## **Supplementary information**

## **Supplementary Figures**



Supplementary Figure S1 The trimeric assembly of Ragulator in the asymmetric unit of the crystal structure. The three Ragulator complexes in the asymmetric unit are denoted A, B, and C, respectively. The Lamtor1, Lamtor2, Lamtor3, Lamtor4, and Lamtor5 subunits of Ragulator<sup>A</sup> are colored in green, magenta, cyan, yellow, and red, respectively. Ragulator<sup>B</sup> and Ragulator<sup>C</sup> are colored in orange and blue, respectively.
a, Top view. b, Side view.



**Supplementary Figure S2 The Ragulator complex is monomeric in solution.** The molecular weight of the Ragulator complex was measured to be 55.10 kDa by the size exclusion chromatography – multi-angle light scattering (SEC-MALS) method. The green, blue, and red profiles represent Ultra-Violet absorption, differential refractive index, and light scattering measurements, respectively.



Supplementary Figure S3 Overall structure of the Lamtor1-Lamtor2 sub-complex of the Ragulator complex. The extended C-terminus of Lamtor1 interacts with the  $\alpha$ 1 and  $\alpha$ 3 helices of Lamtor2. Lamtor1 and Lamtor2 are colored in green and magenta, respectively.



Supplementary Figure S4 Overall structure of the Lamtor1-Lamtor3 sub-complex of the Ragulator complex. The  $\alpha$ 1 helix of Lamtor1 interacts with the  $\alpha$ 1 and  $\alpha$ 3 helices, as well as the  $\beta$ 1- $\beta$ 2 loop of Lamtor3. Lamtor1 and Lamtor3 are colored in green and cyan, respectively.



Supplementary Figure S5 Overall structure of the Lamtor1-Lamtor4 sub-complex of the Ragulator complex. The  $\alpha$ 2 helix of Lamtor1 interacts with the  $\alpha$ 1 helix, as well as the  $\beta$ 3,  $\beta$ 4, and  $\beta$ 5 strands of Lamtor4. Lamtor1 and Lamtor4 are colored in green and yellow, respectively.



Supplementary Figure S6 Overall structure of the Lamtor1-Lamtor5 sub-complex of the Ragulator complex. The  $\alpha$ 3 helix of Lamtor1 interacts with the  $\alpha$ 1 helix, as well as the  $\beta$ 1,  $\beta$ 3,  $\beta$ 4, and  $\beta$ 5 strands of Lamtor5. Lamtor1 and Lamtor5 are colored in green and red, respectively.

								Lam	ntor2	2						
-1		$\alpha$ 1		-	_	β1 —	— <mark>[</mark>	32 -				C	х2			
+++	++	10	ŧ		20 		30 		27 - 53	40	ŧ	<b>↓</b> 50	•	+	60	
MLRE	KAL	TQVI	LSQA-	-NTGG	VQST	LLLNN	EGSLL	AYSGY	0	DTDA	RVTA	AIAS	NIWA	AYDR	NGN	human
MLR	KAL	TQVI	LSQA-	-NTGG	VQST	LLLNN	EGSLL	AYSGY	<u></u> 0	DTDA	RVTA	AIAS	NIWA	AYDK	IGH	trog
MLRE	KAL	TQVI	SQA-	-NTSG	VQST	LLLNN	EGSLL	AYSGY	<u></u>	DTDA	RVTA	AIAS	NIWA	AYDK	IGH	zebrafish
MLKE	KAL	TQVI	SQA-	-NTGG	VQSS	LLLNN	EGSLL	AYSGY	<u></u> 0	DKDAL	RVTA	AIAS	NIWA	AYEK	<b>IGK</b>	sea urchin
MLKE	KAL	TQVI	SQA-	-NTGG	VENT	LLLSQ	EGALL	AYSGY	<u></u>	DKDA	RITA	AIAS	NIWA	AYEK	IGR	fruit fly
MLKE	KAL	TQVI	SQA-	-NTGG	VENT	LLLNQ	EGALL	AYSGY	<u></u>	DRDA	RVTA	ATAS	NIWS	AYEK	NGR	beetle
MLQE	KVL	TSVI	GQA-	-NTGG	VENT	ILLTH	EGALL	AFSGY	<u>c</u>	DRDA	TTA	AIAS	NIWS	AYEK	NGR	mosquito
MLKE	KAL	TRVI	EQA-	-NTGG	IHCT	LLLNQ	EGSLM	AFAGE	(	SEKKER	IVTA	ATAA	NIWM	SYDK	ISN	sponge
MLKE	KVF	AEMI	SQA-	-NTGG	VMSS	MLLNN	EGSLL	AYSGS	D1	/HKDK	1ITA	AVSS	NIWS	AYEK	GGK	hydra
MLRS	KIL	PQII	NQA	INNTD	TKGV	LIMKD	DGSLI	ACSDA	TTS	SHNTSE	KIVA/	AITS	NIWT	AYNR	NS-	slime mold
MLHS	KNV	KGFI	EDF-	-KTSS	LQSS	MIITA	INGIL	SYATS	51	INNNL	MMS	LLIK	DKWS	EDENI	DTE	yeast Ego3
			22		R1		RE				W3					
		70		80	p4	0	ps	10	10		110		1	20	_	
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QAFN	EDN	LKF	IMDO	MEGR	VAIT	RVANL	LLCMY	AKETV	GFG	ILKAK/	QAL	QYL	EEPL	TQVA	AS	human
QAFN	EDN	LKF	IMD	MEGR	VAIT	RVSNL	LLCMY	AKETV	GFG	ILKAK7	AQAL	THYL	EEPL	NQVSS	SS	frog
QAFN	EDK	LKF	IMDO	MEGR	VAIT	RVANL	LLCMY	AKETV	GFG	ILKAK/	EAL	<b>VQYLI</b>	EEPL	TQVA	<b>AS</b>	zebrafish
VAFI	EDD	TKT/	TIMDO	EEWK	SAIT	KVANL	LICIH	AESFV	GFG	LEKK	ISSL	AEYIA	QEPL	QTVAS	SS	sea urchin
NAFF	EGR	LTF\	LID	ENGH	TIAV	QVASV	LLCLY	AKQTV	GLGI	LKQKZ	MSL	ASYL	ERPL	KQISZ	AS	fruit fly
NAFF	HERE	POM	LIE	TDGR	IAIT	QVANL	LICLY	AKNNV	GFG	ILKEK/	MAL	AIYL	ENPL	KQVAT	rs	beetle
QAFF	EDR	LQF	FLQ	EGGN	TIIV	QVANL	LICLY	ARENV	GLGI	LKEK	KAL	ATYL	EGPL	KEIAT	rs	mosquito
ATME	DKP	LEL	VLQ	EDGC	VAIK	KVANL	LICIY	AQKSV	GLGI	LKVK	TTT.	AGYL	KEPL	SQIA	APT	sponge
MAFE	NEG	LQY	FMD	QEGK	VAVT	KVSAA	LICIY	AEETV	GFGI	LKLKI	DTL	AEHLI	KEPL	SASM	Q	hydra
	D	LQY	LID	EEGR	FVIT	RVASL	LLCVY	SDINI	EFGI	LKLK	<b>OKL</b>	REYLI	EEPL	SKVEF	IVQ	slime mold
EQHS	KTK	IYTY	EMEI	DLHTC	VAQI	PNSDL	LLFI	AEGS	PYGI	LVIK	IERA	MREL	TDLF	GIKT	G	yeast Ego3
	1 -	hand I	a tra altra	and to the			an Inid	a state to				the set of		and the second	<b>`</b>	

Lamtor1-binding Lamtor3-binding (side-chain) Lamtor3-binding (main-chain)

Supplementary Figure S7 Structure-based multiple sequence alignment of Lamtor2 (also knows as p14) orthologues from various species.  $\alpha$  helices and  $\beta$  sheets are shown as red cylinders and yellow arrows, respectively. Every ten residue of human Lamtor2 is marked. Conserved residues are shaded in pink. Residues mediating interactions with Lamtor1, Lamtor3 (by side-chains), and Lamtor3 (by main-chains), are highlighted by green arrows, blue arrows, and blue arrowheads, respectively. The places which contain loop-insertions are indicated with orange ovals.

				Lamtor3			
	) α1	β	1	β2	α2		
1	<b>1 1</b> <sup>10</sup>	20	1 1 30	40	<b>5</b> 0	60	
MAD	DEKRFLYKK	LPSVEGLHA	IVVSDRDGVPV	IK-VANDNAPE	ALRPGELSTE	ALATDOGSK	human
MAE	ELRRFLYKK	LTSVEGLHA	IVVSDRDGVPV	IK-VANENAPE	<b>DALRPAFLSTF</b>	ALATDQGSK	frog
MAD	NLRSYLYKQ	LPSVEGLHA	IVVIDRDGAPV	IK-VANDNAPE	YALRPAFL STF	ALATDQGSK	zebrafish
MVE	QLKRYLWSL	LOMVEGLEA	IVITDRDGVPV	IK-VKSDETPER	HAMRPAFLATE	AMAADQASK	sea urchin
MSDI	DIKKYLDGL	LQKVSGLYV.	IQITDRDGVPL	LR-VSQEKNVDI	FALMPSFIPTF	<b>FTACDQASK</b>	fruit fly
MVE	EVKNNLQEL	LSKVSGLYG	VITDRDGVHL	LK-VNSDKAPE	HAMRPNFISTF	GLAVDQGSK	beetle
MTDI	DVKRFFQNM	LQRVSGLRC	MVIDRDGVPL	VN-LEKEKLSE	IVMKPAFLSTF	FLAAEQSNK	mosquito
MDE	GLQAVLKKH	LHLVDGLRC:	ISVSDRDGVQI	VE-VHTDHFPT	<b>GVIKPIFMGTF</b>	AIASEQASK	sponge
MTA	VLGNYFQGL	LSNIDGGLA	FVIDRNGVPI	VK-ITNSKCPES	SATRAS FLSTF	AAATDQASK	hydra
MNIDE	FLNHIKTNV	LDVTIPDTS:	ILSDRDGFVL	GKNIDKNSSVDI	WVDSSLLSTF:	SSCTDQASK	slime mold
	- <mark>β3</mark> -	- <mark>β4</mark>	— <mark>β5</mark> —		α3		
.+	- <mark>β3 -</mark> 70 <b>↓↓↓↓↓</b>	- β4 80	<mark>β</mark> 5 90	100	α3 110	120	
LGLSK	- β3 - 70	- β4 80 Ι	β5 90 I				human
LGLSK	- β3 70 NKSIICYYN	- β4 80 I TYQVVQFNRI TYQVVQFNRI	- β5 90 L PLVVSFIASS	100 I SSA-NTGLIVSLI	α3 110 EKELAPLFEEL	120 ROVVEVS	human frog
LGLSKI LGLSKI LGLSKI	- β3 7044444 NKSIICYYN NKSIICYYS	- β4 80 TYQVVQFNRJ TYQVVQFNQJ TYQIVQFNRJ	- β5 90 LPLVVSFIASS LPLVVSFIASS LPLVVSFIASS	100 I SSA-NTGLIVSLI SNA-NTGLILSLI SNA-NTGLIFSLI	α3 110 ckelaplfeel ckelgslfkel skelvplteel	120 H RQVVEVS RQAVIS ROVVEVA	human frog zebrafish
LGLSKI LGLSKI LGLSKI LGLSAI	- β3 70 NKSIICYYN NKSIICYYS NKSIICYYS NKSIICYYT	B4 80 TYQVVQFNRJ TYQVVQFNRJ TYQIVQFNRJ SHQVVHFNKQ	- β5 90 LPLVVSFIASS LPLVVSFIASS LPLVISFIASS DPLFVSLIASS	100 SSA-NTGLIVSLI SNA-NTGLILSLI SNA-NTGLIFSLI RKA-NTGLIMDLI	03 110 EKELAPLFEEL EKELGSLFKEL EKELVPLIEEL EKELVPLIEEL	120 RQVVEVS RQAVIS RQVVEVA KAAIDI	human frog zebrafish sea urchin
LGLSKI LGLSKI LGLSAI LGLGRI	- β3 70 NKSIICYYN NKSIICYYS NKSIICYYS NKSIICTYT NKTIISMYS	- β4 80 TYQVVQFNRJ TYQVVQFNQJ TYQIVQFNRJ SHQVVHFNKQ NYQVVQMIKJ	β5 90 LPLVVSFIASS LPLVVSFIASS LPLVISFIASS QPLFVSLIASE LPLILTFVGAE	100 SSA-NTGLIVSLI SNA-NTGLILSLI SNA-NTGLIFSLI RKA-NTGLIMDLI INC-NTGHILALI	03 110 EKELAPLFEEL EKELGSLFKEL EKELVPLIEEL ESEVEGLLPEL ENQVDGYLEDI	120 RQVVEVS RQAVIS RQVVEVA KAAIDI KQAVTEA	human frog zebrafish sea urchin fruit fly
LGLSKI LGLSKI LGLSKI LGLGRI LGLGRI	- β3 70 NKSIICYYN NKSIICYYS NKSIICTYT NKTIISMYS INTLICVYS	B4 80 ΓΥΩννΩΕΝΙΚΙ ΤΥΩννΩΕΝΙΚΙ ΣΗΩννΗΕΝΙΚΩ ΝΥΩννΩΜΙΚΙ ΩΥΩνΙΩΜΙΚΙ	β5 90 LPLVVSFIASS LPLVVSFIASS LPLVISFIASS QPLFVSLIASF LPLILTFVGAE LPLILTFIASD	100 SSA-NTGLIVSLI SNA-NTGLILSLI SNA-NTGLIFSLI RKA-NTGLIMDLI SNC-NTGHILALI DTC-NTGHILALI	03 110 SKELAPLFEEL SKELGSLFKEL SEVEGLLPEL SEVEGLLPEL EHQVDGYLEDI SKQLDPIVSNL	120 RQVVEVS RQAVIS RQVVEVA KAAIDI KQAVTEA ALAVAES	human frog zebrafish sea urchin fruit fly beetle
LGLSKI LGLSKI LGLSKI LGLGRI LGLGRI LGLGKI	- β3 70 NKSIICYYN NKSIICYYS NKSIICYYS NKSIICTYT NKTIISMYS INTLICVYS NRNIISVYA	- β4 80 ITYQVVQFNRJ TYQVVQFNRJ SHQVVHFNRJ NYQVVQMIKJ QYQVIQMIKJ QYQVIQMIKJ	β5 90 1 LPLVVSFIASS LPLVVSFIASS LPLVISFIASS 2PLFVSLIASE LPLILTFVGAE LPLIVTFIASD LPFVVTFIGTE	100 SA-NTGLIVSLI SNA-NTGLILSLI SNA-NTGLIFSLI SNA-NTGLIMDLI SNC-NTGHILALI SNC-NTGHILAI SNCLNIGLILAM	03 110 EKELAPLFEEL EKELGSLFKEL EKELVPLTEEL SEVEGLLPEL EKQLDPIVSNL EKQLDPIVSNL	120 RQVVEVS RQAVIS RQVVEVA KAAIDI KQAVTEA ALAVAES KTAVADS	human frog zebrafish sea urchin fruit fly beetle mosquito
LGLSKI LGLSKI LGLSKI LGLGRI LGLGKI LGLGKI LGVGKI	- β3 70 NKSIICYYN NKSIICYYS NKSIICYYS NKTIISMYS INTLICVYS NRNIISVYA NTSIISYYS	- β4 80 TYQVVQFNRJ TYQIVQFNRJ SHQVVHFNKJ NYQVVQMIKJ QYQVIQMIKJ DCIVIQMIKJ GYQVIQFNYJ	β5 90 I LPLVVSFIASS LPLVVSFIASS LPLVISFIASS DPLFVSLIASE LPLIVTFIASD LPFVVTFIGTE LPFVLTFIADS	100 I SSA-NTGLIVSLI SNA-NTGLILSLI SNA-NTGLIFSLI SNA-NTGLIMDLI SNC-NTGHILALI OTC-NTGHILALI SNCLNIGLILAM SKS-NTGMLYSLI	03 110 SKELGSLFKEL SKELGSLFKEL SEVEGLLPEL SEVEGLLPEL SEVEGLLPEL SEQUDPIVSNL QQIDTMLEEL SEQLRDPIGHL	120 RQVVEVS RQAVIS RQVVEVA KAAIDI KQAVTEA ALAVAES KTAVADS QAAVSVP	human frog zebrafish sea urchin fruit fly beetle mosquito sponge
LGLSKI LGLSKI LGLSKI LGLGKI LGLGKI LGLGKI LGVGKI LGLSKI	- β3 70 NKSIICYYN NKSIICYYS NKSIICTYT NKTIISMYS INTLICVYS NRNIISVYA NTSIISYYS NKRMICMYS	B4 80 ΓΥΩΥΥΩΕΝΙΩ ΤΥΩΓΥΩΕΝΙΩ ΣΗΩΥΥΩΕΝΙΩ SHQVYHENKQ MYQVYQMIKI QYQVIQMIKI GYQVIQENYI SYQVYMENYI	β5 90 LPLVVSFIASS LPLVVSFIASS 2PLFVSLIASP LPLILTFVGAE LPLILTFVGAE LPFVVTFIGTE LPFVLTFIADS LPFVLTFIADS	100 SA-NTGLIVSLI SNA-NTGLILSLI SNA-NTGLIFSLI SNA-NTGLIMDLI SNC-NTGHILALI STC-NTGHILALI SKS-NTGMLYSLI SSA-NTGMILFM	03 110 EKELAPLFEEL EKELGSLFKEL ESEVEGLIPEL ENQVDGYLEDI EKQLDPIVSNL QQIDTMLEEL EEDLRDPIGHL EEDLRDPIGHL	120 RQVVEVS RQAVIS RQVVEVA KAAIDI KQAVTEA ALAVAES KTAVADS QAAVSVP KSTILVKN	human frog zebrafish sea urchin fruit fly beetle mosquito sponge hydra
LGLSKI LGLSKI LGLSKI LGLGKI LGLGKI LGLGKI LGLSKI LHAGSI	- β3 70 NKSIICYYN NKSIICYYS NKSIICTYT NKTIISMYS NRTIISVYA NTSIISYYS NKRMICMYS	B4 80 ΓΥΩΥΥΩΕΝΙΩΙ ΤΥΩΓΥΩΕΝΙΩΙ ΤΥΩΓΥΩΕΝΙΩΙ ΣΗΩΥΥΩΕΝΙΩΙ SHQVYLENIKI OYQVIQMIKI GYQVIQENYI SYQVYMENYI DRIVVHIIV	β5 90 LPLVVSFIASS LPLVVSFIASS DPLFVSLIASS LPLILTFVGAF LPLILTFVGAF LPLILTFIGTE LPFVLTFIGTE LPFVLTFIADS LPIVVTVIADS SNVILSIVTDI	100 SSA-NTGLIVSLI SNA-NTGLIVSLI SNA-NTGLILSLI SNA-NTGLIFSLI SNC-NTGHILALI STC-NTGHILALI SKS-NTGHILALI SSA-NTGMILEM TDA-NVGLILGTY	03 110 EKELAPLFEEL EKELGSLFKEL EKELVPLIEEL ESEVEGLLPEL ENQVDGYLEDI EKQLDPIVSNL EKQLDPIVSNL EEDLRDPIGHL EEDLRDPIGHL ETEFSDVIEKM	120 RQVVEVS RQAVIS RQVVEVA KAAIDI KQAVTEA ALAVAES KTAVADS QAAVSVP KSTILVKN SQSIQVDIQ	human frog zebrafish sea urchin fruit fly beetle mosquito sponge hydra slime mold

Lamtor3 (also knows as MP1) orthologues from various species.  $\alpha$  helices and  $\beta$  sheets are shown as red cylinders and yellow arrows, respectively. Every ten residue of human Lamtor3 is marked. Conserved residues are shaded in pink. Residues mediating interactions with Lamtor1, Lamtor2 (by side-chains), and Lamtor2 (by main-chains) are indicated by green arrows, magenta arrows, and magenta arrowheads, respectively.

Supplementary Figure S8 Structure-based multiple sequence alignment of

			Lamior4		
α1	<b></b> f	31	β2	- α2	
10	,	20	30	40 ₩ ₩	
MTSALTQGLER	PDQLGY	LVLSEGA-	VLASSGDLEN	IDEQAASAI SELVS	human
MTSTLTQGLER	PDQMGY	LVMSEDGO	VLASAGDLEN	DERLAGVIREMVA	frog
MTTALTOGLER	PDQLGY	LVISEDG-	VLASAGELEN	IDEHTAGVIMOMVR	zebrafish
MSGPGPHNLDK	PNQEGY	LILNDSGA	VMASSGDLEN	DEATAAI IMKMIR	sea urchin
MLKMDREKLI	PNQIGY	LILKEDGA	VLESGGDLKN	DERSANVIMGLLN	fruit fly
IDK	PGQTGY	LILNEEGA	VLSSSGDLEN	DEKSAVI IMGLIN	beetle
MLDLDGRP	PDQIGY	LVMSEDGA	VLASGGELEN	DERSANIISGLLS	mosquito
MSGSSSLIMGIDK	ADNSGY	FVLNREGA	VLSSAGDLEN	DEETANQILDIIY	hydra
	- 00	-	and the second		
$\alpha \alpha$		<b>↓</b> ——–  }	$4 - \beta$	5	
50 60	• <b>1</b> 3	β 70	64 <b>β</b> 80	90	
50 <b>60</b> <b>1 1</b>	<b>1</b>	70 ₩	$\beta 4 - \beta $	5 90 ↓ Ⅰ	
50 60 TACGERLHRGMNVE	PFKRLSV	70 VFGEHTLI	β <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>80</sup> <sup>8</sup>	90 <b>I</b> VKRQNRGREPIDV	human
50 60 TACGERLHRGMNVE VACTERDLGEQQP	PFKRLSV -FKRMSV	↓ 70 ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓	A A A A A A A A A A A A A A	90 <b>I</b> VKRQNRGREPIDV VKRQNVVREPISV	human frog
50 60 TACGFRLHRGMNVE VACTFRDLGEQQP- TACRFRLHGTAEPE	PFKRLSV FKRMSV FKRMSV	↓ 70 VFGEHTLI VFGEHTFI WFEDYVYA	A 80 4 VTVSGQRVFV VTISGQKIYV VTISGQKVFV	90 VKRQNRGREPIDV VKRQNVVREPISV VKRQNNQREPVIV	human frog zebrafish
50 60 TACGFRLHRGMNVE VACTFRDLGEQQP- TACRFRLHGTAEPE LASKVNITPDNSQS	PFKRLSV FKRMSV FKRMSV FKRLSV	↓ 70 ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓	A B B B B C C C C C C C C C C C C C	90 VKRQNRGREPIDV VKRQNVVREPISV VKRQNNQREPVIV SKRKNIPKEPVMA	human frog zebrafish sea urchin
50 60 TACGFRLHRGMNVE VACTFRDLGEQQP- TACRFRLHGTAEPE LASKVNITPDNSQS LTETIDESFMPSSS	PFKRLSV FKRMSV FKRMSV FKRLSV SFKRLSV SCERITI	VFGEHTLI VFGEHTLI MFEDYVYA IFDDFLYI DYEHHYYS	A B B B C C C C C M C	90 VKRQNRGREPIDV VKRQNVVREPISV VKRQNNQREPVIV SKRKNIPKEPVMA IKIS-KSQNGVTT	human frog zebrafish sea urchin fruit fly
50 60 TACGFRLHRGMNVF VACTFRDLGEQQP- TACRFRLHGTAEPF LASKVNITPDNSQS LTETIDESFMPSSS LTETIDESFMPSSS	PFKRLSV FKRMSV FKRMSV FKRLSV FKRLSV CERITI FKKLSI	VFGEHTLI VFGEHTFI MFEDYVYA IFDDFLYI DYEHHYYS NYDKHCYI	80 WTVSGQRVFV WTISGQKVFV WTISGQKVFV WTISSDKVYI GICMSNRRIYI GICMSNRRIYI	90 VKRQNRGREPIDV VKRQNVVREPISV VKRQNNQREPVIV SKRKNIPKEPVMA IKIS-KSQNGVTT VKKTLDYANGIP	human frog zebrafish sea urchin fruit fly beetle
50 60 TACGFRLHRGMNVE VACTFRDLGEQQP- TACRFRLHGTAEPE LASKVNITPDNSQS LTETIDESFMPSSS LTSHIDRAAFEEG- LTESVDPEVFKKRS	PFKRLSV FKRMSV FKRMSV FKRLSV FKRLSV GCERITI FKKLSI GCQKISI	VFGEHTLI VFGEHTLI MFEDYVYA IFDDFLYI DYEHHYYS NYDKHCYI AYEEHSYI	80 4 80 4 80 4 80 4 80 4 80 4 80 4 80 4 80 80 80 80 80 80 80 80 80 80	90 VKRQNRGREPIDV VKRQNVVREPISV VKRQNNQREPVIV SKRKNIPKEPVMA IKIS-KSQNGVTT VKKTLDYANGIP VKRR-NTPNGIDV	human frog zebrafish sea urchin fruit fly beetle mosquito
50 60 TACGFRLHRGMNVE VACTFRDLGEQQP- TACRFRLHGTAEPE LASKVNITPDNSQS LTETIDESFMPSSS LTSHIDRAAFEEG- LTESVDPEVFKKRS SASKVSLSKKDDEY	PFKRLSV FKRMSV FKRMSV FKRLSV SFKRLSV SFKRLSV SCERITI FKKLSI SCQKISI FQRISL	VFGEHTLI VFGEHTLI MFEDYVYA IFDDFLYI DYEHHYYS NYDKHCYI AYEEHSYI IYSEFVLI	80 WTVSGQRVFV WTISGQKIYV WTISGQKVFV WTISGQKVFV CATISSDKVYI CICLSNRKVYV CICLSNRKVYV CICLSNRKVYV CICLSNRKVYV CICLSNRKVYV CICLSNRKVYV	90 VKRQNRGREPIDV VKRQNVVREPISV VKRQNNQREPVIV SKRKNIPKEPVMA IKIS-KSQNGVTT VKKTLDYANGIP VKRR-NTPNGIDV VKRPLAKSDKNE	human frog zebrafish sea urchin fruit fly beetle mosquito hydra

Supplementary Figure S9 Structure-based multiple sequence alignment of Lamtor4 orthologues from various species.  $\alpha$  helices and  $\beta$  sheets are shown as red cylinders and yellow arrows, respectively. Every ten residue of human Lamtor4 is marked. Conserved residues are shaded in pink. Residues mediating interactions with Lamtor1, Lamtor5 (by side-chains), and Lamtor5 (by main-chains) are indicated by green arrows, red arrows, and red arrowheads, respectively.



Supplementary Figure S10 Structure-based multiple sequence alignment of Lamtor5 orthologues from various species.  $\alpha$  helices and  $\beta$  sheets are shown as red cylinders and yellow arrows, respectively. Every ten residue of human Lamtor5 is marked. Conserved residues are shaded in pink. Residues mediating interactions with Lamtor1, Lamtor2, Lamtor4 (by side-chains), and Lamtor4 (by main-chains) are indicated by green arrows, magenta arrows, brown arrows, and brown arrowheads, respectively.



Supplementary Figure S11 The interaction interface between Lamtor2 and Lamtor3.  $a, \beta$  strands pairing between main-chains of Lamtor2 and Lamtor3 residues. b, van der Waals interactions and hydrogen bonds between side-chains of Lamtor2 and Lamtor3 residues. Carbon atoms of Lamtor2 and Lamtor2 are colored in magenta and cyan, respectively. Nitrogen and oxygen atoms are colored in blue and red, respectively. Hydrogen bonds are shown as black dashed lines.



Supplementary Figure S12 The C-terminal region of Lamtor1 is among the most conserved parts of the Ragulator surface. The sequence conservation of every subunit of the Ragulator complex is scored and mapped onto the surface of its structure, with red and cyan representing the highest and the lowest conservation scores, respectively.



Supplementary Figure S13 The 6×His tag on Lamtor1 (41-161) in the Ragulator complex and the 6×His tag on RagA in the RagA-RagC complex were indeed cleaved off after the cleavage reaction. a, After the cleavage reaction, the 6×His tag on Lamtor1 (41-161) in the Ragulator complex was indeed cleaved off. b, After the cleavage reaction, the 6×His tag on RagA in the RagA-RagC complex was indeed cleaved off.