Supplemental Material and Methods

Virus production and transduction

Lentiviral particles (for stable integration of shRNA or CRISPR/Cas9 constructs) were produced in HEK293T cells with 3rd generation packaging plasmids (Addgene 12259 and 12259) and the respective transfer plasmid using calcium phosphate. Virus containing supernatant was concentrated using Amicon Ultra centrifugal filter units (Merck Millipore) and cells transduced with hexadimethrine bromide (Sigma-Aldrich). Transduced cells were selected with puromycin or sorted by flow cytometry based on expression of blue fluorescent protein (BFP).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the GeneArt Site-Directed Mutagenesis System (Thermo Fisher Scientific) according to manufacturer's protocol.

Immunoblotting

Cells were lysed with RIPA buffer (see below), suspended in 4xLDS loading buffer (Thermo Fisher Scientific) supplemented with 250 mmol/L 1,4-Dithiothreitol (DTT, Sigma-Aldrich). After boiling the samples at 70°C for 5 minutes, proteins were separated by electrophoresis on 4-12% gradient polyacrylamide gels (Life Technologies). Proteins were transferred onto a Protran™ nitrocellulose membrane (GE Healthcare). Membranes were blocked in 5% (w/v) milk powder in Tris-buffered saline (TBS)/0.01% Tween20 (Sigma-Aldrich) and incubated overnight with primary antibodies at 4°C. Membranes were washed in TBS/Tween and incubated with horseradish peroxidase (HRP)-coupled IgG secondary antibody for 1h at room temperature. Membranes were washed in TBS/Tween and proteins were detected using ECL detection reagent (Thermo Fisher Scientific) in a ChemiDoc™ Touch Imaging system (BioRad).

RIPA buffer

Before use, buffer was supplemented with protease inhibitor (Roche Complete Mini)

Tris-Cl pH=7.5	50 mmol/L
Sodium chloride	150 mmol/L
NP-40	1%
Sodium deoxycholate	0.5%

Sodium dodecyl sulfate	0.1%
EGTA	1 mmol/L
Sodium fluoride	50 mmol/L
Beta glycerolphosphate	10 mmol/L
Sodium pyrophosphate	5 mmol/L
Sodium ortho vanadate	1 mmol/L

Co-Immunoprecipitation

Cells were lysed in ice cold Co-IP buffer (see below) and disrupted using syringes. Lysates were incubated with Protein G Dynabeads (Thermo Fisher Scientific)/antibody conjugates or beads alone for 3h at 4°C. Beads were washed and bound protein was released by boiling the beads in LDS buffer (as described above) at 70°C for 5 minutes.

CoIP buffer

Before use, buffer was supplemented with protease inhibitor (Roche Complete Mini)

Tris-Cl pH=7.5	50 mmol/L
Sodium chloride	80 mmol/L
NP-40	0.3%
Glycerol	10%
Magnesium chloride	1.5 mmol/L
Sodium fluoride	25 mmol/L
Beta glycerolphosphate	10 mmol/L
Sodium pyrophosphate	5 mmol/L
Sodium ortho vanadate	2 mmol/L

Generation of shRNA cell lines

Custom designed shRNAs in Lentiviral vectors (Cellecta) to transduce target cells with either the targeting shRNA plasmid (shP3F) or the non-targeting control shRNA plasmid (shscr) were ordered (Suppl.Fig.1A) (Cellecta):

HEK293T cells were co-transfected with the transfer plasmid, the envelope plasmid, and packaging plasmids. 48h after transfection viral supernatant was collected and concentrated using Amicon® Ultra Centrifugal Filters (Merck Millipore) according to manufacturer's protocol. Cell lines were transduced with viral concentrate in medium containing 8 μg/mL Hexadimethrine bromide (Sigma). Cells from PDXs were transduced without Hexadimethrine bromide. After 72h cells were selected with 0.5 μg/mL

Puromycin (Sigma). In advance we tested three different shP3F sequences targeting the breakpoint region for knockdown efficiency and toxicity and continued with shP3F #3.

Targeting sequences

#1 CCTCTCACCTCAGAATTCAAT
#2 CTCTCACCTCAGAATTCAATT
#3 GGCCTCTCACCTCAGAATTCA

qRT-PCR

RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). 1 µg of RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-time PCR was performed using TaqMan probes (see below) and the TaqMan Gene Expression Master Mix (both Thermo Fisher Scientific). Expression changes were calculated using the ΔΔCt method with GAPDH as internal control.

TaqMan probes
All probes were ordered as Assay-on-Demand mix (Thermo Fisher Scientific) containing specific primers and FAM/BHQ-1 probe.

Gene	Assay ID
PAX3-FOXO1	Hs03024825
GAPDH	Hs02758991_g1
PMAIP1 (NOXA)	Hs00560402
MYCN	Hs00232074
MDM2	Hs01066930

Immunohistochemistry (IHC) of tissue microarray (TMA) and analysis of gene expression profiling data

4 micron sections of a previously described TMA [49] representing samples from 81 RMS cases were stained using primary antibodies for Aurora Kinase (1:50 dilution, rabbit anti-AK2-L, JLM28, Leica, UK) or Ki67 (1:100 dilution, mouse anti-MIB-1, M7240, Dako, UK) with the Roche Ventana Benchmark Ultra © automated system. Positive controls included bowel, testis and tissue culture preparations whilst the protocol without the primary antibody was used as a negative control.

Stained TMAs were scored by two histopathologists (AK, GP) with discrepant scoring discussed in real time. Aurora Kinase staining in tumour cells was assessed for intensity of staining (1-weak, 2-intermediate or 3-strong) and the percentage of positive cells (1 (<30%), 2 (30-60%) and 3 (>60%) of cells). Ki67 staining was assessed for percentage of tumor cells positive. A combined metric was created

for AURKA by multiplying the intensity score by the percentage positivity score and averaged over all scorable cores per sample. An overall score of negative (0), weak (<3 combined score), moderate (3-6 combined score) and strong (>6 combined score) was then applied. Our previously described ITCC gene expression profiling data [4] was analyzed using the open access platform R2 Genomics and Visualization Platform (http://r2.amc.nl).

Antibodies

Antigen	Manufacturer	Host (Clone)	Dilution
AURORA A	Genetex	ms mono (35C1)	1:1000
BAD	Cell signaling	rb mono (D24A9)	1:1000
BAK	Cell signaling	rb poly	1:1000
BAX	Cell signalin	rb poly	1:1000
BIM	Santa Cruz	ms mono (H-5)	1 :500
CASP9	Cell signaling	ms mono (C9)	1:1000
Cl. CASP3	Cell signaling	rb mono (D175)	1:1000
Cl. CASP7	Cell signaling	rb poly (D198)	1:1000
FOXO1	Santa Cruz	rb poly (H-128)	1:500
GAPDH	Cell signaling	rb mono (14C10)	1:1000
MYCN	Cell signaling	rb mono	1:1000
NOXA	Cell signaling	rb mono (D8L7U)	1:1000
P21	Cell signaling	rb mono (12D1)	1:1000
P53	Thermo Fisher Sc	ms mono (DO-1)	1:1000
PARP	Cell signaling	rb poly	1:1000
PLK1	Merck Millipore	ms mono	1:1000
pFOXO1$_{(S256 \triangleq S437)}$	Cell signaling	rb poly	1:750
pPLK1$_{(T210)}$	Cell signaling	rb mono (D5H7)	1:750
XIAP	Cell signaling	rb poly	1:1000

ms: mouse / rb: rabbit

Supplemental Table 1: Drug library content

Product Name	Target
Veliparib (ABT-888)	PARP
Axitinib	VEGFR, PDGFR, c-Kit
Saracatinib (AZD0530)	Src, Bcr-Abl
FG-4592	HIF
Afatinib (BIBW2992)	EGFR
Bortezomib (PS-341)	Proteasome
Bosutinib (SKI-606)	Src
Dovitinib (TKI-258, CHIR-258)	c-Kit, FGFR, Flt, VEGFR, PDGFR
Dasatinib	Src, Bcr-Abl, c-Kit
Erlotinib HCl (OSI-744)	EGFR

Gefitinib (ZD1839)	EGFR
Lapatinib (GW-572016)	EGFR, HER2
Lenalidomide (CC-5013)	TNF-alpha
Nilotinib (AMN-107)	Bcr-Abl
Pazopanib HCl	VEGFR, PDGFR, c-Kit
Rapamycin (Sirolimus)	mTOR
Sorafenib Tosylate	VEGFR, PDGFR, Raf
Sunitinib Malate	VEGFR, PDGFR, c-Kit, Flt
Vandetanib (ZD6474)	VEGFR
Vorinostat (SAHA, MK0683)	HDAC
VX-680 (Tozasertib, MK-0457)	Aurora Kinase
Y-27632 2HCl	ROCK
Elesclomol (STA-4783)	HSP
Entinostat (MS-275)	HDAC
Enzastaurin (LY317615)	PKC
Olaparib (AZD2281, Ku-0059436)	PARP
GDC-0941	PI3K
SB431542	TGF-beta/Smad
Crizotinib (PF-02341066)	c-Met, ALK
AUY922 (NVP-AUY922)	HSP
PHA-665752	c-Met
SB216763	GSK-3
MK-2206 2HCl	Akt
Vismodegib (GDC-0449)	Hedgehog, P-gp
KU-55933 (ATM Kinase Inhibitor)	ATM
GSK1904529A	IGF-1R
MLN8054	Aurora Kinase
Danusertib (PHA-739358)	Aurora Kinase, FGFR, Bcr-Abl, c-RET, Src
GSK690693	Akt
JNJ-38877605	c-Met
Palbociclib (PD-0332991) HCl	CDK
Cabozantinib (BMS-907351)	VEGFR, c-Met, Flt, Tie-2, c-Kit
Everolimus (RAD001)	mTOR
BMS-754807	IGF-1R
YM155 (Sepantronium Bromide)	Survivin
Alisertib (MLN8237)	Aurora Kinase
AT9283	Bcr-Abl, JAK, Aurora Kinase
Barasertib (AZD1152-HQPA)	Aurora Kinase
Roscovitine (Seliciclib,CYC202)	CDK
Lenvatinib (E7080)	VEGFR
Valproic acid	GABA Receptor, HDAC
CYC116	Aurora Kinase, VEGFR
XAV-939	Wnt/beta-catenin
Thalidomide	Others
Decitabine	DNA/RNA Synthesis
PIK-75	PI3K, DNA-PK

2-Methoxyestradiol (2-MeOE2)	HIF
Vemurafenib (PLX4032, RG7204)	Raf
Rigosertib (ON-01910)	PLK
Ruxolitinib (INCB018424)	JAK
Resveratrol	Sirtuin
Ispinesib (SB-715992)	Kinesin
AEE788 (NVP-AEE788)	EGFR, Flt, VEGFR, HER2
PHA-793887	CDK
Ponatinib (AP24534)	Bcr-Abl, VEGFR, FGFR, PDGFR, Flt
AT7519	CDK
MK-1775	Wee1
Quizartinib (AC220)	Flt
AZD7762	Chk
R406 (free base)	Syk
Org 27569	Cannabinoid Receptor
EX 527 (Selisistat)	Sirtuin
Pomalidomide	TNF-alpha, COX
KU-60019	ATM
BIRB 796 (Doramapimod)	p38 MAPK
RO4929097	Y-Secretase
Tie2 kinase inhibitor	Tie-2
Azacitidine	DNA/RNA Synthesis
Acadesine	AMPK
Nicorandil	Others
PF-573228	FAK
Lovastatin	HMG-CoA Reductase
LDE225 (Erismodegib)	Smoothened
PF-4708671	S6 Kinase
MLN2238	Proteasome
MLN9708	Proteasome
SGI-1776 free base	Pim
AZ 960	JAK
Apatinib	VEGFR
Volasertib (BI 6727)	PLK
Degrasyn (WP1130)	DUB, Bcr-Abl
BKM120 (Buparlisib)	PI3K
Imatinib (STI571)	PDGFR,c-Kit, v-Abl
Mifepristone	Estrogen/progestogen Receptor
LY2603618	Chk
NU7441 (KU-57788)	DNA-PK, PI3K
MK-0752	Gamma-secretase
Trametinib (GSK1120212)	MEK
Ibrutinib (PCI-32765)	Src
NVP-BSK805 2HCl	JAK
GDC-0980 (RG7422)	mTOR, PI3K
A-769662	AMPK

AMG-900	Aurora Kinase
Crenolanib (CP-868596)	PDGFR
AZ 3146	Kinesin
PHA-767491	CDK
CUDC-907	HDAC, PI3K
NVP-BVU972	c-Met
SB705498	TRPV
Tofacitinib (CP-690550)	JAK
Dabrafenib (GSK2118436)	Raf
GDC-0068	Akt
Torin 2	mTOR
TAE226 (NVP-TAE226)	FAK
TPCA-1	IKK
Carfilzomib (PR-171)	Proteasome
T0070907	PPAR
WZ811	CXCR
IOX2	HIF
Evacetrapib (LY2484595)	CETP
Pazopanib	VEGFR
Rimonabant	Cannabinoid Receptor
Cabozantinib malate	c-met, VEGFR2
Spironolactone	Androgen Receptor
JNK-IN-8	Free Base
QNZ (EVP4593)	NF-κB
Tofacitinib (CP-690550) Citrate	JAK
GDC-0152	IAP
AZD3514	Androgen Receptor
AZ20	ATM/ATR
GSK126	Histone Methyltransferase
EPZ5676	Methyltransferase
GSK J4 HCl	Others
LDK378	ALK
IWP-2	Wnt/beta-catenin
GSK2334470	PDK-1
PF-3758309	PAK
HSP990 (NVP-HSP990)	HSP (e.g. HSP90)
AZD3463	ALK
EPZ-6438	Histone Methyltransferase
PYR-41	E1 Activating
PR-619	DUB
P5091 (P005091)	DUB
BMS-833923	Hedgehog/Smoothened
AZD1080	GSK-3
C646	Histone Acetyltransferase
10058-F4	c-Myc
AVL-292	BTK

IOX1	Histone demethylases
OG-L002	Histone demethylases
SGC-CBP30	Epigenetic Reader Domain
CNX-774	BTK
MM-102	Histone Methyltransferase
JIB-04	Histone demethylases
PFI-2	Histone Methyltransferase
CPI-203	Epigenetic Reader Domain
GSK2606414	PERK
6H05	Rho
K-Ras(G12C) inhibitor 9	Rho
SH-4-54	STAT
OTX015	BET
LEE011	CDK
LDC000067	CDK
PI-1840	Proteasome
JNK Inhibitor IX	JNK
GNF-5837	Trk receptor
Afuresertib (GSK2110183)	Akt
GDC-0994	ERK
UNC0379	Histone Methyltransferase
GSK-LSD1 2HCl	Histone Demethylase
GSK J1	Histone Demethylase
INCB024360	IDO
BRD4770	Histone Methyltransferase
BV-6	IAP
EI1	Histone Methyltransferase
MI-2 (Menin-MLL Inhibitor)	Histone Methyltransferase
LDC1267	Axl
CPI-360	Histone Methyltransferase
CH5183284 (Debio-1347)	FGFR
YK-4-279	DNA/RNA Synthesis
AZD6738	ATM/ATR
Verdinexor (KPT-335)	CRM1
EPZ015666	Histone Methyltransferase
Pexmetinib (ARRY-614)	p38 MAPK
Pexidartinib (PLX3397)	CSF-1R
BI-847325	MEK
PFI-4	Epigenetic Reader Domain
Epacadostat (INCB024360)	IDO
NSC 23766	Rac
BMS-345541	IκB/IKK
Pacritinib (SB1518)	JAK
Idasanutlin	MDM2/p53
iBet762	BET
ABT-263	BCL2, BCL-XL, BCL-w
ABT-199	BCL2
Obatoclax	BCL2-family

Dynasore	Dynamin
Dyngo4a	Dynamin
GDC-0973	MEK
Fenretinide	Retinoid Acid Receptor
JQ-1	BET
Birinapant	IAP
Doxorubicine	DNA
Vincristine	Microtubuli
Etoposide	Topoisomerase