M1-linked ubiquitination facilitates NF-κB 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-κB (NF-κB) 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-κB 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 IκB kinase γ (IKKγ) 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-κB 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, IκB kinase; Imd, immune deficiency; LDD, linear ubiquitin chain-determining domain; LUBAC, linear ubiquitin chain assembly complex; LUBEL, linear ubiquitin E3 ligase; NF-κB, 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 RBCK1 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 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 the 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-κB (NF-κB) 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-κB activation and for mounting a proper immune response in the intestine [20]. Infection in flies leads to robust activation of NF-κB 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-κB-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 IkB kinase (IKK) complex, which consists of the catalytic subunit Ird5 (IKKβ) and the regulatory subunit Kenny (IKKγ) [25,28-31]. For transcriptional activation, the NF-κB 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-κB factors Dorsal and Dorsal-related immunity factor (Dif) [23,37].

In addition to being activated upon recognition of pathogen-associated molecular patterns (PAMPS), NF-κB 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-κB 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-κB.

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 LUBELΔRRB 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 LUBELΔRRB 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 LUBELΔRRB 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 simaΔG07907 mutant flies were unable to induce ldh expression (Fig. 2A). Interestingly, ldh was induced equally well in LUBELΔRRB (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 LUBELΔRRB 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 LUBELΔRRB 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, diptericin [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 diptericin 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 diptericin expression (Fig. 3E) and the sensitivity to hypoxia (Fig. 3F) of LUBEL-RNAi flies. Likewise, LUBELΔRRB 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
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 diptericin 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 contrary to the intestine and the fat body, it has been shown that *drosomycin*, but not *diptericin*, 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* 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 *diptericin*, which is a specific Relish target gene in the fat body [51,55], was induced in control flies during hypoxia, no hypoxia-induced *diptericin* expression could be detected when silencing LUBEL in the fat body (Fig. 5D). On the other hand, *drosomycin* expression, which in contrary 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...
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 LUBELΔRBR and LUBEL-RNAi flies fed with paraquat for two days. While more than half of control flies survived paraquat feeding, most LUBELΔRBR 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 LUBELΔRBR 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 LUBELΔRBR 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-κB 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-κB activation was studied by analysing the expression of diptericin 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 LUBELΔRBR flies, rescue UAS-LUBEL-RNAi;daGal4/UAS-Dredd and LUBELΔRBR;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.
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(A) % survival over time in 5% O2

(B) % survival over time in 5% O2 for different genotypes

(C) Relative Ratio of dip1/epc1/p49 in control vs 5% O2

(D) Relative Ratio of dip1/epc1/p49 in control vs 5% O2 for different genotypes

(E) Blot analysis showing M1-linked Ub chains

(F) Blot analysis showing M1-linked Ub chains
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-κB 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-κB 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-κB 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-κB 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-κB 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

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**Fig. 4.** Intracellular mediators of the Imd pathway are required for NF-κB activation in response to hypoxia, while the receptor PGRP is not. (A) Adult control flies daGal4, mutant LUBELΔRR and PGRPΔS flies and double-mutant LUBELΔRR,PGRPS flies 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. (B) Adult wild-type CantonS flies and mutant fly lines of the Imd pathway mediators Tak1Δ, Diap2Δ, Key1ΔWT and DreddΔ 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 CantonS flies and mutant fly lines of the Imd pathway mediators PGRPS, Tak1Δ, Key1ΔWT and DreddΔ were subjected to low oxygen conditions (5% O₂) for 24 h. Relish and HIF activation was studied by analysing the expression of dipteriscin and ldh, respectively, with qPCR. Error bars indicate SEM from more than 4 independent experimental repeats. (E, F) Adult wild-type CantonS flies and mutant Tak1Δ and Diap2Δ 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.
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; LUBELΔRBR 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 LUBELΔRBR; 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 diptericin 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 LUBELΔRBR (B-D), transgenic UAS-LUBEL-RNAi;daGal4 (B, C) and UAS-Duox-RNAi;daGal4 (B), overexpressing daGal4/UAS-Dredd (C), rescue LUBELΔRBR, daGal4/UAS-Dredd (C) and UAS-LUBEL-RNAi;daGal4/UAS-Dredd (C), mutant PGRPΔ5 (D) and double-mutant LUBELΔRBR;PGRPΔ5 (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.
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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). CantonS wild-type flies, the ubiquitous daGal4 driver line, the fat body-specific c564Gal4 driver line, the Dipt-LacZ reporter line, balancer lines, and keyk, dredd22, diap22 mutant fly lines and UAS-Dredd flies were kindly provided by Prof. Pascal Meier and Dr. François Leulier [69]. The Drosophila fly strains w; Mi[ET1]LUBELOBO0917 (#22725, referred to as LUBELOBR), fatiga16255 (#11561), simet846097 (#14640), PGRP-LC:α5 (#36323, referred to as PGRPα5), tak12 (#26272), UAS-Duox-TRIP (#33975, referred to as DUOX-RNAi) and Dsr-lacZ (#55708) were obtained from Bloomington stock centre. UAS-LUBELO-RNAi (P(GD7269)18055, #18055, referred to as LUBELO-RNAi) were obtained from Vienna Drosophila resource centre. The efficiency of UAS-LUBELO-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 MiniHypox platform (Faculty of Medicine and Health Technology, Tampere University).
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, 15 larvae were frozen at indicated time points after vortexting.

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, Nuaillé, 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 x 10⁶ 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, Pierce™ 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 Laemmi 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 ΔΔCt calculations. The following gene-specific primers were used to amplify cDNA: *diptericin* (5'-ACCGCATCCACCTCAATC-3', 5'-AACCACGTCCGTTCTGGA-3'), *drosomycin* (5'-CGTGAGAACCATGCAGATCATG-3', 5'-TCCCAGGACCACCAAGCAT-3'), *dredd* (5'-ACATTGCCCTTCTCCACAGA-3', 5'-GACGGTCTGCCACCGGACCA-3'), *C. elegans* heat shock protein 18 (5'-ACCCCCCCTCTCTCTCAAGA-3', 5'-CGGAGATTCGTTGATGCT-3'), *ldh* (5'-CAGTTCGCAACGAACGCAGA, 5'-CAGCTCGCTGCAGCTCTTG-3'), *rbr* (5'-CAGGAACCCATTGAGATCCAG AAG, 5'-CGAGCTCCGGTCCAGATCAAAAG) and *rp49* (5'-GACGCTTCAGGGGACAGTATCTG-3', 5'-AACCGCGGTCTGACATGAG-3').

**Fluorometric measurement of caspase-3/7**

Caco2 cells were seeded in 96-well plates at 1 × 10⁴ 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 495/521 with the plate reader HIDEK 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 ΔΔCt value, and graphs depict relative fold induction of the target gene compared to a normalised control sample (ΔΔCt). 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** < 0.01, ***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* ≥ 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 data analysis and writing of the manuscript. JK and PK planned and assembled the MiniHypoxy platform. MB contributed to the design of the experiments, writing and data analysis of this manuscript.

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All data are contained within this paper.

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