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Chuqin Huang, Rui Xu, Samuel Liégeois, Di Chen, Zi Li, et al.. Differential Requirements for Mediator Complex Subunits in *Drosophila melanogaster* Host Defense Against Fungal and Bacterial Pathogens. *Frontiers in Immunology*, 2021, 11, pp.478958. 10.3389/fimmu.2020.478958 . hal-03169200

HAL Id: hal-03169200

<https://hal.science/hal-03169200v1>

Submitted on 30 Nov 2021

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**Differential requirements for Mediator complex subunits in
Drosophila melanogaster host defense against fungal and bacterial
pathogens**

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Running title: The Mediator complex in Drosophila host defense against microbial infections

18 **Abstract**

19 The humoral immune response to bacterial or fungal infections in *Drosophila* relies largely on
20 a transcriptional response mediated by the Toll and Immune deficiency NF- κ B pathways.
21 Antimicrobial peptides are potent effectors of these pathways and allow the organism to
22 attack invading pathogens. Dorsal-related Immune Factor (DIF), a transcription factor
23 regulated by the Toll pathway, is required in the host defense against fungal and some Gram-
24 positive bacterial infections. The Mediator complex is involved in the initiation of
25 transcription of most RNA polymerase B (PolB)-dependent genes by forming a functional
26 bridge between transcription factors bound to enhancer regions and the gene promoter region
27 and then recruiting the PolB pre-initiation complex. Mediator is formed by several modules
28 that each comprises several subunits. The Med17 subunit of the head module of Mediator has
29 been shown to be required for the expression of *Drosomycin*, which encodes a potent
30 antifungal peptide, by binding to DIF. Thus, Mediator is expected to mediate the host defense
31 against pathogens controlled by the Toll pathway-dependent innate immune response. Here,
32 we first focus on the Med31 subunit of the middle module of Mediator and find that it is
33 required in host defense against *Aspergillus fumigatus*, *Enterococcus faecalis*, and injected
34 but not topically-applied *Metarhizium robertsii*. Thus, host defense against *M. robertsii*
35 requires *Dif* but not *Med31* in two distinct infection models. The induction of some Toll-
36 pathway-dependent genes is decreased after a challenge of *Med31* RNAi-silenced flies with
37 either *A. fumigatus* or *E. faecalis*, while these flies exhibit normal phagocytosis and
38 melanization. We have further tested most Mediator subunits using RNAi by monitoring their
39 survival after challenges to several other microbial infections known to be fought off through
40 DIF. We report that the host defense against specific pathogens involves a distinct set of
41 Mediator subunits with only one subunit for *C. glabrata* or *Erwinia carotovora carotovora*, at
42 least one for *M. robertsii* or a somewhat extended repertoire for *A. fumigatus* (at least eight

43 subunits) and *E. faecalis* (eight subunits), with two subunits, Med6 and Med11 being required
44 only against *A. fumigatus*. *Med31* but not *Med17* is required in fighting off injected *M.*
45 *robertsii* conidia. Thus, the involvement of Mediator in *Drosophila* innate immunity is more
46 complex than expected.

47 **Keywords**

48 Mediator complex, *Drosophila* innate immunity, Toll pathway, host defense against fungi and
49 bacteria, RNA interference, survival to infection, humoral immune response

50

51 **Introduction**

52 Fungal invasions represent one of the most difficult infectious diseases to cure nowadays,
53 causing worldwide more than 1.6 million deaths per year. People are constantly exposed to
54 fungi, which are controlled in a first line of defense by the innate immune system through the
55 phagocytosis of inhaled spores by macrophages and especially through neutrophils.
56 Nevertheless, fungi can cause diseases such as airway allergy, bronchitis in healthy patients
57 while it may cause deadly invasive aspergillosis in immunocompromised patients (1, 2).

58 The simpler immune system of the genetic model organism *Drosophila melanogaster* makes
59 the analysis of host-pathogen interactions during infections easier to investigate as it lacks the
60 adaptive immunity arm found in higher vertebrates. The host defense against pathogens in
61 *Drosophila* mainly encompasses three major arms: melanization, the cellular response
62 (essentially phagocytosis) and the humoral immune responses (*Toll* and Immune deficiency
63 (*IMD*) NF- κ B pathways). The *IMD* pathway is required to fight off Gram-negative bacterial
64 infections whereas the *Drosophila* defenses against some Gram-positive bacteria and fungi
65 mostly rely on the Toll pathway. Following the sensing of cell wall compounds or of the
66 proteolytic activity of secreted microbial virulence factors in the hemolymph by circulating
67 receptors, host proteolytic cascades will lead to the production of a mature Toll ligand, which

68 will trigger an intracellular signaling pathway that activates the NF- κ B-like transcription
69 factors Dorsal or Dorsal immune-related factor (DIF) (3-5). DIF mediates *Toll* pathway
70 function in innate immunity in adult flies while it is redundant with Dorsal in larvae (6-8).
71 The *Toll* pathway regulates the expression of tens of genes, including those encoding
72 antimicrobial peptides such as Drosomycin as well as those coding for the less characterized
73 *Drosophila*-induced Immune Molecules (DIMs)/Bomanins, effectors that may act in
74 conjunction with as yet unidentified cofactors (9-12)

75 *Aspergillus fumigatus* is the fungus that was initially used to demonstrate that Toll pathway
76 mutants are sensitive to fungal infections (13). Given its medical relevance, we have
77 implemented an unbiased genetic screen in which we monitor the survival of mutant
78 *Drosophila* lines to injected conidia of this fungus. To this end, we are using transgenic lines
79 that express miRNAs designed to target specific genes under the control of a Gal4 driver
80 expressed ubiquitously (14), as well as other RNAi lines (15). To bypass the developmental
81 lethality potentially caused by the down-regulated expression of the targeted gene, the RNAi
82 transgene is expressed only at the adult stage using the Gal80 thermosensitive system (16). In
83 the screen, we found that flies knocked down by RNAi targeting either of two Mediator
84 complex subunit genes (*Med8*, *Med 31*) by RNAi transgene at the adult stage succumb to *A.*
85 *fumigatus* infection.

86 The Mediator complex, evolutionarily conserved from yeast to plants, invertebrates, and
87 mammals, consists of a multiprotein complex (25 subunits in yeast and 33 subunits in
88 mammals) which plays an essential role for the transcription of almost all genes transcribed
89 by RNA polymerase B (Pol B) (17-19). The Mediator complex is composed of a central
90 module and a CDK8 kinase module (CKM). The central module consists of three complexes
91 of distinct subunits known as the head, the middle, which together form the core module, and
92 the tail parts. The central module associates with the CKM, which contains four subunits, and

93 co-activates the transcription of target genes, yet does not appear to be fully essential for
94 Mediator function. The Mediator core complex serves as a functional bridge connecting a
95 variety of transcription activators bound to enhancer regions to the transcriptional machinery
96 at the basal promoter, which includes Pol B and general transcription factors, to initiate gene
97 transcription. The first step of gene transcription is the binding of transcription factors to the
98 enhancer regions. Then, there is a subsequent recruitment of the Mediator complex to the
99 enhancer regions by its interaction(s) with transcription factors through the tail and middle
100 modules. Finally, general transcription factors and Pol B are recruited through the core
101 module to form the preinitiation complex on the core promoter of the target gene (20). Gene
102 transcription is then initiated, a process facilitated by the Mediator complex. In fact, Mediator
103 complex can contact hundreds of transcription activators through its different subunits,
104 generally the tail ones, and transduces the signals from specific transcription factors under
105 different conditions (21). All subunits of the Mediator complex are recruited to the enhancer
106 region, the CDK8 kinase module is transiently dissociated from the complex during the
107 interaction with core promoters, however, its exact role is still not clear (20). Besides its role
108 in the initiation of transcription, other studies have revealed that the Mediator complex also
109 play roles at some other stages of transcription such as elongation, termination, processing of
110 mRNA, and in epigenetic regulation and noncoding RNA activation (22).

111 Even though Mediator is required for the transcription of nearly all Pol B transcripts, half of
112 its subunits appear to have specific functions. In *Drosophila*, most studies have focused on the
113 role of the Mediator complex during development, *e.g.*, (23, 24). A previous study revealed
114 that one of the *Drosophila* Mediator complex subunits, dTRAP80 (a homologue of the head
115 module component Med17), is required for DIF-dependent transcriptional activation of the
116 *Drosomycin* gene in cultured cells and *in vitro*-translated Med17 has been shown to physically
117 interact with both Dorsal and DIF by GST pull down (25). As stated above, two independent

118 hits of our genetic screen are *Med8* and *Med31*. *Med31* but not *Med8* may bind to DIF and
119 Dorsal and both have been reported to bind to *Med17* in a large-scale effort to map protein-
120 protein interactions in *Drosophila* by coaffinity purification of protein complexes (26).
121 Whereas *Med17* and *Med8* both belong to the head module, *Med31* belongs to the middle
122 module, yet interacts, possibly indirectly, with several head module subunits besides *Med17*:
123 *Med6*, *Med11*, *Med18*, and *Med20* of the head module, and also *Med7* from the middle
124 module and *Med14*. *Med8* appears to associate with subunits from all three central modules,
125 including the *Med14* scaffolding subunit, *Med6*, *Med11*, *Med 17*, and *Med18* of the head
126 module, *Med4*, *Med7*, *Med10* of the middle module and *Med15*, *Med16*, *Med23*, *Med25*,
127 *Med27*, and *Med30* of the tail module (27). We therefore hypothesized that *Med31*, and
128 possibly *Med8*, may play a role in *Drosophila* host defense against *A. fumigatus* infection
129 through the facilitation of transcriptional activation mediated by DIF. We have extensively
130 characterized the *Med31 A. fumigatus* susceptibility phenotype and also investigated the
131 function of *Med31* in host defense against other pathogens including the dimorphic or
132 monomorphic yeasts *Candida albicans* or *Candida glabrata*, the entomopathogenic fungus
133 *Metarhizium robertsii*, the Gram-positive bacterium *Enterococcus faecalis* and the Gram-
134 negative bacterium *Erwinia carotovora carotovora 15 (Ecc15)*. The Toll pathway is required
135 for host defense against all these pathogens, except the last one. We further studied the role of
136 most other identified Mediator subunits in *Drosophila* host defense, with a special emphasis
137 on *Med17*, the subunit reported to directly bind to DIF. Our results delineate an unexpectedly
138 complex picture of the Mediator complex in host defense that does not fit with it acting solely
139 through the DIF transcription factor.

140

141 **Materials and methods**

142 **Fly strains and husbandry**

143 Flies were raised at 25°C, 60% humidity with 12h of light/dark cycle. The flies were fed with
144 a semi-solid medium which consists of 7.77% w/v of corn flour, 6.32% w/v of glucose,
145 3.219% w/v of yeast dry powder, 0.9% w/v of agar, 0.2% w/v of sorbitol (except for *A.*
146 *fumigatus* infected flies because *A. fumigatus* is sensitive to the sorbitol preservative),
147 0.0726% w/v of CaCl₂, 3.162% w/v of sucrose, 0.15% w/v of p-Hydroxybenzoic Acid
148 Methyl Ester and water.

149 The TRiP RNAi lines were obtained from the *TsingHua Fly Center* (THFC), the GD RNAi
150 lines come from the *Shanghai Institute for Biological Sciences* (originally from the Vienna
151 *Drosophila* Research Center, Austria). The insert was checked by sequencing for each line.
152 UAS-mCherry-sh and GD60000 were used as controls for TRiP lines and GD lines,
153 respectively. Supplementary Table 1 lists the RNAi lines used in this study. Males from the
154 RNAi lines or their controls were crossed with Ubi-Gal4, tub-Gal80^{ts} virgins. Crosses were
155 set-up at 25°C for three days to ensure an efficient fertilization of females by males. Adults
156 were then transferred to another tube while the tube containing eggs was moved to 18°C, in
157 order to keep the inhibition of Gal4 by Gal80^{ts} and bypass developmental lethality.
158 Soon after the F1 progeny hatched, flies were shifted at 29°C to inhibit Gal80^{ts} and activate
159 Gal4 to initiate the transgene expression. Flies were kept at 29°C for 5 days to ensure the
160 down-regulation of the genes of interest prior to immune challenge.

161

162 **Microbial strains and growth conditions**

163 The RFP-labeled wild-type *Aspergillus fumigatus* strain was a kind gift from Drs. Anne
164 Beauvais and Jean-Paul Latge (Institut Pasteur, Paris). *A. fumigatus* was cultured on potato
165 dextrose agar (PDA) medium plates supplemented with 0.1g/l chloramphenicol (Huankai
166 Microbio Tech, China) in a tissue culture incubator under 5% CO₂ at 29°C. *Metarhizium*
167 *robertsii* (ARSEF 2575) was grown on PDA plates from BD Company, USA (#213400) at

168 25°C for 7 to 14 days. Of note, we did not use the same PDA plates for *A. fumigatus* and *M.*
169 *robertsii* because the PDA used for *A. fumigatus* contains chloramphenicol, which affects the
170 growth of *M. robertsii*.

171 *Candida albicans* (CAF 2.1) and *Candida glabrata* (28) were cultured on Yeast extract
172 Peptone Dextrose (YPD) Agar plates for two days at 29°C, from which one colony was plated
173 on a new plate again for two days at 29°C, and then this plate was kept at 25°C for infections
174 for four weeks.

175 *E. faecalis* (OG1RF) and *Erwinia carotovora* (*Ecc15*) were cultured in Luria-Bertani (LB)
176 agar plates overnight at 37 °C. A single colony was inoculated in LB liquid medium at 37°C
177 and overnight shaking.

178 **Microbial preparation and infection experiments**

179 Prior to *A. fumigatus* injection or *M. robertsii* natural infection, flies were raised on 100mM
180 sucrose for two days to eliminate sorbitol (an antifungal compound present in the fly food)
181 from the flies. Prior to *M. robertsii* injection, flies were raised on regular food. *M. robertsii*
182 and *A. fumigatus* conidia were collected from the surface of the PDA plate by adding three ml
183 of either PBST (PBS containing 0.01% Tween-20) for injections, or sterilized deionized
184 demineralized water containing 0.01% Tween-20 for natural infections. The concentration of
185 the conidia was counted by using a hemocytometer and then adjusted to the adequate working
186 concentration. The working concentrations were 5×10^7 *A. fumigatus* conidia/ml and 10^7 *M.*
187 *robertsii* conidia/ml in PBST for injections, and 5×10^4 conidia/ml in water containing 0.01%
188 Tween-20 for *M. robertsii* natural infection.

189 For *M. robertsii* natural infection, anesthetized flies were incubated into the conidia solution
190 and shaken for 30 seconds, before being dried on a filter paper adapted to a vacuum pump. *M.*
191 *robertsii*-naturally infected flies were then raised on a vial containing a filter paper with

192 100mM sucrose. *A. fumigatus*-injected flies were kept on food without sorbitol whereas *M.*
193 *robertsii*-injected flies were fed regular food.

194 The overnight cultured *E. faecalis* and *Ecc15* were centrifuged at 3500g, 4 °C for 10 minutes.
195 The pellet was washed twice in PBS. *E. faecalis* and *Ecc15* were prepared in PBS at working
196 concentrations of OD₆₀₀ = 0.1 and 1, respectively.

197 Injection of *A. fumigatus*, *M. robertsii*, *E. faecalis* or *Ecc15* was performed by injecting 4.6
198 nL of working solutions, or the same volume of PBS as a control, into flies by using a
199 microinjector (Nanoject, Drummond) and appropriate capillaries. Infection of *C. albicans* or
200 *C. glabrata* was performed by pricking through a sharpened tungsten needle dipped into the
201 single colony directly taken from the plates (28).

202 **Survival assays**

203 The survival assays were performed with 15-25 females per tube, in triplicates. Infected flies
204 were incubated at 29°C with 60% humidity. Log-Rank statistical tests were performed with
205 GraphPad Prism 6. Experiments were performed at least twice, except for the following
206 experiments that were performed only once: *M. robertsii* natural infections: *Med6*, *Med8*,
207 *Med11*, *Med22*, *Med24*; *C. glabrata* infections: *Med12*, *Med14*, *Med21*; *E. faecalis* infections:
208 *Med11*, *Med12*; *Ecc15* infections: *Med11*, *Med12*.

209

210 **Detection of AMP expression level after infection**

211 RNA was extracted from four flies per sample in triplicates or quadruplicates. Flies were
212 homogenized with 1 mL of Trizol reagent (Thermo Fisher Scientific, #15596018) in a
213 microfuge tube using a pestle and the RNA extraction was performed according to the manual
214 instructions. 200 µL of chloroform was added to the samples, which were vortexed and

215 incubated 5 min at room temperature. Then, samples were centrifuged 13,000 g at 4°C for 10
216 min. The water phase at the top of the samples was collected and mixed with the same volume
217 of isopropanol and vortexed again. Samples were centrifuged at 13,000 g at 4°C for 10 min.
218 The pellet was washed with a solution of 75 % ethanol in water and air dried. Then, RNAs
219 were re-suspended in 35 µL of RNase-free water. The quality and the concentration of the
220 total RNA were measured using a Nanodrop 2000 (Thermo Fisher Scientific). The reverse
221 transcription of 800 ng of total RNA was used for with a cDNA synthesis kit (TransGene
222 Biotech, #AT-341), Quantification of the target gene expression level was performed by
223 quantitative Polymerase Chain Reaction (qPCR) with the SYBR Green Supermix kit (Vazyme
224 Biotech, Q311-02). The relative gene expression was normalized to the expression level of the
225 housekeeping gene *Rpl32*, which encodes ribosomal protein 49. Digital PCR was performed
226 on cDNAs at 1 ng/µL as described (29). The list of primers used for this study is found in
227 Table S2.

228 Wild-type flies pricked with concentrated cultures of *Micrococcus luteus* were used as
229 positive controls for *Drosomycin* and *DIMI*, or *Escherichia coli* for *Diptericin* expression
230 (30). The $\Delta\Delta\text{Ct}$ method was used to normalize the values as following: we gave a value of 1
231 for the expression level obtained for *Drosomycin* and *DIMI* 24h after infection with *M. luteus*
232 and for the expression level obtained for *Diptericin* 6h after infection with *E. coli*.

233 ***Western Blot***

234 Four hours after a challenge with *M. luteus*, the hemolymph from 20 flies was collected into
235 40 µL of PBS containing Protease Inhibitor Cocktail (Thermo Fisher Scientific) by
236 centrifugation (3500 g, 30min, 4°C) after cutting the tip of the abdomen (28). The protein
237 concentration of the sample was measured by using a BCA kit (Beyotime Biotechnology).
238 Samples were mixed with SDS-PAGE loading buffer (Beyotime Biotechnology) and boiled

239 for 5 minutes. A SDS-PAGE electrophoresis gel was performed using 20 µg of protein.
240 Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (0.22 µm).
241 The nitrocellulose membrane with transferred proteins was blocked in PBST with 5% BSA at
242 room temperature for 1h. The membranes were incubated in a 1:10,000 rabbit anti-PPO1
243 primary antibody solution (a kind gift from Erjun Ling, Shanghai) in PBST with BSA
244 overnight at 4°C (31). The membranes were washed and incubated in a secondary anti-rabbit
245 HRP antibody (1:10000) for 1h at room temperature.

246 **Phagocytosis assay**

247 3-7 days old adult females were injected with 69 nL of latex beads (Thermo Fisher Scientific),
248 16% w/v (re-suspended in PBS and sonicated prior injection). Control flies were injected with
249 the same volume of PBS. 24h post-injection, flies were injected with 69 nL pHrodo™ Red E.
250 coli BioParticles™ Conjugate (Thermo Fisher Scientific, P35361). The phagocytic activity
251 was observed under a fluorescence microscope (Zeiss Imager.M2) after 30 min. The red
252 fluorescence was quantified in fields of same size. 10 flies of each line were scored in each
253 experiment and three independent experiments were performed.

254 **Microbial load counts**

255 *A. fumigatus* and *C. glabrata* microbial loads after infection were counted after plating a
256 homogenate of single whole flies. Each single fly was transferred into a 1.5 mL Eppendorf
257 tube containing 50 µL of PBST and smashed by a tissue homogenizer. After a few seconds of
258 centrifugation, the entire homogenate product was plated on PDA, and incubated at 29°C.
259 Colony forming units (CFUs) were counted after 2-3 days of incubation. In the case of *C.*
260 *glabrata*, a 1:100 or 1:1000 dilution of the homogenate product was plated depending on the
261 time after infection.

262 *E. faecalis* loads were counted on hemolymph collected from single flies. A series of dilution
263 was performed from 1 to 1:10⁸ in PBS. 10 μ L of each dilution was plated in duplicate on LB
264 agar and incubated at 37°C. Colony forming units (CFUs) were counted the next day.

265 **Statistical analysis**

266 All statistical analysis was performed by using GraphPad Prism 7. The Mann-Whitney or
267 Kruskal-Wallis tests was used for the statistical analysis of all the data except survival
268 experiments. Survival curves were plotted and analyzed by Log-Rank test (Kaplan-Meier
269 method).

270 **Results**

271

272 ***Med31 RNAi flies are susceptible to injected A. fumigatus conidia***

273 We have established that as little as five *A. fumigatus* conidia injected on average per fly are
274 sufficient to kill *MyD88*-immunodeficient flies and are routinely injecting 250 conidia per fly
275 (Xu *et al.*, *in preparation*). As shown in Fig. 1A, this dose rapidly kills *MyD88* whereas only
276 a moderate proportion of *mCherry* RNAi control flies succumbed to this challenge in most
277 experiments. *Med31* RNAi flies displayed a high sensitivity to injected *A. fumigatus* in ten
278 independent experiments, which was however not as pronounced as in *MyD88* flies. We
279 confirmed this result using two other independent RNAi lines and also checked by classical
280 (not shown) and digital RT-qPCR that all three RNAi lines effectively decreased the steady-
281 state levels of MDE31 transcripts (Fig. S1 A-C). Next, we measured the fungal load to
282 determine whether it increases in the mutant background, as has been reported for immuno-
283 deficient flies challenged with pathogens (28, 32). At 24h after infection, the titer was
284 somewhat higher in *MyD88* and *Med31* RNAi flies than in *mCherry* RNAi control flies (Fig.
285 1B). However, the fungal burden did not increase at 48 h (Fig. 1C). We next monitored the

286 induction of the Toll pathway using the expression level of two of its target genes,
287 *Drosomycin* and *BomSI/DIMI* (33). The injection of 250 *A. fumigatus* conidia mildly induced
288 the expression of these two genes, which was reduced in *Med31* RNAi flies (Fig. 1D-E).
289 Thus, *Med31* is required for the full transcriptional induction of *Drosomycin*, likely by
290 recruiting Pol B to the *Drosomycin* and *DIMI* promoters bound by DIF.

291 ***Med31 RNAi flies display a moderate sensitivity to Enterococcus faecalis infection***

292 When challenged with *E. faecalis*, *Med31* RNAi flies displayed a sensitivity to this infection
293 that was intermediate between those of *MyD88* and wild-type control flies in seven out of
294 nine experiments (Fig. 2A), whereas they behaved almost like wild-type flies in the two other
295 experiments. To corroborate these results, we measured the bacterial load and found that it
296 was increased on average 32-fold in the *Med31* RNAi flies with respect to *mCherry* RNAi
297 control flies (Fig. 2B). Unexpectedly, we did not find a significant decrease in *Drosomycin*
298 induction by *E. faecalis* challenge in three independent experiments, although the mean
299 induction of *Drosomycin* was somewhat decreased when compared to controls (Fig. 2C); in a
300 digital RTqPCR experiment, we indeed found a significant difference. However, there was a
301 significant difference when monitoring another read-out of Toll pathway activation,
302 *BomSI/DIMI* transcript levels (Fig. 2D), which was confirmed by digital RTqPCR. The
303 reduction was however modest. Thus, *Med31* RNAi flies appear to have reduced host
304 defenses against two pathogens known to be effectively killing Toll pathway-deficient flies.

305 ***Test of Med31 RNAi flies in further infection models***

306 The Toll pathway has been reported to play an essential role in host defense against
307 entomopathogenic fungi such as *Metarhizum robertsii* and pathogenic yeasts such as *Candida*
308 *albicans* and *C. glabrata* (4, 28, 34, 35). Entomopathogenic fungi invade the host body cavity
309 upon the deposition of spores on the cuticle of the insect ("natural" infection model) or can be
310 artificially directly injected inside the fly (septic injury model mimicking a wound).

311 Interestingly, we found in nine "natural" infection experiments that *Med31* RNAi flies
312 behaved as wild-type control flies in this infection paradigm (Fig. 3A) whereas they displayed
313 a moderate but reproducible susceptibility to injected *M. robertsii* in five experiments (Fig.
314 3B). With respect to pathogenic yeasts, *Med31* RNAi flies displayed a weak susceptibility to
315 *C. albicans* in two out of four experiments (Fig. 3C) while it was not sensitive to *C. glabrata*
316 in six out of nine survival experiments (Fig. 3D). We found that the *C. glabrata* burden was
317 not differing between *mCherry* RNAi and *Med31* RNAi flies during the course of the
318 infection (Fig. 3E). Thus, *Med31* does not appear to be required to the same extent in host
319 defense against microbial infections depending on the pathogen, even though all of these
320 microbes are controlled, at least to some degree, by the Toll pathway. We also checked
321 whether *Med31* affects the host defense against Gram-negative pathogens. The weakly
322 pathogenic *Escherichia coli* did not kill *Med31* RNAi flies more efficiently than a PBS-
323 injection control in two independent experiments (Fig. 3F). We found that *Med31* RNAi flies
324 were also insensitive to *Ecc15* challenge in seven out of eleven experiments (Fig. 3G). We
325 also checked whether IMD pathway signaling was affected in *Med31* RNAi flies by
326 measuring the steady-state transcript levels of *Diptericin*. No significant difference was
327 recorded (Fig. 3H). In conclusion, the requirement for full *Med31* function in host defense
328 against bacterial or fungal infection varies according to the pathogen and the unique suite of
329 host defenses engaged in each case. As *Dif* has been reported to be required in host defense
330 against *M. robertsii* in the "natural infection" model and against *C. glabrata*, the lack of a
331 requirement for *Med31* in these infections is unexpected given its involvement in the host
332 defense against *A. fumigatus*, *E. faecalis*, and injected *M. robertsii* conidia. We cannot
333 however exclude the possibility that another Mediator subunit mediates an interaction with
334 DIF in the other infections that are not modulated through Med31.

335 ***Med31 does not appear to be required for the melanization nor for the cellular immune***
336 ***response***

337 We have tested whether *Med31* plays a role in two other host defenses, melanization and
338 phagocytosis. We did not notice any alteration of the melanin plug formed at the wounding
339 site in *Med31* RNAi flies. We further tested at the molecular level whether the proteolytic
340 processing of prophenol oxidase into mature phenol oxidase was impaired in these flies, as
341 this represents a key step in the melanization response. We found in four out of five
342 experiments that prophenol oxidase was equally or better cleaved in the mutant flies as
343 compared to wild-type controls whereas in one experiment a minor unprocessed form
344 remained while the control was fully cleaved (Fig. 4A). We conclude that *Med31* does not
345 influence melanization after a septic injury.

346 We next checked whether the basal phagocytic machinery was functional in *Med31* RNAi
347 flies by injecting pH-rodo-labeled *E. coli* that emit red fluorescence when placed in an acidic
348 environment such as that encountered in mature phagolysosomal vesicles. Figs. 4B-C show
349 that the uptake of these particles by hemocytes located on the fly dorsal vessel was not
350 dramatically altered when *Med31* expression was ubiquitously knocked-down.

351 ***A mini-screen to identify other Med subunits involved in host defense against A. fumigatus***

352 To obtain a better understanding of the role of the Mediator complex in host defense in the
353 *Drosophila* model, we decided to test the available RNAi lines targeting the genes encoding
354 other subunits of this complex. A limitation of the RNAi approach is that the efficiency of
355 interference may be varying. As the Mediator complex plays an essential role in development,
356 we reasoned that expressing the RNAi transgene throughout development should severely
357 alter the proportion of adult flies hatching from a cross between the RNAi line and a ubi-Gal4
358 driver line. This strategy should therefore allow us to validate the efficiency of the RNAi lines
359 in blocking their targets. Indeed, we found that this was the case for most tested RNAi lines,

360 *Med4*, *Med9*, *Med10*, *Med18*, and *CDK8* excepted (Table 1). We thus directly measured the
361 efficiency of most of the RNAi lines that did not pass this test by RTqPCR (not shown) and
362 digital RTqPCR (Fig. S2). *Med18* excepted, the tested lines displayed a strong decrease of the
363 targeted transcripts, suggesting that the corresponding Mediator subunits (*Med4*, *Med9*, and
364 *Med10*) may not play an essential role during development.

365 We have performed survival analysis on 30 RNAi lines after *A. fumigatus* challenge by
366 expressing the RNAi transgene only at the adult stage. Eight lines yielded a lethal phenotype,
367 that is, uninfected flies succumbed at the same rate as challenged flies (Table 2, Fig. 5A). Two
368 lines, *Med21* and *Med27*, displayed a heightened sensitivity to the control injection of PBS,
369 indicating that they are highly-wound sensitive (Table 2, Fig. 5B). In both cases, it is thus not
370 possible to assess whether these subunits are specifically involved in host defense. We did not
371 find any enhanced sensitivity to fungal infections for 12 lines (Table 2, Fig. 5C). Thus, ten
372 subunits (*Med4*, *Med9*, *Med10*, *Med19*, *Med20*, *Med23*, *Med 24*, *Med25*, *Med26*, and *CycC*)
373 do not appear to play an essential role in host defense against *A. fumigatus* since the
374 corresponding RNAi transgene is clearly functional. Finally, seven lines displayed a *Med31*-
375 like phenotype, although a significant proportion of PBS-injected controls succumbed in the
376 case of *Med 11* (Table 2, Fig. 5D, Fig. S3).

377 ***The Med17 subunit RNAi mutants display a sensitivity only to A. fumigatus and E. faecalis***
378 ***infections***

379 Among the lines that shared with the *Med31* RNAi line a sensitivity phenotype to *A.*
380 *fumigatus*, the *Med17* RNAi KD line is of special interest since it targets the expression of
381 *Med17*, a Mediator complex subunit that has been shown to bind to DIF *in vitro* and to be
382 required for the Toll-dependent induction of *Drosomycin* expression in cell culture (25). A
383 similar phenotype was observed with two further independent RNAi lines (Fig. S1D-E) and
384 all three KD lines nearly abolished *Med17* expression (Fig. S1F). It was therefore interesting

385 to determine whether the *Med17* RNAi line displayed the same palette of sensitivity to
386 specific microbial infections as the *Med31* one. Whereas the *Med17* KD line indeed displayed
387 an increased sensitivity to *E. faecalis* infections in all six performed experiments (Fig. 6A), it
388 however was as resistant as control flies to injected *M. robertsii* conidia in the six survival
389 experiments (Fig. S4A), unlike the *Med31* RNAi line. Finally, the *Med17* RNAi line was not
390 sensitive to *C. glabrata* in five out of six experiments and not susceptible to *Ecc15* in six
391 experiments (Fig. S4B-C). Thus, even though Med31 and Med17 bind to each other in the
392 Mediator complex, their disruption leads to related but not identical phenotypes. Interestingly,
393 whereas *Drosomycin* induction by a *M. luteus* challenge was reduced in the *Med17* KD line as
394 expected, it did not affect the induction of *BomSI/DIMI* transcripts (Fig. 5E-F), even though
395 the *BomSI* gene contains a canonical DIF binding site in its promoter (33). Also of note, the
396 expression of some other AMP genes by an *E. coli* challenge, namely *Drosocin* and
397 *CecropinA* but not *Diptericin*, appeared to be upregulated in the *Med17* RNAi line (Fig. S4 D-
398 G).

399 ***Role of other Med subunits in host defense against additional pathogens***

400 We next tested the susceptibility of other available RNAi lines against other Med subunit-
401 encoding genes to *E. faecalis* and found that six of them displayed an enhanced sensitivity to
402 this pathogen (Table 3, Fig. S5). We had initially found that *Med12*, *Med15*, and *Med28* RNAi
403 flies succumbed at the same rate whether challenged or not in two experiments with *A.*
404 *fumigatus*. In the case of *E. faecalis* infection, the PBS-injected control did not die as rapidly
405 as previously, whereas the bacteria-challenged ones succumbed much faster (Fig. S5). Indeed,
406 the *E. faecalis* burden was higher than in controls for the *Med 15* and *Med28* RNAi flies,
407 indicating that these lines are indeed susceptible to *E. faecalis* and not solely to the injection
408 procedure. The important observation is that the *Med6* and *Med11* lines displayed a sensitivity
409 phenotype after *A. fumigatus* but not after *E. faecalis* infection.

410 We further challenged these Mediator subunit RNAi lines with *C. glabrata* or *Ecc15*. Most of
411 them tested negative except for *Med30* in the case of the pathogenic yeast (Fig. 6B) and
412 *Med19* for *Ecc15* that displayed intermediate sensitivity to these pathogens (Fig. 6C, Tables 3-
413 4).

414 Finally, we used the *M. robertsii* natural infection model to characterize the Mediator subunit
415 subset and found that *Med7*, *Med27*, and *Med29* RNAi displayed an enhanced sensitivity to
416 this challenge although it should be noted that mock-infected *Med7* and *Med29* RNAi flies
417 also succumbed during the course of this experiment, albeit at a somewhat slower rate (Fig.
418 6D-F). This observation for *Med 7* and *Med 29* is in keeping with the lethality observed in the
419 infections series with *A. fumigatus* (Table 4). Whereas *Med27* is clearly required for host
420 defense against a natural *M. robertsii* infection, we cannot determine unambiguously whether
421 it is also required in the host defense against *A. fumigatus* given its sensitivity to the wound.

422

423 **Discussion.**

424 We have tested the roles of almost all the subunits of Mediator complex in host defense
425 against infectious pathogens including filamentous fungus (*A. fumigatus*), entomopathogenic
426 fungus in its filamentous form (*M. robertsii* natural infection; Wang *et al.*, *in preparation*),
427 entomopathogenic fungus under the hyphal body form (*M. robertsii* conidia injection; Wang
428 *et al.*, *in preparation*), yeast (*C. glabrata*), Gram-positive bacterium (*E. faecalis*), and the
429 Gram-negative bacterium (*Ecc15*). All of the above-listed pathogens, except for *Ecc15*, are
430 controlled, at least to some extent, by the Toll pathway. Our results showed that different
431 Mediator subunits displayed distinct sensitivity to infections (Fig. 7, Fig. S6, Table 4) and
432 reveal differential modes of actions of the Toll pathway.

433

434 A recent structural and genetic analysis of the Mediator complex in Mammals has revealed
435 that 15 subunits are essential for cell viability in three human cell types (Table 4), mostly
436 subunits of the core module (except for Med20 in the head module and Med1, Med9, Med19,
437 Med26 in the middle module) and three tail subunits interfacing with the head and middle
438 modules, namely Med27, Med28, and Med30 as well as the scaffolding subunit Med14.
439 These essential subunits appear to be indispensable for the function of the Mediator complex
440 in globally recruiting RNA Polymerase B to promoters to form the preinitiation complex (36).
441 Here, we find that most subunits are required during development, except for Med4, Med9,
442 Med10, Med18, Med19, Med20, CycC, and CDK8 for which some escaper pupae or adults
443 were obtained (Table 1). It is likely that the *Med18* phenotype is due to a partial attenuation of
444 its expression at the mRNA level as determined by regular and digital RTqPCR. We have not
445 checked the efficiency of RNA interference for *Med16*, *Med19*, *CycC*, and *CDK8*, and thus
446 cannot formally exclude a similar explanation for these subunits. In contrast, the RNAi
447 approach seems to be efficient as regards *Med4*, *Med9*, *Med10*, and *Med20*. While Med9 and
448 Med20 are also not indispensable in Mammals, Med4 and Med10 may in contrast be essential
449 in Mammals but not in insects, although it is difficult to compare viability at the cellular *vs.* at
450 the organismal level. Indeed, the depletion of Med subunits only at the adult stage led to the
451 demise of eight uninfected RNAi mutant lines in the experiments in which susceptibility to *A.*
452 *fumigatus* was tested. This occurred also when subunits not homologous to essential
453 mammalian subunits were targeted, *i.e.*, *Med1*, *Med12*, *Med13*, *Med15*, *Med16*, *Med28*, and
454 *Med29*. It was unexpected that the uninfected controls for the same lines did not reveal any
455 lethality when *E. faecalis* was tested later in another series of survival experiments (Table 4).
456 This uninfected control was not performed for survival experiments with other tested
457 pathogens; however, mock-infected controls were performed in these experiments and did not
458 reveal any unusual viability issue when kept at 29°C, except where indicated in Table 4 (*M.*

459 *robertsii* natural infections). We suspect that conditions were slightly harsher in the *A.*
460 *fumigatus* survival experiment series, resulting in a more efficient RNAi and depletion of the
461 cognate subunit thus revealing their essential function, even in the context of the adult in
462 which cells do not divide, somatic and germinal stem cells excepted. In contrast to classical
463 mutations induced by chemical mutagenesis or CRISPR-Cas9, a fundamental difference with
464 conditional RNAi is that the proteins are made in the cell prior to the induction of the RNAi.
465 Even if the interference is 100% effective, the limiting factor to express the phenotype will be
466 the relative stability of the protein already made, which may also vary from one target to the
467 other, thus modulating the gamut of observable phenotypes. Indeed, it has already been noted
468 in genome-wide genetic screens that the genes identified by RNA interference and those
469 retrieved with random mutagenesis techniques differ extensively (37). Thus, this limitation
470 has to be kept in mind when interpreting the results from this study.

471 One interesting observation relates to the CDK8 module: the Med12 and Med13 subunits
472 appear to be essential in the adult, but not CycC and CDK8, even though the RNAi targeting
473 each of the latter two subunits is effective, at least for *CycC* since no adults were retrieved
474 when the RNAi transgene was expressed continuously throughout development. One
475 possibility would be that the CycC and CDK8 proteins are more stable, although one should
476 note that the survival experiments were monitored for over two weeks while the whole
477 development at 29°C occurs in less than ten days. An alternative is that *Med12* and *Med13*
478 have differing functions from the Cdk8/CycC kinase module of the Mediator complex, in
479 keeping with a previous study (38).

480 We found that mutations affecting some of the Mediator complex subunits were not sensitive
481 to any pathogen we have tested: Med4, Med9, Med10, Med20, Med26 of the core module,
482 Med23, Med24, Med 25 of the tail module, and Med13 and CycC for the CDK8 kinase
483 module (Fig. 7, Fig. S6, Table 4)). Most of these subunits correspond to nonessential subunits

484 in the mammalian complex, except for Med4 and Med10. Med20 is the only subunit of the
485 head module not playing a role in the host defense against the pathogens we have tested and
486 consistently is also the only subunit of the head not essential in the mammalian complex (36).
487 The only RNAi lines displaying a sensitivity to *A. fumigatus* infection affect the expression of
488 genes encoding subunits of the head module with two exceptions, Med31 in the middle
489 module and Med30 in the tail module (Fig. 7 and Fig. S6, Tables 2&4). With respect to *E.*
490 *faecalis* infection, the RNAi susceptible lines correspond to many subunits of the head
491 module (Med8, Med17, Med22, Med28). We cannot formally exclude that Med6 and Med11
492 would also have tested positive had the conditions been as stringent as for the *A. fumigatus*
493 infections. Beyond the head module, Med15, Med28, and Med30 in the tail module and
494 Med31 in the middle module also displayed an *E. faecalis* infection sensitivity phenotype
495 (Fig. 7, Fig. S6, Tables 3-4). As the *Med15* and *Med28* RNAi lines uninfected flies displayed
496 a lethal phenotype in the *A. fumigatus* survival experiment series, we cannot exclude that
497 these subunits may also be required for host defense against this fungus. It follows that it is an
498 open possibility that host defense against these two pathogens involves the same set of
499 Mediator subunits (Fig. 7, Table 4).

500 *Med30* expression down-regulation was the only one that led to a sensitivity to *C. glabrata*
501 infection and corresponds to a subunit of the tail module of Mediator also required for host
502 defense against *A. fumigatus* and *E. faecalis* (Fig. 7, Fig. S6, Table 3&4). This finding
503 suggests that host defense against *C. glabrata* is strikingly distinct from that of the two
504 microbes discussed so far, even though DIF plays a central role in host defense against the
505 fungal and *E. faecalis* infections (6, 28, 39). It would be interesting to determine whether DIF
506 can directly bind also to Med30 since its binding to Med17 is not required in the context of
507 pathogenic yeast infection.

508 Only the *Med19* RNAi line displayed sensitivity to *Ecc15* infection (Fig. 7, Fig. S6, Table
509 3&4). It is not clear at this stage whether this phenotype reflects an impaired function of the
510 IMD pathway in the case of *Med19*. We expected to find a role for Med25 as it has been
511 identified as being required for IMD pathway activation in an RNAi screen performed on
512 cultured cells (40). This observation suggests that the regulation of the IMD pathway in
513 cultured cells and *in vivo* may not be identical, at least as regards the role of the Mediator
514 complex.

515 Med30 and Med31 display unique phenotypes, in that they are required for host defense
516 against three types of infections, including *E. faecalis* and *A. fumigatus*. *Med30* RNAi flies
517 are the only ones to be sensitive to *C. glabrata* whereas *Med31* flies are susceptible to
518 injected *M. robertsii* conidia but not in a natural infection paradigm. Unexpectedly, distinct
519 subunits appear to be involved in the response to this type of infection, in which the pathogen
520 breaks through the cuticle, namely Med27 and possibly Med7 and Med29. This result is
521 striking in that the Toll pathway and DIF are both required in the host defense against *M.*
522 *robertsii* in either infection model (4, Wang et al., in preparation). Future work will tell
523 whether the subunits required for host defense against *M. robertsii* in the natural infection
524 model are required locally, for instance in the hypodermis or whether they mediate the action
525 of DIF in the fat body. Of note, only two subunits, Med31 and Med17, have been tested in the
526 *M. robertsii* conidia injection model (Table 4, Fig. S6).

527 Med17 is the subunit shown to bind directly to DIF *in vitro* (25). DIF is required for the
528 induction of multiple genes regulated by the Toll pathway (41), including *Drosomyacin* and
529 *BomS1/DIM1*. It was therefore surprising to observe that the induced *BomS1* expression was
530 not impaired in *Med17* RNAi KD flies, although that of *Drosomyacin* was affected. This
531 situation is reminiscent of that documented for *Med1* during *Drosophila* development: it
532 recruits GATA transcription factors only for a subset of genes regulated by these factors (24).

533 Thus, it is likely that the context plays an essential role, not only with respect to the regulation
534 of specific subsets of genes through a given set of Mediator subunits, but also depending on
535 the pathogen and infection route. Indeed, there is only a limited overlap of genes with an
536 altered expression in the natural infection or conidia injection models of *M. robertsii*
537 injection. It should also be kept in mind that host defense is not limited to resistance, for
538 instance through AMPs, but involves the dimension of resilience/tolerance whereby the host
539 withstands or repairs damages exerted by pathogen virulence factors or its own immune
540 response (42-44). As exemplified by the finding that the *Caenorhabditis elegans* Med15 is
541 required for host defense against *Pseudomonas aeruginosa* and detoxification of some of the
542 toxins it secretes such as phenazines (45), it will be therefore interesting to determine whether
543 some of the Med subunits identified in this study for their involvement in host defense
544 actually mediate resistance, resilience, or both.

545

546

547 **Acknowledgements**

548 We thank Guiying Lin, Xiaomin Chang, Jin Li, Jianmei Li, Xinzhu Chen, Yuqi Yang,
549 Jianglong Guo, and Xuejun Hong for help in some of the experiments and for the genetic
550 screen. The expert technical contribution of Luong Lin, Yongxin Liao, Fengyi Zhang,
551 Yanzhen He for the screen is gratefully acknowledged. We are indebted to Prof. Ni and his
552 collaborators (Tsinghua University) for giving us access to his resource of Trip RNAi lines
553 (Tsinghua Fly Center) and also the Vienna Drosophila Resource Center (VDRC,
554 www.vdrc.at) and Bloomington Drosophila Stock Center (NIH P40OD018537) for their
555 resource. We thank Anne Beauvais and Jean-Paul Latge for the gift of the RFP-labeled *A.*
556 *fumigatus* wild-type strain, Erjun Ling for the gift of the PPO1 antibody. We thank Dr.
557 Kwang-Zin Lee for critical reading of the manuscript. This work was funded through the
558 Incubation Project for Innovation Team of the Guangzhou Medical University (#
559 B1850004105) and the 111 Project (# D18010) as well as support from the China 1,000 Talent
560 program to DF.

561

562 **Authors contributions**

563 CH performed most experiments described in this work and participated in the genetic screen
564 that led to this work. RX developed the *A. fumigatus* infection model and started the genetic
565 screen that led to the identification of Med subunit genes. SL and ZL provided guidance and
566 supervision for the experiments. DC and DF played a key role in designing the genetic screen
567 and DC organized the actual implementation of the genetic screen and coordinated the
568 logistics. DF and CH designed the experiments, analyzed the data, and wrote the manuscript.

569

570 **Conflicts of interest**

571 The authors report no conflicts of interest.

572

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690

691

692 **Figures legends**

693

694 **Figure 1. Med31 RNAi flies are susceptible to *A. fumigatus* infection**

695 A: Survival of *Drosophila* after *A. fumigatus* infection. *MyD88* is the positive control line
696 (red), *Ubi>mCherry* RNAi line is the wild type control line (green) and *Ubi>Med31* RNAi
697 line is the experimental line (blue). Each infected line has a non-infected (NI, dashed lines)
698 and PBS-injected control (dotted lines). *Med31* RNAi flies succumbed faster to infection than
699 the wild type controls. Infected *Med31* RNAi flies vs. infected *mCherry* RNAi flies: log-rank
700 test, ****, $P < 0.0001$. The survival curves are representative of ten independent experiments.

701 B, C: Fungal load of whole single flies after 24h and 48h of infection. Each dot represents a
702 single fly. The fungal load was higher in *MyD88* and *Med31* RNAi flies than in control flies at
703 24h post infection. It did not increase in *Med31* RNAi flies at 48h post infection compared to

704 24h. ****, $P < 0.0001$. ***, $P < 0.001$. D, E: Expression levels of *Drosomycin* (D) and *DIMI*
705 (E) at 48h post infection, normalized to *Rpl32* (RP49 protein coding gene) housekeeping gene
706 expression. The expression of control flies challenged with the nonpathogenic Gram-positive
707 bacterium *Micrococcus luteus* is given for reference. Each dot represents one sample
708 containing four flies. *Med31* RNAi flies displayed decreased *Drosomycin* and *DIMI*
709 expression levels at 48h post infection. *, $P < 0.05$; **, $P < 0.001$. Mean \pm SEM are indicated (B-
710 E).

711 **Figure 2. *Med31* RNAi flies are susceptible to *E. faecalis* infection.**

712 A: Survival of *Drosophila* after *E. faecalis* infection. *MyD88* was used as a positive control
713 line (red), *Ubi>mCherry* RNAi line represented the wild type control line (green) and the *Ubi*
714 *>Med31* RNAi line survival is shown in blue. For each infected line a PBS-injected control
715 was also performed (dotted lines). *Med31* RNAi flies succumbed faster to infection than the
716 wild type controls. Infected *Med31* RNAi flies vs. infected *mCherry* RNAi flies: log-rank test,
717 ****, $P < 0.0001$. The survival curves are representative of nine independent experiments. B:
718 Bacterial load in the hemolymph after 24h of infection. Each dot represents the burden of a
719 single fly. The bacterial load in the *Med31* RNAi flies was higher than the controls at 24h post
720 infection. ***, $P < 0.001$. C, D: Expression level of *Drosomycin* (C) and *DIMI* (D) normalized
721 to the *Rpl32* house keeping gene 48h post infection. Each dot represents one sample
722 containing four flies. Septic infection with *M. luteus* was a positive control for *Drosomycin*
723 and *DIMI* expression. *Drosomycin* expression level in *Med31* RNAi flies was not
724 significantly different from control flies (C) but *DIMI* expression level was significantly
725 decreased at 24h post infection. *, $P < 0.05$. Mean \pm SEM are indicated (B-D).

726 **Figure 3. Susceptibility of *Med31* RNAi flies to other pathogens.**

727 Survival of *Med31* RNAi flies challenged with other pathogens. *MyD88* represents the
728 positive control line (red) for flies infected with *M. anisopliae*, *C. albicans*, *C. glabrata* and
729 *E. faecalis*; *kenny*, a member of the IMD pathway, is the positive control line (red) for fly
730 lines infected with *Ecc15* or *E. coli*, *Ubi>mCherry* RNAi line corresponds to the wild type
731 control line (green) and *Ubi>Med31* RNAi line (blue). For each infected line a PBS-injected
732 control was also performed (dotted lines). A: Survival of *Med31* RNAi flies after *M. robertsii*
733 natural infection. The survival curve is representative of nine independent experiments. B:
734 Survival of *Med31* RNAi flies following *M. robertsii* conidia injection. Infected *Med31* RNAi
735 flies vs. infected *mCherry* RNAi flies: log-rank test, ****, $P < 0.0001$. The survival curves are
736 representative of five independent experiments. C: Survival of *Med31* RNAi flies following

737 *C. albicans* infection. Infected *Med31* RNAi flies vs. infected mCherry RNAi flies: log-rank
738 test, ****, $P < 0.001$. The survival curves are representative only of two out of four
739 independent experiments, the other two not displaying any difference between *Med31* RNAi
740 and control flies. D, E: Survival of *Med31* RNAi flies and the fungal load following *C.*
741 *glabrata* infection. The survival curves are representative of nine independent experiments. F:
742 Survival of *Med31* RNAi flies following *E. coli* infection. The survival curves are
743 representative of two independent experiments. G. Survival of *Med31* RNAi flies after *Ecc15*
744 challenge. The survival curves are representative of nine experiments. H: *Diptericin*
745 expression level normalized to the *Rpl32* house keeping gene at 24h post infection with
746 *Ecc15*. Each dot represents one sample containing four flies. Septic infection with *E.coli* was
747 a positive control for *Diptericin* expression. Mean \pm SEM are indicated.

748 **Figure 4. Melanization and phagocytosis are not affected in *Med31* RNAi flies.**

749 A: prophenol oxidase (PPO) cleavage four hours after a *M. luteus* challenge was detected by
750 Western blot analysis. The cleavage of PPO into phenol oxidase (PO) is shown in the picture.
751 PPO cleavage into PO was total for both *Med31* RNAi and *mCherry* RNAi control flies, while
752 that induced in another wild-type control, w1118, was only partial. This blot is representative
753 of three out of four independent experiments. The phagocytic activity of flies was detected
754 using injected pH-RODO-labelled *E. coli*, which become fluorescent when internalized into
755 mature phagosomes. B: Flies were injected with latex beads as a phagocytosis-deficient
756 control (left panels). The phagocytic activity of *Med31* RNAi flies was not altered compared
757 to the *mCherry* RNAi control flies (right panels). C: Quantification of the fluorescence
758 emitted by internalized bacteria. The *eater¹* phagocytosis-deficient mutant flies represent a
759 positive control. There was no significant difference between *Med31* RNAi flies and the
760 *mCherry* RNAi control flies. Each dot represents the fluorescence measured in a single fly.
761 Three independent experiments were performed. *, $P < 0.05$. Mean \pm SEM are indicated.

762 **Figure 5. The distinct survival phenotypic categories for other Mediator complex**
763 **subunits RNAi flies challenged with *A. fumigatus*.**

764 Survival of RNAi lines targeting genes encoding other Mediator complex subunits after *A.*
765 *fumigatus* infection. *MyD88* represents the positive control line (red), *Ubi>mCherry* RNAi
766 line the wild type control line (green), and *Ubi>* RNAi line of Mediator complex subunits is
767 shown as a blue line. Each infected line has a non-infected (NI, dashed lines) and a PBS-
768 injected control (dotted lines). A: Example of a lethal phenotype, uninfected *Med15* RNAi
769 flies succumbed at the same rate as challenged flies. B: Example of a wound-sensitive

770 phenotype, *Med21* RNAi flies were sensitive to the injection of PBS. C: Example of an
771 absence of sensitivity phenotype, *Med26* RNAi flies did not show any enhanced sensitivity to
772 infections. D: Example of sensitivity to *A. fumigatus* infection. *Med17* RNAi flies displayed a
773 *Med31*-like phenotype. Infected *Med17* RNAi flies vs. infected *mCherry* flies: log-rank test,
774 ****, $P < 0.001$. E and F: Expression level of *Drosomycin* (E) and *DIMI* (F) at 48h post *A.*
775 *fumigatus* infection measured by digital RTqPCR. Each dot represents one sample containing
776 five flies. Mean \pm SEM are indicated.

777

778 **Figure 6. Survival of RNAi flies targeting different Mediator complex subunits after**
779 **challenge with various pathogens.**

780 Survival of other Mediator complex subunits RNAi lines after challenges with either *E.*
781 *faecalis*, *C. glabrata*, *Ecc15*, or *M. robertsii*. *MyD88* was used as positive control line (red)
782 for *M. robertsii*, *C. glabrata* or *E. faecalis* infection, and *key* was the positive control line
783 (red) for lines infected with *Ecc15*. *Ubi>mCherry* RNAi line was the wild type control line
784 (green) and *Ubi>* RNAi line of Mediator complex subunits is displayed in blue. A: Survival
785 curves of *Med17* RNAi flies, which were susceptible to *E. faecalis* infection. Infected *Med17*
786 RNAi flies vs. infected *mCherry* RNAi flies: log-rank test, ****, $P < 0.0001$. The survival
787 curves are representative of six experiments. B: *Med30* RNAi flies were susceptible to *C.*
788 *glabrata* infection. Infected *Med30* RNAi flies vs. infected *mCherry* RNAi flies: log-rank test,
789 **, $P < 0.01$. The survival curves are representative of two experiments. C: *Med19* RNAi flies
790 were susceptible to *Ecc15* infection. Infected *Med19* RNAi flies vs. infected *mCherry* RNAi
791 flies: log-rank test, ****, $P < 0.0001$. The survival curves are representative of two
792 experiments. D-F: *Med7*, *Med27*, and *Med29* RNAi displayed an enhanced sensitivity to *M.*
793 *robertsii* “natural” infection. Infected *Med7* RNAi flies vs. infected *mCherry* RNAi flies: log-
794 rank test, ****, $P < 0.0001$. The survival curves are representative of three experiments.
795 Infected *Med27* RNAi flies vs. infected *mCherry* RNAi flies: log-rank test, ***, $P < 0.001$. The
796 survival curves are representative of two experiments. Infected *Med29* RNAi flies vs. infected
797 *mCherry* flies: log-rank test, ****, $P < 0.0001$. The survival curves are representative of two
798 experiments. Please, note that the mock-infected flies for *Med7* and *Med29* succumbed also
799 rapidly to the procedure, making it difficult to conclude unambiguously on the role of these
800 two subunits in the host defense against *M. robertsii* “natural” infection. Error bars are SEM.

801

802 **Figure 7. Roles of Mediator complex modules against infectious pathogens**

803 The Mediator complex is composed of a central complex and of the CDK kinase module
804 (CKM). The central complex has three modules: the head (light blue circumference), the
805 middle (black circumference) and the tail (red circumference). Med14 constitutes a
806 scaffolding subunit indicated by dark blue. The CKM (green circumference) consists of four
807 subunits: *CDK8* (or its paralog *CDK19*), *Med12* (or its paralog *Med12L*), *Med13* (or its
808 paralog *Med13L*) and Cyclin C. Subunits sensitive to *A. fumigatus* are shown in yellow;
809 subunits sensitive to *E. faecalis* in green, light green when the phenotype is uncertain due to a
810 lethality observed in the *A. fumigatus* experiments (Med12); subunits sensitive to *M. robertsii*
811 natural infection are displayed in dark purple, light purple when the phenotype is uncertain;
812 subunits sensitive to *M. robertsii* injection are shown in orange (only *Med17* and *Med31*
813 RNAi flies have been tested); subunits sensitive to *C. glabrata* infection are pictured in dark
814 blue whereas subunits sensitive to *Ecc15* infection are light blue. Lines for which the RNAi
815 may not be effective are displayed in gray; a lighter gray indicates lines in which pupae
816 developed upon continuous expression of the RNAi transgene during development, yet did
817 not yield any adult flies (metamorphosis phenotype). Lines that yielded a lethal phenotype in
818 the uninfected controls in the *A. fumigatus* experiments when gene expression was inhibited
819 in the adult display a boxed subunit number, which is dashed when sensitivity was observed
820 in the wounding (PBS-injected) but not the uninfected controls.

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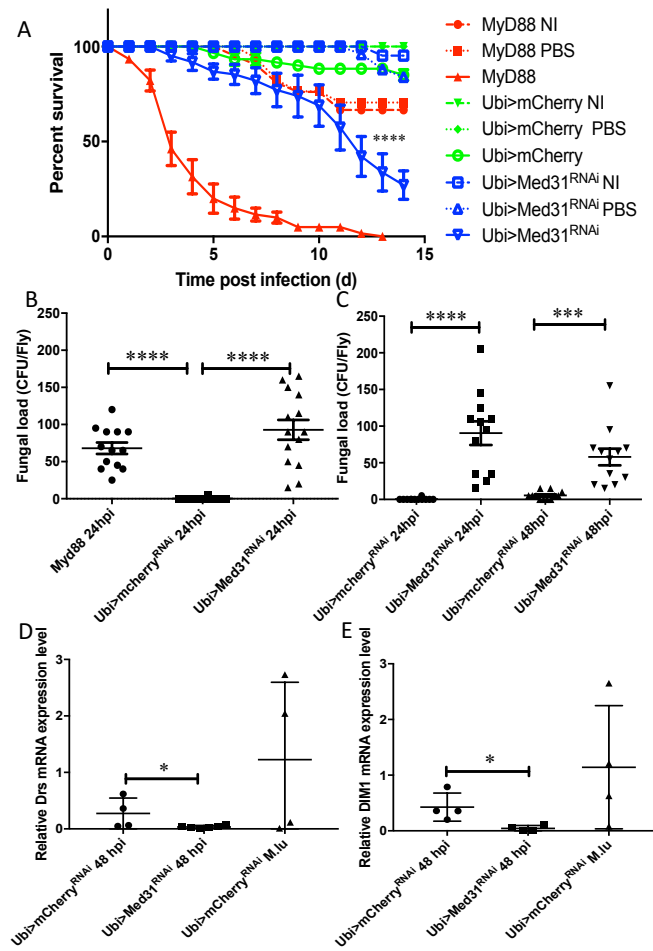


Figure 1

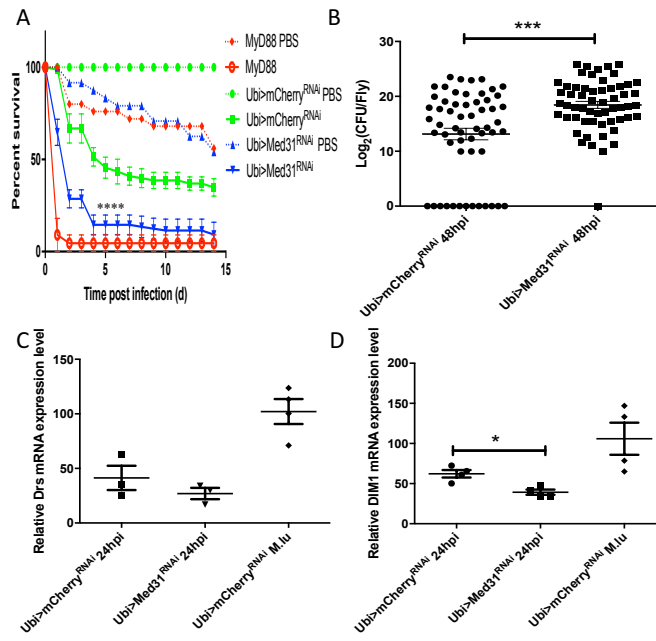


Figure 2

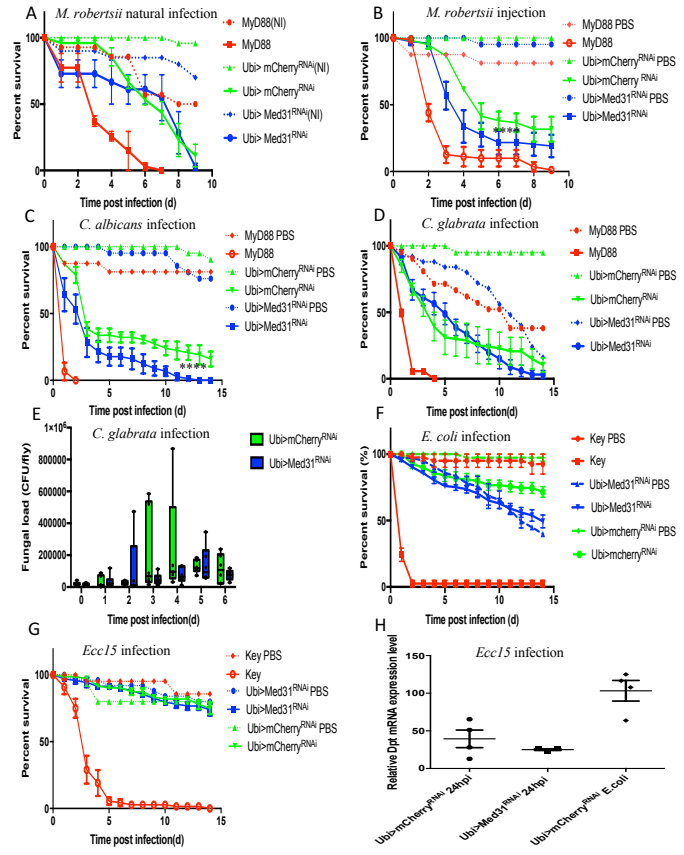


Figure 3

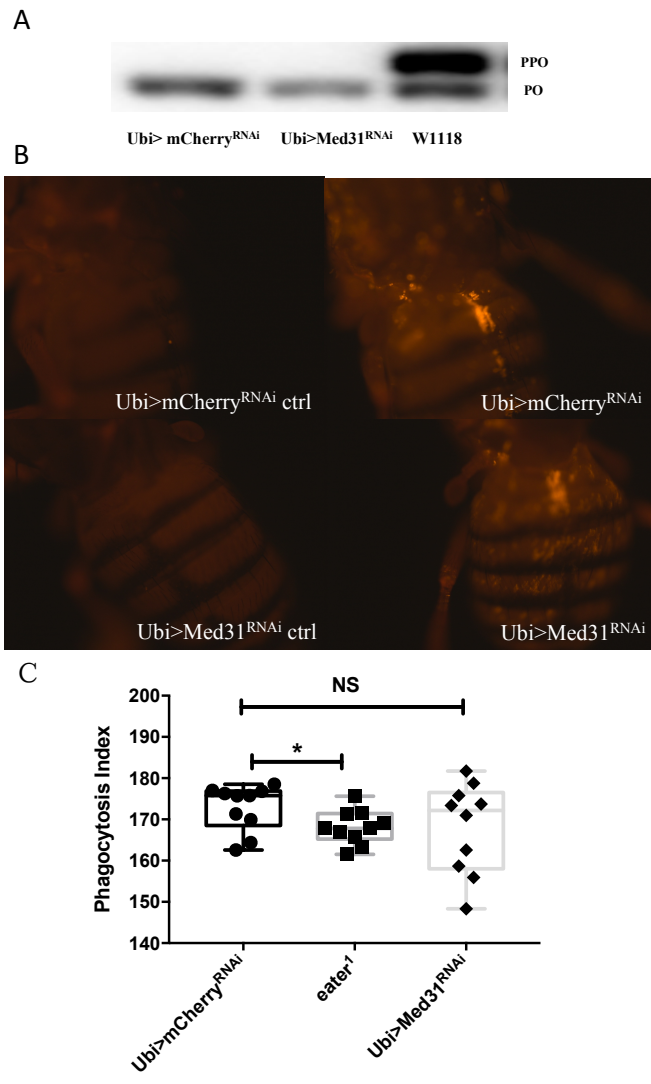


Figure 4

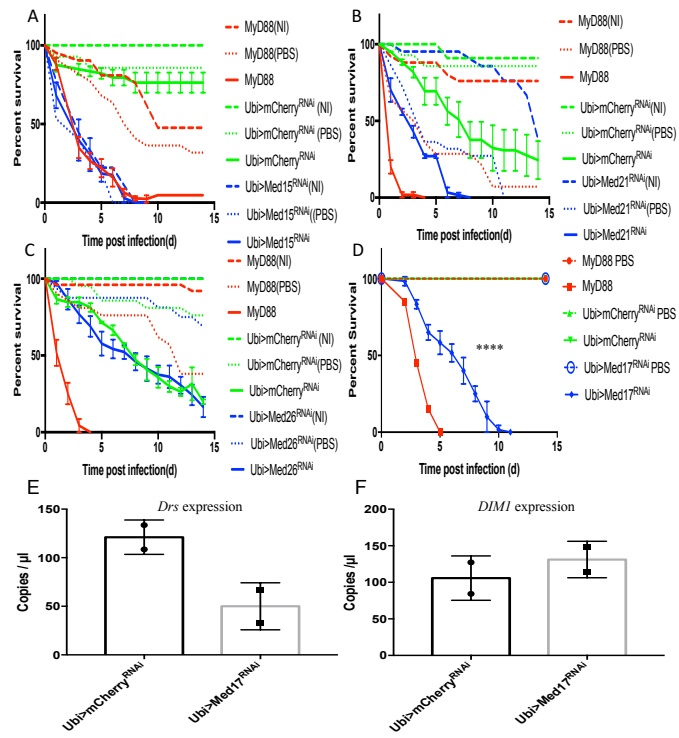


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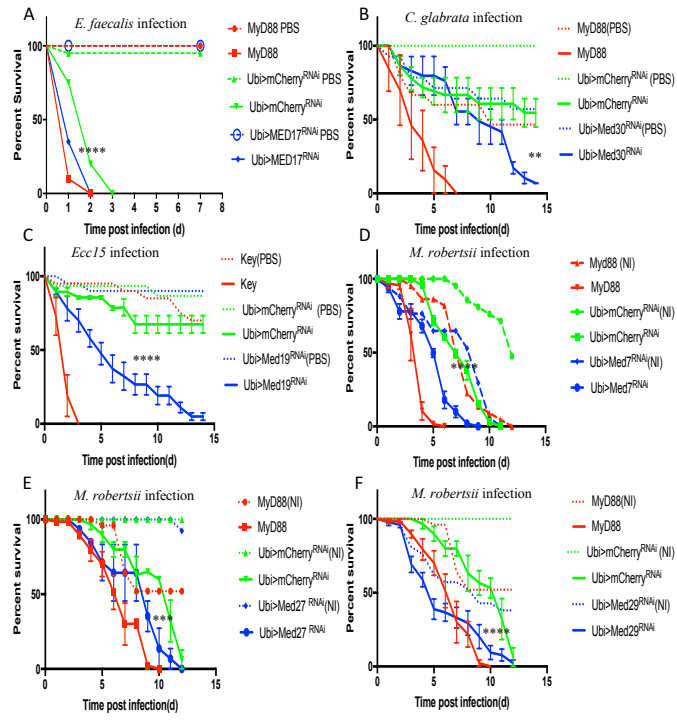


Figure 6

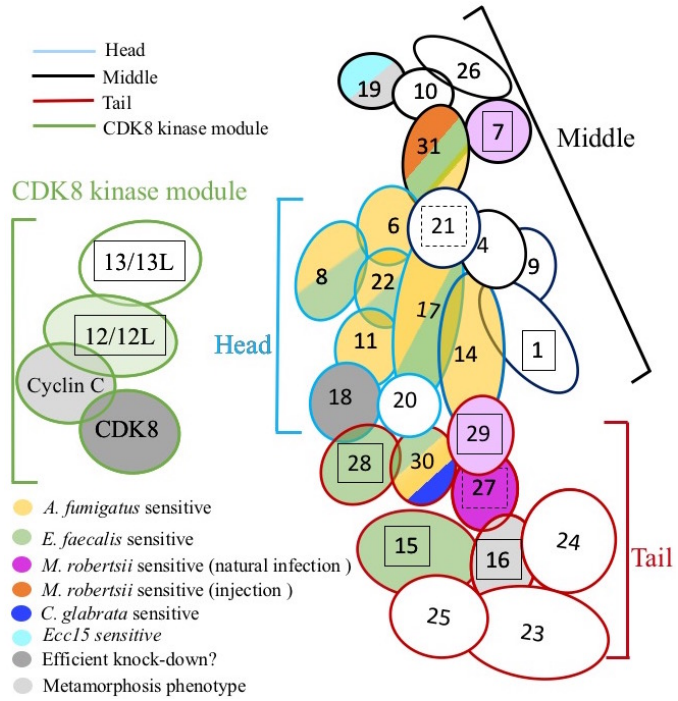


Figure 7

Table 1. Validation of Med RNAi flies lethality at 29 °C.

RNAi strain	Pupae	Hatching flies (%)	RNAi strain	Pupae	Hatching flies (%)
mCherry	51	51 (100%)	Med19	27	0
Med1	0	0	Med20	20	0
#Med4	30	25 (83.33%) 10 (33.33% homozygous) 15 (50% heterozygous)	Med21	0	0
Med6	0	0	Med22	0	0
Med7	0	0	#Med23	0	0
Med8	0	0	Med24	0	0
Med9	4	4 (100%)	Med25	0	0
#Med10	12	100% 4 (33.33% homozygous) 8 (66.67% heterozygous)	Med26	0	0
Med11	0	0	Med27	0	0
Med12	0	0	Med28	0	0
Med13	0	0	Med29	0	0
Med14	0	0	#Med30	0	0
Med15	0	0	Med31	0	0
Med16	8	0	Cyc C	14	0
Med17	0	0	CDK8	14	12 (85.71%)
Med18	12	10 (83.3%)			

Notes:

Flies were crossed with Ubi-Gal4,tub-Gal80^{ts} virgins (two males and four females per tube) at 29°C and parents were discarded five days post crossing. The offspring was raised at 29 °C and their viability was assessed.

#Some UAS-RNAi lines are heterozygous (no homozygous flies found). Therefore, in the progeny of the crosses done with Gal4 lines, there are flies carrying a balancer chromosome and not the UAS-RNAi transgene: these can be identified only at the adult stage thanks to their genetic markers.

Table 2. Other Med subunits in host defense against *A. fumigatus*

Phenotype	MED subunits
With phenotype	Med 6, Med 8, Med 11, Med14, Med17, Med22, Med30
Lethal	Med1, Med7, Med12, Med13, Med15, Med16, Med28, Med29
Wound sensitive	Med21, Med 27
No phenotype	Med4, Med9, Med10, <i>Med18</i> , Med19, Med20, Med23, Med24, Med25, Med 26, Cyc C, <i>CDK8</i>

The subunits indicated in italics may not be efficiently silenced by RNAi and the absence of a phenotype may just reflect this technical problem, thereby preventing a solid conclusion to be drawn.

Table 3. Other Med subunits in host defense against other pathogens

Pathogens	Other MED subunits
<i>E. faecalis</i>	Med 8, Med12?, Med15, Med17, Med 22, Med 28, Med 30
<i>Ecc15</i>	Med 19
<i>C. glabrata</i>	Med 30
<i>M. robertsii</i> <i>Natural infection</i>	Med7?, Med27, Med 29?

The question marks indicate that a sensitivity to the infection procedure may significantly contribute to the phenotype.

Table 4. Summary of Med subunits in host defense against pathogens

	<i>A. fumigatus</i>	<i>E. faecalis</i>	<i>M. robertsii</i> natural infection	<i>C. glabrata</i>	<i>Ecc15</i>	<i>M. robertsii</i> injection	lethality at 29 °C	RNAi efficiency by dPCR
Med1	L	-	-	-	-	/	++	/
<u>Med4</u>	-	-	-	-	-	/	+/-	+
<u>Med6</u>	+	-	-	-	-	/	++	/
<u>Med7</u>	L	-	+?	-	-	/	++	/
<u>Med8</u>	+	+	-	-	-	/	++	/
Med9	-	-	-	-	-	/	+/-	+
<u>Med10</u>	-	-	-	-	-	/	+/-	+
<u>Med11</u>	+	-	-	-	-	/	++	/
Med12	L	+	/	-	-	/	++	/
Med13	L	-	-	-	-	/	++	/
<u>Med14</u>	+	/	-	-	/	/	++	/
Med15	L	+	-	-	-	/	++	/
Med16	L	-	-	-	-	/	+	/
<u>Med17</u>	+	+	-	-	-	-	++	+
<u>Med18</u>	-	-	-	-	-	/	+/-	+/-
Med19	-	-	-	-	+	/	+	/
Med20	-	-	-	-	-	/	+	+
<u>Med21</u>	-?	-	/	-	/	/	++	/
<u>Med22</u>	+	+	-	-	-	/	++	/
Med23	-	-	-	-	-	/	++	/
Med24	-	-	-	-	-	/	++	/
Med25	-	-	-	-	-	/	++	/
Med26	-	-	-	-	-	/	++	/
<u>Med27</u>	-?	-	+	-	-	/	++	/
Med28	L	+	-	-	-	/	++	/
Med29	L	-	+?	-	-	/	++	/
<u>Med30</u>	+	+	-	+	-	/	++	/
<u>Med31</u>	+	+	-	-	-	+	++	+
Cyc C	-	-	-	-	-	/	+	/
<i>CDK8</i>	-	-	-	-	-	/	+/-	/

Table 4 (continued). The underlined Med subunits on the left column correspond to subunits homologous to essential subunits in the mammalian Mediator complex. L in the *A. fumigatus* column indicates that uninfected controls succumbed at the same rate as challenged flies, making it difficult to draw any conclusion with regards to the susceptibility to this challenge. ?: the phenotype is not certain as mock-infected controls displayed some sensitivity to the infection procedure. The column next to the right-most column recapitulates the developmental phenotypes shown in Table 1: ++: no escapers; +: some pupal escapers that did not reach the adult stage; +/-: some adult escapers. The right-most column shows the results of monitoring the steady-state transcripts of the targeted gene; +: efficient RNAi with few transcripts detected; +/-: partial depletion of transcripts. Similar results were obtained by “classical” RTqPCR, which is however not as precise. /: not tested.