

M1-linked ubiquitination facilitates NF- κ B activation and survival during sterile inflammation

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Methionine 1 (M1)-linked ubiquitination plays a key role in the regulation of inflammatory nuclear factor- κ B (NF- κ B) signalling and is important for clearance of pathogen infection in *Drosophila melanogaster*. M1-linked ubiquitin (M1-Ub) chains are assembled by the linear ubiquitin E3 ligase (LUBEL) in flies. Here, we have studied the role of LUBEL in sterile inflammation induced by different types of cellular stresses. We have found that the LUBEL catalyses formation of M1-Ub chains in response to hypoxic, oxidative and mechanical stress conditions. LUBEL is shown to be important for flies to survive low oxygen conditions and paraquat-induced oxidative stress. This protective action seems to be driven by stress-induced activation of the NF- κ B transcription factor Relish via the immune deficiency (Imd) pathway. In addition to LUBEL, the intracellular mediators of Relish activation, including the transforming growth factor activating kinase 1 (Tak1), *Drosophila* inhibitor of apoptosis (IAP) Diap2, the I κ B kinase γ (IKK γ) Kenny and the initiator caspase Death-related ced-3/Nedd2-like protein (Dredd), but not the membrane receptor peptidoglycan recognition protein (PGRP)-LC, are shown to be required for sterile inflammatory response and survival. Finally, we showed that the stress-induced upregulation of M1-Ub chains in response to hypoxia, oxidative and mechanical stress is also induced in mammalian cells and protects from stress-induced cell death. Taken together, our results suggest that M1-Ub chains are important for NF- κ B signalling in inflammation induced by stress conditions often observed in chronic inflammatory diseases and cancer.

Abbreviations

AMP, antimicrobial peptide; daGal4, daughterless-Gal4; DAMP, danger-associated molecular pattern; Diap, *Drosophila* inhibitor of apoptosis; Dif, Dorsal-related immunity factor; Dredd, death-related ced-3/Nedd2-like protein; DUB, deubiquitinating enzyme; Fadd, fas-associated death domain; HIF, hypoxia-inducible factor; HOIL-1L, heme-oxidised iron-responsive element-binding protein 2 ubiquitin ligase-1L; HOIP, HOIL-1-interacting protein; IKK, I κ B kinase; Imd, immune deficiency; LDD, linear ubiquitin chain-determining domain; LUBAC, linear ubiquitin chain assembly complex; LUBEL, linear ubiquitin E3 ligase; NF- κ B, nuclear factor κ -light-chain enhancer of activated B cells; NZF, npl4 zinc finger; PAMP, pathogen-associated molecular pattern; PGRP, peptidoglycan recognition protein; PIM, PUB-interacting motif; PNG, peptide N-glycanase; PRR, pattern-recognition receptor; PUB, UBA or UBX-containing protein; RBR, RING in between RING; RING, really interesting new gene; SAMP, stress-associated molecular pattern; SHARPIN, SH3 and multiple ankyrin repeat domains protein (SHANK)-associated RBRK1 homology (RH) domain-interacting protein; Sima, similar; Tab2, transforming growth factor- β -activated kinase 1 binding protein 2; Tak1, transforming growth factor activating kinase; TGF, transforming growth factor; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TUBE, tandem ubiquitin-binding entity; UAS, upstream activating sequence; UBD, ubiquitin-binding domain; VCP, valosin-containing protein.

Introduction

Ubiquitination is a reversible process involving the addition of ubiquitin, a 76-amino acid-long polypeptide, to the target substrate through a three-step enzymatic process carried out by E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases [1]. Polyubiquitin chains are created when a lysine residue (K6, K11, K27, K29, K33, K48 and K63) or the N-terminal methionine (M1) on ubiquitin itself are ubiquitinated [2–4]. M1-linked ubiquitin (M1-Ub) chain formation is solely catalysed by the linear ubiquitin chain assembly complex (LUBAC) consisting of heme-oxidised iron-responsive element-binding protein 2 ubiquitin ligase-1L (HOIL-1L), HOIL-1-interacting protein (HOIP) and SH3 and multiple ankyrin repeat domains protein (SHANK)-associated RBCK1 homology (RH) domain-interacting protein (SHARPIN) [5–8]. All LUBAC components are needed for properly regulated M1-ubiquitination. However, the catalytic activity required for forming M1-Ub chain resides in the really interesting new gene (RING) in between RING (RBR) domain, and the extended linear ubiquitin chain-determining domain (LDD), of HOIP [5,9–12]. This catalytic domain is responsible for positioning two Ub moieties in the close vicinity and orientation required for forming specific M1-Ub chains [10]. By mutating or inhibiting the activity of the RBR domain of HOIP, LUBAC is unable to form M1-Ub chains and thus inhibits its downstream signalling [13,14]. The role of M1-ubiquitination has been extensively studied in inflammatory signalling, where it has been shown to be a key regulator of the canonical nuclear factor- κ B (NF- κ B) pathway [6,14–18].

In *Drosophila*, M1-Ub chains are formed by the RBR-LDD-containing HOIP-homolog protein called linear ubiquitin E3 ligase (LUBEL) [19,20]. Similarly as in mammals, LUBEL-mediated M1-ubiquitination is induced upon bacterial infection and is required for NF- κ B activation and for mounting a proper immune response in the intestine [20]. Infection in flies leads to robust activation of NF- κ B signalling pathways, leading to the transcription of hundreds of genes, including antimicrobial peptides (AMPs) required to fend off intruding pathogens. During systemic inflammation, the expression and release of AMPs are induced in the haemocytes of the fat body, whereas a more local insult leads to an *in situ* production of AMPs. There are two NF- κ B-activating pathways in *Drosophila*, the immune deficiency (Imd) pathway and the Toll pathway [21–23]. The Imd pathway is activated upon recognition of Gram-negative bacteria by peptidoglycan recognition proteins (PGRPs), followed by the

formation of a signalling complex consisting of Imd, the adaptor protein *Drosophila* Fas-associated death domain (dFadd), the caspase-8 homolog Death-related ced-3/Nedd2-like protein (Dredd), and the E3 ligase *Drosophila* inhibitor of apoptosis protein 2 (Diap2) [24–27]. Transforming growth factor (TGF)-activating kinase 1 (Tak1) and Tak1-binding protein 2 (Tab2) are then recruited to the ubiquitin chains formed by Diap2, leading to phosphorylation and activation of the I κ B kinase (IKK) complex, which consists of the catalytic subunit Ird5 (IKK β) and the regulatory subunit Kenny (IKK γ) [25,28–31]. For transcriptional activation, the NF- κ B transcription factor Relish needs to be phosphorylated by the IKK complex and cleaved by Dredd, allowing its translocation to the nucleus and the induction of target gene expression [32–36]. The Toll receptor, on the other hand, is activated in response to Gram-positive bacteria and induces activation and translocation of the NF- κ B factors Dorsal and Dorsal-related immunity factor (Dif) [23,37].

In addition to being activated upon recognition of pathogen-associated molecular patterns (PAMPs), NF- κ B can also be induced in the absence of pathogens, in both *Drosophila* and mammals. Such sterile inflammation may be induced by molecules recognised as danger-associated molecular patterns (DAMP) or by stress-associated molecular patterns (SAMP) [38–40]. Here, we have studied the role of M1-ubiquitination during sterile inflammation induced by mechanical, hypoxic or oxidative stress in *Drosophila* and mammalian cells. Our results show that M1-Ub chains are required for stress-induced activation of NF- κ B and survival of flies and human cancerous intestinal epithelial (Caco2) cells during hypoxia or oxidative stress. We, hence, suggest that the formation of M1-Ub chains is a conserved, common response to different forms of stresses that enables downstream signalling and activation of NF- κ B.

Results

LUBEL catalyses the formation of M1-Ub chains and is required for survival during hypoxia in *Drosophila*

Hypoxia is a condition where the cellular oxygen level decreases, inducing stress responses to protect cells from damage. To investigate whether M1-Ub chains are induced *in vivo* during hypoxia in *Drosophila*, we placed adult fruit flies in specialised hypoxia chambers on a modified MiniHypoxy platform (Fig. 1A) [41], in which the oxygen content is reduced to 5% within minutes (Fig. 1B). After exposure to hypoxia, we

isolated M1-Ub chains from lysates of whole flies with a GST-tagged recombinant tandem ubiquitin-binding entity (TUBE) specific for M1-Ub chains (M1-TUBE). We found that hypoxia induced an increase in M1-Ub chain formation in control flies (Fig. 1C, lanes 1, 2), but not in *LUBEL*^{ΔRBR} mutant flies that lack the catalytic RBR domain of LUBEL, responsible for M1-ubiquitination in *Drosophila* (Fig. 1C, lanes 5, 6, and 1D). A similar effect was observed when the expression of LUBEL was silenced by inducing expression of RNAi transcripts (*LUBEL-RNAi*) using the daughterless-Gal4 (daGal4) driver, which directs ubiquitous expression of upstream activating sequence (UAS)-regulated sequences (Fig. 1C, lanes 3, 4, and 1D). Interestingly, we did not detect changes in K63-Ub chain formation in control or LUBEL mutant flies in response to hypoxia (Fig. 1C, second panel). As we have previously shown that LUBEL-mediated M1-Ub chains are induced by ingested bacteria [20], we wanted to exclude the possibility that the observed M1-ubiquitination in response to hypoxia was due to increased receptor stimulation by resident commensal bacteria. For this purpose, we generated germ-free axenic flies. Similarly, as in conventionally reared flies, an increase in M1-Ub chains was observed in axenic flies during hypoxic conditions (Fig. 1E), indicating that the hypoxia-induced M1-Ub chain formation is not induced by bacteria.

To investigate whether LUBEL is needed for *Drosophila* to endure hypoxia, we assessed the survival of LUBEL mutant flies under low oxygen conditions. While wild-type flies survived in 5% O₂, both *LUBEL*^{ΔRBR} and *LUBEL-RNAi* flies were more sensitive to the hypoxic conditions (Fig. 1F). The sensitivity to hypoxia was similar as in flies lacking one allele of Similar (Sima), the *Drosophila* hypoxia-inducible factor (HIF)-1α [42]. To ascertain that the hypoxia sensitivity of LUBEL mutant flies was not caused by a defect in the maintenance of the commensal bacteriome, we analysed the survival of flies reared axenic. As expected, also the axenic *LUBEL*^{ΔRBR} mutant flies and *LUBEL-RNAi* flies were more sensitive to hypoxia compared with wild-type axenic flies (Fig. 1G).

LUBEL is not required for activation of the HIF pathway

The hypoxia-inducible factor HIF-1α is a conserved transcription factor, responsible for the activation of the expression of genes controlling oxygen homeostasis. To analyse whether M1-ubiquitination is needed for activation of HIF-mediated hypoxia responses in the fly, we analysed the expression of the hypoxia-inducible Sima target gene *ldh* [43–45] in control and

LUBEL mutant flies. The *ldh* expression was induced in control flies upon hypoxia, whereas *sima*^{KG07607} mutant flies were unable to induce *ldh* expression (Fig. 2A). Interestingly, *ldh* was induced equally well in *LUBEL*^{ΔRBR} (Fig. 2A), *LUBEL-RNAi* flies (Fig. 2B) and in control flies upon hypoxia. The *Drosophila* enzyme Fatiga is an oxygen sensing hydroxylase that marks Sima for degradation under normal oxygen levels. Fatiga is inactivated in the absence of oxygen, leading to a stabilisation of Sima and a subsequent increase in HIF target gene expression [43,46,47]. Interestingly, the hypoxia sensitivity of *LUBEL*^{ΔRBR} mutants were not rescued by constitutive activation of the HIF pathway by genetic deletion of Fatiga (Fig. 2C). Similarly, *ldh* expression was induced to comparable levels in control flies and *LUBEL*^{ΔRBR} mutants in the presence and absence of Fatiga (Fig. 2D). These results indicate that LUBEL-mediated M1-Ub chains are neither required for oxygen sensing by Fatiga nor for the activation of HIF.

LUBEL is required to induce Relish target gene expression during hypoxia

As hypoxia has been shown to induce NF-κB activation in flies [45,48], we investigated whether this activation requires LUBEL. Indeed, expression of the Imd/NF-κB Relish pathway-specific gene, *dipterocin* [23,49,50], was induced during hypoxia in control flies, whereas ubiquitous RNAi silencing of LUBEL prevented this induction (Fig. 3A). In contrast, the expression of *drosomycin*, an AMP specific for the Toll pathway [23,49,50], was similar in control and LUBEL-RNAi flies (Fig. 3B). These results indicate that LUBEL is exclusively required for Relish activation in response to hypoxia. The Relish-induced *dipterocin* expression is suggested to depend on Dredd [33]. To investigate whether the LUBEL-mediated Relish activation during hypoxia is mediated via Dredd, we introduced transgenic expression of Dredd in LUBEL-RNAi flies (Fig. 3C and D). Dredd overexpression, as expected, rescued both the inability to induce *dipterocin* expression (Fig. 3E) and the sensitivity to hypoxia (Fig. 3F) of *LUBEL-RNAi* flies. Likewise, *LUBEL*^{ΔRBR} flies did not die during hypoxia when Dredd expression was induced (Fig. 3F). Overall, these data indicate that LUBEL indeed regulates hypoxia-induced Relish activation upstream of Dredd.

Intracellular, but not extracellular mediators are required for Relish activation in response to hypoxia

To assess how the Imd pathway is engaged during hypoxia, we examined whether the transmembrane

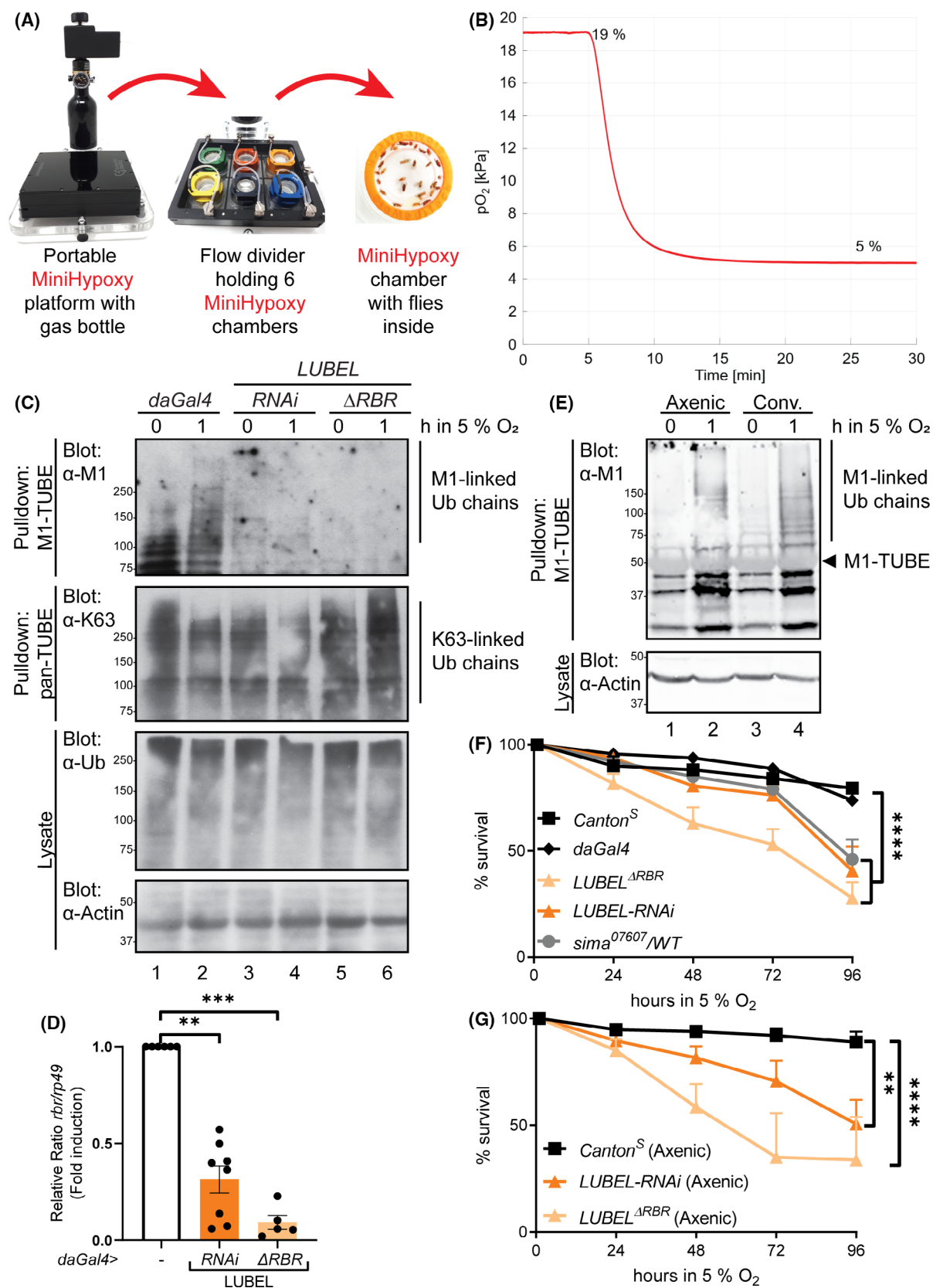


Fig. 1. LUBEL catalyses formation of M1-Ub chains and is required for survival during hypoxia in *Drosophila*. (A) A picture of the modified MiniHypoxia platform connected to a gas bottle, the 6-well flow divider and a MiniHypoxia chamber with flies. (B) The dynamics of gas supply to the MiniHypoxia chamber was monitored by measuring the partial oxygen pressure (pO₂) while a 5-mL·min⁻¹ gas flow was supplied to the chamber. (C) Adult control *daGal4* flies and mutant LUBEL^{ΔRBR} and UAS-LUBEL-RNAi;*daGal4* flies were subjected to low oxygen conditions (5% O₂). M1-Ub and pan-Ub chains were isolated at denaturing conditions from fly lysates with M1-TUBE and pan-TUBE respectively. Ubiquitin chains from samples were analysed by Western blotting with α-M1, α-K63 and α-pan-Ub antibodies, and equal loading was controlled with α-Actin antibody, *n* = 3. (D) *Rbr* mRNA transcripts of adult control *daGal4* flies and mutant LUBEL^{ΔRBR} and UAS-LUBEL-RNAi;*daGal4* flies were analysed with qPCR. Error bars indicate SEM from more than 5 independent experimental repeats. (E) Conventionally reared control and axenic wild-type *Canton^S* flies were subjected to low oxygen conditions (5% O₂). M1-Ub chains were isolated and analysed as in C, *n* = 3. (F) Adult *Canton^S* and *daGal4* control flies, mutant LUBEL^{ΔRBR}, UAS-LUBEL-RNAi;*daGal4* and heterozygote mutant *sima*^{KG07607} flies were subjected to low oxygen conditions (5% O₂), and their survival was monitored over time. Error bars indicate SEM from more than 10 independent experimental repeats. (G) Axenic adult wild-type *Canton^S* and mutant LUBEL^{ΔRBR} and UAS-LUBEL-RNAi;*daGal4* flies were subjected to low oxygen conditions (5% O₂), and their survival was monitored over time. Error bars indicate SEM from more than 3 independent experimental repeats. Statistical significance was calculated using the Student's *t*-test (D) or two-way ANOVA and the Tukey's multiple comparison test (F, G), ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

pattern recognition receptor (PRR) PGRP-LCx is required for hypoxia-induced Relish activation. Interestingly, flies with loss of function of PGRP-LCx were not sensitive to low oxygen levels, and loss of PGRP-LCx could not significantly rescue the sensitivity induced by loss of function of LUBEL (Fig. 4A). On the contrary, the intracellular, key Imd pathway mediators Dredd, Diap2 and Kenny were required for flies to survive hypoxic conditions, as flies lacking one allele of Kenny, both alleles of Diap2, or carrying a mutation disturbing the catalytic activity of Dredd, died during hypoxia (Fig. 4B). Similarly, loss of both Tak1 alleles decreased viability after hypoxia exposure (Fig. 4B). Furthermore, PGRP-LCx, but not Tak1, Dredd, Diap2 or Kenny mutant flies were able to induce *dipterocin* expression in response to hypoxia (Fig. 4C), while the hypoxia-induced *ldh* expression was comparable in both control and all Imd pathway mutant flies (Fig. 4D). This indicates that the Imd pathway is engaged downstream of the receptor during hypoxia. When analysing M1-ubiquitination in Tak1 and Diap2 mutant flies after exposure to hypoxia, we found that both Tak1 and Diap2 are required for augmented M1-Ub chain formation (Fig. 4E and F). Hence, our results indicate that hypoxia-induced Relish activation is activated by intracellular cues at the level of Tak1 and Diap2.

LUBEL is required for both local and systemic AMP expression in response to hypoxia

In flies, NF-κB responses are activated locally upon infection or injury in epithelial tissues, such as the intestine and trachea, and systemically upon septic infection in the fat body [22,51,52]. To study local hypoxia-induced NF-κB activation in the trachea, the *Drosophila* organ for oxygen uptake and distribution, we dissected 3rd instar larvae carrying either a

Drosomycin-LacZ or *Diptericin-LacZ* reporter. In contrast to the intestine and the fat body, it has been shown that *drosomycin*, but not *dipterocin*, is expressed upon activation of Relish via the Imd pathway in the trachea [51,53,54]. Indeed, *drosomycin* was induced in the trachea of *Drosomycin-LacZ* larvae, but not in LUBEL^{ΔRBR} mutant *Drosomycin-LacZ* larvae during hypoxia (Fig. 5A). Similarly as others [51], we could not detect any specific *Diptericin-LacZ* reporter activation in the trachea (Fig. 5B). These data indicate that LUBEL is required for activation of Relish in the trachea during hypoxia.

To analyse whether LUBEL is required also for systemic fat body-mediated NF-κB responses, we specifically silenced LUBEL in the fat body using the fat body-specific driver *c564Gal4* (Fig. 5C). While *dipterocin*, which is a specific Relish target gene in the fat body [51,55], was induced in control flies during hypoxia, no hypoxia-induced *dipterocin* expression could be detected when silencing LUBEL in the fat body (Fig. 5D). On the other hand, *drosomycin* expression, which in contrast to the trachea is mainly induced via the Toll pathway in the fat body, was not affected by the fat body-specific loss of LUBEL (Fig. 5E). Similarly, the *ldh* expression in the fat body was not affected by the loss of LUBEL (Fig. 5F). These results suggest that LUBEL is specifically required for activation of Relish both in the trachea and the fat body during hypoxia.

LUBEL catalyses the formation of M1-Ub chains in response to oxidative and mechanical stress

Since the Imd pathway did not seem to be induced via the canonical extracellular receptor during hypoxia, we wanted to address whether other types of sterile cellular stresses induce a similar Relish activation and

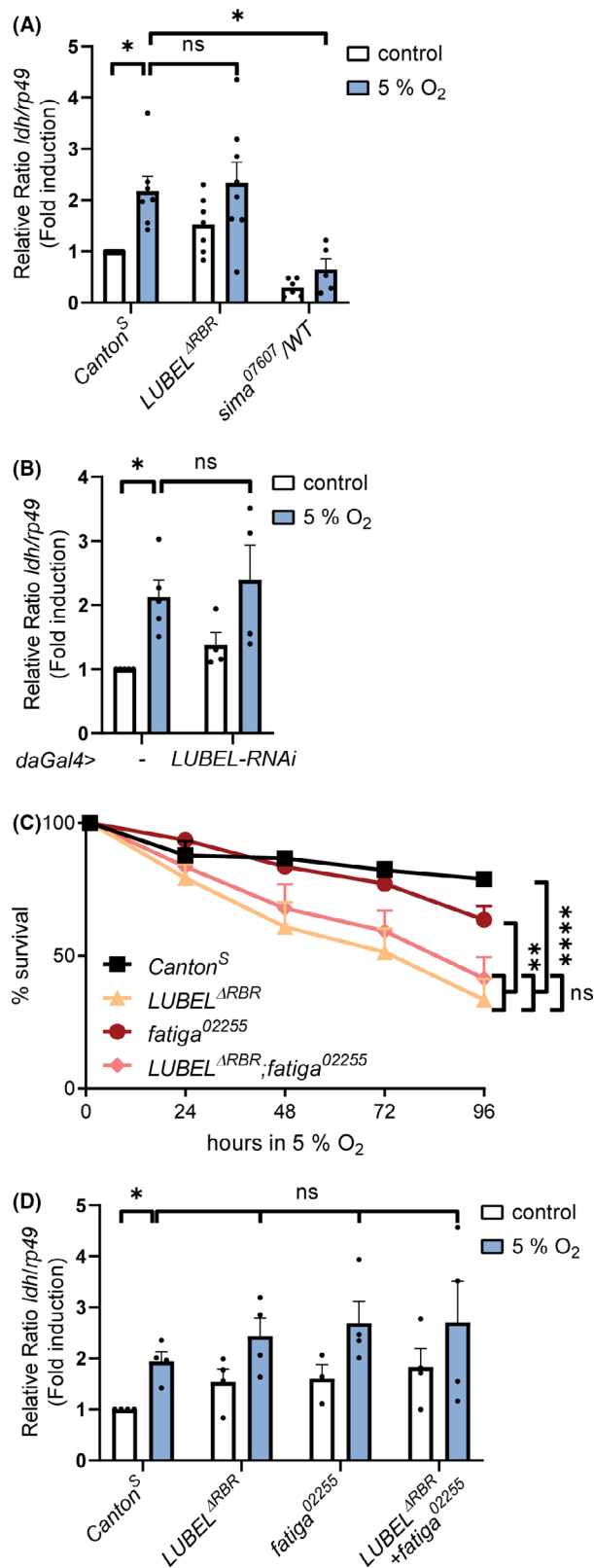


Fig. 2. LUBEL is not required for activation of the HIF pathway. (A, B) Adult wild-type *Canton^S*, mutant *LUBEL^{ΔRBR}* and heterozygote mutant *sima^{KG07607}* flies (A), or control *daGal4* and transgenic *UAS-LUBEL-RNAi;daGal4* flies (B) were subjected to low oxygen conditions (5% O₂) for 24 h. HIF pathway activation was studied by analysing the expression of *Idh* with qPCR. Error bars indicate SEM from more than 3 independent experimental repeats. (C) Adult wild-type *Canton^S* flies, mutant *LUBEL^{ΔRBR}*, mutant *fatiga⁰²²⁵⁵* and double-mutant *LUBEL^{ΔRBR};fatiga⁰²²⁵⁵* flies were subjected to low oxygen conditions (5% O₂), and their survival was monitored over time. Error bars indicate SEM from more than 6 independent experimental repeats. (D) Adult wild-type *Canton^S* flies, mutant *LUBEL^{ΔRBR}*, mutant *fatiga⁰²²⁵⁵* and double-mutant *LUBEL^{ΔRBR};fatiga⁰²²⁵⁵* flies were subjected to low oxygen conditions (5% O₂) for 24 h. HIF pathway activation was studied by analysing the expression of *Idh* with qPCR. Error bars indicate SEM from more than 3 independent experimental repeats. Statistical significance was calculated using the Student's *t*-test (A, B, D) or two-way ANOVA and the Tukey's multiple comparison test (C), ns nonsignificant, **P* < 0.05, ***P* < 0.01, *****P* < 0.0001.

whether these responses are mediated by LUBEL. To induce oxidative stress, we fed flies with the pesticide paraquat [56,57] and found that paraquat feeding was able to induce the formation of M1-Ub chains in control flies (Fig. 6A). To elucidate whether LUBEL is important for surviving oxidative stress, we monitored the survival of *LUBEL^{ΔRBR}* and *LUBEL-RNAi* flies fed with paraquat for two days. While more than half of control flies survived paraquat feeding, most *LUBEL^{ΔRBR}* and *LUBEL-RNAi* flies succumbed (Fig. 6B). The sensitivity was similar in Dual oxidase (Duox) RNAi silenced flies, which are susceptible to oxidative stress as they are unable to produce reactive oxygen species (ROS) (Fig. 6B). During hypoxia, ectopic expression of Dredd was able to rescue the stress-sensitive phenotype of the *LUBEL^{ΔRBR}* and *LUBEL-RNAi* flies (Fig. 6C). This suggests that LUBEL induces Imd signalling also in response to oxidative stress. Similarly, loss of function of the receptor PGRP-LCx did not affect sensitivity to paraquat feeding (Fig. 6D) and did not rescue the sensitivity induced by *LUBEL^{ΔRBR}* mutation, indicating that the Relish activating receptor PGRP-LCx is not required for protection against oxidative stress. As it has been shown that mechanical stress activates NF-κB in *Drosophila* [58], we wanted to investigate whether M1-Ub chains are induced in response to mechanical shear stress. Shear stress was mimicked in 3rd instar larvae by vortexing. Similarly as hypoxic and oxidative stress, mechanical stress increased M1-Ub chain formation in *Drosophila* larvae (Fig. 6E).

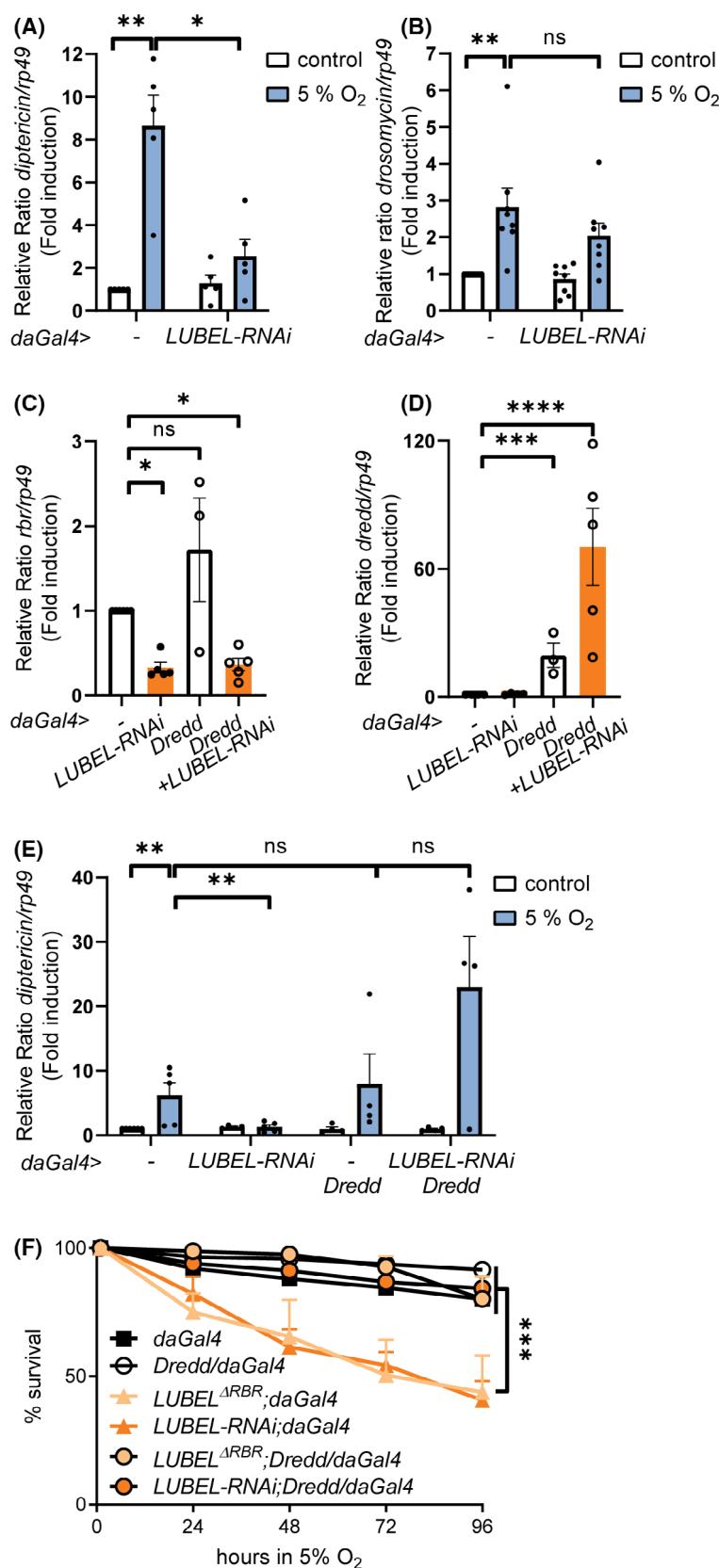


Fig. 3. LUBEL mutant flies are unable to induce NF- κ B target gene expression during hypoxia. (A-E) Adult control *daGal4* flies (A-E), mutant *UAS-LUBEL-RNAi*; *daGal4* flies (A-E), overexpression *daGal4/UAS-Dredd* flies (C-E) and rescue *UAS-LUBEL-RNAi*; *daGal4/UAS-Dredd* flies (C-E) were subjected to low oxygen conditions (5% O₂) for 24 h. NF- κ B activation was studied by analysing the expression of *dipterizin* and *drosomycin* with qPCR. *Rbr* and *dredd* mRNA transcripts were studied by analysing *rbr* and *dredd* with qPCR from nontreated flies. Error bars indicate SEM from more than 4 independent experimental repeats. (F) Adult control *daGal4* flies, overexpressing *daGal4/UAS-Dredd* flies, mutant *UAS-LUBEL-RNAi*; *daGal4* and *LUBEL^{ΔRBR}* flies, rescue *UAS-LUBEL-RNAi*; *daGal4/UAS-Dredd* and *LUBEL^{ΔRBR}*; *daGal4/UAS-Dredd* flies were subjected to low oxygen conditions (5% O₂), and their survival was monitored over time. Error bars indicate SEM from more than 4 independent experimental repeats. Statistical significance was calculated using the Student's *t*-test (A-E) or two-way ANOVA and the Tukey's multiple comparison test (F), ns nonsignificant, * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, **** *P* < 0.0001.

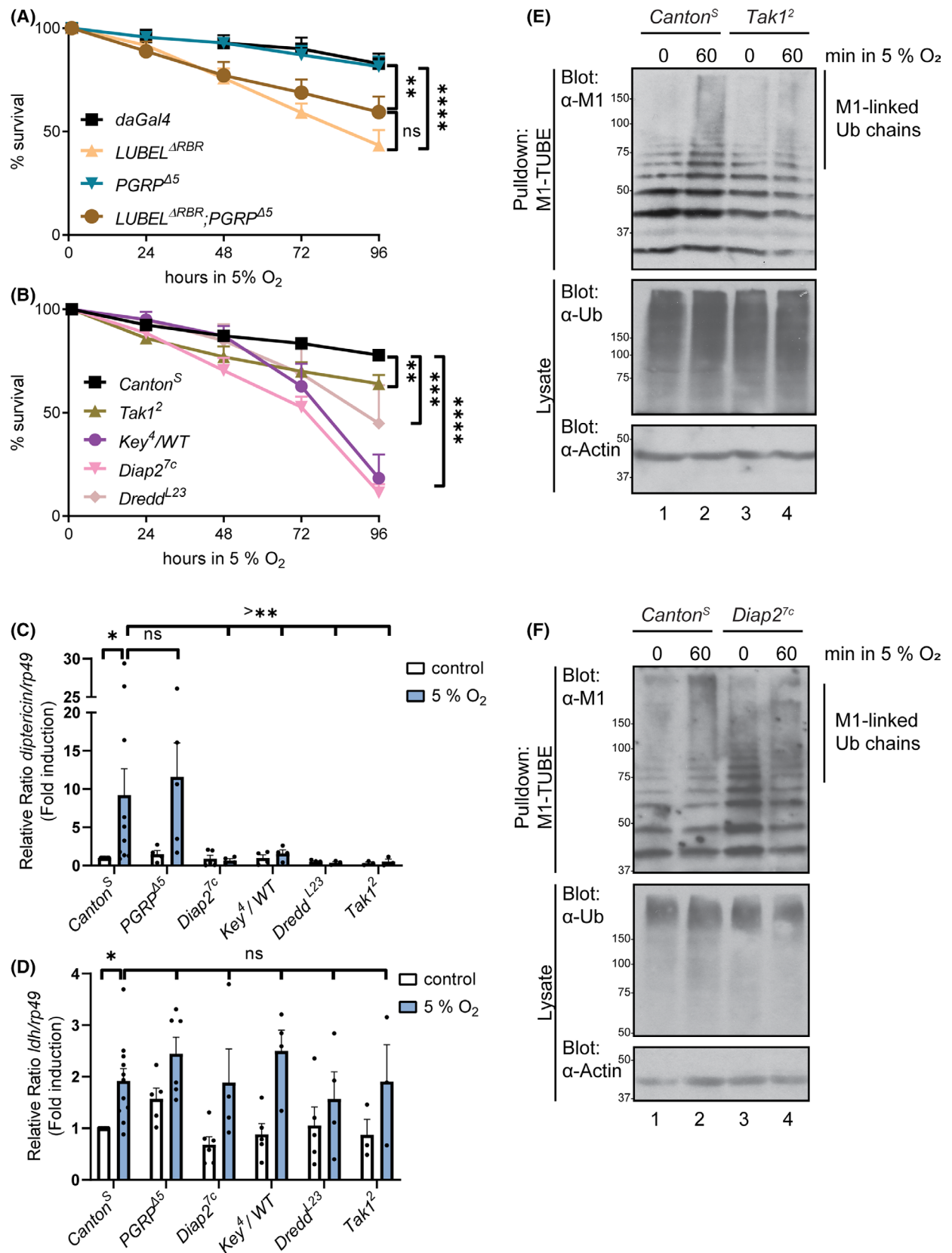


Fig. 4. Intracellular mediators of the Imd pathway are required for NF- κ B activation in response to hypoxia, while the receptor PGRP is not. (A) Adult control flies *daGal4*, mutant *LUBEL^{ΔRBR}* and *PGRP^{Δ5}* flies and double-mutant *LUBEL^{ΔRBR};PGRP^{Δ5}* flies were subjected to low oxygen conditions (5% O₂), and their survival was monitored over time. Error bars indicate SEM from more than 3 independent experimental repeats. (B) Adult wild-type *Canton^S* flies and mutant fly lines of the Imd pathway mediators *Tak1²*, *Diap2^{7c}*, *Key⁴/WT* and *Dredd^{L23}* were subjected to low oxygen conditions (5% O₂), and their survival was monitored over time. Error bars indicate SEM from more than 5 independent experimental repeats. (C, D) Adult wild-type *Canton^S* flies and mutant fly lines of the Imd pathway mediators *PGRP^{Δ5}*, *Tak1²*, *Diap2^{7c}*, *Key⁴/WT* and *Dredd^{L23}* were subjected to low oxygen conditions (5% O₂) for 24 h. Relish and HIF activation was studied by analysing the expression of *dipteracin* and *Idh*, respectively, with qPCR. Error bars indicate SEM from more than 4 independent experimental repeats. (E, F) Adult wild-type *Canton^S* flies and mutant *Tak1²* and *Diap2^{7c}* flies were subjected to low oxygen conditions (5% O₂). M1-Ub chains were isolated at denaturing conditions from fly lysates with M1-TUBE. Ubiquitin chains from samples were analysed by Western blotting with α -M1 and α -pan-Ub antibodies, equal loading was controlled with α -Actin antibody, *n* = 3. Statistical significance was calculated using the two-way ANOVA and the Tukey's multiple comparisons test (A, B) or Student's *t*-test (C, D), ns nonsignificant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Overall, these results indicate that M1-ubiquitination is induced in the fly as a response to sterile stress conditions, particularly hypoxia, oxidative and mechanical stress.

M1-Ub chains are formed in response to stress in human intestinal epithelial cells and protects from stress-induced cell death

All the stress conditions that we studied in *Drosophila* are known to activate evolutionary conserved stress-mediated inflammatory responses. To investigate whether the stress-induced M1-Ub chain formation is a conserved response present in mammals, we exposed human Caco2 cells to the same stress conditions used in flies. Interestingly, all these stress conditions led to increased M1-Ub chain formation in the mammalian cells (Fig. 7A,B,C). Importantly, the commercial HOIP inhibitor HOIPIN-1 [13] reduced M1-Ub chain formation during hypoxia and oxidative stress (Fig. 7A and B). While we were not able to detect any significant changes in NF- κ B activation during hypoxia and oxidative stress in the Caco2 cells (data not shown), we investigated whether disrupted M1-Ub chain formation affects cell survival. For this purpose, we measured activation of the apoptotic effector caspase-3 in response to hypoxia and oxidative stress in the presence and absence of HOIPIN-1. While both hypoxia and oxidative stress lead to activation of caspase-3, HOIPIN-1 treatment further induced caspase activation (Fig. 7D and E). Taken together, our findings indicate that the formation of M1-Ub chains is an evolutionary conserved stress response, important to protect the organism and its cells from stress-induced damage.

Discussion

During pathological conditions, specific stress responses are induced to provide the cell with tools to repair or eliminate damaged proteins and promote

survival. In addition, inflammatory signalling responses, such as the NF- κ B pathways, are engaged and contribute to the restoration of cellular homeostasis. In this study, we have shown that M1-linked ubiquitination is augmented in response to distinct noxious and aseptic stimuli like hypoxia, oxidative stress, and mechanical stress in *Drosophila*. We also demonstrate that M1-ubiquitination is induced in response to sterile stresses in human cells. We show that LUBEL-mediated M1-ubiquitination is required for activation of NF- κ B and for the fly to survive stress conditions, which is in line with previous research showing that LUBEL-mediated M1-Ub chains are essential for survival when flies are subjected to heat shock [19]. In addition, NF- κ B target gene expression has been shown to be induced in the fat body after sterile mechanical pinching of *Drosophila* larvae [58]. Similarly, we found that LUBEL is required for Relish activation both locally in the trachea and systemically in the fat body of the adult fly during hypoxia. This is interesting, as we have previously shown that M1-Ub chains are indispensable for mounting local immune responses in the epithelial tissues, but not for Relish activation in the fat body in response to pathogen infection [20]. This suggests that the requirement of LUBEL-mediated ubiquitination differs in septic and sterile NF- κ B activation.

While PAMPs, DAMPs and SAMPs may be patterns of extracellular origin, our results indicate that the Imd pathway is activated downstream of the conventional PRR PGRP-LCx during sterile stress, through Imd regulators such as Tak1 and Diap2. In addition to activation through PGRP-LCx, Tak1 can be activated by the Ca²⁺/calcium/calmodulin-dependent kinases 2 (CaMKII) pathway [59], by the *Drosophila* TNF receptor Wengen [60,61], and by the intracellular PRR PGRP-LE [62–64], and it would be interesting to analyse if these pathways contribute to LUBEL activation and M1-ubiquitination during cell

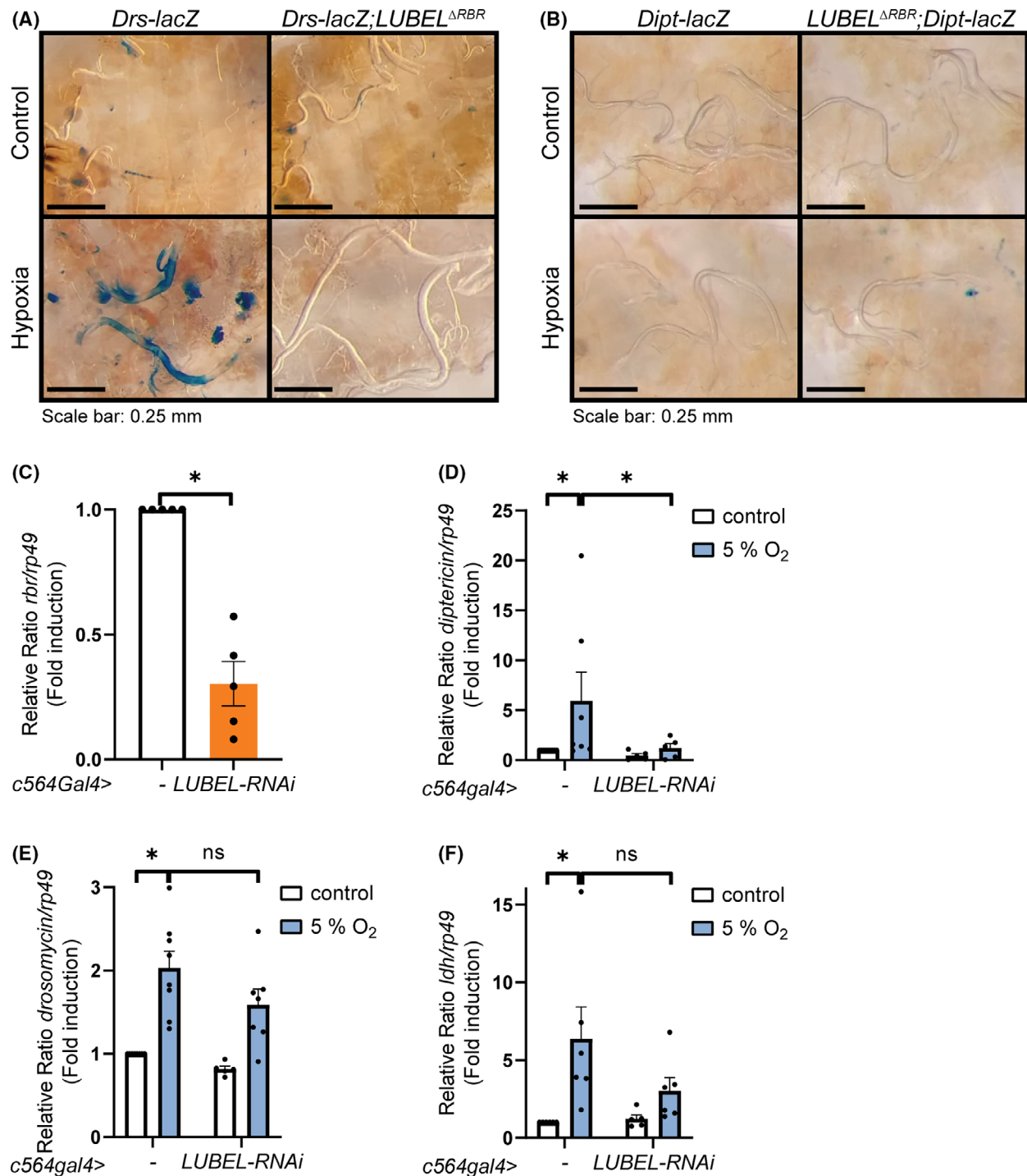


Fig. 5. LUBEL is required for both local and systemic AMP expression in response to hypoxia. (A) Dissected larval trachea from control *Drosomycin-lacZ* and mutant *Drosomycin-lacZ;LUBEL^{ΔRBR}* reporter flies stained for β-galactosidase activity after 24 h of low oxygen treatment (5% O₂). The images are representatives of 4 independent experimental repeats. (B) Dissected larval trachea from control *Diptericin-lacZ* and mutant *LUBEL^{ΔRBR}; Diptericin-lacZ* reporter flies stained for β-galactosidase activity after 24 h of low oxygen treatment (5% O₂). The images are representatives of 3 independent experimental repeats. (C-F) Adult control *c564Gal4* flies, mutant *UAS-LUBEL-RNAi/c564Gal4* flies were subjected to low oxygen conditions (5% O₂) for 24 h. *Rbr* mRNA transcripts were studied by analysing *rbr* with qPCR from nontreated flies (C). NF-κB activation was studied by analysing the expression of *dipteracin* with qPCR (D). The same samples were also analysed for expression of *drosomycin* (E) and *ldh* (F) with qPCR. Error bars indicate SEM from more than 4 independent experimental repeats. Statistical significance was calculated using the Student's *t*-test, ns nonsignificant, **P* < 0.05.

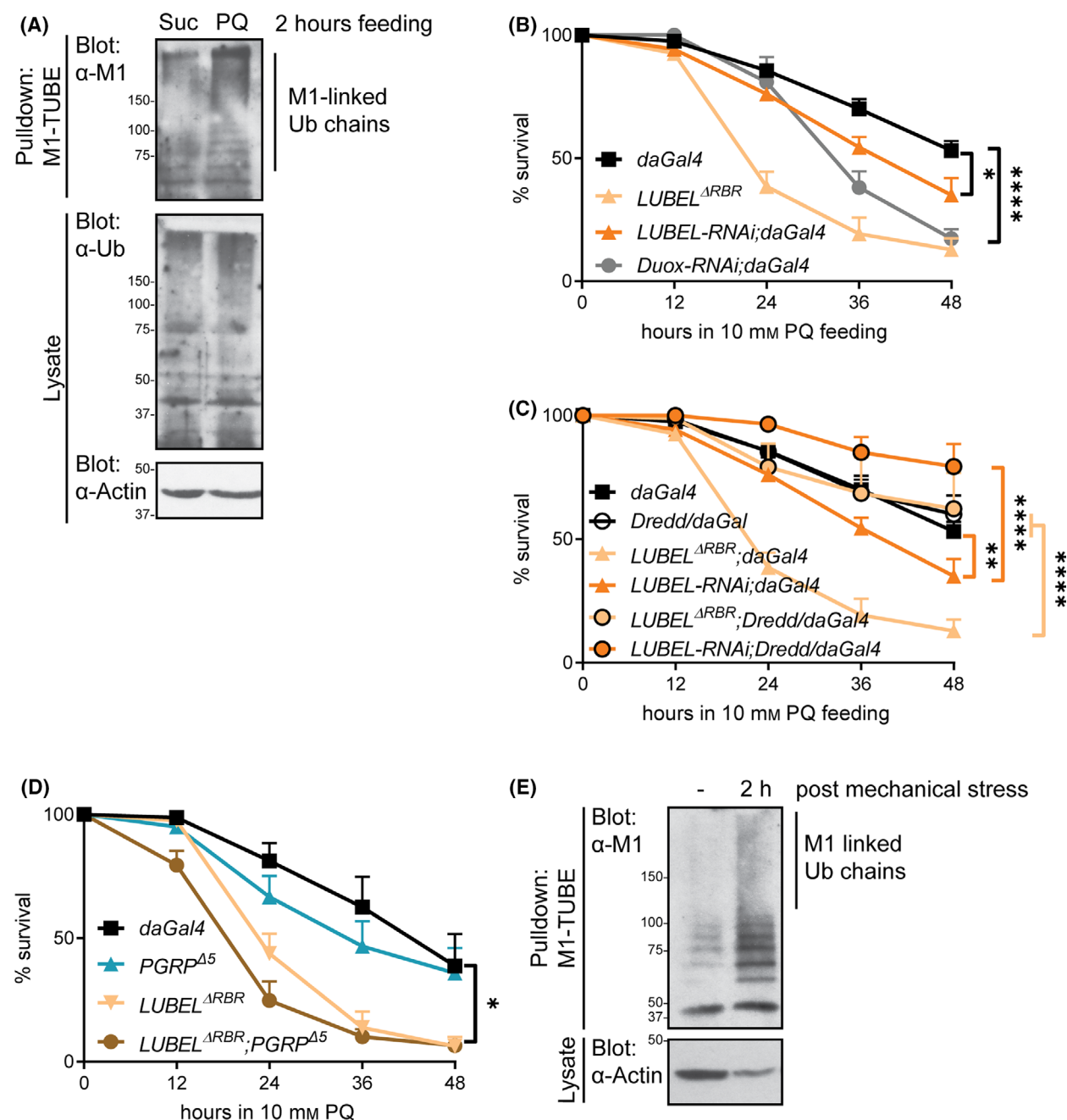


Fig. 6. LUBEL catalyses formation of M1-Ub chains in response to oxidative and mechanical stress in *Drosophila*. (A) Adult control flies *daGal4* were fed with 5% sucrose (Suc) or 5% sucrose with 20-mM paraquat (PQ) for 2 h. M1-Ub chains were isolated at denaturing conditions from fly lysates with M1-TUBE. Ubiquitin chains from samples were analysed by Western blotting with α-M1 and α-pan-Ub antibodies, equal loading was controlled with α-Actin antibody, $n = 3$. (B-D) Adult control *daGal4* flies (B-D), mutant *LUBEL^{ΔRBR}* (B-D), transgenic *UAS-LUBEL-RNAi;daGal4* (B, C) and *UAS-Duox-RNAi;daGal4* (B), overexpressing *daGal4/UAS-Dredd* (C), rescue *LUBEL^{ΔRBR};daGal4/UAS-Dredd* (C) and *UAS-LUBEL-RNAi;daGal4/UAS-Dredd* (C), mutant *PGRP^{Δ5}* (D) and double-mutant *LUBEL^{ΔRBR};PGRP^{Δ5}* (D) flies were fed with 10-mM paraquat in 5% sucrose, and their survival was monitored over time. Error bars indicate SEM from more than 3 independent experimental repeats. (E) Wild-type *CantonS* larvae were vortexed for 10 s and then placed in normal food to recover for 2 h. M1-Ub chains were isolated at denaturing conditions from fly lysates with M1-TUBE. Ubiquitin chains from samples were analysed by Western blotting with α-M1 antibody, equal loading was controlled with α-Actin antibody, $n = 3$. Statistical significance was calculated using the two-way ANOVA and Tukey's multiple comparison test, * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

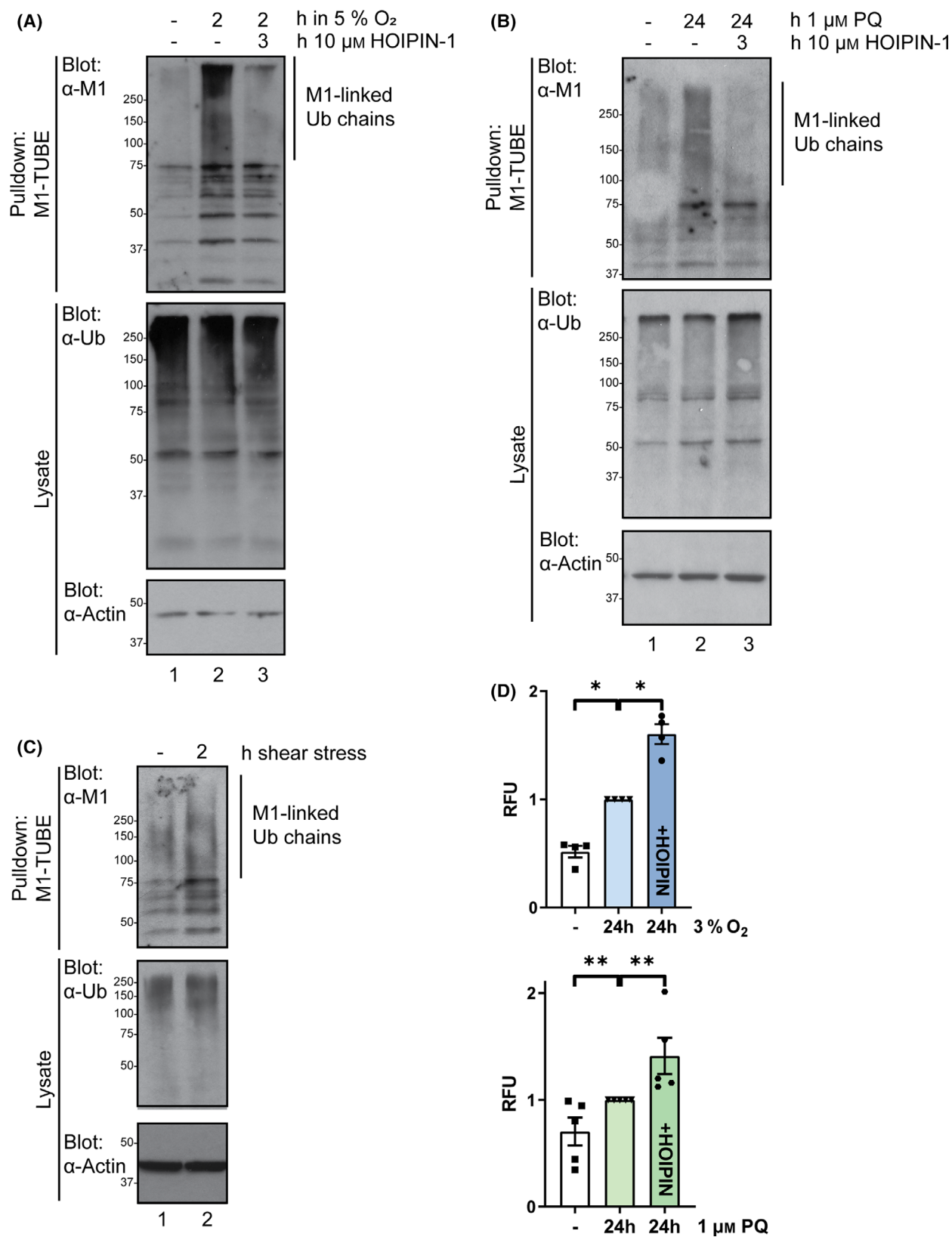


Fig. 7. M1-ubiquitination is induced upon hypoxic, oxidative and mechanical stress and protects human intestinal epithelial cells against cell death. (A–C) Human Caco2 cells were (A) exposed to low oxygen conditions, 5% O₂, for 2 h, (B) treated with 10 µM of paraquat for 24 h or (C) subjected to shear stress by placing the plates on an orbital shaker, 100 r.p.m., for 2 h. To inhibit M1-ubiquitination, Caco2 cells were additionally treated with 10-µM HOIPIN-1 inhibitor for 3 h (A, B). M1-Ub chains were isolated at denaturing conditions from cell lysates with M1-TUBE. Ubiquitin chains from samples were analysed by Western blotting with α-M1 and α-pan-Ub antibodies, equal loading was controlled with α-Actin antibody, *n* = 3. (D, E) Human Caco2 cells were (D) exposed to low oxygen conditions, 3% O₂, for 24 h, or (E) treated with 1 µM of paraquat for 24 h. To inhibit M1-ubiquitination, Caco2 cells were additionally treated with 10-µM HOIPIN-1 inhibitor for 24 h. The caspase-3/7 activity was assessed after addition of Apo-ONE reagent by measuring fluorescence at 499/521 nm and portrayed as relative fluorescence unit (RFU). Error bars indicate SEM from 4 independent experimental repeats. Statistical significance was calculated using the Mann–Whitney *U* test, **P* < 0.05, ***P* < 0.01.

stress. Furthermore, we cannot exclude that activation of Tak1 and Diap2 is induced through Toll in response to stress. The stress-induced activation of M1-ubiquitination may also be caused by recognition of changes in intracellular patterns. Cell stress causes protein misfolding and aggregation, followed by ubiquitin-marking of aberrant protein structures. Interestingly, HOIP has been shown to be recruited to ubiquitin-marked molecules through the evolutionary conserved ubiquitin-binding chaperone Valosin-containing protein (VCP)/p97 [65–67]. HOIP has also been shown to be directly recruited to intracellular bacteria marked by ubiquitin via its ubiquitin-binding Npl4 zinc finger (NZF) [68]. Similar to HOIP, LUBEL contains several NZF motives in its N-terminus [19,20] that could potentially recognise ubiquitinated molecules during stress conditions in the fly.

It is still unknown how sterile cell stress is recognised and converted into activation of inflammatory transcription factors and what the physiological relevance of stress-induced activation of immune responses is in both flies and mammals. Sterile immune responses may function to prime cells to respond to pathogenic invasion, to maintain cellular homeostasis or to support stress-specific protective responses by inducing expression of additional survival-promoting genes. As stress-activated inducers of survival-promoting NF-κB activation, M1-Ub chains may be important protectors of cells that are damaged by protein aggregation, for example in neurodegenerative disorders. On the other hand, M1-Ub chains could contribute to cancer development by protecting cells growing in hypoxic conditions, a state common for growing tumours. M1-Ub chain induction may also protect cancerous cells from mechanical stress caused by changes in cell density and cell–cell adhesion during cancer cell growth and metastasis. In addition, M1-Ub chains might contribute to drug resistance by activating protective measures in response to genotoxic and proteotoxic stress that is induced by anti-cancer drugs and by components released from cells killed by cytotoxic agents. Finally, M1-Ub chains may contribute to inflammation-

induced cancer by maintaining NF-κB-mediated expression of growth-promoting and antiapoptotic genes. The highly editable ubiquitin and its regulators serve as interesting targets when tuning inflammatory diseases and cancer. However, further examination of the role of M1-ubiquitination during these pathological conditions is needed to unravel the details of ubiquitin-mediated induction of sterile inflammation.

Materials and methods

Fly husbandry and strains

Drosophila melanogaster was maintained at 25 °C with a 12-h light–dark cycle on Nutri-fly BF (Dutscher Scientific, Essex, UK). *Canton^S* wild-type flies, the ubiquitous *daGal4* driver line, the fat body-specific *c564Gal4* driver line, the *Dipt-LacZ* reporter line, balancer lines, and *key⁴*, *dredd^{L23}*, *diap2^{7c}* mutant fly lines and *UAS-Dredd* flies were kindly provided by Prof. Pascal Meier and Dr. François Leulier [69]. The *Drosophila* fly lines *yw*; *Mi{ETI}LUBEL^{MB00197}* (#22725, referred to as *LUBEL^{ΔRBR}*), *fatiga⁰²²⁵⁵* (#11561), *sima^{KG07607}* (#14640), *PGRP-LC^{Δ5}* (#36323, referred to as *PGRP^{Δ5}*), *tak1²* (#26272), *UAS-Duox-TRiP* (#33975, referred to as *DUOX-RNAi*) and *Drs-lacZ* (#55708) were obtained from Bloomington stock centre. *UAS-LUBEL-RNAi* (*P{GD7269}v18055*, #18055, referred to as *LUBEL-RNAi*) were obtained from Vienna *Drosophila* resource centre. The efficiency of *UAS-LUBEL-RNAi* silencing was analysed by detecting mRNA transcripts of the C-terminal catalytic RBR region by qPCR. Similarly, the efficiency of *UAS-Dredd* overexpression was confirmed with primers for *dredd* by qPCR. For qPCR analysis, 10 flies were used. Axenic flies were reared germ-free according to the previously published protocol [70]. Flies were confirmed to be axenic by 16S PCR and by growing fly homogenates on Luria Bertani (LB) plates and checking for bacterial growth.

Stress treatments and survival experiments in *Drosophila*

Hypoxia experiments were performed by placing adult flies or larvae in a modified portable MiniHypoxia platform (Faculty of Medicine and Health Technology, Tampere

University, Finland) [41]. The modified MiniHypoxy platform can hold six individual MiniHypoxy chambers, and a single chamber can hold up to 80 flies (Fig. 1A). With the modified portable MiniHypoxy platform, flies can be treated with a desired gas mixture, it enables, for example, live monitoring of flies under a microscope throughout an experiment. In this study, flies were exposed to a gas mixture of 5% O₂ with 95% N₂. The readymade gas mixture is supplied either directly from a large cylinder obtained from Linde Gas (Oy Linde Gas Ab, Espoo, Finland) or as in this study, to support portability, from a small refillable gas bottle (Fig. 1A). The refillable gas bottles can be loaded with any gas composition, and thus flies can be exposed and maintained in various gas environments. 5-mL·min⁻¹ gas flow was supplied to each chamber, which renders the chambers hypoxic within minutes (with 3 min 20 s fall time) (Fig. 1B). To validate the functionality of the platform, the partial oxygen pressure (pO₂) was measured using an in-house made optical oxygen sensor [71]. In short, oxygen-sensitive fluorescent dyes embedded in thin polymer film on a glass plate at the bottom of the chamber provide a noninvasive way to measure oxygen partial pressure in the chamber through the glass. The establishment of the correct oxygen level in the chamber and the gas exchange dynamics were demonstrated using readymade mixtures of 19% and 5% oxygen. For isolation of M1-Ub chains, 20–40 adult flies per genotype were incubated for 1 h and qPCR 10 flies per genotype were incubated 24 h at 25 °C in the MiniHypoxy chambers with 5% oxygen. For X-Gal staining, larvae were exposed to 5% oxygen for 24 h before dissection at 25 °C. For hypoxia survival assays, 20 flies per fly genotype were counted at indicated time points. To induce oxidative stress, adult flies were fed with paraquat mixed in 5% sucrose pipetted on a Whatman paper. For isolation of M1-Ub chains, 20–40 adult flies per genotype were fed with 20-mM paraquat for 2 h. For survival assays, more than 10 flies per fly genotype were fed with 10-mM paraquat and counted at indicated time points. For mechanical stress, 3rd instar larvae were subjected to mechanical stress by vortexing for 10 s at 3200 r.p.m. For isolation of M1-Ub chains, 15 larvae were frozen at indicated time points after vortexing.

Cell culture and stress treatments of human Caco2 cells

Human epithelial colon adenocarcinoma (Caco2) cells (ACC 169, DSMZ, Leipzig, Germany) were grown in DMEM/F-12 (Gibco, ThermoFisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% heat-inactivated fetal bovine serum (Biowest, Nuaille, France), 100-IU·mL⁻¹ penicillin and 100-μg·mL⁻¹ streptomycin (Sigma-Aldrich, Missouri, USA) at 37 °C humidified atmosphere with 5% CO₂ until use. For hypoxia and oxidative

stress experiments, cells were plated at 1×10^6 cells on 10-cm-diameter dishes with serum-free DMEM/F-12 supplemented with 0.1% bovine serum albumin (BSA, Sigma-Aldrich) and 100-IU·mL⁻¹ penicillin and 100-μg·mL⁻¹ streptomycin. Hypoxic conditions were achieved by exposing 80–90% confluent cells to 5% O₂, 5% CO₂ and 90% pure N₂ (AGA, Finland) by placing the plates in a hypoxic chamber (Galaxy 14S; Eppendorf, Hamburg, Germany) for 2 h at 37 °C. Oxidative stress was induced by treating cells with 1-μM paraquat (Sigma-Aldrich) for 24 h. To inhibit M1-ubiquitination, Caco2 cells were treated with 10-μM HOIPIN-1 inhibitor (Axon Medchem BV, Groningen, Netherlands) for 3 h. For the shear stress experiments, Caco2 cells were seeded in 6-well plates. The flow setup for inducing shear stress was prepared as previously described [72] with minor alterations. Briefly, when 100% confluency of Caco2 cells was reached, these were subjected to shear stress by placing the plates on an orbital shaker, 100 r.p.m., for maximum 2 h at 37 °C. Only the outer part of the well was collected to ensure a flow with less oscillatory shear index. The exclusion of the inner part was based on measurements done previously [72]. After exposure, cells were lysed for M1-TUBE pulldown assay.

Plasmids and antibodies

M1-Ub conjugates were purified using a recombinant protein containing the UBAN region of NEMO (residues 257–346) fused to GST and His (referred to as M1-TUBE), and pan-Ub chains were purified using a protein consisting of four UBA domains in tandem fused to GST and His (referred to as pan-TUBE) [73,74], kindly provided by Dr. Mads Gyrd-Hansen. The following antibodies were used: α-M1 (clone IE3, #MABS199, Millipore, Burlington, Massachusetts, USA, or clone 1F11/3F5/Y102L, Genentech, South San Francisco, California, USA), α-K63 (clone Apu3, #05-1308, Millipore), α-pan-Ub (P4D1, #sc8017, Santa Cruz Biotechnology, Dallas, Texas, USA) and α-Actin (C-11, #sc-1615, Santa Cruz Biotechnology).

Purification of His/GST-tagged M1-TUBE and pan-TUBE fusion protein

His/GST-tagged M1-TUBE and pan-TUBE expression was induced in *E. coli* BL21 (Novagen/Merck Millipore, Burlington, Massachusetts, USA) by the addition of 0.2-mM IPTG to an overnight culture of bacteria in LB medium at 18 °C. Bacteria were lysed by sonication in lysis buffer containing 50-mM Tris-HCl (pH 7.4), 150-mM NaCl, 2-mM β-mercaptoethanol (Sigma-Aldrich), 40-mM imidazole, PierceTM Protease inhibitor (ThermoFisher Scientific) and 3-mg lysozyme. Clarified lysate was added to a column with Ni-NTA agarose (QIAGEN, Hilden, Germany) and washed with cold lysis buffer. Bound protein was eluted with cold lysis buffer supplemented with 300-mM imidazole

(pH 7.4) and dialysed into PBS (Medicago, Uppsala, Sweden) containing 10% glycerol and 1-mM DTT (Sigma-Aldrich) using a 10 K Slide-A-Lyzer dialysis cassette (ThermoFisher Scientific).

Purification of ubiquitin conjugates from flies and cells

Flies and cells were lysed using a buffer containing 50-mM Tris pH 7.5, 150-mM NaCl, 1% TritonX, 1-mM EDTA, 10% glycerol supplemented with 1-mM DTT, 5-mM NEM, 1× Pierce™ Protease and Phosphatase Inhibitor (ThermoFisher Scientific), 5-mM chloroacetamide and 1% SDS. Lysates were sonicated, diluted to 0.1% SDS and cleared before incubation with Glutathione Sepharose™ 4B (GE Healthcare) and M1-TUBE (10–20 mg·mL⁻¹) or pan-TUBE (5 mg·mL⁻¹) for minimum of 2 h or o/n under rotation at 4 °C. The beads were washed 3 times with ice-cold wash buffer containing 10-mM Tris pH 7.5, 150-mM NaCl, 0.1% TritonX, 5% glycerol and eluted using Laemmli sample buffer.

Quantitative RT-PCR (qPCR)

Flies were homogenised using QIAshredder (QIAGEN); total RNA was extracted with RNeasy Mini Kit (QIAGEN); and cDNA was synthesised with SensiFast cDNA synthesis kit (Bioline, London, UK) according to the manufacturers' protocols. qPCR was performed using SensiFast SYBR Hi-ROX qPCR kit (BioLine). *rp49* was used as a housekeeping gene for $\Delta\Delta C_t$ calculations. The following gene-specific primers were used to amplify cDNA: *dipteracin* (5'-ACCGCAGTACCCACTCAATC-3', 5'-ACTTTCCAGCTCGGTTCTGA-3'), *drosomycin* (5'-CGTGAGAACCCTTTTCCAATATGATG-3', 5'-TCCCAGGACCACCAGCAT-3'), *dredd* (5'-ACATTGCCCTTCTCCACAGA-3', 5'-CATGGCGATGCTGTTGGATG-3'), *ldh* (5'-CAGTTCGCAACGAACGCGCA, 5'-CAGCTCGCCCTGCAGCTTG-3'), *rbr* (5'-CGGAACCCATGCAGATC AAG, 5'-CGCAGTCCGTGATCAAAG) and *rp49* (5'-GACGCTTCAAGGGACAGTATCTG-3', 5'-AAACGCGGTTCTGCATGAG-3').

Fluorometric measurement of caspase-3/7

Caco2 cells were seeded in 96-well plates at 1×10^4 cells·well⁻¹. When reaching 70–80% confluency, cells were exposed to hypoxia (3% O₂, 5% CO₂ and 92% N₂) for 24 h or oxidative stress (1-μM paraquat) for 24 h. To inhibit M1-ubiquitination, Caco2 cells were additionally treated with 10-μM HOIPIN-1 inhibitor for 24 h. Caspase-3/7 activity was analysed using Apo-ONE® Homogenous Caspase-3/7 Assay (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. Fluorescence was measured at 499/521 with the plate reader HIDEX sense (HIDEX, Turku, Finland).

X-gal staining of *Drosophila* trachea

Trachea from 3rd instar fly larvae was dissected in PBS and fixed for 15 min with PBS containing 0.4% glutaraldehyde (Sigma-Aldrich) and 1-mM MgCl₂ (Sigma-Aldrich). The samples were washed with PBS and incubated with a freshly prepared staining solution containing 5-mg·mL⁻¹ X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 5-mM potassium ferrocyanide trihydrate (Sigma-Aldrich), 5-mM potassium ferrocyanide crystalline (Sigma-Aldrich) and 2-mM MgCl₂ in PBS at 37 °C. After washing with PBS, the samples were mounted using Mowiol (Sigma) and imaged with brightfield microscopy (Leica, Wetzlar, Germany).

Statistical analysis

Results from survival assays were analysed by two-way analysis of variance (ANOVA) with the Tukey's *post hoc* test for 95% confidence intervals. Results from qPCR were analysed by the two-tailed Student's t-test on the $-\Delta C_t$ value, and graphs depict relative fold induction of the target gene compared to a normalised control sample ($\Delta\Delta C_t$). In comparison with normalised control values, the Mann–Whitney *U* test was applied. Statistical analyses were performed using GRAPHPAD Prism version 9.1.0 for Windows (GRAPHPAD Software, San Diego, California, USA). In figures, ns stands for nonsignificant, $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Error bars in figures specify SEM from the indicated number of independent experimental repeats. Experiments were performed indicated number of times ($n \geq 3$), and statistics were calculated for each individual experiment.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

ALA designed and executed most of the experiments, data analysis and writing of the manuscript. CK planned and performed caspase assays. NT planned and performed the mechanical stress experiments in flies and human cells. GMC planned and performed experiments with human cells and contributed to data analysis and writing of the manuscript. JK and PK planned and assembled the MiniHypoxia platform. MB contributed to the design of the experiments. AM contributed to the design of the experiments, writing and data analysis of this manuscript.

Peer review

The peer review history for this article is available at <https://publons.com/publon/10.1111/febs.16425>.

Data availability statement

All data are contained within this paper.

References

- Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem.* 1998;**67**:425–79.
- Dikic I, Wakatsuki S, Walters KJ. Ubiquitin-binding domains from structures to functions. *Nat Rev Mol Cell Biol.* 2009;**10**:659–71.
- Swatek KN, Komander D. Ubiquitin modifications. *Cell Res.* 2016;**26**:399–422.
- Komander D, Rape M. The Ubiquitin Code. *Annu Rev Biochem.* 2012;**81**:203–29.
- Kirisako T, Kamei K, Murata S, Kato M, Fukumoto H, Kanie M, et al. A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J.* 2006;**25**:4877–87.
- Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, et al. Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature.* 2011;**471**:591–6.
- Ikeda F, Deribe YL, Skånland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, et al. SHARPIN forms a linear ubiquitin ligase complex regulating NF- κ B activity and apoptosis. *Nature.* 2011;**471**:637–41.
- Tokunaga F, Nakagawa T, Nakahara M, Saeki Y, Taniguchi M, Sakata SI, et al. SHARPIN is a component of the NF- κ B-activating linear ubiquitin chain assembly complex. *Nature.* 2011;**471**:633–6.
- Stieglitz B, Morris-Davies AC, Koliopoulos MG, Christodoulou E, Rittinger K. LUBAC synthesizes linear ubiquitin chains via a thioester intermediate. *EMBO Rep.* 2012;**13**:840–6.
- Stieglitz B, Rana RR, Koliopoulos MG, Morris-Davies AC, Schaeffer V, Christodoulou E, et al. Structural basis for ligase-specific conjugation of linear ubiquitin chains by HOIP. *Nature.* 2013;**503**:422–6.
- Smit JJ, Monteferrario D, Noordermeer SM, Van Dijk WJ, Van Der Reijden BA, Sixma TK. The E3 ligase HOIP specifies linear ubiquitin chain assembly through its RING-IBR-RING domain and the unique LDD extension. *EMBO J.* 2012;**31**:3833–44.
- Tokunaga F, Sakata S, Saeki Y, Satomi Y, Kirisako T, Kamei K, et al. Involvement of linear polyubiquitylation of NEMO in NF- κ B activation. *Nat Cell Biol.* 2009;**11**:123–32.
- Oikawa D, Sato Y, Ohtake F, Komakura K, Hanada K, Sugawara K, et al. Fukai S & Tokunaga F (2020) Molecular bases for HOIPINs-mediated inhibition of LUBAC and innate immune responses. *Commun Biol.* 2020;**31**:1–17.
- Peltzer N, Rieser E, Rodriguez TA, Correspondence HW. HOIP deficiency causes embryonic lethality by aberrant TNFR1-mediated endothelial cell death. *Cell Rep.* 2014;**9**:153–65.
- Shimizu Y, Taraborrelli L, Walczak H. Linear ubiquitination in immunity. *Immunol Rev.* 2015;**266**:190–207.
- Jahan AS, Elbæk CR, Damgaard RB. Met1-linked ubiquitin signalling in health and disease: inflammation, immunity, cancer, and beyond. *Cell Death Differ.* 2021;**28**:473–92.
- Fiil BK, Gyrd-Hansen M. Met1-linked ubiquitination in immune signalling. *FEBS J.* 2014;**281**:4337–50.
- Emmerich CH, Ordureau A, Strickson S, Arthur JSC, Pedrioli PGA, Komander D, et al. Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains. *Proc Natl Acad Sci USA.* 2013;**110**:15247–52.
- Asaoka T, Almagro J, Ehrhardt C, Tsai I, Schleiffer A, Deszcz L, et al. Linear ubiquitination by LUBEL has a role in Drosophila heat stress response. *EMBO Rep.* 2016;**17**:1624–40.
- Aalto AL, Mohan AK, Schwintzer L, Kupka S, Kietz C, Walczak H, et al. M1-linked ubiquitination by LUBEL is required for inflammatory responses to oral infection in Drosophila. *Cell Death Differ.* 2019;**26**:860–76.
- Ferrandon D, Imler JL, Hetru C, Hoffmann JA. The Drosophila systemic immune response: Sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol.* 2007;**7**:862–74.
- Charroux B, Royet J. Drosophila immune response: From systemic antimicrobial peptide production in fat

- body cells to local defense in the intestinal tract. *Fly (Austin)*. 2010;**4**:40–7.
- 23 Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. *Annu Rev Immunol*. 2007;**25**:697–743.
 - 24 Gottar M, Gobert V, Michel T, Belvin M, Duyk G, Hoffmann JA, et al. The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. *Nature*. 2002;**416**:640–4.
 - 25 Paquette N, Broemer M, Aggarwal K, Chen L, Husson M, Ertürk-Hasdemir D, et al. Caspase-mediated cleavage, IAP binding, and ubiquitination: linking three mechanisms crucial for *Drosophila* NF-kappaB signaling. *Mol Cell*. 2010;**37**:172–82.
 - 26 Choe KM, Lee H, Anderson KV. *Drosophila* peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor. *Proc Natl Acad Sci USA*. 2005;**102**:1122–6.
 - 27 Meinander A, Runchel C, Tenen T, Chen L, Kim C-HH, Ribeiro PS, et al. Ubiquitylation of the initiator caspase DREDD is required for innate immune signalling. *EMBO J*. 2012;**31**:2770–83.
 - 28 Silverman N, Zhou R, Erlich RL, Hunter M, Bernstein E, Schneider D, et al. Immune activation of NF-kappaB and JNK requires *Drosophila* TAK1. *J Biol Chem*. 2003;**278**:48928–34.
 - 29 Kleino A, Valanne S, Ulvila J, Kallio J, Myllymäki H, Enwald H, et al. Inhibitor of apoptosis 2 and TAK1-binding protein are components of the *Drosophila* Imd pathway. *EMBO J*. 2005;**24**:3423–34.
 - 30 Zhuang ZH, Sun L, Kong L, Hu JH, Yu MC, Reinach P, et al. *Drosophila* TAB2 is required for the immune activation of JNK and NF-kappaB. *Cell Signal*. 2006;**18**:964–70.
 - 31 Chen L, Paquette N, Mamoor S, Rus F, Nandy A, Leszyk J, et al. Innate immune signaling in *Drosophila* is regulated by transforming growth factor β (TGF β)-activated kinase (Tak1)-triggered ubiquitin editing. *J Biol Chem*. 2017;**292**:8738.
 - 32 Ertürk-Hasdemir D, Broemer M, Leulier F, Lane WS, Paquette N, Hwang D, et al. Two roles for the *Drosophila* IKK complex in the activation of Relish and the induction of antimicrobial peptide genes. *Proc Natl Acad Sci USA*. 2009;**106**:9779–84.
 - 33 Silverman N, Zhou R, Stöven S, Pandey N, Hultmark D, Maniatis T. A *Drosophila* I κ B kinase complex required for relish cleavage and antibacterial immunity. *Genes Dev*. 2000;**14**:2461–71.
 - 34 Stöven S, Silverman N, Junell A, Hedengren-Olcott M, Ertürk D, Engström Y, et al. Caspase-mediated processing of the *drosophila* NF- κ B factor relish. *Proc Natl Acad Sci USA*. 2003;**100**:5991–6.
 - 35 Rutschmann S, Jung AC, Zhou R, Silverman N, Hoffmann JA, Ferrandon D. Role of *Drosophila* IKK γ in a Toll-independent antibacterial immune response. *Nat Immunol*. 2000;**1**:342–7.
 - 36 Lu Y, Wu LP, Anderson KV. The antibacterial arm of the *Drosophila* innate immune response requires an I κ B kinase. *Genes Dev*. 2001;**15**:104–10.
 - 37 Valanne S, Wang J-H, Rämet M. The *Drosophila* Toll Signaling Pathway. *J Immunol*. 2011;**186**:649–56.
 - 38 Rider P, Voronov E, Dinarello CA, Apte RN, Cohen I. Alarmins: Feel the Stress. *J Immunol*. 2017;**198**:1395–402.
 - 39 Asri RM, Salim E, Nainu F, Hori A, Kuraishi T. Sterile induction of innate immunity in *Drosophila melanogaster*. *Front Biosci - Landmark*. 2019;**24**:1390–400.
 - 40 Rubartelli A, Sitia R. Stress as an intercellular signal: the emergence of stress-associated molecular patterns (SAMP). *Antioxid Redox Signal*. 2009;**11**:2621–9.
 - 41 Metsälä O, Kreutzer J, Högel H, Miikkulainen P, Kallio P, Jaakkola PM. Transportable system enabling multiple irradiation studies under simultaneous hypoxia *in vitro*. *Radiat Oncol*. 2018;**13**:220.
 - 42 Nambu JR, Chen W, Hu S, Crews ST. The *Drosophila melanogaster* similar bHLH-PAS gene encodes a protein related to human hypoxia-inducible factor 1 α and *Drosophila* single-minded. *Gene*. 1996;**172**:249–54.
 - 43 Lavista-Llanos S, Centanin L, Irisarri M, Russo DM, Gleadle JM, Bocca SN, et al. Control of the hypoxic response in *Drosophila melanogaster* by the basic helix-loop-helix PAS protein similar. *Mol Cell Biol*. 2002;**22**:6842–53.
 - 44 Dekanty A, Romero NM, Bertolin AP, Thomas MG, Leishman CC, Perez-Perri JJ, et al. *Drosophila* genome-wide RNAi screen identifies multiple regulators of HIF-dependent transcription in hypoxia. *PLoS Genet*. 2010;**6**:e1000994.
 - 45 Liu G, Roy J, Johnson EA. Identification and function of hypoxia-response genes in *Drosophila melanogaster*. *Physiol Genomics*. 2006;**25**.
 - 46 Arquier N, Vigne P, Duplan E, Hsu T, Therond PP, Frelin C, et al. Analysis of the hypoxia-sensing pathway in *Drosophila melanogaster*. *Biochem J*. 2006;**393**:471–80.
 - 47 Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science (80-)*. 2001;**294**:1337–40.
 - 48 Bandarra D, Biddlestone J, Mudie S, Muller HA, Rocha S. Hypoxia activates IKK-NF- κ B and the immune response in *Drosophila melanogaster*. *Biosci Rep*. 2014;**34**:429–40.
 - 49 Boutros M, Agaisse H, Perrimon N. Sequential activation of signaling pathways during innate immune responses in *Drosophila*. *Dev Cell*. 2002;**3**:711–22.
 - 50 Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B. *Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation. *Cell Host Microbe*. 2009;**5**:200–11.

- 51 Tzou P, Ohresser S, Ferrandon D, Capovilla M, Reichhart J-M, Lemaitre B, et al. Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* Surface Epithelia. *Immunity*. 2000;**13**:737–48.
- 52 Davis MM, Engström Y. Immune response in the barrier epithelia: Lessons from the fruit fly *drosophila melanogaster*. *J Innate Immun*. 2012;**4**:273–83.
- 53 Ferrandon D, Jung AC, Crique MC, Lemaitre B, Uttenweiler-Joseph S, Michaut L, et al. A drosomycin-GFP reporter transgene reveals a local immune response in *Drosophila* that is not dependent on the Toll pathway. *EMBO J*. 1998;**17**:1217–27.
- 54 Akhouayri I, Turc C, Royet J, Charroux B. Toll-8/Tollo negatively regulates antimicrobial response in the *Drosophila* respiratory epithelium. *PLoS Pathog*. 2011;**7**:e1002319.
- 55 Hedengren M, Åsling B, Dushay MS, Ando I, Ekengren S, Wihlborg M, et al. Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. *Mol Cell*. 1999;**4**:827–37.
- 56 Hosamani R, Muralidhara N. Acute exposure of *drosophila melanogaster* to paraquat causes oxidative stress and mitochondrial dysfunction. *Arch Insect Biochem Physiol*. 2013;**83**:25–40.
- 57 Bus JS, Gibson JE. Paraquat: model for oxidant-initiated toxicity. *Environ Health Perspect*. 1984;**55**:37–46.
- 58 Kenmoku H, Hori A, Kuraishi T, Kurata S. A novel mode of induction of the humoral innate immune response in *Drosophila* larvae. *DMM Dis Model Mech*. 2017;**10**:271–81.
- 59 Culver C, Sundqvist A, Mudie S, Melvin A, Xirodimas D, Rocha S. Mechanism of hypoxia-induced NF- κ B. *Mol Cell Biol*. 2010;**30**:4901–21.
- 60 Geuking P, Narasimamurthy R, Lemaitre B, Basler K, Leulier F. A Non-Redundant Role for *Drosophila* Mkk4 and Hemipterous/Mkk7 in TAK1-Mediated Activation of JNK. *PLoS One*. 2009;**4**:e7709.
- 61 Palmerini V, Monzani S, Laurichesse Q, Loudhaief R, Mari S, Cecatiello V, et al. Andersen DS & Mapelli M (2021) *Drosophila* TNFRs Grindelwald and Wengen bind Eiger with different affinities and promote distinct cellular functions. *Nat Commun*. 2021;**12**:1–12.
- 62 Takehana A, Yano T, Mita S, Kotani A, Oshima Y, Kurata S. Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity. *EMBO J*. 2004;**23**:4690.
- 63 Bosco-Drayon V, Poidevin M, Boneca IG, Narbonne-Reveau K, Royet J, Charroux B. Peptidoglycan sensing by the receptor PGRP-LE in the *drosophila* gut induces immune responses to infectious bacteria and tolerance to microbiota. *Cell Host Microbe*. 2012;**12**:153–65.
- 64 Kaneko T, Yano T, Aggarwal K, Lim JH, Ueda K, Oshima Y, et al. Kurata S & Silverman N (2006) PGRP-LC and PGRP-LE have essential yet distinct functions in the *drosophila* immune response to monomeric DAP-type peptidoglycan. *Nat Immunol*. 2006;**7**:715–23.
- 65 van Well EM, Bader V, Patra M, Sánchez-Vicente A, Meschede J, Furthmann N, et al. A protein quality control pathway regulated by linear ubiquitination. *EMBO J*. 2019;**38**:55.
- 66 Schaeffer V, Akutsu M, Olma MH, Gomes LC, Kawasaki M, Dikic I. Binding of OTULIN to the PUB Domain of HOIP Controls NF- κ B Signaling. *Mol Cell*. 2014;**54**:349–61.
- 67 Takiuchi T, Nakagawa T, Tamiya H, Fujita H, Sasaki Y, Saeki Y, et al. Suppression of LUBAC-mediated linear ubiquitination by a specific interaction between LUBAC and the deubiquitinases CYLD and OTULIN. *Genes Cells*. 2014;**19**:254–72.
- 68 Noad J, Von Der Malsburg A, Pathe C, Michel MA, Komander D, Randow F. LUBAC-synthesized linear ubiquitin chains restrict cytosol-invading bacteria by activating autophagy and NF- κ B. *Nat Microbiol*. 2017;**2**:1–10.
- 69 Leulier F, Rodriguez A, Khush RS, Abrams JM, Lemaitre B. The *Drosophila* caspase Dredd is required to resist Gram-negative bacterial infection. *EMBO Rep*. 2000;**1**:353–8.
- 70 Kietz C, Pollari V, Meinander A. Generating germ-free *drosophila* to study gut-microbe interactions: protocol to rear *drosophila* under axenic conditions. *Curr Protoc Toxicol*. 2018;**77**:e52.
- 71 Välimäki H, Verho J, Kreutzer J, Kattiparambil Rajan D, Ryyänen T, Pekkanen-Mattila M, et al. Fluorimetric oxygen sensor with an efficient optical read-out for *in vitro* cell models. *Sensors Actuators B Chem*. 2017;**249**:738–46.
- 72 Driessen R, Zhao F, Hofmann S, Bouten C, Sahlgren C, Stassen O. Computational characterization of the dish-in-a-dish, a high yield culture platform for endothelial shear stress studies on the orbital shaker. *Micromachines*. 2020;**11**:552.
- 73 Fiil BK, Damgaard RB, Wagner SA, Keusekotten K, Fritsch M, Bekker-Jensen S, et al. OTULIN Restricts Met1-linked ubiquitination to control innate immune signaling. *Mol Cell*. 2013;**50**:818–30.
- 74 Keusekotten K, Elliott PR, Glockner L, Fiil BK, Damgaard RB, Kulathu Y, et al. OTULIN antagonizes LUBAC signaling by specifically hydrolyzing met1-linked polyubiquitin. *Cell*. 2013;**153**:1312.