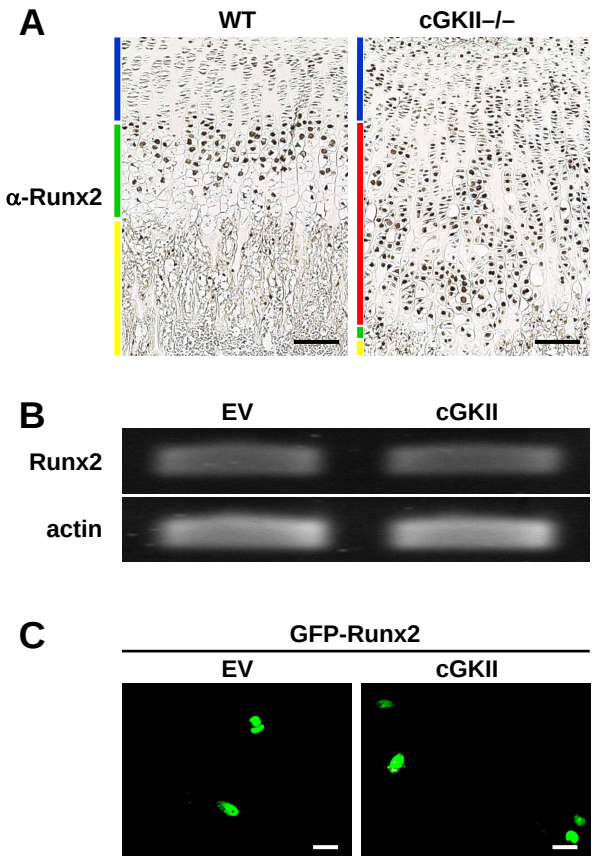
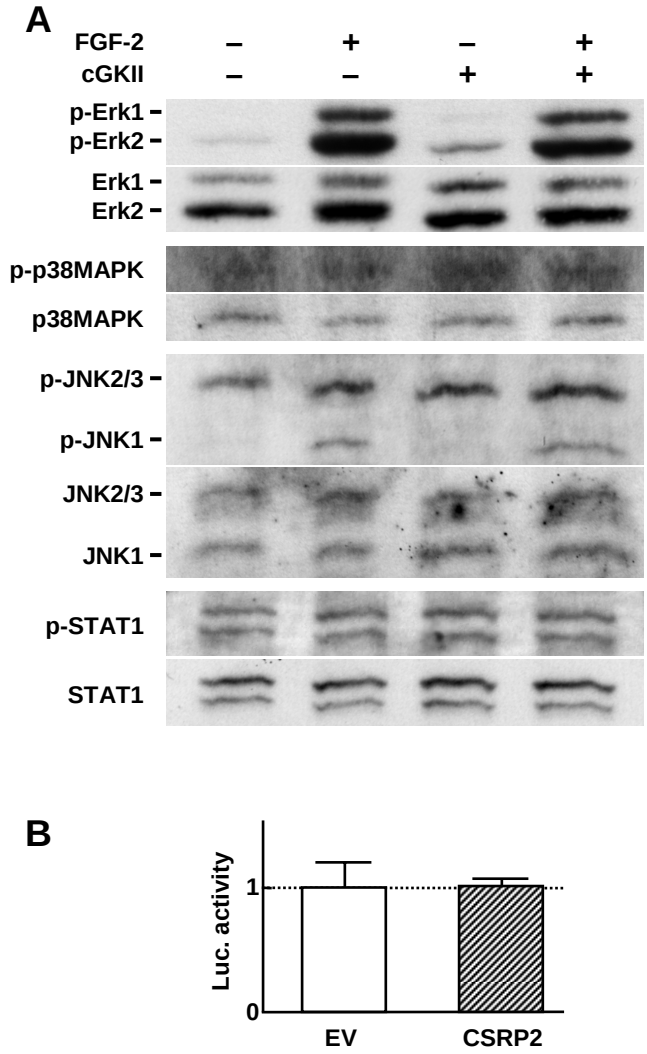
Supplemental Table 1. Screening of phosphorylation targets of cGKII using a serine/threonin kinase substrate array

Peptide target	Phospho specific antibody	Positive	Negative	cGKII	%
Caspase9 (Ser196)	p-(Ser) Arg-X-Tyr/Phe-X-pSer Motif Antibody	2.816	0.46	3.209	109.0
Bad (Ser112)	p-Bad (Ser112) Antibody	3.108	-0.365	3.359	107.2
PLK (Ser137)	p-(Ser) PKC Substrate Antibody	3.126	-0.088	3.112	99.6
p90RSK (Ser380)	p-p90RSK (Ser380)	2.977	-0.379	2.917	98.2
p90RSK (Ser380)	p-(Ser/Thr) Phe Antibody	3.112	-0.298	3.051	98.2
Bad (Ser155)	p-Bad (Ser155) Antibody	3.192	-0.388	3.054	96.1
eNOS (Ser1177)	p-eNOS (Ser1177) Antibody	3.219	-0.334	3.301	94.7
GSK-3β (Ser9)	p-GSK-3β (Ser9) Antibody	2.351	1.161	2.248	91.3
PLK (Ser137)	p-PLK (Ser137) Antibody	3.289	2.205	3.109	83.4
VASP (Ser239)	p-VASP (Ser239) Antibody	3.346	-0.307	2.706	82.5
cdc25 (Ser216)	p-(Ser) 14-3-3 Binding Motif Antibody	3.014	1.942	2.809	80.8
p38MAPK (Thr180/Tyr182)	p-p38 MAPK (Thr180/Tyr182) Antibody	3.028	0.241	2.376	76.6
MAPKAPK2 (Thr334)	p-Threonine (42H4) Monoclonal Antibody	3.028	0.241	2.376	76.6
GSK-3α/β (Ser21/9)	p-GSK-3α/β (Ser21/9) Antibody	2.005	-0.426	1.329	72.2
Chk2 (Thr68)	p-Threonine (42H4) Monoclonal Antibody	3.203	0.167	2.162	65.7
c-Abl (Thr735)	p-Threonine (42H4) Monoclonal Antibody	2.988	0.062	1.806	59.6
PTEN (Ser380)	p-PTEN (Ser380) Antibody	2.988	0.062	1.806	59.6
MSK1 (Ser376)	p-(Ser/Thr) Phe Antibody	3.202	-0.241	1.734	57.4
Akt (Thr308)	p-Threonine (42H4) Monoclonal Antibody	3.263	0.225	1.96	57.1
cdc25 (Ser216)	p-cdc25C (Ser216) Antibody	2.992	-0.073	1.606	54.8
Bad (Ser136)	p-(Ser) 14-3-3 Binding Motif Monoclonal Antibody	3.022	1.752	2.41	51.8
eIF4G (Ser1108)	p-eIF4G (Ser1108) Antibody	3.079	-0.391	1.085	42.5
SAPK/JNK (Thr183/Tyr185)	p-SAPK/JNK (Thr183/Tyr185) Monoclonal Antibody	3.106	1.119	1.945	41.5
HSP27 (Ser82)	p-HSP27 (Ser82) Antibody	3.233	2.181	2.574	37.4
VASP (Ser157)	p-(Ser/Thr) PKA Substrate Antibody	3.139	-0.361	0.635	28.5
p53 (Ser15)	p-(Ser/Thr) ATM/ATR Substrate Antibody	3.018	0.921	1.498	27.5
Stat3 (Ser727)	p-Stat3 (Ser727) Monoclonal Antibody	3.018	0.921	1.498	27.5
eIF4E (Ser209)	p-eIF4E (Ser209) Antibody	3.055	-0.188	0.599	24.3
PKCII (Ser633)	p-(Ser/Thr) PKA Substrate Antibody	3.016	-0.314	0.491	24.2
IkappaB-α (Ser32)	p-IkappaB-α (Ser32/36) Monoclonal Antibody	3.324	0.234	0.958	23.4
p90RSK (Thr573)	p-p90RSK (Thr573) Antibody	2.922	0.024	0.632	21.0
ATF2 (Thr71)	p-Threonine-Proline Monoclonal Antibody	2.922	0.024	0.632	21.0
p53 (Ser20)	p-p53 (Ser20) Antibody	3.188	2.55	2.645	14.9
MEK1/2 (Ser217/221)	p-MEK1/2 (Ser217/221) Antibody	3.039	-0.315	0.147	13.8
Phospho-(Thr) PDK1 Substrate	p-(Ser/Thr) Phe Antibody	3.35	-0.315	0.184	13.6
AMPK (Thr172)	p-AMPK-α (Thr172) Antibody	3.35	-0.315	0.184	13.6
SAPK/JNK (Thr183/Tyr185)	p-Threonine-Proline Monoclonal Antibody	3.499	1.102	1.396	12.3
CaMKII (Thr286)	p-(Ser/Thr) PKA Substrate Antibody	3.499	1.102	1.396	12.3
FKHR (Thr24) /FKHRL1 (Thr32)	p-FKHR (Thr24)/FKHRL1 (Thr32) Antibody	2.852	2.999	2.981	12.2
Chk2 (Thr68)	p-Chk2 (Thr68) Antibody	3.028	0.915	1.125	9.9
CEBPβ (Ser105)	p-CEBPβ (Ser105) Antibody	2.964	1.128	1.274	8.0
Ezrin (Thr567) /Radixin (Thr564) /Moesin (Thr558)	p-Ezrin (Thr567) /Radixin (Thr564) /Moesin (Thr558) Antibody	2.852	-0.238	-0.056	5.9
c-Abl (Thr735)	p-c-Abl (Thr735) Antibody	3.213	-0.363	-0.21	4.3
cdc2 (Thr161)	p-cdc2 (Thr161) Antibody	3.331	-0.372	-0.266	2.9
p44/42 MAPK (Thr202/Tyr204)	p-p44/42 MAPK (Thr202/Tyr204) Monoclonal Antibody	3.034	0.78	0.84	2.7
SAPK/JNK (Thr183/Tyr185)	p-SAPK/JNK (Thr183/Tyr185) Antibody	3.036	0.283	0.346	2.3
CaMKII (Thr286)	p-CaMKII (Thr286) Antibody	2.97	-0.308	-0.235	2.2
ATF2 (Thr71)	p-ATF2 (Thr71) Antibody	2.961	-0.227	-0.176	1.6
Cofilin (Ser3)	p-Cofilin (Ser3) Antibody	0.383	-0.343	-0.334	1.2

The details of this assay are described in the Supplemental Methods.
We considered the peptides whose phosphorylation level was more than 80% as the potential phosphorylation targets of cGKII.

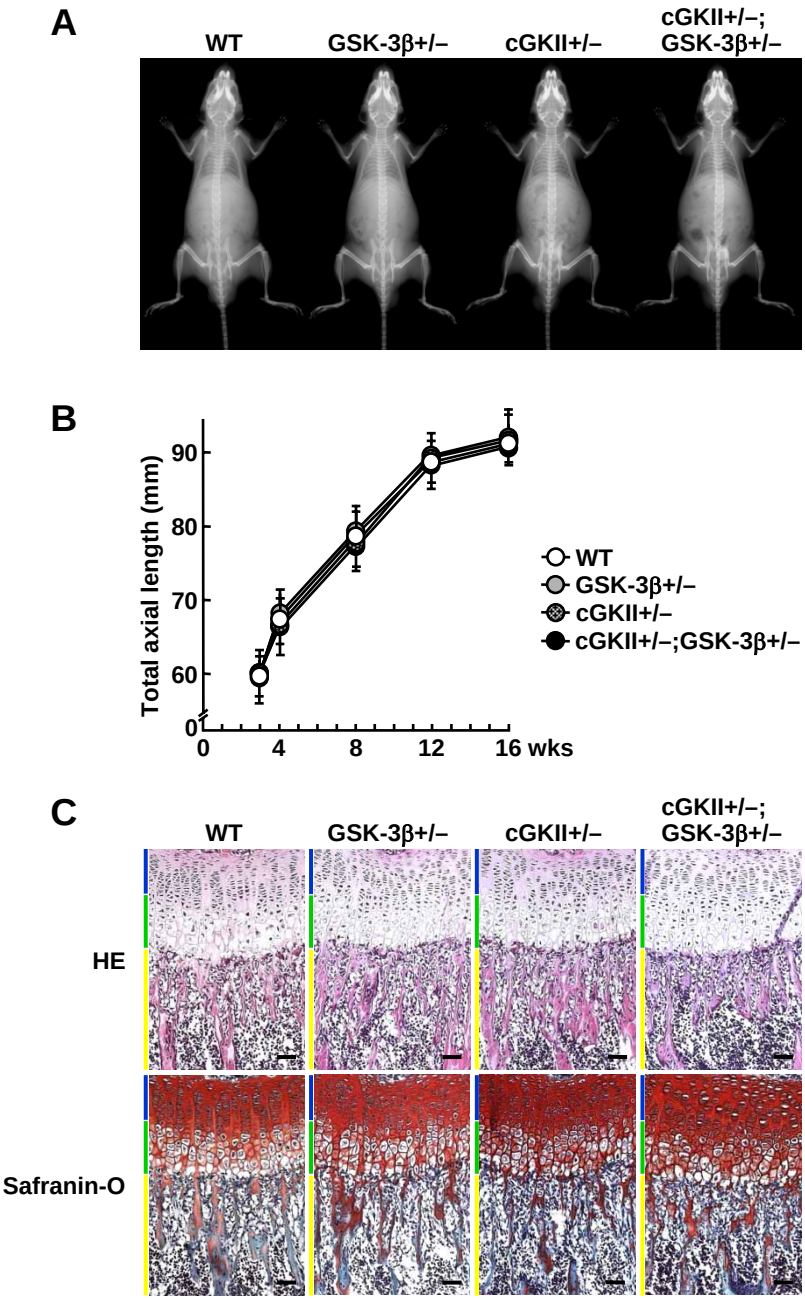


Supplemental Figure 1
Effect of cGKII on expression and subcellular localization of Runx2. (**A**) Localization of Runx2 by immunohistochemistry in the growth plates of the proximal tibias of WT and cGKII–/– mice at 2 weeks of age. Blue, red, green, and yellow bars indicate proliferative zone, abnormal intermediate layer, hypertrophic zone, and primary spongiosa, respectively. Scale bars, 50 μm. (**B**) mRNA levels of Runx2 in cultured ATDC5 cells transfected with cGKII or the empty vector (EV) measured by RT-PCR analysis. (**C**) Subcellular localization of Runx2 shown by fluorescent images of HeLa cells transfected with GFP-tagged Runx2 (GFP-Runx2) in combination with cGKII or the empty vector (EV). Scale bars, 10 μm.



Supplemental Figure 2

Interaction of cGKII with MAPK/STAT signalings or CSRP2. (**A**) Immunoblotting for Erk1/2, p38MAPK, JNK2/3, JNK1, STAT1, and the phosphorylated proteins in ATDC5 cells transfected with cGKII or the empty vector (–) in the presence or absence of FGF-2. (**B**) COL10 promoter activity by transfection of CSRP2 or the empty vector (EV) in HuH-7 cells with the luciferase reporter gene construct containing a 4.5 kb promoter fragment of COL10. Data are expressed as means (bars) ± standard deviation (S.D.; error bars) of the fold change compared to EV.



Supplemental Figure 3

Skeletal phenotypes of GSK-3β +/–, cGKII+/–, and the compound hetero-knockout (cGKII+/–;GSK-3β +/–) littermates. (**A**) Radiographs of WT and the three genotypes of littermates at 8 weeks of age. (**B**) Growth curves of mice of the four genotypes from 3 to 16 weeks postnatal determined by the total axial length (from nose to tail end). Data are expressed as means (symbols) ± S.D. (error bars) for 4-9 mice/genotype. No significant difference among the genotypes. (**C**) HE and Safranin-O stainings of the tibial growth plates of 3-week-old mice of the four genotypes. Blue, green, and yellow bars indicate proliferative zone, hypertrophic zone, and primary spongiosa, respectively. None of the genotypes showed the abnormal intermediate layer. Scale bars, 50 μm.

Supplemental Methods

Screening of phosphorylation targets of cGKII using a serine/threonin kinase substrate array. Screening of the phosphorylation targets of cGKII was performed using a serine/threonin kinase substrate screening kit (#7400, Cell Signaling). The kit provided 87 nonphospho-peptides of diverse sequences as kinase substrates. Also included were phospho and nonphospho-peptides of identical sequences as positive and negative controls, respectively. Potential substrate phosphorylation was detected by the corresponding phospho-specific antibodies. Ten μM biotinylated nonphospho-peptide substrates were combined with 200 μM ATP in the presence of the 100 nM cGKII recombinant protein (Sigma, St. Louis, MO) and were incubated at 30°C for 30 min. Each reaction was transferred to a 96-well streptavidin-coated plate and incubated at room temperature for 30 min. Phospho-specific antibody was added to matched wells of the plate and was incubated at room temperature for 60 min. HRP-conjugated IgG antibody (Cell Signaling) was added and was incubated at 37°C for 30 min. TMB substrate (BioFX laboratories, Owing Mills, MD) was used for color development. Absorbance at 405 nm was read on an ELISA plate reader MTP-300 (CORONA Electric, Ibaraki, Japan). The phosphorylation level of the each peptide substrate (Y%) was calculated as follows:

$Y = (C-A) / (B-A) \times 100$

A = the absorbance of negative control

B = the absorbance of positive control

C = the absorbance of the candidate peptide