Supplemental materials and methods:

Computer Algorithm Predictions for Candidate Protein Kinases of Olig2

Four computer algorithms were used in the study for Olig2 kinases predication, Scansite (Obenauer et al., 2003), GSP2.1 (Xue et al., 2008), KinasePhos2.0 (Wong et al., 2007) and NetPhorest (Horn et al., 2014). Kinases that were predicted to phosphorylate Olig2 at S10, S13 and S14 were selected and further studied.

Antibodies and Immunoblotting

Immunoblotting was performed according to standard protocols. Rabbit anti-P-Olig2 antibody (1:500) was generated in the Stiles lab and described previously (Sun et al., 2011). Anti-Olig2 antibody used in this study is either a rabbit polyclonal anti-Olig2 antibody (1:1,000, Millipore) or a monoclonal mouse anti-Olig2 antibody (1:2,000, Millipore). For other antibodies, we used rabbit anti-P-Rb (S780) antibody (1:1000, Cell Signaling), rabbit anti-P-Rb (S807/811) antibody (1:1000, Cell Signaling), mouse anti-Rb antibody (1:250, BD Biosciences), mouse anti-CDK1 antibody (1:1000, Abcam), mouse anti-CDK2 antibody (1:1000, Millipore), mouse anti-V5 antibody (1:5000, Invitrogen), rabbit anti-Thiophosphate-ester antibody (1:4000, Abcam), rabbit anti-Flag antibody (1:2000, Sigma), sheep anti-CK2β antibody (1:1000, R&D systems), rabbit anti-GSK3α/β antibody (1:1000, Cell Signaling) and mouse anti-p21 antibody (1:500, BD Biosciences). Immunoblotting by either mouse vinculin antibody (1:5000, Sigma) or mouse α-tubulin antibody (1:5000, Sigma) served as loading control.

CDK2-Knockout NPC Lines

To generate *CDK2*-knockout and control NPC lines, we crossed female and male *CDK2*$^{+/-}$ mice (Berthet et al., 2003) to obtain *CDK2*$^{-/-}$ and *CDK2*$^{+/+}$ embryos. We derived neural progenitor cell lines from the lateral ganglionic eminence (LGE) of individual *CDK2*-null or CDK2 wildtype embryos at E13.5.

MALDI-MS and –MS/MS Analysis of in vitro Kinase Assays

An aliquot corresponding to 25 pmol peptide was acidified with 0.1% TFA, desalted using µC18 ZipTips (Millipore), and spotted on an Opti-TOF 384 well plate. Matrix (1 µL of 5 mg/mL HCCA in 70% acetonitrile, 0.1% TFA with 120 µg/mL diammonium citrate) was added and sample allowed to air dry. Peptides were analyzed using a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA) in reflection mode averaging 1500 laser shots in a random, uniform pattern (30 subspectra, pass or fail, 50 shots/subspectrum) with a laser intensity of ~4000. MS/MS experiments to confirm phosphorylation site(s) were also performed in reflectron mode and averaged 5000 laser shots in a random uniform pattern (100 subspectra, pass or fail, 50 shots/subspectrum) with CID gas on and the precursor mass window set to relative with a value of 200 (fwhm).

shRNA Knockdown:

The shRNAs that target *mCK2*β were designed and cloned into the pLKO lentiviral vector that expresses a puromycin selection marker. Two independent shRNAs that target *mCK2*β were used in this study (shCK2β-1, 5'-GCTCCGTGGTAATGAATTCTT-3'; shCK2β-2, 5'-CTTTCAGGAAGTCTATGGTTTT-3'), and a non-target shRNA was

used as control (shConr, 5'-CAACAAGATGAAGAGCACCAA-3'). The lenti-viruses were produced and used to transduce NPCs for stably expressing shRNAs targeting *mCK2β*. Adeno associated virus (AAV) was used to express shRNAs that target *mCDK1* for transiently knocking-down *mCDK1* expression in NPCs. Two independent shRNAs that target *mCDK1* were used in this study (shCDK1-1, 5'-GCTGTATCTCATCTTTGAGTT-3'; shCDK1-2, 5'-GCCAAACGAATCTCTGGCAAA-3'), and a non-target shRNA was used as control (shConr, 5'-CAACAAGATGAAGAGCACCAA-3'). The shRNAs were cloned into an AAV vector that co-express a *Red Fluorescent Protein* (*RFP*) gene. Virus titer and transduction efficiency were controlled and monitored trough RFP signals. P-Olig2 and Olig2 levels were examined 48 hr after virus transduction.

Analog-Sensitive Kinase Assay with CDK2

We transfected 293 cells with WTOlig2-V5 or S14GOlig2-V5 together with either WT-CDK2-Flag or AS-CDK2-Flag. Briefly, forty-eight hours after transfection, cells were washed twice with PBS and incubated for 20 min in phosphorylation buffer (20 mM HEPES pH 7.3, 100 mM KOAc, 5 mM NaOAc, 2 mM $MgOAc_2$, 1 mM EGTA, 10 mM $MgCl_2$, 0.5 mM DTT, 30 μg/ml Digtonin, 0.5 mM GTP, 0.1 mM ATP, 0.1 mM 6-Fu-ATP-γ-S (Axxora), complete protease inhibitors (EDTA-Free, Roche), 1 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4O_7P_2$ and 1 mM β−glycerophosphate). Cells were then lysed with 2X RIPA (100 mM Tris, 300 mM NaCl, 2% NP-40, 0.2% SDS, and 40 mM EDTA) containing 2.5 mM p-nitrobenzyl mesylate (PNBM) (Abcam). The cell lysates were then used for immunoprecipitation with V5 conjugated agarose beads (Sigma). Immunoprecipitated samples were size fractioned by SDS-PAGE and immunoblotted by anti-Olig2 and anti-thiophosphate-ester antibody

Digestion and NanoLC-ESI-MS Analysis of Olig2 Peptides

In order to improve recovery of higher-order phosphorylated peptides, we processed Olig2 directly on beads for subsequent proteomic analysis. IP resin was washed 3 times with 200 μL 100 mM HEPES buffer (pH 7.4), and then resuspended in 100 mM ammonium bicarbonate. After reduction with 10 mM DTT at 56°C for 30 minutes, cysteine residues were alkylated with 22.5 mM IAA for 30 minutes protected from light and proteins subjected to digestion with AspN overnight at 37°C. AspN fragments were further digested with trypsin for 3 hours. After digestion, the supernatant was extracted, acidified, and desalted using C18 resin (Adelmant et al., 2011). Phosphopeptides were enriched by Fe-NTA IMAC as described (Ficarro et al., 2011), acidified with 10% TFA, loaded onto a C18 precolumn (360 μm O.D. x 100 μm I.D. fused silica packed with 8 cm 7 μm SYMMETRY C18; Waters, Milford, MA), and washed with 10 column volumes of 0.2 M acetic acid in water. The pre-column was connected to an analytical column (360 μm O.D. x 30 μm I.D. fused silica packed with 12 cm 5 μm Monitor C18; Column Engineering, Ontario, CA) with a low volume union (Valco, Houston, TX) and peptides were gradient eluted (0-30% B in 120 minutes; A=0.2 M acetic acid in water, B=0.2 M acetic acid in acetonitrile; spray voltage=2.2 kV) into the mass spectrometer (Orbitrap Velos, ThermoFisher Scientific, San Jose, CA) at a flow rate of ~30 nL/min. Relative quantification was performed by comparing peptide signal intensities across analyses. Raw values were corrected for differences in loading amounts using additional Olig2 phospho-peptide signals (Olig2 28-45+pT and Olig2 70-95+pS).

References:

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Supplemental figures and tables:

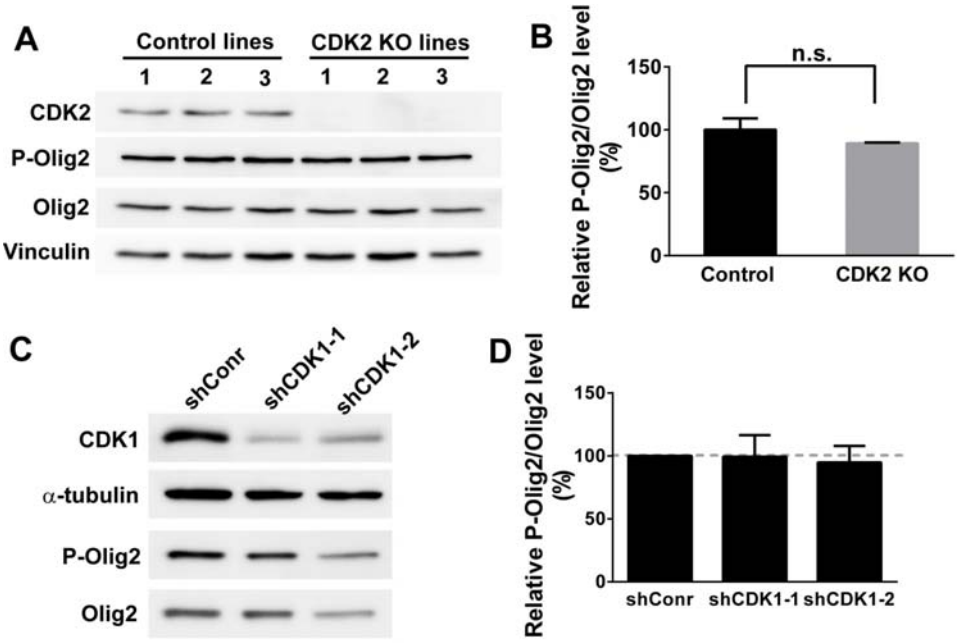

Figure S1. Individually knocking-out *CDK2* or knocking-down *CDK1* in NPCs does not affect Olig2 phosphorylation, Related to Figure 3.
(A and B) Knocking-out *CDK2* alone in NPCs does not affect Olig2 phosphorylation. P-Olig2 levels were examined in both wild-type and *CDK2* knock-out NPC lines by immunoblot assay. Relative P-Olig2/Olig2 levels were quantified and compared between wild-type and knockout group. Data were analyzed by *t*-test and are represented as mean ± SEM. n=5 repeats. (C and D) Acute knockdown of *CDK1* alone in NPCs does not affect Olig2 phosphorylation. *mCDK1* was knocked down for 48 hr in wild-type NPCs with AAV that express shRNAs targeting *mCDK1*. Relative P-Olig2/Olig2 levels were quantified and compared between wild-type and knockdown group. Data were analyzed by *t*-test and are represented as mean ± SEM. n=3 repeats. n.s., not significant.

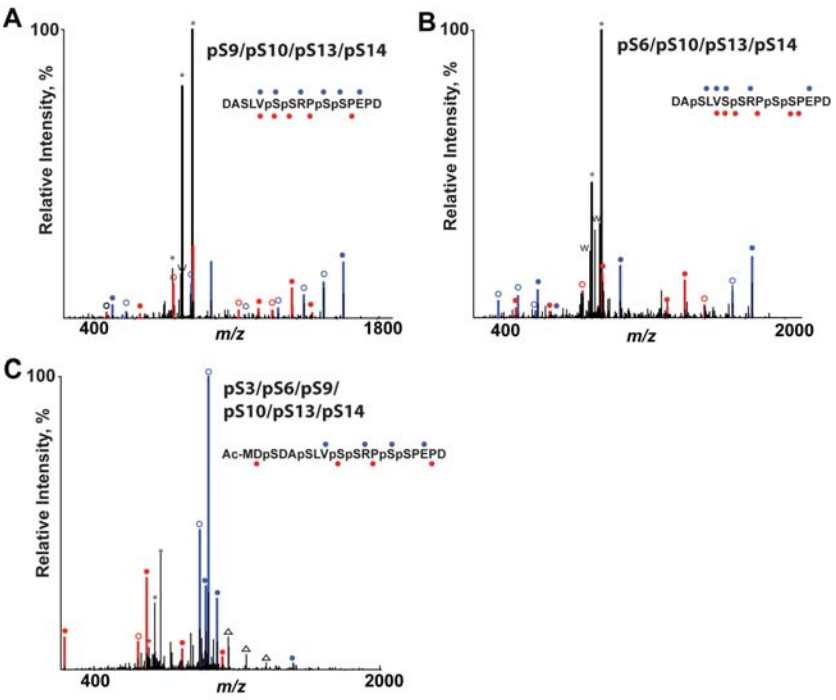

Figure S2. Mass spectrometric analyses show that additional serines in Olig2 N-terminus are phosphorylated, Related to Figure 4.

ESI-MS/MS spectra corresponding to Olig2-pS9/pS10/pS13/pS14 (A), Olig2-pS6/pS10/pS13/pS14 (B), and Olig2-pS3/pS6/pS9/pS10/pS13/pS14 (C) were recorded during analysis of NPC derived Olig2 phosphopeptides and reveal hyperphosphorylation of the protein N-terminus (pS, phosphoserine). Fragment ions containing the original N-terminus (b-type) or C-terminus (y-type) are shown. Lons of type b and y are shown in blue and red, respectively, and marked by filled circles, while those corresponding to phosphate losses are labeled with unfilled circles. Evidence for observed b- and y- type ions are depicted by circles above, and below the sequence, respectively. *, loss of one or more phosphate moieties from precursor; w, loss of water from precursor or b/y ion; Δ, internal fragment.

Sites	Scansite	GPS2.1	KinasePhos2.0	NetPhorest
S10	GSK3	GSK3B	AKT1	ATM/ATR
		STE11	ATM	GSK3
		PDK1	GSK3	DNAPK
		RSK	Aurora	NEK1
			PKG	
			PLK1	
			RSK	
			STK4	
			CHK1	
			CK1	
S13		CAMK1/4	AKT1	PAK
		DYRK	ATM	PKA
		Aurora	GSK3	CLK
		RSK2/5	Aurora	DMPK
			PKG	TGFbR2
			PLK1	CK1
			MAPK	CK2
			RSK	
			STK4	
			CHK1	
			CK2	
S14		MAPKAPK	AKT1	RCK
		CHK	ATM	CLK
		CK1	GSK3	MAPK
		DYRK	Aurora	CDK
		GSK3	IKK	
		IRAK	PKG	
		IKK	CAMK	
		PLK	PLK1	
		CDK	MAPK	
		MAPK(ERK, JNK, P38)	RSK	
		FRAP	STK4	
			CHK1	
			CK2	

Table S1. Protein kinases candidates for S10, S13 and S14 sites predicted by computer algorithm, Related to Figures 1, 2 and 3.
Kinases highlighted in green, the kinase candidates that have been tested; kinases highlighted in red, the kinase candidates that have been verified for the corresponding site.

Name	Consensus Motif	Inhibitors	Putative Candidates for Olig2 Kinase
Erk1/2	PXS/TP	PD98059 (MEK1/2)	No
JNK	(P)XS/TP	SP600125	No
		JNK-IN-8	No
p38MAPK	PXS/TP	SB203580	No
		SB202190	No
CDKs	S/TPX(R/K)	PD0332991 (CDK4/6)	No
		AZD5438 (CDK1/2/9)	Yes
		R547 (CDK1/2/4)	Yes
		CVT313 (CDK1/2)	Yes
		Roscovitine (CDK2/5)	N.A.
		BS-181 (CDK7)	No
PKA	RRXS/TZ	K-252a	N.A.
		H-89	N.A.
CKI	pSXXS/T	IC261	N.A.
CKII	S/TXXD/E/pS or S/TD/E/pS	CX-4954	Yes
PLK	D/EXS/TZ(XD/E)	BI-2536	N.A.
DNA-PK	S/TQ	NU-7441	No
DYRK	RPXS/TP	Harmine	No
GSK3	S/TXXXpS/T	CHIR-99021	Yes
		SB216763	Yes
		Lithium	Yes
Aurora	R/K/NRXS/TB	VX-680	No

Table S2. A summary of kinase inhibitor screen for Olig2 kinase, Related to Figures 1, 2 and 3.

S/T in red, the target Serine/Threonine of the kinase; X, any amino acid; pS, phosphorylated serine; Z, a hydrophobic residue; B, any hydrophobic residue with the exception of Proline. N.A., kinase inhibitor reduces both Olig2 and P-Olig2 level, and does not show specific effect on P-Olig2.

Name	Inhibitors	CNS MPO Score
CDKs	AZD5438	5.8
	R547	3.9
	CVT313	4.3
CK2	CX-4945	5.3
GSK3	CHIR-99021	2.1
	SB216763	3.5

Table S3. **Central nervous system multiparameter optimization (CNS MPO) algorithm predicts potential for successful drug development for Olig2 kinase inhibitors, Related to Figure 7.**
The scale is 0-6, and higher score implies higher probability of successful clinical potential of the compound.