Supplementary Information

The PB1 and the ZZ domain of the autophagy receptor p62/SQSTM1 regulate the interaction of p62/SQSTM1 with the autophagosome protein LC3B

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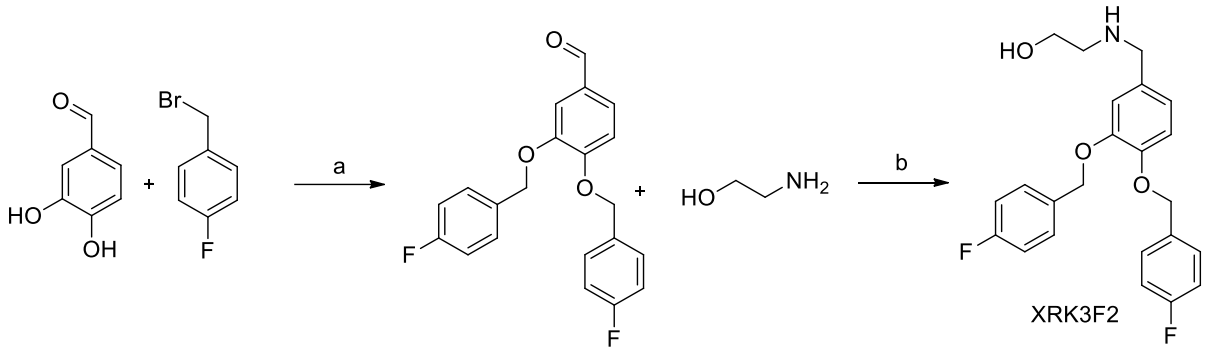
Running Title: Regulation of p62/SQSTM1 interaction with LC3B

Supplementary Methods

Synthesis of XRK3F2

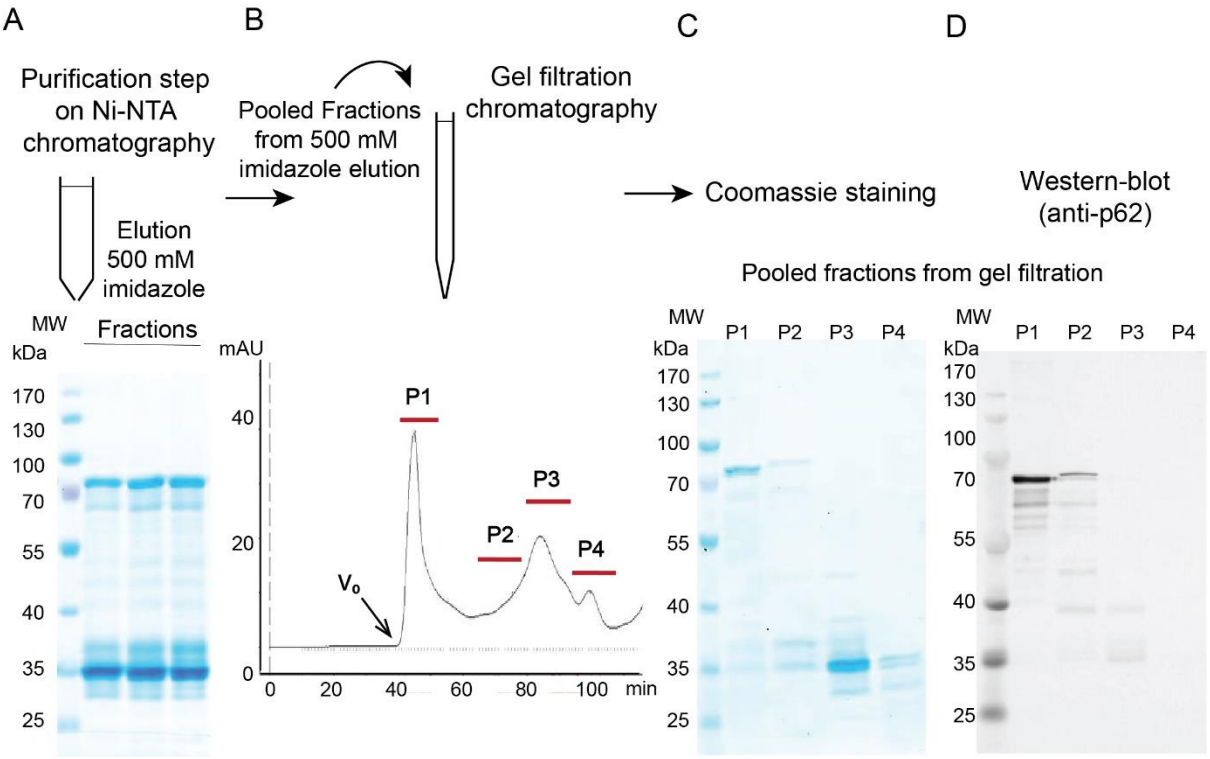


Procedure a. 3,4-Dihydroxybenzaldehyde (276 mg, 2 mmol) was dissolved in acetonitrile (20 ml) and then cesium carbonate (1.63 g, 5 mmol, 2.5 eq) was added. After brief stirring, 4-fluorobenzyl bromide (0.55 ml, 4.4 ml, 2.2 eq) was added and the reaction mixture was stirred overnight at room temperature. After complete reaction, the reaction mixture was filtered and the filtrate was concentrated on the rotary evaporator. The residue was absorbed on Celite and purified by flash chromatography. **Yield:** 653 mg (1.84 mmol, 92%). **^{1}H-NMR** (400 MHz, DMSO-d_6):δ= 9.82 (s, 1H, CHO), 7.48-7.57 (m, 6H, H_{ar}), 7.20-7.30 (m, 5H, H_{ar}), 5.25 (s, 2H, O-CH_2), 5.19 (s, 2H, O-CH_2) ppm

Procedure b. A solution of 3,4-bis[(4-fluorophenyl)methoxy]benzaldehyde (652 mg, 1.84 mmol) in methanol (35 ml) was cooled in an ice bath. 2-Aminoethanol (101 mg, 1.66 mmol, 0.9 eq) was then added, and the mixture was stirred at room temperature for 8 h. Sodium borohydride (139 mg, 3.68 mmol, 2.0 eq) was added slowly to the reaction mixture, which was maintained at 8 °C by using an ice bath. After the addition, the mixture was stirred at room temperature for 12 h. The reaction was quenched by the addition of aqueous HCl (6 M) with cooling until the pH was adjusted to 4. The solvent was removed under reduced pressure. The residue was dissolved in water (25 ml) and washed three times with dichloromethane (25 ml each) to remove organic impurities. The aqueous phase was separated and its pH was adjusted to 10 by using solid sodium carbonate. It was then extracted with dichloromethane (35 ml). The organic portion was dried over anhydrous magnesium sulphate and evaporated in vacuo to give the product XRK3F2 as a colorless oil. The crude product was purified by preparative HPLC. **Yield:** 540 mg (1.35 mmol, 82%). **MS** (ESI+) *m/z*: 400,08 $[M+H]^+$. **^{1}H-NMR** (400 MHz, DMSO-d_6):δ= 8.32 (s, 1H, NH), 7.45-7.51 (m, 4H, H_{ar}), 7.18-7.23 (m, 5H, H_{ar}), 7.03 (d, *J* = 8.4 Hz, 1H, H_{ar}), 6.93 (dd, *J* = 2,0 Hz/8,4 Hz, 1H, H_{ar}), 5.09 (s, 2H, O-CH_2), 5.08 (s, 2H, O-CH_2), 3.82 (s, 2H, **CH_2**-NH), 3.54 (t, *J* = 5.6 Hz, 2H, CH_2-OH), 2.69 (t, *J* = 5.6 Hz, 2H, NH-CH_2) ppm. **^{13}C-NMR** (101 MHz, DMSO-d_6): δ= 165.0, 161.7 (d, *J* = 243.6 Hz), 161.6 (d, *J* =

243.5 Hz), 147.9, 147.6, 133.4 (d, *J* = 3.0 Hz), 133.3 (d, *J* = 3.0 Hz), 129.7 (d, *J* = 8.3 Hz), 129.6 (d, *J* = 8.3 Hz), 121.9, 115.4, 115.1 (d, *J* = 21.4 Hz), 115.0 (d, *J* = 21.4 Hz), 114.3, 69.4, 69.3, 58.3, 50.9, 49.3 ppm.

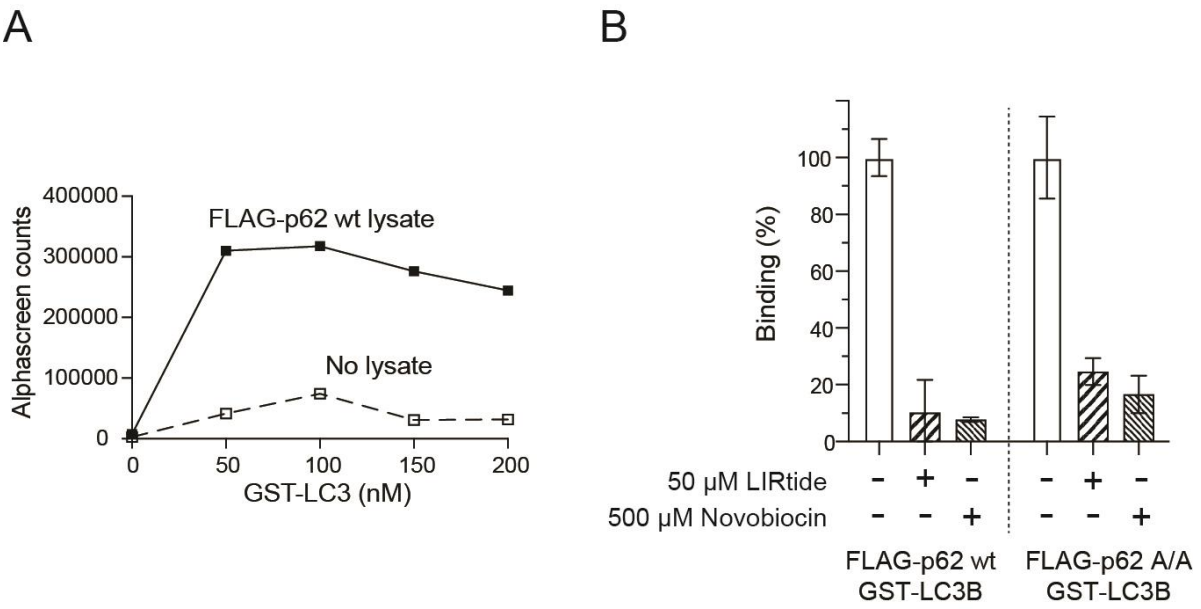
Supplementary Figure 1.



Supplementary Figure 1. Size exclusion chromatography of purified His-Trx-p62/SQSTM1. Analysis of the oligomerization stage of p62/SQSTM1 employed in this study. The His-Trx-p62/SQSTM1 expressing the wild type sequence could not be eluted from Ni-NTA resin. **A)** The mutant protein His-Trx-p62/SQSTM1 A/A with key Lys and Asp residues at both “sides” of the PB1 domain elutes from Ni-NTA chromatography (400-500 mM imidazole). Fractions from elution with 500 mM imidazole are separated by SDS-PAGE and stained by Coomassie brilliant blue. **B)** The protein obtained from (A) was subjected to gel filtration/size exclusion chromatography (Superdex S200 HiLoad 16/60). Elution profile from size exclusion chromatography revealed a large peak of protein eluting close to V_0, implying a large MW, as well as low MW proteins. **C-D)** The different aliquots corresponding to the peaks from (B) were analyzed by SDS-PAGE and stained with Coomassie brilliant blue (C) and western-blot with anti-p62/SQSTM1 antibodies (D), confirm that His-Trx-p62/SQSTM1 A/A retains the ability to form oligomers, as it elutes from size exclusion chromatography as a large oligomer. The

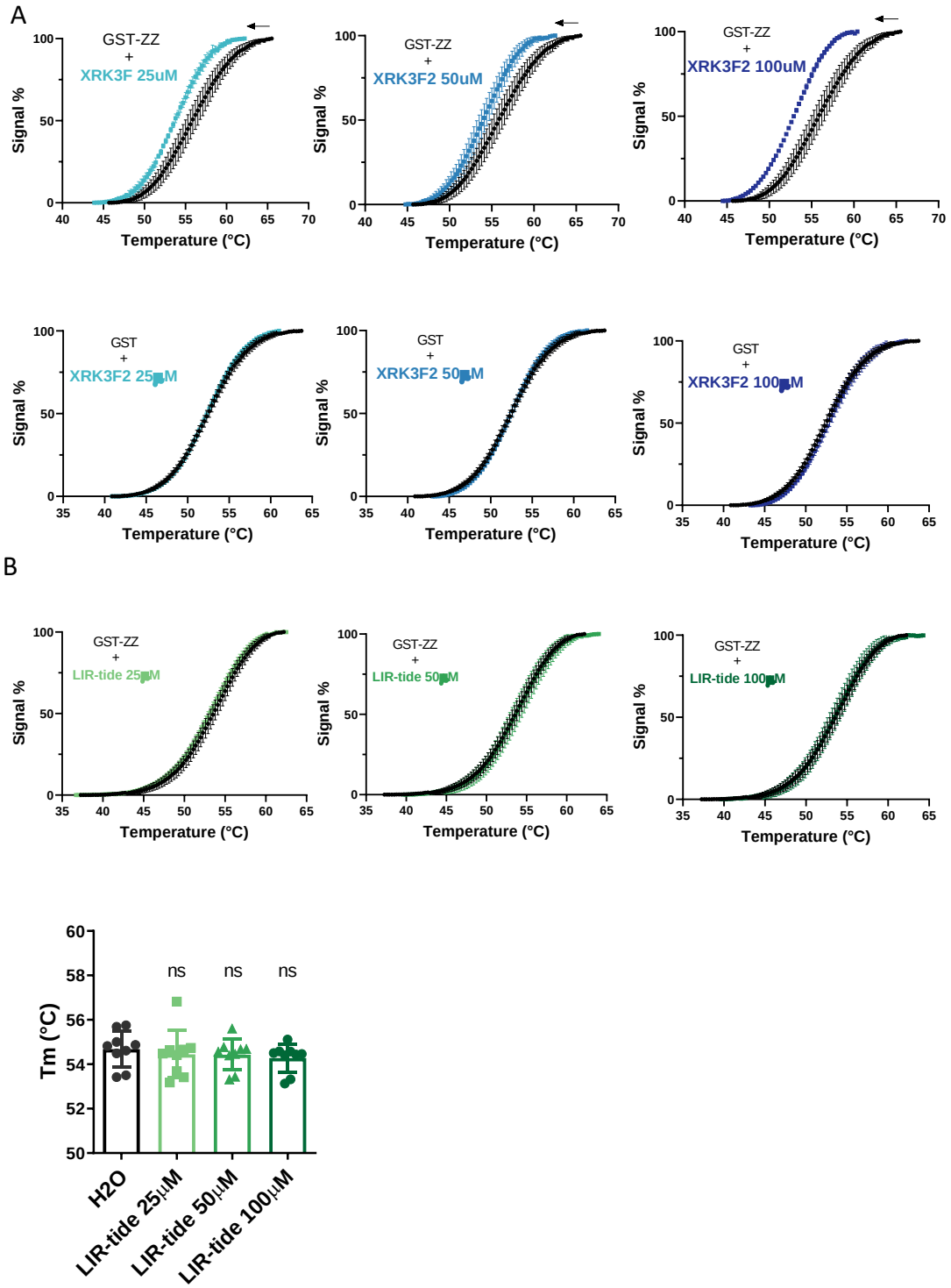
theoretical mass of p62/SQTM1 is approximately 47 kDa. However, in SDS-PAGE of mammalian extracts, the protein migrates with an apparent higher molecular weight, corresponding to 62 kDa. Similarly, the bacterially expressed His-Trx-p62/SQTM1 protein also exhibits an apparent larger size in SDS-PAGE compared to the expected theoretical prediction.

Supplementary Figure 2



Supplementary Figure 2. Further characterization of the AlphaScreen interaction assay between GST-LC3B and non-purified FLAG-p62/SQTM1 constructs. A) Dependancy of GST-LC3B and FLAG-p62/SQTM1 wt to oberve signal. **B)** The interaction between GST-LC3B and non-purified FLAG-p62/SQTM1 forms are displaced by LIRtide and Novobiocin.

Supplementary Figure 3.



Supplementary Figure 3. XRK3F2 binds to the ZZ domain of p62/SQSTM1. Temperature stability assay in the absence or presence of the indicated concentrations of ligands. **A)** Top: Melting curves of purified ZZ domain in the presence of XRK3F2. Graphic depicts 3 replicates of 1 out of 3 independent experiments. Results expressed as Mean ± SD. Bottom**:** Melting

curves of purified GST in the presence of XRK3F2. Graphic depicts 3 replicates of 1 out of 3 independent experiments. Results expressed as Mean ± SD. **B)** Top: Melting curves of purified ZZ domain in the presence of LIR-tide showing lack of interaction of LIRtide with GST- ZZ domain. Graphic depicts 3 replicates of 1 out of 3 independent experiments. Results expressed as Mean ± SD. Bottom: Quantification of melting temperatures of 3 independent experiments. Results expressed as Mean ± SD with n.s (non-significant) by one-way ANOVA followed by Bonferroni multiple comparison test.