**Supporting Information**

# C7orf59/Lamtor4 phosphorylation and structural flexibility modulate Ragulator assembly

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## Supporting Table 1. Mutagenic primers.

| *Mutation* | *Protein* | *Primer Sequence (5' – 3')* | *Type* |
|---|---|---|---|
| *K31* | p18 | CTCCATTGAGAGCTTAGGTAGGGGGGCTGC | Forward |
| *K31* | p18 | GCAGCCCCCCTACCTAAGCTCTCAATGGAG | Reverse |
| *G35* | p18 | GGGCTCGGCTCAATTGAGAGCTTTGGTAGGG | Forward |
| *G35* | p18 | CCCTACCAAAGCTCTCAATTGAGCCGAGCCC | Reverse |
| *K60* | p18 | ATGTTGCTGGCTGTCTAGGCAAGGATGGAAGAG | Forward |
| *K60* | p18 | CTCTTCCATCCTTGCCTAGACAGCCAGCAACAT | Reverse |
| *Q74* | p18 | CTCCATGCCCTATGAGTCTGCAGCAGACACATC | Forward |
| *Q74* | p18 | GATGTGTCTGCTGCAGACTCATAGGGCATGGAG | Reverse |
| *E80* | p18 | CGGTCCATGTACTAATGCTGCTCCATGCCC | Forward |
| *E80* | p18 | GGGCATGGAGCAGCATTAGTACATGGACCG | Reverse |
| *K103* | p18 | CGGTGGCAGCTTCTACCAATGGGTCAGGC | Forward |
| *K103* | p18 | GCCTGACCCATTGGTAGAAGCTGCCACCG | Reverse |
| *L108* | p18 | GCTGGTAAGAGACGGCTACGGTGGCAGCTTCTTC | Forward |
| *L108* | p18 | GAAGAAGCTGCCACCGTAGCCGTCTCTTACCAGC | Reverse |
| *Q130* | p18 | TCCTGGAGACCTGCTACAAATCAGAGAACGGG | Forward |
| *Q130* | p18 | CCCGTTCTCTGATTTGTAGCAGGTCTCCAGGA | Reverse |
| *D149* | p18 | AACCAGCTCCTCTTTTGCCTACACACGGATCTGAGAAAG | Forward |
| *D149* | p18 | CTTTCTCAGATCCGTGTGTAGGCAAAAGAGGAGCTGGTT | Reverse |
| *E34A/N35A* | C7orf59 | GGCTGCCTGCTCATCAGCCGCCAGGTCCCCAGATGA | Forward |
| *E34A/N35A* | C7orf59 | TCATCTGGGGACCTGGCGGCTGATGAGCAGGCAGCC | Reverse |
| *D36A/E37A* | C7orf59 | CACTGGCTGCCTGCGCAGCATTCTCCAGGTCC | Forward |
| *D36A/E37A* | C7orf59 | GGACCTGGAGAATGCTGCGCAGGCAGCCAGTG | Reverse |

| | | | |
|---|---|---|---|
| *S67D* | C7orf59 | GTTCTCCAAAGACCACATCCAGGCGCTTGAAGGGCA | Forward |
| *S67D* | C7orf59 | TGCCCTTCAAGCGCCTGGATGTGGTCTTTGGAGAAC | Reverse |
| *S67A* | C7orf59 | CTCCAAAGACCACAGCCAGGCGCTTGAAGGG | Forward |
| *S67A* | C7orf59 | CCCTTCAAGCGCCTGGCTGTGGTCTTTGGAG | Reverse |
| *C7orf59ΔN* | C7orf59 | CTCGGCTACCTGGTACTGAGTGAAGGT | Forward |
| *C7orf59ΔN* | C7orf59 | GGGCCCCTGGAACAGAACTTCCAGGCT | Reverse |

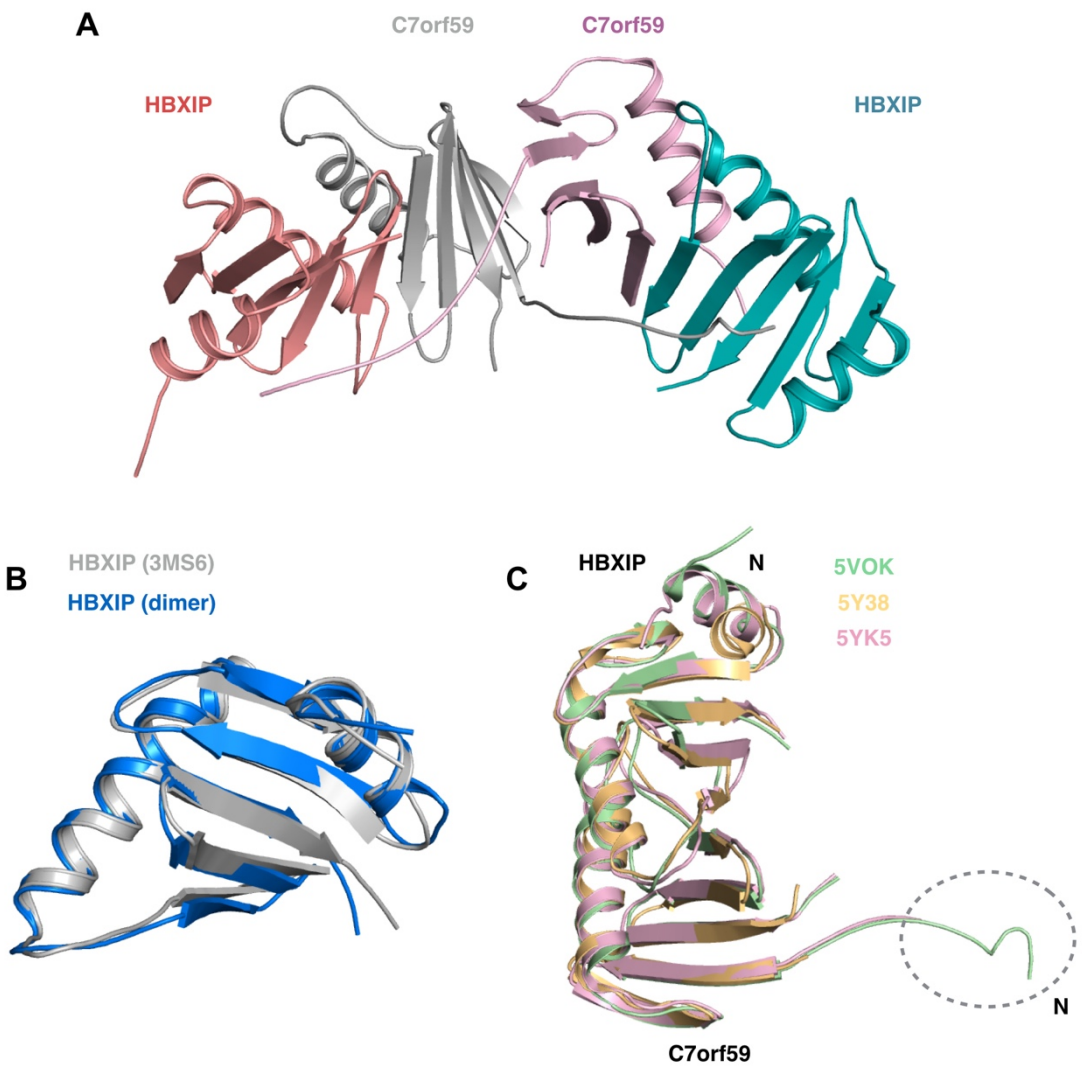


**Figure S1.** Packing of the dimers in the crystal lattice stabilizes the flexible N-terminal region of C7orf59. Two of the four dimers present in the asymmetric unit are shown. **B:** Superposition of the HBXIP structure reported previously (PDB:3MS6, in grey) and the HBXIP structure from this study in the complex with C7orf59 (blue) with rmsd = 0.622, indicating that it does not undergo major conformational changes upon binding to C7orf59. **C:** Superposition of the crystal structures of HBXIP-C7orf59 dimer from this study (5VOK), Zhang et al (5Y38) and Mu et al (5YK5). The dotted line indicates residues 1–8 of C7orf59 which are visible in the electron density of 5VOK. "N" indicates the N-terminal extremity of each monomer.

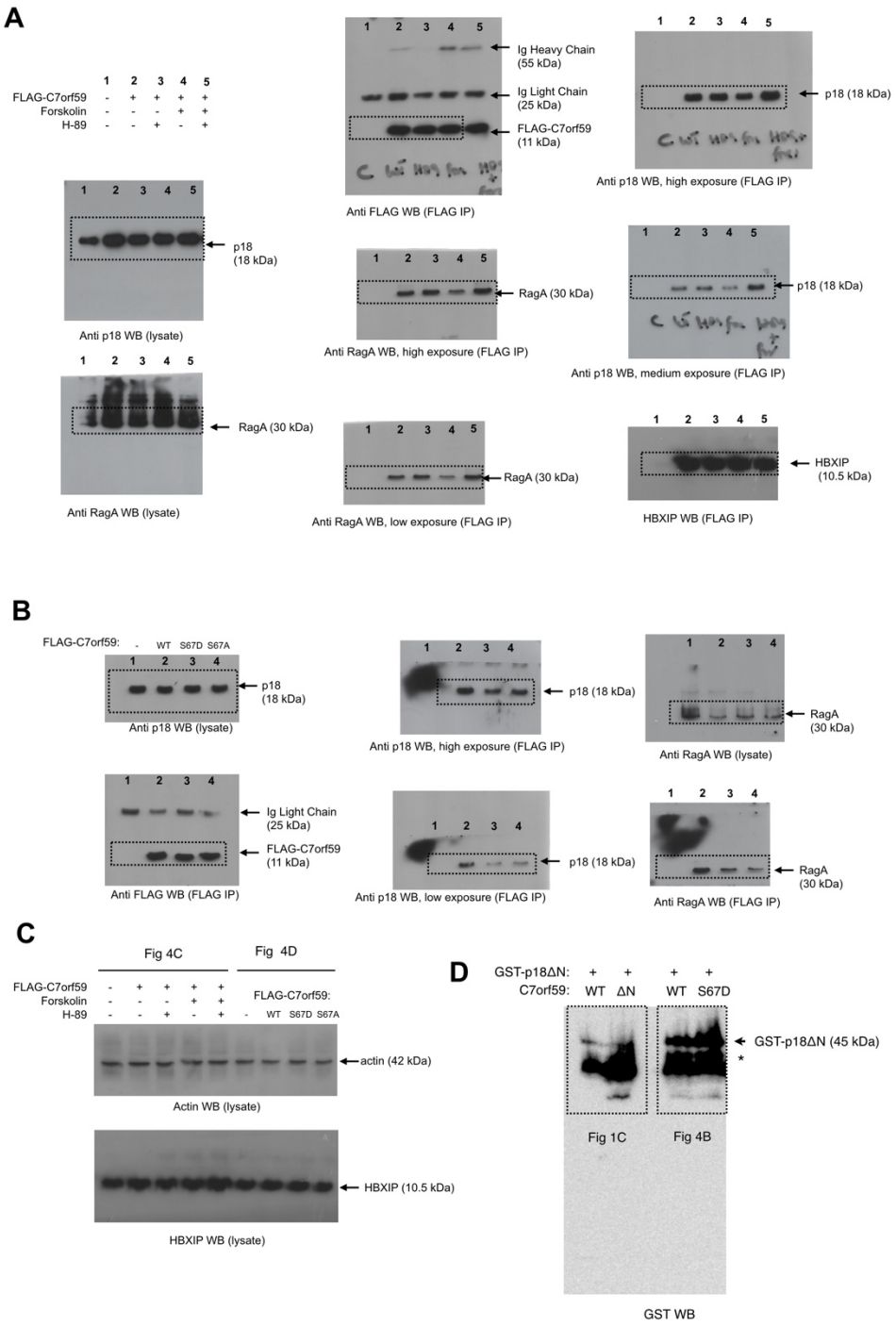


**Figure S2.** Uncropped versions of Western Blots shown in Fig. 4C (**A**), 4D **(B)**, 4 C/D **(C)** and Fig. 1C/4B **(D)**.

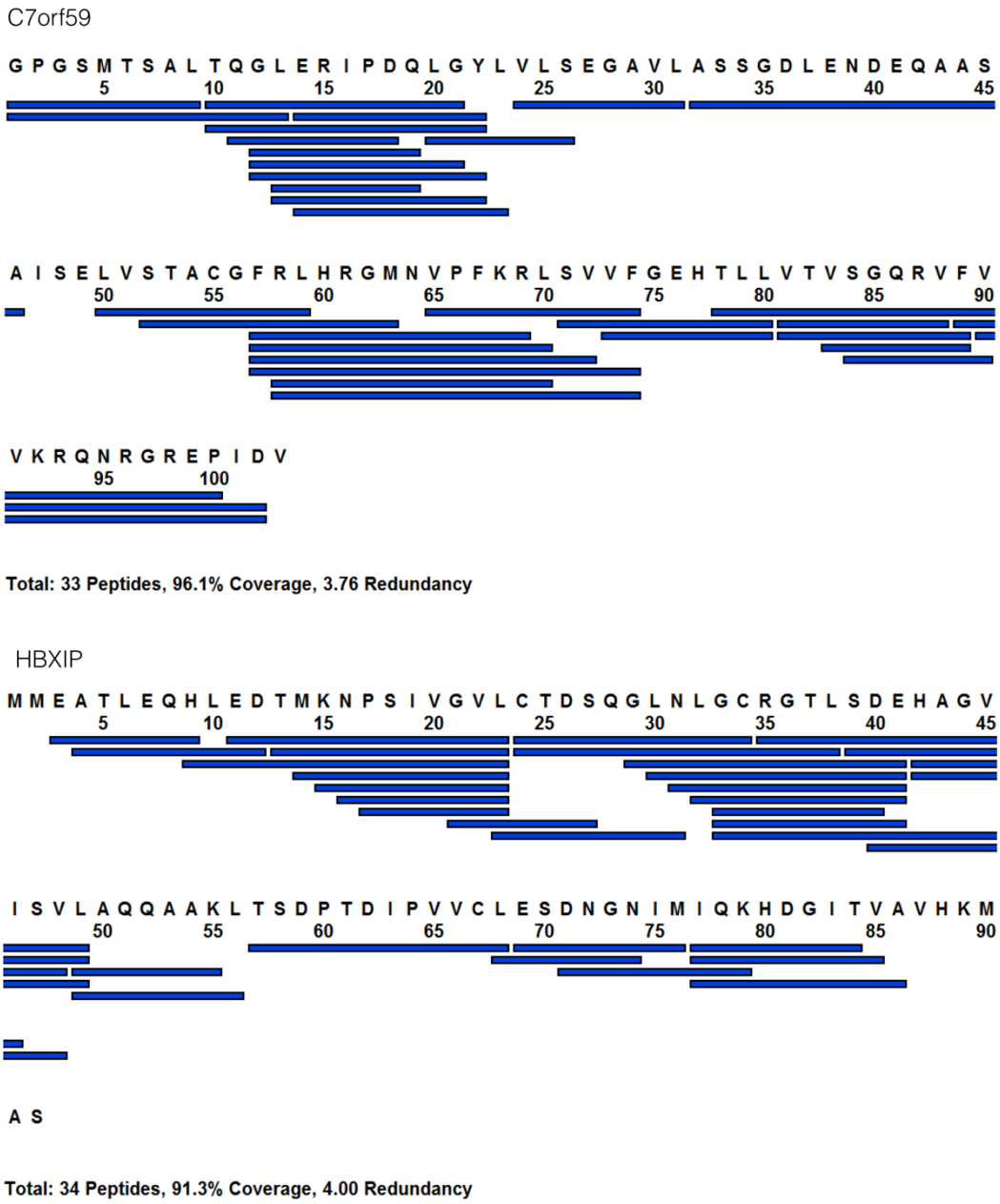


**Figure S3.** Sequence coverage of HBXIP and C7orf59 in the HDX-MS experiment.

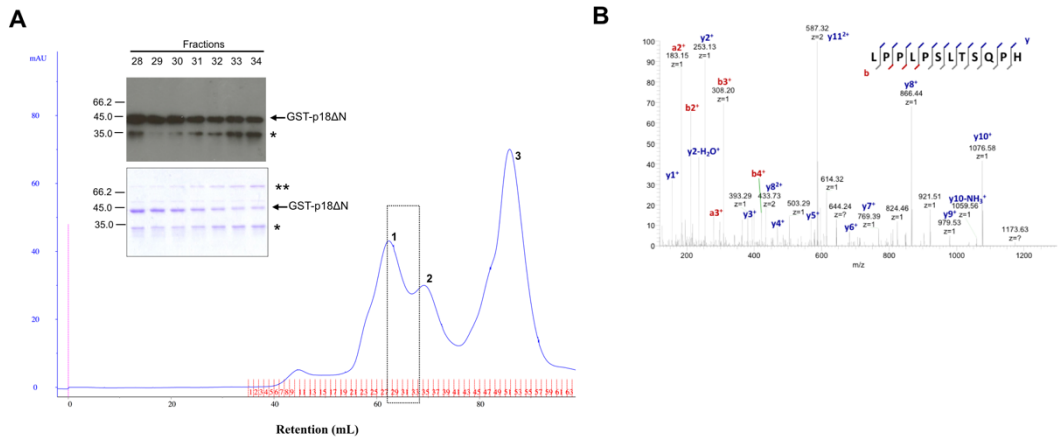


**Figure S4.** Identification of p18 degradation products after spontaneous proteolysis and dissociation of the GST-p18ΔN/HBXIP-C7orf59 complex. **A:** Size exclusion elution profile of the GST-p18ΔN + HBXIP-C7orf59 complex on a Superdex 200 16/60 column and identification of the ˜35 kD band, identified by an asterisk, as GST-positive. The inset shows GST Western blot (upper panel) and Coomassie-stained SDS-PAGE analysis of selected fractions highlighted by the rectangle. Another unidentified band migrating approximately at 66 kD, marked with a double asterisk, was not recognized by the GST antibody and probably corresponds to a contaminant from *E. coli*. **B:** Mass spectrometry analysis of one of the HBXIP-C7orf59-bound nontryptic peptides ($_{105}$LPPLPSLTSQPH$_{116}$) detected from peak 3.

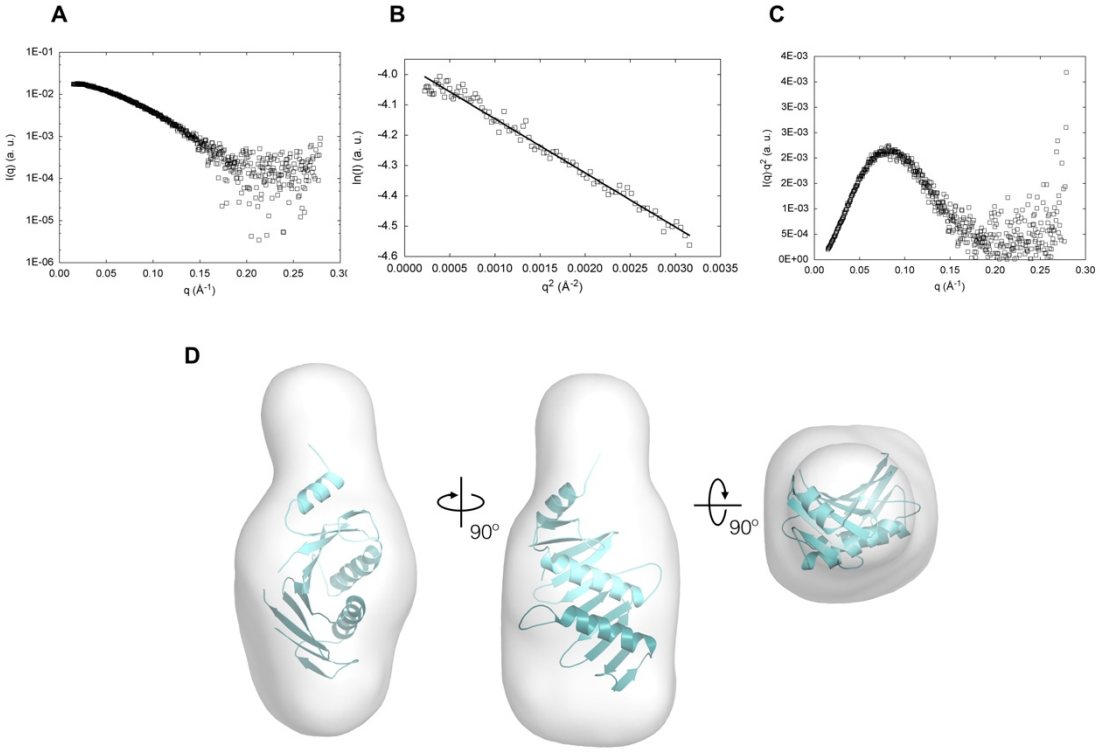


**Figure S5.** Small angle X-ray scattering analysis of the HBXIP-C7orf59 dimer bound to p18-derived peptide, obtained from spontaneous dissociation of the HBXIP-C7orf59/GST-p18ΔN trimer. **A:** Scattering curve **B:** Guinier region. **C:** Krakty plot. **D:** SAXS envelope fitted to the HBXIP-C7orf59 dimer. The flexible N-terminal residues of C7orf59 were removed for this analysis. HBXIP is in light cyan and C7orf59 is in dark cyan.

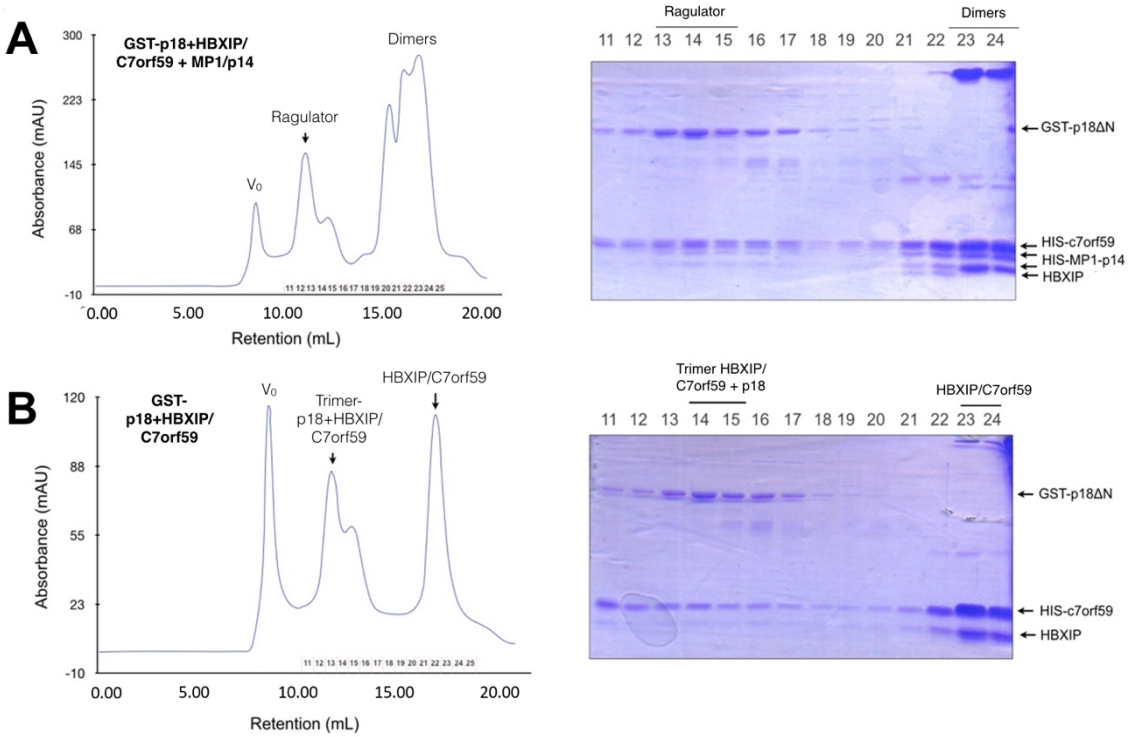


**Figure S6. A:** Size exclusion chromatography showing assembly of pentameric Ragulator from recombinant, affinity-purified dimer and trimer. Elution profile at 280 nm (left panel) and 16% SDS-PAGE analysis of the fractions eluted from Superdex 200 16/10 size exclusion column (right panel), starting from the affinity-purified MP1-p14 dimer and HBXIP-C7orf59/GST-p18ΔN trimer. **B**: Control run of size exclusion chromatography of HBXIP-C7orf59/GST-p18ΔN trimer under the same conditions, using the same trimer sample that was used for the assembly of pentamer showed above. Left panel: UV (280 nm) elution profile. Right panel: SDS-PAGE analysis of the trimer and dimer fractions.