficiency is physiologically relevant in at-risk populations (61). It remains to be seen whether ascorbate deficiency is a protective mechanism from the pathology of schistosomiasis outside laboratory settings or in humans. In addition to schistosomes, other pathogens have lost GULO during evolution (1) (Fig. 1*B*) and thus could require host ascorbate for their survival, development, or reproduction. It is also possible the effects we described are highly specific to schistosomes. Vitamin C supplementation should be avoided in infections caused by pathogens that use host-derived ascorbate for their development.

Genes are often mutated or lost in evolution despite substantial costs to the organism because this protects from infection (62). The widespread disease caused by vitamin C deficiency in diverse societies and contexts (14, 15) is hard to reconcile with the prevailing notion that over millions of years of evolution repeated independent *GULO* losses in many animal lineages have been evolutionarily neutral. Our work reporting a physiological benefit of vitamin C deficiency provides evidence against the neutrality idea and for the idea that loss of vitamin C synthesis can be beneficial. Schistosomes are generalist parasites that infect many species (63) including most primates. Schistosomiasis or other helminth infections have been prevalent in untreated populations in endemic areas (64), premodern societies (65), and wild primates (66); therefore, it is possible these infections have exerted evolutionary pressures on the primate lineage. It remains possible that another pathogen drove *GULO* loss or that *GULO* loss resulted from neutral drift. In addition to vitamin C, the activity of several other vitamin synthesis or vitamin metabolism pathways has been lost or reduced in different species, and this has traditionally been ascribed to metabolic costs (67). We propose the general idea that vitamin synthesis may be lost, or vitamin levels may be reduced as a protection mechanism against pathogens which obtain these vitamins from the host.

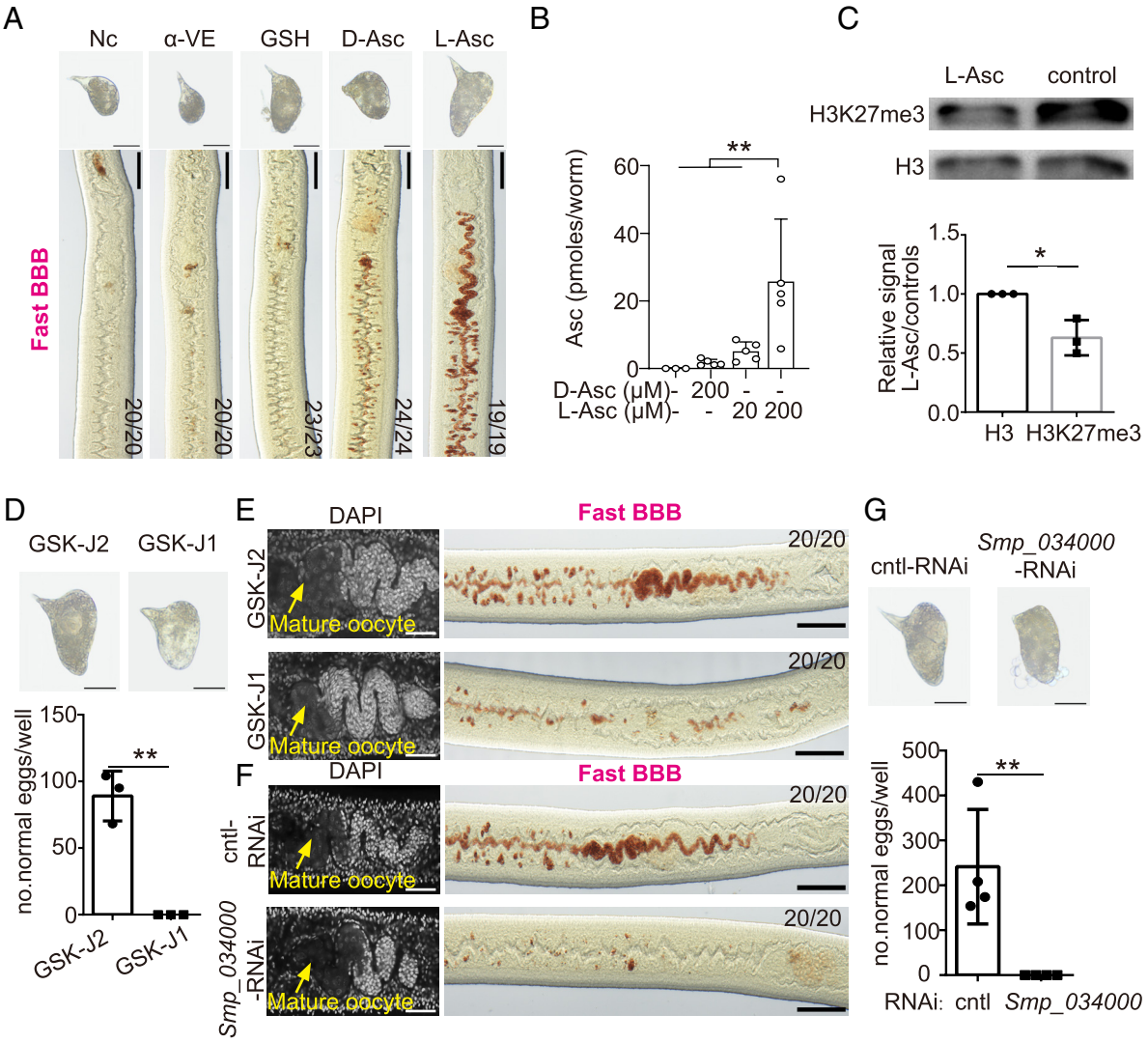

Fig. 5. Ascorbate promotes schistosome reproduction via KDM6. (*A*) Eggs and vitellaria of virgin females treated with BATT in medium supplemented with different antioxidants. Vitellaria were labeled by Fast BBB staining. Nc= no treatment control; α-VE: vitamin E (alpha tocopherol); GSH=glutathione; D-Asc= D-ascorbate; L-Asc=L-ascorbate. (*B*) Ascorbate uptake in female worms. Paired worms were incubated in BM169 media containing L-ascorbate or D-ascorbate of the indicated concentrations for 4 d and ascorbate levels of single female worms were measured after separation. (*C*) Evaluation of H3K27me3 levels in female parasites treated with or without ascorbate. (*Top*) Representative western blot image using the H3K27me3 antibody. (*Bottom*) Quantitation of H3K27me3 levels from Western blot images. (*D*) Morphology and number of normal eggs per well laid by virgin females treated with BATT and either GSK-J2 or GSK-J1. (*E*) DAPI and Fast BBB staining showing the ovary (*Left*) and vitellaria (*Right*) of virgin females treated with BATT and either GSK-J2 or GSK-J1. (*F*) DAPI and Fast BBB staining showing the ovary (*Left*) and vitellaria (*Right*) of virgin females treated with BATT and either control or *Smp_034000* dsRNA. (*G*) Morphology and number of normal eggs per well laid by virgin females treated with BATT and either control or *Smp_034000* dsRNA. (Scale bar, 50 μm for the ovary and egg or 100 μm for the vitellaria.) All graphs show means ± SD. Statistical significance was assessed with the Mann–Whitney test. $*P < 0.05$ and $**P < 0.01$.

Methods

***S. mansoni* Parasites and Mouse Infections.** For experiments performed at UT Southwestern, *S. mansoni* worm maintenance and infections were performed as described (36). *S. mansoni* Naval Medical Research Institute (NMRI) strain was provided by the NIAID Schistosomiasis Resource Center. Infected *Biomphalaria glabrata* snails (M-line) and 6 to 7 wk old female Swiss-Webster mice were used for in-house life cycle maintenance. Worm pairs were harvested from mice with mixed infections of male and female cercaria shed by several *B. glabrata* snails. For harvesting, adult worms were perfused from mice 7 to 9 wk after infection using DMEM with 5% horse serum and heparin warmed at 37 °C. Recovered worms were washed several times with perfusion solution and cultured in Basch Medium 169 containing 10% fetal bovine serum (BM169) (26) supplemented with 1 × Antibiotic-Antimycotic (Sigma-Aldrich A5955, MO, USA). Five worm pairs were cultured in 12-well plates containing 2 mL media per in 5% CO_2 at 37 °C. Media were changed every other day. To separate worm pairs, worms were suspended in BM169 with 0.25% tricaine and agitated for 5 min. Unpaired worms were processed further as indicated in the manuscript or washed two times in BM169 and returned to culture. For metabolomics, DNA and RNA analysis, and knockdown experiments, worms were cultured in ABC169 media (26), with or without ascorbate. For experimental mouse infections, each mouse was infected with 200 *S. mansoni* cercariae released from infected *B. glabrata* snails by tail exposure. Mice were restrained, and their tail was dipped in water with cercariae for 30 min. Both male and female mice were used in all studies. Mice infected for experiments were on the C57BL/Ka background. For some experiments, wild-type or $Gulo^{-/-}$ mice were bred from homozygous crosses. In the long-term intermittent supplementation experiments to assess survival, $Gulo^{-/-}$ mice and their littermate $Gulo^{+/-}$ controls were bred from heterozygous to homozygous ($Gulo^{+/-} \times Gulo^{-/-}$) crosses. Ascorbate plasma concentrations of $Gulo^{+/-}$ mice are similar to those in wild-type ($Gulo^{+/+}$) mice (12). $Gulo^{-/-}$ mice were maintained on 1% ascorbate-supplemented chow (Envigo) and switched to a standard mouse diet for ascorbate depletion before or after infection as noted in the results. In experiments with short-term intermittent ascorbate depletion (*SI Appendix*, Fig. S1), mice were infected at 9 to 13 wk of age. In experiments with time-restricted or complete ascorbate depletion, mice were infected at 14 to 21 wk of age. In survival experiments with long-term alternating ascorbate depletion/repletion cycles, mice were infected

at 23 to 32 wk of age. Mice were analyzed 7 to 9 wk after infection unless otherwise noted. In ascorbate supplementation periods, *Gulo*$^{-/-}$ mice were fed 1% ascorbate-supplemented chow unless otherwise noted. For *Gulo*$^{-/-}$ mice in the complete depletion (CD) treatment which developed scurvy, 20 mg/L ascorbate in the drinking water was provided in the final days of the experiment, a supplementation dose lower than those needed to replenish plasma ascorbate (8) or rescue schistosomiasis pathology. Mice were housed in the Animal Resource Center of University of Texas Southwestern Medical Center and all procedures were approved by the UT Southwestern Institutional Animal Care and Use Committee (IACUC) (approval APNs: 2017-102092, 2018-102427, and 2020-102931).

For experiments performed at Fudan University, female BALB/c mice (6 wk old, 16 to 24 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China) and used for *S. mansoni* maintenance. *Gulo*$^{-/-}$ mice on a C57BL/6JGpt background were generated by GemPharmatech (Jiangsu, China) using CRISPR/Cas9 technology. Three-week-old female *Gulo*$^{-/-}$ mice were provided with 50 mg/L L-ascorbic acid (Sigma-Aldrich A0278) in the drinking water for 1 wk, with the solution changed twice per week. At 4 wk of age, they were infected with cercariae (*S. mansoni*, 300 cercariae; *S. japonicum*, 40 cercariae). After *S. mansoni* infection, *Gulo*$^{-/-}$ mice remained on 50 mg/L L-Ascorbic acid drinking water for 2 wk and then divided into an L-ascorbic acid treatment group, which continued to receive the same dose of ascorbate acid, and a control group which received water without ascorbate acid until analysis at 7 wk postinfection. For *S. japonicum* infection, *Gulo*$^{-/-}$ mice treated with or without 50 mg/L L-ascorbic acid were analyzed at 6 wk postinfection. To obtain virgin female worms or adult paired *S. mansoni*, female BALB/c mice were infected with either female-only or mixed-sex *S. mansoni* cercariae. At 7 wk postinfection, mice were perfused through the hepatic portal vein using 0.9% sodium chloride solution (plus 200 to 350 U/mL heparin). Parasites were washed three times with BM169 medium before being transferred to the appropriate culture medium for in vitro cultivation. Experiments with animals at Fudan University were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China (2006398) and were approved by the Animal Care and Use Committee of Fudan University (Fudan IACUC 2021JS0078).

Cell Isolation and Hematopoietic Analysis. Hematopoietic analysis was performed as previously described (28). Bone marrow cells were obtained by flushing femurs and tibia with a 25G needle in staining medium consisting of Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS; Gibco), supplemented with 2% heat-inactivated bovine serum (Gibco). Spleens were mechanically dissociated by crushing between frosted plates and trituration in staining medium. Livers or lungs were enzymatically digested for 30 min at 37 °C in 1.5 mL RPMI-1640 (Sigma), containing 250 μg/mL liberase (Roche) and 100 μg/mL DNase I (Roche). Cell suspensions were filtered through a 40 μm strainer. Cell number was assessed with a Vi-CELL cell viability analyzer (Beckman Coulter). Blood was collected by cardiac puncture using a 25G needle or by tail vein bleeds and mixed in a tube containing 5 μL 0.5M EDTA. Complete blood cell counts were determined using a hemavet HV950 (Drew Scientific). For flow cytometric analysis of blood, 40 μL was lysed in 1 mL of ammonium chloride buffer (ACK; 155mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA) for 10 min at 4 °C. Cells were incubated with fluorescently conjugated antibodies for 90 min on ice when using CD34 antibody or for 30 min at 4 °C. Cells were washed with staining media and resuspended in staining media containing 1 μg/mL DAPI or 1 μg/ml propidium iodide for live/dead discrimination. Cell populations were defined with the following surface markers: $CD150^+CD48^+Lineage^-Sca\text{-}1^+Kit^+$ hematopoietic progenitor cells (HPC-2), $CD34^-CD16/32^-Lineage^-Sca\text{-}1^-Kit^+$ megakaryocyte–erythroid progenitors (MEPs), $Mac1^+CD115^-Ly6C^{mid/+}SiglecF^+$ eosinophils, $Mac1^-SiglecF^+$ eosinophil precursors, $Mac1^-B220^-CD3^-CD71^{mid}Ter119^-$ immature erythroid progenitors, $Mac1^-B220^-CD3^-D71^+Ter119^-$ erythroid progenitors, $Mac1^-B220^-CD3^-CD71^+Ter119^+$ erythroid progenitors, $Mac1^+Ly\text{-}6g^+$ neutrophils, $Mac1^+CD115^+Ly\text{-}6c^+Ly\text{-}6g^-$ inflammatory monocytes, $CD4^+CD3^+$ T cells, $CD8^+CD3^+$ T cells, $B220^+Mac1^-$ B cells. The lineage cocktail for progenitor analysis consisted of CD2, CD3, CD5, CD8, Gr1, B220, Ter119. Analysis was performed using the LSRFortessa flow cytometer (BD Biosciences) or FACSCanto (BD Biosciences). Data were analyzed using FlowJo (Flowjo LLC) or FACSDiva (BD Biosciences).

RNA interference (RNAi). dsRNA production and RNAi treatment were performed as previously described (68, 69). Oligo sequences used to generate dsRNA templates are listed in *SI Appendix*, Table S3. For RNAi experiments in *SI Appendix*, Fig. S10, worm pairs were treated with 50 μg/mL dsRNA in BM169 medium containing 200 μM ascorbic acid and 0.2% V/V bovine washed RBCs. The medium containing dsRNA was changed every two days for up to 20 d before analysis. Controls were treated with dsRNA transcribed from pJC53.2. To quantify egg production, eggs were removed from the culture media at each media change. Two days later, egg number and the number of worm pairs were counted to calculate daily egg production. For RNAi experiments in Fig. 5, approximately 30 virgin females were cultured in a 6-well plate in wells containing 6 mL of BM169 medium supplemented with 50 μM BATT. Worms were treated with 50 μg/mL dsRNA for target genes or control. The culture medium, BATT, and dsRNA were changed every other day. Each treatment included at least three biological replicates. Parasites were collected after 12 d of culture. For RNAi experiments in *SI Appendix*, Fig. S13, around six virgin females and six adult males were cultured in a 12-well plate containing 3 mL of BM169 medium supplemented with 200 μM ascorbate. Paired worms were treated with 50 μg/mL dsRNA for target genes or control. The culture medium and dsRNA were changed every other day. Each treatment included three biological replicates. Parasites were collected after 10 d of culture.

Additional methods are in *SI Appendix*.

Data, Materials, and Software Availability. RNAseq data (*SI Appendix*, Table S1) have been deposited in GEO with accession number GSE310725 (38) and BioProject # 1338474 (37). Metabolomics data (*SI Appendix*, Table S2) have been deposited in Metabolomics Workbench ST004274 (40). Other data are included in the article and/or *SI Appendix*.

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