ATP-sensitive potassium channel (K$_{ATP}$)–dependent regulation of cardiotropic viral infections

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The effects of the cellular environment on innate immunity remain poorly characterized. Here, we show that in *Drosophila* ATP-sensitive potassium channels (K$_{ATP}$) mediate resistance to a cardiotropic RNA virus, Flock House virus (FHV). FHV viral load in the heart rapidly increases in K$_{ATP}$ mutant flies, leading to increased viremia and accelerated death. The effect of K$_{ATP}$ channels is dependent on the RNA interference genes *Dcr-2*, *AGO2*, and *r2d2*, indicating that an activity associated with this potassium channel participates in this antiviral pathway in *Drosophila*. Flies treated with the K$_{ATP}$ agonist drug pinacidil are protected against FHV infection, thus demonstrating the importance of this regulation of innate immunity by the cellular environment in the heart. In mice, the Coxsackievirus B3 replicates to higher titers in the hearts of *mayday* mutant animals, which are deficient in the Kir6.1 subunit of K$_{ATP}$ channels, than in controls. Together, our data suggest that K$_{ATP}$ channel deregulation can have a critical impact on innate antiviral immunity in the heart.

ion channel | myocarditis | potassium efflux | aging | tolbutamide

Viral infections represent a major threat for living organisms, from prokaryotes to complex eukaryotes, including humans. Therefore, the characterization of the cellular and molecular mechanisms determining the outcome of viral infections is a focus of intense investigation.

A primary determinant of survival to virus infection in animals is the innate immune response, which allows the host to interfere with viral replication and to control the viral load (1). Considerable efforts on different experimental models have led to the realization that dsRNAs, which are produced in the course of viral replication, are a major trigger of innate antiviral responses. In vertebrates, foreign dsRNA molecules are recognized by proteins of the Toll-like receptor (TLR) and RIG-I–like receptor (RLR) families, leading to the production of type I and III interferons, which in turn mediate induction of antiviral molecules (2). In both plants and invertebrates, dsRNAs are recognized by RNase III enzymes of the Dicer (Dcr) family, and trigger antiviral RNAi (3). Interestingly, the cytosolic RLRs and the Dicer enzymes share an evolutionary conserved DExD/H box helicase domain, revealing some similarities in the sensing of viral nucleic acids by plants, invertebrates, and vertebrates (4). In the fruit fly *Drosophila melanogaster*, Dcr-2 accounts for the production of siRNAs of viral origin, which are loaded on the Argonaute (AGO) protein family member AGO2, with the help of the dsRNA binding protein R2D2. These siRNAs guide the slicer enzyme AGO2 toward cRNA sequences, thus achieving antiviral silencing. In agreement with the essential roles of Dcr-2 and AGO2 in the siRNA pathway, flies mutant for these genes are highly susceptible to viral infections (5–7). Though our information on the biochemistry of RNA interference is rapidly expanding, a central and as yet poorly explored area is the control of RNA interference by the cellular environment.

In addition to the immune response, which controls the viral load, it is becoming apparent that homeostatic mechanisms enable the host to control the cellular or tissue damage caused by a given dose of virus (8, 9). In the course of a collaborative effort aimed at understanding the genetic basis of antiviral defenses, one of our groups recently identified an *N*-ethyl-*N*-nitrosourea (ENU)-induced strain of mutant mice, *mayday*, which show an increased frequency of sudden death upon infection with the mouse cytomegalovirus (MCMV). These mice produce normal levels of cytokines upon infection, and die with viral titers in the spleen similar to wild-type controls, indicating that their death does not result from a failure to control the viral burden (9). The *mayday* mutation is an allele of the gene *Kcnj8*, which encodes the inwardly rectifying ATP-sensitive potassium (K$_{ATP}$) channel Kir6.1. K$_{ATP}$ channels are octameric complexes composed of four pore-forming Kir subunits and four sulfonylurea receptor (SUR) subunits that regulate the opening of the channel (10) (Fig. 1*A*). In mice, these channels allow smooth muscle cells in coronary arteries to adapt to the vasoconstriction triggered by the inflammatory cytokines produced by the innate immune system in response to infection (9, 11). K$_{ATP}$ channels are evolutionarily ancient, and we previously reported that silencing of the SUR ortholog (dSUR) in the *Drosophila* heart reduces the survival following infection by the RNA virus Flock House virus (FHV) (9). This observation suggests that K$_{ATP}$ channels play an evolutionarily conserved role in host–virus interactions, and raises the question of the exact mechanism by which they regulate survival to viral infection in organisms with physiologies as different as flies and mammals.

Here we have characterized the role of K$_{ATP}$ channels in FHV-infected *Drosophila*. Using both genetics and pharmacology, we show that K$_{ATP}$ channels are involved in an antiviral resistance mechanism, as impairment of their expression or function leads to a strong increase of the viral titer and accelerated death of the FHV-infected flies. We further show that FHV is a cardiotropic virus and that K$_{ATP}$ channels control the FHV viral load in the heart. Potassium efflux does not affect an intrinsic property of the viral replication cycle, but rather modulates antiviral RNA interference in the heart. These results indicate that tissue-specific regulation of resistance mechanisms by the cellular environment can have dramatic consequences on the outcome of an infection. We illustrate this point by showing that the K$_{ATP}$ agonist drug pinacidil exerts a spectacular protective effect against FHV infection in *Drosophila*.
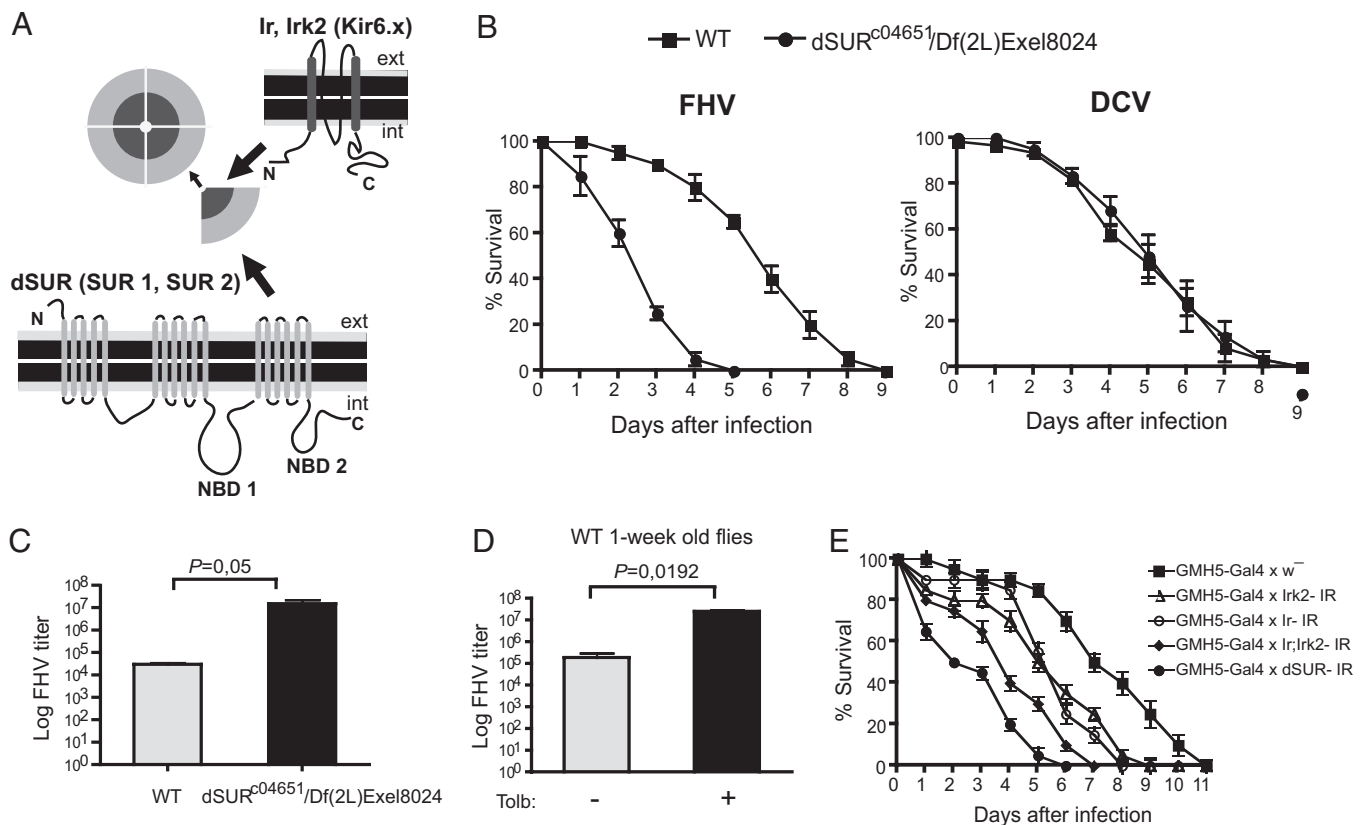
Fig. 1. dSUR, Ir, and Irk2 are important for resistance to FHV infection in *Drosophila*. (*A*) Structural organization of the cardiac K_{ATP} channel. Kir and SUR are the constitutive subunits of the cardiac K_{ATP} channel. Four subunits of the inwardly rectifying K^+ channel, encoded by the genes *Ir* or *Irk2*, associate with the ABC regulatory protein encoded by *dSUR*, to form a functional K_{ATP} channel octamer. SUR possesses two cytoplasmic nucleotide binding domains (NBD1 and NBD2). The names of the mammalian orthologs are given in parentheses. (*B*) Survival curves of *dSUR* mutant and control flies after infection with 2×10^6 pfu/ mL FHV or 2×10^6 DCV particles of a dose lethal to 50% of flies tested, per milliliter. (*C*) FHV titers in *dSUR* mutant and control flies at day 3 following virus infection. (*D*) FHV titers in wild-type (WT) 1-wk-old flies fed on the K_{ATP} antagonist tolbutamide and control flies fed on sucrose solution only, at day 3 following virus infection. (*E*) Survival curves of flies obtained from crosses between UAS-*dSUR* RNAi, UAS-*Ir* RNAi, UAS-*Irk2* RNAi, and UAS-*Ir;Irk2* RNAi lines (IR, inverted repeat) with the heart-specific GMH5-Gal4 driver and control flies [GMH5-Gal4 line crossed with white (w⁻) flies] after infection with FHV. Data represent the mean ± SDs of three (*C* and *D*) or two (*B* and *E*) independent experiments each involving two groups of 10 flies.

Results

***Drosophila* K_{ATP} Channels Are Involved in the Resistance to FHV Infection.** We previously reported that knockdown of the expression of the gene *dSUR*, which encodes the regulatory subunit of K_{ATP} potassium channels (Fig. 1*A*), increases the lethality of *Drosophila* after infection with FHV (9). Flies mutant for *dSUR* [c04651/Df(2L)Exel8024] are viable, indicating that *dSUR* does not carry essential developmental functions. When infected with FHV, *dSUR* mutant flies succumb rapidly, 4–5 d before control flies. By contrast, these mutant flies do not show increased lethality compared with controls when challenged with *Drosophila* C virus (DCV), the entomopathogenic bacteria *Enterococcus faecalis*, *Enterobacter cloacae*, or the fungus *Beauveria bassiana* (Fig. 1*B*) (9). To see if the rapid death of FHV-infected *dSUR* mutant flies reflects a defect in homeostasis or resistance, we monitored the viral load in *dSUR* mutant and control flies. *dSUR* mutant flies show increased accumulation of viral RNAs, and contain up to two logs more infectious viral particles than control flies 3 d after infection, indicating that dSUR regulates the resistance to FHV infection (Fig. 1*C*). Consistent with the survival data, DCV-infected *dSUR* mutant flies contain similar viral titers as control flies.

As an independent test of the requirement for K_{ATP} channels during infection, we investigated the effect of the drug tolbutamide, which acts as an antagonist of K_{ATP} channels. We previously reported that flies fed on a tolbutamide solution died more rapidly than control flies after infection with FHV (9). The tolbutamide treatment leads to increased accumulation of viral RNAs and infectious particles in infected flies, with a two-log increase in viral load 3 d after infection (Fig. 1*D*).

SUR molecules function as the regulatory subunit of Kir6.x channels in mammals. The *D. melanogaster* genome contains three genes encoding channels related to mammalian *Kir* genes, known as *Ir*, *Irk2*, and *Irk3* (Fig. S1*A*). Among these, *Ir* and *Irk2* are closely related to one another, and belong to the same clade as mammalian *Kir2*, *Kir3*, *Kir5*, and *Kir6*, whereas *Irk3* is more distantly related to these molecules. Based on their expression in the hindgut and the Malpighian tubules, these genes have been proposed to function in the osmoregulation of the fly (12). We monitored expression of *Ir* and *Irk2* by RT-PCR, and found that these genes are indeed expressed in the excretory system of *Drosophila*, but that they are mainly expressed, like *dSUR*, in the heart (Fig. S1*B*). We knocked down expression of these genes in the heart using the heart-specific GMH5-Gal4 driver (13), and observed that both *Ir* and *Irk2* are required to slow infection by FHV (Fig. 1*E*). Knockdown of both *Ir* and *Irk2* caused a more severe phenotype than single knockdowns, but not as severe as the knockdown of dSUR. By contrast, silencing of *Irk3* expression did not affect resistance to FHV infection (Fig. S1*C*).

Overall, both genetic and pharmacological data point to an essential role for K_{ATP} channels in the resistance to FHV infection, raising the question of the mechanism involved.

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FHV Is a Cardiotropic Virus. We monitored the presence of virus in the heart of infected wild-type flies by immunofluorescence, and observed that FHV efficiently infects heart muscle cells. By contrast, we did not detect the presence of DCV in the heart, even though the virus can readily be observed in surrounding fat body cells (Fig. 2A). Typical FHV-induced changes in the morphology of mitochondria (14) were observed in longitudinal fibers and cardiomyocytes (Fig. 2B). We noted a more rapid accumulation of FHV capsid proteins in the heart of *dSUR*-silenced flies compared with controls (Fig. S2A). Similar results were obtained when the amount of viral RNAs in the heart or the rest of the body was monitored by quantitative RT-PCR (Fig. 2C). Finally, tolbutamide treatment of wild-type flies led to an increase of FHV viral RNAs mostly in the heart (Fig. 2D) and to increased viremia at the early time points of infection (Fig. 2E). Together, these data indicate that FHV is a cardiotropic virus and that dSUR participates in a tissue-autonomous manner in the control of viral load in the heart, thus limiting viremia and spreading to other tissues.

K_{ATP} Channels Regulate Antiviral Innate Immunity in the Heart. To test whether potassium efflux regulates FHV infection at the level of viral replication, as opposed to other steps of the viral cycle, such as viral entry, viral uncoating, or capsid synthesis and assembly, we used a transgenic viral replicon. In this system, sequences corresponding to RNA1 from FHV and encoding the viral RNA-dependent RNA polymerase (RdRP) are placed under the control of the UAS_{Gal4} system in transgenic flies (Fig. S2B). We previously reported that RNA1 produced from this transgene, even at low levels (in the absence of a Gal4 driver), is rapidly amplified due to the expression of the viral RdRP (5). We monitored RNA1 expression by quantitative RT-PCR in UAS-FHV RNA1 transgenic flies treated with tolbutamide and

untreated controls, and observed a strong increase in the levels of RNA1 in the treated flies. The majority of the increase of RNA1 in flies treated with the drug was attributed to the heart (Fig. S2C). Thus, K_{ATP} channels act at the level of the accumulation of viral RNAs in infected cells.

To test whether K_{ATP} channels have a direct effect on the viral polymerase or affect the host resistance to infection, we analyzed the effect of tolbutamide on FHV-infected flies carrying mutations in essential genes of the antiviral RNAi pathway. Whereas addition of tolbutamide resulted in an increase by two orders of magnitude in the FHV titer in wild-type flies, it had no effect in *Dcr-2*, *AGO2*, or *r2d2* mutant flies (Fig. 3A). Consistent with these data, tolbutamide treatment did not aggravate the survival of *Dcr-2*, *AGO2*, or *r2d2* mutant flies infected by FHV (Fig. S3A). We then tested for a genetic interaction between *AGO2* and *dSUR*. For this, we compared the resistance to FHV infection (survival and viral load) of flies heterozygous for *AGO2* and *dSUR*, as well as flies double heterozygous for both *AGO2* and *dSUR*. Flies heterozygous for both *AGO2* and *dSUR* die more rapidly (Fig. S3B) and contain higher viral loads than flies heterozygous for one of the mutants (Fig. S3C), pointing to a genetic interaction between *AGO2* and *dSUR*. Similar results were observed when *Dcr-2* was used instead of *AGO2* (Fig. S3 D and E). These data reveal that cardiac potassium channels do not affect an intrinsic property of the viral RdRP, but rather affect the antiviral RNAi pathway.

The K_{ATP} Agonist Drug Pinacidil Protects Flies Against FHV infection. Based on our findings, we reasoned that drugs activating potassium channels in the *Drosophila* heart may exert protective effects upon FHV infection. We investigated the effect of the K_{ATP} agonist drug pinacidil, and did not observe an effect in our standard assay conditions with young flies (1 wk old; Fig. S4A).
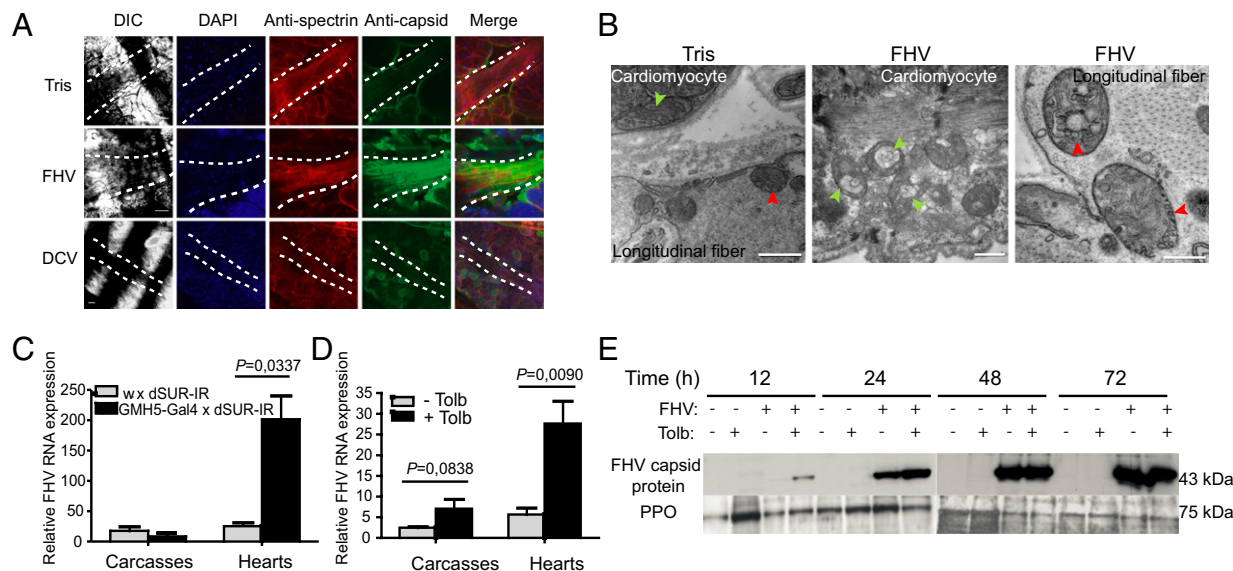
Fig. 2. FHV, but not DCV, infects the *Drosophila* heart. (A) Confocal microscopy of the colocalization of spectrin and FHV or DCV in the hearts of virus-infected WT 1-wk-old flies double-labeled with anti-spectrin (red; Alexa Fluor 546) and antibody to viral capsid protein (green; Alexa Fluor 488) at 3 d following virus challenge. Injections with Tris buffer were used as negative controls. Data are representative of three experiments with at least 10 flies for each treatment. (Scale bar: 35 μm.) (B) Electron micrographs showing FHV-induced spherular invaginations of the outer mitochondrial membrane (arrowheads) of infected, but not control (Tris) cardiomyocytes (green arrowheads) and longitudinal fibers (red arrowheads) that are associated ventrally with the *Drosophila* heart (33). (Scale bar: 500 nm.) (C) Quantitative RT-PCR of FHV RNA levels in the hearts and carcasses from *dSUR*-silenced flies and control flies at 3 d after virus infection. (D) Quantitative RT-PCR of FHV RNA levels in the hearts and carcasses from WT 1-wk-old flies fed on the K_{ATP} blocker tolbutamide and control flies fed on sucrose solution only, at day 3 following infection with FHV. For C and D, data represent the mean ± SD of three independent experiments involving at least 30 flies per replicate. (E) Immunoblot for expression of FHV capsid protein in the hemolymph of 1-wk-old WT flies treated with tolbutamide and control flies kept on sucrose solution only at different times after virus infection. Prophenoloxidase (PPO) protein levels in the hemolymph of flies served as a loading control. Data are representative of two independent experiments involving at least 30 flies for each treatment.

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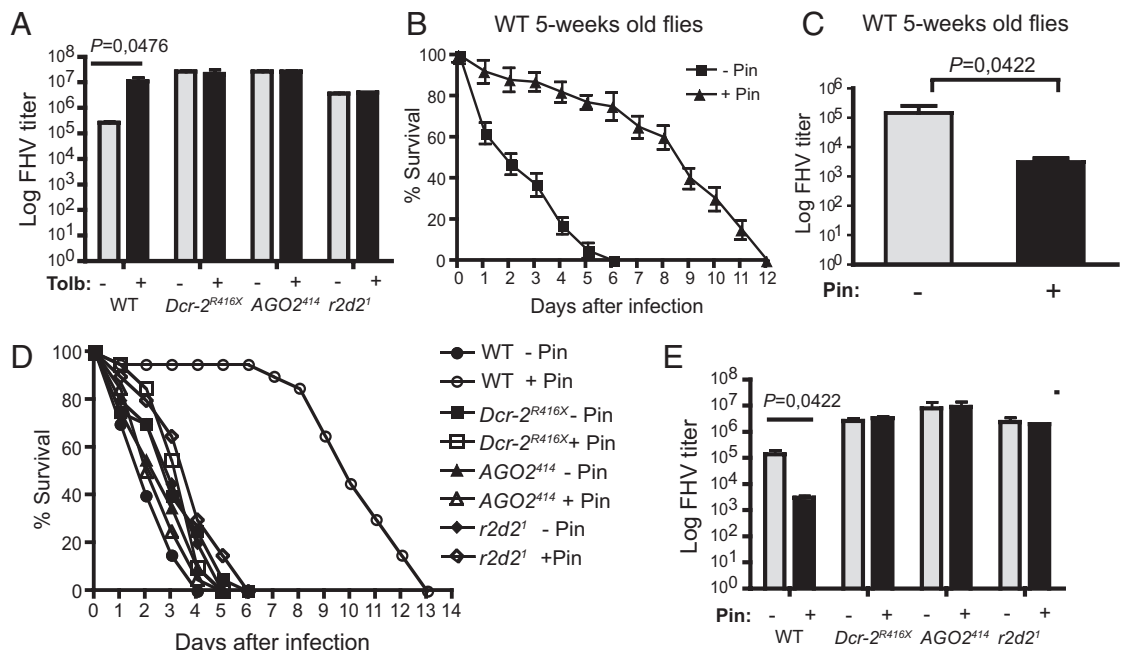
Fig. 3. Potassium channels regulate antiviral RNA interference in the *Drosophila* heart. (*A*) FHV titers in 1-wk-old *Dcr-2*, Argonaute-2 (*AGO2*), *r2d2* mutant, and WT control flies at day 3 following feeding on tolbutamide or control sucrose solution alone and virus infection. (*B*) Survival curves of WT 5-wk-old flies fed on the K$_{ATP}$ agonist pinacidil and control flies fed on sucrose solution alone after infection with FHV. (*C*) FHV titers in WT 5-wk-old flies fed on pinacidil and control flies fed on sucrose solution alone at 3 d following virus infection. (*D*) Survival curves of 5-wk-old *Dcr-2*, *AGO2*, *r2d2* mutant, and WT control flies following treatment with pinacidil and infection with FHV. (*E*) FHV titers in 5-wk-old *Dcr-2*, *AGO2*, *r2d2* mutant, and WT control flies at day 3 after feeding on pinacidil or sucrose solution and virus infection. Data represent the mean ± SD of three independent experiments each involving at least 30 flies (viral load) or two groups of 10 flies (survival) per treatment.

Previous work has shown that *dSUR* expression decreases with aging, and that this decrease in *dSUR* expression is associated with increased pacing-induced heart failure (15). We monitored resistance to FHV infection in aging flies, and observed a strong effect of aging on resistance to FHV infection (Fig. S4*B*). In particular, 5-wk-old wild-type flies were highly susceptible to FHV infection, with 50% of the flies dying within the first 24 h of the infection, reaching 100% mortality in 5–6 d. We treated 5-wk-old flies with pinacidil 3 h before infection with FHV, and observed a dramatic increase in their survival (Fig. 3*B*). Pinacidil treatment of *dSUR* knockdown old flies did not protect them against FHV infection, confirming that the drug was acting on K$_{ATP}$ channels and not on other targets (Fig. S4*C*). Pinacidil-treated old, wild-type flies, but not *dSUR* knockdown old flies, exhibited a significantly reduced viral load compared with old control flies, indicating that the drug strongly increased the resistance to infection (Fig. 3*C* and Fig. S4*D*). Five-wk-old flies also died more rapidly than young flies when challenged with DCV or bacteria. In these cases, however, pinacidil treatment had only weak or no effect on the survival of the flies (Fig. S5 *A–C*).

Pinacidil treatment did not improve the resistance to FHV infection of *Dcr-2*, *AGO2*, and *r2d2* null mutant flies, indicating that a functional antiviral RNAi pathway is required for the effect of the drug (Fig. 3 *D* and *E*). These results confirm that potassium channels regulate innate immunity in the heart, and reveal that the K$_{ATP}$ agonist drug pinacidil can be used to protect flies against a cardiotropic virus.

Mammalian K$_{ATP}$ Channels Are Involved in the Control of Cox-sackievirus B3 (CVB3) Infection in the Heart. Because K$_{ATP}$ channels limit a cardiotropic infection in flies, we decided to test the importance of mammalian *Kcnj8* in the containment of a cardiotropic infection in mice. We assessed virus loads in the heart and serum of *mayday* mutants compared with WT C57BL/6J

mice. Mice were infected with 500 pfu of CVB3 i.p., and virus loads were compared at 3 d postinfection (dpi). Though there was no significant difference in viremia between *mayday* and control mice, a significant increase in virus load in heart tissue was observed in both homozygous and heterozygous *mayday* mutants, compared with the control mice (Fig. 4). We note that exaggerated CVB3 proliferation is observed even in heterozygous *mayday* mutant mice, whereas the deleterious effect of the *mayday* mutation (hypersensitivity to MCMV infection or LPS administration, leading to acute lethality) is strictly recessive (9). The cellular mechanism of the antiviral effect may therefore be quite different from the cellular mechanism of protection against coronary artery vasoconstriction, despite the likelihood that the same hetero-octameric channel complex mediates both phenomena.

Discussion

Our findings reveal that the evolutionarily conserved cardiac K$_{ATP}$ channels play an essential role in the resistance to infection by the cardiotropic RNA virus FHV in *Drosophila*, through modulation of RNA interference. RNAi plays a major role in the regulation of several biological processes, including the control of infections. As such, it does not come as a surprise that RNA silencing must be regulated. Indeed, it was recently reported that molecules from the RNAi pathway, such as TRBP, AGO, or PIWI, can be regulated by phosphorylation, hydroxylation, or methylation (16). We now show that ion channels can also affect antiviral RNAi. It will be interesting in future studies to investigate whether potassium channels also regulate other RNAi pathways in the heart, such as the miRNA or the endosiRNA pathway, which may regulate heart physiology in the absence of viral infection (17).

K$_{ATP}$ channels were previously shown to play a role in the survival to viral infection in mice, but through a different mechanism—namely, the modulation of the response to cytokines in coronary

Fig. 4. Higher virus load in the heart of *Kcnj8* (*mayday*) mutant mice. Eight-wk-old *mayday* and C57BL/J control mice were infected with 500 pfu of CVB3 i.p. At 3 dpi, mice were bled for sera, and hearts were collected after perfusion. Virus titers were determined by the plaque assay on HeLa cells. *$P = 0.0001$, **$P = 0.0020$.

arteries (9, 11). This discrepancy may reflect the differences existing between insects and mammals both at the level of antiviral innate immunity (e.g., the prominent role of RNAi in flies vs. IFN-mediated inducible response in mammals) and host physiology (e.g., the important effect of inflammatory cytokines on the vasculature in mammals). An alternative hypothesis is that K_{ATP} channels may play a previously unnoticed dual role in virus–host interactions, modulating the effects of cytokines and also participating in the regulation of innate immunity mechanisms.

K^+ ions have previously been shown to regulate the induction of the NRLP3 inflammasome, which converts procaspase-1 into active caspase-1, an important aspect of the innate immune response (18). The inflammasome processes prointerleukin (IL)-1β into the active 17-kDa IL-1β inflammatory cytokine. One signal activating the NRLP3 inflammasome is the extracellular release of ATP, which activates the purinergic receptor P2X7R, leading to K^+ efflux and NRLP3 inflammasome activation. Treatment of cells with the K^+ ionophore toxin nigericin is sufficient to trigger the NRLP3 inflammasome, pointing to the important role of potassium ions in the regulation of the production of the essential inflammatory cytokine IL-1β. Interestingly, the sulfonyl urea drug glyburide (also known as glibenclamide), which functions like tolbutamide as an inhibitor of K_{ATP} channels, was recently reported to inhibit the NRLP3 inflammasome in bone marrow-derived macrophages (19). In a more direct connection to the context of viral infections, RIG-I appears to play a dual role upon recognition of RNA virus infection, activating on one hand the transcription factors of the NF-κB and IRF families through the signaling adaptor MAVS, and triggering inflammasome activation through the adaptor ASC on the other. Treat-

ment of bone marrow-derived dendritic cells with glyburide completely abrogates vesicular stomatitis virus-induced secretion of IL-1β, providing further support for a role of K^+ ions in the regulation of antiviral innate immunity in mammals (20). In addition to the inflammasome, antiviral apoptosis can also be triggered by the voltage-dependent K^+ channels Kv2.1 in mammals (21). We now add RNA interference to the list of innate immunity mechanisms regulated by K^+ ions (Fig. S6). An intriguing question in all these cases pertains to the mechanism by which K^+ ions regulate these targets, and whether this function is directly regulated by K^+ ions, or indirectly through modified concentrations of other ions, such as Ca^{2+} or Na^+. Of note, inhibition of the sodium-potassium pump by cardiac glycosides was recently shown to block induction of IFN-β gene expression (22). This effect is mediated by the direct inhibition by Na^+ ions of the ATPase activity of RIG-I, an observation that is particularly interesting in light of the conservation of the DExD/H box helicase domains of RIG-I and Dcr-2 (23).

Potassium channels play an important role in cardiac repolarization both in flies and mammals (24, 25). An unexpected finding of our study is that FHV is a cardiotropic virus in *Drosophila*, providing a useful model to study virus–cardiomyocyte interaction in vivo in this genetically tractable model. Cardiotropic viruses play an important role in the onset of myocarditis in humans, which can lead to sudden death by cardiac arrest (26). However, the mechanisms leading to cardiomyopathy following viral infection in mammals are still poorly understood. One striking observation is that the cardiotropic viruses causing myocarditis are common and will be encountered by the vast majority of the population in their lifetime. However, only some 10% of the infected subjects will develop myocarditis (27, 28), which points to an important role for the genetic background and/or environmental factors in the onset of this disease. For example, defects in an antiviral immunity pathway may be associated with increased viral replication and tissue damage. Curiously, not much is known at this stage about antiviral host defense in cardiomyocytes, and the available data point to complex regulation (29–31). The fact that patients suffering from myocarditis do not appear to be particularly prone to other types of viral infections suggest that either heart-specific mechanisms of antiviral host defense are affected in these patients, or that certain heart-specific conditions regulate the interaction of the cardiomyocyte with the virus. Our findings in *Drosophila*, in conjunction with the established role of K^+ ions in the regulation of the inflammasome discussed above, suggest that control of K^+ ion efflux could be one such condition. Moreover, we observed a higher virus load in the hearts of both homozygous and heterozygous *mayday* mutant mice after CVB3 infection, although they did not show exaggerated viremia compared with control mice. These data are consistent with an ancestral antiviral function served by K_{ATP} channels, now retained in the mammalian heart, but not necessarily other tissues. Moreover, K_{ATP} channels appear to have been adapted to other functions, including the preservation of cardiovascular homeostasis during infection.

In summary, our data highlight a unique layer of complexity in the regulation of antiviral immunity. In addition to the general (e.g., type I IFN in mammals, or RNAi in invertebrates) and the tissue-specific [e.g., TLR-mediated gene induction in plasmacytoid dendritic cells in mammals (32) or *Vago* induction in the *Drosophila* fat body (23)] mechanisms of antiviral host defense, we describe here a tissue-specific regulation by the cellular environment of antiviral innate immunity. Our data indicate that genetic defects or drugs acting on this cellular environment, as opposed to direct effects on the virus or the immune system, can have major effects on the outcome of the infection. Therefore, the characterization of tissue-specific regulatory mechanisms of innate immunity represents an original and promising avenue of research for the development of novel therapeutics against infectious diseases.

Materials and Methods

***Drosophila* Strains and Infections.** w^- and *yw* flies were used as wild-type controls. Most mutant lines used in the experiments (UAS-dSUR RNAi lines; GMH5-Gal4 driver; UAS-RNA1 FHV; $Dcr2^{R416x}$, $AGO2^{414}$, and $r2d2^1$ mutants) have been previously described. SUR^{c04651} mutant flies and the deficiency Df (2L)Exel8024 were obtained from the Bloomington Stock Centre and the Exelixis Collection (Harvard Medical School), respectively. UAS-Ir (v28430 and v28431), UAS-Irk2 (v4341), and UAS-Irk3 (v3886) RNAi lines were purchased from the Vienna *Drosophila* RNAi Center (VDRC). All fly lines were tested for *Wolbachia* infection and cured whenever necessary. Raising of fly stocks and infections were done as previously described (5, 9). Two replicates of 10 flies were used for each treatment, and every assay was replicated three times.

Mice and Virus. $Kcnj8^{mayday/mayday}$ and $Kcnj8^{+/mayday}$ mice (C57BL/6J background) were used for Coxsackievirus B3 (CVB3) infection. Age-matched C57B/6J mice were used as controls. Mice were i.p. injected with 500 pfu of CVB3 (Woodruff strain), which was generously provided by L. Whitton (The Scripps Research Institute). Heart and serum were harvested at 3 dpi and subjected to virus titration by the conventional plaque assay on HeLa cells. All animal protocols were approved by The Scripps Research Institute Department of Animal Resources in compliance with Institutional Animal Care and Use Committee guidelines.

Drug Treatment. Stock solutions (1 M) of tolbutamide and pinacidil (Sigma) were initially prepared in dimethylsulfoxyde. Adult flies were fed on a 1% sucrose solution supplemented with tolbutamide or pinacidil (2 mM final concentration) for 3 h before infection with FHV.

RNA and Protein Analysis. Analysis of RNA expression was performed by real-time quantitative RT-PCR as previously described (23). For hemolymph protein analysis, hemolymph was collected from flies at various time points following infection using thin glass capillaries adjusted to the Nanoject II apparatus. Electrophoresis and immunoblot analysis, as well as immunostaining, were performed as described (23).

Median Tissue Culture Infective Dose Assays. Infected flies were homogenized in Schneider's *Drosophila* media (BioWest) and following centrifugation of fly parts, supernatants were filtered and dilutions of virus suspensions were used to infect *D. melanogaster* Kc167 cells in a 96-well plate format. The presence of virus was scored 24 h later by immunofluorescence.

Statistical Analysis. Data were analyzed using GraphPad Prism software. The mean values were compared by nonparametric unpaired *t* test. In all tests, $P < 0.05$ was considered significant.

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