Supporting Information

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SI Materials and Methods

Antibodies and Cells. Antibodies to clock proteins (PER1-1-GP, PER2-1-GP, P3-28R, CLK-1-GP, CK1δ-GP, and CK1ε-GP) were described previously (1, 2). Rabbit anti-ACTIN antibody was purchased from Sigma. Anti-MYC and -HA were from Roche, anti-FLAG from Sigma, and anti-V5 from Invitrogen. Anti-PP2A catalytic subunit and PP1 catalytic subunit (PPP1CA) were from Millipore and Santa Cruz Biotech, respectively.

A mixture of proteasome inhibitors [40 μM MG132 + 40 μM Z-Ile-Glu(OBu^t)-Ala-Leu-H (PSI) + 30 μM lactacystin; BIOMOL International] was used to inhibit the proteasome in Fig. 2*B* and Fig. S5*D*. In Fig. 1*D*, cells were treated with 40 μg/mL cycloheximide and harvested at indicated times. In Fig. 3*D*, 40 μg/mL cycloheximide was added into the medium for 10 h to inhibit translation. The 10-h duration ensures complete degradation of endogenous PER; this was determined by monitoring the endogenous proteins at various times during CHX treatment. Translation was allowed to resume by washing off CHX. A total of 100 nM okadaic acid (OA; EMD Chemicals) and 20 nM calyculin A (CA; EMD Chemicals) were used for cells, and 500 nM CA was used for lung explants. For Fig. 3*A*, OA and CA were added for 2 h and washed off, because continuous incubation with these drugs caused cell death within a day. For Fig. 3*B*, CA was added for 6 h and washed off. However, these drugs were not removed from the medium when the cells were used for immunoblots.

Recombinant Plasmids, Adenoviral Constructs, and Virus Production. pcDNA constructs for *wtCKIεMyc*, *Per1V5*, and *Per2V5* were described previously (3, 4). pcDNA-V5-*GSK3*β was generated by cloning a full-length mouse GSK3β cDNA (MGC 68385) into pcDNA-V5. Tetracycline-inducible CK2α/β dual pBI plasmid (TRE-CK2α-HA/CK2β-Myc pBI) was obtained from Myra Hurt (Florida State University, Tallahassee, FL) and David Litchfield (University of Western Ontario, London, ON, Canada) (5). CK2α/β controlled by TRE was inducibly expressed by cotransfecting the cells with pAd-Track-CMV-rtTA. pAd-Track-CMV-rtTA (tetracycline transactivator) was generated by cloning TA cDNA into the pAd-Track-CMV vector. A construct for expressing wt3xFlag-CKIα was generated by subcloning a full-length CKIα cDNA (accession no. BC019740; Open Biosystems) into KpnI and XbaI sites of pAd-Track-CMV (ATCC) after PCR amplification of the cDNA using the following primers: forward, 5′ ATCCGGTACCGCCACCATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGGCGAGCAGCAGCGGCTCCAAGGCC 3′; reverse, 5′ TAGGTCTAGATTAGAAACCTGTGGGGGTTTGGGCCTG 3′.

A construct for wt3xFlag-CK1γ was generated by subcloning a full-length CK1γ cDNA into KpnI and XbaI sites of pAd-Track-CMV after PCR using the following primers: forward, 5′ ATCCGGTACCGCCACCATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGGACCATTCTAATAGGGAAAAGGATGATAGA 3′; reverse, 5′ TAGGTCTAGATCACTTGTGTCGCTGGGCTGTCTTCTT 3′.

To generate adenoviral *cre*, a *cre* fragment from the pRho-*cre* plasmid (Addgene 13779) was subcloned into EcoRI/NotI sites of pcDNA3.1, and then the KpnI/PmeI fragment from the pcDNA3.1 was subcloned into KpnI/EcoRV sites of pAd-Track-CMV. The cloned pAdTrack-CMV shuttle vectors were subsequently cut with PmeI for linearization and then transformed into the *Escherichia coli* BJ5183 strain along with the pAdEasy-1 adenoviral backbone vector to generate a complete adenoviral vector by in vivo recombination. Adenovirus was produced as described by He et al. (6).

Adenoviruses expressing GFP, wtCK1ε, DNCK1ε, or *Per2* promoter luciferase were described previously (3, 7). To generate tetracycline-inducible dominant negative PP1 mutants, an inducible adenoviral vector (pAdTrack-TRE) was generated as follows. A BglII/NotI fragment of pLVX-Tight-Puro vector (Clontech) containing the tetracycline operator linked to a cytomegalovirus minimal immediate-early promoter (TRE) was subcloned into the same sites of the promoterless pAdTrack to generate pAdTrack-TRE. cDNA encoding wtPP1CA (Ppp1ca), the α isoform of the PP1 catalytic subunit (accession no. BC014828; Open Biosystems), was cloned into XhoI and XbaI sites of 3xFlag-pCS2 vector after PCR with the following primers: sense, 5′ ATCCCTCGAGTCCGACAGCGAGAAGCTCAACCTG 3′; antisense, 5′ TAGGTCTAGACTATTTCTTGGCTTTGGCAGAATTGCG 3′.

Two dominant negative PP1 mutants (D64N and D95N) were generated by a two-step PCR (8). Because PER1/2 are predominantly nuclear, an exogenous nuclear localization signal (NLS) sequence was added to these mutants and cloned into XbaI site of the pAd-Track-TRE using the following primers: sense, 5′ATCCTCTAGAGCCACC*ATGGATCCAAAAAAGAAGAGAAAGGTA*GATTACAAGGATGACGATGACAAG3′ (NLS underlined); antisense, 5′ TAGGTCTAGACTATTTCTTGGCTTTGGCAGAATTGCG 3′.

All of the constructs were verified by sequencing. Complete adenoviral vectors and adenovirus was produced as described previously. The adenoviral-*DNPP1* was infected into *ROSA-rtTA; Per2^{Luc}* mouse embryonic fibroblasts where DNPP1 expression can be induced by 1 μg/mL doxycycline (Fig. 4). *ROSA-rtTA;Per2^{Luc}* mice were generated by crossing *Per2^{Luc}* with *ROSA-rtTA* (006965; The Jackson Laboratory) mice. In Fig. 4*C*, *ROSA-rtTA;Per2^{Luc}* MEFs were infected with adenoviral-*DNPP1* or *gfp* for 24 h before 2-h serum shock, followed by 1 μg/mL Dox treatment. In Fig. 4*D*, Dox was added into the medium without serum shock.

Transfection, Immunocytochemistry, Immunoblotting, Immunoprecipitation, and Quantitative Real-Time RT-PCR. These procedures were performed as described previously (1, 4, 7). pAdTrack-GFP was used for a control DNA for transfection in Fig. 4*G*. Immunocytochemistry for PER1/2 was performed 6 d after adenoviral *cre* infection. Real-time PCR was performed using the same primers and conditions on an iCycler iQ PCR System (Bio-Rad) as described previously (7).

Kinase Assay and Phosphatase Treatment. In vivo CK1δ/ε:PER1/2 complexes were purified by coimmunoprecipitation using anti-CK1δ or ε antibody described in Lee et al. (1). The immune complexes were washed in a kinase buffer [20 mM Hepes (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, and 0.1% Triton X-100] three times without ATP and split into two aliquots. PP1 was barely detectable in the immune complexes after the repeated washes, whereas CK1δ/ε levels were not reduced, suggesting that interaction of PP1 with PER is not stable compared with that between PER and CKIδ/ε. A total of 0.1 mM ATP was added only into one aliquot and incubated at 30 °C for 30 min. The reactions were stopped by adding 2× sample buffer. Phosphatase treatment of immunoprecipitated samples was performed as described previously (9).

Genotyping PCR. Genomic DNA was extracted from WT and double-floxed fibroblasts 3 d after infection with adenoviral *cre* or *gfp*. Genomic DNA was then subjected to PCR using a three-primer mixture that would amplify all three alleles, as described in Etchegaray et al. (10).

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11. Rivers A, Gietzen KF, Vielhaber E, Virshup DM (1998) Regulation of casein kinase I epsilon and casein kinase I delta by an in vivo futile phosphorylation cycle. *J Biol Chem* 273:15980–15984.

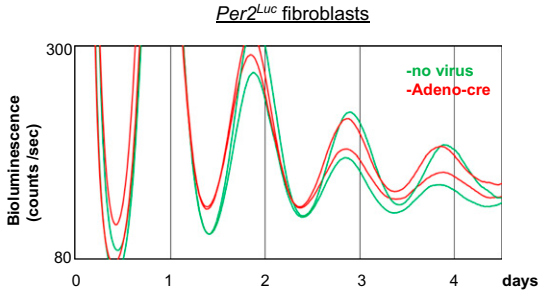

Fig. S1. Adenovirus *cre* infection did not affect circadian bioluminescence rhythms in *Per2*^{Luc} cells. The cells were infected with adenoviral *cre* (Adeno-cre), serum shocked for 2 h, and set up in the Lumicycle. Control cells were not infected. Two traces are shown for each condition (infected and uninfected) and are representative of several experiments.

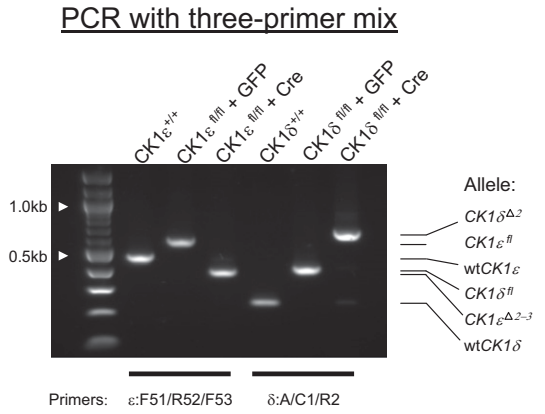

Fig. S2. *CK1*δ and ε were successfully deleted by introducing adenoviral *cre* into the double-floxed mutant cells. The mutant cells were harvested 3 d after adenoviral *cre* or *gfp* infection and then assessed for deletion of floxed alleles by genotyping PCR as described in Etchegaray et al. (10). CK1δ or ε$^{+/+}$: genomic DNA from WT fibroblasts.

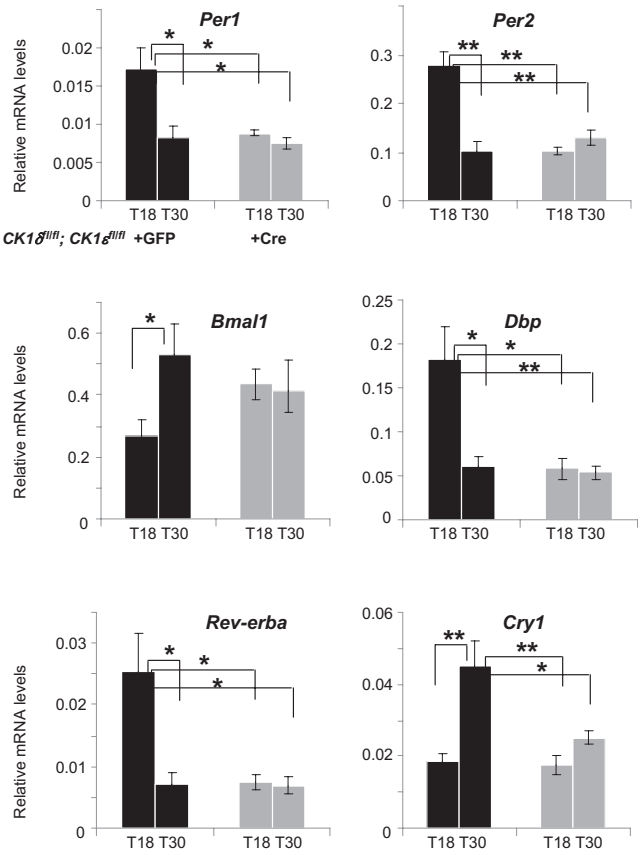

Fig. S3. Clock gene mRNA levels in adenoviral *gfp*- or *cre*-infected floxed mutant cells. Double-mutant cells and control cells were generated as described in *Materials and Methods* and then serum shocked for 2 h to synchronize cells. Cells were collected at peak (T18) and trough (T30) time points, which were selected based on our previous studies (7). Clock gene mRNA levels were determined using real-time quantitative PCR, and compared between control and double-mutant cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

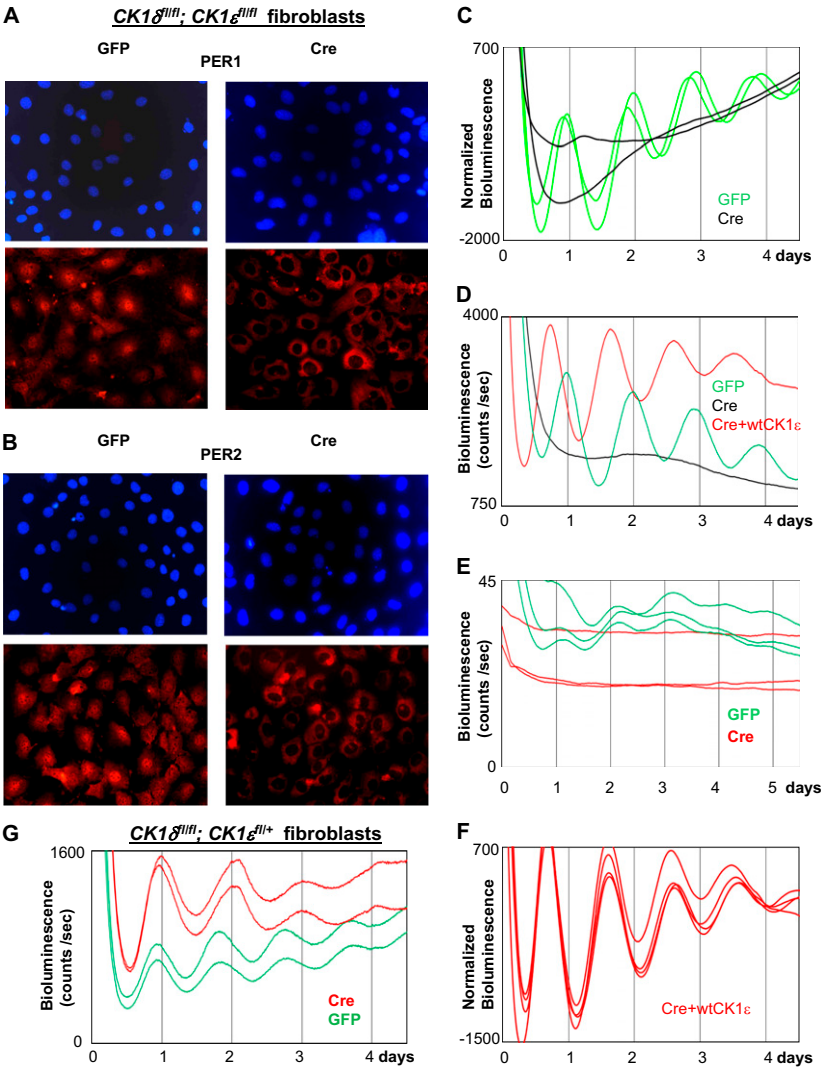

Fig. S4. PER1/2 are predominantly cytoplasmic and bioluminescence rhythms are arrhythmic in CK1δ/ε-deficient cells, but the defects can be rescued by exogenous expression of wtCK1ε. (*A* and *B*) Subcellular localization of PER in control (GFP) and CK1δ/ε-deficient (Cre) cells. (*C*) Bioluminescence rhythms of the double-floxed mutant cells before and after CK1δ/ε are deleted. Traces are from different experiments than those in Fig. 2*C*. (*D*) Raw data of traces shown in Fig. 2*C*. (*E*) Raw data of traces shown in Fig. 2*D*. Traces that are within a close range are shown. (*F*) Rescue of circadian rhythms by exogenous expression of wtCK1ε in the mutant cells. Traces are from different experiments than those in Fig. 2*C*. (*G*) Period is lengthened in $CK1\delta^{\Delta 2/\Delta 2}$:$CK1\varepsilon^{-/+}$ cells. Mouse embryonic fibroblasts were isolated from $CK1\delta^{fl/fl}$:$CK1\varepsilon^{fl/+}$ embryos. The floxed alleles were deleted by adenovirus *cre* as described in *Materials and Methods*. The period of GFP- and Cre-infected cells was 22.03 ± 0.12 (n = 5) and 24.55 ± 0.22 (n = 4) h, respectively. Values presented are mean ± SEM from two experiments. $P < 0.001$ by Student t test.

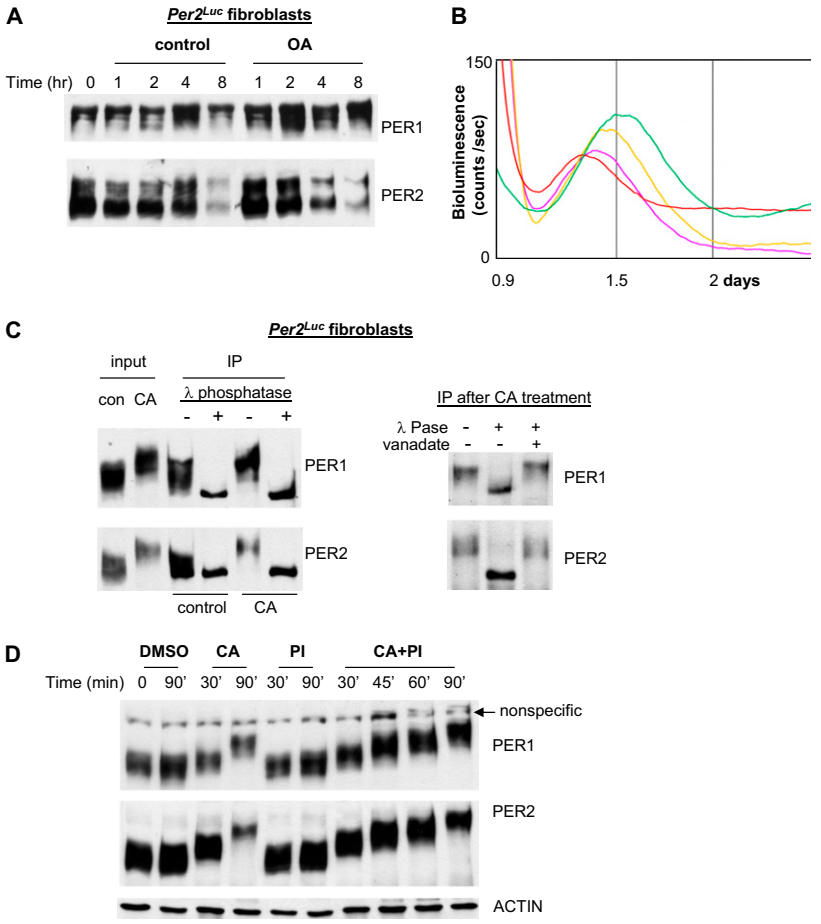

Fig. S5. Effects of OA and CA treatment on PER. (*A*) Intact PER phosphorylation by OA treatment. (*B*) Raw data of traces shown in Fig. 3*B*. (*C*) The slow mobility shift of PER induced by CA treatment is due to hyperphosphorylation. PER was immunoprecipitated from control and CA-treated cells and subjected to λ-phosphatase treatment as described previously (1). Vanadate is a phosphatase inhibitor. (*D*) The hyperphosphorylated PER induced by CA treatment is stabilized by proteasome inhibitor drugs (MG132 + PSI + lactacystin).

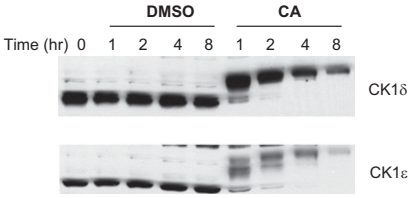

Fig. S6. CK1δ/ε are hyperphosphorylated by CA treatment. *Per2*^{Luc} cells were treated with CA and harvested at the indicated times. Immunoblots show that CKIδ/ε are hyperphosphorylated by CA treatment as shown previously (11).

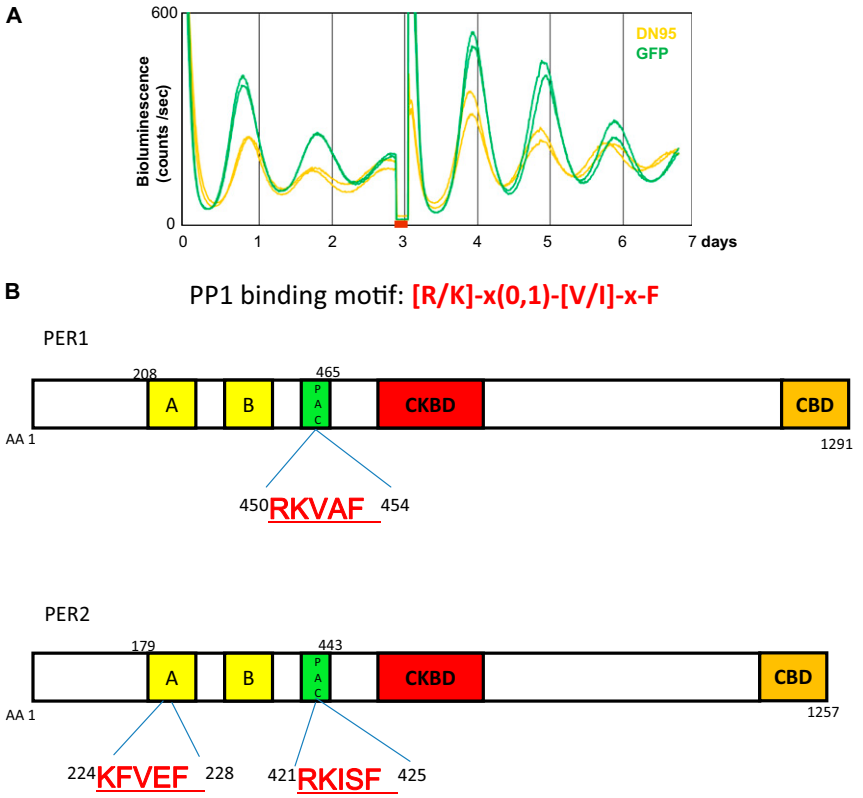

Fig. S7. Inhibition of PP1 induces period shortening. (*A*) The period shortening is rescued by turning off transgenic expression of DNPP1. Adenoviral *DN95* or *gfp* was infected into *ROSA-rtTA;Per2*^{Luc} fibroblasts, and expression of DN was initiated by doxycycline. Half of the adenoviral titers used in Fig. 4*C* was added into the cells to facilitate accurate assessment of period changes before and after Dox treatment. The cells were placed into the Lumicycle after a 2-h serum shock. DN expression was turned off by washing off Dox for 2 h at the indicated time (red line). The cells were washed four times with 30 min incubation of fresh DMEM, serum shocked again, and then placed into the Lumicycle again. The period (measured from the first two peaks) of the bioluminescence rhythms was ~3 h shorter before wash-off compared with after wash-off in DN-expressing cells. However, the period was not altered in GFP cells before and after wash-off. DN95: $n = 6$; period before wash-off, 19.53 ± 0.17 h; after wash-off, 22.86 ± 0.47; $P < 0.001$. GFP: $n = 4$; period before wash-off, 23.29 ± 0.10 h; after wash-off, 23.37 ± 0.21; $P > 0.05$. The periods of DN and GFP cells were not significantly different after wash-off ($P > 0.05$). Values presented are mean ± SEM from two experiments. *P* values were calculated by Student *t* test. (*B*) PP1 binding motifs in PER1 and 2. These motifs are conserved in human, mouse, Rat PER1, and PER2. A and B, PAS A and PAS B domains; PAC, C-terminal domain of PAS; CKBD, CK1 binding domain; CBD, CRY binding domain.

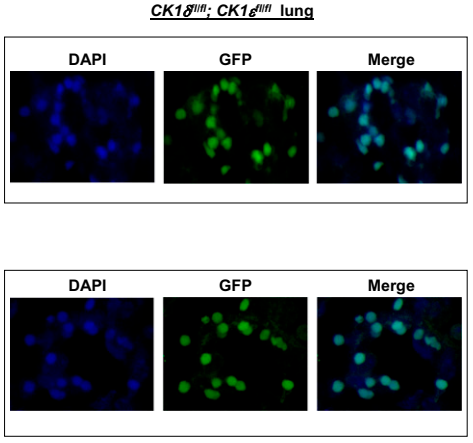

Fig. S8. Adenoviral *gfp* efficiently infects lung explants. Lung explants were infected with adenoviral *gfp*, fixed after DAPI staining, and subjected to confocal microscopy. Two visual fields are shown.