Supplementary Information

CUL4A-DDB1-DCAF10 is an N-recognin for N-terminally acetylated Src kinases

Nora Kremer[1,§], Franziska Mueller[2,§], Hang Nguyen[1], Tanja Popp[1], Elena Artes[1], Julian Wolters[1], Michael Renner[1], Ingrid Vetter[2], Stefano Maffini[2], Maria S. Robles[1], Andrea Musacchio[2,3], Tanja Bange[1,*]

1, Institute of Medical Psychology and Biomedical Center (BMC), Faculty of Medicine, LMU Munich, 80336 Munich, Germany

2, Department of Mechanistic Cell Biology, Max Planck Institute of Molecular Physiology, 44227 Dortmund, Germany

3, Centre for Medical Biotechnology, Faculty of Biology, University of Duisburg-Essen, 45141 Essen, Germany

§ these authors contributed equally

* address correspondence to

T.B.: tanja.bange@med.uni-muenchen.de, +49-89-218075654, ORCID 0000-0002-9680-8586

Keywords: N-degron pathway, Src family kinases, Nt-acetylation, N-myristoylation, ubiquitination, CUL4A-DDB1-DCAF10

Table of Contents:

Supplementary Methods

Purification of GST-DCAF10

DCAF10 was amplified from pcDNA3.1+/C-(K)-DYK (GenScript; Piscataway, New Jersey, USA) and via Gibson cloning integrated in the first cassette of pGEX-6P-2rbs (GenBank accession code KM817768)[46]. BL21(DE3) cells (Thermo Fisher Scientific, Waltham, MA, USA) transformed with pGEX-6P-2rbs-DCAF10 were inoculated from a single colony picked from a Lysogeny Broth (LB) agar (LB medium with 1.5% (w/v) agar) plate containing 100 ng/mL ampicillin and 34 ng/mL chloramphenicol, then cultured overnight in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.4) with the appropriate antibiotics. Terrific broth (TB) medium (24 g/L yeast extract, 20 g/L tryptone, glycerol 4 mL/L, 100 mL/L phosphate buffer containing 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) was used to prepare a 1:100 dilution of the starter culture, and OD_{600} (600 nm) was measured. At OD_{600} = 1, protein induction was initiated by adding 1:1000 dilution of 1 M IPTG for overnight expression at 16°C for 18 h with shaking (120 rpm). The following day, pellets were homogenized and lysed in glycerol lysis buffer (20 mM Tris-HCl pH 6.8, 300 mM NaCl, 10% (v/v) glycerol, 5 mM 2-mercaptoethanol supplemented with protease inhibitor cocktail). Lysis was performed on ice for 30 min, followed by sonication (7 × 20 sec pulses with 20 sec breaks on ice) using a Bandelin sonoplus sonicator (Sonotrode MS1.5, 70% output, 20W). Lysates were centrifuged at 4°C for 1 h at 14.000 × g, and the supernatant was transferred in a new tube. 1 L of TB growth medium culture yielded 20 mL of supernatant (collected in 20 × 1 mL tubes).

GST beads were used according to manufacturer's instructions (Pierce™ Glutathione magnetic agarose bead; Thermo Fisher Scientific, Waltham, MA, USA). For each 1 mL of supernatant, 100 µL GST magnetic beads were used. 100 µL GST-magnetic beads were washed twice with 500 µL wash buffer (125 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM EDTA) in a 2 mL tube. The lysis supernatant was diluted 1:1 with wash buffer, and incubated with the beads on an end-over-end rotator for 1 h at 25°C. After incubation, the supernatant was removed, and

the beads were washed twice with wash buffer (without DTT and EDTA). Protein elution was then performed by incubating the beads with glutathione elution buffer (10 mM reduced glutathione, 125 mM Tris-HCl pH 7.4, 250 mM NaCl, 0.3% Triton X-100) for 30 min at 25°C on an end-over-end rotator. This process was repeated with glutathione elution buffer containing 0.1% Triton X-100 under the same conditions. Elution fractions were pooled and concentrated by centrifugation (4°C, 25 min, 4500 rpm) using a 30 kD cut-off concentrator (Merck Millipore, #UFC803008, Darmstadt, Germany). Purified proteins were analyzed by colloidal Coomassie stain, WB and MS.

Cloning, expression, and purification of hNatA

Human NatA (hNatA) full-length (FL) was cloned in a pFL vector expressing both genes, NAA15 with a 6× C-term histidine tag (NAA15-6xHIS) and NAA10. The expression was carried out in insect cells (Tnao38) for 72 h infected 1:20 with baculovirus. Washed cell pellets were lysed by sonication in a lysis buffer containing 200 mM NaCl, 50 mM HEPES pH 8.0, 20 mM Imidazole, and 2 mM TCEP. The protein purification was performed as a three-step purification, starting with an affinity nickel-column, followed by an ion-exchange column with a gradient of 50–300 mM NaCl, and finished with size exclusion chromatography (SEC) using an S200 10/300 column. The main peaks were collected, and the protein was concentrated in SEC buffer (150 mM NaCl, 50 mM HEPES pH 8.0, 1 mM EDTA). The purified hNatA was flash-frozen in liquid nitrogen and stored at -80°C.

SiRNA knockdown and overexpression experiments in HeLa, DLD-1, and RPE-1 cell lines

HeLa, DLD-1, and RPE-1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were counted and diluted to 100,000 cells/mL. For siRNA knockdown, the transfection reagent Lipofectamine RNAiMAX (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used at a final dilution of

1:333 (3 µL/mL of medium). Pools of siRNAs, including siNMT1, siNMT2, siDCAF10, siZYG11B, and siZER1, or combinations of different siRNAs, were reverse transfected at a concentration of 25 nM for each siRNAi and incubated as indicated (48–72 h). As control, cells were treated only with Lipofectamine RNAiMAX. Both siRNAs and RNAiMAX were diluted in Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA). For overexpression of DCAF10, the transfection reagent Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used at a final dilution of 1:200 (2 µL/mL of medium). For one 6-well, 4 µg of DNA were combined with Lipofectamine 2000 and diluted in Opti-MEM. The medium was changed 4–6 h post-transfection. The transfection was performed one day after plating, and the cells were incubated for an additional 24 h. For proteasomal or translational inhibition, the cells were treated with 10 µM MG132 or 100 µg/mL cycloheximide for 4 and 8 h before lysis, respectively.

High-pH fractionation for DCAF10 and ZYG11B MS measurements

10 µg of cellular lysates were reduced, alkylated, and digested with LysC/Trypsin (protein:enzyme ratio of 1:100) according to MS standard in-solution digest[55]. Peptides were then separated with a high-pH fractionation kit (eight fractions for DCAF10 and four fractions for ZYG11B; Thermo Fisher Scientific, Waltham, MA, USA). Each fraction was again separated using a 50 min gradient from 5–60% acetonitrile with 0.1% formic acid using a self-packed 50 cm column (ReprosilC18, 75 µM inner diameter), and directly sprayed via a nano-electrospray source in an Orbitrap Exploris™ 480 (Thermo Fisher Scientific, Waltham, MA, USA). Data were acquired in a data-independent mode, acquiring one survey scan (MS scan) with 120 k resolution and subsequently 34 windows with an isolation width of 18.7 Th with 1 Dalton overlap from 350 to 1000 m/z at a resolution of 15 k (1.5 sec cycle time). The target value was set to 300% for the MS scan and 1000% for the MS/MS scans. The maximum injection time was 45 msec for MS. HCD Collision energy was set to 30%. Resulting raw files

were processed with DIA-NN (version 1.8.2 beta 22 and 27) using a Uniprot human database (reference January 2023)[91]. Oxidation (M), M excision, Nt-acetylation, and carbamidomethylation (C) were given as modifications. One miscleavage and two modifications per peptide were allowed. A false discovery rate cut-off of 1% was applied. Intensities of fractions from the report.pg_matrix.tsv were then summed and log_2-transformed (log_2) for overall protein quantities. DCAF10 and ZYG11B were detected in control cells but not in KD cells. To not overestimate the KD, we considered that the protein amounts of ZYG11B and ZER in the siRNA treated cells must be below the detection limit of these MS runs. Therefore, we used for our knock-down estimation the log_2 intensity of DCAF10 or ZYG11B, respectively, in the control cells and subtracted it from the lowest measured proteins. For DCAF10: 22.37 (mean log_2 intensity of DCAF10) - 14.35 (log_2 intensity of lowest quantified protein) means at least a log_2 reduction of 8. For ZYG11B: 19.7 (mean log_2 intensity of Zyg11B) - 15.7 (log_2 intensity of lowest measured proteins) means at least a log_2 reduction of 4.

Site-directed mutagenesis

To generate Lyn variants with mutated N-terminal amino acid, site-directed mutagenesis was performed on the pcDNA5/FRT/TO-Lyn^{WT}-EGFP-IRES construct using primers (listed in **Supplementary Table S4**) that carry single (for G2A) or double (for G2P) mismatches to wild-type Lyn in PCR reactions. The resulting PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega; Madison, WI, USA) according to the manufacturer's instructions, followed by DpnI digestion, phosphorylation, and ligation. Subsequently, half of the ligation reaction was transformed into DH5α competent cells. After isolation, the plasmid DNA from single colonies was sent for Sanger sequencing (Eurofins Genomics, Munich, Germany) to confirm successful mutagenesis.

Fluorescence microscopy

Cells were seeded on coverslips to be 90% confluent on the day of fixation. Lyn-GFP expression was induced by 24 h of doxycycline treatment (100 ng/mL for Lyn^{WT}, and 10 ng/mL for Lyn^{G2A} and Lyn^{G2P}), or media was just replaced for the controls (without doxycycline). Cells were washed once with PBS before fixation with 4% PFA in PBS for 10 min at RT. After three washes with PBS, cells were permeabilized with PBS-T (PBS with 0.1% (v/v) Tween-20) for 10 min at RT. Afterwards, cover slips were incubated in 0.5 µg/mL DAPI diluted in PBS-T for 5 min at RT. After a final wash in ddH_2O, the coverslips were dried on the air and mounted with Mowiol as a mounting medium on glass slides. Image acquisition of cells was performed at RT using a Deltavision Elite System (GE Healthcare, Chicago, IL, USA) equipped with an IX-71 inverted microscope (Olympus, Tokyo, Japan), a 60x/1.42 Plan Apo N objective and a pco.edge sCMOS camera (PCO-TECH Inc., Kelheim, Germany). Images were acquired as z-stacks containing 16 sections with a distance of 200 nm using the software softWoRx (GE Healthcare, Chicago, IL, USA). Raw data were then deconvolved and converted into average intensity projections, exported, and saved as 16-bit TIFF files. Figures were edited and arranged using ImageJ. Acquisition settings: Green channel: 0.1 sec, 50% transmission; DAPI channel: 0.01 sec, 32% transmission.

Supplementary Methods References

55. Shevchenko, A., Tomas, H., Havli, J., Olsen, J. V. & Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **1**, 2856–2860 (2006).

Supplementary Tables

Peptides have been purchased from GenScript (Piscataway, NJ, USA).

Supplementary Table S1.. Peptides

Gene name	Peptide sequence	Protein name / UniProt ID	Nt modification	C-terminal modification
ARF1	GNIFANLFKGKK	ADP-ribosylation factor 1 / P84077	/	K(biotin)
ARF1	GNIFANLFKGKK	ADP-ribosylation factor 1 / P84077	acetyl-group	K(biotin)
COX17	PGLVDSNPAPKK	Cytochrome c oxidase copper chaperone / Q14061	/	K(biotin)
EF1B	GFGDLKSPAGKK	Elongation factor 1-beta / P24534	/	K(biotin)
EF1B	GFGDLKSPAGKK	Elongation factor 1-beta / P24534	acetyl-group	K(biotin)
FYN	GCVQCKDKEAKK	Tyrosine-protein kinase Fyn / P06241	/	K(biotin)
FYN	GCVQCKDKEAKK	Tyrosine-protein kinase Fyn / P06241	acetyl-group	K(biotin)
FYN	GCVQCKDKEAKK	Tyrosine-protein kinase Fyn / P06241	myristoyl-group	K(biotin)
GNAI3	GCTLSAEDKAKK	Guanine nucleotide-binding protein G(i) subunit alpha-3 / P08754	/	K(biotin)
GNAI3	GCTLSAEDKAKK	Guanine nucleotide-binding protein G(i) subunit alpha-3 / P08754	acetyl-group	K(biotin)
LYN	GCIKSKGKDSKK	Tyrosine-protein kinase Lyn / P07948	/	K(biotin)
LYN	GCIKSKGKDSKK	Tyrosine-protein kinase Lyn / P07948	acetyl-group	K(biotin)
LYN	GCIKSKGKDSKK	Tyrosine-protein kinase Lyn / P07948	myristoyl-group	K(biotin)
LYN G2A	ACIKSKGKDSKK	Tyrosine-protein kinase Lyn / P07948	acetyl-group	K(biotin)
LYN P2A	PCIKSKGKDSKK	Tyrosine-protein kinase Lyn / P07948	/	K(biotin)
NatA target peptide	SASEAGVRWGRPVGRRRR	/	/	/
NDUFAF4	GALVIRGIRNKK	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 4 / Q9P032	/	K(biotin)
NDUFAF4	GALVIRGIRNKK	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 4 / Q9P032	acetyl-group	K(biotin)
SRC	GSNKSKPKDAKK	Proto-oncogene tyrosine-protein kinase Src / P12931	/	K(biotin)
SRC	GSNKSKPKDAKK	Proto-oncogene tyrosine-protein kinase Src / P12931	acetyl-group	K(biotin)
THOC7	GAVTDDEVIRKK	THO complex subunit 7 / Q6I9Y2	/	K(biotin)

THOC7	GAVTDDEVIRKK	THO complex subunit 7 / Q6I9Y2	acetyl-group	K(biotin)
TMEM97	GAPATRRCVEKK	Sigma intracellular receptor 2 / Q5BJF2	/	K(biotin)
TMEM97	GAPATRRCVEKK	Sigma intracellular receptor 2 / Q5BJF2	acetyl-group	K(biotin)
YES	GCIKSKENKSKK	Tyrosine-protein kinase Yes / P07947	/	K(biotin)
YES	GCIKSKENKSKK	Tyrosine-protein kinase Yes / P07947	acetyl-group	K(biotin)

171 **Supplemenatry S2. Cell lines**

Cell line	Plasmid	Gene	Tag	Reference
DLD-1 Flp-In™ T-REx™	/	/	/	A gift from AG Musacchio, Dr. Stefano Maffini (MPI Dortmund)
DLD-1 Flp-In™ T-REx™ Lyn KO	/	/	/	this study
DLD-1 KO Lyn Flp-In™ T-REx™ Lyn^{G2A}-GFP	pCDNA5/FRT/TO-EGFP-IRES	LYN-G2A	GFP (C-term)	this study
DLD-1 KO Lyn Flp-In™ T-REx™ Lyn^{G2P}-GFP	pCDNA5/FRT/TO-EGFP-IRES	LYN-G2P	GFP (C-term)	this study
DLD-1 KO Lyn Flp-In™ T-REx™ Lyn^{WT}-GFP	pCDNA5/FRT/TO-EGFP-IRES	LYN-WT	GFP (C-term)	this study
Hela Flp-In™ T-REx™	/	/	/	A gift from S. Taylor (University of Manchester)
RPE-1 Flp-In™ T-REx™	/	/	/	A gift from AG Musacchio, Dr. Stefano Maffini (MPI Dortmund)

172

173 **Tsupplementary S3. siRNAs**

siRNA	Company	Identifier
ON-TARGETplus siRNA Human DCAF10	Dharmacon	L-014344-01-0005
ON-TARGETplus siRNA Human NMT1	Dharmacon	L-004316-01-0005
ON-TARGETplus siRNA Human NMT2	Dharmacon	L-004317-01-0005
ON-TARGETplus siRNA Human ZER1	Dharmacon	L-019424-02-0005
ON-TARGETplus siRNA Human ZYG11B	Dharmacon	L-021798-02-0005

174 **Supplementary Table S4. Oligonucleotides and primers (F: Forward, R: Reverse)**

Sequence 5´to 3´	Company	Purpose
GTTCTGTTCCAGGGGCCCCTGGGATCCATGTTTCCCTTTGGGCCCCATAG	Metabion	F primer DCAF10 to clone in pGEX-6P-2rbs for Gibson cloning
CAGTCAGTCACGATGCGGCCGCTCGAGCTACTAAAACTTTGGCTGTACAAAGAAACCCG	Metabion	R primer DCAF10 to clone in pGEX-6P-2rbs for Gibson cloning
TTCTTCCAGTTGCCCCCTCT	Metabion	crRNA for Lyn KO (sequence 1)
GGAGGCCTCATACCCATTACA	Metabion	crRNA for LYN KO (sequence 2)
GCCACCATGGTGAGCAAG	Metabion	pcDNA5-F to linearize pcDNA5/FRT/TO-EGFP-IRES
GGTACCAAGCTTAAGTTTAAACGC	Metabion	pcDNA5-R to linearize pcDNA5/FRT/TO-EGFP-IRES
TTAAACTTAAGCTTGGTACCATGGGATGTATAAAATCAAAAGGG	Metabion	F primer to amplify LYN cDNA from pCR4-TOPO-LYN for Gibson assembly
CCCTTGCTCACCATGGTGGCAGGCTGCTGCTGGTATTG	Metabion	R primer to amplify LYN cDNA from pCR4-TOPO-LYN for Gibson assembly

CTTAAGTTTAAACGCTAGAGTCCG	Metabion	LYN-R Site-directed mutagenesis of LYN in the pcDNA5/FRT/TO-LYN^{WT}-EGFP-IRES construct
CTTGGTACCATGGCATGTATAAAATC	Metabion	LYN G2A-F Site-directed mutagenesis of LYN in the pcDNA5/FRT/TO-LYN^{WT}-EGFP-IRES construct
CTTGGTACCATGCCATGTATAAAATC	Metabion	LYN G2P-F Site-directed mutagenesis of LYN in the pcDNA5/FRT/TO-LYN^{WT}-EGFP-IRES construct
GCTACCCGGGATGAACATCCGCAATGCC	Sigma	NAA10 F - Amplifying full-length wildtype human NAA10 for pFH vector
TGCAGCTAGCTTAGCTGGCGCTATCGC	Sigma	NAA10 R - Amplifying full-length wildtype human NAA10 for pFH vector
GATCGGATCCATGCCAGCCGTGTCCCTG	Sigma	NAA15 F - Amplifying full-length wildtype human NAA15-6xHIS for pFH vector
CGTAGTCGACCTAGTGATGGTGATGGTGATG GATCTCGTTGGCCAGCTC	Sigma	NAA15-6xHIS R - Amplifying full-length wildtype human NAA15-6xHIS for pFH vector
TTGTTTCAAGGTCCTGGATCCTCGTACAACTA CGTGGTAACGGC	Sigma	DDB1 F StrepII - Gibson cloning in pLIB
TCCTCTAGTACTTCTCGACAAGCTTTTACTAA TGGATCCGAGTTAGCTCCTCC	Sigma	DDB1 R - Gibson cloning in pLIB
CCACCATCGGGCGCGGATCCATGCATCACCA TCACCATCACTTTCCCTTTGGGCCCCATAGC	Sigma	DCAF10 6xHIS F - Gibson cloning in pLIB
CTGTTCCAGGGGCCCGGATCCTTTCCCTTTGG GCCCCATAGC	Sigma	DCAF10 F MBP - Gibson cloning in pLIB
TCCTCTAGTACTTCTCGACAAGCTTTTATCAA AACTTTGGCTGATACAAAGAAACCCG	Sigma	DCAF10 R - Gibson cloning in pLIB
CCACCATCGGGCGCGGATCCATGGCGGACGA GGCCC	Sigma	CUL4A F - Gibson cloning in pLIB
TCCTCTAGTACTTCTCGACAAGCTTTTATCAG GCCACGTAGTGGTACTG	Sigma	CUL4A R - Gibson cloning in pLIB
CCACCATCGGGCGCGGATCCATGGCGGCGGC GATG	Sigma	RBX1 F - Gibson cloning in pbig1a
TCCTCTAGTACTTCTCGACAAGCTTTTACTAA TGCCCATACTTCTGGAACTCCC	Sigma	RBX1 R - Gibson cloning in pbig1a
AACGCTCTATGGTCTAAAGATTTAAATCGAC CTACTCCGGAATATTAATAGATC	Sigma	Cas I F - Gibson cloning in pbig1a
AAACGTGCAATAGTATCCAGTTTATTTAAAT GGTTATGATAGTTATTGCTCAGCG	Sigma	Cas I R - Gibson cloning in pbig1a
AAACTGGATACTATTGCACGTTTAAATCGAC CTACTCCGGAATATTAATAGATC	Sigma	Cas II F - Gibson cloning in pbig1a
AAACATCAGGCATCATTAGGTTTATTTAAAT GGTTATGATAGTTATTGCTCAGCG	Sigma	Cas II R - Gibson cloning in pbig1a
AAACCTAATGATGCCTGATGTTTAAATCGAC CTACTCCGGAATATTAATAGATC	Sigma	Cas III F - Gibson cloning in pbig1a
AACCCCGATTGAGATATAGATTTATTTAAAT GGTTATGATAGTTATTGCTCAGCG	Sigma	Ω R - Gibson cloning in pbig1a
GTACGGTTGCATGGGGAGTCGCCAACGCTGC TTTTCATTTCCACTGCATC	Sigma	RBX1 mut - Gibson cloning in pbig1a
ATTGGAGACATTTTGATGGCTTG	Metabion	F - Diagnostic primers for LYN KO exon 2
TTTCTTCACACAAAAGAATGTGACC	Metabion	R - Diganositc primers for LYN KO exon 2
TTCTTCCAGTTGCCCCCTCT	Metabion	F - Diagnostic primers for LYN KO exon 4
GGAGGCCTCATACCCATTACA	Metabion	R - Diganositc primers for LYN KO exon 4

176 **Supplemenatry Table S5. Plasmids**

Plasmid backbone	Gene	Reference
pbiG1a	/	A gift from AG Musacchio, (MPI Dortmund)
pbiG1a	DDB1, CUL4A, RBX1 human	this study
pbiG1a	DDB1, CUL4A, RBX1$^{C75A/H77A}$ human	this study
pcDNA3.1+/c-(k)-DYK	DCAF10 human	Genscript ORF #OHu10624D
pcDNA5/FRT/TO--EGFP-IRES	LYN^{G2A} human	this study
pCDNA5/FRT/TO-EGFP-IRES	/	A gift from AG Musacchio, Dr. Stefano Maffini (MPI Dortmund)
pcDNA5/FRT/TO-EGFP-IRES	LYN^{WT} human	this study
pcDNA5/FRT/TO-EGFP-IRES	LYN^{G2P} human	this study
pCR4-TOPO-LYN	LYN human	Horizon Discovery/Dharmacon (#MHS6278-202856900)
pFL	/	A gift from AG Musacchio (MPI Dortmund)
pFL	NAA15-6xHIS + NAA10 human	this study
pGEX-6P-2rbs	DCAF10 human	this study
pGEX-6P-2rps	/	GenBank accession code KM817768
pLIB	/	A gift from AG Musacchio (MPI Dortmund)
pLIB	CUL4A human	this study
pLIB	RBX1 human	this study
pLIB-6xHis-MBP	DCAF10 human	this study
pLIB-HIS-MBP	/	A gift from AG Musacchio, Dr. John Weir (MPI Dortmund)
pLIB-StrepII	DDB1 human	this study
pLIB-StrepII-3C	/	A gift from AG Musacchio (MPI Dortmund)
pOG44	/	A gift from AG Musacchio, Dr. Stefano Maffini (MPI Dortmund)

177

178 **Supplementary Table S6. Antibodies**

Antibody	Company	Identifier
anti-CUL4A Rabbit pAb	Cell Signaling	#2699
anti-DCAF10/ WDR32 Rabbit pAb	Thermo Fisher Scientific	#PA5-24133
anti-DDB1 (D4C8) Rabbit mAb	Cell Signalling	#6998

anti-FLAG (DYKDDDK) Mouse mAb	Cell Signaling	#8146S
anti-Fyn Rabbit pAb	Cell Signaling	#4023S
anti-GFP Rabbit pAb	In-house	AG Musacchio
anti-GFP Mouse mAb	Roche	#11814460001
anti-GST Mouse mAb	Merck Millipore	#71097
anti-Lyn (C13F9) Rabbit mAb	Cell Signaling	#2796S
anti-mouse IgG HRP linked antibody	Cell Signaling	#CST 7076S
anti-NMT1 Rabbit pAb	abcam	#AB186123
anti-NMT2 Rabbit pAb	abcam	#AB230028
anti-rabbit IgG HRP-linked Antibody	Cell Signaling	#7074S
anti-Src (36D10) Rabbit mAb	Cell Signaling	#2109S
anti-Streptavidin Rabbit pAb	Rockland immunochemicals	#100-4195
anti-THOC7 Rabbit pAb	abcam	#ab155218
anti-Ubiquitin-HRP (P4D1) Mouse mAb	Cell Signaling	#14049
anti-Vinculin Mouse mAb	Sigma	#V9131
anti-ZER1 Rabbit pAb	Proteintech	#16647-1-AP
anti-ZYG11B Rabbit pAb	antibodies-online.com	#ABIN4916715
VeriBlot Detection Reagent (for IP samples)	abcam	#ab131366

Supplementary Table S7. Antibiotics used in bacterial and cell culture

Reagent	Working concentration	Company
Ampicillin	100 µg/mL	Sigma
Blasticidin	8 µg/mL	Sigma
Chloramphenicol	34 µg/mL	Sigma
Doxycycline	10–100 ng/mL	Sigma
Gentamicin	10 µg/mL	Sigma
Hygromycin B	400 µg/mL	Sigma
Kanamycin	50 µg/mL	Sigma
Penicillin-streptomycin	100 U/mL	Gibco/Thermo Fisher Scientific
Tetracyclin	7 µg/mL	Sigma

Tsupplementary Table S8. Reagents and Kits

Reagent	Company	Reference number
Acetyl-Coenzym A	Sigma Aldrich	A2056
Biotin	iba	2-1016-002
BL21(DE3)	Thermo Fisher Scientific	EC0114
CoA	Sigma Aldrich	C4282
CPM (7-Diethylamino-3-(4'-Maleimidylphenyl)-4-Methylcoumarin)	Invitrogen	D346
Cycloheximide	Sigma Aldrich	C4859-1mL
Dulbecco's Modified Eagle Medium (DMEM), high glucose, GlutaMAX Supplement, pyruvate	Gibco/Thermo Fisher Scientific	31966047
Dynabeads ™ MyOne™ Streptavidin	Invitrogen/ Thermo Fisher Scientific	65601
Dynabeads™ Protein A Immunoprecipitation Kit	Invitrogen/ThermoFisher Scientific	10006D
Fetal bovine serum (FBS)	Pan Biotech	P13-3001
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific	K0502
GFP-Trap magnetic agarose	ChromoTek	gtma
Gluthatione	Thermo Fisher Scientific	78259
GST magnetic agarose beads	Pierce/ Thermo Fisher Scientific	#78602
IPTG	Thermo Fisher Scientific	15529019
Lipofectamine RNAiMAX	Invitrogen/ Thermo Fisher Scientific	13778-150
Lipofectamine® 2000	Invitrogen/ Thermo Fisher Scientific	11668-027
LysC	Wako Chemicals	129-02541
MG132	Sigma-Aldrich	474787
Monarch DNA Gel Extraction Kit	New England BioLabs	T1120S

Monarch PCR & DNA Cleanup Kit	New England BioLabs	T1130S/L
Opti-MEM I reduced-Serum medium	Gibco/Thermo Fisher Scientific	31985062
PageRuler Plus Prestained Protein Ladder	Thermo Fisher Scientific	26619
Phsophostop	Roche	04906845001
Pierce ™ Glutathione Magnetic Agarose	Thermo Fisher Scientific	78602
Precision Plus Protein Standard Dual color	BIO-RAD	1610374
Protease Inhibitors	Roche	04693132001
PureYield Plasmid Miniprep System	Promega	A1223
Recombinant Human UbcH5a/UBE2D1 Protein (E2)	Bio-Techne GmbH	E2-622
Recombinant Human Ubiquitin Activating Enzyme (UBE1)	Bio-Techne GmbH	E-304-050
Recombinant Human Ubiquitin Protein (Ub)	Bio-Techne GmbH	P0CG47.1
SF Cell Line 4D-Nucleofector X Kit S	Lonza	V4XC-2032
Strep-Tactin®XT 4Flow® beads	iba	2-5010-010
Trypsine	Sigma-Aldrich	T6567
Wizard SV Gel and PCR Clean-Up System	Promega	A9281
X-tremeGNE 360 Transfection reagent	Sigma-Aldrich	XTG360RO

183 **Supplementary Table S9. List of enzymes**

Enzyme	**Supplier**	**Reference Number**
*BamH*I HF	New England Biolabs GmbH	R3136S
Benzonase® Nuclease	Sigma-Aldrich	9025-65-4
*Dpn*I restriction enzyme	New England Biolabs GmbH	RO176S
Gibson Assembly Master Mix (containing T5 Exonuclease, Phusion polymerase, Tag ligase)	New England Biolabs GmbH/ *in-house;* AG Musacchio	E2611S
*Hind*III HF	New England Biolabs GmbH	R3104S
Phusion® High-Fidelity PCR 2x Master Mix	New England Biolabs GmbH	M0530S
*Pme*I	New England Biolabs GmbH	R0560S
Q5® High-Fidelity 2x Master Mix	New England Biolabs GmbH	M0494S
*Swa*I	New England Biolabs GmbH	R0604S
T4 DNA Ligase	New England Biolabs GmbH	M0202S
T4 Polynucleotide Kinase	New England Biolabs GmbH	M0201S

184 **Supplementary Table S10. Software and Algorithms**

Software	**Version**	**Supplier**
BioRender	-	BioRender
DIA-NN	1.8.2. beta 22 and 27	https://github.com/vdemichev/DiaNN[43]
GraphPad Prism	Version 8.4.3	GraphPad Software, LLC.
ImageJ	2.0.0-rc-69/1.52p	National Institutes of Health
Image Lab Software	6.0.1	BIO-RAD
Perseus	1.6.50, 2.0.11	https://maxquant.net/perseus/[44]
PyMOL: The PyMOL molecular Graphics System	Version 3.0	Schrödinger, LLC
Resolve3D softWoRx-Acquire	Version: 7.2.0	Release RC4
Rstudio	version 2023.06.0	Posit Software, PBC
SnapGene	7.1.2	GSL Biotech LLC

185