Supporting Information

Discovery and Characterization of a Highly Potent and Selective Aminopyrazoline-Based *in vivo* Probe (BAY-598) for the Protein Lysine Methyltransferase SMYD2

Erik Eggert,[†] Roman C. Hillig,[†] Silke Koehr,[†] Detlef Stöckigt,[†] Jörg Weiske,[†] Naomi Barak,[†] Jeffrey Mowat,[†] Thomas Brumby,[†] Clara D. Christ,[†] Antonius ter Laak,[†] Tina Lang,[†] Amaury E. Fernandez-Montalvan,[†] Volker Badock,[†] Hilmar Weinmann,[†] Ingo V. Hartung,[†] Dalia Barsyte-Lovejoy,[‡] Magdalena Szewczyk,[‡] Steven Kennedy,[‡] Fengling Li,[‡] Masoud Vedadi,[‡,#] Peter J. Brown,[‡] Vijayaratnam Santhakumar,[‡] Cheryl H. Arrowsmith,[‡,§] Timo Stellfeld,[*,†] and Carlo Stresemann[†,*]

[†]Drug Discovery, Bayer AG, 13353 Berlin, Germany

[‡]Structural Genomics Consortium, University of Toronto, Toronto, ON, M5G 1L7

[#]Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON, M5S 1A8.

[§]Princess Margaret Cancer Centre and Department of Medical Biophysics, University of Toronto, Toronto, ON, M5G 2M9.

[*]Corresponding authors: carlo.stresemann@bayer.com; timo.stellfeld@bayer.com

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1. Additional Selectivity Information for (S)-4 (BAY-598)

1.1. **Table S1 KINOMEscan Data.** The KINOMEscan was performed at DiscoveRx. For a more detailed description of this assay technology, see: Fabian, M. A.; Biggs, W. H., III; Treiber, D. K.; Atteridge, C. E.; Azimioara, M. D.; Benedetti, M. G.; Carter, T. A.; Ciceri, P.; Edeen, P. T.; Floyd, M.; Ford, J. M.; Galvin, M.; Gerlach, J. L.; Grotzfeld, R. M.; Herrgard, S.; Insko, D. E.; Insko, M. A.; Lai, A. G.; Lélias, J.-M.; Mehta, S. A.; Milanov, Z. V.; Velasco, A. M.; Wodicka, L. M.; Patel, H. K.; Zarrinkar, P. P.; Lockhart, D. J. A small molecule–kinase interaction map for clinical kinase inhibitors. *Nat. Biotechnol.* **2005**, *23*, 329–336.

Target Gene Symbol	%Ctrl @ 1000 nM BAY-598
AAK1	78
ABL1(E255K)-phosphorylated	85
ABL1(F317I)-nonphosphorylated	100
ABL1(F317I)-phosphorylated	98
ABL1(F317L)-nonphosphorylated	100
ABL1(F317L)-phosphorylated	93
ABL1(H396P)-nonphosphorylated	92
ABL1(H396P)-phosphorylated	88
ABL1(M351T)-phosphorylated	91
ABL1(Q252H)-nonphosphorylated	66
ABL1(Q252H)-phosphorylated	100
ABL1(T315I)-nonphosphorylated	77
ABL1(T315I)-phosphorylated	100
ABL1(Y253F)-phosphorylated	100
ABL1-nonphosphorylated	79
ABL1-phosphorylated	90
ABL2	88
ACVR1	96
ACVR1B	100
ACVR2A	100
ACVR2B	100

ACVRL1	74
ADCK3	98
ADCK4	81
AKT1	84
AKT2	100
AKT3	100
ALK	93
ALK(C1156Y)	100
ALK(L1196M)	92
AMPK-alpha1	68
AMPK-alpha2	85
ANKK1	92
ARK5	100
ASK1	88
ASK2	93
AURKA	96
AURKB	93
AURKC	77
AXL	91
BIKE	80
BLK	88
BMPR1A	100
BMPR1B	100
BMPR2	100
BMX	89
BRAF	100
BRAF(V600E)	100
BRK	73
BRSK1	100
BRSK2	74
BTK	79
BUB1	100
CAMK1	85
CAMK1D	90
CAMK1G	98
CAMK2A	100
CAMK2B	100
CAMK2D	96
CAMK2G	89
CAMK4	82
CAMKK1	84
CAMKK2	84
CASK	61

CDC2L1	97
CDC2L2	92
CDC2L5	81
CDK11	85
CDK2	100
CDK3	91
CDK4-cyclinD1	93
CDK4-cyclinD3	100
CDK5	82
CDK7	98
CDK8	92
CDK9	74
CDKL1	76
CDKL2	100
CDKL3	96
CDKL5	100
CHEK1	98
CHEK2	99
CIT	63
CLK1	78
CLK2	97
CLK3	94
CLK4	94
CSF1R	100
CSF1R-autoinhibited	47
CSK	91
CSNK1A1	63
CSNK1A1L	94
CSNK1D	100
CSNK1E	86
CSNK1G1	84
CSNK1G2	94
CSNK1G3	79
CSNK2A1	78
CSNK2A2	56
CTK	100
DAPK1	100
DAPK2	100
DAPK3	94
DCAMKL1	67
DCAMKL2	93
DCAMKL3	75
DDR1	96

DDR2	100
DLK	94
DMPK	100
DMPK2	66
DRAK1	100
DRAK2	100
DYRK1A	100
DYRK1B	74
DYRK2	100
EGFR	99
EGFR(E746-A750del)	66
EGFR(G719C)	86
EGFR(G719S)	78
EGFR(L747-E749del, A750P)	87
EGFR(L747-S752del, P753S)	86
EGFR(L747-T751del,Sins)	70
EGFR(L858R)	91
EGFR(L858R,T790M)	100
EGFR(L861Q)	85
EGFR(S752-I759del)	100
EGFR(T790M)	86
EIF2AK1	100
EPHA1	86
EPHA2	84
EPHA3	100
EPHA4	90
EPHA5	100
EPHA6	99
EPHA7	91
EPHA8	94
EPHB1	94
EPHB2	74
EPHB3	99
EPHB4	97
EPHB6	100
ERBB2	87
ERBB3	84
ERBB4	93
ERK1	100
ERK2	98
ERK3	77
ERK4	100
ERK5	80
ERK8	96

ERN1	93
FAK	99
FER	68
FES	92
FGFR1	59
FGFR2	86
FGFR3	88
FGFR3(G697C)	75
FGFR4	71
FGR	100
FLT1	95
FLT3	92
FLT3(D835H)	93
FLT3(D835Y)	78
FLT3(ITD)	91
FLT3(K663Q)	100
FLT3(N841I)	95
FLT3(R834Q)	100
FLT3-autoinhibited	100
FLT4	87
FRK	77
FYN	71
GAK	100
GCN2(Kin.Dom.2,S808G)	100
GRK1	93
GRK4	98
GRK7	100
GSK3A	88
GSK3B	88
HASPIN	100
HCK	68
HIPK1	51
HIPK2	100
HIPK3	100
HIPK4	100
HPK1	88
HUNK	94
ICK	99
IGF1R	97
IKK-alpha	92
IKK-beta	100
IKK-epsilon	99
INSR	78

INSRR	85
IRAK1	92
IRAK3	93
IRAK4	100
ITK	94
JAK1(JH1domain-catalytic)	85
JAK1(JH2domain-pseudokinase)	100
JAK2(JH1domain-catalytic)	100
JAK3(JH1domain-catalytic)	48
JNK1	96
JNK2	100
JNK3	100
KIT	100
KIT(A829P)	98
KIT(D816H)	95
KIT(D816V)	86
KIT(L576P)	85
KIT(V559D)	99
KIT(V559D,T670I)	100
KIT(V559D,V654A)	100
KIT-autoinhibited	90
LATS1	90
LATS2	100
LCK	66
LIMK1	100
LIMK2	62
LKB1	83
LOK	69
LRRK2	100
LRRK2(G2019S)	98
LTK	65
LYN	80
LZK	83
MAK	74
MAP3K1	100
MAP3K15	69
MAP3K2	100
MAP3K3	73
MAP3K4	75
MAP4K2	96
MAP4K3	90
MAP4K4	100
MAP4K5	100

MAPKAPK2	91
MAPKAPK5	100
MARK1	71
MARK2	99
MARK3	69
MARK4	75
MAST1	83
MEK1	98
MEK2	90
MEK3	100
MEK4	100
MEK5	100
MEK6	74
MELK	99
MERTK	73
MET	100
MET(M1250T)	86
MET(Y1235D)	100
MINK	100
MKK7	99
MKNK1	100
MKNK2	96
MLCK	100
MLK1	100
MLK2	77
MLK3	69
MRCKA	93
MRCKB	100
MST1	68
MST1R	81
MST2	88
MST3	100
MST4	81
MTOR	100
MUSK	97
MYLK	86
MYLK2	100
MYLK4	100
MYO3A	100
MYO3B	96
NDR1	82
NDR2	76
NEK1	93

NEK10	100
NEK11	77
NEK2	99
NEK3	95
NEK4	100
NEK5	85
NEK6	91
NEK7	100
NEK9	100
NIK	100
NIM1	100
NLK	100
OSR1	78
p38-alpha	86
p38-beta	100
p38-delta	72
p38-gamma	70
PAK1	84
PAK2	89
PAK3	100
PAK4	91
PAK6	100
PAK7	100
PCTK1	98
PCTK2	91
PCTK3	92
PDGFRA	100
PDGFRB	100
PDPK1	59
PFCDPK1(P.falciparum)	76
PFPK5(P.falciparum)	100
PFTAIRE2	100
PFTK1	84
PHKG1	97
PHKG2	99
PIK3C2B	100
PIK3C2G	77
PIK3CA	97
PIK3CA(C420R)	76
PIK3CA(E542K)	92
PIK3CA(E545A)	53
PIK3CA(E545K)	71
PIK3CA(H1047L)	92

PIK3CA(H1047Y)	92
PIK3CA(I800L)	90
PIK3CA(M1043I)	68
PIK3CA(Q546K)	86
PIK3CB	100
PIK3CD	81
PIK3CG	90
PIK4CB	100
PIM1	82
PIM2	90
PIM3	88
PIP5K1A	70
PIP5K1C	86
PIP5K2B	100
PIP5K2C	85
PKAC-alpha	74
PKAC-beta	100
PKMYT1	100
PKN1	61
PKN2	100
PKNB(M.tuberculosis)	88
PLK1	100
PLK2	83
PLK3	89
PLK4	56
PRKCD	89
PRKCE	92
PRKCH	80
PRKCI	63
PRKCQ	100
PRKD1	86
PRKD2	100
PRKD3	99
PRKG1	100
PRKG2	84
PRKR	84
PRKX	100
PRP4	90
PYK2	100
QSK	94
RAF1	100
RET	91
RET(M918T)	80

RET(V804L)	84
RET(V804M)	100
RIOK1	77
RIOK2	100
RIOK3	46
RIPK1	76
RIPK2	75
RIPK4	100
RIPK5	90
ROCK1	98
ROCK2	95
ROS1	68
RPS6KA4(Kin.Dom.1-N-terminal)	100
RPS6KA4(Kin.Dom.2-C-terminal)	93
RPS6KA5(Kin.Dom.1-N-terminal)	93
RPS6KA5(Kin.Dom.2-C-terminal)	99
RSK1(Kin.Dom.1-N-terminal)	100
RSK1(Kin.Dom.2-C-terminal)	100
RSK2(Kin.Dom.1-N-terminal)	99
RSK2(Kin.Dom.2-C-terminal)	100
RSK3(Kin.Dom.1-N-terminal)	91
RSK3(Kin.Dom.2-C-terminal)	100
RSK4(Kin.Dom.1-N-terminal)	92
RSK4(Kin.Dom.2-C-terminal)	100
S6K1	87
SBK1	88
SGK	100
SG110	92
SGK2	100
SGK3	98
SIK	98
SIK2	99
SLK	66
SNARK	100
SNRK	85
SRC	79
SRMS	94
SRPK1	57
SRPK2	95
SRPK3	79
STK16	84
STK33	83
STK35	100

STK36	81
STK39	77
SYK	59
TAK1	100
TAOK1	89
TAOK2	91
TAOK3	92
TBK1	82
TEC	99
TESK1	94
TGFBR1	100
TGFBR2	59
TIE1	99
TIE2	100
TLK1	100
TLK2	93
TNIK	100
TNK1	66
TNK2	96
TNNI3K	73
TRKA	93
TRKB	100
TRKC	55
TRPM6	80
TSSK1B	79
TTK	82
TXK	73
TYK2(JH1domain-catalytic)	88
TYK2(JH2domain-pseudokinase)	95
TYRO3	100
ULK1	64
ULK2	100
ULK3	74
VEGFR2	100
VRK2	93
WEE1	100
WEE2	100
WNK1	100
WNK3	93
YANK1	77
YANK2	98
YANK3	100
YES	100

YSK1	90
YSK4	93
ZAK	95
ZAP70	100

1.2. Table S2 LeadProfilingScreen Data. Assays were performed at Eurofins Panlabs, Inc. Methods employed were adapted from the scientific literature to maximize reliability and reproducibility. Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. Items meeting criteria for significance (≥50% stimulation or inhibition) are highlighted. * Batch: represents compounds tested concurrently in the same assay(s); ham = hamster, hum = human, % Inh. = % inhibition.

Assay Name	Batch*	Spec.	Rep.	Conc.	% Inh.
Histamine H_3	340806	hum	2	10μM	-5
Imidazoline I_2, Central	340848	rat	2	10μM	31
Interleukin IL-1	340820	mouse	2	10μM	5
Leukotriene, Cysteinyl $CysLT_1$	340778	hum	2	10μM	-2
Melatonin MT_1	340780	hum	2	10μM	12
Muscarinic M_1	340869	hum	2	10μM	-4
Muscarinic M_2	340870	hum	2	10μM	11
Muscarinic M_3	340871	hum	2	10μM	-1
Neuropeptide Y Y_1	340785	hum	2	10μM	5
Neuropeptide Y Y_2	340787	hum	2	10μM	8
Nicotinic Acetylcholine	340838	hum	2	10μM	-15
Nicotinic Acetylcholine α1, Bungarotoxin	340839	hum	2	10μM	13
Opiate δ_1 (OP1, DOP)	340789	hum	2	10μM	9
Opiate κ(OP2, KOP)	340791	hum	2	10μM	19
Opiate μ(OP3, MOP)	340874	hum	2	10μM	25
Phorbol Ester	340847	mouse	2	10μM	-19
Platelet Activating Factor (PAF)	340846	hum	2	10μM	9
Potassium Channel [K_{ATP}]	340875	ham	2	10μM	3
Potassium Channel hERG	340876	hum	2	10μM	57
Prostanoid EP_4	340877	hum	2	10μM	15
Purinergic P2X	340793	rabbit	2	10μM	34
Purinergic P2Y	340795	rat	2	10μM	-5
Rolipram	340845	rat	2	10μM	3
Serotonin (5-Hydroxytryptamine) 5-HT_{1A}	340844	hum	2	10μM	-6
Serotonin (5-Hydroxytryptamine) 5-HT_{2B}	340878	hum	2	10μM	21
Serotonin (5-Hydroxytryptamine) 5-HT_3	340797	hum	2	10μM	7
Sigma σ_1	340879	hum	2	10μM	44
Sodium Channel, Site 2	340843	rat	2	10μM	69
Tachykinin NK_1	340783	hum	2	10μM	14
Thyroid Hormone	340801	rat	2	10μM	14
Transporter, Dopamine (DAT)	340812	hum	2	10μM	71
Transporter, GABA	340767	rat	2	10μM	15
Transporter, Norepinephrine (NET)	340840	hum	2	10μM	78
Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	340804	hum	2	10μM	-1

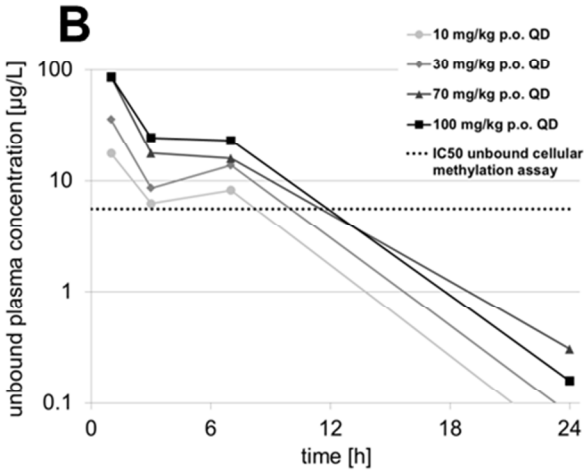
Adenosine A_1	340858	hum	2	10μM	21
Adenosine A_{2A}	340860	hum	2	10μM	-11
Adenosine A_3	340751	hum	2	10μM	13
Adrenergic α_{1A}	340857	rat	2	10μM	19
Adrenergic α_{1B}	340856	rat	2	10μM	13
Adrenergic α_{1D}	340859	hum	2	10μM	21
Adrenergic α_{2A}	340853	hum	2	10μM	22
Adrenergic β_1	340841	hum	2	10μM	3
Adrenergic β_2	340861	hum	2	10μM	25
Androgen (Testosterone) AR	340799	rat	2	10μM	13
Bradykinin B_1	340747	hum	2	10μM	9
Bradykinin B_2	340749	hum	2	10μM	-9
Calcium Channel L-Type, Benzothiazepine	340753	rat	2	10μM	33
Calcium Channel L-Type, Dihydropyridine	340982	rat	2	10μM	46
Calcium Channel N-Type	340761	rat	2	10μM	-5
Cannabinoid CB_1	340863	hum	2	10μM	45
Dopamine D_1	340865	hum	2	10μM	18
Dopamine D_{2S}	340866	hum	2	10μM	0
Dopamine D_3	340808	hum	2	10μM	21
Dopamine $D_{4.2}$	340810	hum	2	10μM	8
Endothelin ET_A	340757	hum	2	10μM	12
Endothelin ET_B	340760	hum	2	10μM	2
Epidermal Growth Factor (EGF)	340763	hum	2	10μM	5
Estrogen ERα	340765	hum	2	10μM	-1
$GABA_A$, Flunitrazepam, Central	340851	rat	2	10μM	-23
$GABA_A$, Muscimol, Central	340867	rat	2	10μM	11
$GABA_{B1A}$	340770	hum	2	10μM	-6
Glucocorticoid	340835	hum	2	10μM	14
Glutamate, Kainate	340772	rat	2	10μM	7
Glutamate, NMDA, Agonism	340774	rat	2	10μM	5
Glutamate, NMDA, Glycine	340776	rat	2	10μM	13
Glutamate, NMDA, Phencyclidine	340850	rat	2	10μM	9
Histamine H_1	340868	hum	2	10μM	20
Histamine H_2	341160	hum	2	10μM	-2

2. Figure S1 DMPK Properties of *(S)*-4 and Exposure Studies

A

CL_{int} Hepatocytes	Rat	2.5 L/h/kg	**Rat PK in vivo**	
CL_{int} Microsomes	Mouse	3.5 L/h/kg	Cl_b	1.6 L/h/kg
	Human	0.79 L/h/kg	V_{SS}	2.1 L/kg
Caco2	P_{app} AB	34 nm/sec	MRT	1.6 h
	Efflux ratio	5.1 fold	F	24 %

B



(A) In vitro and in vivo DMPK properties of (*S*)-**4**. (B) Exposure in mice following 10 to 100 mg/kg po q.d. of (*S*)-**4** .

3. Figure S2 Validation of AHNAK as SMYD2 substrate

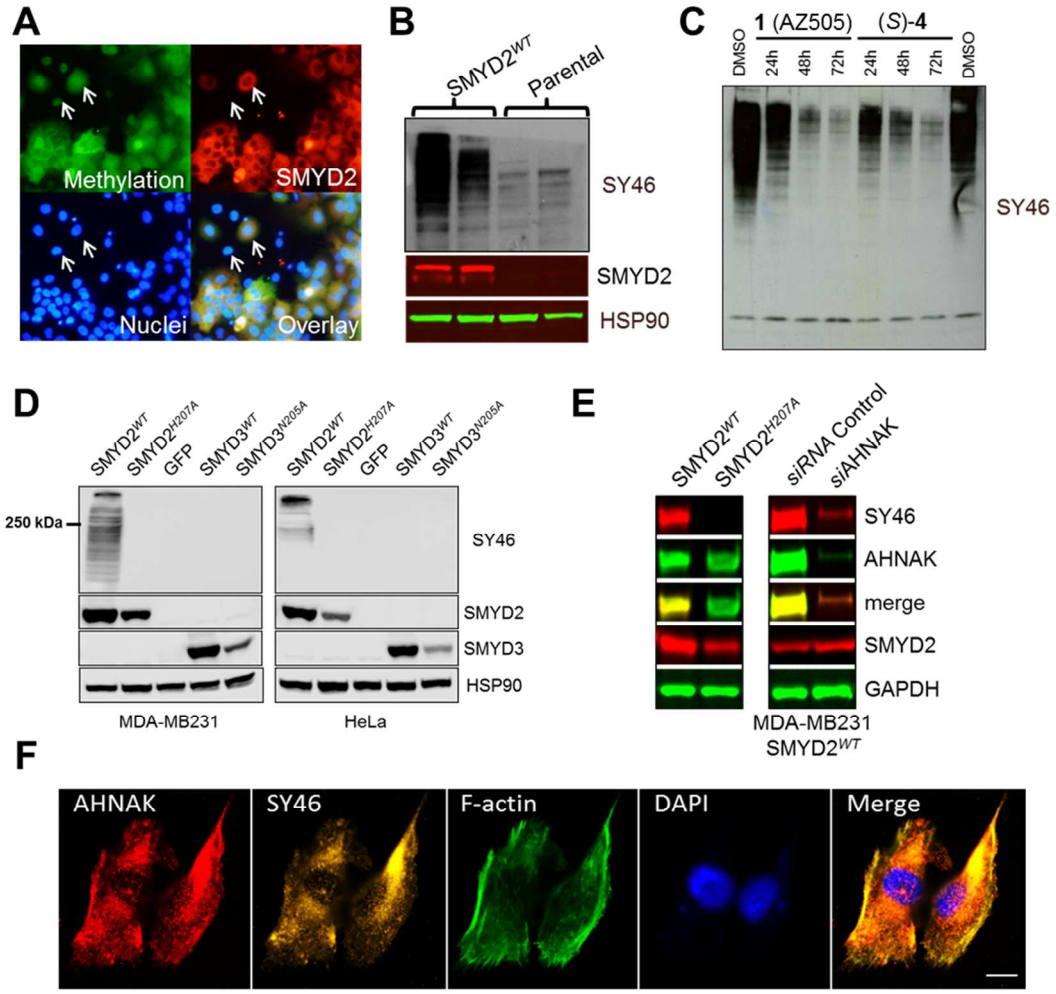


Figure S2 (A) Overexpression of SMYD2 in KYSE-150 cells. SMYD2 is primarily detected in the cytosol. One SMYD2 overexpressing and one non-overexpressing cell are highlighted by arrows. The methylation signal detected by SY46 is overlapping with the SMYD2 overexpressing cells (B) Western blot analysis of SMYD2 overexpressing or parental KYSE150 cells. SY46 methylation antibody detects a strong increase in methylation signal in the high molecular weight region in SMYD2 overexpressing cells. (Later we discovered AHNAK as the high molecular substrate of around 630 kDa. Due to the high molecular weight of AHNAK and partial degradation different fragments are detected.) (C) Western blot analysis of SMYD2 overexpressing KYSE150 cells treated with DMSO or 10 µM of either AZ505 or *(S)*-**4** and harvested after the indicated time points. The methylation signal detected by SY46 was reduced in a time dependent manner**.** (D) Only expression of SMYD2 leads to specific methylation signals detected by SY46. Western blot analysis of a transient overexpression of wildtype ($SMYD2^{WT}$ /$SMYD3^{WT}$) or enzymatically point mutants ($SMYD2^{H207A}$/$SMYD3^{N205A}$) of either SMYD2 or SMYD3 in MDA-MB231 or HeLa cells. Only in $SMYD2^{WT}$ cells a methylation signal is detected. Expression of green fluorescence protein (GFP) was used as control (E)

Identification of AHNAK as high molecular weight methylation target of SMYD2. Western blot analysis of MDA-MB231 cells stably overexpressing either $SMYD2^{WT}$ or $SMYD2^{H207A}$ mutant protein. Only in $SMYD2^{WT}$ cells a methylation signal, overlapping with the signal derived from the AHNAK specific antibody was detected by SY46. A siRNA against AHNAK (siAHNAK) efficiently reduced AHNAK expression. Methylation signal detected with SY46 is lost upon AHNAK knockdown. A control siRNA was directed against renilla luciferase. (C) Immunofluorescence staining of MDA-MB231 cells overexpressing $SMYD2^{WT}$ using AHNAK antibody (red) and SY46 methylation antibody (yellow). F-actin was stained with Phalloidin-A555 and nuclei were visualized by DAPI staining. The methylation signal co-localized with AHNAK staining further confirming AHNAK as a new SMYD2 target.

4. Table S3 Crystallographic Data: Data Collection and Refinement Statistics

	SMYD2:SAM: compound **6**	SMYD2:SAM: *(S)*-**4** (BAY-598)
PDB Accession Code	5ARF	5ARG
Data Collection		
Source	BL 14.1 (Helmholtz-Zentrum Berlin)	
Wavelength [Å]	0.91841	
Space group (no.)	$P2_12_12_1$	
Unit cell parameters, *a*, *b*, *c* [Å]	51.8, 69.6, 130.5	52.3, 69.6, 131.1
Resolution limit [Å]	1.92–48.11 (1.92–2.03)	1.99–48.61 (1.99–2.11)
No. of reflections	133348	221439
No. of unique reflections	36156	33656
Multiplicity	3.69	6.58
$I/\sigma(I)$	12.83 (2.30)	14.39 (2.41)
R_{meas} [%]	8.3 (69.5)	10.1 (81.5)
Completeness [%]	97.9 (97.4)	99.9 (99.5)
B (Wilson) [Å^2]	33.05	35.1
Mosaicity [deg]	0.099	0.129
Refinement		
Resolution limit [Å]	1.92–47.59 (1.92–1.97)	1.99–47.72 (1.99–2.04)
Completeness [%]	97.9 (95.9)	99.9 (99.5)
No. of reflections	34348	31972
R_{work}/R_{free} [%]	25.8/30.1 (32.5/36.7)	23.0/27.3 (33.2/38.1)
Mean *B* value [Å^2]	53.0	55.5
rmsd bond length [Å]	0.017	0.017
rmsd bond angles [deg]	1.99	2.03

Values in brackets refer to the highest resolution shell.

5. Supplementary Material and Methods

Cell Culture. Additional cell lines used in the supplementary data were obtained from the American Type Culture Collection (MDA-MB231), and the German Collection of Microorganisms and Cell Cultures (HeLa).

Additional antibodies used in supplementary western blot data were raised against HSP90 (BD Pharmingen, #610418, 1:2000), SMYD3 (abcam, #ab183498, 1:500), GAPDH (abcam, #ab9484, 1:2000).

Cell imaging. Immunofluorescence staining was done with cells cultured in 8-well glas chamber slides (Millipore, #PEZGS0816). Cells were fixed for 10 min with 4% paraformaldehyde (Santa Cruz), permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) and blocked with 1.0% bovine serum albumin (Sigma-Aldrich) all in PBS buffer. Staining was done using AHNAK antibody (1:200), SY46 antibody (1:200) for detection of methylation and c-myc antibody (Santa Cruz Biotechnology, #sc-40, 1:500) (for detection of the c-Myc tag of the SMYD2 protein) combined with secondary antibodies Alexa Fluor 488 goat anti-rabbit (Thermo Fisher Scientific, #A-11008, 1:500) and Alexa Fluor 647 goat anti-mouse (Thermo Fisher Scientific, # A-21236, 1:500). F-actin was stained with Alexa Fluor 555 Phalloidin (Thermo Fisher Scientific, #A34055, 1:30 in PBS). 1 µg/ml DAPI (Thermo Fisher Scientific, #62248) in PBS was used for nuclear staining. Specimens were covered with cover slides (ROTH, #1871) and mounting medium (Vector, VECTASHIELD Antifade Mounting Medium, #H-1000) and images were acquired with an LSM 700 confocal microscope (Zeiss).

Transient gene knockdown. Transient knockdown of AHNAK expression was achieved by siRNA mediated down regulation of AHNAK transcripts. For that purpose cells were seeded in 6-well plates one day before transfection to reach a confluency on the day of transfection of around 80%. Cells in a 6-well were then transfected with 75 pmol of either specific hAHNAK MISSION esiRNA (Sigma-Aldrich, #EHU058201) or MISSION esiRNA against Renilla Luciferase as unspecific control (Sigma-Aldrich, #EHURLUC) using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, #13778-150, Lipid:siRNA ratio was 2:1) according to the manufactures instructions. For the generation of the transfection mix serum-free Opti-MEM medium (Thermo Fisher Scientific, #31985062) was used. Cells were harvested for western blot analysis 3 days after transfection.