Supplementary Material

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Urm1 – An essential regulator of JNK signaling and oxidative stress in *Drosophila melanogaster*.

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Supplementary Figure legends

Supplementary Figure 1. Urm1 and Uba4 is localized in the cytoplasm of larval imaginal disc cells.

a. Coomassie blue stained gel depicting the input control for Fig. 1D, which demonstrates a localization of Urm1 in the cytoplasmic fraction of *Drosophila* embryonic S2 cells. **b.** 3x-Flag-tagged Urm1 and 6x-Myc-tagged Uba4 was expressed in the dorsal part of third instar larval wing imaginal discs using the MS1096-GAL4 driver (UAS/GAL4 system). Immunohistochemical staining using commercial anti-Flag and anti-Myc (9E10) antibodies reveals a cytoplasmic localization of Urm1 as well as its E1 activating enzyme Uba4. Scale bar = 10 μm. **c.** Urm1 appears to be expressed at comparable levels throughout

embryogenesis. Immunoblotting of embryonic protein lysates of the age spans indicated in the figure. **d.** Endogenous Urm1 protein is strongly expressed in third instar imaginal discs, where it primarily is localized to the cytoplasmic compartment. FLP/FRT-mediated clonal expression of an RNAi transgene targeting Urm1 (marked by GFP), indicates the specificity of the Urm1 antibody. Scale bar = 10 μm. **e.** Quantitative PCR on cDNA extracted from the w^{1118} control strain at the developmental stages indicated. The levels of Urm1 mRNA is displayed as a relative amount equalized to the ribosomal protein RpL32. The result shown represents an average of two biological replicates.

Supplementary Figure 2. *Drosophila* Uba4 shares high similarities with both its mammalian and yeast counterparts.

Sequence alignment of *Drosophila Uba4 (CG13090)* with its homologous counterparts in *S. cerevisiae, C. elegans M. musculus* and *H. sapiens,* clearly pinpointing the identity of *CG13090* as the fly orthologue of the Urm1 E1 activating enzyme Uba4/MOCS3.

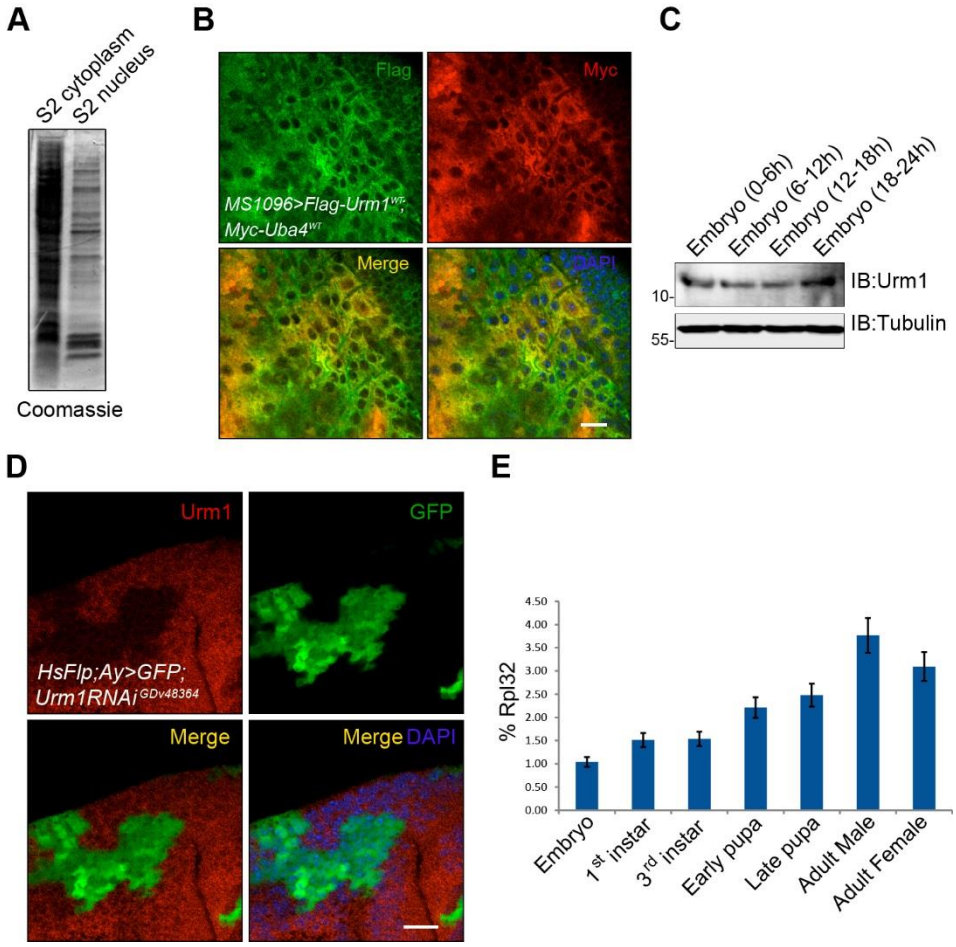
Supplementary Figure 3. Urm1 interacts genetically with Uba4.

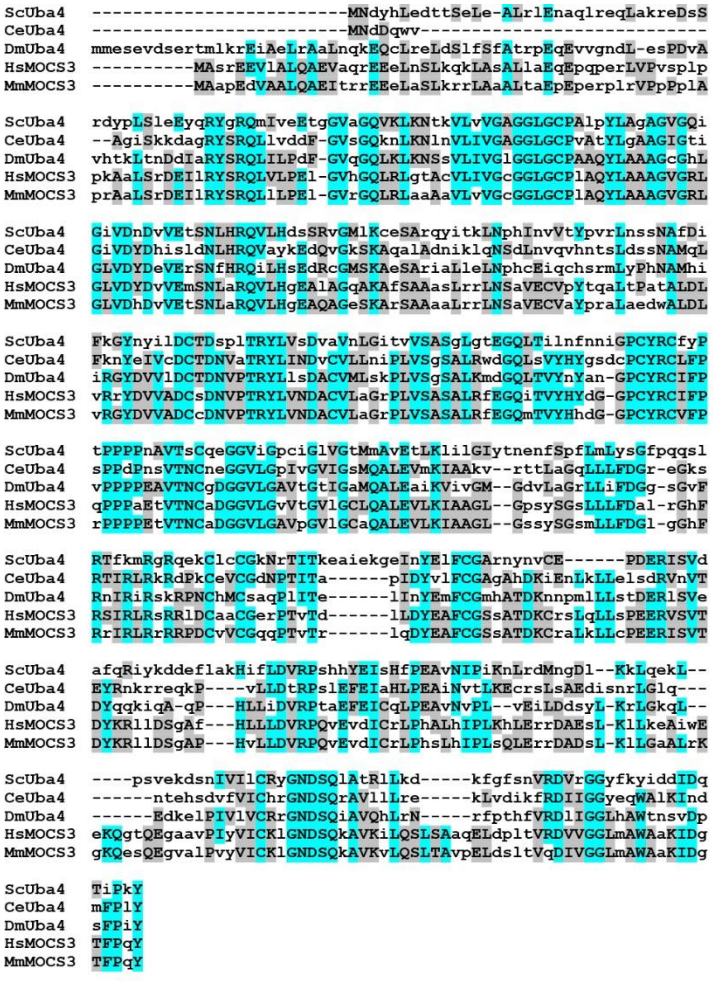
a. Survival into adulthood was monitored in homozygous $Urm1^{n123}$ mutants, as compared with $Urm1^{n123}$ flies in which one copy of the Uba4 genomic locus has been removed using two separate fly strains carrying deficiencies uncovering *Uba4* (*Df(2L)Exel7038/CyO* and *Df(2L)BSC215/CyO*, respectively). Removal of one copy of Uba4 reduces the survival of $Urm1^{n123}$ mutant flies from 10,4% to 6.9% when using *Df(2L)BSC215/CyO* and 4.4% for *Df(2L)Exel7038/CyO*. **b.** Uba4 is not affected at the protein level by loss of Urm1 (total fly lysates from homozygous zygotic $Urm1^{n123}$ flies, as well as $Urm1^{rv164}$ and w^{1118} controls). **c.** Misexpression of Urm1 and Uba4 cause similar wing vein defects, a phenotype that is potentiated in flies co-expressing Urm1 and Uba4. MS1096-GAL4 was used to trigger

excessive expression of Urm1 and Uba4, respectively or simultaneously, in developing *Drosophila* wings. Misexpression of Urm1 and Uba4 both cause a specific loss of the anterior cross-vein (acv), in addition to an appearance of excessive wing vein material in other places. The quantification to the left depicts the percentage of flies lacking the acv, and the pictures to the right are representative photographic images of, the different genotypes indicated. Control MS1096> flies represents the progeny of MS1096-GAL4 flies crossed to w^{1118} controls. Fly crosses were incubated at 25°C for moderate expression or at 29°C for strong expression. *n* > *38* for all genotypes tested. Scale bar = 500 μm.

Supplementary Figure 4. Flies with reduced levels of Urm1 display an increased resistance when exposed to oxidative stress induced by H_2O_2.

a. Flies in which the level of Urm1 has been strongly reduced by ubiquitous expression of an Urm1-RNAi transgenic construct, show an increased survival when exposed to oxidative stress induced by 2% H_2O_2 (equivalent to ~0.6 M). Flies with reduced Urm1 levels displayed a median survival of 112 hours on 2% H_2O_2, as compared with 74 hours in the *Act5C>*w^{1118} controls. **b.** Prx5 is covalently conjugated by Urm1 in response to oxidative stress induced by 10 mM paraquat, as indicated by a size shift of Prx5 from ~17 kDa to ~30 kDa, which is in agreement with the addition of one Flag-tagged Urm1 moiety. The immunoblot to the left displays the Flag- IP showing urmylated Prx5, whereas the right immunoblot depicts non-conjugated Prx5.


A
S2 cytoplasm
S2 nucleus
Coomassie
B
Flag
Myc
MS1096>Flag-Urm1WT;
Myc-Uba4WT
Merge
DAPI
C
Embryo (0-6h)
Embryo (6-12h)
Embryo (12-18h)
Embryo (18-24h)
10-
IB:Urm1
55-
IB:Tubulin
D
Urm1
GFP
HsFlp;Ay>GFP;
Urm1RNAiGDv48364
Merge
Merge DAPI
E
% Rpl32
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Embryo
1st instar
3rd instar
Early pupa
Late pupa
Adult Male
Adult Female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CeUba4 ----------MNdDqwv----------
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HsMOCS3 ----------MAsrEEVlALQAEVaqrEEeLnSLkqkLAsALlaEqEpqperLVPvsplp
MmMOCS3 ----------MAapEdVAALQAEItrrEEeLaSLkrrLAaALtaEpEperplrVPpPplA
ScUba4 rdypLSleEyqRYgRQmIveEtgGVaGQVKLKNtkVLvVGAGGLGCPAlpYLAgAGVGQi
CeUba4 --AgiSkkdagRYSRQLlvddF-GVsGQknLKNlnVLIVGAGGLGCPvAtYLgAAGIGti
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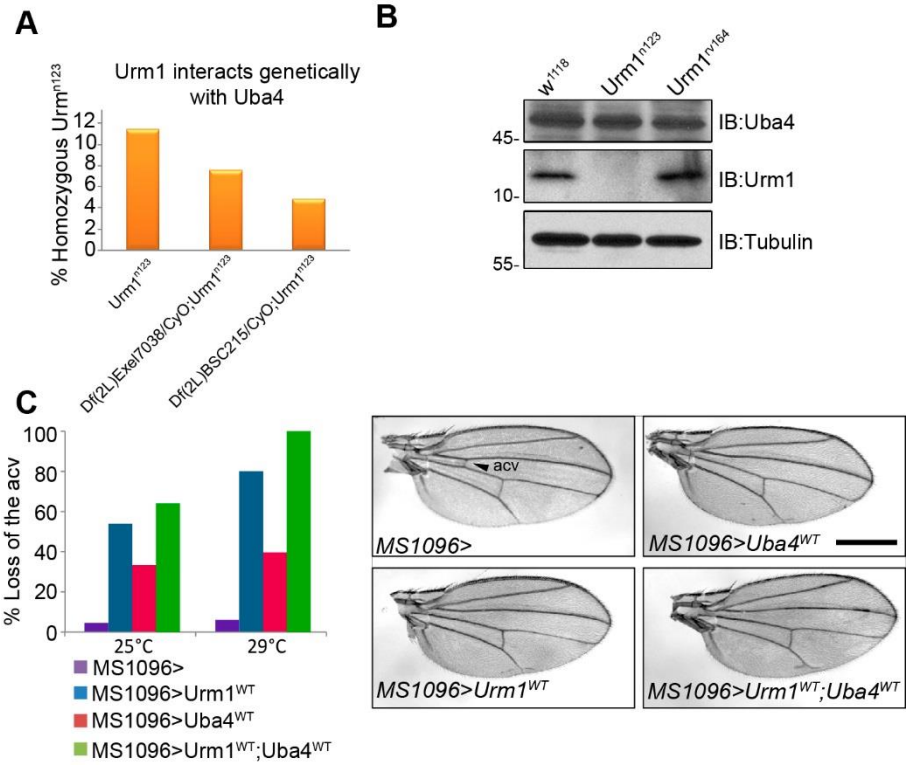
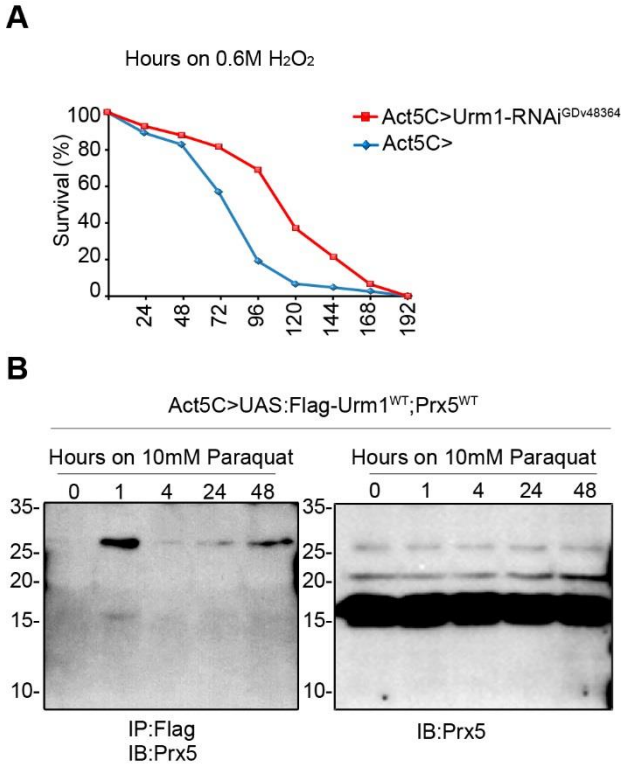

A
Urm1 interacts genetically with Uba4
% Homozygous Urm1n123
12
10
8
6
4
2
0
Urm1n123
Df(2L)Exel7038/CyO:Urm1n123
Df(2L)BSC215/CyO:Urm1n123
B
w1118
Urm1n123
Urm1rv164
45-
10-
55-
IB:Uba4
IB:Urm1
IB:Tubulin
C
% Loss of the acv
100
80
60
40
20
0
25°C
29°C
MS1096>
MS1096>Urm1WT
MS1096>Uba4WT
MS1096>Urm1WT;Uba4WT
acv
MS1096>
MS1096>Uba4WT
MS1096>Urm1WT
MS1096>Urm1WT;Uba4WT


A
Hours on 0.6M H2O2
Survival (%)
100
80
60
40
20
0
24
48
72
96
120
144
168
192
Act5C>Urm1-RNAi GDv48364
Act5C>
B
Act5C>UAS:Flag-Urm1 WT ;Prx5 WT
Hours on 10mM Paraquat
0 1 4 24 48
Hours on 10mM Paraquat
0 1 4 24 48
35-
25-
20-
15-
10-
IP:Flag
IB:Prx5
IB:Prx5