# **Expanded View Figures**

## Figure EV1. Linkage types of Ub chains and predicted domains in CG11321.

- A Schematics of eight different linkage types of Ub chains. Ub can form different types of polymers by conjugating via intrinsic Lys residues (K 6, K 11, K 27, K 29, K 33, K 48, and K 63) and M 1 (linear).
- B A multiple amino acid sequence alignment of RBR-C in HOIP family members. Conserved residues are colored according to the ClustalX coloring scheme. Long unaligned regions in *A. mellifera* and *P. humanus* were replaced by the number of deleted residues in squared brackets. R1, IBR, R2, and LDD domains are indicated with gray bars above the sequences. The zinc (Zn)-coordinating residues known in *H. sapiens* HOIP-RBR-C are labeled below. A black triangle indicates the Cys (C) residue for the thioester intermediate, two \* are Zn-coordinating Cys residues in R2 targeted to create catalytically dead mutants, and two O are residues mutated in LDD.
- C-F Multiple amino acid sequence alignments of N-terminal HOIP domains, UBA1 (C), UBA2 (D), B-box (E), and NZF (F).

Α	Intrinsic Lys-I	inked chain	s Linear (Met 1-linked) chain					
	K 6 K 11		M 1					
	K 27							
	K 33							
	K 48 K 63	Pubatrat						
		Substrati						
В	RBR-C D. melanog	aster 2514	R1 CELCMNSYPMNQMVSMLKCLHKCCKQCAKSYFTVQITDRSINDCSCPFCKLPELSNEAQHEDEHLEYFSNLDIFLKSILDNDVHELFQRKLRDRSLLQ 2011					
	A. me P. hun	ninera 3233 nanus 1590 ornioa 1520	CELCTGRFAMSONSMLKCHRCCNECARNIFTIQISDMNITDAVEPEKEENLKDANEDEVLETFSNLDIQLKTLLDPFIELFORARDATILMO 5326 CELCMGRYPMKONISMLKCTHRCCKECAKNYFTLIITDRNISDAICPFCKEPELNEEDVILLEYSNLDILLKNILDFNIELFORARDATILMO 5326 CELCMGRYPMKONISMLKCTHRCCKECAKNYFTLIITDRNISDAICPFCKEPELNEEDVILLEYSNLDILLKNILDFNIELFORARDATILMO 5326					
	A. Calif. T. rul	pripes 717	CPCCLCIFPISKNCSLCSCCSVCHCCFRMHFTIAVRDRHIRDMVCPVCCEPDINDIGVABBLIGFISHDUPTIHEEDHILFFIARDHALFFI CPCCLCIFPISKNCSLCSCCSVCHCFRMHFTIAVRDRHIRDMVCPVCCEPDINDGQGDISYFSTLDIQLRDCLADVYELFHKKITEHAMK 810					
	M. mus H. sa	culus 693 piens 699	CAVCGWALPENRMQALISCECTICPECFRQHFTIALKEKHITDMVCPACGRPDLTNBALLARISILDIALGARELDPAYALFHKKITEAVLMR CAVCGWALPENRMQALISCECTICPECFRQHFTIALKEKHITDMVCPACGRPDLTDDAQLISYFSTLDIQLRESLDPAYALFHKKITEAVLMR CAVCGWALPHNRMQALISCECTICPECFRQHFTIALKEKHITDMVCPACGRPDLTDDTGLISYFSTLDIQLRESLDPAYALFHKKITEAVLMR 792					
	D. melanog	aster 2612	IBR * * R2 DPNFKWCIQCSSGFFARPK©KRLIC <mark>PDCG</mark> SVTCAQCRKPWERQHEGSSCEA¥LEW <mark>KRENDPELQAQG</mark> VQEHLAQNGIDCPKCKFR¥SLARGGCMHFTC 2709					
	A. me P. hun	llifera 3329 nanus 1684	DPNFKWCIQCSSGFYADPDQKRLICPDCRSVTCAQCRRPWEKQHEGITCEQFAAWKDENDPDNQAAGLAKHLADNGIDCPKCKFRYSLSRGGCMHFTC 3426 DPNFKWCVQCSSGFIANPRQKKLTCPDCKSVSCAICRLPWEKQHEGISCAQFKNWKDANDPERQAEGVAKHLQENGIECPNCKFQYSLSRGGCMHFTC 1781					
	A. callo T. rul	pripes 811	DENRRWCAHCAAGFIADNRRLCHTCFACGGKTTYSCKKWEDDHEGDTEGOROWNIDHDFINGSVGGAKHDDDGGIDCFACKWRFDDANGGCHFFAC 1/11 DPKFWCHCTSGFIYDGDQLKVTCFSCRKSFCAQCKKPWEPOHODISCEOFOLWKEDDFEYGCGLAGYLRDNGITCFHCRFQVALTKGGCMHFAC 908					
	M. mus H. sa	culus 787	DPKFLWCACCFGFITERDIDARCFCCHQFFCVDrVBCWEEQHRGRSCEDFQNWKRTNDPEYQAQGLAWILQENGIDCPKCKFSYALARGCCMFFLC DPKFLWCACCFGFITEREQLEATCPCCHQFFCVDrVBCWEEQHRGRSCEDFQNWKRTNDPEYQAQGLAWILQENGIDCPKCKFSYALARGCCMFFLC DFKFLWCACCFGFITEREQLEATCPCCHQFFCVDrVBCWEEQHRGRSCEDFQNWKRTNDPEYQAQGLAWILQENGIDCPKCKFSYALARGCCMFFLC DFKFLWCACCFGFITEREQLEATCPCCHQFFCVDrVBCWEEQHRGRSCEDFQNWKRTNDPEYQAQGLAWILQENGIDCPKCKFSYALARGCCMFFLC					
	D. melanog	aster 2710	OO LDD TQCKFEFCYGCARPFMMGAKCTVSTYCAKLGLHAHHPRNCLFYLRDKIFLQLQFLLKEQNVKFDTEPMQIKDESSSSSKARAQARCPIPLQKETPQGL 2807					
	A. me P. hun	llitera 3427 nanus 1782	SQCKYEFCCGCGCAFMMGAKGSVSPYCAKLGLHAHHPRNCLFYLRDKEFQQLQQLLRDNGIEYDTEGPAGERKCKVQLQKETPTGV 3512 TQCKYEFCCGCGKPFKMGTKCDVSQYCAKLGLHAHHPRNCLFYLRDKEPGELQHLLKEHKIDFLVDPPE-NTKSPNKNNELNKLRCPIPIQKENPKGL 1878					
	A. califo T. rul	ornica 1712 oripes 909	PECCHEFCSGCNEAYHHKNYCQXXKNCFQGLCCHCPROCFSYLRDNSYTQLQOLLKQKKVDFNVDIPSDQGDACCPVMEQKEDDRS 1/99 SQCRVQFCSGCNNPYH-TTACKAAQCSY-TGLHAHHPROLFYLRDNSYTQLQOLLKQKVDFNVDIPSQGDACCPMEQKEDDRS 1/99					
	M. mus H. sa	culus 885 piens 891	TOCREGESSOLINFERANKCPDPNCKVKKSLEGHPROCLFYLRDWTAARLOKLLODNVVFNTEPPAGRAVPGGGCKVEGREFPDG TOCREGESGCVAFYAKNKCPDPNCKVKKSLEGHPROCLFYLRDWTAARLOKLLODNVVFNTEPPA					
	D. melanog	aster 2808	LDD VDTVCNTEVPDKHAGMCRTHYVEYLAGKVAKAGIDPLPIFDLTDCVQELR <mark>RRGIALPERGPWDTDEIYKNMCSEVIK</mark> KHIPLKSA 2892					
	A. me P. hun	llifera 3513 nanus 1879	VDAVCNSDVVEGHAGLCRNHYIE(55)YLAGLVLKGKLDPVAIFDLNDAKQELRRRGKVPPAKDQEMSERDYLEACIQIVKKEIPLE					
	A. cano T. rul X	orrica 1800 oripes 995 laovis 954	REEKGEDTERGENGLEELHING					
	M. mus H. sa	culus 976 piens 982	RDEACGRETPROVAGLOGAHYKELUVGLINAHSLDPATLYEVELETATERVLHURPOHAGEDLPAYOARLUVLHURPUFAGATRERK 1066 DEACGRETPROVAGLOGAHYKEYLVGLINAHSLDPATLYEVERLETATERVLHURPOHAGEDLPAYOARLUVLTEVERGSTERRK 1072					
С	UBA1 D. melanoga A. mell	ister 1042 ifera 1053	MHIIL <mark>KELELYKFTVEELEAALKYCS-<mark>PETHP</mark>IQWL<mark>R</mark>ENWHKLVQTVQSLSTKYGQERGENTIGTVSQNE 1110 TVETLREAEKHGYSTDDVOVALSOGA-SNPIDWLKTOMPHLVETVOVLVTTOGREMKENNIGMLSGVE 1119</mark>					
	P. huma A. califor	anus 941 mica 1272	FVRLLREAEKNNFTTEDLEIAFEHCE-NE-NPIVWLKNNYKNIIDTVÝTLATNÝGRECKENSVGTVSQLE 1008 LAOWYKMADRECFEVDAMSTALAOCSLHSLDPVOWLETNWTNNYTOVALRACKKGREEDONCVGELSLAE 1341					
	T. rubi X. la	ipes 478 ievis 463	LIHQI <mark>KEAEMSGISPEEVYAAIVCSG-NSIKPCVWLKSELPHLLDEICAIAA</mark> S[17]DKEERPQQSD <mark>G</mark> [8]KLSRAE 571 IVALIKKGEKNGVLPEEVCSAIRYSG-TEV-PERWLOTELPYVLERLLDAASOKARDMVGALWVEE 526					
	M. muso H. sap	ulus 485 iens 491	LVSMIQEGETAGASPEEVFSALQYSG-TEV-PLQWLASELSYVLEMVAELAGQQDPELGAFSCQE 547 LVSMIREGEAAGACPEEIFSALOYSG-TEV-PLOWLASELPYVLEMVAELAGOODPGLGAFSCQE 553					
	D. melanoga	ster 1111	AREALRNSGGNVWQAVADCIQQ <mark>RQOK</mark> YRKLAAK <mark>G</mark> NFL <mark>R</mark> DDIVNALTAHQ <mark>G</mark> NVEQALVELN <mark>R</mark> TQLKPFLM <mark>R</mark> IWGSPNG 1187					
	A. meli P. huma	<i>ifera</i> 1120 anus 1009	A <mark>KEALRVAKG</mark> DVWKAVAMAAQR <mark>RQLKCEEIMKKG</mark> NFTMMEVVKALENNAGAEDAALFELQKNQLKPFLM <mark>RIWGPPVG</mark> 1196 A <mark>RKSLHASKG</mark> VIWAAVNDCVEN <mark>RQKKFNELASRGNFTREDILTVLTANHGDIEIAYSELKK</mark> ISNE-FVNN <mark>PP</mark> DKIEK 1084					
	A. califo T. rubi	nica 1342 ipes 572	A <mark>K</mark> AAYMSCN <mark>GNIKEAVNLCVRNRKDLYGKLSALGEFPREEILDAMQQSCGD</mark> QAACEHFLQASKLYPFIGRIWAQRES 1418 A <mark>K</mark> VAWLAAGGNTDRAVRQLLRD <mark>RQRK</mark> MKELHAL <mark>G</mark> FRDVSQCEEALRLS <mark>GG</mark> QVTGALSLLQ <mark>RP</mark> LLEPFHQRIWTDQPE 648					
	X. la M. muso	evis 527 sulus 548	A <mark>R</mark> EAWVSS <mark>GG</mark> DMDAAVILCLTE <mark>RRRK</mark> VDALSTLGFPDKDKVVAALYESAGDVGRALSILQKPLLEPFLIRMWEESQP603 A <mark>R</mark> KAWLDRH <mark>GN</mark> LDEAVEECVRA <mark>RRK</mark> VHELQSL <mark>GFGPKEGSLQALFQHGGDVARALTELQRQRLEPFHQR</mark> LWDRDPE624					
_	H. sap	iens 554	A <mark>R</mark> RAWLDRH <mark>GNLDEAVEECVRT<mark>R</mark>RKVQELQSL<mark>GFGPEEGS</mark>LQALFQH<mark>GGD</mark>VSRALTELQRQRLEPFRQRLWDS<mark>GP</mark>E 630</mark>					
D	UBA2 D. melanog A. mel	aster 2457 lifera 3176	DPAILARKYVDOELVTNIAEAQIAATLVSMKFSEDVALWAARECSDLDOAIAMLOOE 2513 ERERIARRLLAEGKASNYDEAEVAASLLALKFGDVEALQAAKECSSIESALAFLOOE 3232					
	P. hum A. califo	<i>anus</i> 1533 <i>rnica</i> 1458	NFERQA <mark>RRYLAEGIVPSYEKAEILVKLLNFGFEEEDAEQAALECGSVD</mark> SALA <mark>YLQOE</mark> 1589 DLDRRT <mark>R</mark> MILVEGRLKSWGRAEMVISILD <mark>Q</mark> GVPRTEVSLEDVVEAV <mark>R</mark> NCRD <mark>R</mark> QSALAYLQOE 1519					
	T. rub X. k	<i>ripes</i> 657 aevis 611	DKORMCRRLLALYDL <mark>PSWGRCELVLSLLOEPDISY</mark> SLEDVVOAVKESHDKDFIRRLLNNE 716 DROAVLRRLLAEHSLHSWGRAELALSLLLEGEGRYELODVVEAVRESODRDFIKRMLTOE 670					
	M. mus H. saj	culus 633 biens 639	DROSLV <mark>RRLLAVYTLPSWGRAELALALLOETPRNYELLDVVEAVR</mark> HSODRAFLRRLLA <mark>DE</mark> 692 DKOSLV <mark>RRLLAVYALPSWGRAELALSLLOETPRNYELGDVVEAVR</mark> HSODRAFLRRLLA <mark>DE</mark> 698					
E B-box								
	D. melanogaster 90 CTLCGSQNPWVTCAECAGQIFCASCDDMFHKHPKRKQHMRKAV 132 D. melanogaster 733 TPDHEWECEFCTFVNEPNIKICSICCKTPS A. mellifera 93 CALCGAENVYARCDTCNGN-YCEACDDMNHKHPKRKSHVRRRI 134 A. mellifera 783 IPKEEWACEHCTFINNVKDRVCVVCCKTRS							
	P. humanus 59 A. californica 348	CDLCGSS CDVCGD-	EPTVRCEKCSSQVFCLSCDDMYHRHFKRQSHVRKGI 101 P. humanus 478 IPDHKWECEHCTFVNKPGVRVCAICCKTPT 507 DAVAFCVECQRKTLCDGCNVRWHQHPHRRGHKIQQI 389 A. californica 1029 VKPKPWHCEHCTFINQASSHACEMCHKISD 105	/ 58				
	T. rubripes 223 X. laevis 204	CKLCGG- CFLCGTL	PSSVVCPSCDSPSFCDACDDLYHRHPSFASHKRDKI 264 T. rubripes 412 NTHRQWICQFCTYVNTGLTLACEMCNLSCK 441 ACSVFCCSCNE-MLCEECDKRAHSHPARAEHVRLPC 245 X. laevis 403 AKSEGWQCSHCTFFNTQNGRVCEICDRIRE 432	2				
	M. musculus 214 CFLCGSAPGTLHCPACNQ-VSCPACDILFHGHPSRAHHLRQAL 255 M. musculus 402 AQPQVWYCDHCTFCNSGPVWVCAMCNRTRD 431 H. sapiens 217 CFLCGSAPGTLHCPSCKQ-ALCPACDHLFHGHPSRAHHLRQTL 258 H. sapiens 408 AQSQVWYCIHCTFCNSSPGWVCVMCNRTSS 437							

Figure EV1.

### Figure EV2. LUBEL-RBR-C specifically synthesizes linear Ub chains.

- A In vitro ubiquitination assay of predicted LUBEL-RBR-C in combination with Ube1 and two different *Drosophila* E2s, UbcD10 and Effete/UbcD1, using nontagged or N-terminally His<sub>6</sub>-tagged Ub. Synthesized Ub chains were analyzed by immunoblotting using anti-linear Ub antibody or anti-Ub antibody. Protein loading was visualized by Ponceau S staining. \*: nonspecific band.
- B Mass spectrometry analysis of the Ub chains generated by LUBEL-RBR-C and UbcD10. MS/MS spectra acquired from the linear Ub chain peptide using an identical sample as Fig 2A lane 4 is shown.
- C, D In vitro deubiquitinating activities of vOTU (C), or OTULIN (D). Recombinant vOTU, OTULIN (WT or a catalytically dead C129A mutant) was incubated with K 48-, K 63-linked, or linear Ub<sub>2</sub> chains for indicated times. Subsequently, proteins were resolved on SDS–PAGE gels and stained with Coomassie dye. \*: nonspecific band.
- E In vitro ubiquitination assay of LUBEL-RBR-C C2690S/C2693S (CC/SS) mutant compared to WT. Linear Ub chain formation was analyzed by immunoblotting using anti-linear Ub antibody. Total amount of proteins was analyzed by Ponceau S staining. \*: nonspecific band.
- F Linear Ub chains in *Drosophila* S2 cells transfected with LUBEL-RBR-C. Myc-RBR-C WT or Myc-RBR-C CC/SS was transfected in S2 cells. Linear Ub chains in TCL were visualized by immunoblotting using anti-linear Ub antibody. Expression of RBR-C (WT or C2690/2693S) was analyzed by using anti-Myc-antibody, and tubulin was blotted to examine protein loading.
- G In vitro ubiquitination assay using full-length LUBEL. In vitro ubiquitination assay was performed using recombinant full-length LUBEL purified by a baculovirusbased insect expression method, in combination with Ube1 and UbcD10. Amount of E3 ligase was determined by using an antibody raised against LUBEL-RBR-C. LUBEL-RBR-C was used as positive control.
- H Catalytic activity of LUBEL-RBR-C with extended N-terminal UBA2 *in vitro*. *In vitro* ubiquitination assay was performed using LUBEL-RBR-C or LUBEL-UBA2-RBR-C in combination with Ube1 and UbcD10. Linear Ub chain formation was analyzed as (E), and amount of E3 ligase was determined by anti-RBR-C antibody. \*: nonspecific band.



Figure EV2.

Α		*		В
D. melanogaster A. mellifera P. humanus C. elegans	270 418 242 760	ICCKFKGIQGHHNSCYLDATLFSMFTFTSVFDSILYRPGPQDIRNYSEVQKVLRDEIVNPLRKNVFVRSD ICCKYRGIQGHHNSCYLDATLFSMFTFTSVFDNLLFRPPNEKDCPQYEEVQRVLREEIVNPLRKNMFVRAD ICCKNRGIQGHNSCYLDATLFSMFAFTCVFDSLLYRPFTKHDVSQYSEVQKVLRDEIVNPLRKNFYVRAD IVCROKGIQGYCNSCYLDATLYAMFVQTTCFPFLEKSIKGSETAQOFOKILAHEIVFPLRVHYVRAD	340 488 312 828	w
A. californica M. musculus H. sapiens	528 583 587	ICCKHHGIQGHHNSCYLDATLFCMFYFTTVFDFIFNRQPRGDLPQYPEVQRVLKDGIVNPLRKFNYVRAD MICKKKGIQGHYNSCYLDSTLFCLFAFSSALDTVLLRPKEKNDIEYYSETQELLRTEIVNPLRIYGYVCAT MICKKKGIQGHYNSCYLDSTLFCLFAFSSVLDTVLLRPKEKNDVEYYSETQELLRTEIVNPLRIYGYVCAT UCH2-1	598 653 657	(kDa) 150 -
D. melanogaster A. mellifera P. humanus	341 489 313	RVMKLRELLDOL-SSVSGLTCEEKDPEEFLNSLLSQIMRVEPFLKLSSGODSYFYQLFVEKDEKLTL RVMKLRTLLEKL-SSVSGLTSEEKDPEEFLTSLVAQILNAEPFLKLSSGODSYFYQLFVEKDEKLT RVMKLRTLDKL-SSVSGLTSEEKDPEEFLVSLUAQILKAEPFLKLSSGODSHYQLFVEKDEHLVE	406 554 378	100 - 75 -
C. elegans A. californica M. musculus H. sapiens	829 599 654 658	HVMKLRKLLABLMPHVTGLTNEEKDPEBILGFIFSKVFHAEPFIKLIGQNHAKDSQYLVPIVVD-DWLGGA KVLRSLDRL-ATVEGMMSBEKDPEFLSALLTDIMKADPLHLSSGEHTYFYQFMEKDDKLL XIMKLRKILEKV-EAASGFTSEEKDPEEFLNILFHDILRVEPLLKIRSAGGKVQDCNFYQIFMEKNEKVGV XIMKLRKILEKV-EAASGFTSEEKDPEEFLNILFHHLRVEPLLKIRSAGGKVQDCNFYQIFMEKNEKVGV	898 664 723 727	50 -
				37 -
D. melanogasler A. mellifera P. humanus	407 555 379	PSVQQLFEQSFHSSDIKLKEVPSCFIIQMPRFGKNYKMYPRILPSQVLDVTDIIENSPRQCSLCGKLAEYE PTVQQLLEQSFLTSNIRLKEVPSCLIIQMPRFGKSFKMYQKIQPTLLLDVTDIIEDSPRQCTVCGKLAEYE PSVQQLFEQSFVTSDIKLKQVPPCLIIQMPRFGKSFKMYQKILPPQLLDVTDVIENSPRQCTVCGKSAQFE	477 625 449	25 -
A. californica M. musculus	665 724 728	ATOCHLLEKRINKSOVITAKAPPVLIMOLPRIGOU-AVIDKILPLETIDITPIVAGAPACSKOACSEVI PTOCLLEWSFINSNLKFAEAPSCLIIOMPRIGKDFKLFKKIPPSLELNITDLLEDTPROCICGGLAMYE PTIOQLLEWSFINSNLKFAEAPSCLIIOMPRIGKDFKLFKKIPPSLELNITDLLEDTPROCICGGLAMYE	968 735 794 798	50 -
п. зарюно	, 20	CxxC	,	37 -
D. melanogaster A. mellifera P. humanus C. elegans A. californica M. musculus	478 626 450 969 736 795	CRDCFGSLQAGSGLECTAFCPKCLKTFHSHIKTNHVSKKIYSPKEFKIMA-EHM	531 677 503 1015 803 848	25 -
H. sapiens	799	CRECYDDPDISAG-KIKOFCKTONTOVHLHPKRLNHKYNPVSLPKDLPDWDWRHGCXXC CXXC CXXC	852	(kDa 50
D. melanogaster A. mellitera P. humanus C. elegans A. californica M. musculus H. sapiens	532 678 504 1016 804 849 853	VVPRLYMELFAVVCIETSHVAFVKSGSGPDAPWCFFDSMADRKGEQNGYNIPEITCVPELTQWL PVPRLYLELSAVVCIETSHVVAFVKCGSGSBAPWCFFDSMADRKGEQNGYNIPEMVPCPDFPWL ITPRLYMELFAVVCIETSHVVAFVKCGSGLDAPWCFFDSMADRKGEQNGYNIPEIVPCPLFYWL GUAISLPREVMELFAVLCIETSHVVAFVKTSSNQWVFFDSMADRGEQSGSUNIPEVVHVPMSNWL CIPCQKMELFAVLCIETSHVVAFVKYGKD-DSAWLFFDSMADRGGSGVNIPEVVHVPMSNWL CIPCQKMELFAVLCIETSHVVAFVKYGKD-DSAWLFFDSMADRGGSGVNIPQVTPCPEVGEYL CXXC UCH2-2	596 742 568 1077 873 912 916	
C	out	Pulldown D GST GST-dCYLD RBR-C		
(kDa) 50 -	F	- Flag-RBR-C (kDa) WB: Flag 50		



#### 100 WB: RBR 37 GST-dCYLD 100 75 GST-dCYLD 75 50 50 37 37 GST 25 GST 25 Ponceau S 20 Ponceau S Pulldown Pulldown

# Figure EV3. Characteristics of Drosophila CYLD.

- A A multiple amino acid sequence alignment of the CYLD catalytic domain in different species. \* indicates predicted catalytic Cys residue, while C-X-X-C pairs (labeled CxxC) and the UCH catalytic domains, UCH2-1 and UCH2-2 (in gray), are shown below the sequences.
- B Endogenous level of K 63-linked Ub chains in *dCYLD* mutant flies. Poly-Ub chains in the total protein extracts of  $w^-$  and *dCYLD* mutant were enriched by GST-TR-TUBE pulldown, and the samples were resolved and detected using anti-K 63-linked Ub chains. GST was used as control for the pulldown. Input of GST proteins was visualized by Ponceau S, and total protein extracts were blotted with anti-tubulin antibody. \*: nonspecific band.
- C Interaction between dCYLD and LUBEL-RBR-C. Flag-RBR-C was transfected into S2 cells and total cell lysate was incubated with either agarose-immobilized GST or GST-dCYLD. After GST pulldown, samples were analyzed by immunoblotting using anti-Flag antibody. Loading of GST proteins was visualized by Ponceau S staining.
- D Protein-protein interaction of recombinant dCYLD and recombinant LUBEL-RBR-C. LUBEL-RBR-C purified from *E. coli* was incubated with immobilized GST or GSTdCYLD for pulldown assay. The interaction was analyzed by immunoblotting using anti-LUBEL-RBR antibody. Loading of GST proteins was visualized by Ponceau S staining, \*: nonspecific band.



Figure EV4.

## Figure EV4. Establishing LUBEL mutant fly strains.

- A Endogenous mRNA expression of LUBEL detected in different embryonic stages of  $w^-$  flies by qPCR. RNA was isolated from embryos and the LUBEL expression levels were measured by qPCR. TATA binding protein (TBP) was used as a reference, and two sets of primers, N-terminal region or catalytic region, were used to detect all isoforms or only the long isoforms, respectively. Representative data are shown from three independent experiments.
- B Endogenous mRNA fragments of LUBEL detected in S2 cells (above) and  $w^-$  male adult flies (bottom) by RNA-Seq. The aligned transcripts were visualized using The Integrative Genomics Viewer (IGV\_2.3.40 software) and screenshots of the CG11321 region are shown. Representative alignments of three repeats are shown.
- C Negative geotaxis assay of aged male (top panel) and female (bottom panel) flies. The assays were repeated five times for each group (between 47 to 55 flies per group), and the results were combined to create the graphs. *t*-test analysis showed no significant difference between the lines. Representative videos can be found in Movies EV2 and EV3.
- D, E Survival of LUBEL mutant and *dCYLD* mutant flies upon Gram-negative bacterial infection. Septic injury was performed using *E. coli* in 20 young adult male flies. Recovered flies were kept in 25°C and counted every 24 h until indicated time. *Rel<sup>E20</sup>* was used as positive control for the assay. Curve comparison tests indicated there is no statistical difference except for *Rel<sup>E20</sup>* > 0.0001. Representative survival curve is shown for the LUBEL mutant flies, *CC/SS* #1 and #2, and *delR2* (D; from four independent experiments), and catalytically dead dCYLD flies (E; from three independent experiments).
- F Survival of adult male LUBEL mutant and *dCLYD* mutant flies by septic injury with Gram-positive *M. luteus* bacteria. Septic injury with *M. luteus* was performed and recovered flies were kept in 29°C and counted every 24 h for indicated time. Curve comparison tests indicated the difference is not significant between the lines. Representative data are shown from three independent experiments.
- G Antimicrobial peptide induction upon septic injury in *LUBEL* mutant flies. Septic injury with *E. coli* was performed, and after 10-h recovery, RNA was isolated and mRNA levels of *Attacin C, Diptericin,* and *Drosomycin* were measured by qPCR. *Rp49* was used as a reference, and  $w^-$  unpricked sample was used as calibrator to calculate the expression ratio. Multiple comparisons were performed using one-way ANOVA. Representative data are shown from three independent experiments.

Data information: Data in (A, C, G) are presented as mean ± SD. \*P < 0.05, \*\*P < 0.005, \*\*P < 0.001, \*\*\*\*P < 0.0001. Data were analyzed using unpaired *t*-test.



#### Figure EV5. Heat shock responses in $w^-$ , dCYLD mutant, and LUBEL knockdown (KD) flies.

- A Immunoblotting for total Ub chains in heat-shocked  $w^-$  flies in Fig 6A using anti-pan Ub antibody.
- B mRNA expression of poly-ubiquitin gene *ubi-p63E* in LUBEL mutant flies, untreated, or heat shocked. 10 male and 10 female adult flies were heat shocked for 1 h and RNA was isolated and *Ubi-p63E* mRNA level was measured by qPCR. *Rp49* was used as reference and untreated samples were used as calibrator for each fly line to calculate the expression ratio. Data are analyzed by two-way ANOVA with multiple comparison, and presented as mean  $\pm$  SD (\*\*\*\**P* < 0.001). Representative of three experiments is shown.
- C Expression of LUBEL mRNA in heterozygous flies (*Tub-Gal4*/+ and *Mef2-Gal4*/+) and UAS-shLUBEL (*Tub-Gal4* > LUBEL and *Mef2-Gal4* > LUBEL) flies detected by qPCR. The primers that target N-terminal region (left panel) or catalytic region (right panel) of LUBEL were used. *Rp49* was used as reference, and GD control fly line was used as calibrator to calculate the expression ratio. Data were analyzed using unpaired t-test; values represent mean  $\pm$  SD (\*\**P* < 0.01, \*\*\**P* < 0.001), (*n* = 3). Representative of three independent experiments are shown.
- D Survival of *dCYLD* mutant flies upon heat shock. Survival curve of heat-treated  $w^-$  and *dCYLD* mutant flies is shown. 15 male and 15 female files per each fly line were used in this assay. Curve comparison tests indicated the difference is not significant. Representative data are shown from four independent experiments.
- E Heat-induced mRNA expression of HSP70.  $w^-$ , CC/SS #2, and delR2 flies were heat treated for 30 min and mRNA HSP70 was quantified by qPCR. Rp49 was used as a reference and  $w^-$  untreated sample was used as calibrator to calculate the expression ratio. Data are analyzed by two-way ANOVA with multiple comparison, and presented as mean  $\pm$  SD (\*\*\*\*P < 0.0001). Representative of three experiments is shown.
- F Repeat of muscle-specific LUBEL KD using *B24-Gal4* driver. A heat-hock survival assay was performed as Fig 6C, using *24B-Gal4* driver line to knockdown LUBEL in the muscle. *P*-values calculated by Gehan–Breslow–Wilcoxon test: *Tub-Gal4* < 0.0001 (\*\*\*\*), *24B-Gal4* = 0.0066 (\*\*). knockdown efficiency for the catalytic region, analyzed as in (C), is shown on the right graph. Representative of three experiments is shown.