SUPPLEMENTARY INFORMATION

Phosphorylation at distinct subcellular locations underlies specificity in mTORC2 activation of SGK1 and Akt

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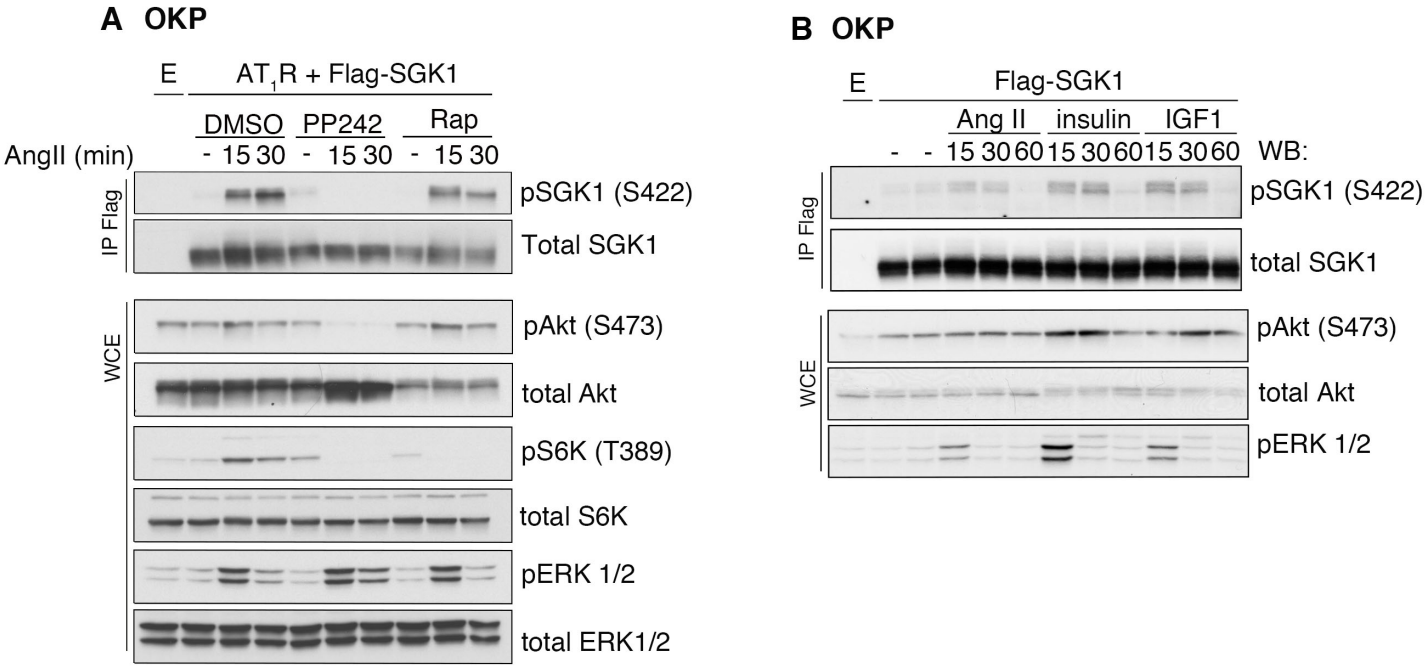


Figure S1, related to Figure 1. AngII stimulates mTORC2-dependent SGK1 S422 phosphorylation in OKP proximal tubule cells.
(A) Western blot of immunoprecipitates and WCE derived from AT1R and Flag-SGK1-transfected OKP cells, serum-starved overnight and then stimulated with 200 nM AngII for the indicated times. E, empty vector.
(B) Western blot of immunoprecipitates and WCE derived from Flag-SGK1 transfected OKP cells, serum-starved overnight and then stimulated with either 400 nM AngII, 200 nM insulin or 50 ng/ml IGF1 for the indicated times. E, empty vector.
(A and B, 3 biological replicates)

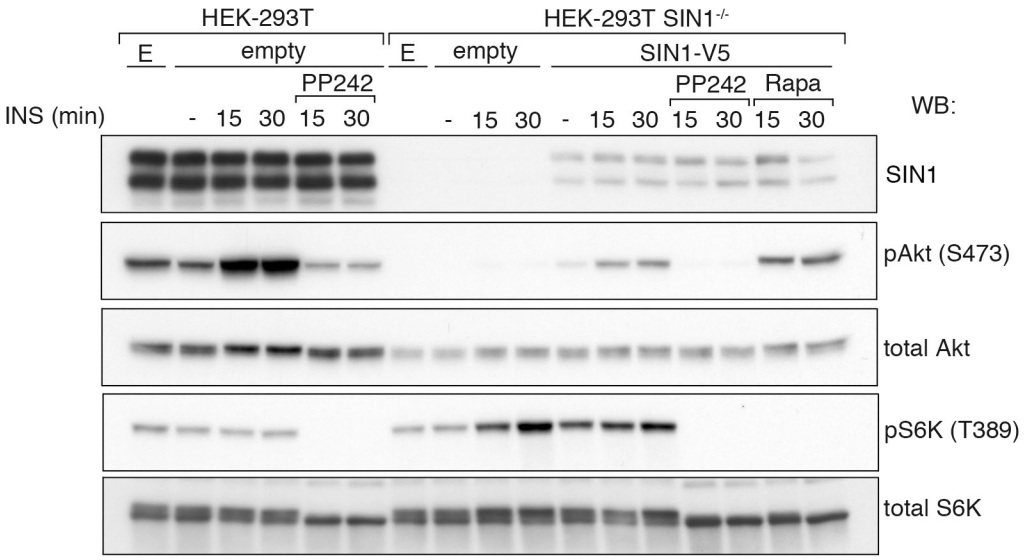


Figure S2, related to Figure 1. Characterization of HEK-293T SIN1$^{-/-}$ cells. Western blot analysis of WCE derived from either HEK-293T cells transfected with empty vector or HEK-293T SIN1$^{-/-}$ cells transfected with empty vector or WT SIN1-V5 as indicated (E, empty vector). Cells were serum-starved overnight before treatment with 200 nM insulin. Where indicated,300 nM PP242 or 25 nM rapamycin were added. (n=3 biological replicates).

A. Tryptic digests (83.7% coverage)

1 **Acc. #:** Q8BKH7 **Uniprot ID:** SIN1_MOUSE **Species:** MOUSE **Name:** Target of Rapamycin Complex 2 subunit MAPKAP1
Organism: Mus musculus **Gene:** Mapkap1 **Existence:** Evidence at protein level **Version:** 1
Protein MW: 59009.2 **Protein pI:** 7.2 **Protein Length:** 522 **Index:** 416897

```
1   MAFLDNPTII LAHIRQSHVT SDDTGMCEMV LIDHDVDLEK THPPSVPGDS GSEVQGSSGE TQGYIYAQSV DITSSWDFGI
81  RRRSNTAQRL ERLRKERQNQ IKCKNIQWKE RNSKQSAQEL KSLFEKKSLK EKPPSSGKQS ILSVRLEQCP LQLNNPFNEY
161 SKFDGKGHVG TTATKKIDVY LPLHSSQDRL LPMTVVTMAS ARVQDLIGLI CWQYTSEGRE PKLNDNVSAY CLHIAEDDGE
241 VDTDFPPLDS NEPIHKFGFS TLALVEKYSS PGLTSKESLF VRINAAHGFS LIQVDNTKVT MKEILLKAVK RRKGSQKISG
321 PQYRLEKQSE PNIAVDLEST LESQNAWEFC LVRENSSRAD GVFEEDSQID IATVQDMLSS HHYKSFKVSM IHRLRFTTDV
401 QLGISGDKVE IDPVTNQKAS TKFWIKQKPI SIDCDLLCAC DLAEEKSPSH AVFKLTYLSS HDYKHLYFES DAATVSEIVL
481 KVNYILESRA STARADYLAQ KQRKLNRRTS FSFQKEKKSG QQ
```

B. GluC digests (63.2% coverage).

Acc. #: Q8BKH7 **Uniprot ID:** SIN1_MOUSE **Species:** MOUSE **Name:** Target of Rapamycin Complex 2 subunit MAPKAP1
Organism: Mus musculus **Gene:** Mapkap1 **Existence:** Evidence at protein level **Version:** 1
Protein MW: 59009.2 **Protein pI:** 7.2 **Protein Length:** 522 **Index:** 416897

```
1   MAFLDNPTII LAHIRQSHVT SDDTGMCEMV LIDHDVDLEK THPPSVPGDS GSEVQGSSGE TQGYIYAQSV DITSSWDFGI
81  RRRSNTAQRL ERLRKERQNQ IKCKNIQWKE RNSKQSAQEL KSLFEKKSLK EKPPSSGKQS ILSVRLEQCP LQLNNPFNEY
161 SKFDGKGHVG TTATKKIDVY LPLHSSQDRL LPMTVVTMAS ARVQDLIGLI CWQYTSEGRE PKLNDNVSAY CLHIAEDDGE
241 VDTDFPPLDS NEPIHKFGFS TLALVEKYSS PGLTSKESLF VRINAAHGFS LIQVDNTKVT MKEILLKAVK RRKGSQKISG
321 PQYRLEKQSE PNIAVDLEST LESQNAWEFC LVRENSSRAD GVFEEDSQID IATVQDMLSS HHYKSFKVSM IHRLRFTTDV
401 QLGISGDKVE IDPVTNQKAS TKFWIKQKPI SIDCDLLCAC DLAEEKSPSH AVFKLTYLSS HDYKHLYFES DAATVSEIVL
481 KVNYILESRA STARADYLAQ KQRKLNRRTS FSFQKEKKSG QQ
```

Figure S3, related to Figure 5. Sequence coverage maps of the MS analysis of SIN1. A) Tryptic digestion; B) GluC digestion. Peptides identified in the MS analysis of the enzymatic digestions are shown in red. Unambiguous phosphorylation sites are highlighted in dark blue. Ambiguous assignments are shown in green.

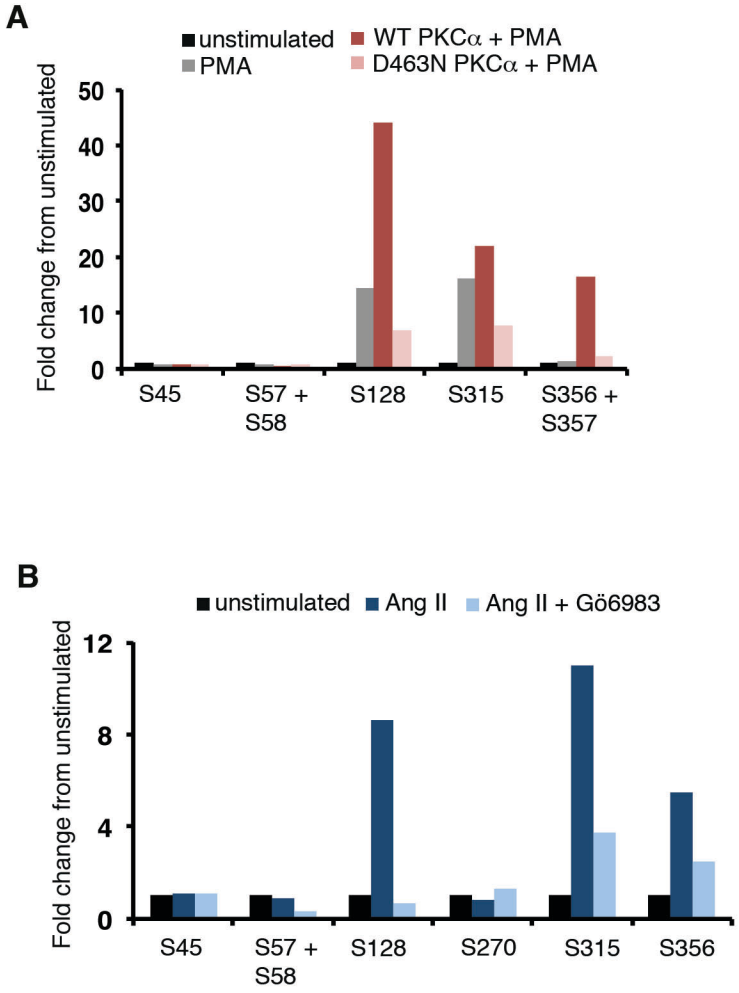
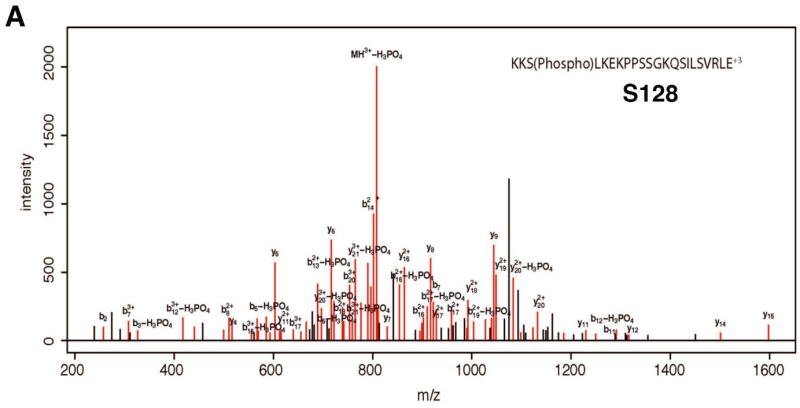


Figure S4, related to Figure 5. Identification of SIN1 phosphorylation sites by LC-MS/MS phospho-site mapping.

(A) Relative quantification of phosphopeptide abundance. HEK-293 cells were transfected with empty vector, SIN1-Flag, and either WT PKCα or PKCαD463N. Cells were left unstimulated or stimulated with 1 mM PMA as indicated for 15 min.

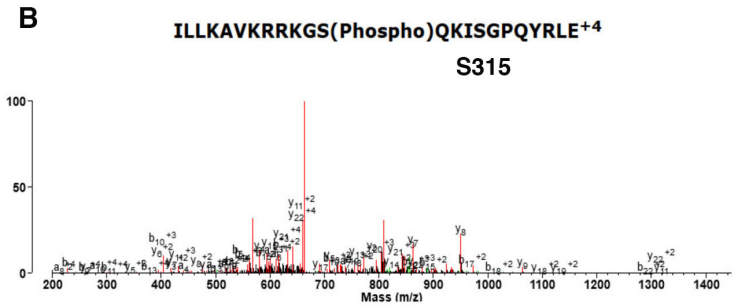
(B), HEK-293-AT_1R cells were transfected with empty vector or SIN1-Flag followed by stimulation with 200 nM Ang II either in the presence of DMSO (vehicle) or the PKC inhibitor, Gö6983 (5 mM) for 60 min.

Both A and B show MS results for GluC SIN1-flag digestion. Graphs depict fold change in phosphopeptide abundance from the unstimulated condition for one representative experiment. (A and B, 3 biological replicates)



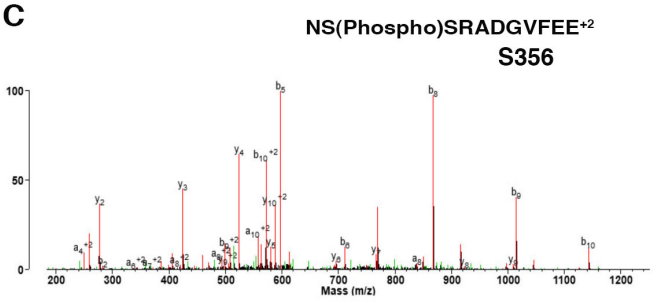
Main Sequence Ions

b-H_3PO_4	b-$H_3PO_4^{+2}$	b	b^{+2}				y	y^{+2}	y-H_3PO_4	y-$H_3PO_4^{+2}$
---	---	---	---	1	K	22	---	---	---	---
---	---	257.1972	*129.1022*	2	K	21	*2391.3007*	1196.1540	*2293.3238*	1147.1655
326.2187	*163.6130*	424.1956	*212.6014*	3	S(Phospho)	20	*2263.2057*	1132.1065	*2165.2288*	1083.1181
439.3027	*220.1550*	537.2796	269.1435	4	L	19	*2096.2074*	1048.6073	---	---
567.3977	284.2025	665.3746	333.1909	5	K	18	1983.1233	992.0653	---	---
696.4403	348.7238	794.4172	397.7122	6	E	17	1855.0284	928.0178	---	---
824.5352	412.7713	922.5121	461.7597	7	K	16	1725.9858	863.4965	---	---
921.5880	461.2976	1019.5649	510.2861	8	P	15	1597.8908	799.4490	---	---
1018.6408	509.8240	1116.6177	558.8125	9	P	14	1500.8380	750.9227	---	---
1105.6728	553.3400	1203.6497	602.3285	10	S	13	1403.7853	702.3963	---	---
1192.7048	596.8561	1290.6817	645.8445	11	S	12	1316.7532	658.8803	---	---
1249.7263	625.3668	1347.7032	674.3552	12	G	11	1229.7212	615.3642	---	---
1377.8213	689.4143	1475.7982	738.4027	13	K	10	1172.6997	586.8535	---	---
1505.8798	753.4436	1603.8567	802.4320	14	Q	9	1044.6048	522.8060	---	---
1592.9119	796.9596	1690.8888	845.9480	15	S	8	916.5462	458.7767	---	---
1705.9959	853.5016	1803.9728	902.4901	16	I	7	829.5142	415.2607	---	---
1819.0800	910.0436	1917.0569	959.0321	17	L	6	716.4301	358.7187	---	---
1906.1120	953.5596	*2004.0889*	1002.5481	18	S	5	603.3461	302.1767	---	---
2005.1804	1003.0939	*2103.1573*	1052.0823	19	V	4	516.3140	258.6606	---	---
2161.2815	1081.1444	*2259.2584*	1130.1329	20	R	3	417.2456	*209.1264*	---	---
2274.3656	1137.6864	*2372.3425*	1186.6749	21	L	2	261.1445	---	---	---
---	---	---	---	22	E	1	*148.0604*	---	---	---



Main Sequence Ions

b-H_3PO_4	b-$H_3PO_4^{+2}$	b	b^{+2}				y	y^{+2}	y-H_3PO_4	y-$H_3PO_4^{+2}$
---	---	---	---	1	I	23	---	---	---	---
---	---	227.1754	---	2	L	22	*2635.4920*	1318.2496	*2537.5151*	1269.2612
---	---	340.2595	---	3	L	21	*2522.4079*	1261.7076	*2424.4310*	1212.7191
---	---	468.3544	234.6809	4	K	20	*2409.3238*	1205.1656	*2311.3469*	1156.1771
---	---	539.3915	270.1994	5	A	19	*2281.2289*	1141.1181	*2183.2520*	1092.1296
---	---	638.4600	319.7336	6	V	18	*2210.1918*	1105.5995	*2112.2149*	1056.6111
---	---	766.5549	383.7811	7	K	17	*2111.1233*	1056.0653	*2013.1465*	1007.0769
---	---	922.6560	461.8317	8	R	16	*1983.0284*	992.0178	*1885.0515*	943.0294
---	---	1078.7571	539.8822	9	R	15	*1826.9273*	913.9673	*1728.9504*	864.9788
---	---	1206.8521	603.9297	10	K	14	*1670.8262*	835.9167	*1572.8493*	786.9283
---	---	1263.8736	632.4404	11	G	13	*1542.7312*	771.8692	1444.7543	722.8808
1332.8950	666.9512	1430.8719	715.9396	12	S(Phospho)	12	*1485.7097*	743.3585	1387.7328	694.3701
1460.9536	730.9804	*1558.9305*	779.9689	13	Q	11	1318.7114	659.8593	---	---
1589.0486	795.0279	*1687.0255*	844.0164	14	K	10	1190.6528	595.8300	---	---
1702.1326	851.5700	*1800.1095*	900.5584	15	I	9	1062.5578	531.7826	---	---
1789.1647	895.0860	*1887.1416*	944.0744	16	S	8	949.4738	475.2405	---	---
1846.1861	923.5967	*1944.1630*	972.5852	17	G	7	862.4417	431.7245	---	---
1943.2389	972.1231	*2041.2158*	1021.1115	18	P	6	805.4203	403.2138	---	---
2071.2975	1036.1524	*2169.2744*	1085.1408	19	Q	5	708.3675	354.6874	---	---
2234.3608	1117.6840	*2332.3377*	1166.6725	20	Y	4	580.3089	290.6581	---	---
2390.4619	1195.7346	*2488.4388*	1244.7230	21	R	3	417.2456	209.1264	---	---
2503.5460	1252.2766	*2601.5229*	1301.2651	22	L	2	261.1445	---	---	---
---	---	---	---	23	E	1	*148.0604*	---	---	---



Main Sequence Ions

b-H_3PO_4	b-$H_3PO_4^{+2}$	b	b^{+2}				y	y^{+2}	y-H_3PO_4	y-$H_3PO_4^{+2}$
---	---	---	---	1	N	11	---	---	---	---
184.0717	---	282.0486	---	2	S(Phospho)	10	1176.4569	588.7321	1078.4800	539.7436
271.1037	---	369.0806	---	3	S	9	1009.4585	505.2329	---	---
427.2048	214.1060	525.1817	263.0945	4	R	8	922.4265	461.7169	---	---
498.2419	249.6246	596.2188	298.6130	5	A	7	766.3254	---	---	---
613.2689	307.1381	711.2458	356.1265	6	D	6	695.2883	---	---	---
670.2903	335.6488	768.2672	384.6372	7	G	5	580.2613	---	---	---
769.3587	385.1830	867.3356	434.1715	8	V	4	523.2399	---	---	---
916.4272	458.7172	1014.4041	507.7057	9	F	3	424.1714	---	---	---
1045.4697	523.2385	1143.4466	572.2270	10	E	2	277.1030	---	---	---
---	---	---	---	11	E	1	*148.0604*	---	---	---

Figure S5, related to Figure 5. Annotated LC-MS/MS identifying Ang II-sensitive phosphorylation sites.
A) S128; B) S315; C) S356. Observed *b* and *y* ions are shown in red in the ion spectra plots and tables.

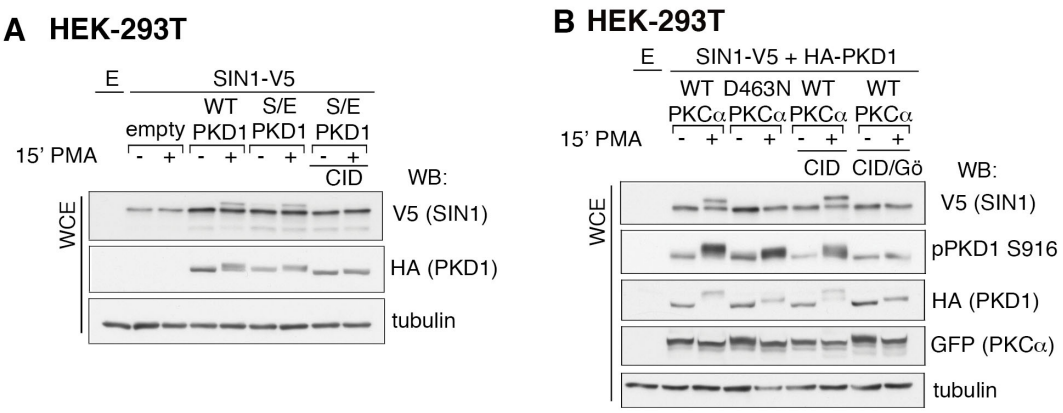


Figure S6, related to Figure 5. PKD activation induces phosphorylation of SIN1.
(A) WB analysis of WCE derived from HEK-293T cells transfected with empty vector, WT PKD1 or constitutively active PKD1 (S738/742E) and WT SIN1-V5. Cells were serum-starved overnight before stimulation with 1 μM PMA either in the presence of vehicle (DMSO) or 25 μM CID655763 (CID).E, empty vector.
(B) WB analysis of WCE derived from HEK-293T cells transfected with WT SIN1-V5 and HA-PKD1 and either WT PKCα or PKCαD463N. Cells were stimulated with PMA in the presence of the indicated inibitors (5 μM Gö6976 (Gö), 25 μM CID655763 (CID)) as described in 'B'. E, empty vector.
(A and B, 2 biological replicates)

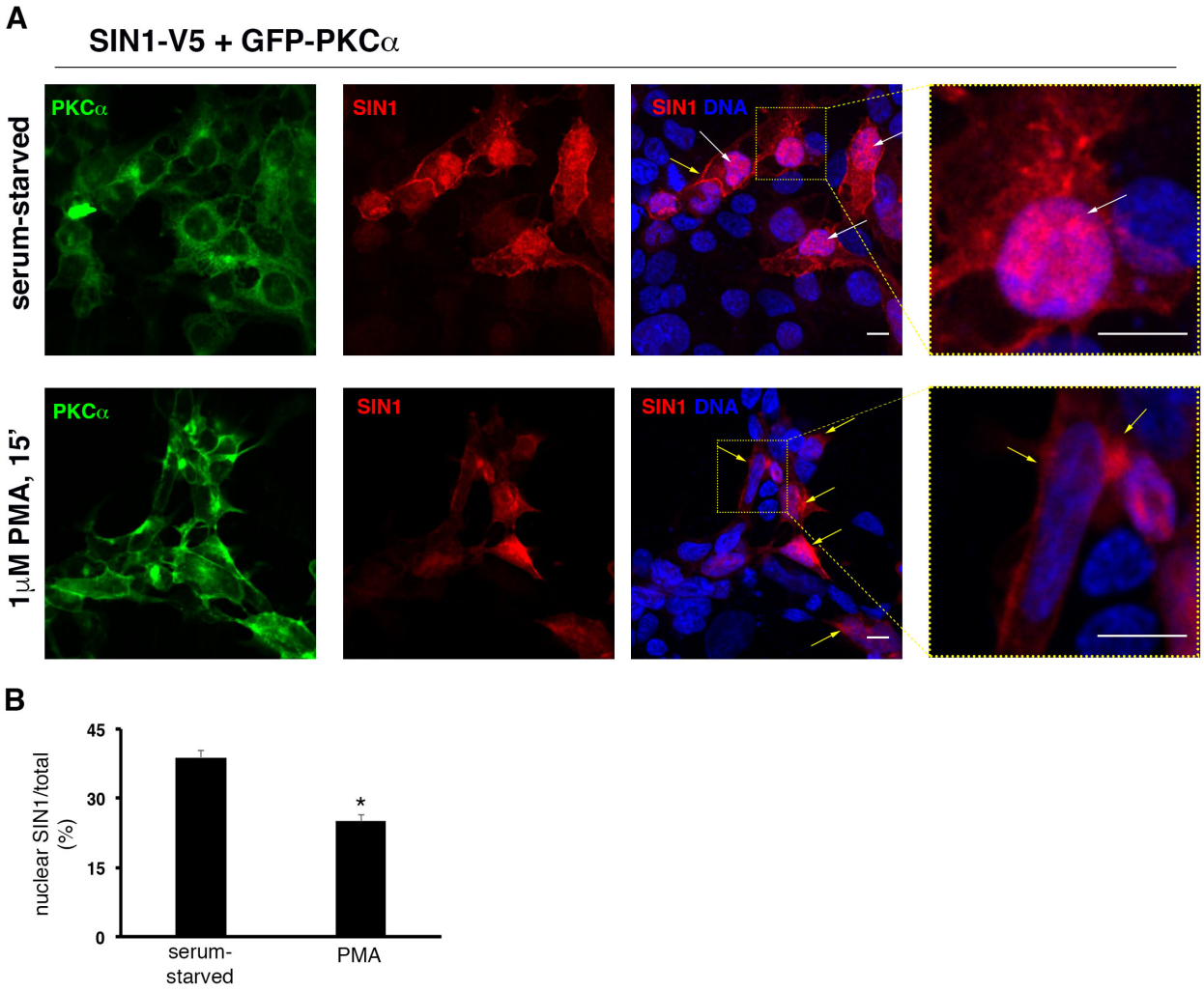


Figure S7, related to Figure 7. PKCα activation relocates SIN1 from the nucleas to a perinuclear compartment and plasma membrane.
(A) SIN1$^{-/-}$ HEK-293T cells expressing WT GFP-PKCα and WT SIN1-V5 were serum-starved overnight and then left unstimulated or stimulated with 1 μM PMA for 15 min. Cells were then processed in a immunofluorescence assay to detect SIN1, costained with DAPI to detect nuclei and imaged by confocal microscopy. White arrows indicate nuclear localized SIN1 and yellow arrows indicate plasma membrane and peri-nuclear localized SIN1. Scale Bar, 10 μM.
(B) Quantification of nuclear SIN1 localization as a percantage of total SIN1. Graph represents mean +/- SEM *$p < 0.001$. (n= 2 biological replicates; serum-starved n=32 cells; PMA-treated, n=53 cells)

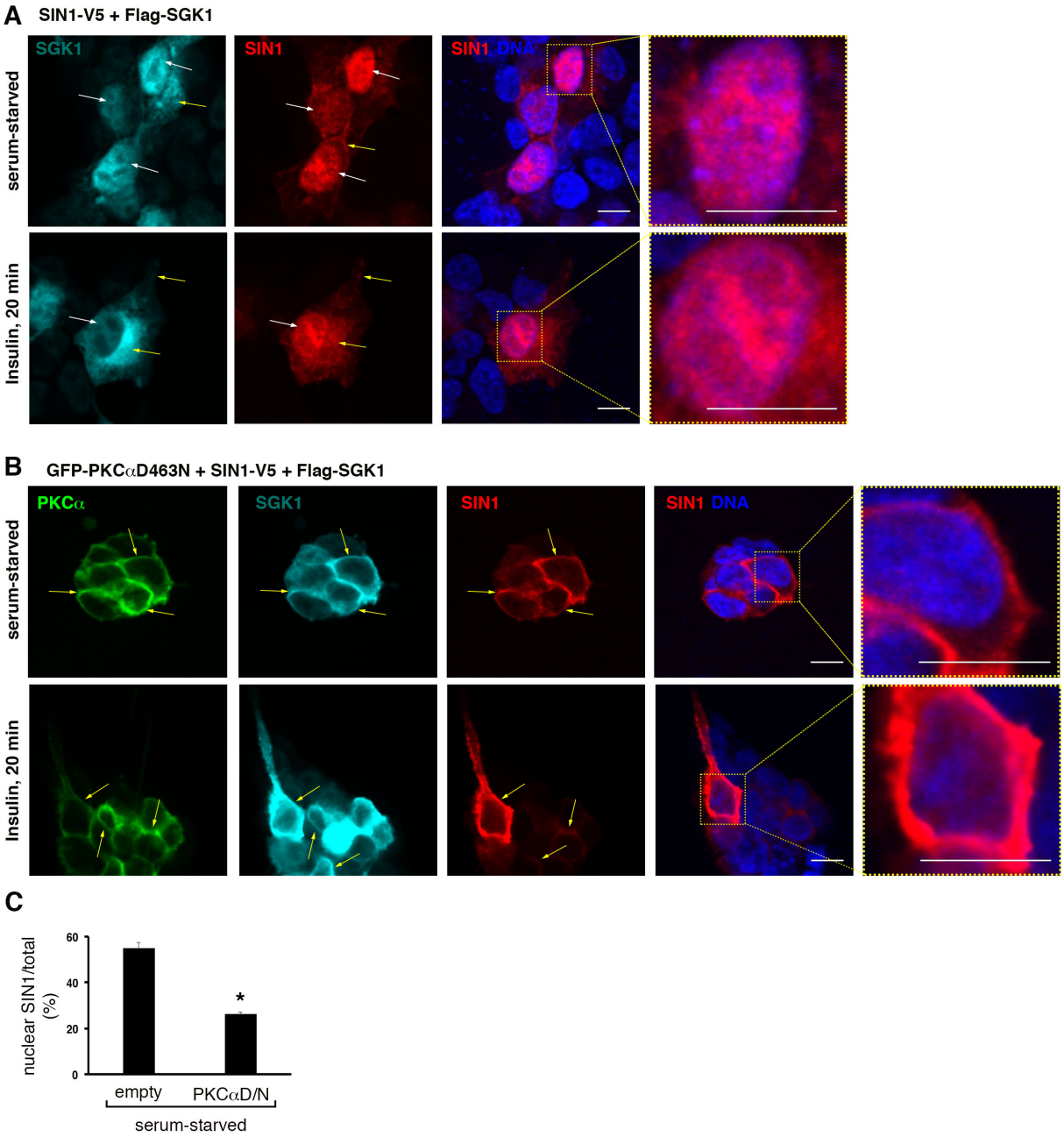


Figure S8, related to Figure 7. PKCαD463N relocates SIN1 and SGK1 from the nucleus and peri-nuclear compartment to the plasma membrane in the absence of hormonal stimulation. SIN1$^{-/-}$ HEK-293T cells expressing WT SIN1-V5, Flag-SGK1 and either empty vector (A) or GFP-PKCαD463N (PKCαD/N) (B) were serum-starved over night and then left unstimulated or stimulated with 200nM insulin for 20 min. Cells were then processed in an immunofluorescence assay to detect SIN1 or SGK1, costained with DAPI to detect nuclei and imaged by confocal microscopy. White arrows indicate nuclear localized SIN1 and SGK1 and yellow arrows indicate plasma membrane and peri-nuclear staining. (n=4 biological replicates for empty vs PKCDN, serum-starved; n=3 biological replicates for empty vs PKCDN, insulin stimulated) (C) Quantification of nuclear localized SIN1 as a percentage of total in serum-starved cells. Graph represents the mean +/- SEM. *$p < 0.0001$ (WT serum-starved, n=38 cells; PKCαD/N serum-starved, n=54 cells).

Table S1. List of phosphopeptides identified in SIN1 by LC-MS/MS (tryptic digestions)

Expectation value	Phosphopeptide	Phosphorylation	*m/z*	Charge
5.30E-05	M(Met-loss+Acetyl)AFLDNPT*IILAHIR	T8	858.4505	2+
6.60E-04	SLFEKKS*LK	S128	580.3101	2+
9.20E-03	EKPPS*SGKQSILSVR	135I136 (ambiguous)	846.9397	2+
6.00E-05	FGFS*TLALVEK	S260	646.3203	2+
1.20E-04	YSS*PGLTSK	S270	510.2264	2+
4.60E-05	YSSPGLTS*KESLFVR	274I275 (ambiguous)	875.9246	2+
3.30E-04	YSSPGLTSKES*LFVR	S278	875.9249	2+
3.10E-05	INAAHGFS*LIQVDNTK	S290	904.4411	2+
1.80E-05	INAAHGFS*LIQVDNTKVTMK	S290	756.3809	3+
1.10E-03	KGS*QKIS*GPQYR	S315	754.8399	2+
5.70E-04	GSQKIS*GPQYR	S319	650.8057	2+
1.10E-03	KGS*QKIS*GPQYR	S315 and S319	754.8399	2+
1.70E-04	ENSS*RADGVFEEDSQIDIATVQDM+LSSHHYK	356I357 (ambiguous)	901.8951	4+
3.00E-03	ENSS*RADGVFEEDSQIDIATVQDMLSSHHYK	356I357 (ambiguous)	897.8961	4+
1.70E-03	ADGVFEEDS*QIDIATVQDMLSSHHYK	S367	1005.7702	3+
2.60E-03	RTS*FSFQK	S510	540.7474	2+

S* or T* denotes phosphorylated serine or threonine. M+ denotes oxidized methionine.

Table S2. List of phosphopeptides identified in SIN1 by LS-MS/MS (GluC digestions)

Expectation value	Phosphopeptide	Phosphorylation	*m/z*	Charge
1.80E-05	KTHPPS*VPGDSGSE	S45	737.8126	2+
1.30E-04	KTHPPSVPGDSGSEVQGS*S*GE	S57;S58	1099.9342	2+
2.30E-04	KKS*LKEKPPSSGKQSILSVRLE	S128	840.4711	3+
1.60E-03	KYSS*PGLTSKE	S270	638.7895	2+
2.60E-03	ILLKAVKRRKGS*QKISGPQYRLE	S315	687.9012	4+
1.10E-03	NS*SRADGVFEE	S356	645.7535	2+

S* or T* denotes phosphorylated serine or threonine.