Functional proteomics and deep network interrogation reveal a complex mechanism of action of midostaurin in lung cancer cells

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Running title:

Analysis of midostaurin mechanism by functional proteomics

Supplemental Figure S1. Publicly available IC_{50} data for inhibition of cell viability across large cancer cell line panel, grouped by tissue classification, generated by the Massachusetts General Hospital and the Sanger Institute. Plasma concentration of midostaurin is depicted in grey. Cell lines with a lower IC_{50} value than 0.1 μM are labeled. *B*, Induction of apoptosis determined by Caspase 3/7 cleavage in A427 cells upon treatment with indicated concentrations of midostaurin, sotrastaurin or ruboxistaurin or vehicle control (DMSO) over a period of 72 hrs utilizing IncuCyte live cell analysis. Data was acquired every 3 hrs. Data points represent biological triplicates each performed in technical triplicate (mean). *C*, Detailed histogram representation of percent of cells in G1, S or G2 phase after 6 hrs of midostaurin treatment (0.5 and 1 μM) compared to vehicle control (DMSO) in A427 cells. Histograms are representative of three biological replicates.

Supplemental Figure S2. *A-C*, Replicate correlations of IRON-normalized intensity values for biological replicates of phosphoproteomics experiments (*A* = pY vehicle control (DMSO), *B* = pY midostaurin treated, *C* = global TMT data). Scale is $\log_2$ intensity, histogram shows distribution of peptides. *D*, Boxplot showing the intensity distribution of IRON-normalized intensity values of the individual reporter ions from the global TMT phosphoproteomics experiment.

Supplemental Figure S3. Replicate correlations of IRON-normalized intensity values for dose-dependent midostaurin competition and sotrastaurin cross-competition pulldown experiments. Percent confidence interval (% CI) is shown.

Supplemental Figure S4. *A*, Viability of A427 cells following 72 hrs treatment with combinations of increasing concentrations of STO-609 (CAMKK2i) and 0.5 μM of GSK2334470 (PDPK1i), BX795 (TBK1i) and alisertib (AURKAi) determined by CellTiterGlo. Data depicts three biological replicates (mean ± SD). *B*, Evaluation of midostaurin effects on phosphorylation of AKT and the CAMKK2 downstream substrate AMPK in A427 cells following 3 hrs of treatment with increasing drug concentrations (0.1 μM, 0.5 μM, 1

μM). Image is representative of three biological replicates. *C*, Relative A427 cell counts upon 96 hrs siRNA-mediated knockdown of *TBK1*, *PDPK1* and/or *CAMKK2* and/or 72 hrs treatment with 0.5 μM alisertib. Data depicts three biological replicates each performed in technical triplicate (mean ± SD). Knockdown efficiency was determined using immunoblotting. NT, non-targeting siRNA. *D*, IC_{50} values for inhibition of *in vitro* kinase activity of AURKA, TBK1 and PDPK1 by midostaurin and sotrastaurin are shown. DMSO: vehicle control.

Supplemental Table S1. Global phosphoproteomics (pSTY) dataset. Localization probability, phosphorylated amino acid, number of phosphorylation sites, peptide sequence, PEP score, phosphorylation probabilities, PTM position in the peptide, peptide charge, mass error, intensity and identification as potential contaminant is shown. Reporter ion 0 (channel 126, vehicle (DMSO) 1), reporter ion 1 (channel 127, vehicle (DMSO) 2), reporter ion 2 (channel 128, vehicle (DMSO) 3), reporter ion 3 (channel 129, Midostaurin 1), reporter ion 4 (channel 130, Midostaurin 2), reporter ion 5 (channel 131, Midostaurin 3).

Supplemental Table S2. Tyrosine phosphoproteomics (pY) dataset. Localization probability, phosphorylated amino acid, number of phosphorylation sites, peptide sequence, PEP score, phosphorylation probabilities, PTM position in the peptide, peptide charge, mass error, intensity and identification as potential contaminant is shown.

Supplemental Table S3. Chemical proteomics (CP) dataset. Uniprot accession identifiers, Q value, exclusive unique peptide count and ion intensities are shown. M: midostaurin; S: sotrastaurin; CT: competition treatment (using indicated compound concentration); PD: pulldown; PDoPD: pulldown of pulldown. Suffix indicates replicate series.

Supplemental Table S4. KEGG pathways enriched using the significantly modulated pY, pSTY phosphopeptides and direct midostaurin kinase targets. Shown is the DAVID output in addition with respective modules (Fig 4*A*) of the assigned genes.

Supplemental Information

MaxQuant Parameters (Chemical Proteomics)

Version 1.5.2.8
Fixed modifications Carbamidomethyl (C)
Decoy mode revert
Special AAs KR
Include contaminants True
MS/MS tol. (FTMS) 20 ppm
Top MS/MS peaks per 100 Da. (FTMS) 12
MS/MS deisotoping (FTMS) True
MS/MS tol. (ITMS) 0.5 Da
Top MS/MS peaks per 100 Da. (ITMS) 8
MS/MS deisotoping (ITMS) False
MS/MS tol. (TOF) 40 ppm
Top MS/MS peaks per 100 Da. (TOF) 10
MS/MS deisotoping (TOF) True
MS/MS tol. (Unknown) 0.5 Da
Top MS/MS peaks per 100 Da. (Unknown) 8
MS/MS deisotoping (Unknown) False
PSM FDR 0.01
Protein FDR 0.01
Site FDR 0.01
Use Normalized Ratios For Occupancy True
Min. peptide Length 7
Min. score for unmodified peptides 0
Min. score for modified peptides 40
Min. delta score for unmodified peptides 0
Min. delta score for modified peptides 6
Min. unique peptides 0
Min. razor peptides 1
Min. peptides 1

Use only unmodified peptides and True
Modifications included in protein quantification Acetyl (Protein N-term);Oxidation (M)
Peptides used for protein quantification Razor
Discard unmodified counterpart peptides True
Min. ratio count 1
Re-quantify True
Use delta score False
iBAQ False
iBAQ log fit False
Match between runs True
Matching time window [min] 10
Alignment time window [min] 20
Find dependent peptides False
Fasta file C:\MaxQuant 1.5.2.8\Databases\SwissProt_Human_2018_05.fasta
Labeled amino acid filtering True
Site tables Oxidation (M)Sites.txt
Decoy mode revert
Special AAs KR
Include contaminants True
RT shift False
Advanced ratiosTrue
AIF correlation 0.47
First pass AIF correlation 0.8
AIF topx 20
AIF min mass 0
AIF SIL weight 4
AIF ISO weight 2
AIF iterative True
AIF threshold FDR 0.01

MaxQuant Parameters (Tyrosine Phosphoproteomics)

Version 1.2.2.5
Fixed modifications Carbamidomethyl (C)
Randomize FALSE
Special AAs KR
Include contaminants TRUE
MS/MS tol. (FTMS) 20 ppm
Top MS/MS peaks per 100 Da. (FTMS) 10
MS/MS deisotoping (FTMS) TRUE

MS/MS tol. (ITMS) 0.5 Da
Top MS/MS peaks per 100 Da. (ITMS) 6
MS/MS deisotoping (ITMS) FALSE
MS/MS tol. (TOF) 0.1 Da
Top MS/MS peaks per 100 Da. (TOF) 6
MS/MS deisotoping (TOF) FALSE
MS/MS tol. (Unknown) 0.5 Da
Top MS/MS peaks per 100 Da. (Unknown) 6
MS/MS deisotoping (Unknown) FALSE
Peptide FDR 0.05
Max. peptide PEP 1
Protein FDR 0.2
Site FDR 0.05
Use Normalized Ratios For Occupancy TRUE
Apply site FDR separately TRUE
Min. peptide Length 6
Min. score 0
Min. unique peptides 0
Min. razor peptides 1
Min. peptides 1
Use only unmodified peptides and TRUE
Modifications included in protein quantification Oxidation (M);Acetyl (Protein N-term)
Peptides used for protein quantification Razor
Discard unmodified counterpart peptides TRUE
Min. ratio count 1
Lfq min. ratio count 1
Site quantification Use least modified peptide
Re-quantify TRUE
Keep low-scoring versions of identified peptides No
Label-free protein quantification TRUE
iBAQ FALSE
iBAQ log fit TRUE
MS/MS recalibration FALSE
Match between runs TRUE
Time window [min] 10
Find dependent peptides FALSE
Fasta file C:\MaxQuant 1.2.2.5\Database\SwissProt_HUMAN_2015_12.fasta
Experimental design file C:\MaxQuant 1.2.2.5\Data\Rix_Claudia_pY\combined\experimentalDesignTemplate.txt
Labeled amino acid filtering TRUE
Site tables Oxidation (M)Sites.txt;Phospho (STY)Sites.txt
Cut peaks TRUE

Randomize	FALSE
Special AAs	KR
Include contaminants	TRUE
AIF correlation	0.8
AIF topx	50
AIF min mass	0
AIF SIL weight	4
AIF ISO weight	2
AIF iterative	FALSE
AIF threshold FDR	0.01

Tyrosine Phosphoproteomics Scaffold parameters

Scaffold: Version: Scaffold_4.3.4 Protein Grouping Strategy: Experiment-wide grouping with protein cluster analysis

Peptide Thresholds: 95.0% minimum

Protein Thresholds: 20.0% minimum and 1 peptide minimum

Peptide FDR: 0.6% (Prophet)

Protein FDR: 2.7% (Prophet)

Tyrosine Phosphoproteomics sample specification

Category	Bio Sample	#Prot	#IDs	#Spec	%IDs
M	M1	1057	7489	42446	0.17643595
M	M2	1060	8359	45038	0.18559882
M	M3	1095	9096	47186	0.19276904
D	D1	1078	7415	41529	0.17854993
D	D2	1136	8239	45699	0.1802884
D	D3	1065	8329	46004	0.18104948

MaxQuant Parameters (Global Phosphoproteomics)

Version	1.5.2.8
Fixed modifications	Carbamidomethyl (C)
Decoy mode	revert
Special AAs	KR
Include contaminants	True
MS/MS tol. (FTMS)	20 ppm
Top MS/MS peaks per 100 Da. (FTMS)	12
MS/MS deisotoping (FTMS)	True
MS/MS tol. (ITMS)	0.5 Da
Top MS/MS peaks per 100 Da. (ITMS)	8
MS/MS deisotoping (ITMS)	False
MS/MS tol. (TOF)	40 ppm

Top MS/MS peaks per 100 Da. (TOF) 10
MS/MS deisotoping (TOF) True
MS/MS tol. (Unknown) 0.5 Da
Top MS/MS peaks per 100 Da. (Unknown) 8
MS/MS deisotoping (Unknown) False
PSM FDR 0.05
Protein FDR 0.2
Site FDR 0.05
Use Normalized Ratios For Occupancy True
Min. peptide Length 7
Min. score for unmodified peptides 0
Min. score for modified peptides 40
Min. delta score for unmodified peptides 0
Min. delta score for modified peptides 6
Min. unique peptides 0
Min. razor peptides 1
Min. peptides 1
Use only unmodified peptides and True
Modifications included in protein quantification Acetyl (Protein N-term);Oxidation (M)
Peptides used for protein quantification Razor
Discard unmodified counterpart peptides True
Min. ratio count 1
Re-quantify True
Use delta score False
iBAQ False
iBAQ log fit False
Match between runs False
Find dependent peptides False
Fasta file C:\MaxQuant 1.5.2.8\Databases\SwissProt_Human_2018_05.fasta
Labeled amino acid filtering True
Site tables Oxidation (M)Sites.txt;Phospho (STY)Sites.txt
Decoy mode revert
Special AAs KR
Include contaminants True
RT shift False
Advanced ratiosTrue
AIF correlation 0.47
First pass AIF correlation 0.8
AIF topx 20
AIF min mass 0
AIF SIL weight 4
AIF ISO weight 2

AIF iterative True

AIF threshold FDR 0.01

Global Phosphoproteomics Scaffold parameters

Scaffold: Version: Scaffold_4.8.7

Peptide Thresholds: 95,0% minimum

Protein Thresholds: 20,0% minimum and 1 peptide minimum

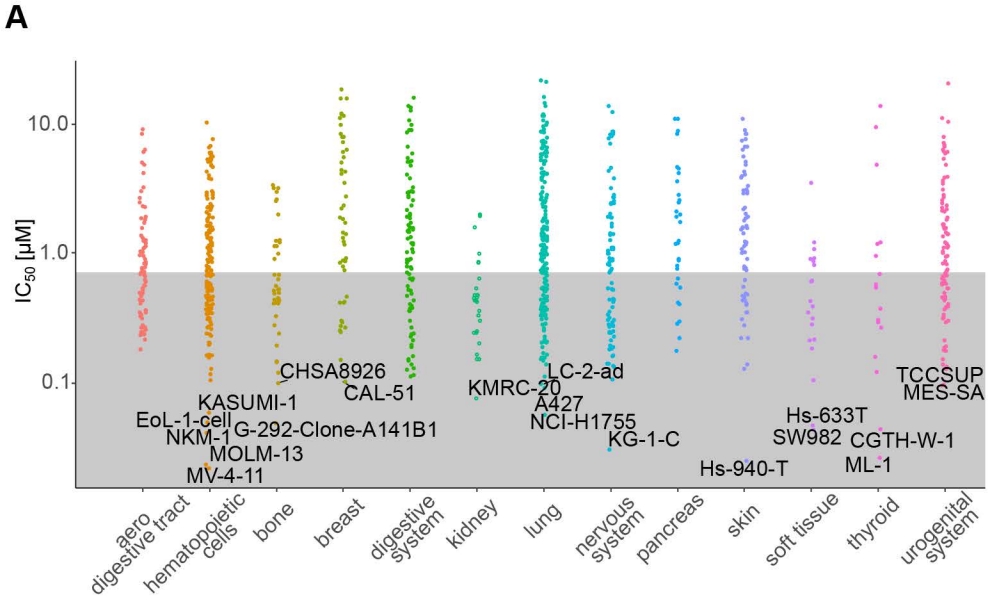
Peptide FDR: 3,5% (Prophet)

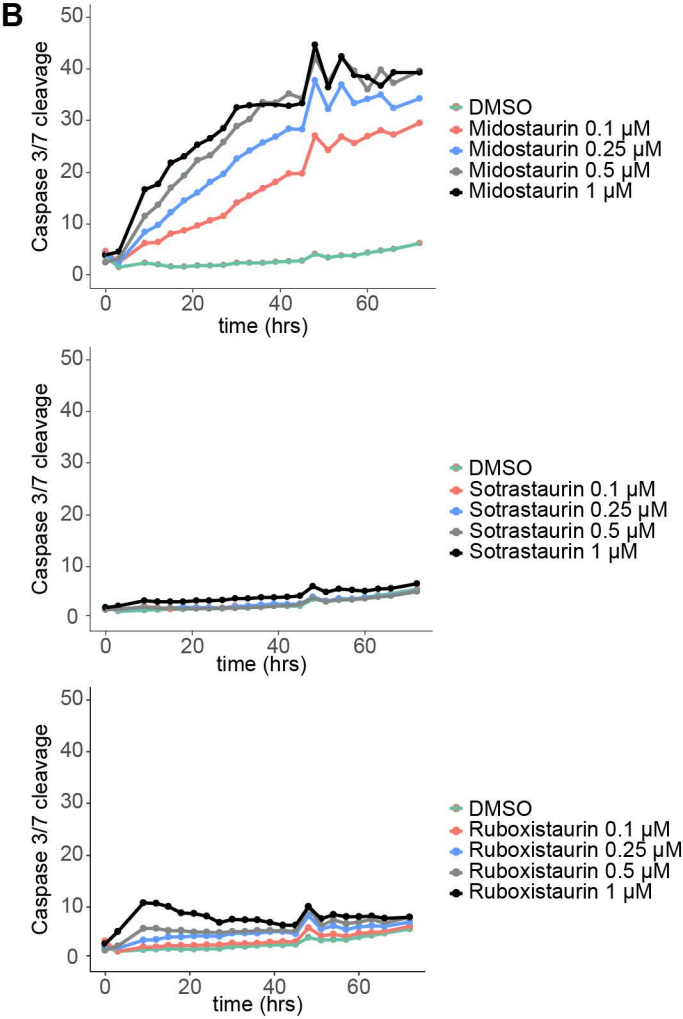
Protein FDR: 6,1% (Prophet)

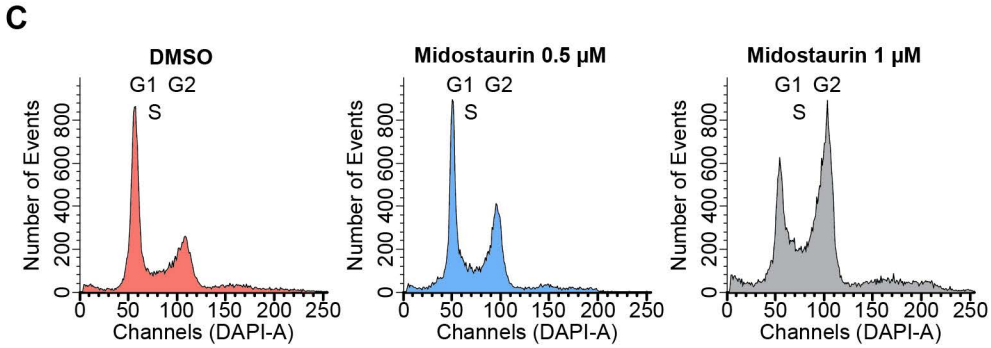
Global Phosphoproteomics sample specifications

Bio Sample	#Prot	#IDs	#Spec	%IDs
MidoTMT-IMAC_Fx1_run1	229	9	25023	3.60E-04
MidoTMT-IMAC_Fx1_run2	225	5	25169	1.99E-04
MidoTMT-IMAC_Fx2_run1	258	17	25390	6.70E-04
MidoTMT-IMAC_Fx2_run2	252	4	25610	1.56E-04
MidoTMT-IMAC_Fx3_run1	238	17	24634	6.90E-04
MidoTMT-IMAC_Fx3_run2	219	26	24963	0.0010415
MidoTMT-IMAC_Fx4_run1	231	9	24779	3.63E-04
MidoTMT-IMAC_Fx4_run2	241	36	24477	0.0014708
MidoTMT-IMAC_Fx5_run1	330	124	25324	0.0048965
MidoTMT-IMAC_Fx5_run2	319	114	25189	0.0045258
MidoTMT-IMAC_Fx6_run1	276	46	23068	0.0019941
MidoTMT-IMAC_Fx6_run2	280	54	22808	0.0023676
MidoTMT-IMAC_Fx7_run1	266	60	23253	0.0025803
MidoTMT-IMAC_Fx7_run2	255	40	22626	0.0017679
MidoTMT-IMAC_Fx8_run1	279	30	24910	0.0012043
MidoTMT-IMAC_Fx8_run2	269	25	25196	9.92E-04
MidoTMT-IMAC_Fx9_run1	208	2	24769	8.07E-05
MidoTMT-IMAC_Fx9_run2	234	15	25324	5.92E-04
MidoTMT-IMAC_Fx10_run1	244	10	25129	3.98E-04
MidoTMT-IMAC_Fx10_run2	258	21	25735	8.16E-04
MidoTMT-IMAC_Fx11_run1	252	10	26248	3.81E-04
MidoTMT-IMAC_Fx11_run2	232	18	25372	7.09E-04
MidoTMT-IMAC_Fx12_run1	227	12	24967	4.81E-04
MidoTMT-IMAC_Fx12_run2	224	7	25100	2.79E-04

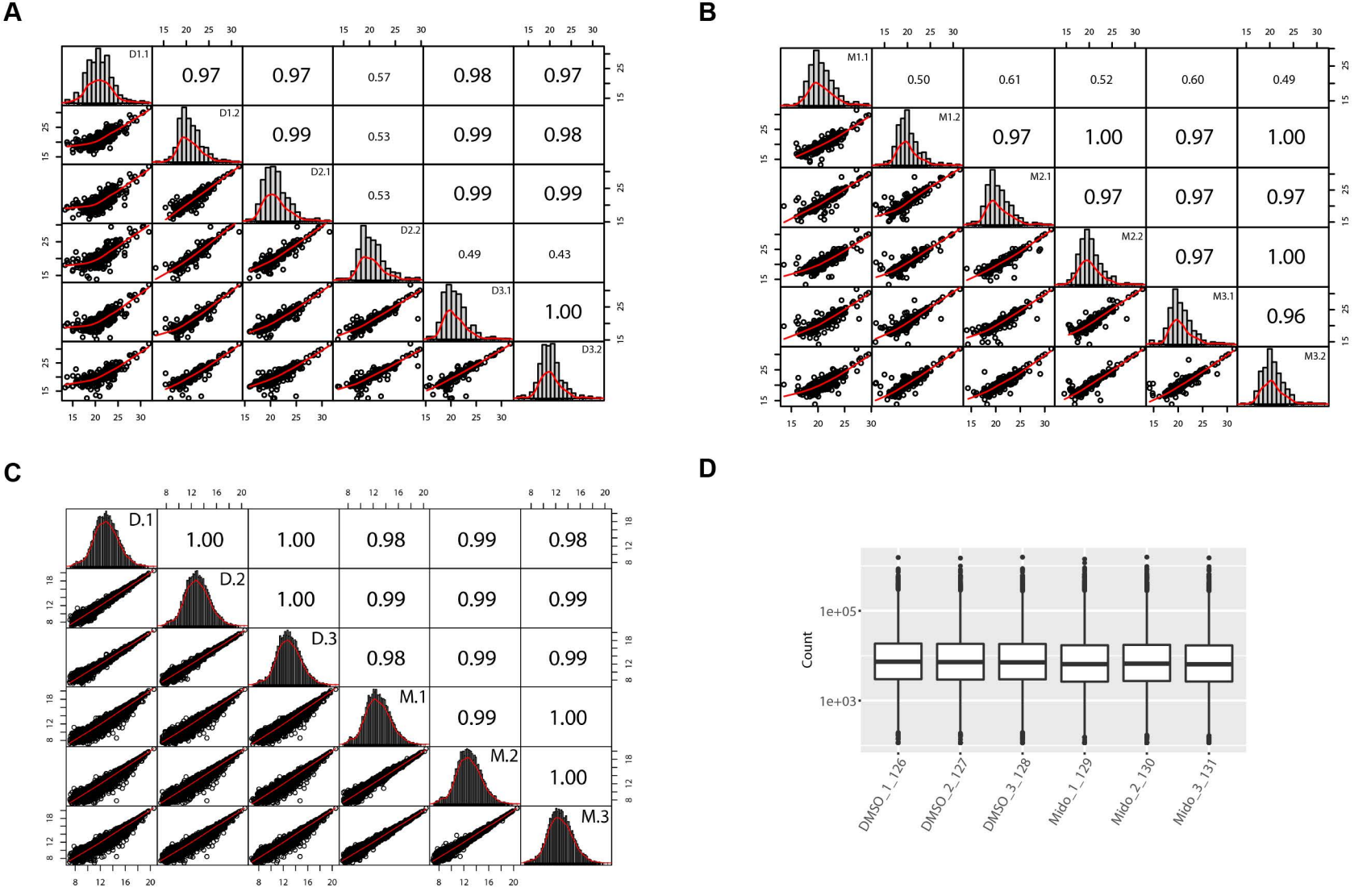
Ctortecka et al., Figure S1



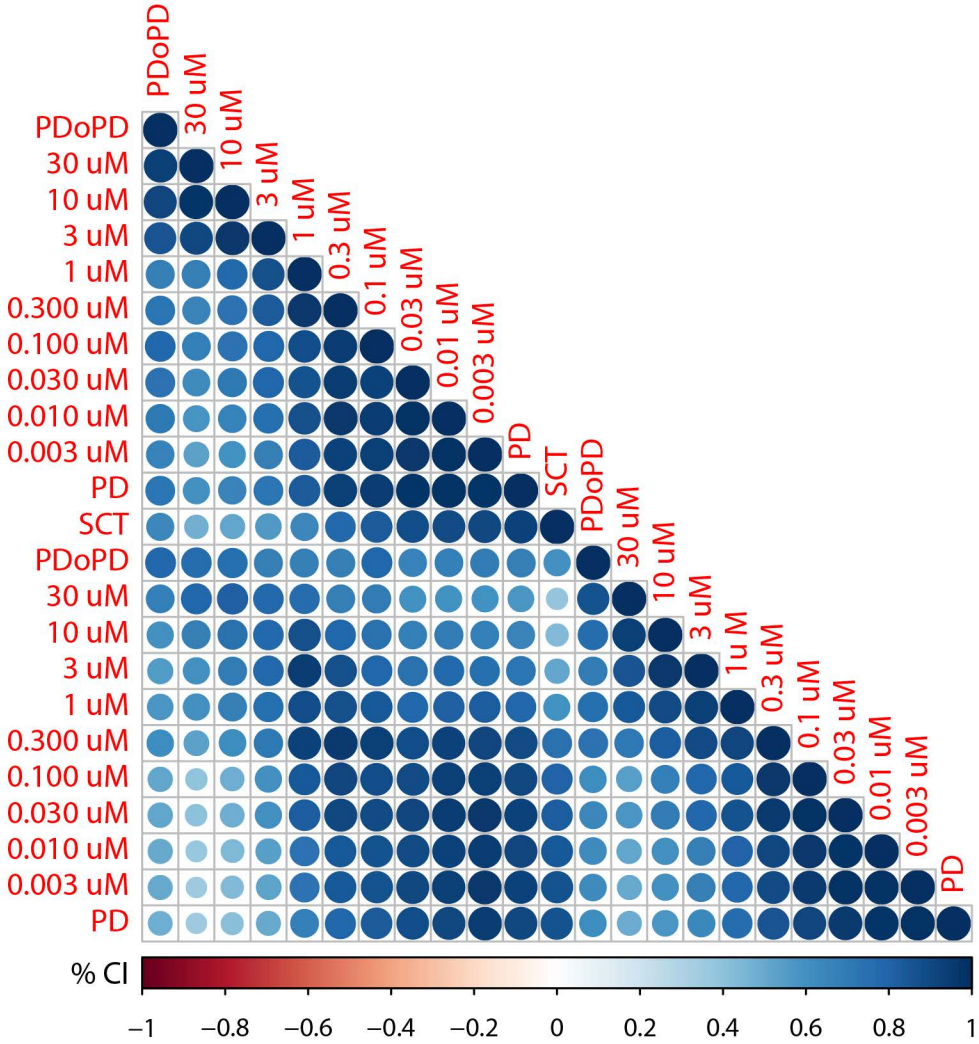




Ctortecka et al., Figure S2



Ctortecka et al., Figure S3



Ctortecka et al., Figure S4