

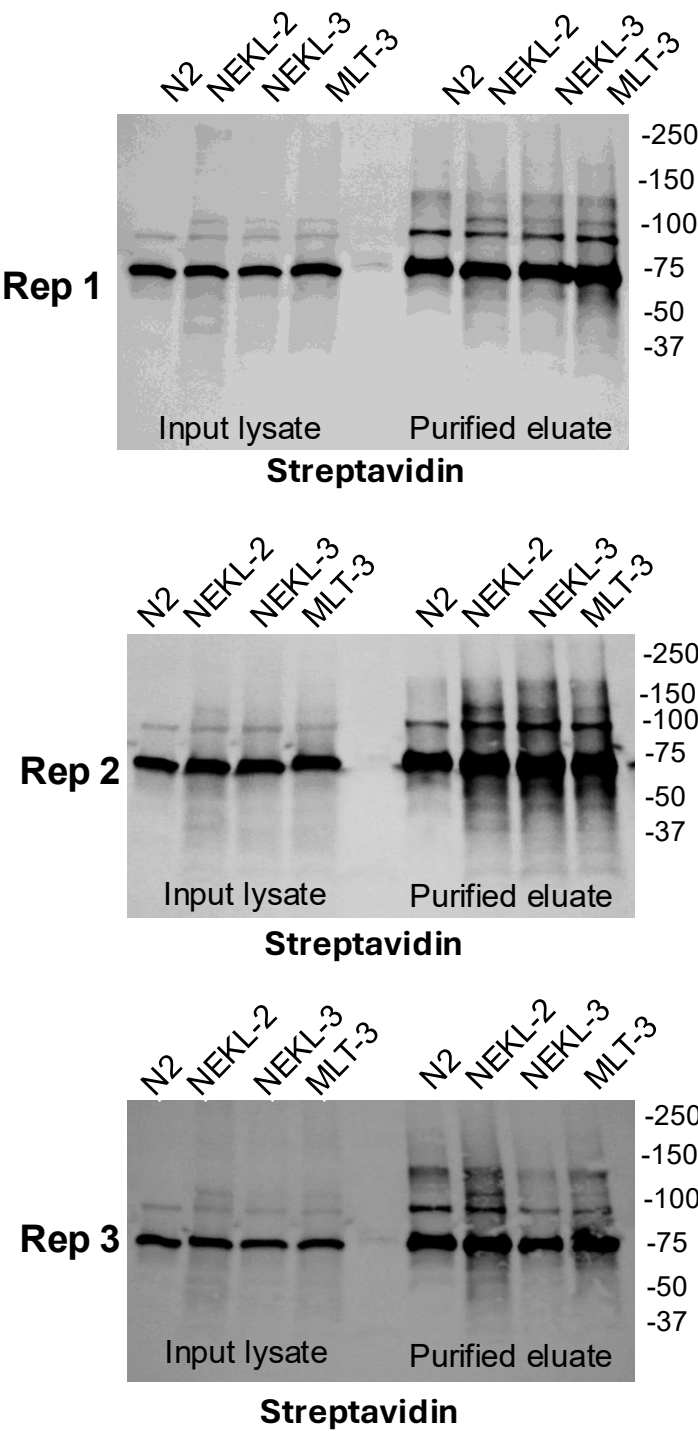
Appendix

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Appendix Figure S1. Western blot data for Exp 1–8

Experiment 1

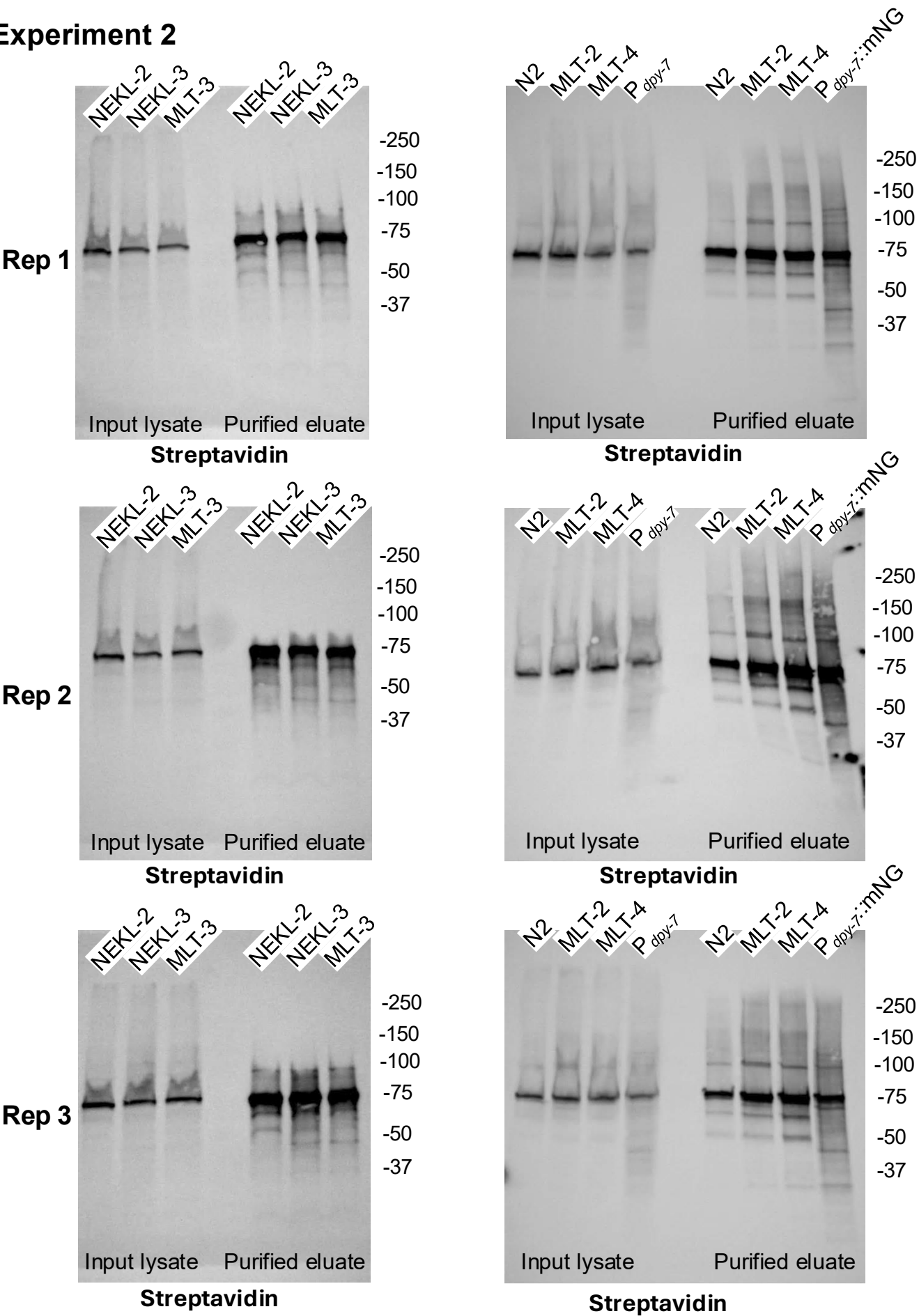


PYC-1a, 129.3 kDa; PYC-1b, 67.8 kDa; PCCA-1, 79.7 kDa; MCCC-1, 73.7 kDa; POD-2a, 230.6 kDa; POD-2b, 91.4 kD

MS Intensity: PYC-1 > PCCA-1 > MCCC-1 >> POD-2

CT-TurboID: NEKL-2 80.9 kD; NEKL-3 75.0 kD; MLT-2 115.3 kD; MLT-3 83.5 kD; MLT-4 109.9 kD

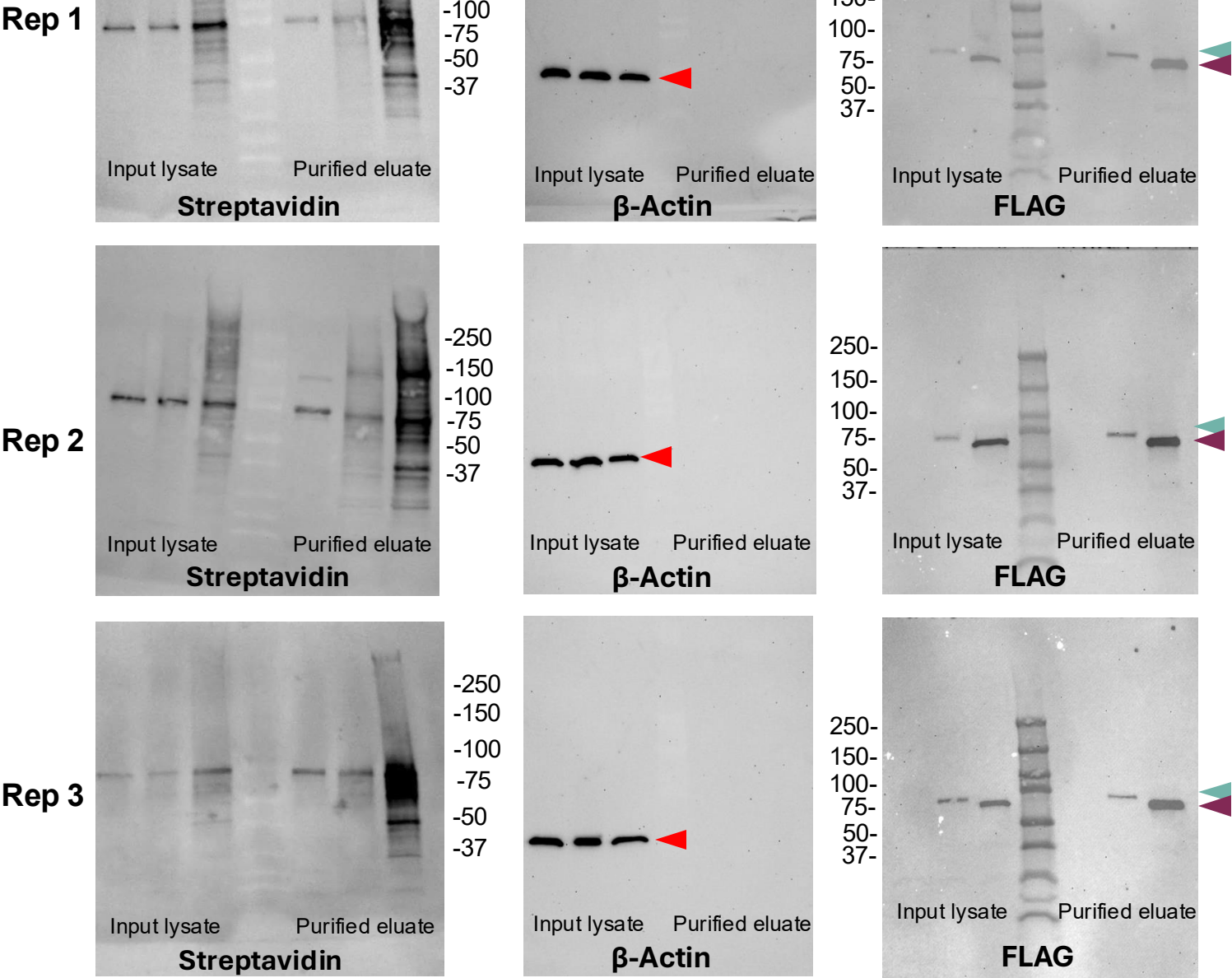
Experiment 2



PYC-1a, 129.3 kDa; PYC-1b, 67.8 kDa; PCCA-1, 79.7 kDa; MCCC-1, 73.7 kDa; POD-2a, 230.6 kDa; POD-2b, 91.4 kD

Experiment 3

◀ β-Actin ◀ NEKL-2 ◀ NEKL-3

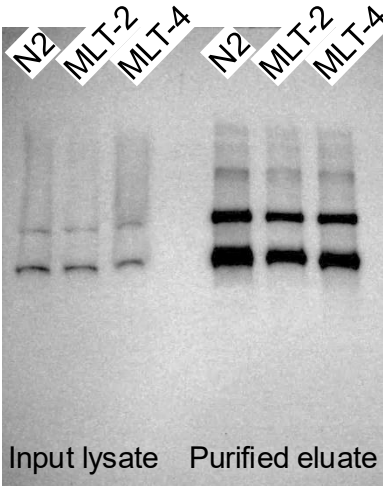


PYC-1a, 129.3 kDa; PYC-1b, 67.8 kDa; PCCA-1, 79.7 kDa; MCCC-1, 73.7 kDa; POD-2a, 230.6 kDa; POD-2b, 91.4 kD
CT-TurboID: NEKL-2 80.9 kDa; NEKL-3 75.0 kDa

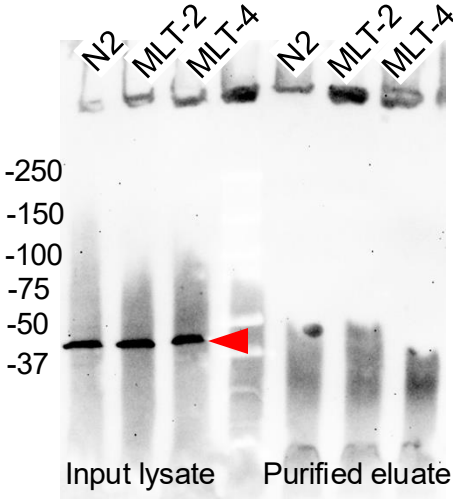
Experiment 4

◀ β-Actin ▶ MLT-2 ▶ MLT-4

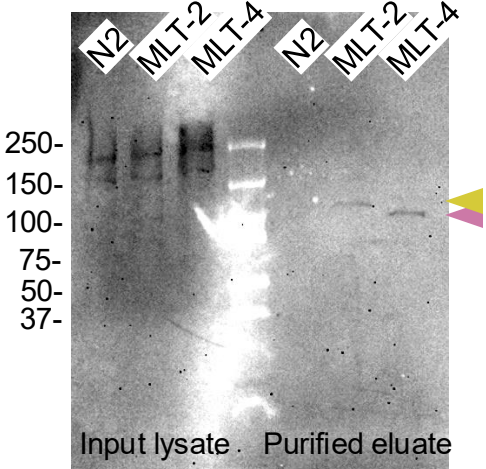
Rep 1



Streptavidin

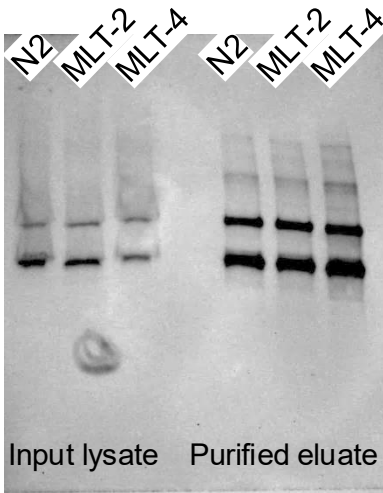


β-Actin

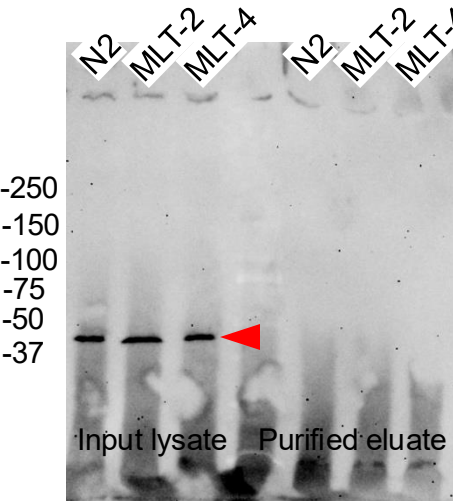


FLAG

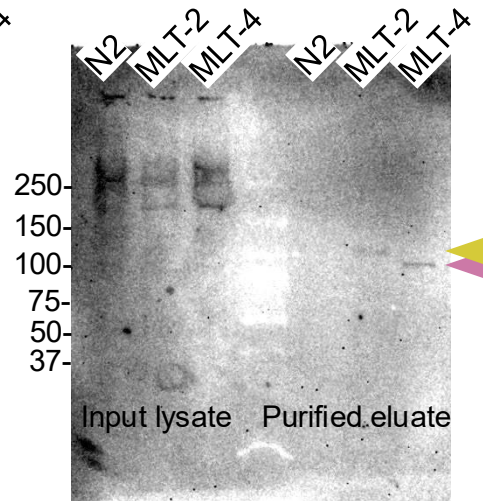
Rep 2



Streptavidin

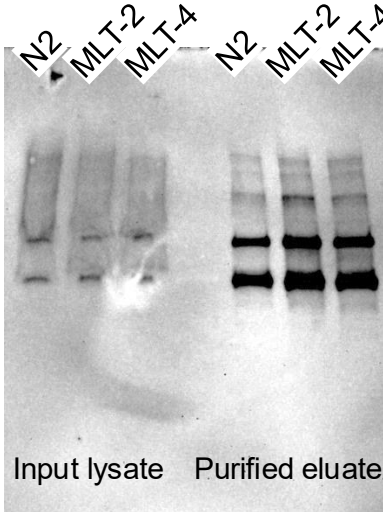


β-Actin

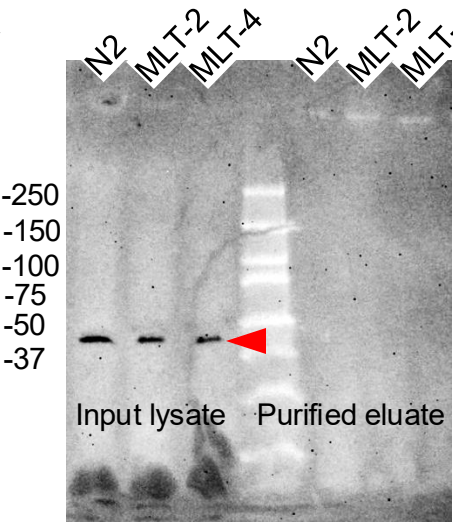


FLAG

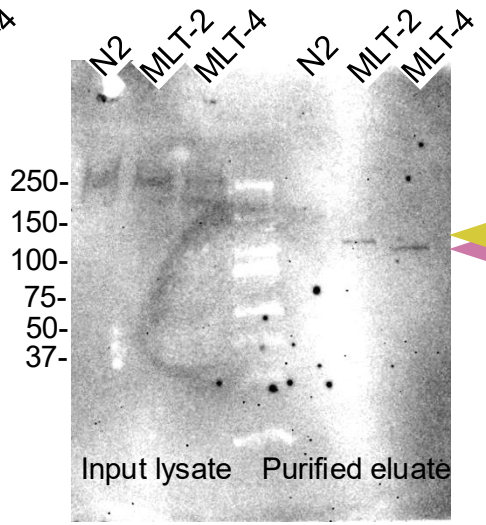
Rep 3



Streptavidin



β-Actin



FLAG

MLT-2 74.9 kDa; MLT-4 69.5 kDa

PYC-1a, 129.3 kDa; PYC-1b, 67.8 kDa; PCCA-1, 79.7 kDa; MCCC-1, 73.7 kDa; POD-2a, 230.6 kDa; POD-2b, 91.4 kD

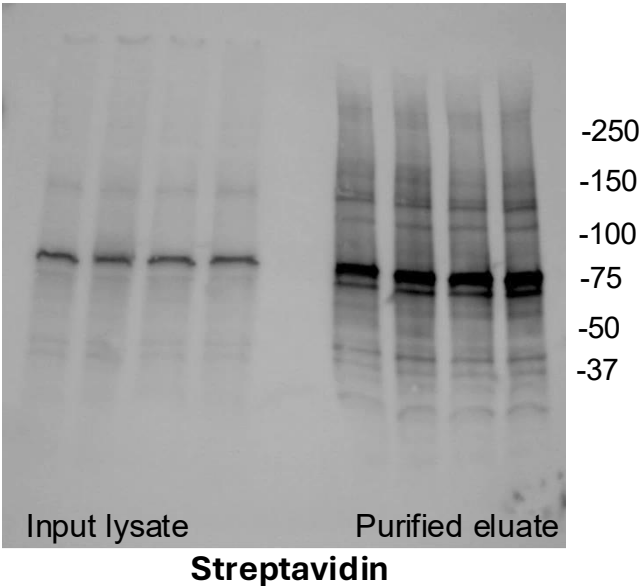
CT-TurboID: MLT-2 115.3 kDa; MLT-4 109.9 kDa

Experiment 5

◀ β-Actin R=Replicate

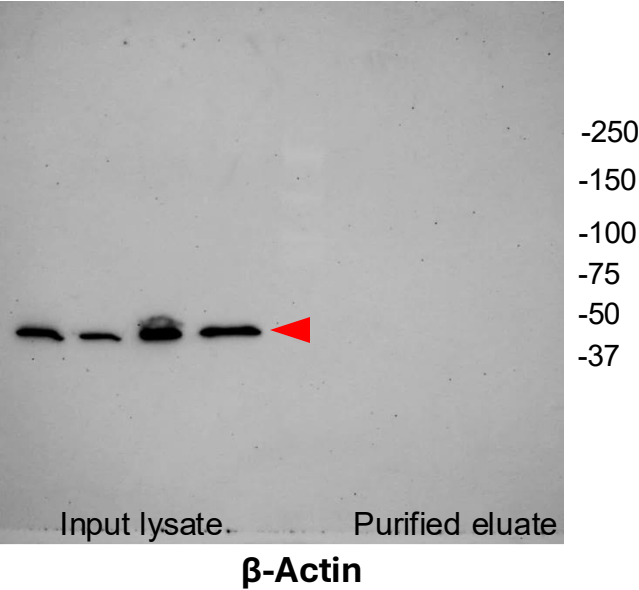
P_{dpy-7}::mNG::TurboID

R1 R2 R3 R4 R1 R2 R3 R4



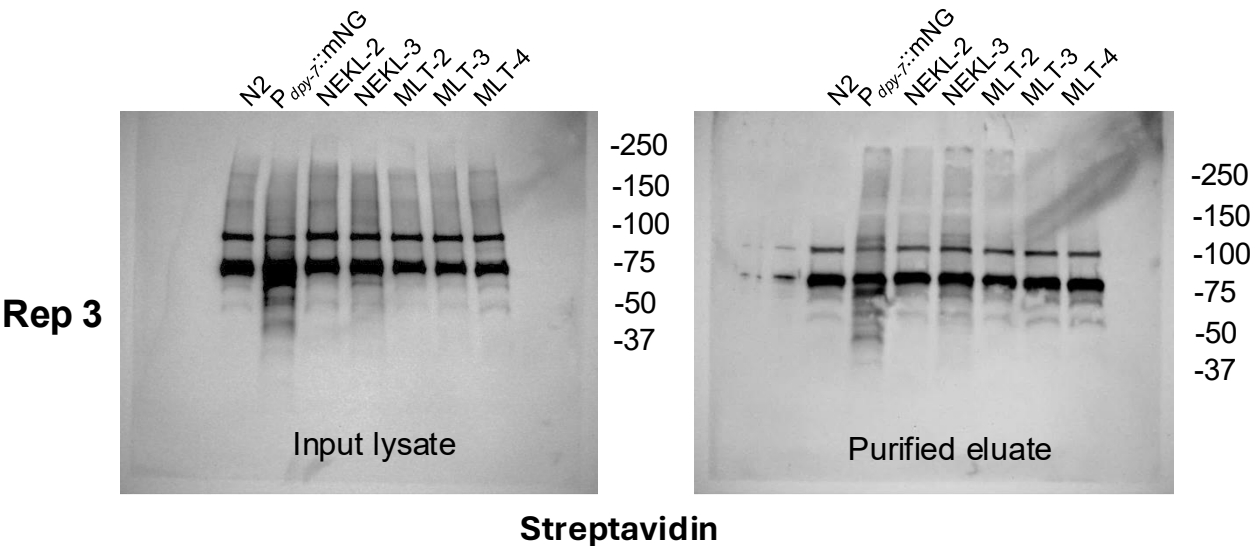
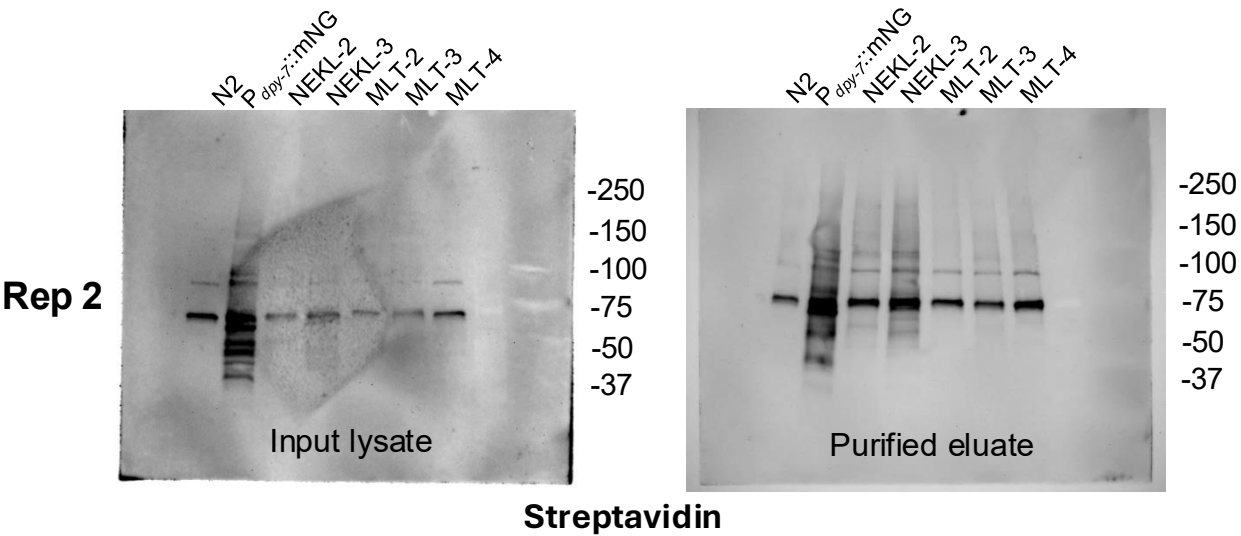
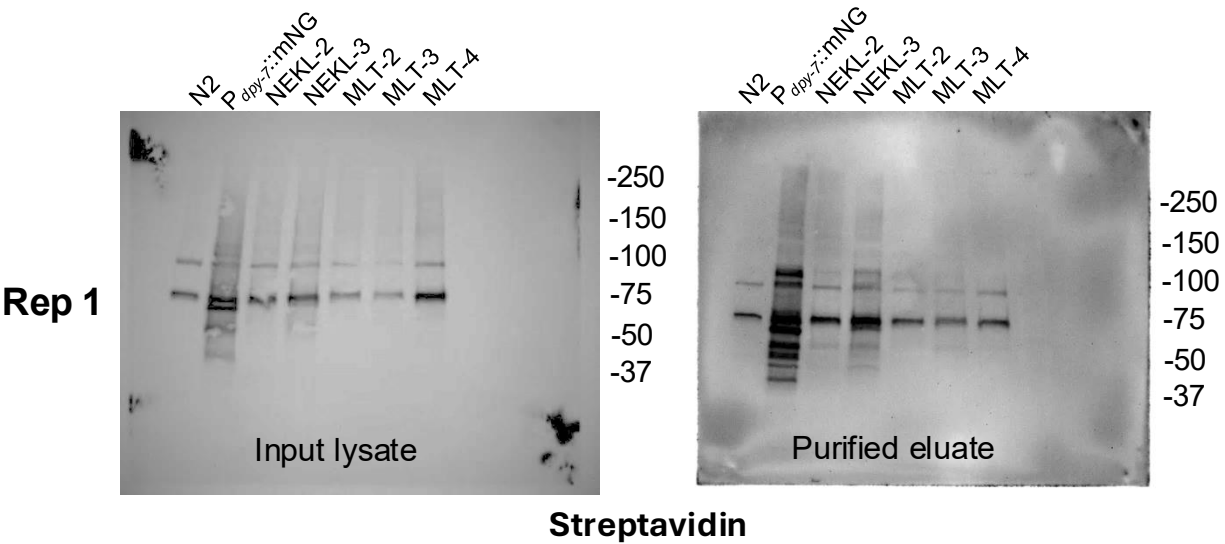
P_{dpy-7}::mNG::TurboID

R1 R2 R3 R4 R1 R2 R3 R4



PYC-1a, 129.3 kDa; PYC-1b, 67.8 kDa; PCCA-1, 79.7 kDa; MCCC-1, 73.7 kDa; POD-2a, 230.6 kDa; POD-2b, 91.4 kD

Experiment 6

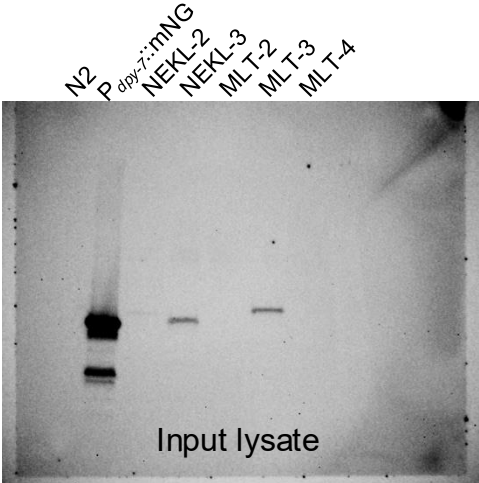


PYC-1a, 129.3 kDa; PYC-1b, 67.8 kDa; PCCA-1, 79.7 kDa; MCCC-1, 73.7 kDa; POD-2a, 230.6 kDa; POD-2b, 91.4 kD

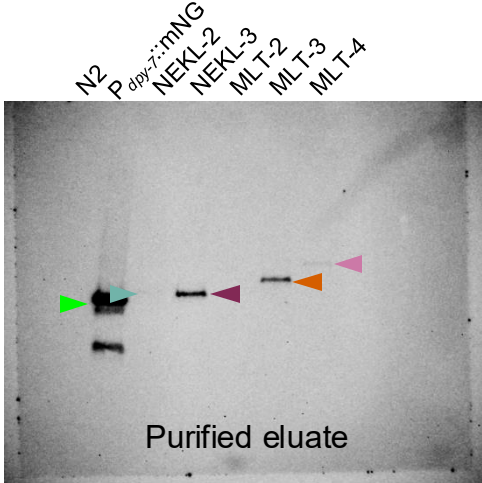
Experiment 6

◀ NEKL-2 ◀ NEKL-3 ◀ MLT-2 ◀ MLT-3 ◀ MLT-4 ◀ mNG::TurboID

Rep 1



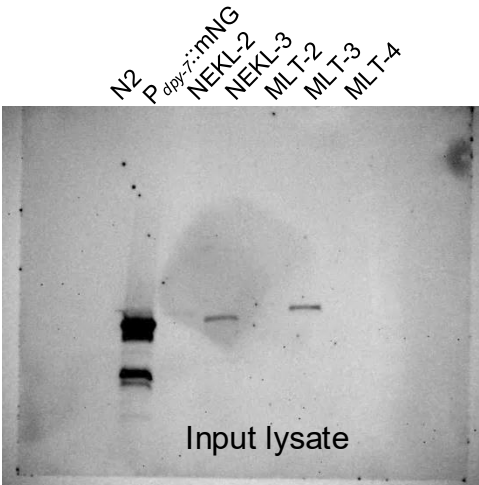
-250
-150
-100
-75
-50
-37



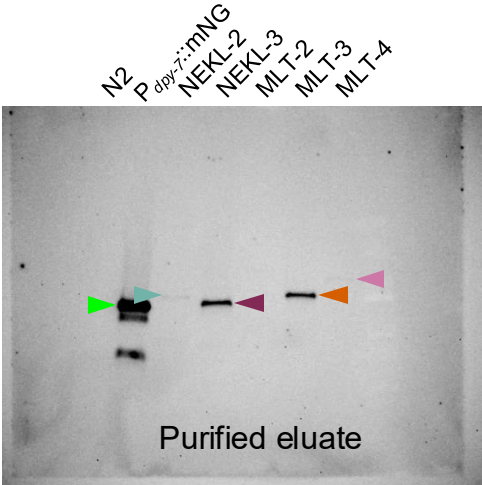
-250
-150
-100
-75
-50
-37

FLAG

Rep 2



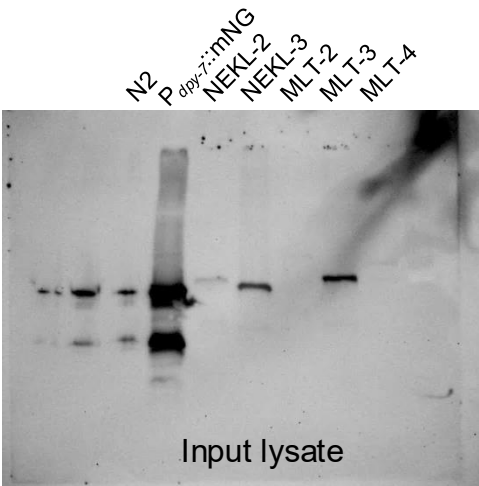
-250
-150
-100
-75
-50
-37



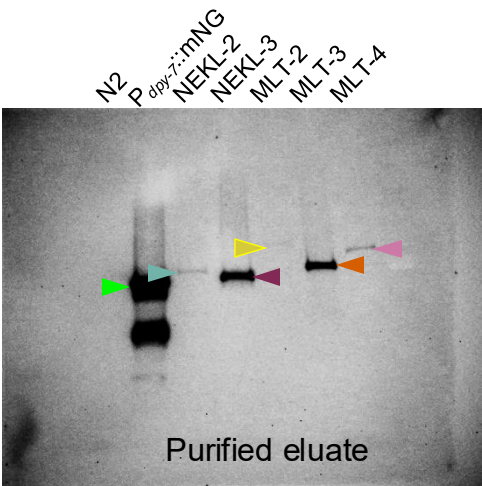
-250
-150
-100
-75
-50
-37

FLAG

Rep 3



-250
-150
-100
-75
-50
-37

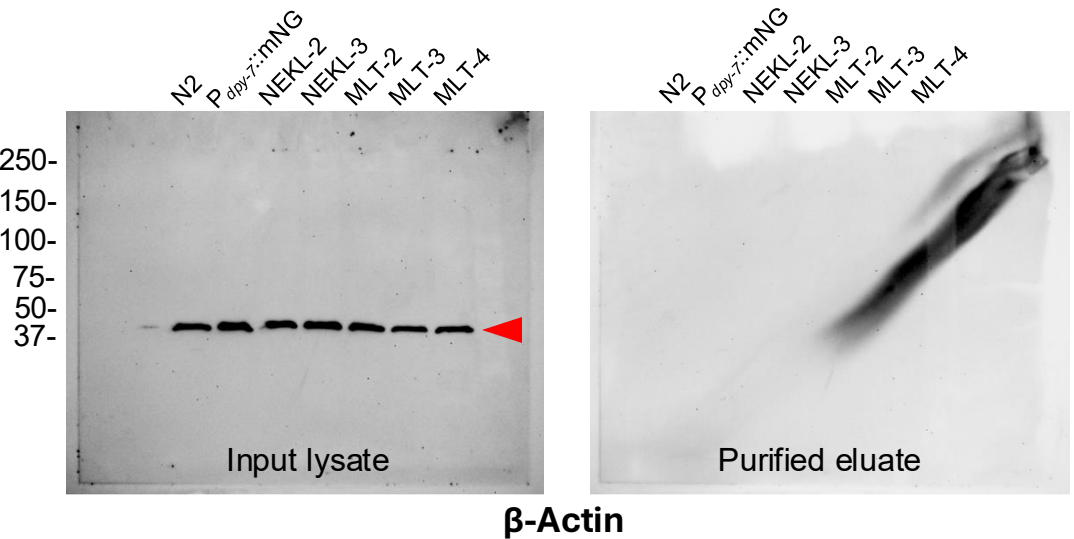
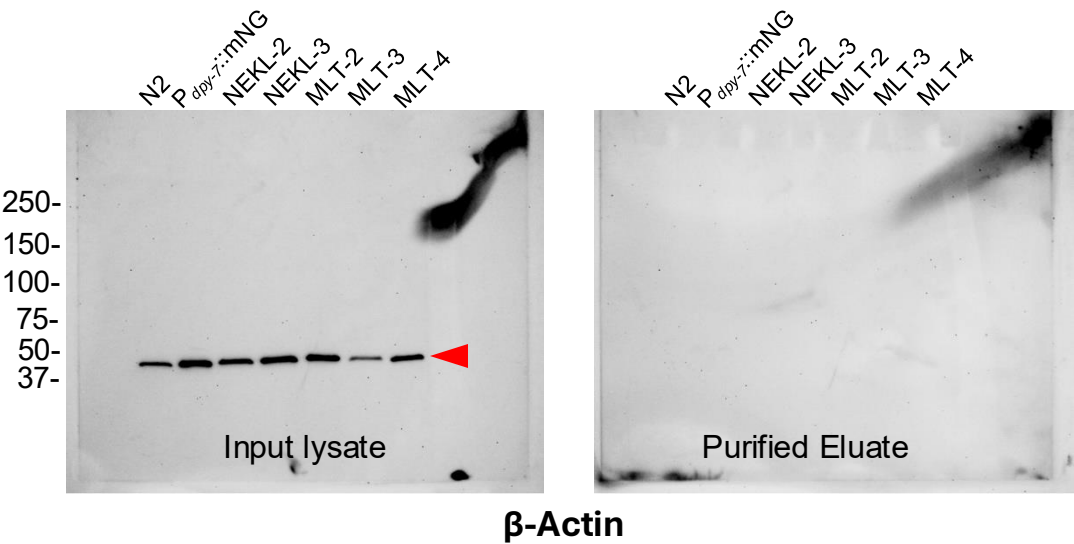
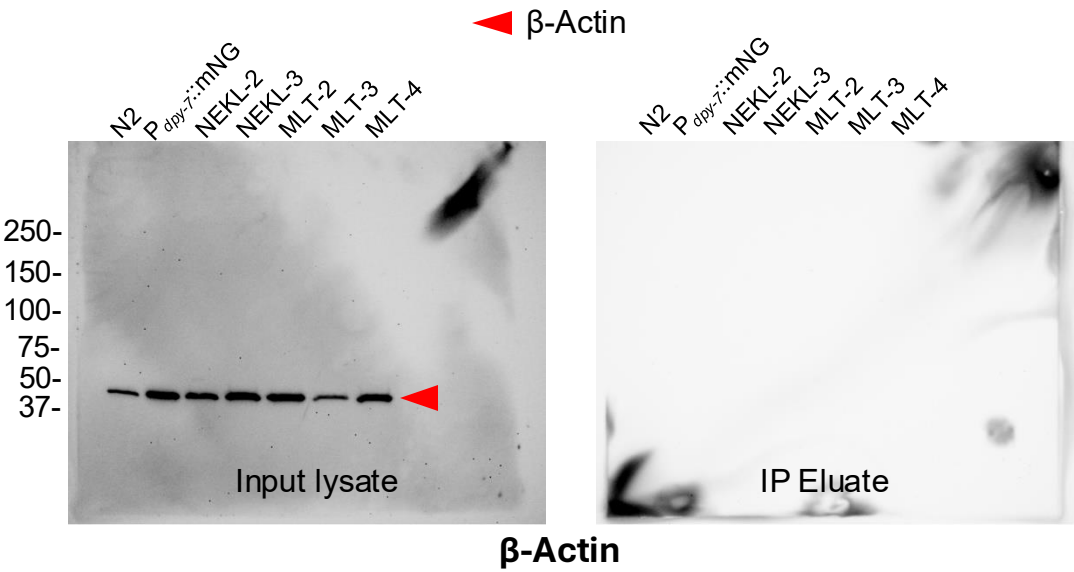


-250
-150
-100
-75
-50
-37

FLAG

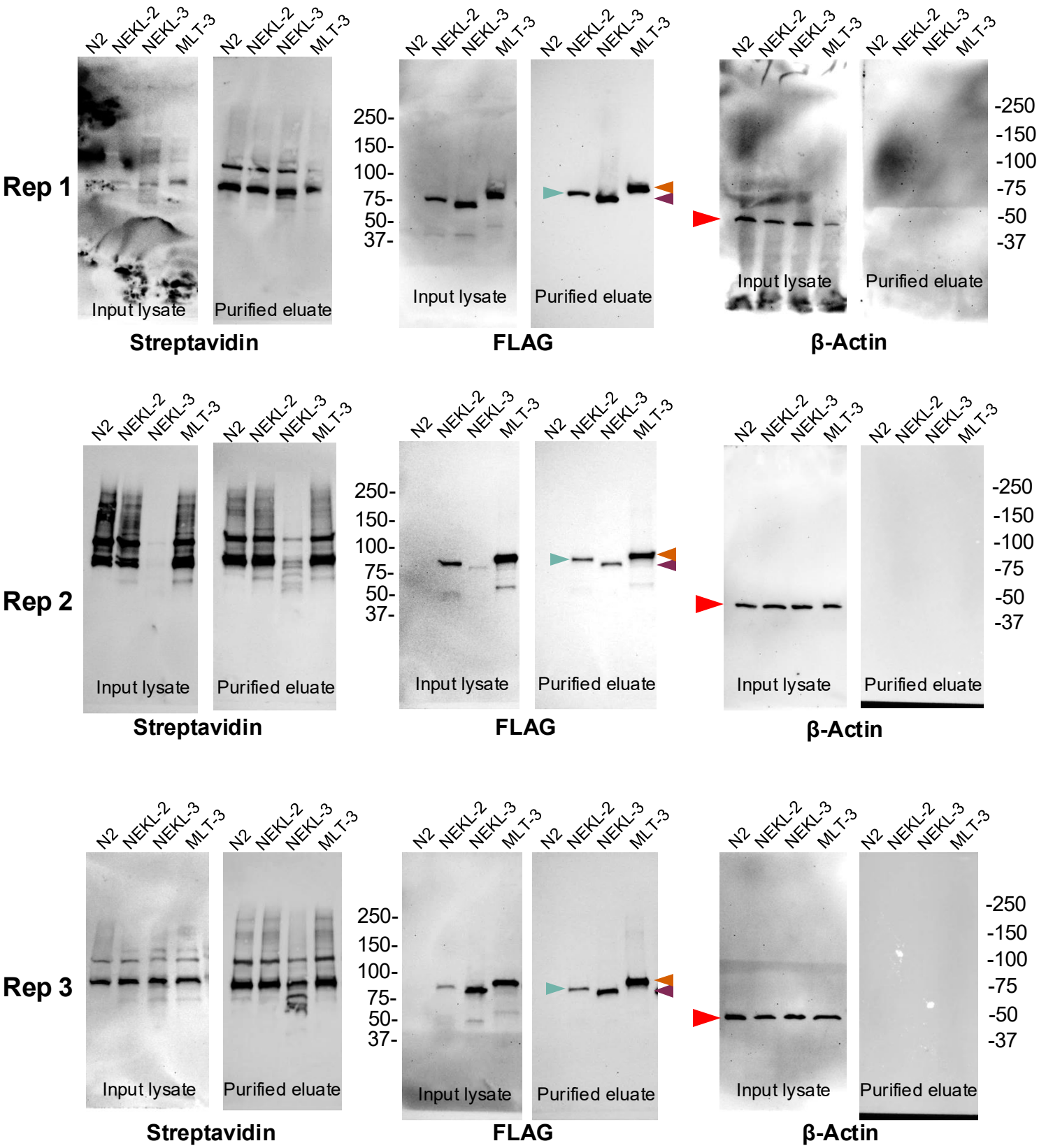
CT-TurboID: NEKL-2 80.9 kDa; NEKL-3 75.0 kDa; MLT-2 115.3 kDa; MLT-3 83.5 kDa; MLT-4 109.9 kDa
mNeonGreen::TurboID 68k Da

Experiment 6



Experiment 7

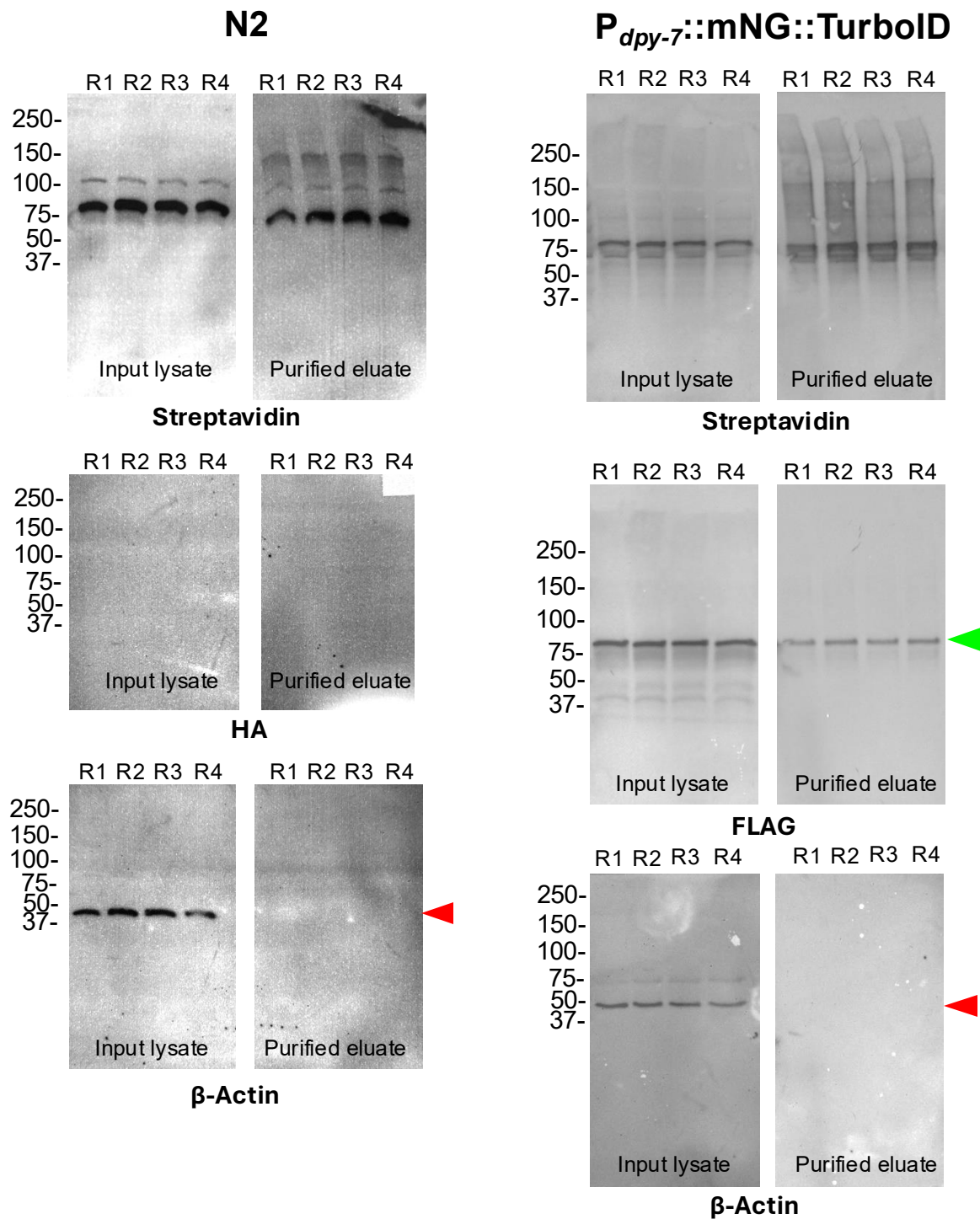
◀ NEKL-2 ◀ NEKL-3 ▶ MLT-3 ▶ β-Actin



PYC-1a, 129.3 kDa; PYC-1b, 67.8 kDa; PCCA-1, 79.7 kDa; MCCC-1, 73.7 kDa; POD-2a, 230.6 kDa; POD-2b, 91.4 kD
CT-TurboID: NEKL-2 80.9 kDa; NEKL-3 75.0 kDa; MLT-3 83.5 kDa

Experiment 8

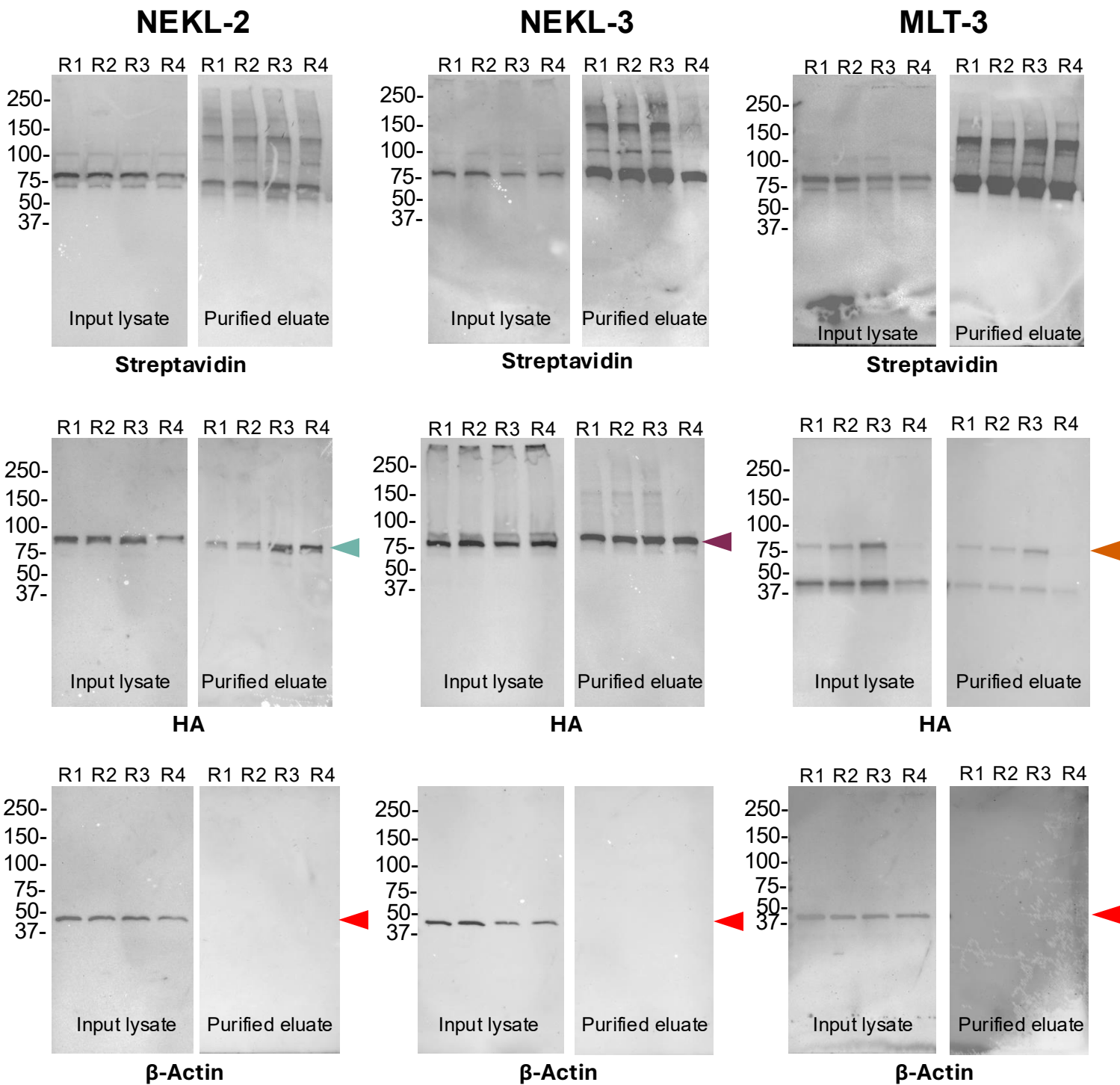
◀ β-Actin ▶ mNG::TurboID R=Replicate



PYC-1a, 129.3 kDa; PYC-1b, 67.8 kDa; PCCA-1, 79.7 kDa; MCCC-1, 73.7 kDa; POD-2a, 230.6 kDa; POD-2b, 91.4 kDa
mNeonGreen::TurboID 68k Da

Experiment 8

◀ NEKL-2 ◀ NEKL-3 ◀ MLT-3 ◀ β-Actin R=Replicate



PYC-1a, 129.3 kDa; PYC-1b, 67.8 kDa; PCCA-1, 79.7 kDa; MCCC-1, 73.7 kDa; POD-2a, 230.6 kDa; POD-2b, 91.4 kD

NT-TurboID: NEKL-2 81.0 kDa; NEKL-3 75.1 kDa; MLT-3 83.4 kDa

Appendix Supplementary Methods S1

C-terminal fusions of TurboID

Linkers TurboID FLAG-3x 40.4 kDa

Protein

GGGGSGSGGGRGSGSGSGSGSGSGSGSGSGSKDNTVPLKLIALLANGFHSGEQLGETLGMSRAAI
NKHIQTLRDWGVDFVFTVPKGYSLEPEIPLLNKQILGQLDGGSVAVLPVVDSTNQYLLDRIGE
LKSGDACIAEYQQAGRGRGRKWFSPFGANLYLSMFWRLKRGPAAI GLGPVIGIVMAEALRKLG
ADKVRVKWPNDLYLQDRKLAGILVELAGITGDAAQIVIGAGINVAMRRVEESVNVNQWITLQEA
GINLDRNTLAATLIRELRAALELFEQEGLAPYLPRWEKLDNFINRPVKLIIGDKEIFGISRGID
KQGALLLEQDGVIKPWMGGEISLRSAEKAGGDYKDDDDKRDYKDDDDKRDYKDDDDK

DNA

GGAGGTGGTGGATCAGGCTCGGGAGGTCGAGGCTCAGGATCCGGTTCCGGCTCCGGCTCTGGTT
CCGGTTCCGGGTTCCGGTTCTGGA AAGGATAACACCGTTCCACTTAAGCTTATCGCCCTTCTTGC
CAACGGAGAATTCCACTCTGGAGAGCAACTTGGAGAGACTCTTGAATGTCCCGTGCTGCCATC
AACAAGCATATCCAAACCCTTCGTGATTGGGGAGTTGATGTTTTCACTGTTCCAGGAAAGgtaa
gtttaaacatatataactaactaacctgattatttaaattttcagGGATACTCCCTTCCAGA
GCCAATCCCCTTCTTAACGCCAAGCAAATCCTTGGACAACCTGATGGAGGATCCGTCGCTGTC
CTTCCAGTTGTTGATTCCACCAACCAATACCTTCTTGACCGTATCGGAGAGCTTAAGTCTGGAG
ACGCCTGCATCGCTGAGTACCAACAAGCTGGACGCGGATCTCGCGGACGCAAGTGGTTCTCCCC
ATTCGGAGCCAACCTTTACCTTTCTATGTTCTGGCGTCTTAAGCGTGGACCAGCTGCTATCGGA
CTTGGACCAGTTATCGGAATCGTTATGGCTGAGGCCCTTCGTAAGCTTGGAGCTGATAAGgtaa
gtttaaacagttcggtaactaactaacatacatatttaaattttcagGTTTCGTGTTAAGTGGCC
AAACGATCTTTACCTTCAAGACCGTAAGCTTGCTGGAATCCTTGTCGAGCTTGCTGGAATCACC
GGAGACGCCGCTCAAATCGTTATCGGAGCTGGAATCAACGTTGCCATGCGTCGTGTTGAGGAGT
CTGTTGTTAACCAAGGATGGATCACTCTTCAAGAGGCTGGAATCAACCTTGATCGTAACACCCT
TGCTGCCACCCTTATCCGTGAGCTTCGTGCTGCCCTTGAGCTTTTCGAGCAAGAGGGACTTGCC
CCATACCTTCCACGCTGGGAGAAGCTTGACAACCTTCATCAACCGCCCAGTTAAGCTTATCATCG
GAGATAAGGAAATCTTCGGAATCTCTCGCGAATCGACAAGCAAGGAGCTCTTCTTCTTGAGCA
AGATGGAGTCATTAAGCCATGGATGGGAGGAGAGATTTCCCTTCGTTCCGCTGAGAAGGCCGGA
GGA GATTATAAAGACGATGACGATAAGCGTGACTACAAGGACGACGACGACAAGCGTGATTACA
AGGATGACGATGACAAG

Lower case in TurboID sequence indicates synthetic introns.

Final coding codon of NEKL-MLT bait proteins directly precedes the N-terminal linker sequence.

Endogenous stop codon of NEKL-MLT bait proteins directly follows the C-terminal FLAG-3x sequence.

N-terminal fusions of TurboID

Linkers	TurboID	HA-3x	40.5 kDa
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Protein

PYDVDPDYAGSYPYDVDPDYAGSYPYDVDPDYAGGGKDNTVPLKLIALLANGEFHSGEQLGETLGMS
 RAAINKHIQTLRDWGVDFVTPVGKGYSLPEPIPLLNAKQILGQLDGGSVAVLPVVDSTNQYLLD
 RIGELKSGDACIAEYQQAGRGRGRKWFSPFGANLYLSMFWRLLKGPAAIGLGPVIGIVMAEAL
 RKLGADKVRVKWPNDLYLQDRKLAGILVELAGITGDAAQIVIGAGINVAMRRVEESVVNQWIT
 LQEAGINLDRNTLAATLIRELRAALELFEQEGLAPYLPRWEKLDNFINRPVKLIIGDKEIFGIS
 RGIDKOGALLLEODGVIKPMMGGEISLRSAEKGGGGSGSGGRGSGSGSGSGSGSGSGSGSGSG

DNA

TACCCATATGACGTTCCAGACTACGCGGGTAGTTATCCGTACGACGTTCCGGATTACGCTGGAT
CTTACCCTTACGACGTACCTGACTACGCTGGAGGTGGTAAGGATAACACCGTTCCACTTAAGCT
TATCGCCCTTCTTGCCAACGGAGAATTCACCTCTGGAGAGCAACTTGGAGAGACTCTTGGAATG
TCCCGTGCTGCCATCAACAAGCATATCCAAACCTTTCGTGATTGGGGAGTTGATGTTTTCTACTG
TTCCAGGAAAGgtaagtttaaacaatatataactaactaaccctgattatttaaattttcagGG
ATACTCCCTTCCAGAGCCAATCCCACCTTCTTAACGCCAAGCAAATCCTTGACAACCTTGATGGA
GGATCCGTGCTGTCCTTCCAGTTGTTGATTCCACCAACCAATACCTTCTTGACCGTATCGGAG
AGCTTAAGTCTGGAGACGCCTGCATCGCTGAGTACCAACAAGCTGGACGCGGATCTCGCGGACG
CAAGTGGTTTCTCCCATTCGGAGCCAACCTTTACCTTTCTATGTTCTGGCGTCTTAAGCGTGGA
CCAGCTGCTATCGGACTTGGACCAGTTATCGGAATCGTTATGGCTGAGGCCCTTCGTAAGCTTG
GAGCTGATAAGgtaagtttaaacagttcggtaactaactaaccatacatatttaaattttcagGT
TCGTGTTAAGTGGCCAAACGATCTTTACCTTCAAGACCGTAAGCTTGCTGGAATCCTTGTCGAG
CTTGCTGGAATCACCGGAGACGCCGCTCAAATCGTTATCGGAGCTGGAATCAACGTTGCCATGC
GTCGTGTTGAGGAGTCTGTTGTTAACCAAGGATGGATCACTCTTCAAGAGGCTGGAATCAACCT
TGATCGTAACACCCTTGCTGCCACCCTTATCCGTGAGCTTCGTGCTGCCCTTGAGCTTTTTCGAG
CAAGAGGGACTTGCCCCATACCTTCCACGCTGGGAGAAGCTTGACAACCTTCATCAACCGCCAG
TTAAGCTTATCATCGGAGATAAGGAAATCTTCGGAATCTCTCGCGGAATCGACAAGCAAGGAGC
TCTTCTTCTTGAGCAAGATGGAGTCATTAAGCCATGGATGGGAGGAGAGATTTCCCTTCGTTC
GCTGAGAAGGGAGGTGGTGGATCAGGCTCGGGAGGTCGAGGCTCAGGATCCGGTTCGGGCTCCG
GCTCTGGTTCCGGTTCGGGTTCCGGTTCTGGA

Lower case in TurboID sequence indicates synthetic introns.

First codon of NEKL-MLT bait proteins (ATG) directly precedes the HA-3x tag sequence.

Second codon of NEKL-MLT bait proteins directly follows the C-terminal linker sequence.

Exp 1 FASP Methods – Orbitrap Exploris DIA

Protein samples were reduced, alkylated, and digested on-bead using filter-aided sample preparation [*Nature Methods* 6: 359-62 (2009)] with sequencing grade modified porcine trypsin (Promega).

Tryptic peptides were trapped and eluted on 3.5µm CSH C18 resin (Waters) (4mm x 75µm) then separated by reverse phase XSelect CSH C18 2.5 µm resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted at a flow rate of 0.300µL/min using a 60 min gradient from 98% Buffer A:2% Buffer B to 95:5 at 2.0 minutes to 80:20 at 39.0 minutes to 60:40 at 48.0 minutes to 10:90 at 49.0 minutes and hold until 53.0 minutes and then equilibrated back to 98:2 at 53.1 minutes until 60 minutes. Eluted peptides were ionized by electrospray (2.4 kV) through a heated capillary (275°C) followed by data collection on an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific).

Precursor spectra were acquired with a scan from 385-1015 Th at a resolution set to 60,000 with 100% AGC, max time of 50 msec, and an RF parameter at 40%. DIA was configured on the Orbitrap 480 to acquire 50 x 12 Th isolation windows at 15,000 resolution, normalized AGC target 500%, maximum injection time 40 ms). A second DIA was acquired in a staggered window (12 Th) pattern with optimized window placements.

Buffer A = 0.1% formic acid, 0.5% acetonitrile

Buffer B = 0.1% formic acid, 99.9% acetonitrile

Following data acquisition, data were searched using Spectronaut (Biognosys version 18.3) against the UniProt *Caenorhabditis elegans* database (August 2023) using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to maxLFQ, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Protein MS2 intensity values were assessed for quality using ProteinNorm (Graw *et al.*). The data was normalized using VSN (Huber *et al.*) and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (limma) with empirical Bayes (eBayes) smoothing to the standard errors (Thurman *et al.*, Ritchie *et al.*). Proteins with an FDR adjusted p-value < 0.05 and a fold change > 2 were considered significant.

Acknowledgement:

IDeA National Resource for Quantitative Proteomics and NIH/NIGMS grant R24GM137786.

References:

1. Searle, B.C., Pino, L.K., Egertson, J.D. *et al.* Chromatogram libraries improve peptide detection and quantification by data independent acquisition mass spectrometry. *Nat Commun* 9, 5128 (2018). <https://doi.org/10.1038/s41467-018-07454-w>
2. Graw S, Tang J, Zafar MK, Byrd AK, Bolden C, Peterson EC, and Byrum SD. proteiNorm – A user-friendly tool for normalization and analysis of TMT and label-free protein quantification. *ACS Omega* 2020 5(40), 25625-25633. DOI: 10.1021/acsomega.0c02564.

3. Thurman TJ, Washam CL, Alkam D, Bird JT, Gies A, Dhusia K, Robeson II MS, and Byrum SD. proteoDA: a package for quantitative proteomics. 2023. Journal of Open Source Software. DOI: 10.21105/joss.05184.
4. Bolstad, B., *preprocessCore: A collection of pre-processing functions*. 2019.
5. Ritchie, M.E., et al., *limma powers differential expression analyses for RNA-sequencing and microarray studies*. Nucleic Acids Res, 2015. **43**(7): p. e47.
6. Chawade, A., E. Alexandersson, and F. Levander, *Normalyzer: a tool for rapid evaluation of normalization methods for omics data sets*. J Proteome Res, 2014. **13**(6): p. 3114-20.
7. Huber, W., et al., *Variance stabilization applied to microarray data calibration and to the quantification of differential expression*. Bioinformatics, 2002. **18 Suppl 1**: p. S96-104.
8. Alhamdoosh, M., Ng, M., Wilson, N.J., Sheridan, J. M., Huynh, H., Wilson, M.J., Ritchie, M. E. 2016. Combining multiple tools outperforms individual methods in gene set enrichment analyses. bioRxiv

Exp 2 FASP Methods – Orbitrap Exploris DIA

Protein samples were reduced, alkylated, and digested on-bead using filter-aided sample preparation [*Nature Methods* 6: 359-62 (2009)] with sequencing grade modified porcine trypsin (Promega). Tryptic peptides were then separated by reverse phase XSelect CSH C18 2.5 μ m resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 60 min gradient from 98:2 to 65:35 buffer A:B ratio. Eluted peptides were ionized by electrospray (2.2 kV) followed by mass spectrometric analysis on an Orbitrap Exploris 480 mass spectrometer (Thermo). To assemble a chromatogram library, six gas-phase fractions were acquired on the Orbitrap Exploris with 4 m/z DIA spectra (4 m/z precursor isolation windows at 30,000 resolution, normalized AGC target 100%, maximum inject time 66 ms) using a staggered window pattern from narrow mass ranges using optimized window placements. Precursor spectra were acquired after each DIA duty cycle, spanning the m/z range of the gas-phase fraction (i.e. 496-602 m/z, 60,000 resolution, normalized AGC target 100%, maximum injection time 50 ms). For wide-window acquisitions, the Orbitrap Exploris was configured to acquire a precursor scan (385-1015 m/z, 60,000 resolution, normalized AGC target 100%, maximum injection time 50 ms) followed by 50x 12 m/z DIA spectra (12 m/z precursor isolation windows at 15,000 resolution, normalized AGC target 100%, maximum injection time 33 ms) using a staggered window pattern with optimized window placements. Precursor spectra were acquired after each DIA duty cycle.

Buffer A = 0.1% formic acid, 0.5% acetonitrile

Buffer B = 0.1% formic acid, 99.9% acetonitrile

Following data acquisition, data were searched using Spectronaut (Biognosys version 18.5) against the UniProt *Caenorhabditis elegans* database (April 2023) using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to maxLFQ, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Protein MS2 intensity values were assessed for quality using ProteiNorm (Graw *et al.*). The data was normalized using VSN (Huber *et al.*) and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (lrimma) with empirical Bayes (eBayes) smoothing to the standard errors (Thurman *et al.*, Ritchie *et al.*). Proteins with an FDR adjusted p-value < 0.05 and a fold change > 2 were considered significant.

Acknowledgement:

IDeA National Resource for Quantitative Proteomics and NIH/NIGMS grant R24GM137786.

References:

1. Searle, B.C., Pino, L.K., Egertson, J.D. *et al.* Chromatogram libraries improve peptide detection and quantification by data independent acquisition mass spectrometry. *Nat Commun* 9, 5128 (2018). <https://doi.org/10.1038/s41467-018-07454-w>
2. Graw S, Tang J, Zafar MK, Byrd AK, Bolden C, Peterson EC, and Byrum SD. proteiNorm – A user-friendly tool for normalization and analysis of TMT and label-free protein quantification. *ACS Omega* 2020 5(40), 25625-25633. DOI: 10.1021/acsomega.0c02564.

3. Thurman TJ, Washam CL, Alkam D, Bird JT, Gies A, Dhusia K, Robeson II MS, and Byrum SD. proteoDA: a package for quantitative proteomics. 2023. Journal of Open Source Software. DOI: 10.21105/joss.05184.
4. Bolstad, B., *preprocessCore: A collection of pre-processing functions*. 2019.
5. Ritchie, M.E., et al., *limma powers differential expression analyses for RNA-sequencing and microarray studies*. Nucleic Acids Res, 2015. **43**(7): p. e47.
6. Chawade, A., E. Alexandersson, and F. Levander, *Normalyzer: a tool for rapid evaluation of normalization methods for omics data sets*. J Proteome Res, 2014. **13**(6): p. 3114-20.
7. Huber, W., et al., *Variance stabilization applied to microarray data calibration and to the quantification of differential expression*. Bioinformatics, 2002. **18 Suppl 1**: p. S96-104.
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Exp 3 FASP Methods – Orbitrap Exploris DIA

Protein samples were reduced, alkylated, and digested on-bead using filter-aided sample preparation [*Nature Methods* 6: 359-62 (2009)] with sequencing grade modified porcine trypsin (Promega). Tryptic peptides were then separated by reverse phase XSelect CSH C18 2.5 μ m resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 60 min gradient from 98:2 to 65:35 buffer A:B ratio. Eluted peptides were ionized by electrospray (2.2 kV) followed by mass spectrometric analysis on an Orbitrap Exploris 480 mass spectrometer (Thermo). To assemble a chromatogram library, six gas-phase fractions were acquired on the Orbitrap Exploris with 4 m/z DIA spectra (4 m/z precursor isolation windows at 30,000 resolution, normalized AGC target 100%, maximum inject time 66 ms) using a staggered window pattern from narrow mass ranges using optimized window placements. Precursor spectra were acquired after each DIA duty cycle, spanning the m/z range of the gas-phase fraction (i.e. 496-602 m/z, 60,000 resolution, normalized AGC target 100%, maximum injection time 50 ms). For wide-window acquisitions, the Orbitrap Exploris was configured to acquire a precursor scan (385-1015 m/z, 60,000 resolution, normalized AGC target 100%, maximum injection time 50 ms) followed by 50x 12 m/z DIA spectra (12 m/z precursor isolation windows at 15,000 resolution, normalized AGC target 100%, maximum injection time 33 ms) using a staggered window pattern with optimized window placements. Precursor spectra were acquired after each DIA duty cycle.

Buffer A = 0.1% formic acid, 0.5% acetonitrile

Buffer B = 0.1% formic acid, 99.9% acetonitrile

Following data acquisition, data were searched using Spectronaut (Biognosys version 18.5) against the UniProt *Caenorhabditis elegans* database (September 2023) using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to maxLFQ, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Protein MS2 intensity values were assessed for quality using ProteiNorm (Graw *et al.*). The data was normalized using VSN (Huber *et al.*) and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (lrimma) with empirical Bayes (eBayes) smoothing to the standard errors (Thurman *et al.*, Ritchie *et al.*). Proteins with an FDR adjusted p-value < 0.05 and a fold change > 2 were considered significant.

Acknowledgement:

IDeA National Resource for Quantitative Proteomics and NIH/NIGMS grant R24GM137786.

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Exp 4 FASP Methods – Orbitrap Exploris DIA

Protein samples were reduced, alkylated, and digested on-bead using filter-aided sample preparation [*Nature Methods* **6**: 359-62 (2009)] with sequencing grade modified porcine trypsin (Promega). Tryptic peptides were then separated by reverse phase XSelect CSH C18 2.5 μ m resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 60 min gradient from 98:2 to 65:35 buffer A:B ratio. Eluted peptides were ionized by electrospray (2.2 kV) followed by mass spectrometric analysis on an Orbitrap Exploris 480 mass spectrometer (Thermo). To assemble a chromatogram library, six gas-phase fractions were acquired on the Orbitrap Exploris with 4 m/z DIA spectra (4 m/z precursor isolation windows at 30,000 resolution, normalized AGC target 100%, maximum inject time 66 ms) using a staggered window pattern from narrow mass ranges using optimized window placements. Precursor spectra were acquired after each DIA duty cycle, spanning the m/z range of the gas-phase fraction (i.e. 496-602 m/z, 60,000 resolution, normalized AGC target 100%, maximum injection time 50 ms). For wide-window acquisitions, the Orbitrap Exploris was configured to acquire a precursor scan (385-1015 m/z, 60,000 resolution, normalized AGC target 100%, maximum injection time 50 ms) followed by 50x 12 m/z DIA spectra (12 m/z precursor isolation windows at 15,000 resolution, normalized AGC target 100%, maximum injection time 33 ms) using a staggered window pattern with optimized window placements. Precursor spectra were acquired after each DIA duty cycle.

Buffer A = 0.1% formic acid, 0.5% acetonitrile

Buffer B = 0.1% formic acid, 99.9% acetonitrile

Following data acquisition, data were searched using Spectronaut (Biognosys version 18.5) against the UniProt *Caenorhabditis elegans* database (September 2023) using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to maxLFQ, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Protein MS2 intensity values were assessed for quality using ProteiNorm (Graw *et al.*). The data was normalized using VSN (Huber *et al.*) and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (lrimma) with empirical Bayes (eBayes) smoothing to the standard errors (Thurman *et al.*, Ritchie *et al.*). Proteins with an FDR adjusted p-value < 0.05 and a fold change > 2 were considered significant.

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Exp 5 Methods – FASP - Eclipse -DDA:

Protein samples were reduced, alkylated, and digested on-bead using filter-aided sample preparation [*Nature Methods* **6**: 359-62 (2009)] with sequencing grade modified porcine trypsin (Promega).

Tryptic peptides were separated by reverse phase XSelect CSH C18 2.5 μ m resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 90 min gradient from 98:2 to 65:35 buffer A:B ratio. Eluted peptides were ionized by electrospray (2.4 kV) followed by mass spectrometric analysis on an Orbitrap Eclipse Tribrid mass spectrometer (Thermo). MS data were acquired using the FTMS analyzer in profile mode at a resolution of 120,000 over a range of 375 to 1200 m/z. Following HCD activation, MS/MS data were acquired using the ion trap analyzer in centroid mode and normal mass range with a normalized collision energy of 30%. Proteins were identified by database search using MaxQuant (Max Planck Institute) label-free quantification with a parent ion tolerance of 2.5 ppm and a fragment ion tolerance of 0.5 Da. Scaffold Q+S (Proteome Software) was used to verify MS/MS based peptide and protein identifications. Protein identifications were accepted if they could be established with less than 1.0% false discovery and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [*Anal. Chem.* **75**: 4646-58 (2003)].

Buffer A = 0.1% formic acid, 0.5% acetonitrile

Buffer B = 0.1% formic acid, 99.9% acetonitrile

Data Analysis

Proteins were identified and quantified by searching the UniprotKB database restricted to *Caenorhabditis elegans* (February 2024) using MaxQuant (version 2.2.0.0, Max Planck Institute). The database search parameters included selecting the MS1 reporter type, trypsin digestion with up to two missed cleavages, fixed modifications for carbamidomethyl of cysteine, variable modifications for oxidation on methionine and acetyl on N-terminus, the precursor ion tolerance of 5 ppm for the first search and 3 ppm for the main search, and label-free quantitation with iBAQ. Peptide and protein identifications were accepted using the 1.0% false discovery rate identification threshold. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al 2003).

MaxQuant iBAQ intensities for each sample were assessed for quality and differential abundance using proteoDA (Thurman et al. 2023; Graw et al. 2020). The data was normalized using VSN (Huber et al. 2002) and statistical analysis was performed using linear models for microarray data (limma) with empirical Bayes (eBayes) smoothing to the standard errors (Ritchie et al. 2015). Proteins with an FDR adjusted p-value < 0.05 and a fold change > 2 were considered significant.

Acknowledgement:

IDeA National Resource for Quantitative Proteomics and NIH/NIGMS grant R24GM137786.

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Exp 6 FASP Methods – Orbitrap Exploris DIA

Protein samples were reduced, alkylated, and digested on-bead using filter-aided sample preparation [*Nature Methods* 6: 359-62 (2009)] with sequencing grade modified porcine trypsin (Promega).

Tryptic peptides were trapped and eluted on 3.5µm CSH C18 resin (Waters) (4mm x 75µm) then separated by reverse phase XSelect CSH C18 2.5 µm resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted at a flow rate of 0.300µL/min using a 60 min gradient from 98% Buffer A:2% Buffer B to 95:5 at 2.0 minutes to 80:20 at 39.0 minutes to 60:40 at 48.0 minutes to 10:90 at 49.0 minutes and hold until 53.0 minutes and then equilibrated back to 98:2 at 53.1 minutes until 60 minutes. Eluted peptides were ionized by electrospray (2.4 kV) through a heated capillary (275°C) followed by data collection on an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific).

Precursor spectra were acquired with a scan from 385-1015 Th at a resolution set to 60,000 with 100% AGC, max time of 50 msec, and an RF parameter at 40%. DIA was configured on the Orbitrap 480 to acquire 50 x 12 Th isolation windows at 15,000 resolution, normalized AGC target 500%, maximum injection time 40 ms). A second DIA was acquired in a staggered window (12 Th) pattern with optimized window placements.

Buffer A = 0.1% formic acid, 0.5% acetonitrile

Buffer B = 0.1% formic acid, 99.9% acetonitrile

Following data acquisition, data were searched using Spectronaut (Biognosys version 19.1) against the UniProt *Caenorhabditis elegans* database (April 2024) using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to maxLFQ, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Protein MS2 intensity values were assessed for quality using ProteiNorm (Graw *et al.*). The data was normalized using VSN (Huber *et al.*) and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (limma) with empirical Bayes (eBayes) smoothing to the standard errors (Thurman *et al.*, Ritchie *et al.*). Proteins with an FDR adjusted p-value < 0.05 and a fold change > 2 were considered significant.

Acknowledgement:

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Exp 7 FASP Methods – Orbitrap Exploris DIA

Protein samples were reduced, alkylated, and digested on-bead using filter-aided sample preparation [*Nature Methods* 6: 359-62 (2009)] with sequencing grade modified porcine trypsin (Promega).

Tryptic peptides were trapped and eluted on 3.5µm CSH C18 resin (Waters) (4mm x 75µm) then separated by reverse phase XSelect CSH C18 2.5 µm resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted at a flow rate of 0.300µL/min using a 60 min gradient from 98% Buffer A:2% Buffer B to 95:5 at 2.0 minutes to 80:20 at 39.0 minutes to 60:40 at 48.0 minutes to 10:90 at 49.0 minutes and hold until 53.0 minutes and then equilibrated back to 98:2 at 53.1 minutes until 60 minutes. Eluted peptides were ionized by electrospray (2.4 kV) through a heated capillary (275°C) followed by data collection on an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific).

Precursor spectra were acquired with a scan from 385-1015 Th at a resolution set to 60,000 with 100% AGC, max time of 50 msec, and an RF parameter at 40%. DIA was configured on the Orbitrap 480 to acquire 50 x 12 Th isolation windows at 15,000 resolution, normalized AGC target 500%, maximum injection time 40 ms). A second DIA was acquired in a staggered window (12 Th) pattern with optimized window placements.

Buffer A = 0.1% formic acid, 0.5% acetonitrile

Buffer B = 0.1% formic acid, 99.9% acetonitrile

Following data acquisition, data were searched using Spectronaut (Biognosys version 19.1) against the UniProt *Caenorhabditis elegans* database (April 2024) using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to maxLFQ, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Protein MS2 intensity values were assessed for quality using ProteinNorm (Graw *et al.*). The data was normalized using VSN (Huber *et al.*) and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (limma) with empirical Bayes (eBayes) smoothing to the standard errors (Thurman *et al.*, Ritchie *et al.*). Proteins with an FDR adjusted p-value < 0.05 and a fold change > 2 were considered significant.

Acknowledgement:

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Exp 8 FASP Methods – Orbitrap Exploris DIA

Protein samples were reduced, alkylated, and digested on-bead using filter-aided sample preparation [*Nature Methods* 6: 359-62 (2009)] with sequencing grade modified porcine trypsin (Promega).

Tryptic peptides were trapped and eluted on 3.5µm CSH C18 resin (Waters) (4mm x 75µm) then separated by reverse phase XSelect CSH C18 2.5 µm resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted at a flow rate of 0.300µL/min using a 60 min gradient from 98% Buffer A:2% Buffer B to 95:5 at 2.0 minutes to 80:20 at 39.0 minutes to 60:40 at 48.0 minutes to 10:90 at 49.0 minutes and hold until 53.0 minutes and then equilibrated back to 98:2 at 53.1 minutes until 60 minutes. Eluted peptides were ionized by electrospray (2.4 kV) through a heated capillary (275°C) followed by data collection on an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific).

Precursor spectra were acquired with a scan from 385-1015 Th at a resolution set to 60,000 with 100% AGC, max time of 50 msec, and an RF parameter at 40%. DIA was configured on the Orbitrap 480 to acquire 50 x 12 Th isolation windows at 15,000 resolution, normalized AGC target 500%, maximum injection time 40 ms). A second DIA was acquired in a staggered window (12 Th) pattern with optimized window placements.

Buffer A = 0.1% formic acid, 0.5% acetonitrile

Buffer B = 0.1% formic acid, 99.9% acetonitrile

Following data acquisition, data were searched using Spectronaut (Biognosys version 19.5) against the UniProt *Caenorhabditis elegans* database (June 2024) using the directDIA method with an identification precursor and protein q-value cutoff of 1%, generate decoys set to true, the protein inference workflow set to maxLFQ, inference algorithm set to IDPicker, quantity level set to MS2, cross-run normalization set to false, and the protein grouping quantification set to median peptide and precursor quantity. Protein MS2 intensity values were assessed for quality using ProteiNorm (Graw *et al.*). The data was normalized using VSN (Huber *et al.*) and analyzed using proteoDA to perform statistical analysis using Linear Models for Microarray Data (limma) with empirical Bayes (eBayes) smoothing to the standard errors (Thurman *et al.*, Ritchie *et al.*). Proteins with an FDR adjusted p-value < 0.05 and a fold change > 2 were considered significant.

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