Figure S1

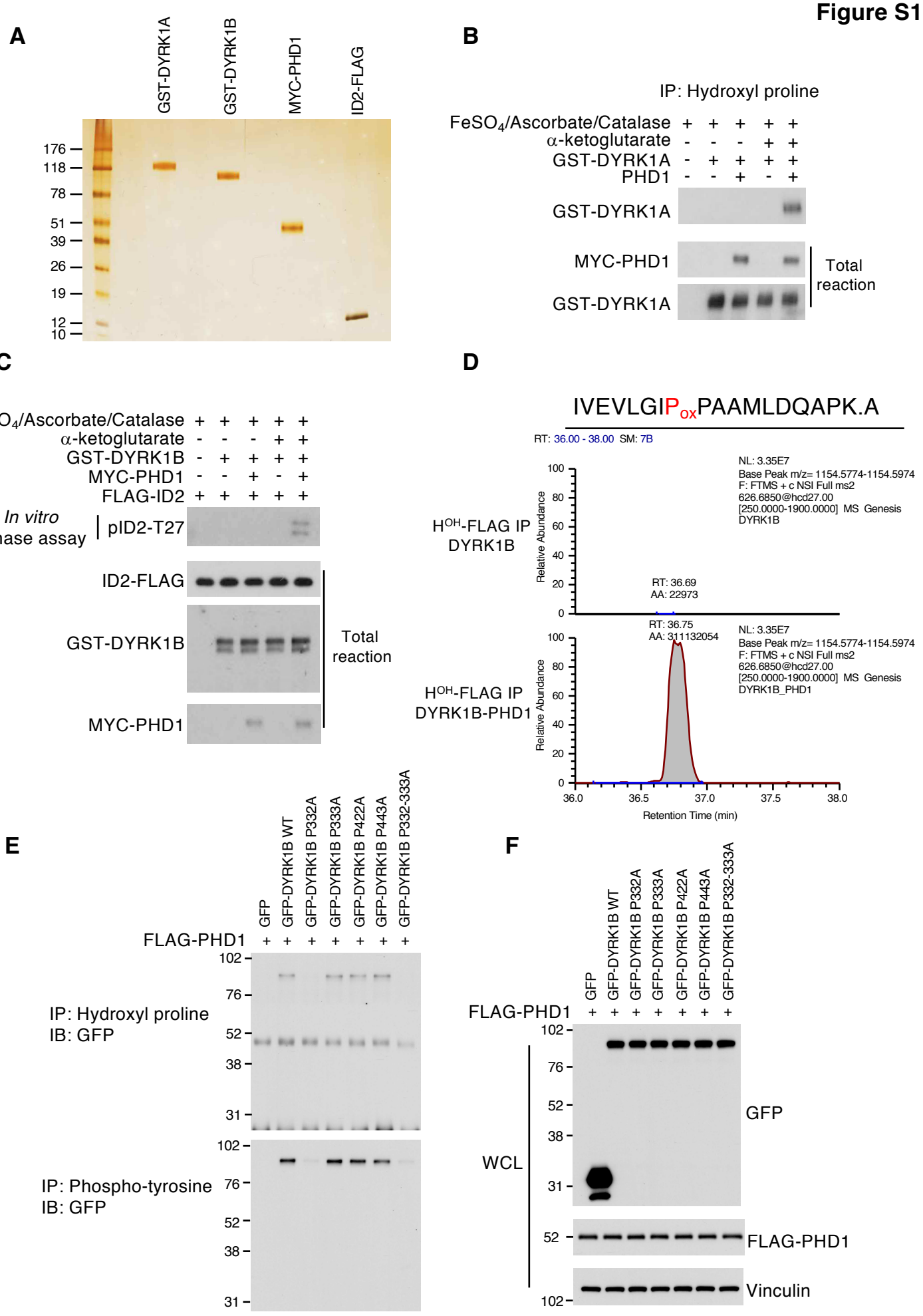

A
GST-DYRK1A
GST-DYRK1B
MYC-PHD1
ID2-FLAG
176
118
78
51
39
26
19
12
10
B
IP: Hydroxyl proline
FeSO4/Ascorbate/Catalase + + + + +
α-ketoglutarate - - - + +
GST-DYRK1A - + + + +
PHD1 - - + - +
GST-DYRK1A
MYC-PHD1
GST-DYRK1A
Total reaction
C
FeSO4/Ascorbate/Catalase + + + + +
α-ketoglutarate - - - + +
GST-DYRK1B - + + + +
MYC-PHD1 - - + - +
FLAG-ID2 + + + + +
In vitro kinase assay
pID2-T27
ID2-FLAG
GST-DYRK1B
MYC-PHD1
Total reaction
D
IVEVLGIPoxPAAMLDQAPK.A
RT: 36.00 - 38.00 SM: 7B
HOH-FLAG IP DYRK1B
NL: 3.35E7
Base Peak m/z= 1154.5774-1154.5974
F: FTMS + c NSI Full ms2
626.6850@hcd27.00
[250.0000-1900.0000] MS Genesis
DYRK1B
RT: 36.69
AA: 22973
Relative Abundance
HOH-FLAG IP DYRK1B-PHD1
RT: 36.75
AA: 311132054
NL: 3.35E7
Base Peak m/z= 1154.5774-1154.5974
F: FTMS + c NSI Full ms2
626.6850@hcd27.00
[250.0000-1900.0000] MS Genesis
DYRK1B_PHD1
Retention Time (min)
E
GFP
GFP-DYRK1B WT
GFP-DYRK1B P332A
GFP-DYRK1B P333A
GFP-DYRK1B P422A
GFP-DYRK1B P443A
GFP-DYRK1B P332-333A
FLAG-PHD1 + + + + + + +
IP: Hydroxyl proline
IB: GFP
IP: Phospho-tyrosine
IB: GFP
F
FLAG-PHD1 + + + + + + +
WCL
GFP
FLAG-PHD1
Vinculin

Figure S1 (Related to Figure 1) DYRK1 kinase proline hydroxylation *in vitro* and mass spectrometry analysis of DYRK1B protein. (A) Silver staining of 100 ng of recombinant GST-DYRK1A and GST-DYRK1B purified from baculovirus, 100 ng of MYC-PHD1 purified from HEK-293T cells and 150 ng of ID2-FLAG purified from E. coli. Purified proteins were used for *in vitro* prolyl hydroxylation and kinase assays. (B) DYRK1A is prolyl hydroxylated *in vitro* by PHD1. Recombinant GST-DYRK1A produced in baculovirus was incubated in the presence or absence of PHD1 and immunoprecipitated using anti-hydroxyl proline antibody. Immunoprecipitated proteins were processed by SDS-PAGE and immunoblot using DYRK1A antibody. Total proteins in the *in vitro* reaction were included as control. (C) Bacterially expressed GST-DYRK1B was incubated in the presence or the absence of PHD1 and used to phosphorylate recombinant ID2-FLAG (*in vitro* kinase assay). Total proteins in the *in vitro* reaction were included as control. (D) Elution profiles of DYRK1B hydroxylated peptide IVEVLGIP#PAAMLDQAPK from cells expressing DYRK1B in the absence (upper panel) or presence (lower panel) of PHD1. The retention time profile of the peptide was generated by tracing a specific fragment, y11 (m/z 1154.5874) of peptide IVEVLGIP#PAAMLDQAPK (m/z 636.685) over time. The area underneath curve was automatically calculated by QualBrowser. Y axis was kept constant. (E) GFP-DYRK1B wild type and proline to alanine point mutants and double mutant P332A-P333A were co-expressed with PHD1 in U87 cells. Cell lysates were analyzed for the presence of proline hydroxylation and tyrosine phosphorylation by immunoprecipitation using anti-hydroxyl proline or anti-phospho-tyrosine antibody followed by western blot for GFP-DYRK1B. (F) Whole cellular lysate (WCL) from the experiment in E.

Figure S2

A

DYRK1A

B

DYRK1B

acidic
aliphatic
aliphatic (small)
amide
aromatic
basic
hydroxyl
imino
sulfur

X non conserved
X similar
X ≥ 80% conserved
X all match

Figure S2 (Related to Figure 2) Evolutionary conservation of proline 380 in DYRK1A and proline 332 in DYRK1B. (A) Amino acid sequence flanking P380 of DYRK1A is evolutionarily conserved. (B) Amino acid sequence flanking P332 of DYRK1B is evolutionarily conserved. The prolyl hydroxylation motif is boxed in red.

Figure S3

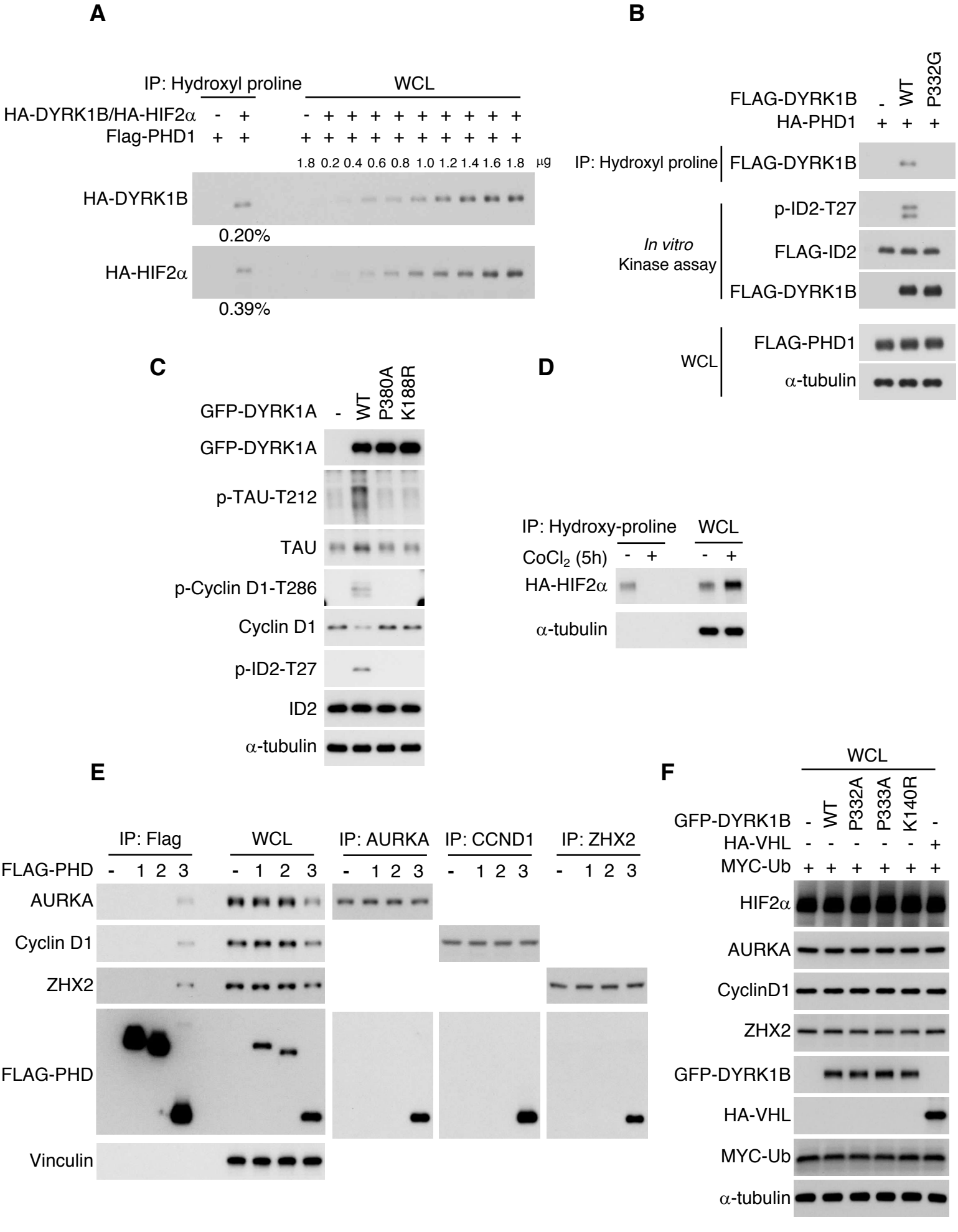

A
IP: Hydroxyl proline
WCL
HA-DYRK1B/HA-HIF2α - + - + + + + + + + +
Flag-PHD1 + + + + + + + + + + +
1.8 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 μg
HA-DYRK1B
0.20%
HA-HIF2α
0.39%
B
FLAG-DYRK1B - WT P332G
HA-PHD1 + + +
IP: Hydroxyl proline
FLAG-DYRK1B
p-ID2-T27
In vitro Kinase assay
FLAG-ID2
FLAG-DYRK1B
WCL
FLAG-PHD1
α-tubulin
C
GFP-DYRK1A - WT P380A K188R
GFP-DYRK1A
p-TAU-T212
TAU
p-Cyclin D1-T286
Cyclin D1
p-ID2-T27
ID2
α-tubulin
D
IP: Hydroxy-proline
WCL
CoCl2 (5h) - + - +
HA-HIF2α
α-tubulin
E
IP: Flag
WCL
IP: AURKA
IP: CCND1
IP: ZHX2
FLAG-PHD - 1 2 3 - 1 2 3 - 1 2 3 - 1 2 3 - 1 2 3
AURKA
Cyclin D1
ZHX2
FLAG-PHD
Vinculin
F
WCL
GFP-DYRK1B - WT P332A P333A K140R -
HA-VHL - - - - - +
MYC-Ub + + + + + +
HIF2α
AURKA
CyclinD1
ZHX2
GFP-DYRK1B
HA-VHL
MYC-Ub
α-tubulin

Figure S3 (Related to Figure 4) Stoichiometry, functional analysis of hydroxylated P332 of DYRK1B and analysis of VHL substrates. (A) Hydroxyl-proline immunoprecipitation of HA-DYRK1B or HA-HIF2α expressed in 293T cells (500 μg of cellular lysates; 100% or 50% of DYRK1B or HIF2α reactions, respectively) and serial dilutions of total extract (μg: total μg of lysates) were processed by western blot using HA antibodies and analyzed by densitometry. Indicated is the fraction of hydroxylated proline in DYRK1B and HIF2α in the presence of FLAG-PHD1. (B) FLAG-DYRK1B proteins immunoprecipitated from U87 cells transfected with plasmids coding for DYRK1B wild type and the mutant P332G were used for hydroxyl-proline immunoprecipitation followed by western blot for FLAG. DYRK1B wild type and DYRK1B-P332G were immunopurified from the same lysates and used in kinase assay *in vitro* towards recombinant ID2. (C) GFP-DYRK1A wild type or mutants P380A and K188R were transfected into U87. Cellular lysates were analyzed for total levels and phosphorylation of endogenous DYRK1 kinase substrates as described in Figure 1G. (D) Western blot analysis of HIF2α from cells treated with vehicle or $CoCl_2$. HIF2α protein was used for GST-VHL pull-down experiment in Figure 4A. (E) Interaction between AURKA, cyclin D1, ZHX2 with PHD3. U87 cells were transfected with plasmids expressing FLAG-PHD1, FLAG-PHD2, FLAG-PHD3 or the empty vector. Cellular lysates were immunoprecipitated using FLAG antibody followed by western blot for the indicated endogenous proteins (left panels). Reciprocal immunoprecipitations were also performed to test interaction of endogenous VHL substrates with FLAG-PHD proteins (right panels). (F) Western blot analysis of whole cellular lysates used in the ubiquitylation assay in Figure 4E. WCL, whole cellular lysate.

Figure S4

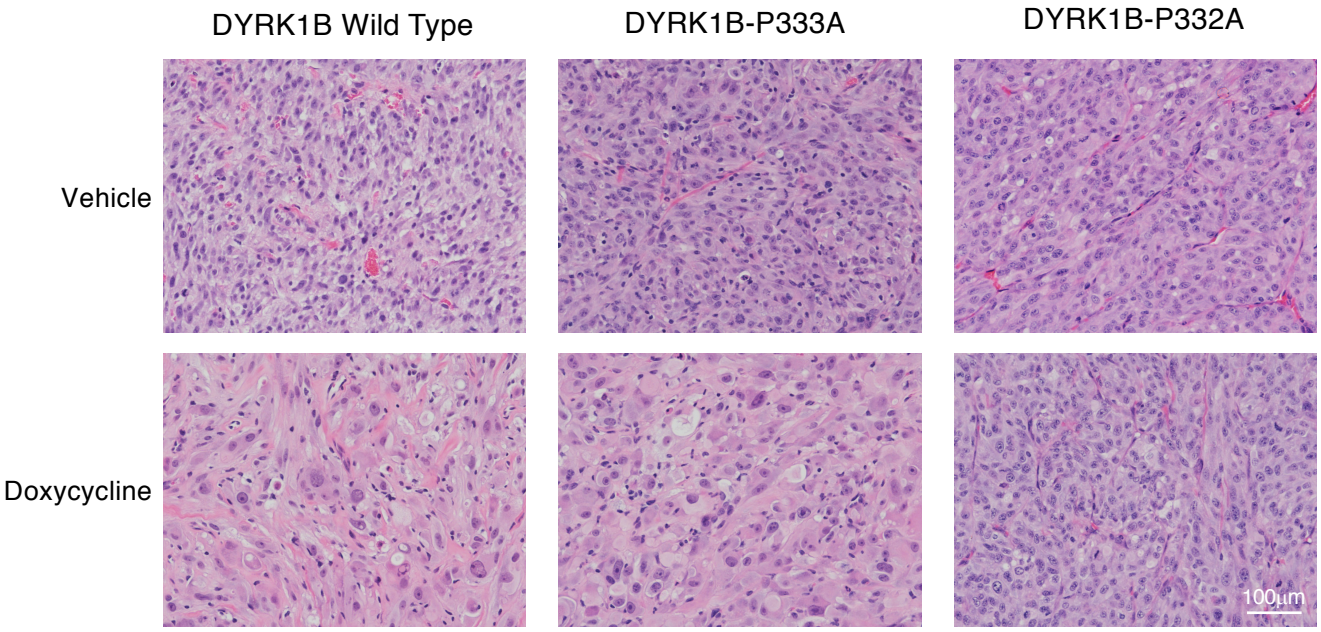
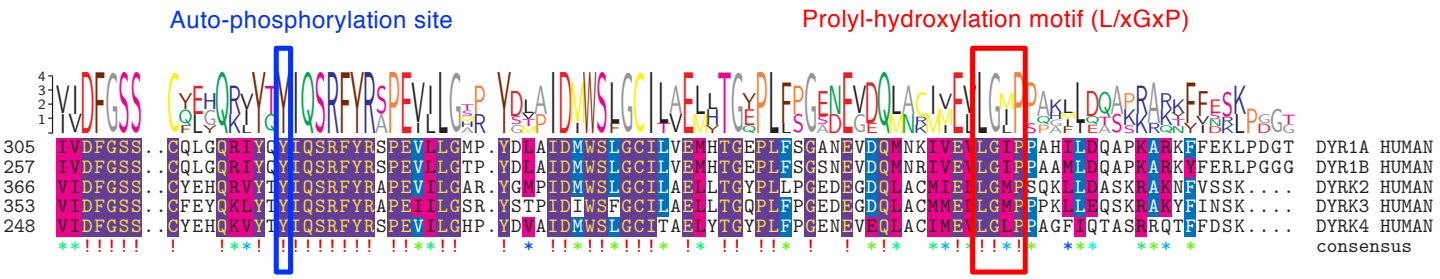

DYRK1B Wild Type
DYRK1B-P333A
DYRK1B-P332A
Vehicle
Doxycycline
100µm

Figure S4 (Related to Figure 5) Histological analysis of DYRK1B expressing glioblastoma xenografts. Hematoxylin and Eosin staining shows enlarged tumor cells after doxycycline-induced expression of DYRK1B wild type and DYRK1B-P333A but not DYRK1B-P332A. Scale bar: 100 μM.

Figure S5

A


Auto-phosphorylation site
Prolyl-hydroxylation motif (L/xGxP)
305 IVDFGSS..CQLGQRIYQYIQSRFYRSPEVLLGMP.YDLAIDMWSLGCILVEMHTGEPLFSGANEVDQMNKIVEVLGIPPAHLLDQAPKARKFFEKLPDGT DYR1A HUMAN
257 IVDFGSS..CQLGQRIYQYIQSRFYRSPEVLLGTP.YDLAIDMWSLGCILVEMHTGEPLFSGSNEVDQMNRIVEVLGIPPAAMLDQAPKARKYFERLPGGG DYR1B HUMAN
366 VIDFGSS..CYEHQRVYTYIQSRFYRAPEVILGAR.YGMPIDMWSLGCILAELLTGYPLLPGEDEGDQLACMIEILGMPSQKLLDASKRAKNFVSSK.... DYRK2 HUMAN
353 VIDFGSS..CFEYQKLYTYIQSRFYRAPEIILGSR.YSTPIDIWSFGCILAELLTGQPLFPGEDEGDQLACMMEILGMPPKLLEQSKRAKYFINSK.... DYRK3 HUMAN
248 VIDFGSS..CYEHQKVYTYIQSRFYRSPEVILGHP.YDVAIDMWSLGCITAELYTGYPLFPGENEVEQLACIMEVLGLPPAGFIQTASRRQTFFDSK.... DYRK4 HUMAN
consensus

B

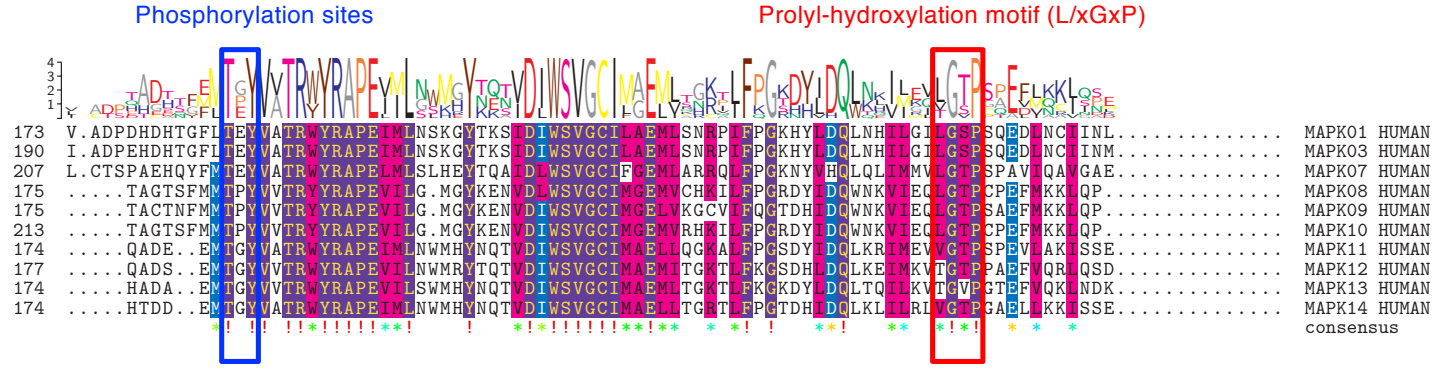

Phosphorylation sites
Prolyl-hydroxylation motif (L/xGxP)
173 V.ADPDHDHTGFLTEYVATRWYRAPEIMLNSKGYTKSIDIWSVGCILAEMLSNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINL..... MAPK01 HUMAN
190 I.ADPEHDHTGFLTEYVATRWYRAPEIMLNSKGYTKSIDIWSVGCILAEMLSNRPIFPGKHYLDQLNHILGILGSPSQEDLNCIINM..... MAPK03 HUMAN
207 L.CTSPAEHQYFMTEYVATRWYRAPELMLSLHEYTQAIDLWSVGCIFGEMLARRQLFPGKNYVHQLQLIMMVLGTPSPAVIQAVGAE..... MAPK07 HUMAN
175 .....TAGTSFMMTPYVVTRYYRAPEVILG.MGYKENVDLWSVGCIMGEMVCHKILFPGRDYIDQWNKVIEQLGTPCPEFMKKLQP..... MAPK08 HUMAN
175 .....TACTNFMMTPYVVTRYYRAPEVILG.MGYKENVDIWSVGCIMGELVKGCVIFQGTDHIDQWNKVIEQLGTPSAEFMKKLQP..... MAPK09 HUMAN
213 .....TAGTSFMMTPYVVTRYYRAPEVILG.MGYKENVDIWSVGCIMGEMVRHKILFPGRDYIDQWNKVIEQLGTPCPEFMKKLQP..... MAPK10 HUMAN
174 .....QADE..EMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLQGKALFPGSDYIDQLKRIMEVVGTPSPEVLAKISSE..... MAPK11 HUMAN
177 .....QADS..EMTGYVVTRWYRAPEVILNWMRYTQTVDIWSVGCIMAEMITGKTLFKGSDHLDQLKEIMKVTGTPPAEFVQRLQSD..... MAPK12 HUMAN
174 .....HADA..EMTGYVVTRWYRAPEVILSWMHYNQTVDIWSVGCIMAEMLTGKTLFKGKDYLDQLTQILKVTGVPGTEFVQKLNDK..... MAPK13 HUMAN
174 .....HTDD..EMTGYVATRWYRAPEIMLNWMHYNQTVDIWSVGCIMAELLTGRTLFPGTDHIDQLKLILRIVGTPGAELLKKISSE..... MAPK14 HUMAN
consensus
acidic
aliphatic
aliphatic (small)
amide
aromatic
basic
hydroxyl
imino
sulfur
non conserved
similar
≥80% conserved
all match

Figure S5 (Related to Figure 6 and Table S1, S2) A prolyl hydroxylation motif is present in DYRK and MAPK kinase family members. (A) The prolyl hydroxylation motif (L/xGx**P**) is conserved across DYRK family members. (B) The prolyl hydroxylation motif (L/xGx**P**) is conserved across MAPK family members. Prolyl hydroxylation motif and auto-phosphorylation sites are boxed in red and blue, respectively.

Figure S6

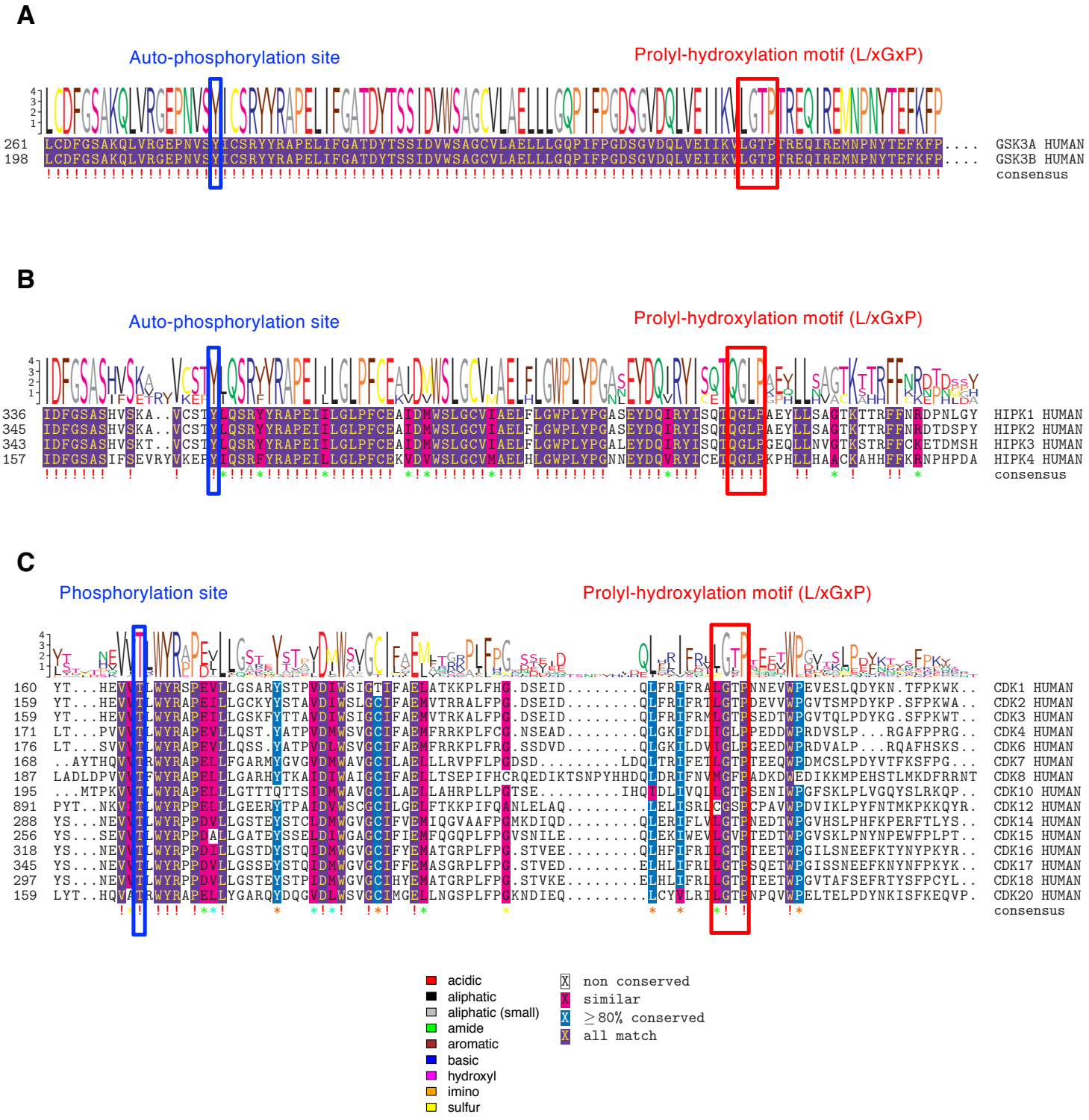

A
Auto-phosphorylation site
Prolyl-hydroxylation motif (L/xGxP)
261 LCDFGSAKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSIDVWSAGCVLAELLLGQPIFPGDSGVDQLVEIIKVLGTPTREQIREMNPNYTEFKFP.... GSK3A_HUMAN
198 LCDFGSAKQLVRGEPNVSYICSRYYRAPELIFGATDYTSSIDVWSAGCVLAELLLGQPIFPGDSGVDQLVEIIKVLGTPTREQIREMNPNYTEFKFP.... GSK3B_HUMAN
consensus
B
Auto-phosphorylation site
Prolyl-hydroxylation motif (L/xGxP)
336 IDFGSASHVSKA..VCSTYLQSRYYRAPEIILGLPFCEAIDMWSLGCVIAELFLGWPLYPGASEYDQIRYISQTQGLPAEYLLSAGTKTTRFFNRDTDSPY HIPK1_HUMAN
345 IDFGSASHVSKA..VCSTYLQSRYYRAPEIILGLPFCEAIDMWSLGCVIAELFLGWPLYPGASEYDQIRYISQIQGLPAEYLLSAGTKTTRFFNRDTDSPY HIPK2_HUMAN
343 IDFGSASHVSKT..VCSTYLQSRYYRAPEIILGLPFCEAIDMWSLGCVIAELFLGWPLYPGALEYDQIRYISQIQGLPGEQLLNVGTKSTRFFCKETDMSH HIPK3_HUMAN
157 IDFGSASIFSEVRYVKEPYIQSRFYRAPEILLGLPFCEKVDVWSLGCVMAELHLGWPLYPGNNEYDQVRYICEIQGLPKPHLLHAACKAHHFFKRNPHPDA HIPK4_HUMAN
consensus
C
Phosphorylation site
Prolyl-hydroxylation motif (L/xGxP)
160 YT...HEVVTLWYRSPEVLLGSARYSTPVDIWSIGTIFAELATKKPLFHG.DSEID.....QLFRIFRALGTPNNEVWPEVESLQDYKN.TFPKWK.. CDK1_HUMAN
159 YT...HEVVTLWYRAPEILLGCKYYSTAVDIWSLGCIFAEMVTRRALFPG.DSEID.....QLFRIFRTLGTPDEVVWPGVTSMPDYKP.SFPKWA.. CDK2_HUMAN
159 YT...HEVVTLWYRAPEILLGSKFYTTAVDIWSIGCIFAEMVTRKALFPG.DSEID.....QLFRIFRMLGTPSEDTWPGVTQLPDYKG.SFPKWT.. CDK3_HUMAN
171 LT...PVVVTLWYRAPEVLLQST.YATPVDMWSVGCIFAEMFRRKPLFCG.NSEAD.....QLGKIFDLIGLPPEDDWPRDVSLP...RGAFPPRG.. CDK4_HUMAN
176 LT...SVVVTLWYRAPEVLLQSS.YATPVDLWSVGCIFAEMFRRKPLFRG.SSDVD.....QLGKILDVIGLPGEEDWPRDVALP...RQAFHSKS.. CDK6_HUMAN
168 ..AYTHQVVTRWYRAPELLFGARMYGVGVDMWAVGCILAELLLRVPFLPGDSD.....LDQLTRIFETLGTPTEEQWPDMCSLPDYVTFKSFPG... CDK7_HUMAN
187 LADLDPVVVTFWYRAPELLLGARHYTKAIDIWAIGCIFAELLTSEPIFHCRQEDIKTSNPYHHDQLDRIFNVMGFPADKDWEDIKKMPEHSTLMKDFRRNT CDK8_HUMAN
195 ...MTPKVVTLWYRAPELLLGTTTQTTSIDMWAVGCILAELLAHRPLLPGTSE.....IHQIDLIVQLLGTPSENIWPGFSKLPLVGQYSLRKQP.. CDK10_HUMAN
891 PYT..NKVITLWYRPPELLLGEERYTPAIDVWSCGCILGELFTKKPIFQANLELAQ.....LELISRLCGSPCPAVWPDVIKLPYFNTMKPKKQYR. CDK12_HUMAN
288 YS...NEVVTLWYRPPDVLLGSTEYSTCLDMWGVGCIFVEMIQGVAAFPGMKDIQD.....QLERIFLVLGTPNEDTWPGVHSLPHFKPERFTLYS.. CDK14_HUMAN
256 YS...SEVVTLWYRPPDALLGATEYSSELDIWGAGCIFIEMFQGQPLFPGVSNILE.....QLEKIWEVLGVPTEDTWPGVSKLPNYNPEWFPLPT.. CDK15_HUMAN
318 YS...NEVVTLWYRPPDILLGSTDYSTQIDMWGVGCIFYEMATGRPLFPG.STVEE.....QLHFIFRILGTPTEETWPGILSNEEFKTYNYPKYR.. CDK16_HUMAN
345 YS...NEVVTLWYRPPDVLLGSSEYSTQIDMWGVGCIFFEMASGRPLFPG.STVED.....ELHLIFRLLGTPSQETWPGISSNEEFKNYNFPKYK.. CDK17_HUMAN
297 YS...NEVVTLWYRPPDVLLGSTEYSTPIDMWGVGCIHYEMATGRPLFPG.STVKE.....ELHLIFRLLGTPTEETWPGVTAFSEFRTYSFPCYL.. CDK18_HUMAN
159 LYT..HQVATRWYRAPELLYGARQYDQGVDLWSVGCIMGELLNGSPLFPGKNDIEQ.....LCYVLRILGTPNPQVWPELTELPDYNKISFKEQVP. CDK20_HUMAN
consensus
acidic
aliphatic
aliphatic (small)
amide
aromatic
basic
hydroxyl
imino
sulfur
non conserved
similar
≥80% conserved
all match

Figure S6 (Related to Figure 7 and Tables S3) A prolyl hydroxylation motif is present in GSK, HIPK and CDK kinases. (A) The prolyl hydroxylation motif (L/xGx**P**) is conserved across GSK3 family members. (B) The prolyl hydroxylation motif (L/xGx**P**) is conserved across HIPK family members. (C) The prolyl hydroxylation motif (L/xGx**P**) is conserved across CDK family members. Prolyl hydroxylation motif and auto-phosphorylation sites are boxed in red and blue, respectively.

Figure S7

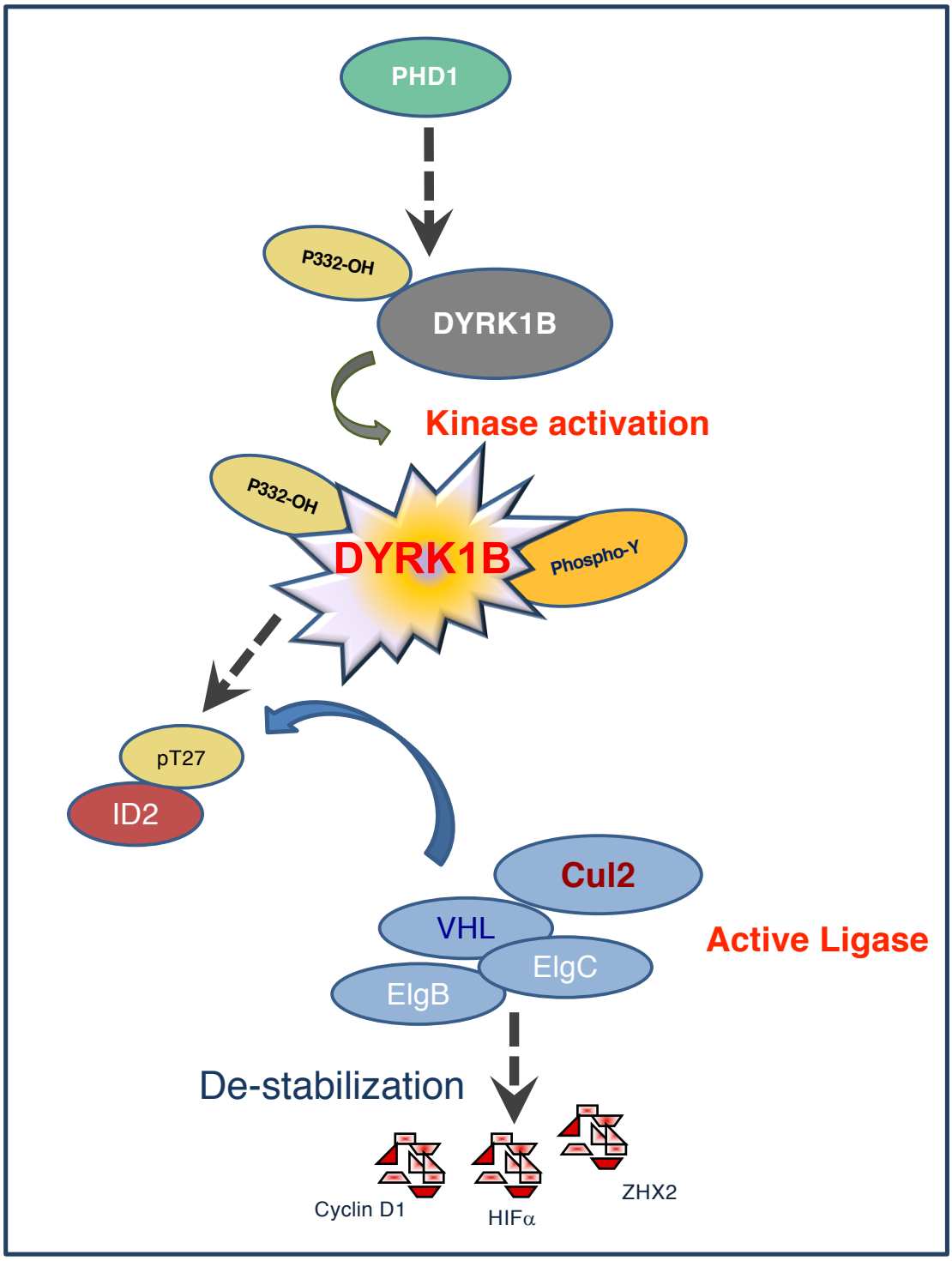

PHD1
P332-OH
DYRK1B
Kinase activation
P332-OH
DYRK1B
Phospho-Y
pT27
ID2
Cul2
VHL
ElgC
ElgB
Active Ligase
De-stabilization
Cyclin D1
HIFα
ZHX2

Figure S7 (Related to Figure 4) PHD-dependent activation of DYRK1 kinases sustains tumor suppression by VCB^{CRL}. In cellular contexts that favour prolyl hydroxylase activity, PHD1 functions on newly synthesized DYRK1 kinases causing hydroxylation of proline 332 of DYRK1B (shown) and proline 380 of DYRK1A. This event is required for tyrosine phosphorylation and full activation of DYRK1. The activity of DYRK1 kinases on threonine 27 of ID2 imposes a functional constraint on ID2 ability to interact with the VHL-Elongin C-Elongin B (VCB complex) and inactivate the ubiquitin ligase activity of VCB^{CRL} by displacing CUL2 from the complex. With functional PHDs and DYRK1 kinases, the VCB^{CRL} ubiquitin ligase complex remains competent to efficiently ubiquitylate its substrates (HIF-α, Cyclin D1, ZHX2).

Table S1. (Related to Figure 1, 7 and Figure S2, 5) List of prolyl hydroxylation motifs, sites of autophosphorylation in the activation loop and relative distance are reported for DYRK family members.

Protein	Threonine / Tyrosine phosphorylation		Proline hydroxylation		Distance
	Position	Motif (YxY)	Position	Motif (L/xGxP)	
DYRK1A	319-321	YQY	377-380	LGIP	59
DYRK1B	271-273	YQY	329-332	LGIP	59
DYRK2	380-382	YTY	438-441	LGMP	59
DYRK3	367-369	YTY	425-428	LGMP	59
DYRK4	262-264	YTY	320-323	LGLP	59

Table S2. (Related to Figure 6 and Figure S5). List of prolyl hydroxylation motifs, sites of autophosphorylation in the activation loop and relative distance are reported for MAPK family members.

Protein	Threonine / Tyrosine Phosphorylation		Proline hydroxylation		Distance
	Position	Motif (TxY)	Position	Motif (L/xGxP)	
MAPK01	185-187	TEY	244-247	LGSP	60
MAPK03	202-204	TEY	261-264	LGSP	60
MAPK07	219-221	TEY	278-281	LGTP	60
MAPK08	183-185	TPY	241-244	LGTP	59
MAPK09	183-185	TPY	241-244	LGTP	59
MAPK10	221-223	TPY	279-282	LGTP	59
MAPK11	180-182	TGY	239-242	VGTP	60
MAPK12	183-185	TGY	242-245	TGTP	60
MAPK13	180-182	TGY	239-242	TGVP	60
MAPK14	180-182	TGY	239-242	VGTP	60

Table S3. (Related to Figure 7 and Figure S6). List of prolyl hydroxylation motifs, sites of autophosphorylation in the activation loop and relative distance are reported for GSK3 and HIPK family members.

Protein	Threonine / Tyrosine Phosphorylation		Proline hydroxylation		Distance
	Position	Motif (xxY)	Position	Motif (L/xGxP)	
GSK3A	277-279	VSY	336-339	LGTP	60
GSK3B	214-216	VSY	273-276	LGTP	60
HIPK1	350-352	STY	408-411	QGLP	59
HIPK2	359-361	STY	417-420	QGLP	59
HIPK3	357-359	STY	415-418	QGLP	59
HIPK4	173-175	EPY	231-234	QGLP	59

Table S4. (Related to Figure 7 and Figure S6). List of prolyl hydroxylation motifs, sites of autophosphorylation in the activation loop and relative distance are reported for CDK family members.

Protein	Threonine phosphorylation		Proline hydroxylation		Distance
	Position	Motif (VxT)	Position	Motif (L/xGxP)	
CDK1	164-166	VVT	220-223	LGTP	57
CDK2	163-165	VVT	219-222	LGTP	57
CDK3	163-165	VVT	219-222	LGTP	57
CDK4	175-177	VVT	230-233	IGLP	56
CDK6	180-182	VVT	235-238	IGLP	56
CDK7	173-175	VVT	229-232	LGTP	57
CDK8	194-196	VVT	259-262	MGFP	66
CDK10	199-201	VVT	255-258	LGTP	57
CDK12	896-898	VIT	952-955	CGSP	57
CDK14	292-294	VVT	349-352	LGTP	58
CDK15	260-262	VVT	317-320	LGVP	58
CDK16	322-324	VVT	378-381	LGTP	57
CDK17	349-351	VVT	405-408	LGTP	57
CDK18	301-303	VVT	357-360	LGTP	57
CDK20	164-166	VAT	220-223	LGTP	57