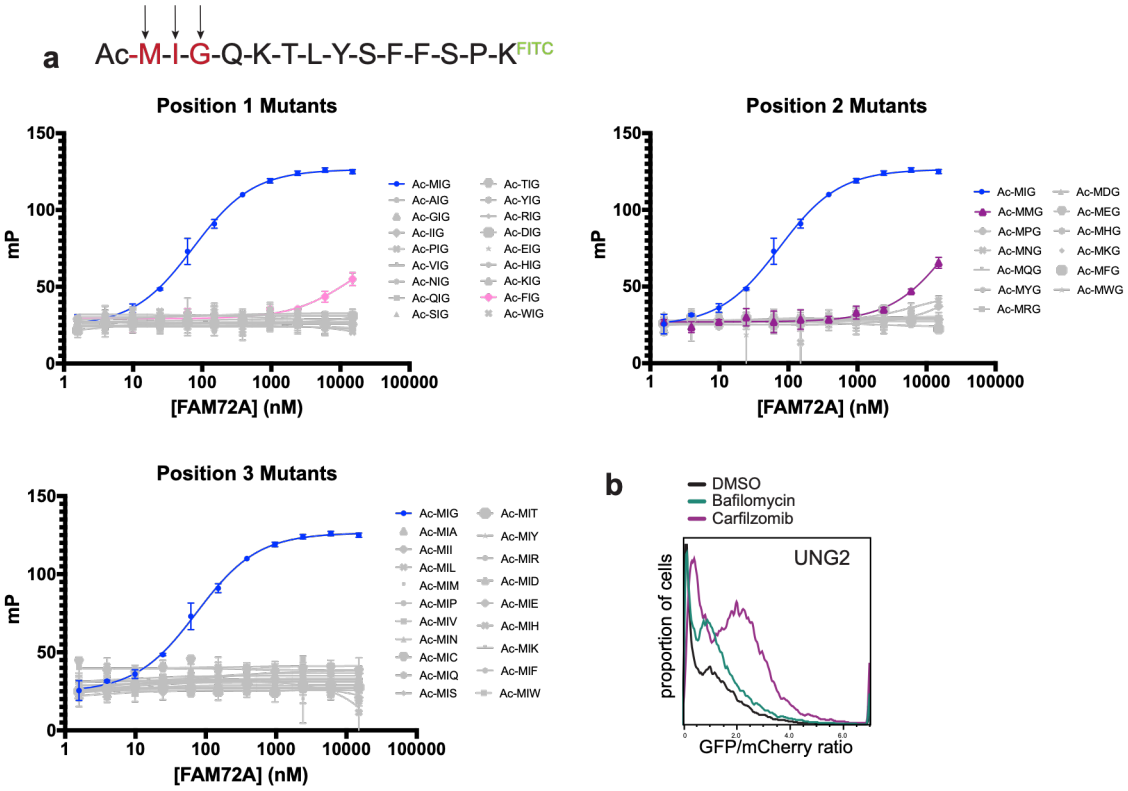
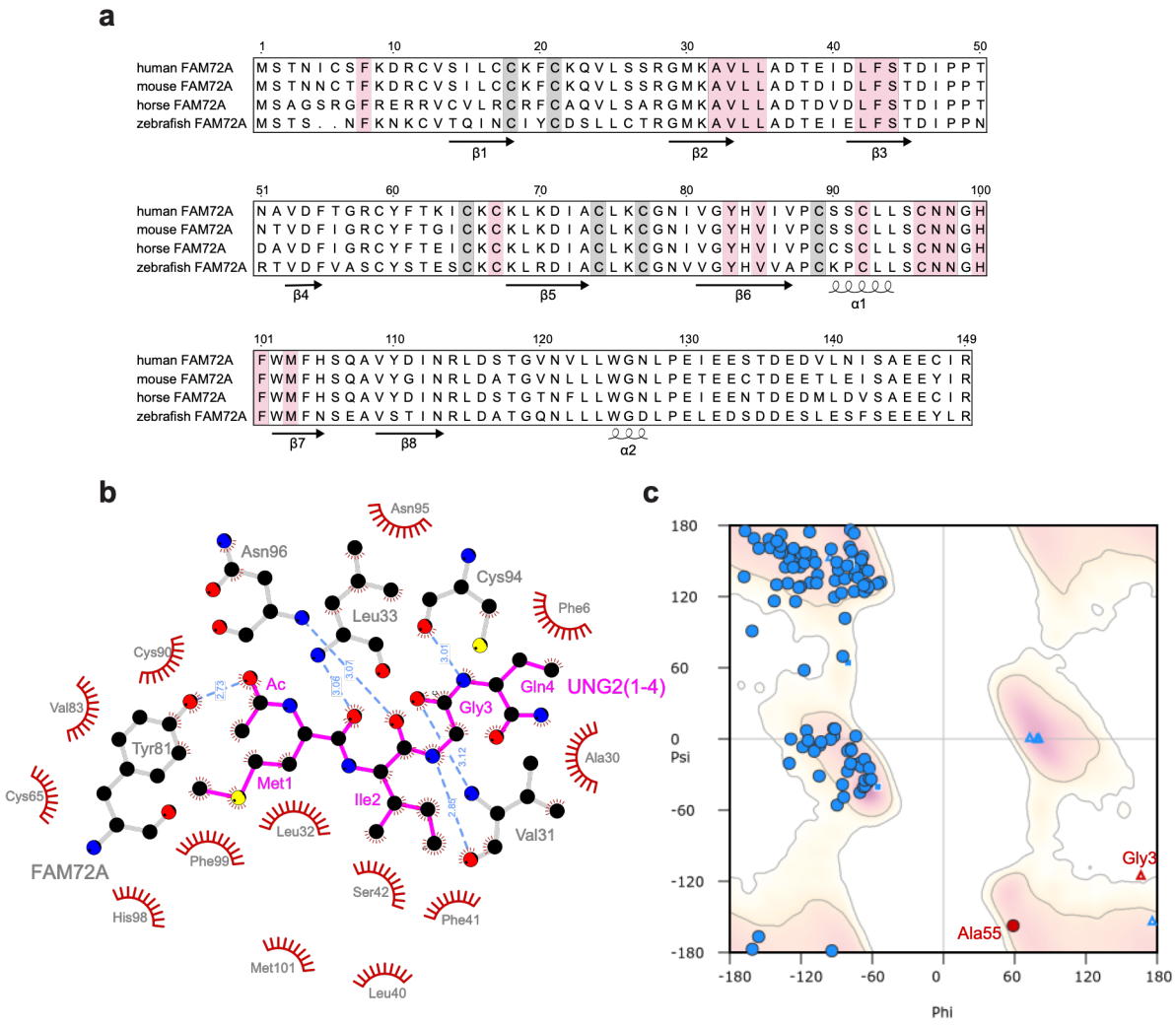
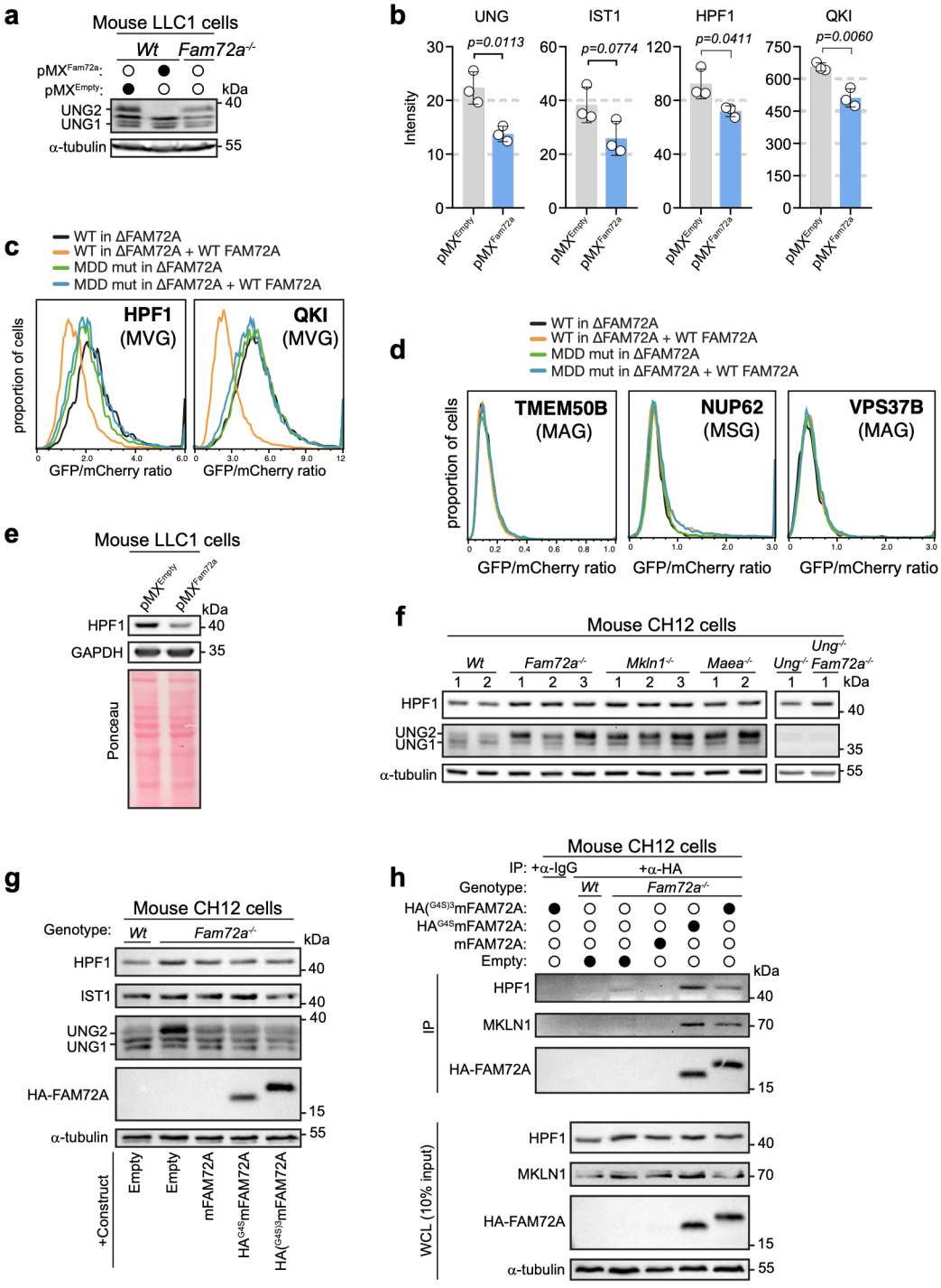


Supplementary Fig. 1: a Associated profiles of zFAM72A non-binding mutants in fluorescence polarization assay in Figure 2a. **b.** UNG2 stability was assessed using the UNG2-GFP-IRES–mCherry reporter following treatment with bafilomycin (700 nM) or carfilzomib (2 μM) for 6 h. Flow cytometry profiles show the distribution of GFP/mCherry fluorescence ratios, with higher ratios indicating increased UNG2 reporter stability.



Supplementary Fig. 2: X-ray crystal Structure of zFAM72A.

a. Sequence alignment of human, mouse, horse and zebrafish FAM72A orthologues. Residues lining the degron binding pocket are invariantly conserved and highlighted in pink. Cysteines coordinating Zn^{2+} ions are invariantly conserved and highlighted in grey. **b.** 2D representation of hydrogen bonding (blue dashed line) and hydrophobic interactions (red spokes) between zFAM72A (grey) and the UNG2(1-4) degron peptide (magenta). Figure was prepared using Ligplot. **c.** Ramachandran plot of the refined zFAM72A and UNG2(1-4) x-ray crystal structure. The plot was generated using Coot based on the coordinates of PDB entry (39LZ).



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22 **Supplementary Figure 3. IST1, QKI and HPF1 are novel substrates of FAM72A-MKLN1-**

23 **CTLH.**

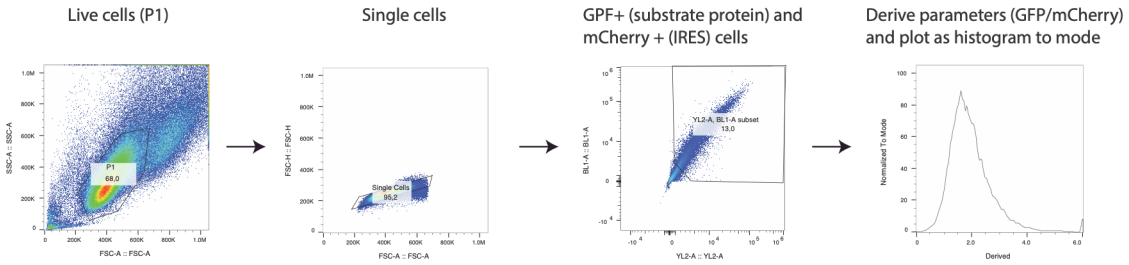
a. Western blotting of UNG from whole cell lysates of WT LLC1 cells retrovirally transduced with a pMX-PIE-FAM72A expression construct (pMX^{Fam72a}) or empty vector control (pMX^{empty}) and *Fam72a*$^{-/-}$ LLC1 cells. **b.** Raw intensity values of UNG, IST1, HPF1, and QKI from the LLC1 whole proteome screen. **c.** HPF1 and QKI stability was assessed by flow cytometry using GFP fusion reporters with IRES-driven mCherry expression in FAM72A-knockout cells, with or without re-expression of wild-type FAM72A. Profiles show GFP/mCherry fluorescence ratios for wild-type HPF1 and QKI, both bearing an N-terminal MVG sequence, and their corresponding MDD degron mutants. **d** same as **c**. for wild-type TMEM50B (MAG), NUP62 (MSG) and VPS37B (MAG) and their corresponding MDD N-terminal mutants. **e.** Western blotting of HPF1 and GAPDH as control from lysates of pMX^{empty} and pMX^{Fam72a} LLC1 cells. **f.** Western blotting of HPF1 and UNG from lysates of mouse CH12 cells of the indicated genotype. **g.** Western blotting of lysates from Wt and *Fam72a*$^{-/-}$ CH12 cells retrovirally transduced with the indicated pMX-PIE construct. Short $(G4S)_1$ and long $(G4S)_3$ flexible linkers were included in the HA-tagged FAM72A constructs. **h.** Co-immunoprecipitation of HA-tagged FAM72A in Wt and *Fam72a*$^{-/-}$ cells transduced with the indicated constructs. Cells were treated with 20 μM MG132 3 hours prior to harvesting (n=1 independent experiments).

Fragment Ion	Observed m/z	Intensity (arb.)
b1	174.0583172	14828.41
b2	287.1424835	237636.5
b3	344.1640256	233711.5
b4	472.2239911	33497.29
b6	701.3661534	191188.5
b7	814.448547	65228.82
b9	1064.539888	7928.021
b10	1211.61621	8314.995
Fragment Ion	Observed m/z	Intensity (arb.)
y1	175.1187	127832.9
y2	246.1557	7311.741
y3	343.2094	161773.4
y4	430.2414	32792.74
y5	527.2947	554076.4
y6	614.3269	315016.1
y7	761.3957	245557.3
y8	908.4634	277923
y9	995.4951	655806.4
y10	1158.558	755164.9
y11	1271.642	525893.3
y12	1372.69	925484.7
y13	1500.783	247707.3
y14	1628.839	36680.38
y15	1685.862	111875.1
y16	1798.947	3260.376

Supplementary Table 1: Fragment intensities for the MS2 spectra (see **Figure 1**) of the N-terminal acetylated MIGQKTLYSFFSPAR peptide fragments compiled from the average of 19 data-dependent scans of the precursor ion 968.5003 m/z (the [M+2H]2+ ion of the peptide).

Supplementary Table 2 (Provided as separate file): Potential FAM72A substrates conforming to the cell-validated consensus [M]-[ILV]-G. ScanProsite [53] was used to search the motif in the UniProtKB (Swiss-Prot) Homo sapien and Mus musculus database excluding isoforms.

Supplementary Table 3 (Provided as separate file): List of plasmids, peptides, and primers used.



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78 **Supplementary Information 1:** Gating strategy for flow cytometry experiments