Supplemental data

Material and methods

Flow cytometry

Mouse ES cells ($1x10^6$ cells in 6 well plates) were transfected with shRNA or overexpression plasmids. On the 4^{th} day after selection with puromycin or zeocin, cells suspension in medium and adherent on wells were harvested and fixed with 70% ethanol for 30 minutes. Then these fixed cells were washed with PBS and treated with propidium iodide (20 μg/ml) and RNase A (0.2 mg/ml) for 30 minutes. The ratio of sub-G1 was determined and analyzed by CellQuest software (Becton Dickinson).

Quantification of Alkaline phosphatase (ALP) activity

Mouse ES cells incubated with 10% Alamar blue for Alamar blue assay were washed with PBS three times and fixed with 4% formaldehyde for 10 minutes. After fixation, cells were re-washed with PBS three times and incubated with the ALP substrate, p-Nitrophenyl phosphate (#N-7653; Sigma-Aldrich), within 10 minutes. Then the ALP activities were measured at OD405 in the substrate solution. To obtain the ALP/AB (alkaline phosphatase activity/Alamar blue activity) value, the ALP activities acquired were divided by the Alamar blue values.

761 ## Figure Legends

762 **Figure S1. Down-regulation of DLK enhances mouse ES cell renewal**

763 (A) Apoptosis of cells was slightly decreased in sh-DLK mouse ES cells compared to
764 sh-Control mouse ES cells. Sub-G1 level of D3 mouse ES cells transfected with
765 sh-Control, sh-DLK-1 or sh-DLK-2 was analyzed on 4^{th} day after transfection. (B)
766 Relative cell numbers increased in sh-DLK transfectants in R1 ES cells. Alamar blue
767 assays were performed. (C) The transfection of two independent sh-DLKs increased
768 expression of Nanog protein in R1 mouse ES cells. Expression of self-renewal
769 markers, Nanog and Oct4 proteins in mouse ES cells transfected with sh-Control,
770 sh-DLK-1 or sh-DLK-2 were measured by Western blotting. The error bars in the
771 figures represent standard error of the mean (mean±SEM). *P* values were obtained
772 from two-tailed Student's *t*-tests (***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.05$).

773

774 **Figure S2. DLK Protein expression levels in mouse ES cells, EBs, MEFs and in**
775 **the withdrawal of LIF.**

776 (A) DLK protein expression was upregulated in mouse EBs compared to mouse ES
777 cells and MEFs. DLK protein was detected in wild type D3 mouse ES cells, MEFs
778 and mouse EBs (5^{th}, 10^{th}, 15^{th} and 20^{th} days). No obvious difference in DLK protein
779 was observed in mouse ES cells and MEFs. (B) A decrease of phosphorylated Akt was

780 observed upon the removal of LIF. The protein expression levels of DLK,
781 phosphorylated Akt, and total Akt were detected by Western blot analysis.
782
783 **Figure S3. DLK activity increases upon the treatment of PI3K/Akt signaling**
784 **inhibitor in R1 mouse ES cells**
785 (A) LY-294002 treatment down-regulated Akt activity and expression amounts of
786 Nanog. Western blotting was performed to detect DLK, phospho-Akt at S473,
787 phospho-Akt at T308, total Akt, Nanog and actin after R1 mouse ES cells were treated
788 with 0, 0.25 and 1.0 μM of LY-294002. Under the same condition, (B) DLK activity
789 increased does-dependently upon the treatment of LY-294002. Western blotting and
790 kinase assay using MBP as substrates were carried out as DLK protein was
791 immunoprecipitated.
792
793 **Figure S4. Up-regulation of DLK in mouse ES cells induces cell death and the**
794 **down-regulation of Nanog protein**
795 Mouse ES cells were transfected with GFP, DLK wild type and DLK mutants (DLK
796 S584A, DLK T659A, or DLK S584A/T659A) plasmids. (A) Apoptotic cells increased
797 significantly in D3 mouse ES cells transfected with DLK mutant plasmids. Sub-G1
798 levels of cells were analyzed on 4^{th} day after transfection. Cells were fixed and stained

799 with propidium iodine. (B) Expression of wild type and mutants of DLK did not affect
800 expression levels of phospho-Akt and Akt. Western blot analyses were performed. (C)
801 Expression of wild type and mutants of DLK did not alter the ALP/AB value. After
802 Alamar blue assay and alkaline phosphatase (ALP) activity were performed, values
803 represented to alkaline phosphatase activity were divided by values obtained from
804 Alamar blue assay in each cells. (D) Relative cell numbers decreased in R1 cells
805 transfected with DLK mutant plasmids. (E) Nanog and Oct4 proteins were
806 downregulated upon transfection of DLK mutant plasmids in R1 cells. Western blot
807 analyses were performed. The error bars in the figures represent standard error of the
808 mean (mean±SEM). *P* values were obtained from two-tailed Student's *t*-tests (***, *P*
809 < 0.0001; **, *P* < 0.001; *, *P* < 0.05).
810
811
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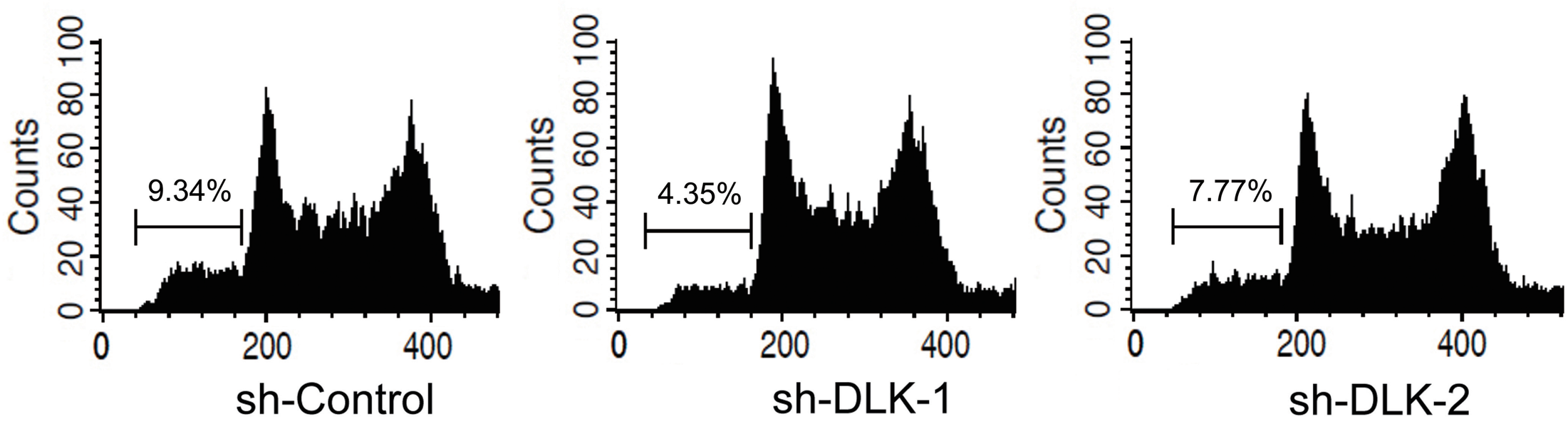
815
816
817

Figure S1, Wu

A



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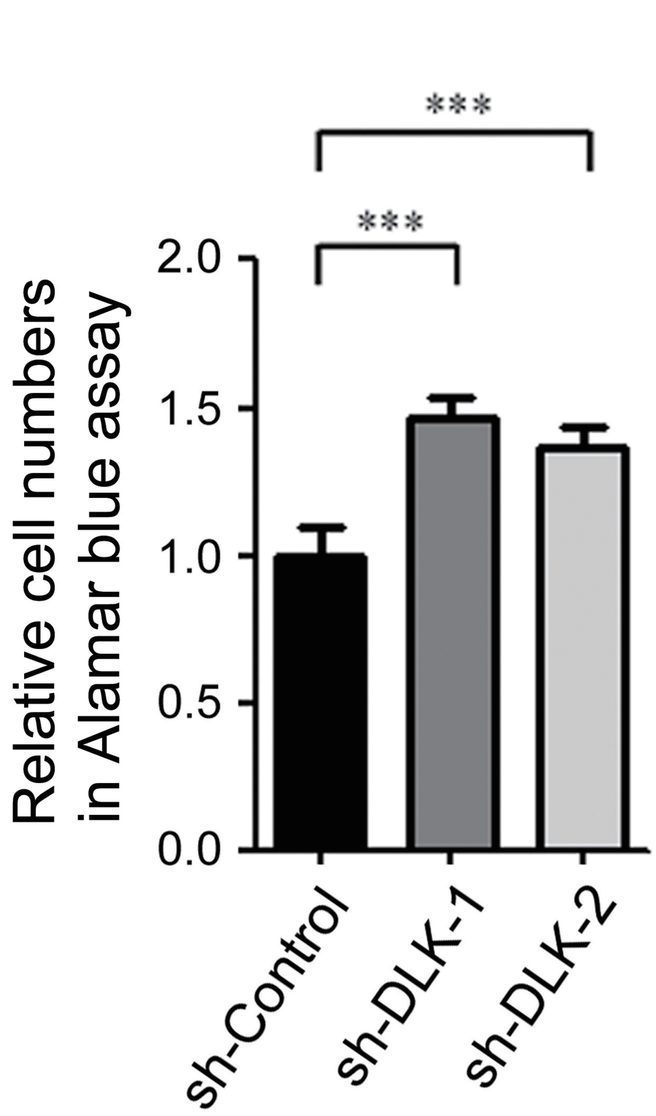

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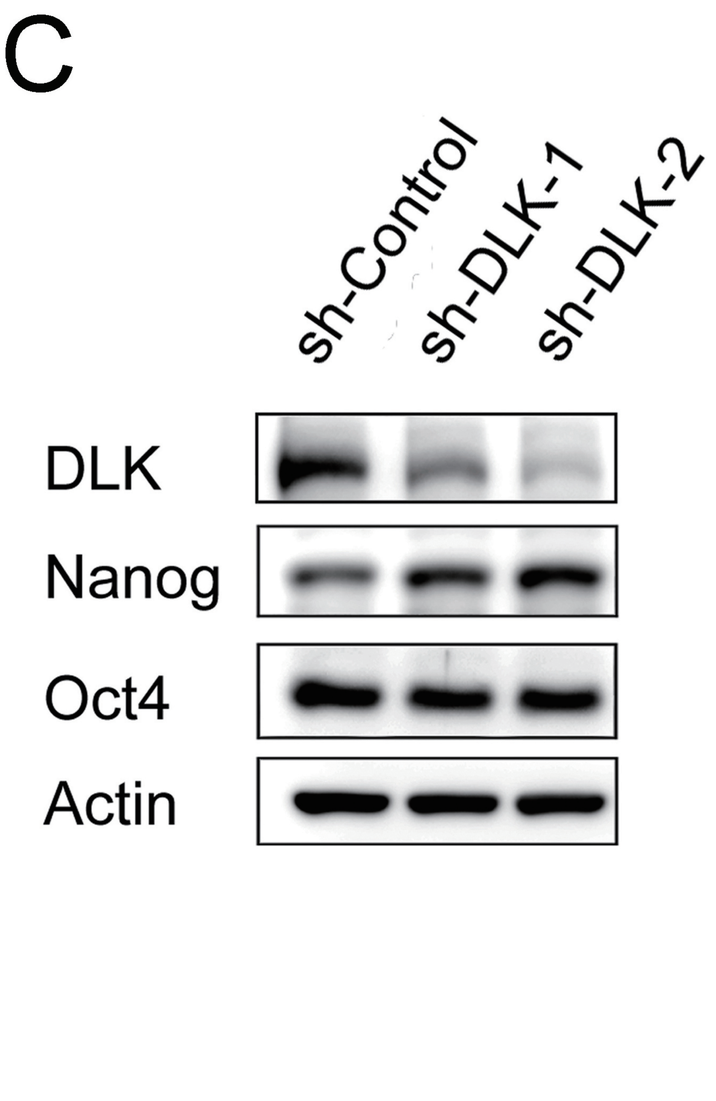


Figure S2, Wu

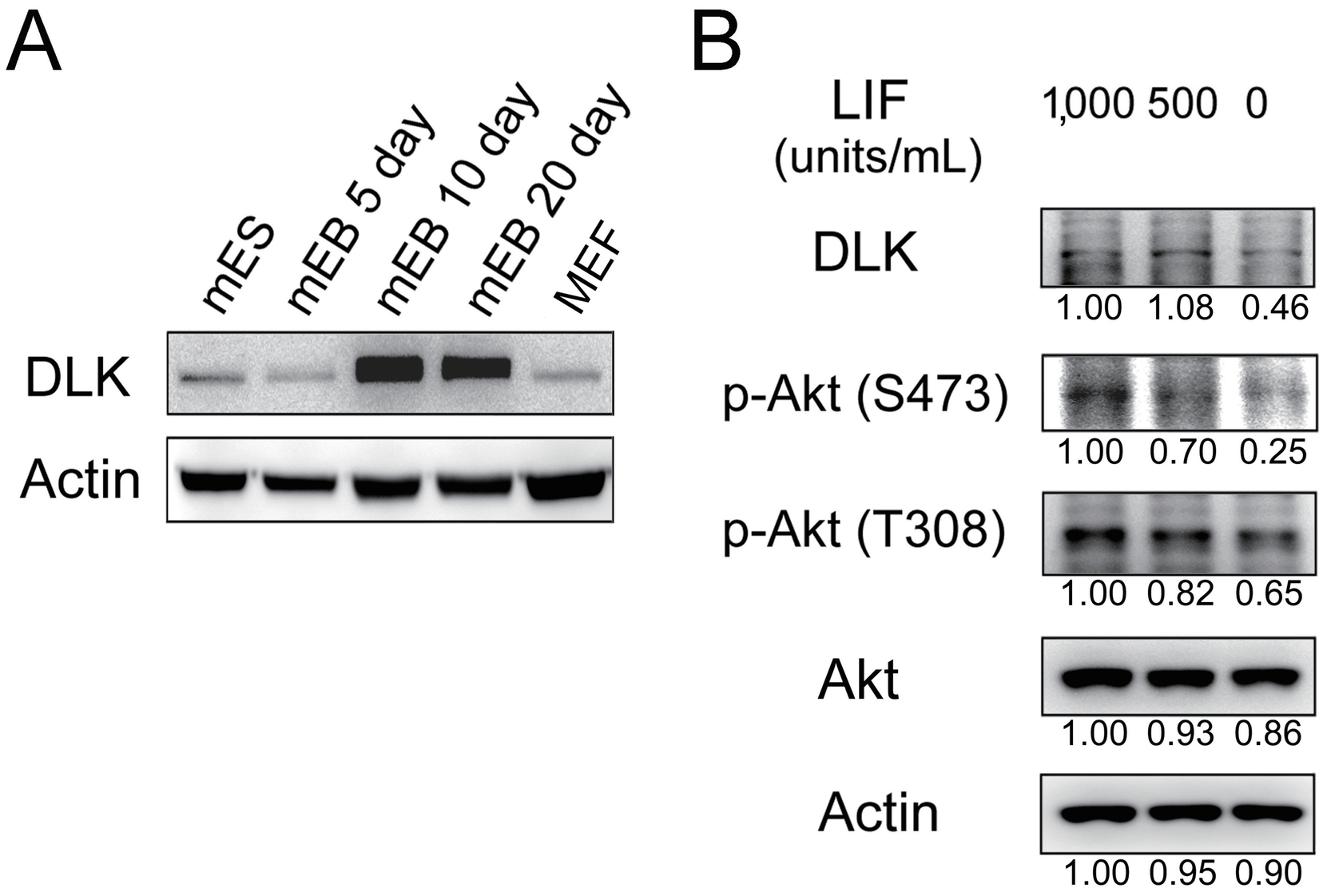
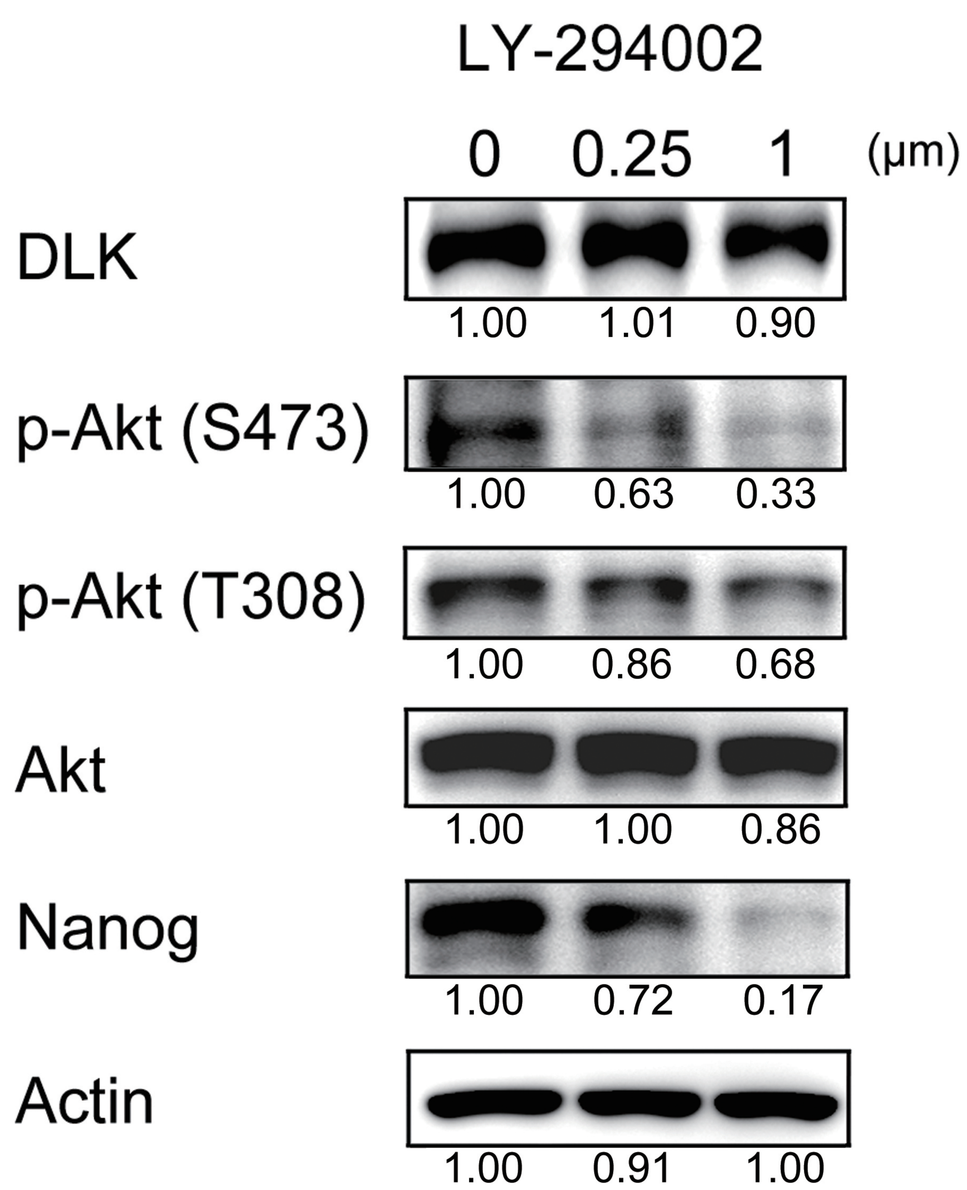

A
mES
mEB 5 day
mEB 10 day
mEB 20 day
MEF
DLK
Actin
B
LIF
(units/mL)
1,000 500 0
DLK
1.00 1.08 0.46
p-Akt (S473)
1.00 0.70 0.25
p-Akt (T308)
1.00 0.82 0.65
Akt
1.00 0.93 0.86
Actin
1.00 0.95 0.90

Figure S3, Wu


A
LY-294002
0 0.25 1 (μm)
DLK
1.00 1.01 0.90
p-Akt (S473)
1.00 0.63 0.33
p-Akt (T308)
1.00 0.86 0.68
Akt
1.00 1.00 0.86
Nanog
1.00 0.72 0.17
Actin
1.00 0.91 1.00

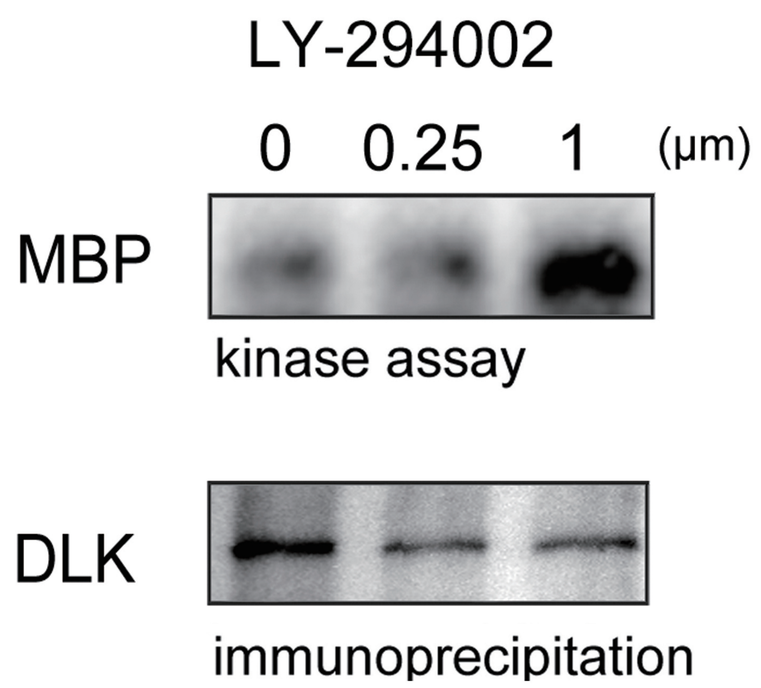
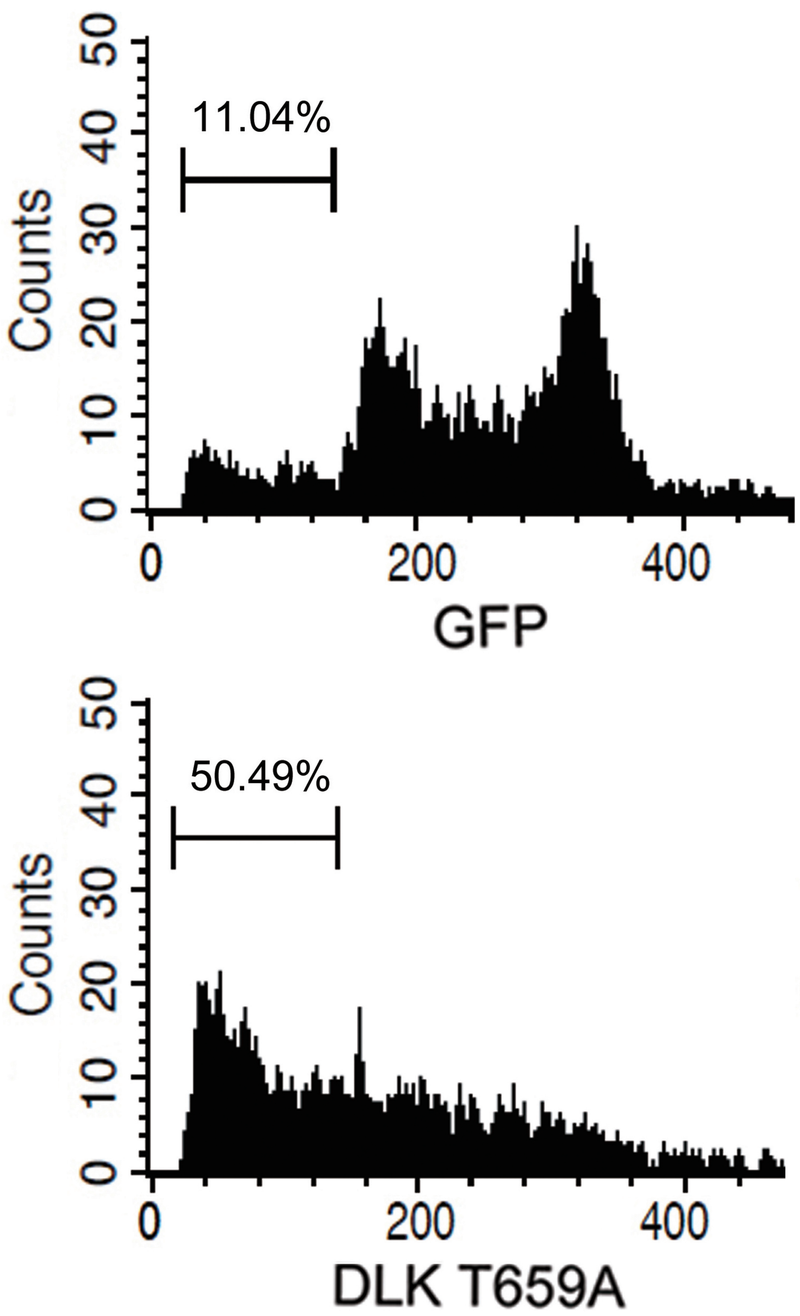
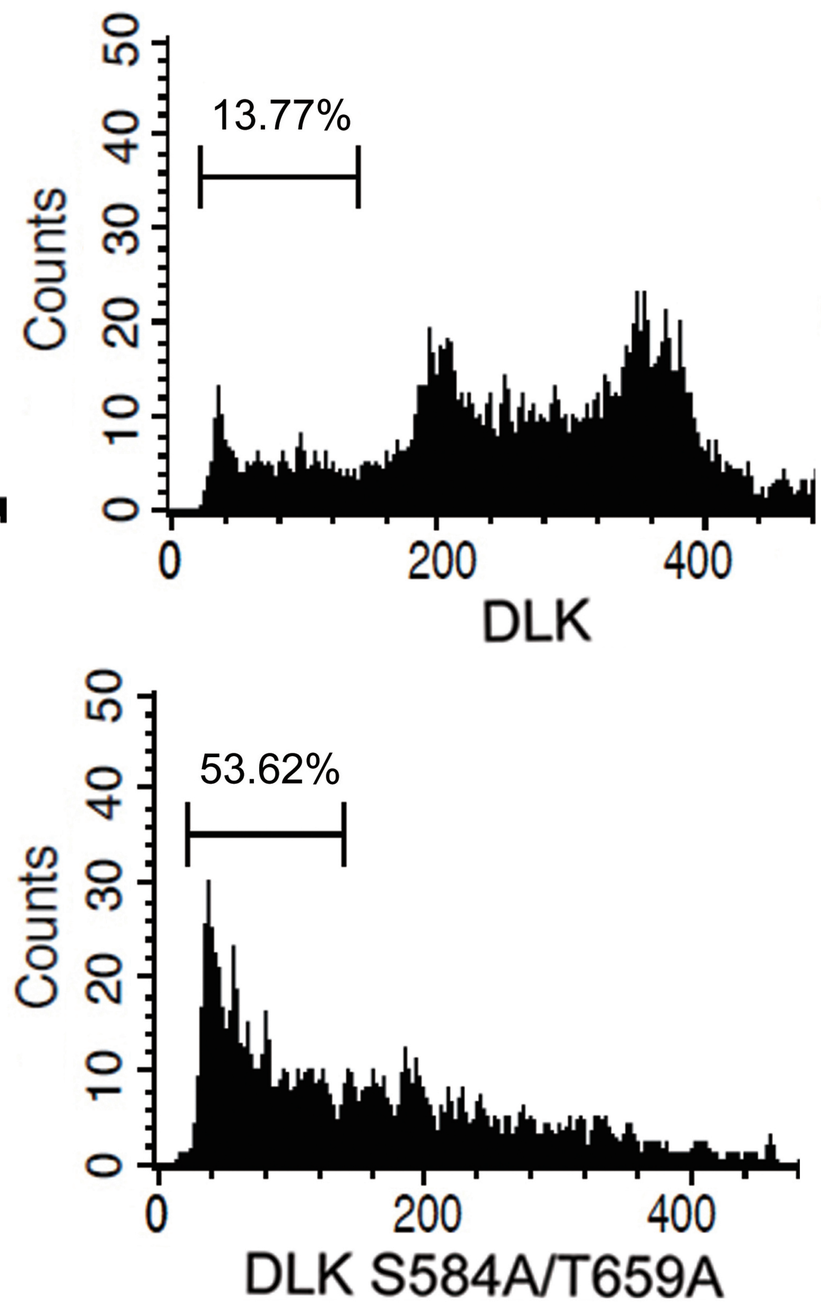
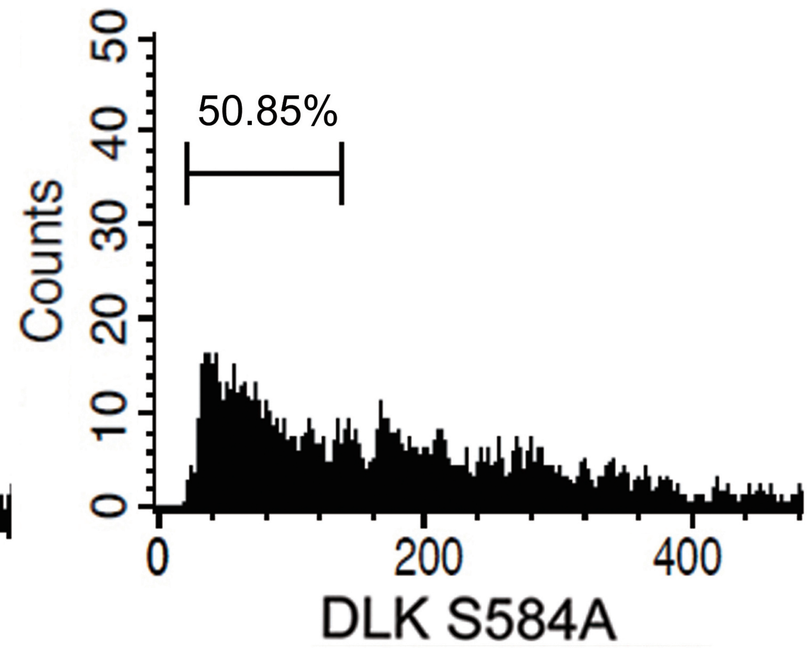

B
LY-294002
0 0.25 1 (μm)
MBP
kinase assay
DLK
immunoprecipitation

Figure S4, Wu

A







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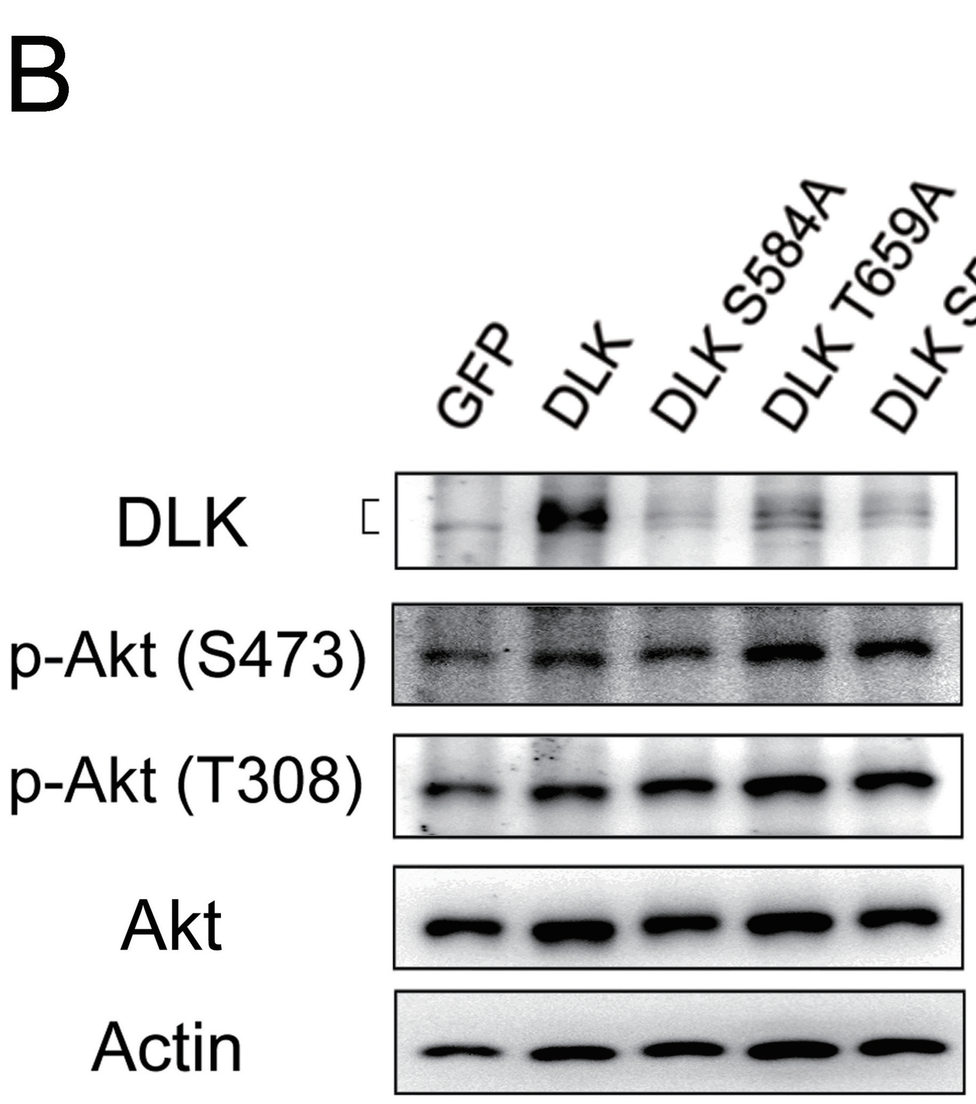

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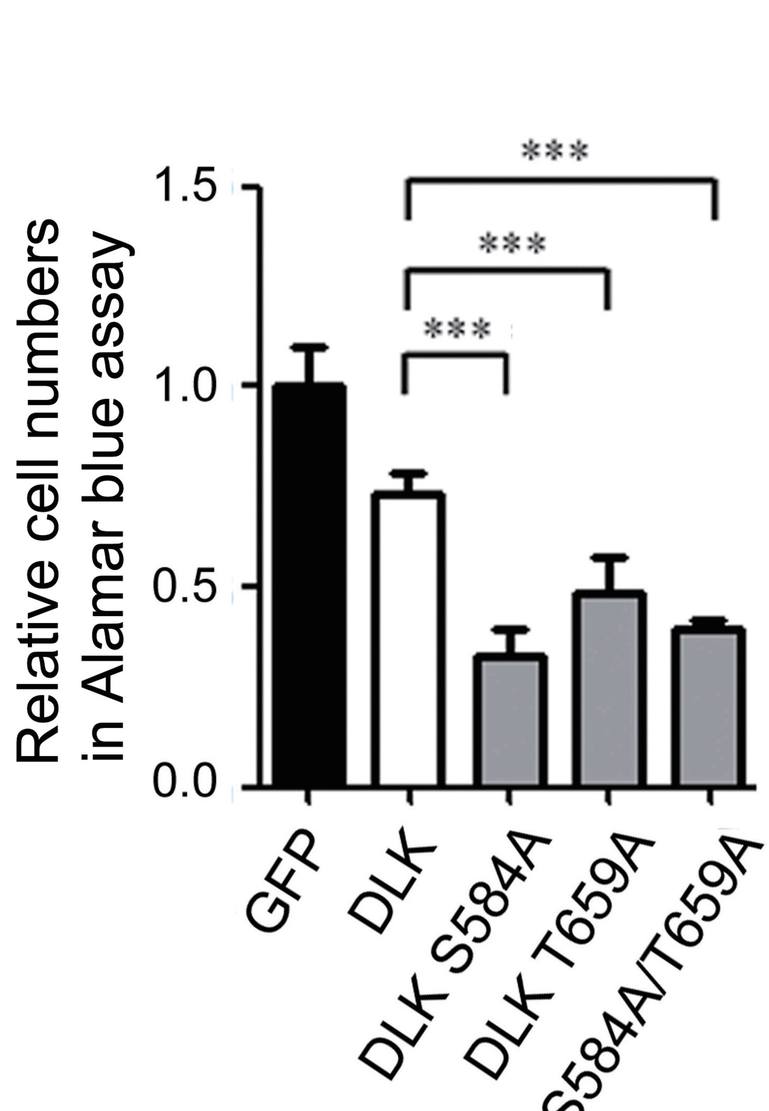

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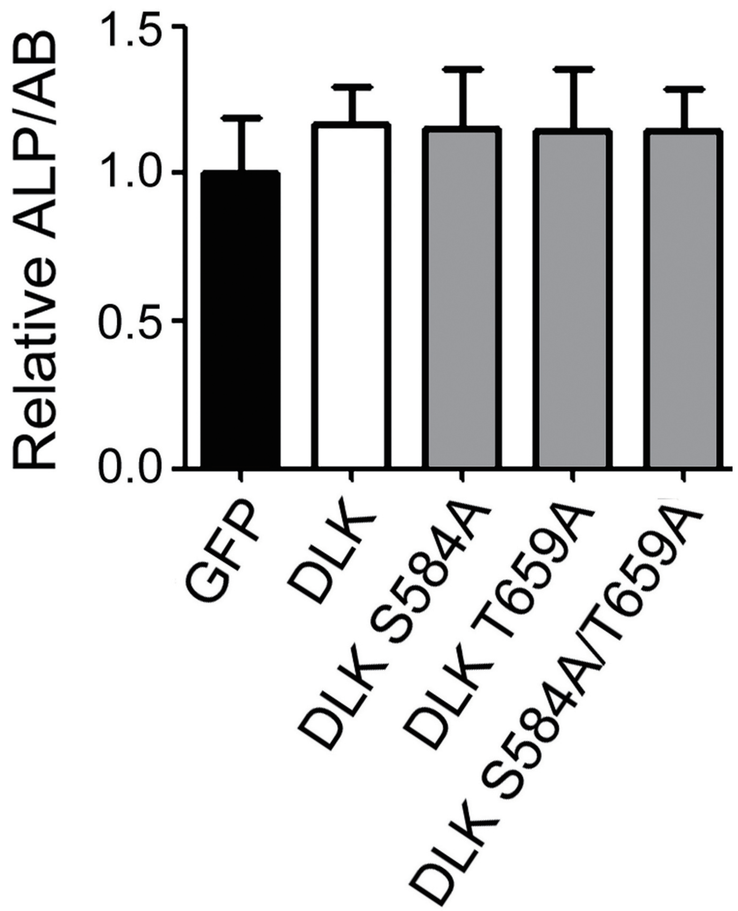

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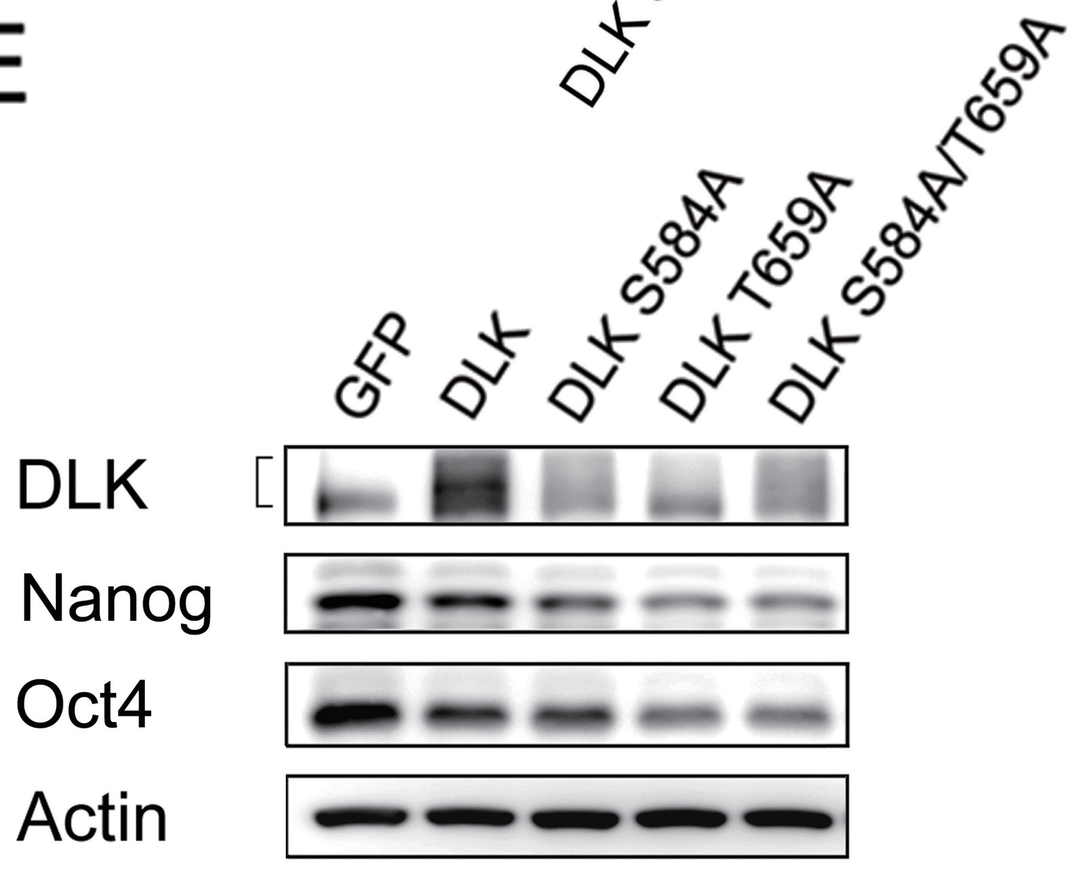

Table 1. Primers used in quantitative real-time PCR

Primer name	Primer sequence
DLK forward	5’-AGCACCAAGATGTCCTTTGC
DLK reverse	5’-ATGATGGGCGATTTCGTG
Nanog forward	5’-ATGCGGACTGTGTTCTCTCA
Nanog reverse	5’-GCAATGGATGCTGGGATACT
Oct4 forward	5’-ATGAGAACCTTCAGGAGATATG
Oct4 reverse	5’-CTCGTTGGGAATACTCAATACT
GAPDH forward	5’-ACCACAGTCCATGCCATCAC
GAPDH reverse	5’-TCCACCACCCTGTTGCTGTA