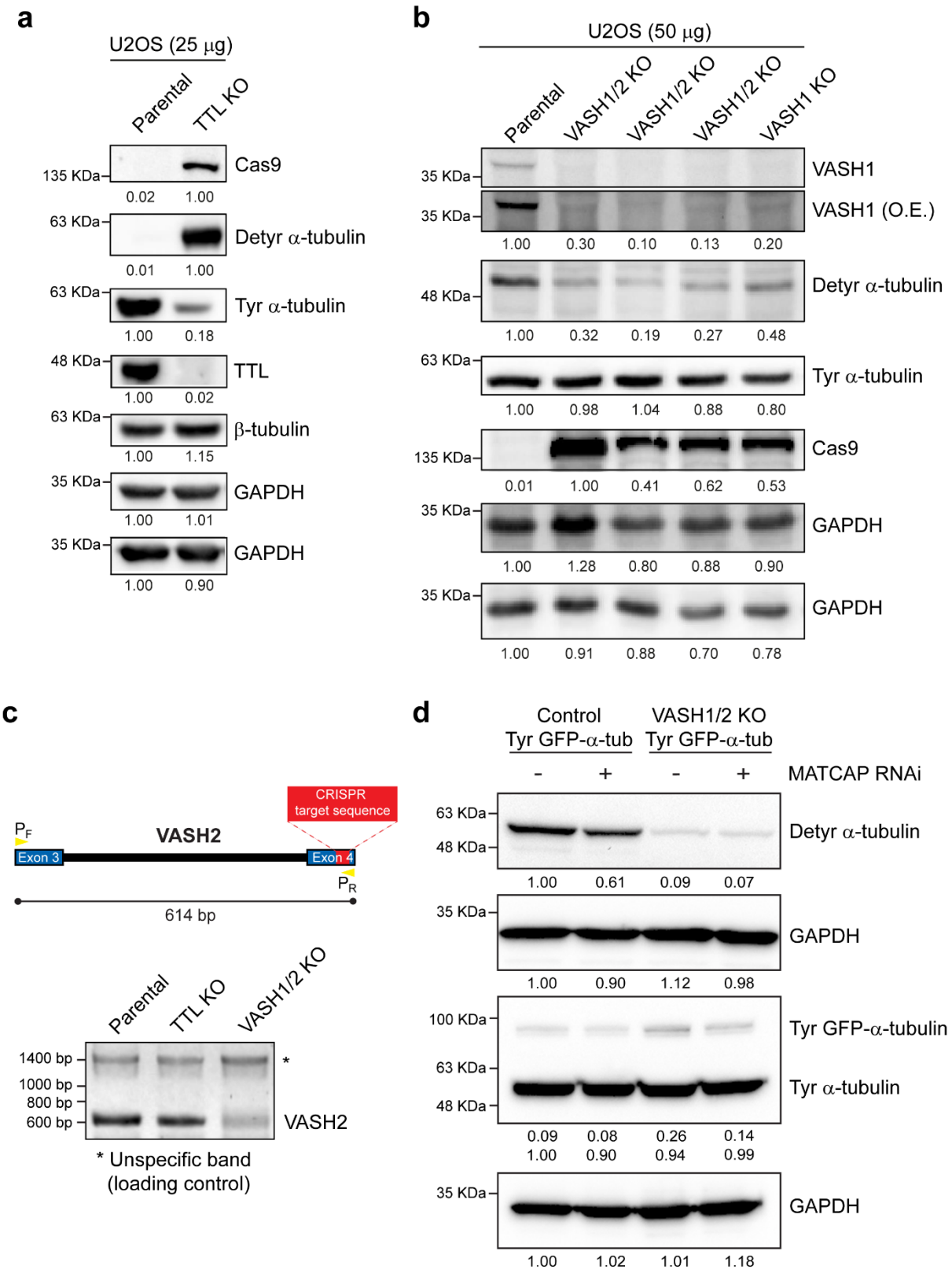
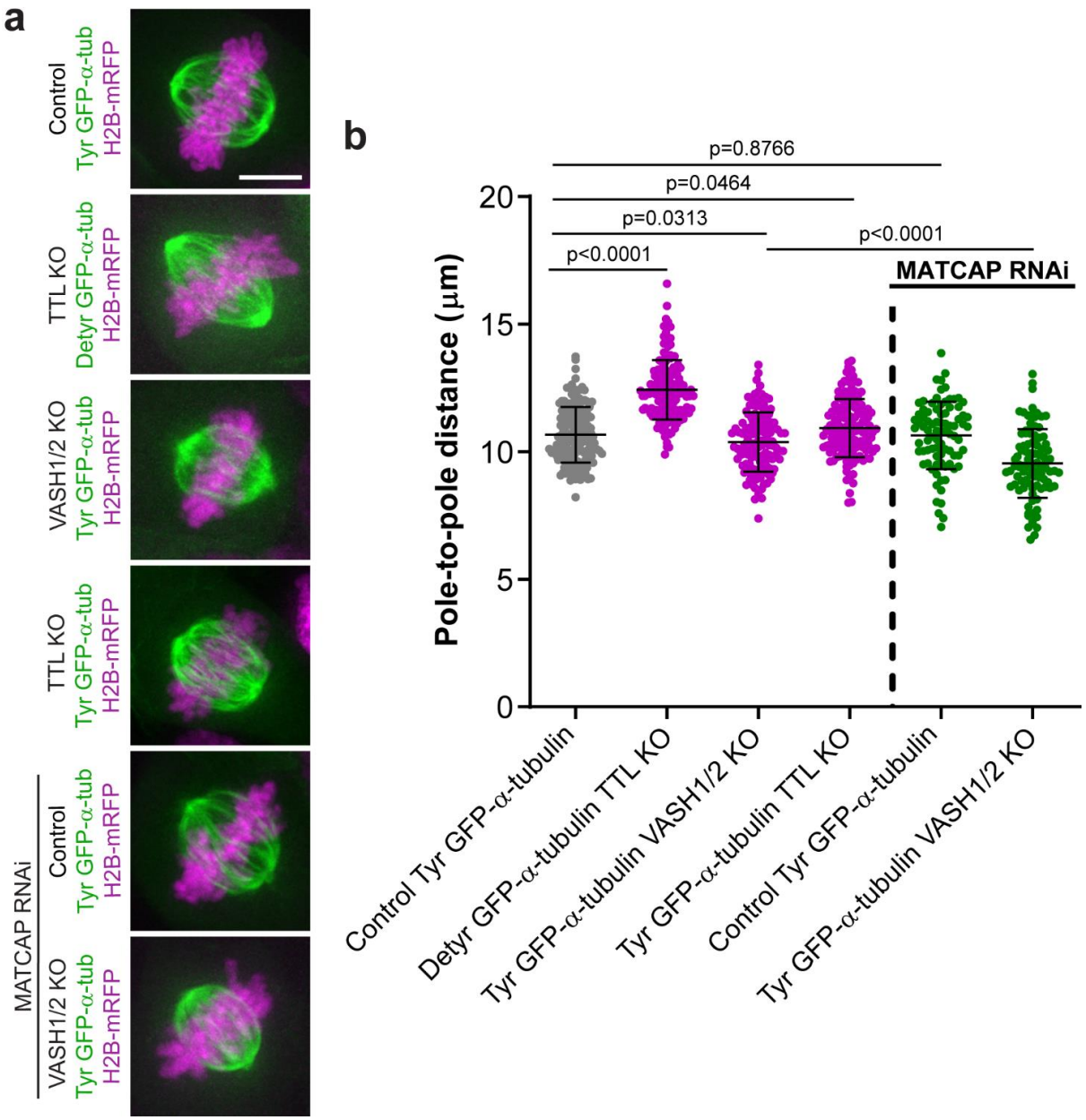
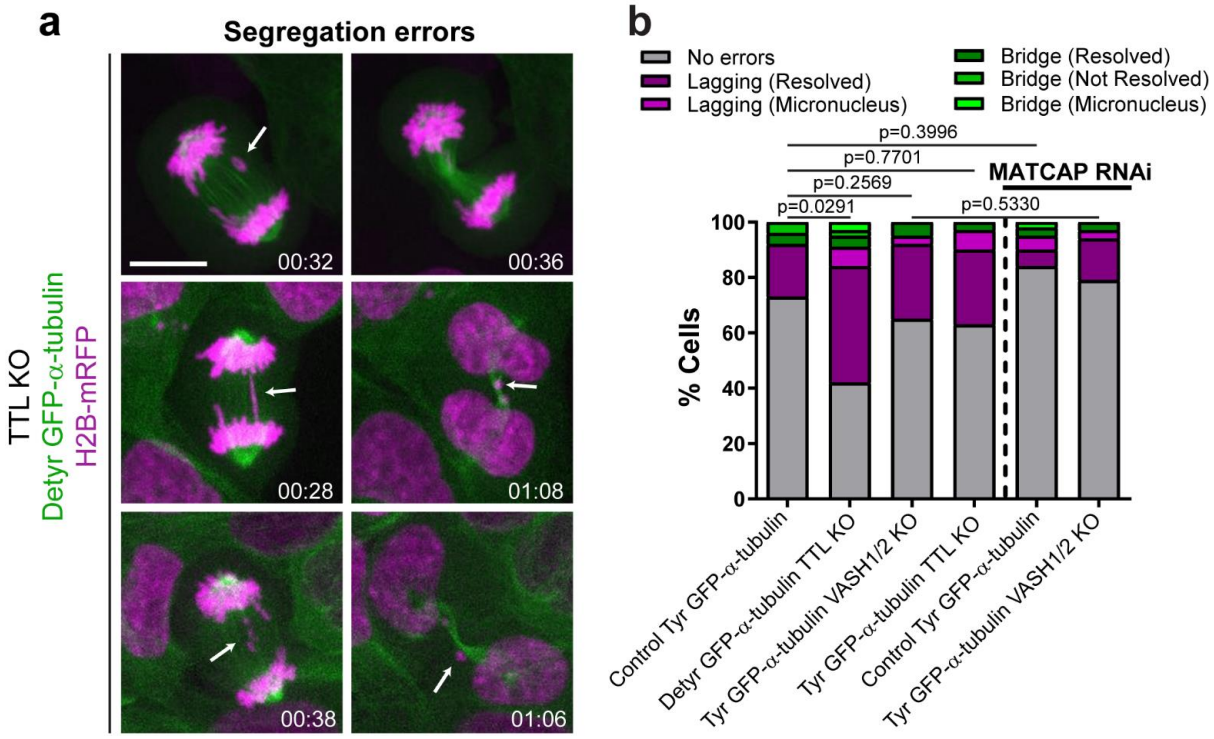


Supplementary Fig. 1. Generation of KO cell lines and characterization of combined perturbation of tubulin carboxypeptidases. (a) Western blot analysis (relative quantification, reference = 1.00) showing the validation of the generation of a non-clonal TTL knockout (KO) cell line. Note that the lack of detyrosinated (detyr) α-tubulin signal in parental cells was due to suboptimal loading of the gel (25 μg of

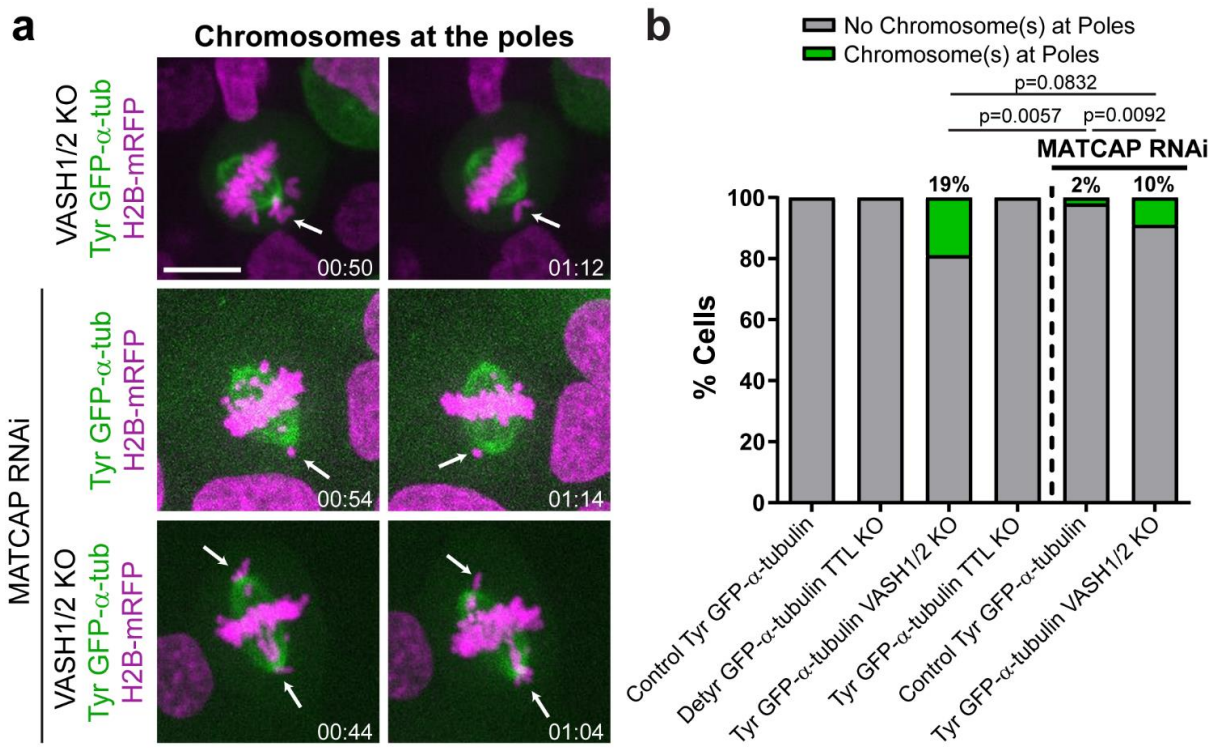
protein) to avoid signal saturation in the TTL KO line. **(b)** Western blot analysis (relative quantification, reference = 1.00) showing the validation of the generation of non-clonal VASH1 KO and VASH1/2 double KO cell lines (values refer to relative levels obtained from the illustrated western blot, which was qualitatively validated by at least 2 independent experiments). Several extracts from the double KO line are run in parallel for comparison. Note that VASH2 targeting could only be indirectly inferred by monitoring detyrosinated α-tubulin levels relative to the single VASH1 KO line due to the lack of a useful antibody against VASH2. O.E. - overexposed. **(c)** PCR analysis of the genomic locus of VASH2 covering the CRISPR-Cas9-targeted region in parental, as well as in non-clonal TTL KO and VASH1/2 KO lines. P_F and P_R indicate forward and reverse primers, respectively. bp – base pairs. **(d)** Western blot analysis of the expression levels (relative quantification, reference = 1.00) of detyrosinated and tyrosinated (tyr) α-tubulin in the Control Tyr GFP-α-tubulin and VASH1/2 KO Tyr GFP-α-tubulin cell lines without (scramble RNAi) and with MATCAP RNAi (values refer to relative levels obtained from the illustrated western blot, which was qualitatively validated by at least 2 independent experiments). GAPDH was used as loading control in all panels assembled from different gels that were run in parallel.



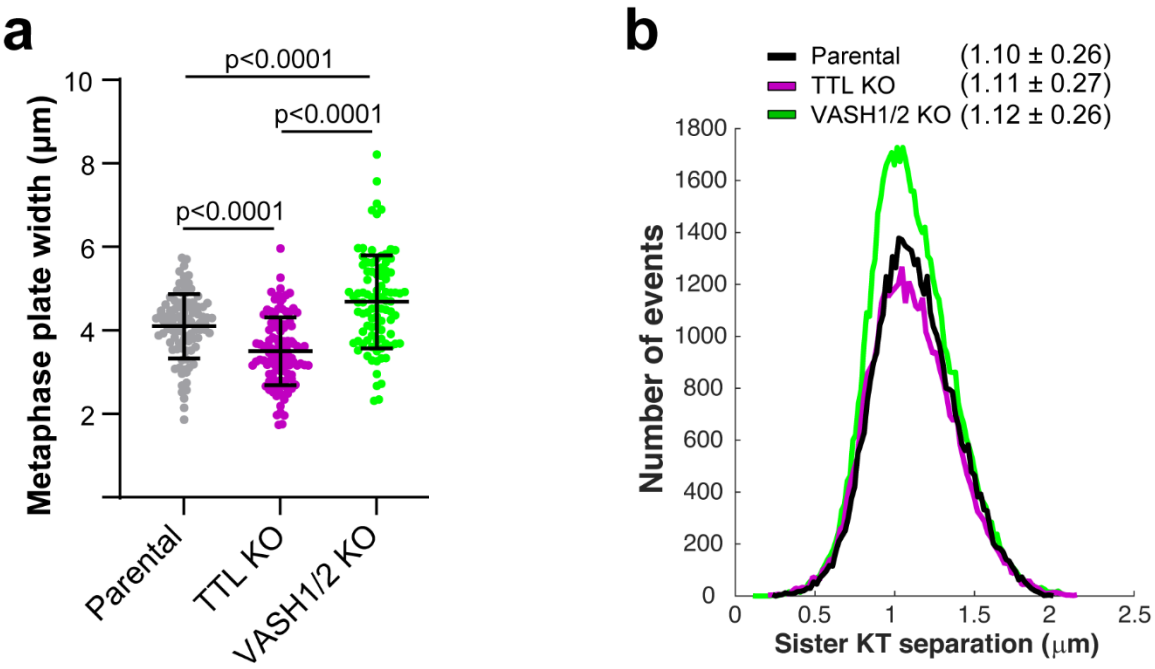
Supplementary Fig. 2. Genetic manipulation of α-tubulin detyrosination/tyrosination impacts mitotic spindle length. (a) Immunofluorescence analysis of spindle length in U2OS Control tyrosinated (Tyr) GFP-α-tubulin, TTL knockout (KO) detyrosinated (Detyr) GFP-α-tubulin, VASH1/2 KO Tyr GFP-α-tubulin and TTL KO Tyr GFP-α-tubulin cell lines in metaphase; also displayed Control Tyr GFP-α-tubulin and VASH1/2 KO Tyr GFP-α-tubulin cell lines after MATCAP RNAi; GFP-α-tubulin is in green and histone H2B-mRFP in magenta; tub – tubulin; scale bar is 5 µm. **(b)** Quantification of mitotic spindle length in each cell line; each data point represents an individual cell; bars represent mean and standard deviation; quantifications from a pool of 3 independent experiments: Control Tyr GFP-α-tubulin =160 cells, TTL KO Detyr GFP-α-tubulin = 161 cells, VASH1/2 KO Tyr GFP-α-tubulin = 126 cells, TTL KO Tyr GFP-α-tubulin = 141 cells, Control Tyr GFP-α-tubulin w/MATCAP RNAi = 84 cells, VASH1/2 KO Tyr GFP-α-tubulin w/MATCAP RNAi = 94 cells; exact p values are displayed above each respective data set; significance $p<0.05$, unpaired two-tailed t-test. Source data are provided as a Source Data file.



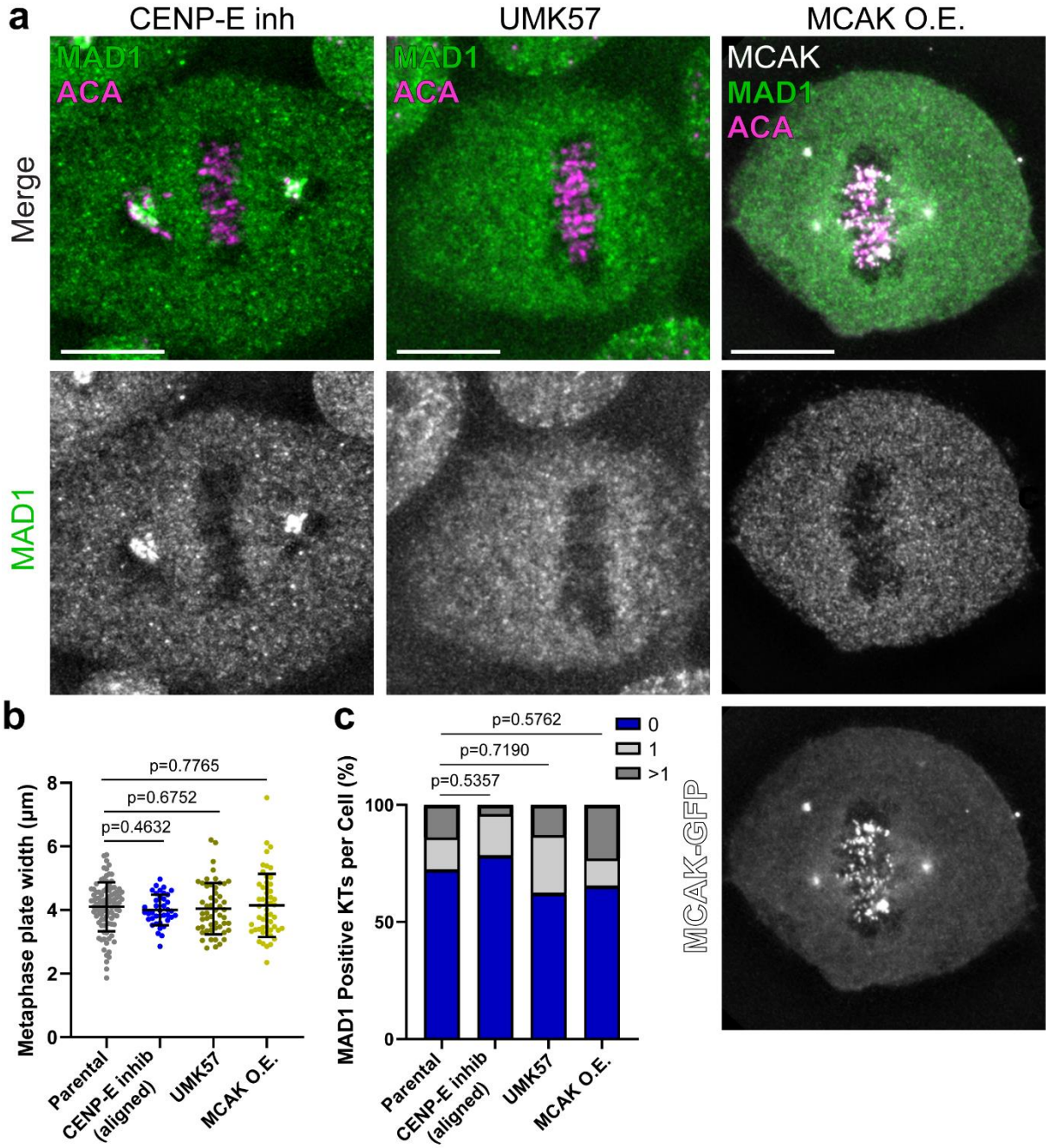
Supplementary Fig. 3. Genetic manipulation of α-tubulin detyrosination/tyrosination impacts segregation fidelity during human cell division. (a) Live-cell imaging of chromosomes and spindle microtubules, highlighting segregation errors (arrows) in the TTL knockout (KO); GFP-α-tubulin is in green and histone H2B-mRFP in magenta; time is in h:min from nuclear envelope breakdown; scale bar is 5 µm. **(b)** Quantification of the percentage of cells with anaphase segregation errors during live cell imaging for the same dataset as in Fig. 2b, c, with discrimination of each error type and fate (resolved vs. micronuclei); Control tyrosinated (Tyr) GFP-α-tubulin = 57 cells, pool of 16 independent experiments, TTL KO detyrosinated (Detyr) GFP-α-tubulin = 67 cells, pool of 18 independent experiments, VASH1/2 KO Tyr GFP-α-tubulin = 72 cells, pool of 20 independent experiments, TTL KO Tyr GFP-α-tubulin = 59 cells, pool of 12 independent experiments, Control Tyr GFP-α-tubulin + MATCAP RNAi = 68 cells, pool of 16 independent experiments, VASH1/2 KO Tyr GFP-α-tubulin + MATCAP RNAi = 102 cells, pool of 25 independent experiments; exact p values are displayed above each respective data set; significance $p<0.05$, unpaired two-tailed t-test. Source data are provided as a Source Data file.



Supplementary Fig. 4. Genetic manipulation of α-tubulin detyrosination/tyrosination impacts polar chromosome alignment during human cell division. (a) Live-cell imaging of chromosome and microtubule behavior, showing alignment defects in the VASH1/2 knockout (KO) with/without MATCAP RNAi; GFP-α-tubulin is in green and histone H2B-mRFP in magenta; misaligned chromosomes are indicated with arrows; time is in h:min from nuclear envelope breakdown; tub – tubulin; scale bar is 5 µm. **(b)** Quantification of the percentage of cells with enduring chromosomes at the poles during live cell imaging for the same dataset as in Fig. 2b, c; Control tyrosinated (Tyr) GFP-α-tubulin = 57 cells, pool of 16 independent experiments, TTL KO detyrosinated (Detyr) GFP-α-tubulin = 67 cells, pool of 18 independent experiments, VASH1/2 KO Tyr GFP-α-tubulin = 72 cells, pool of 20 independent experiments, TTL KO Tyr GFP-α-tubulin = 59 cells, pool of 12 independent experiments, Control Tyr GFP-α-tubulin + MATCAP RNAi = 68 cells, pool of 16 independent experiments, VASH1/2 KO Tyr GFP-α-tubulin + MATCAP RNAi = 102 cells, pool of 25 independent experiments; exact p values are displayed above each respective data set; significance $p<0.05$, unpaired two-tailed t-test. Source data are provided as a Source Data file.

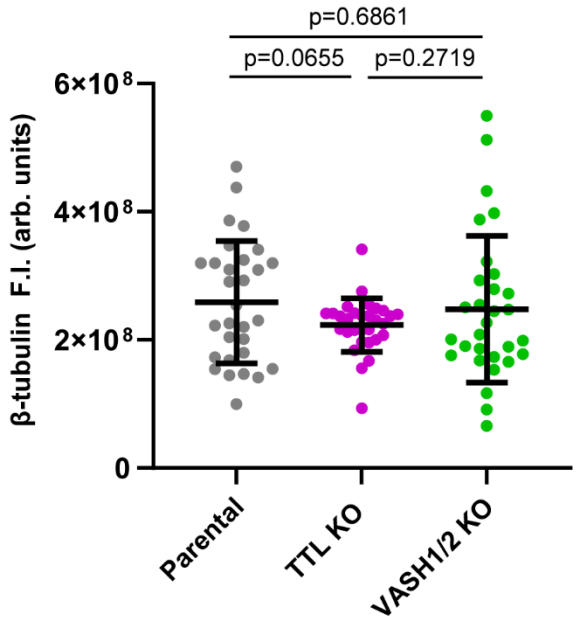


Supplementary Fig. 5. Quantification of metaphase plate width and inter-kinetochore distance upon genetic manipulation of α-tubulin detyrosination/tyrosination. (a) Quantification of the metaphase plate width from the same data set from Figure 4h (as inferred from the dispersion of anti-centromere antibodies signal). Parental = 95 cells, TTL knockout (KO) = 111 cells, VASH1/2 KO = 92 cells, pool of 3 independent experiments; p values are displayed above each respective data set; significance $p<0.05$, unpaired two-tailed t-test. **(b)** The graph reports the average value of the distances per cell between all the sister-kinetochores (KTs) from all the frames for the control condition (black), VASH1/2 KO (green) and TTL KO (magenta). Values within parenthesis indicate mean and standard deviation for all conditions. Parental = 59 cells, 7 independent experiments, TTL KO = 65 cells, 7 independent experiments; VASH1/2 KO = 47 cells, 5 independent experiments. Source data are provided as a Source Data file.



Supplementary Fig. 6. α-tubulin detyrosination, but not CENP-E inhibition, or MCAK enhancement increases metaphase plate width. (a) Immunofluorescence of the U2OS Parental cell line upon treatment with CENP-E inhibitor (inhib.), MCAK agonist UMK57 and overexpression (O.E.) of MCAK-GFP using anti-MAD1 antibodies (green) and anti-centromere antibodies (ACA; magenta); MCAK-GFP is in white; scale bar is 5 µm. **(b)** Quantification of the metaphase plate width (as inferred from the dispersion of ACA signal) from the same parental data set from figure 4h, with the additional condition of CENP-E inhibition, UMK57 and MCAK overexpression (3 independent experiments, Parental = 95 cells; CENP-E inhib. = 39 cells; UMK57 = 52 cells; MCAK OE = 52 cells); exact p values are displayed above each respective data set; significance $p<0.05$, unpaired two-tailed t-test. **(c)** Comparative analysis of the percentage of cells with one or more MAD1 positive kinetochores (KTs) per cell from the same pool as in (b); exact p values are displayed above each respective data set;

significance $p<0.05$, unpaired two-tailed t-test. Source data are provided as a Source Data file.



Supplementary Fig. 7. Quantification of spindle microtubule density upon genetic manipulation of α-tubulin detyrosination/tyrosination. Absolute fluorescence intensity of β-tubulin in the spindle region for each cell line; error bars represent mean and standard deviation; quantifications from a pool of 3 independent experiments: Parental = 30 cells, TTL KO = 30 cells, VASH1/2 KO = 30 cells; exact p values are displayed above each respective data set; significance $p<0.05$, unpaired two-tailed t-test. Source data are provided as a Source Data file. F.I. – fluorescence intensity.

Supplementary Figure 1a (uncropped blots)

Anti Cas9

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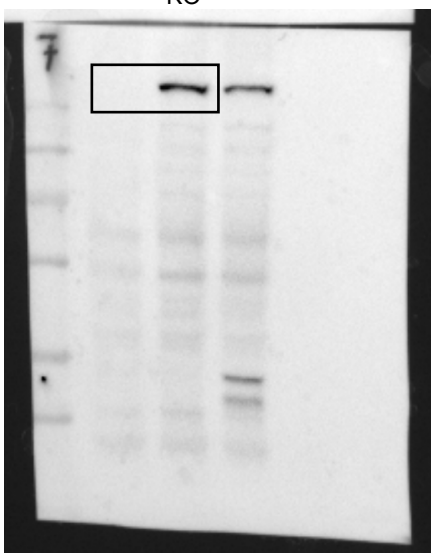
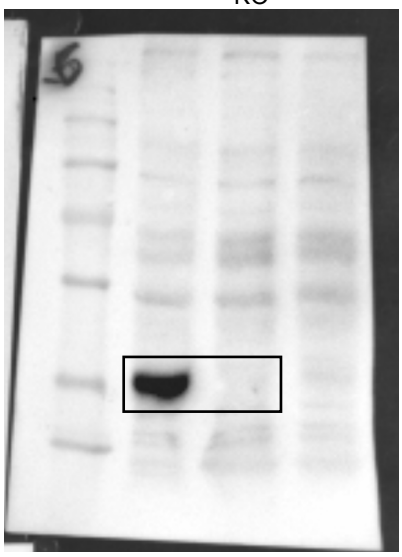


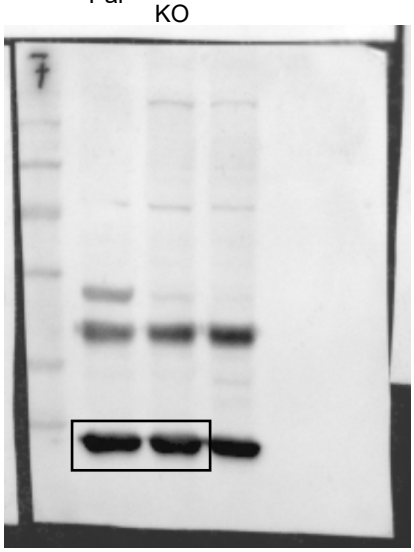
Anti TTL

Par TTL KO



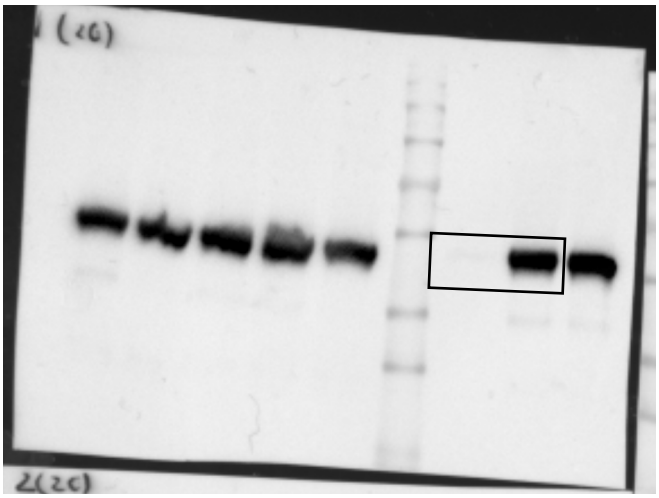
Anti GAPDH

Par TTL KO



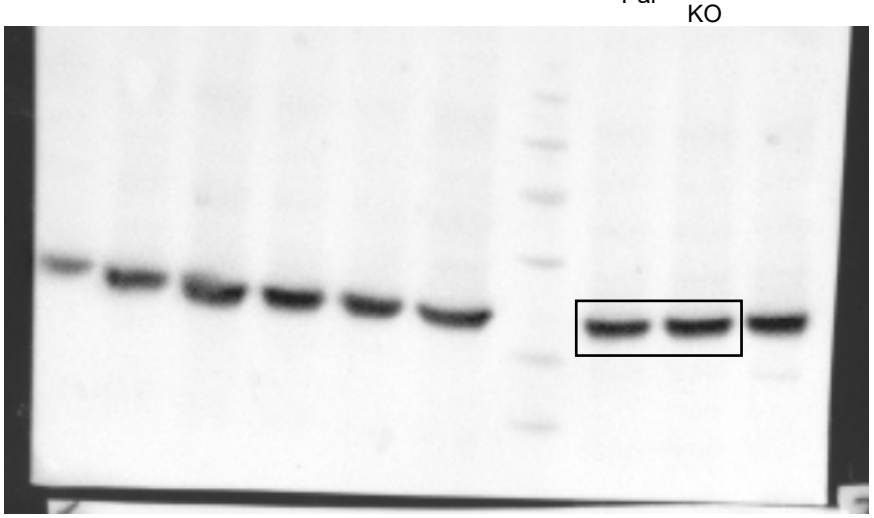
Anti Detyrosinated α-Tubulin

Par TTL KO



Anti β-Tubulin

Par TTL KO



Anti GAPDH

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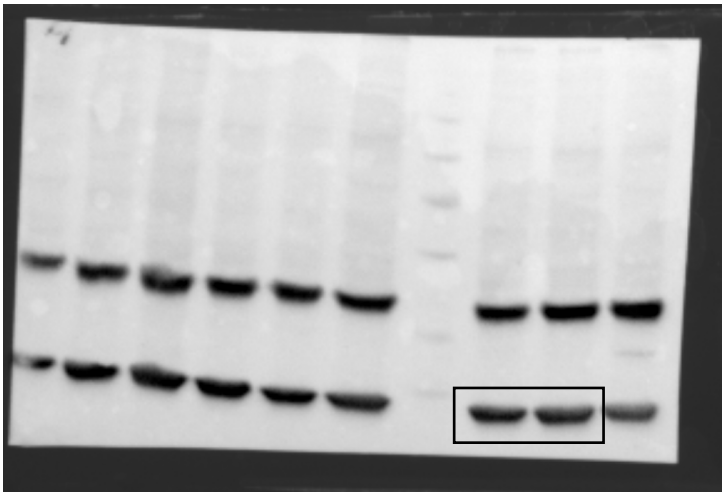
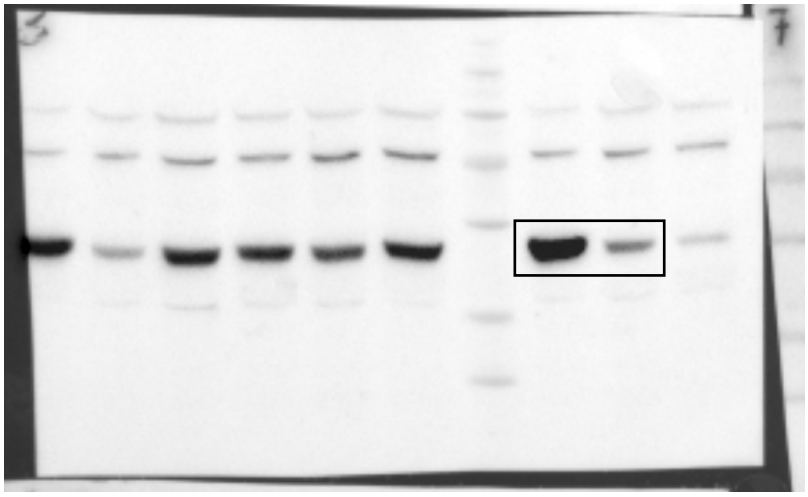


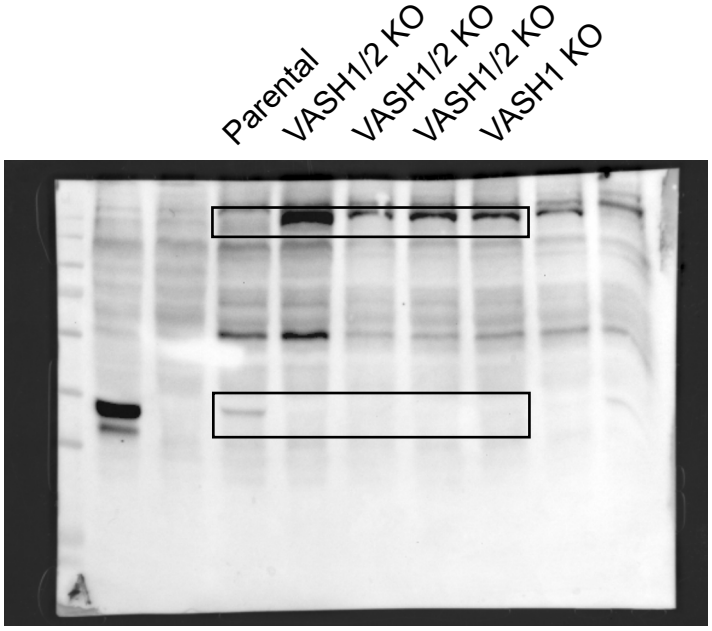
Anti Tyrosinated α-Tubulin

Par TTL KO

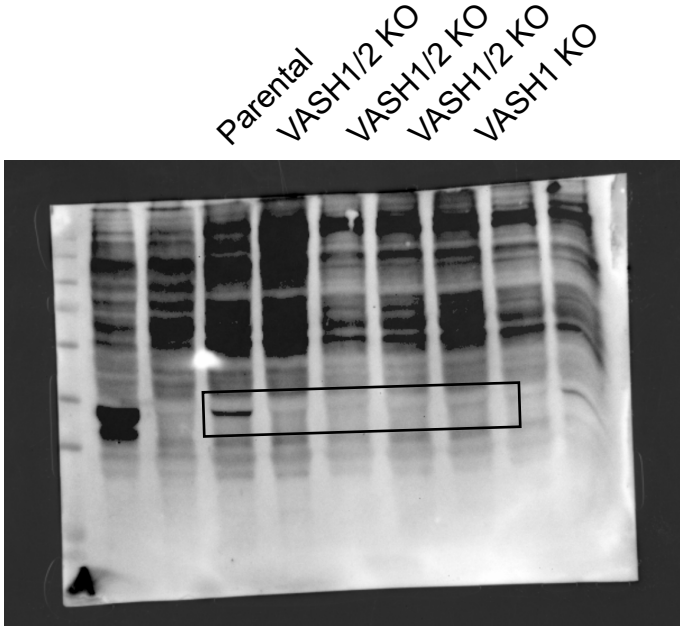


Supplementary Figure 1b (uncropped blots)

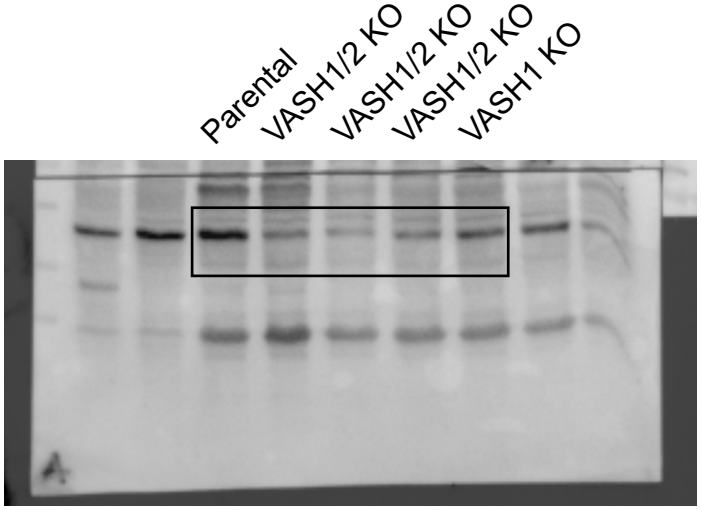
Anti Cas9 + Anti VASH1



Anti VASH1 - OE



Anti Detyrosinated α-Tubulin



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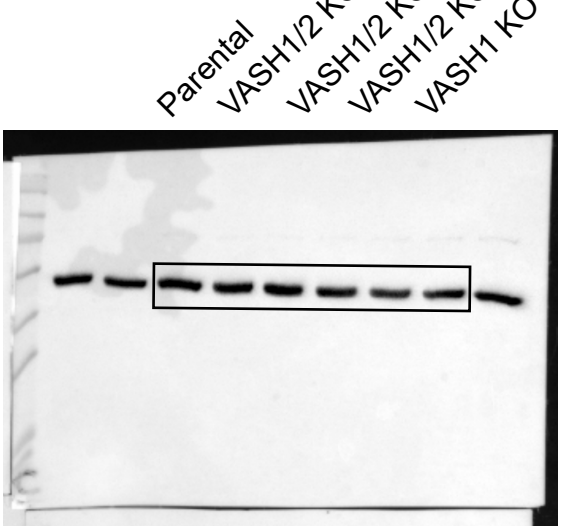

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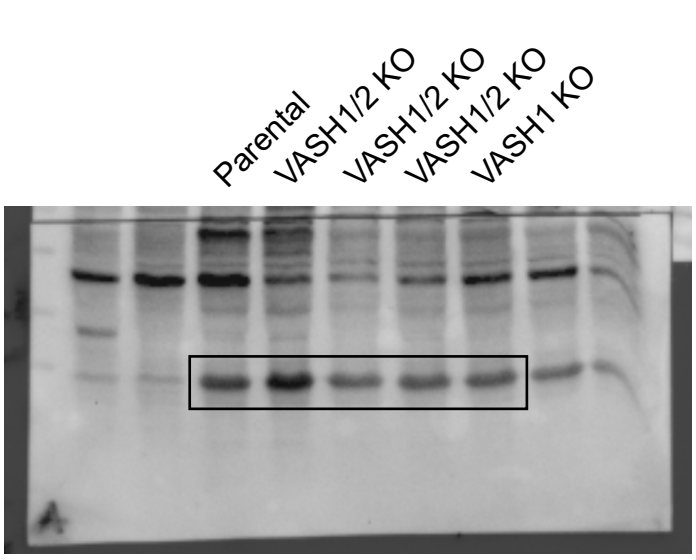

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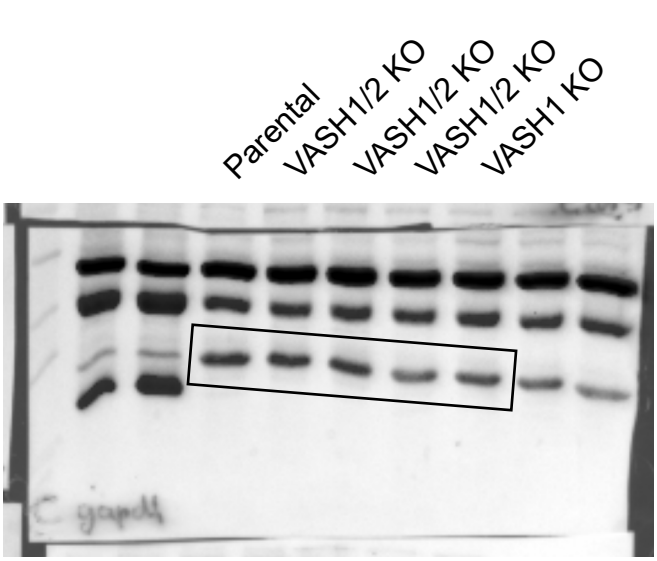
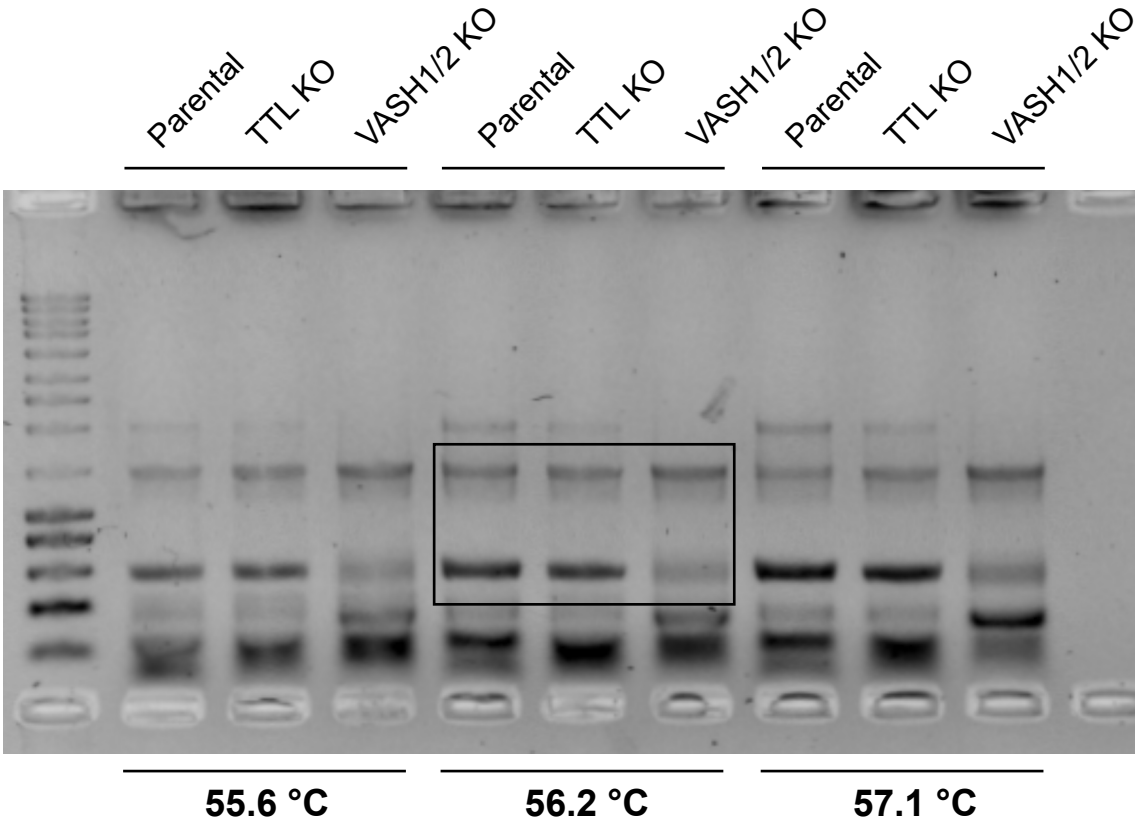
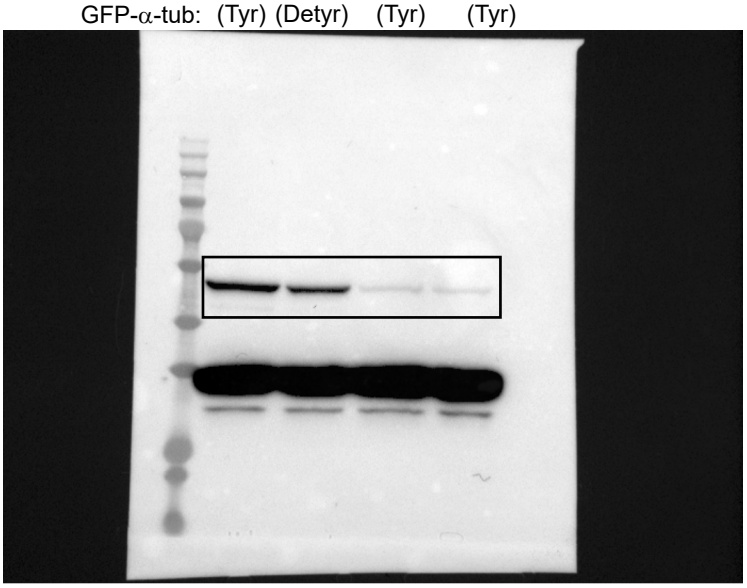


Supplementary Figure 1c (uncropped gel)

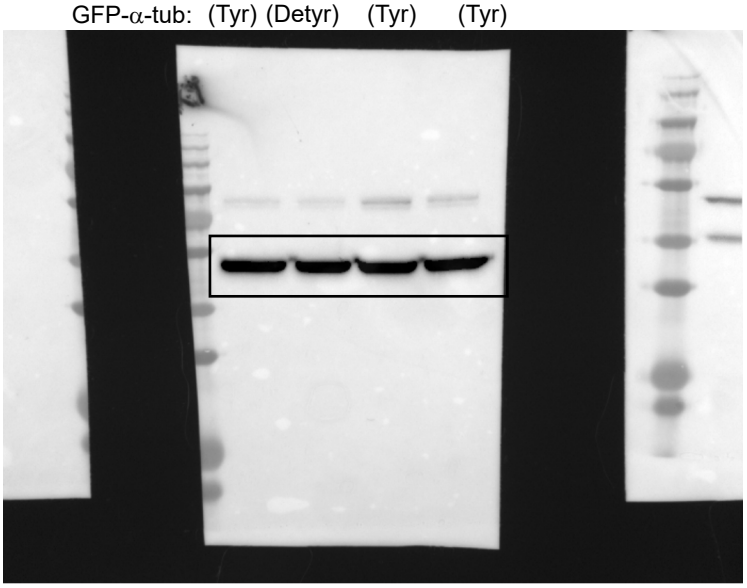


Supplementary Figure 1d (uncropped blots)

Anti Detyrosinated α-Tubulin



Anti Tyrosinated α-Tubulin



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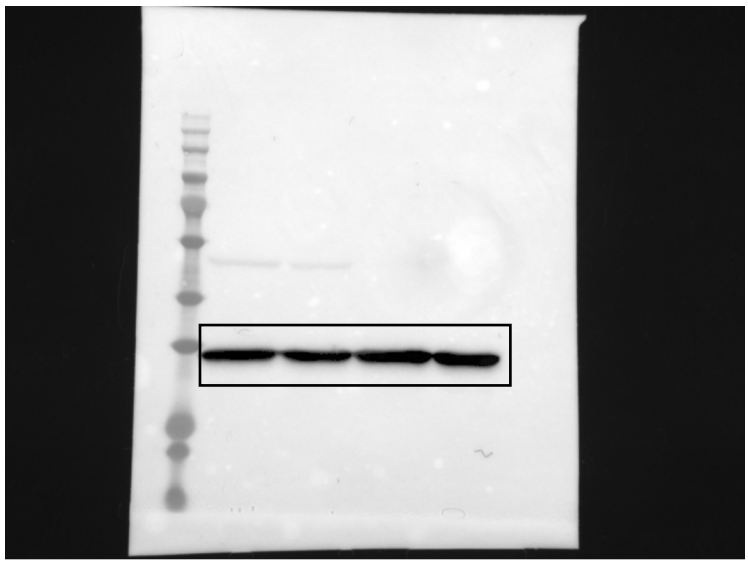

Anti GAPDH