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Supplementary Materials for

Engineering light-controllable CAR T cells for cancer immunotherapy

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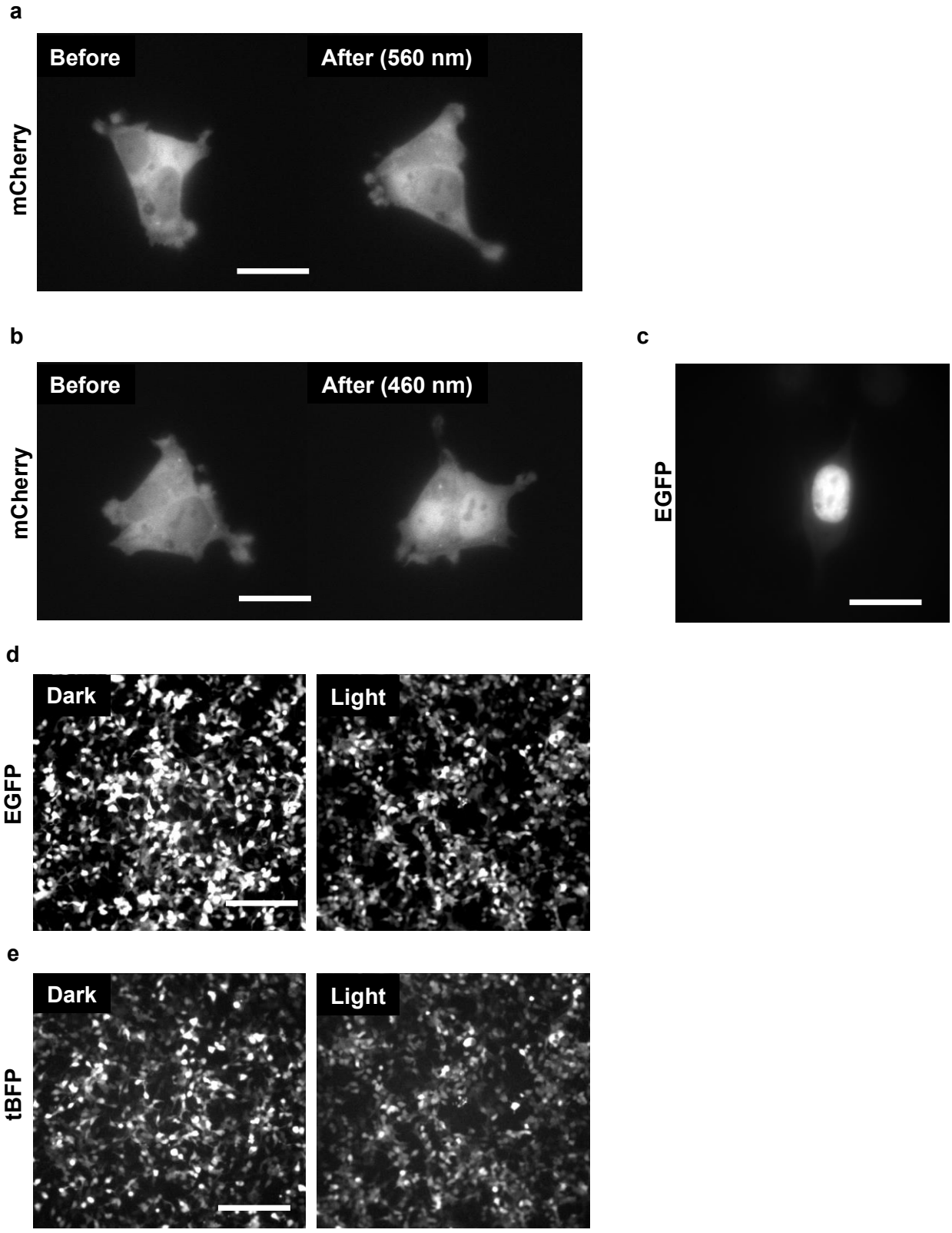

a
Before
After (560 nm)
mCherry
b
Before
After (460 nm)
mCherry
c
EGFP
d
Dark
Light
EGFP
e
Dark
Light
tBFP

Fig. S1. Nuclear translocation of LCmB is specific to blue light stimulation. (**a**) Fluorescence images of HEK 293T cells expressing LexA-CIB1-mCherry-biLINuS (LCmB) before and after 10 min of 560 nm light stimulation (0.5 s/ 30 s). Scale bar, 20 μm. (**b**) Fluorescence images of the same cells in **a** before and after 10 min of 460 nm light exposure (0.5 s/ 30 s). Scale bar, 20 μm. (**c**) A representative image showing the nuclear localization of EGFP-CV in HEK 293T cells. Scale bar, 20 μm. (**d**) Fluorescence images of HEK 293T cells expressing eGFP with (Light) or without (Dark) 24 hr light stimulation. Scale bar, 200 μm. (**e**) Fluorescence images of HEK 293T cells expressing tBFP with (Light) or without (Dark) 24 hr light stimulation. Scale bar, 200 μm. Photobleaching was observed in both cases.

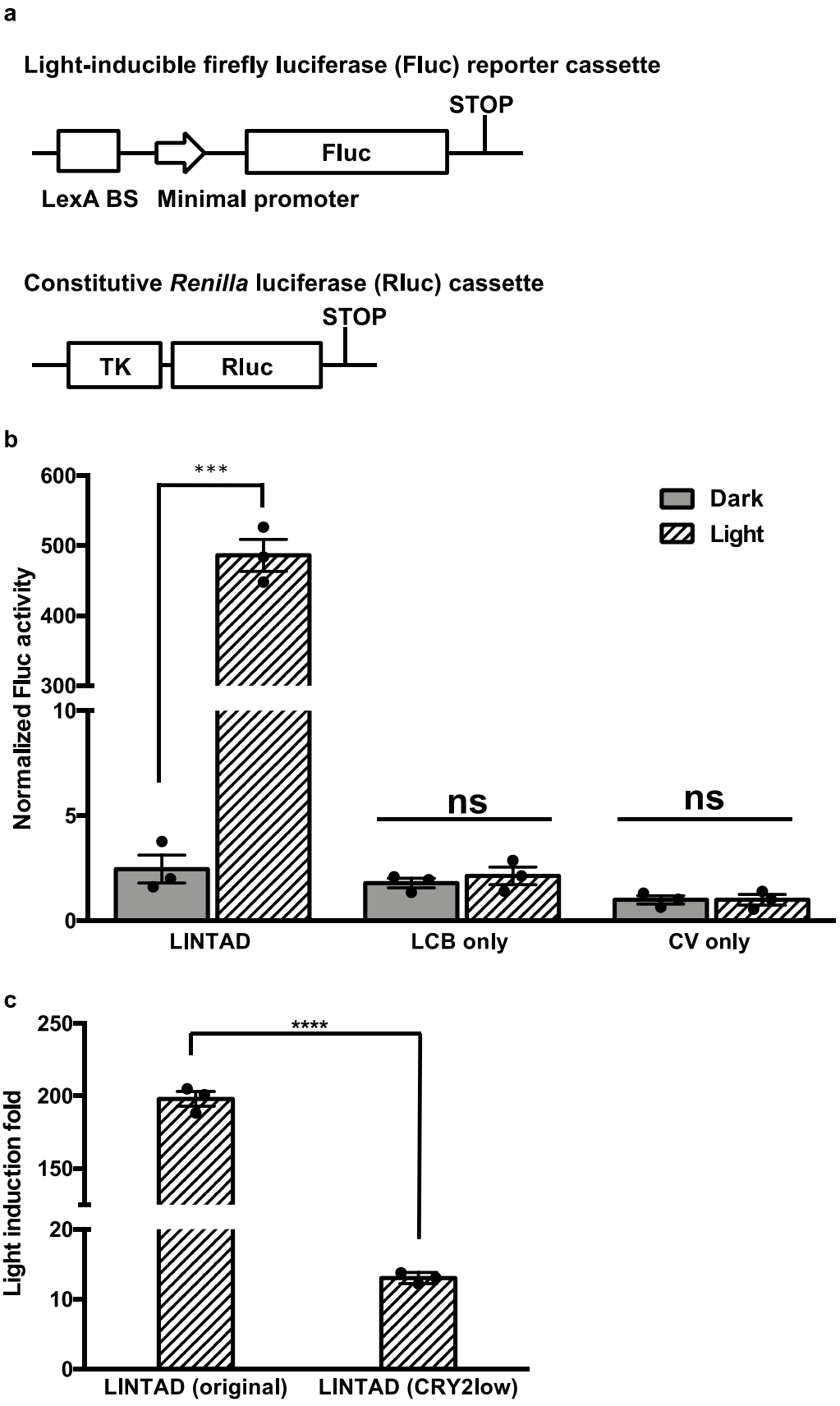

a
Light-inducible firefly luciferase (Fluc) reporter cassette
STOP
Fluc
LexA BS
Minimal promoter
Constitutive Renilla luciferase (Rluc) cassette
STOP
TK
Rluc
b
***
Dark
Light
Normalized Fluc activity
600
500
400
300
10
5
0
ns
ns
LINTAD
LCB only
CV only
c
****
Light induction fold
250
200
150
20
10
0
LINTAD (original)
LINTAD (CRY2low)

Fig. S2. Characterization of LINTAD-mediated gene induction in HEK 293T cells using dual-luciferase reporter system. (**a**) DNA cassettes used in the dual-luciferase reporter system. LexA BS: LexA-binding DNA sequence. Fluc, firefly luciferase gene. TK, constitutive HSV-thymidine kinase promoter. Rluc, *Renilla* luciferase gene. Stop codon after each gene is indicated. (**b**) Light-inducible gene activation using LINTAD in HEK 293T cells. Cells were transfected with intact LINTAD system (LCB and CV, shown as "LINTAD" in figure) or LCB ("LCB only") or CV ("CV only"), together with the dual-luciferase reporter constructs shown in **a**. Twenty-four hr after transfection, cells were stimulated with blue light for 24 hr (1s/30s, "Light") or kept in dark for 24 hr ("Dark"), $n = 3$ independent experiments. Ns, not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. Two-tailed Student's t-test with Bonferroni correction. Error bar: SEM. (**c**) Comparison of LINTAD systems using CRY2PHR and CRY2low in HEK 293T cells. For the original LINTAD group, cells were co-transfected with LCB and CV-expressing plasmids. For the LINTAD (CRY2low) group, cells were transfected with LCB and CRY2low-VPR. Both groups were also transfected with the light-inducible firefly luciferase reporter and the constitutive *Renilla* luciferase-expressing plasmid as internal control. Data represent mean values ± standard deviation ($n = 3$ experiments). **** $P < 0.0001$; two-tailed Student's t-test.

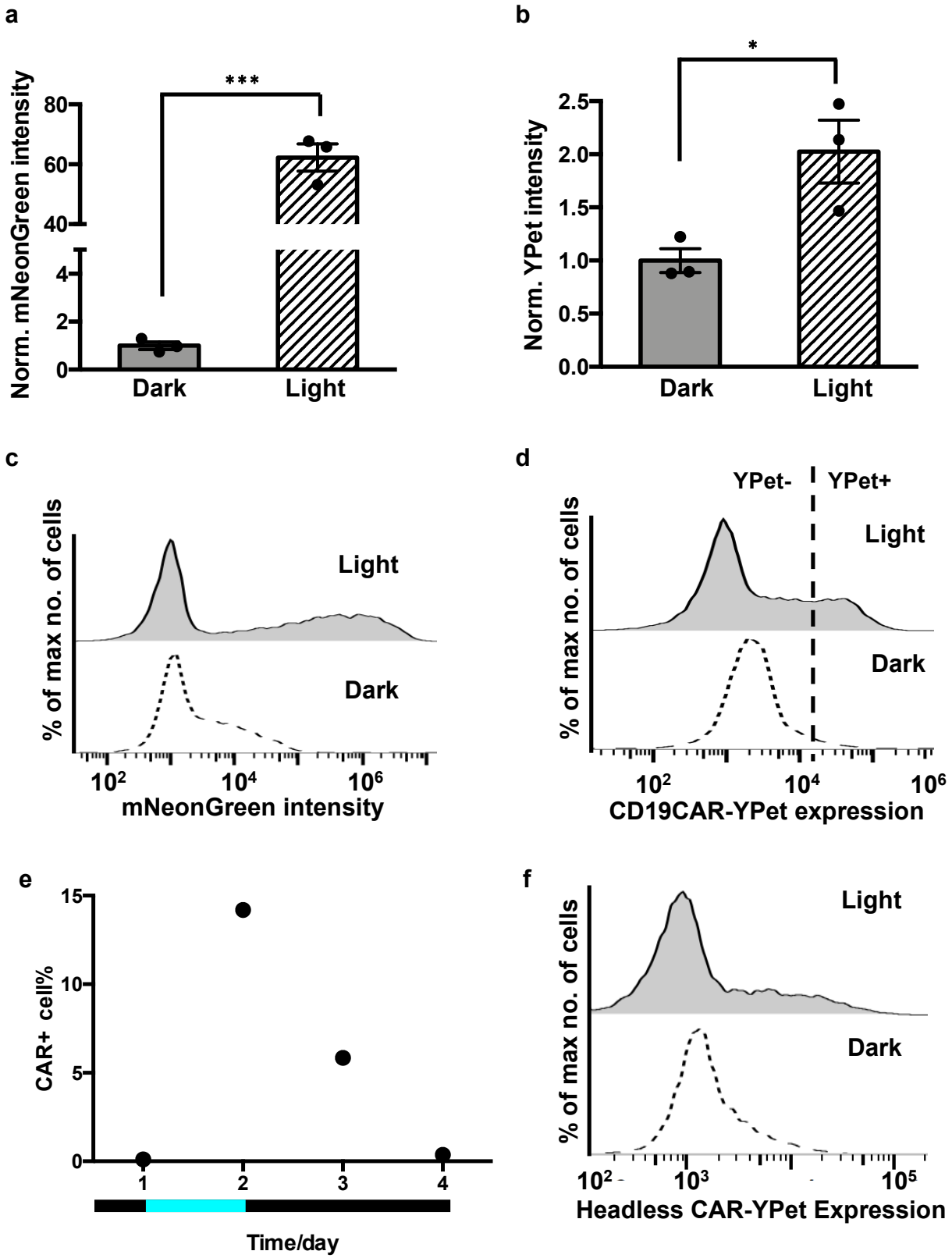
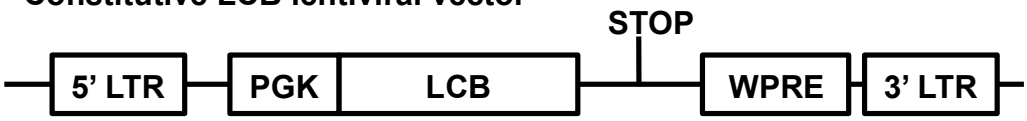

a
***
Norm. mNeonGreen intensity
80
60
40
4
2
0
Dark
Light
b
*
Norm. YPet intensity
2.5
2.0
1.5
1.0
0.5
0.0
Dark
Light
c
% of max no. of cells
Light
Dark
10^2
10^4
10^6
mNeonGreen intensity
d
YPet-
YPet+
% of max no. of cells
Light
Dark
10^2
10^4
10^6
CD19CAR-YPet expression
e
CAR+ cell%
15
10
5
0
1
2
3
4
Time/day
f
% of max no. of cells
Light
Dark
10^2
10^3
10^5
Headless CAR-YPet Expression

Fig. S3. LINTAD-mediated light-inducible gene expression in Jurkat cells. Jurkat cells were transfected with LINTAD system (LCB and CV) with different light-inducible reporter cassettes (mNeonGreen reporter in **a** and **c**; CD19CAR-YPet in **b** and **d**). Cells were stimulated with blue light for 24 hr (Light) or kept in dark for 24 hr (Dark) before fluorescence measurement. (a-b) Comparison of mean fluorescent intensity, $n = 3$ independent experiments, 10,000 cells in each experiment. Two-tailed Student's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars, SEM. (c-d) Representative fluorescence profiles of light-induced or non-induced cells shown in a or b. The YPet+ gate was indicated with dotted line in d. (**e**) Dynamics of CD19CAR expression after light induction in Jurkat cells. Light stimulation was applied from day 1 to day 2 for 24 hr as indicated by the bar below the x-axis. CD19CAR expression was measured by myc-tag staining. (**f**) Representative expression (YPet fluorescence) profiles of Jurkat cells transfected with LCB, CV and the light-inducible YPet-tagged headless CAR (without CD19 recognition domain) reporter with (Light) or without (Dark) 24 hr light stimulation.

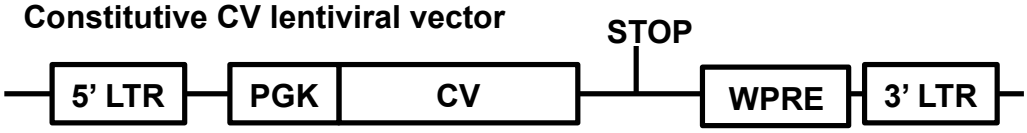
a

Constitutive LCB lentiviral vector



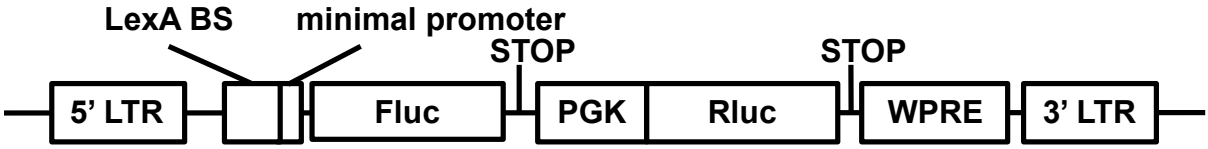
b

Constitutive CV lentiviral vector



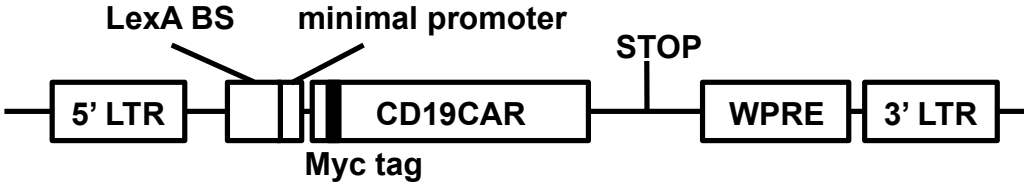
c

Light-inducible Fluc reporter lentiviral vector with constitutive Rluc (dual-luciferase reporter system)



d

Light-inducible CD19CAR reporter lentiviral vector



e

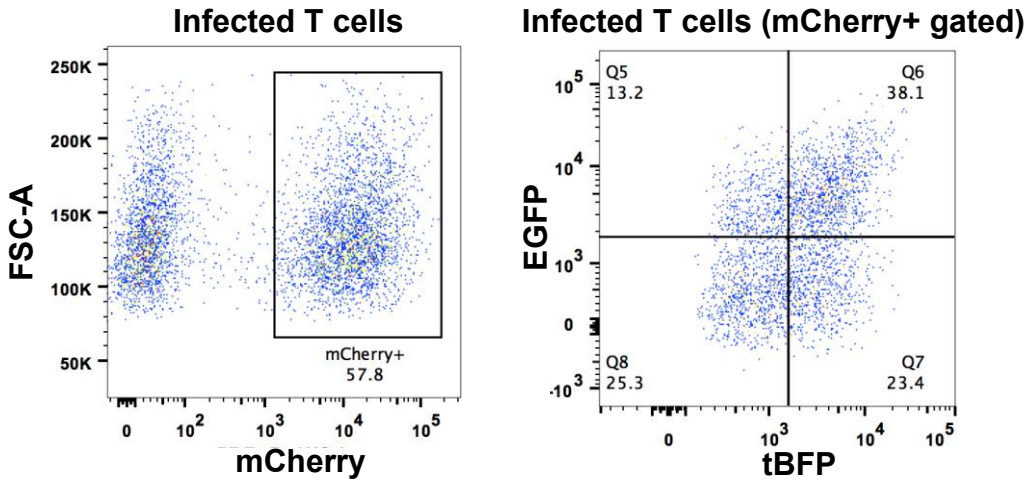
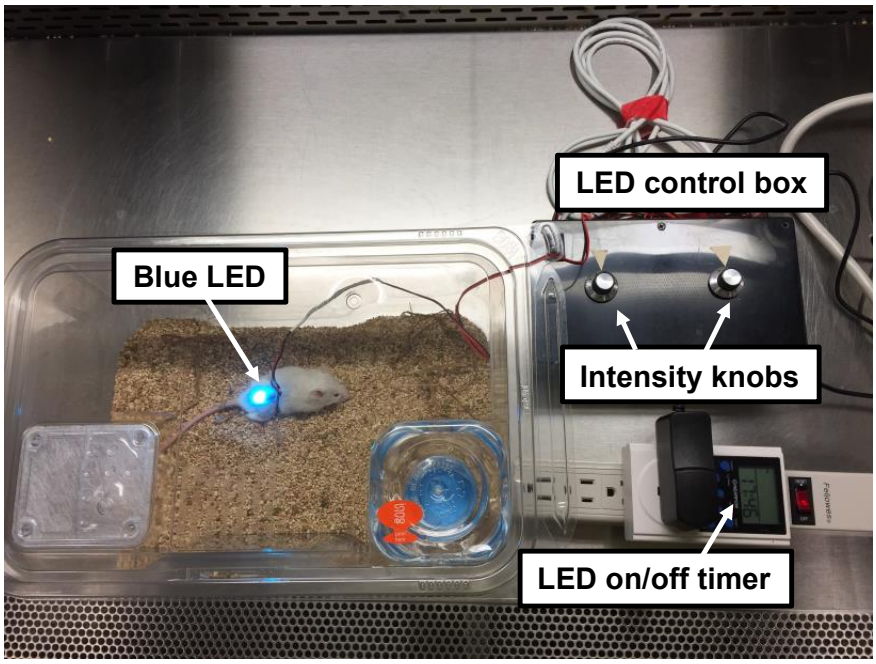
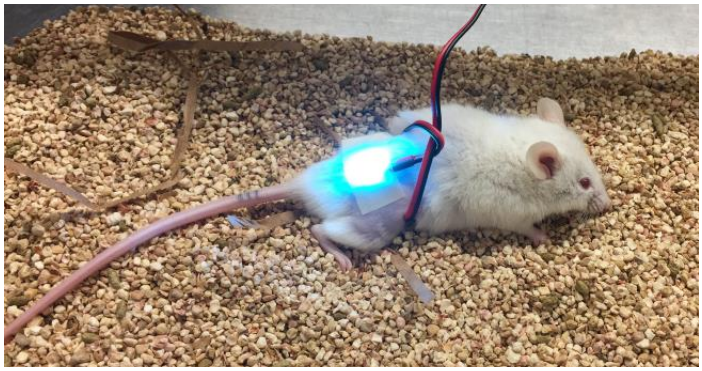


Fig. S4. Lentiviral vectors used for T cell infection. (**a**) The constitutive LCB lentiviral vector contains PGK promoter followed by LCB gene, WPRE element, flanked by 5'LTR and 3'LTR. (**b**) The constitutive CV viral vector has the same design as LCB lentiviral vector, except containing the CV gene instead of LCB. (**c**) The light-inducible Fluc reporter lentiviral vector contains the LexA binding sequence (LexA BS), followed by minimal promoter, Fluc gene, PGK promoter, and Rluc gene (for Fluc normalization). (**d**) The light-inducible CD19CAR reporter lentiviral vector contains LexA BS, minimal promoter and Myc-tagged CD19CAR gene and WPRE element, flanked by 5'LTR and 3'LTR. (**e**) Infection efficiency of LINTAD system in primary T cells. Primary T cells were co-infected with lentiviruses encoding LCB-P2A-tBFP, EGFP-P2A-CV, and LIP-myc-CD19CAR-PGK-mCherry. Left, the mCherry expression profile in the infected T cells. Right, the EGFP-tBFP expression profile of mCherry+ cells.

a


LED control box
Blue LED
Intensity knobs
LED on/off timer

b



c

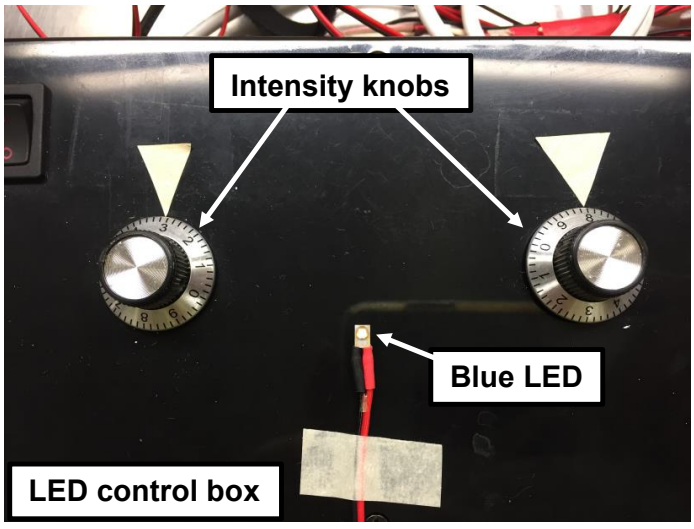

Intensity knobs
Blue LED
LED control box

Fig. S5. Light stimulation system used for in vivo studies. a, overview of the light stimulation system containing home-built LED control box with intensity knobs (for two LEDs), on/off timer (for controlling on/off cycle pattern of LEDs), and blue-light emitting LED. Each item was indicated in the figure. Photo Credit: Ziliang Huang, University of California, San Diego. **b**, a representative mouse with a blue LED attached on the desired stimulation region. Photo Credit: Ziliang Huang, University of California, San Diego. **c**, Home-built LED control box with intensity knobs. The blue LED used in **b** is also shown here (wavelength 460 nm, diameter 3mm). Photo Credit: Ziliang Huang, University of California, San Diego.

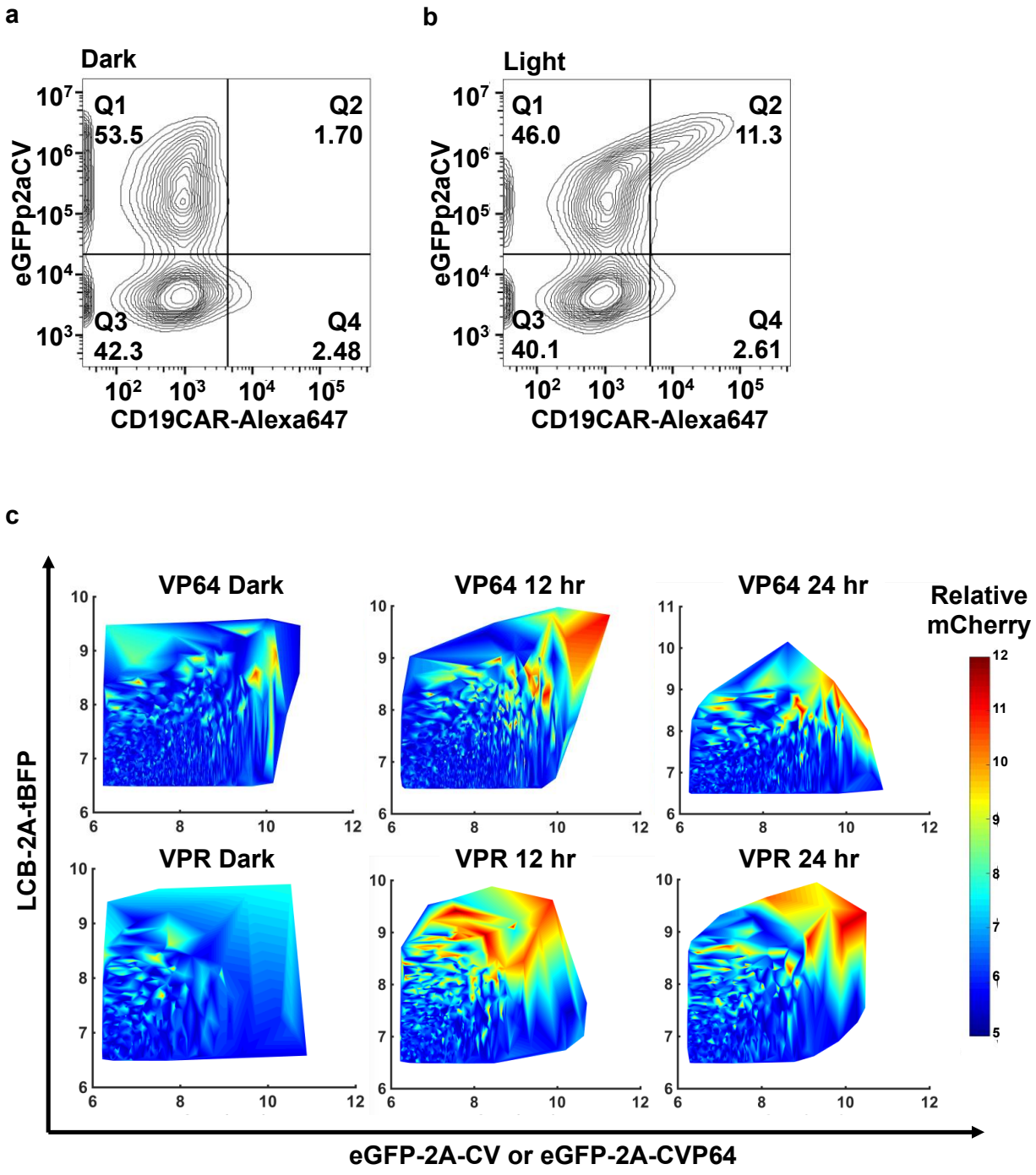
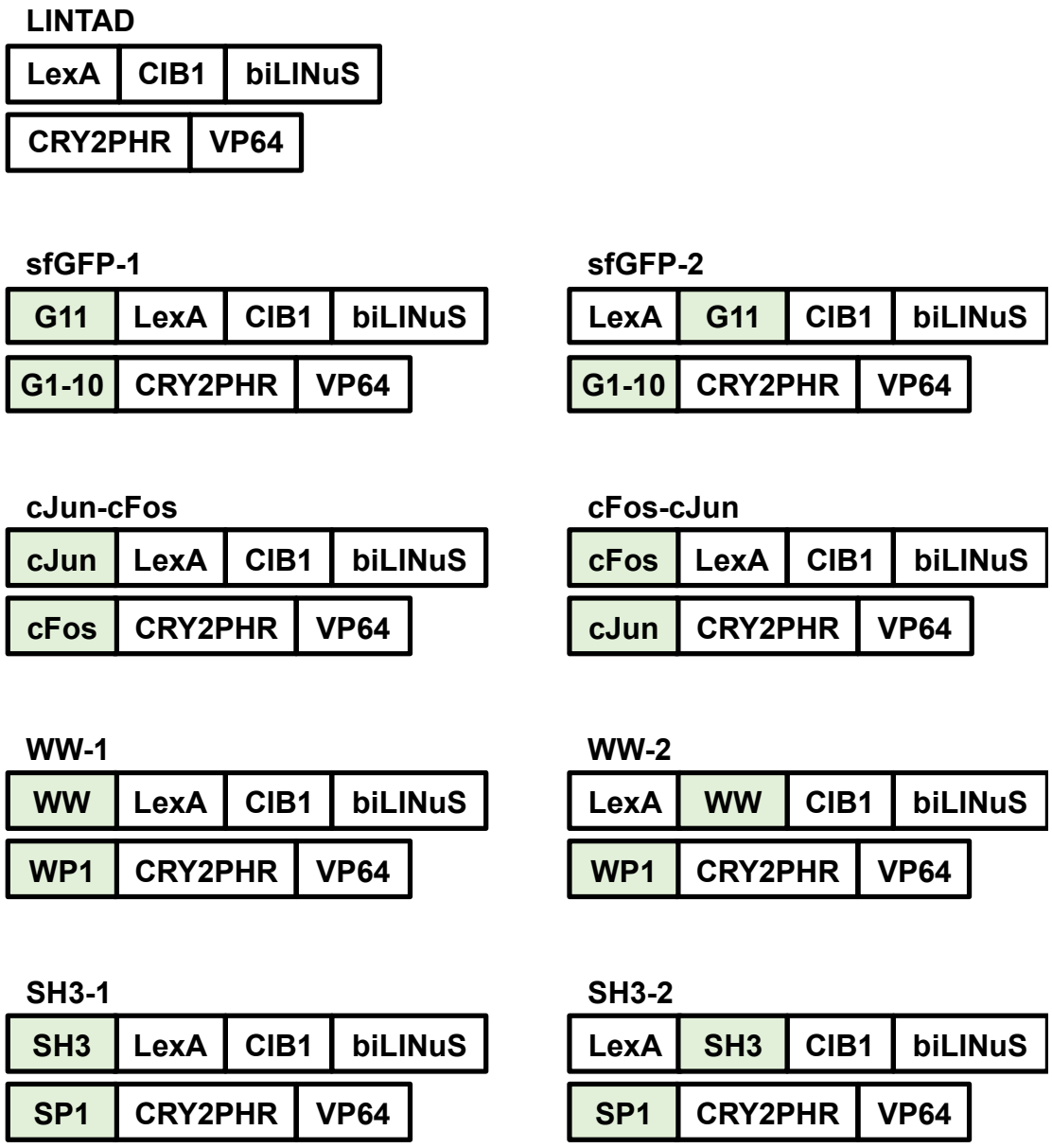

a
Dark
Q1
53.5
Q2
1.70
Q3
42.3
Q4
2.48
eGFPp2aCV
CD19CAR-Alexa647
b
Light
Q1
46.0
Q2
11.3
Q3
40.1
Q4
2.61
eGFPp2aCV
CD19CAR-Alexa647
c
VP64 Dark
VP64 12 hr
VP64 24 hr
VPR Dark
VPR 12 hr
VPR 24 hr
Relative mCherry
LCB-2A-tBFP
eGFP-2A-CV or eGFP-2A-CVP64

Fig. S6. The level of LINTAD gene activation depends on the expression level of regulators. (**a**-**b**) HEK 293T cells transfected with eGFP-2A-CV, and LCB-2A-tBFP and CD19CAR reporter cassette were stimulated with light ("Light") or kept in dark ("Dark") for 24 hr before flow cytometry measurement. Induced CAR expression were measured by anti-CAR antibody staining (goat anti-mouse IgG $F(ab')_2$ fragment, Jackson Immuno-Research Laboratories, # 115-606-072). A threshold on eGFP-2A-CV expression for efficient CAR induction can be clearly seen from the flow cytometry chart. (**c**) Three-dimensional plots showing the relationship between gene induction and regulator expression levels. Human primary T cells were infected with viral vectors expressing LCB-2A-tBFP and eGFP-2A-CV (or eGFP-2A-CVP64 using VP64 instead of VPR as the transcriptional activator as indicated in figure) and the viral vector of light-inducible mCherry reporter. Number on axes indicate relative expression levels of each regulator component. mCherry expression level is represented by color, with cold and hot colors representing low and high levels, respectively.

a


LINTAD
LexA
CIB1
biLINuS
CRY2PHR
VP64
sfGFP-1
G11
LexA
CIB1
biLINuS
G1-10
CRY2PHR
VP64
sfGFP-2
LexA
G11
CIB1
biLINuS
G1-10
CRY2PHR
VP64
cJun-cFos
cJun
LexA
CIB1
biLINuS
cFos
CRY2PHR
VP64
cFos-cJun
cFos
LexA
CIB1
biLINuS
cJun
CRY2PHR
VP64
WW-1
WW
LexA
CIB1
biLINuS
WP1
CRY2PHR
VP64
WW-2
LexA
WW
CIB1
biLINuS
WP1
CRY2PHR
VP64
SH3-1
SH3
LexA
CIB1
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SH3
CIB1
biLINuS
SP1
CRY2PHR
VP64

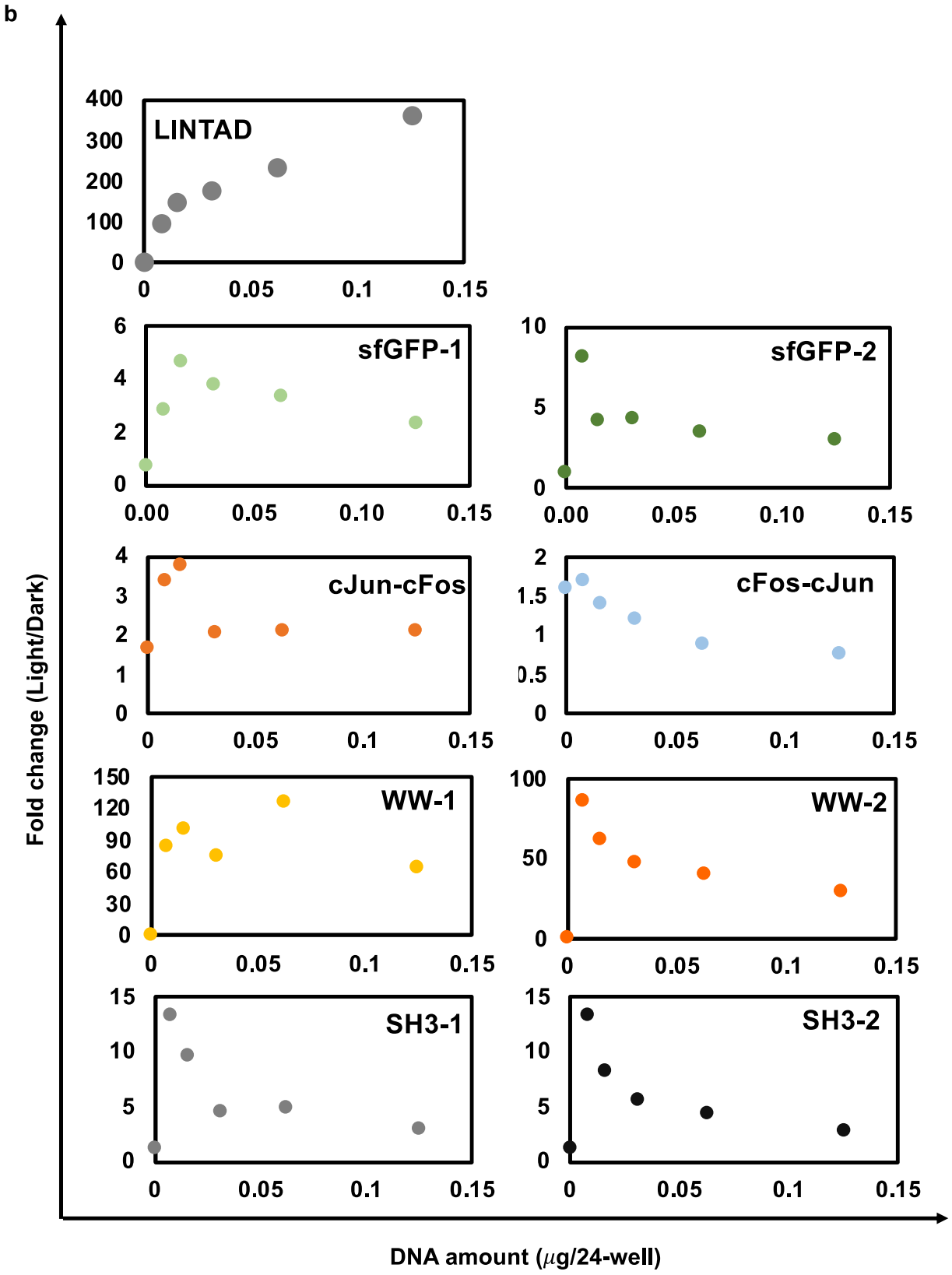

b
LINTAD
sfGFP-1
sfGFP-2
cJun-cFos
cFos-cJun
WW-1
WW-2
SH3-1
SH3-2
Fold change (Light/Dark)
DNA amount (μg/24-well)

Fig. S7. Engineering of LINTAD with weak dimer helpers. (**a**) Construct designs of LINTAD with various weak dimer helpers. LINTAD, original LINTAD system without weak dimer helper. G1-10, split super folder GFP1-10 fragment. G11, split super folder GFP11 fragment. Number (-1 or -2) after name of each group indicates different designs of the same weak dimer pair. (**b**) Comparison of light-induction capability of the above systems at different regulator expression levels. HEK 293T cells cultured in 24-well plates were transfected with different amounts of regulator-expressing constructs (DNA amounts were indicated at X-axis) and dual-luciferase reporter cassettes (same amount for all the groups). Cells were stimulated with blue light (Light) or kept in dark (Dark) for 24 hr before luciferase measurement. "Fold change" represents the ratio of $(Fluc/Rluc)_{Light}/(Fluc/Rluc)_{Dark}$, to compare the light induction capability of each design, where Fluc and Rluc represent the relative luciferase activities of firefly luciferase and *Renilla* luciferase, respectively.

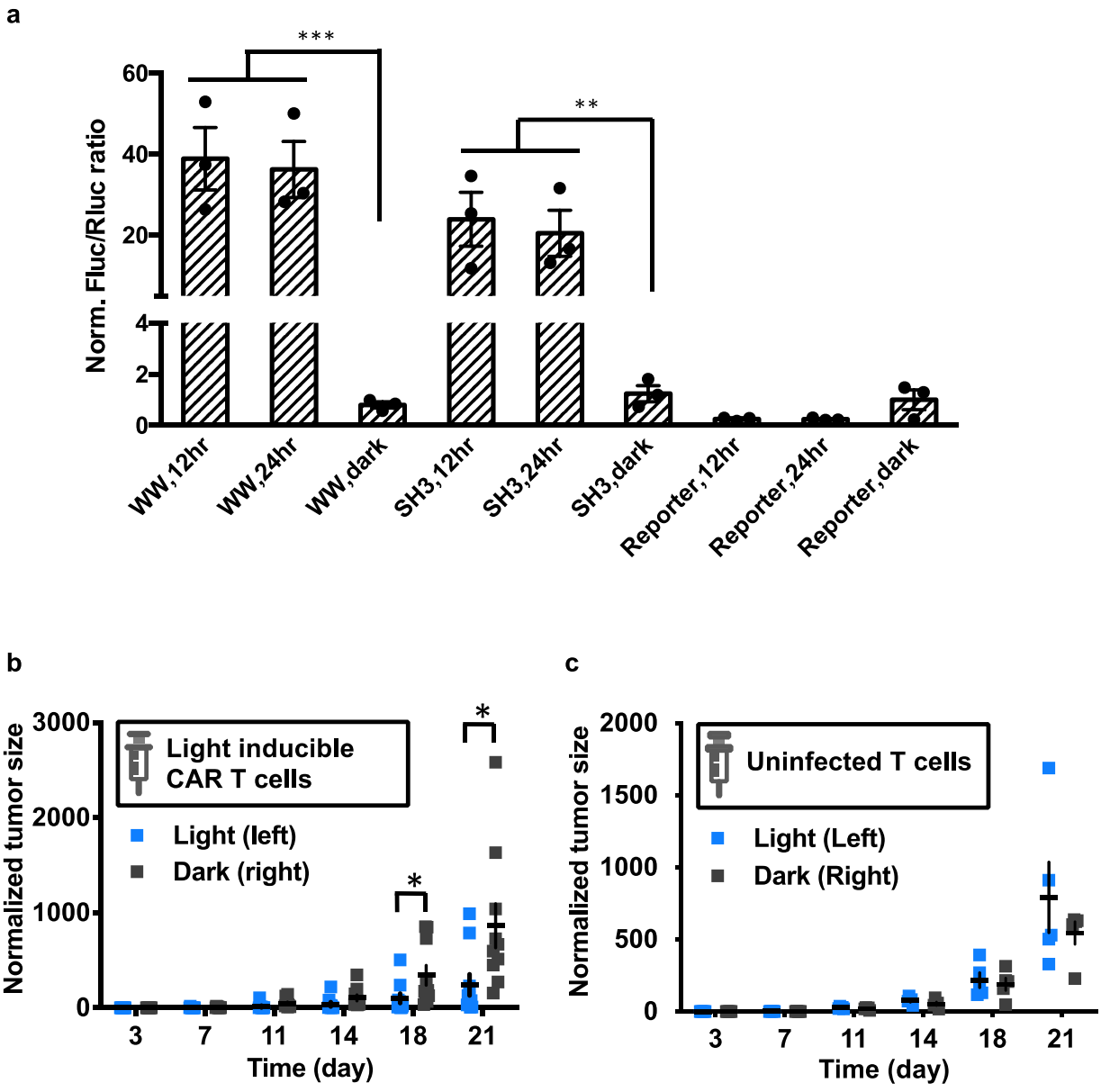
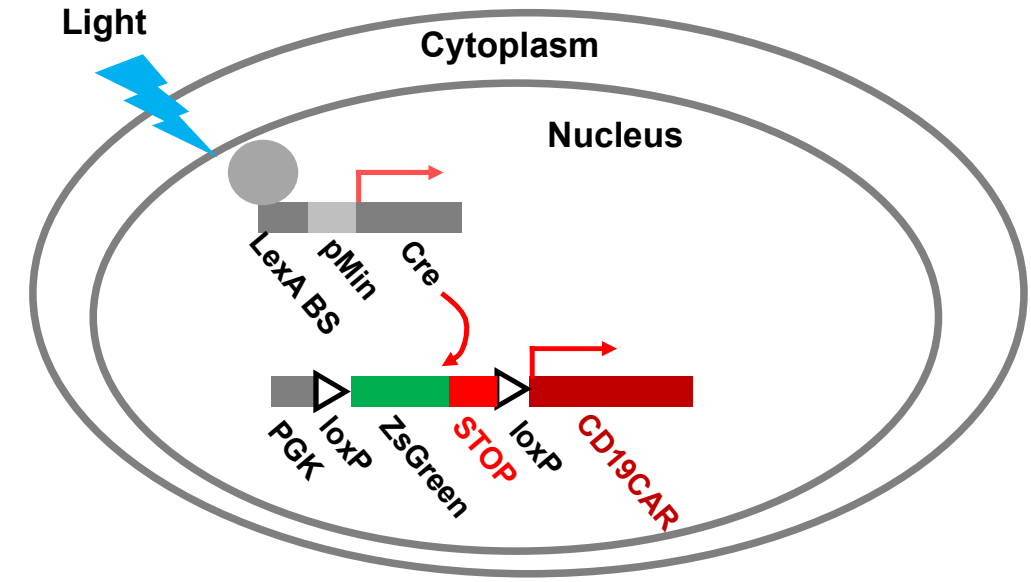

a
***
**
60
40
20
4
2
0
Norm. Fluc/Rluc ratio
WW,12hr
WW,24hr
WW,dark
SH3,12hr
SH3,24hr
SH3,dark
Reporter,12hr
Reporter,24hr
Reporter,dark
b
Light inducible CAR T cells
Light (left)
Dark (right)
Normalized tumor size
3000
2000
1000
0
*
*
3
7
11
14
18
21
Time (day)
c
Uninfected T cells
Light (Left)
Dark (Right)
Normalized tumor size
2000
1500
1000
500
0
3
7
11
14
18
21
Time (day)

Fig. S8. LINTAD with weak dimer helpers in T cells. Primary human T cells were infected with viral vectors expressing WW-2 (indicated as "WW" in figure) or SH3-2 (indicated as "SH3" in figure, see **Fig. S7a**) and viral vector of dual-luciferase reporter. Cells were stimulated with light (12 hr or 24 hr) or kept in dark (Dark) before measurement. Reporter, cells infected with reporter viral vector only. Error bar: standard deviation. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. Error bars, SEM. Two-tailed Student's t-test. (**b-c**) *In vivo* light stimulation of LINTAD CAR T cells can control cytotoxicity with high spatial resolution (plots showing individual data points for Fig. 6c-d). (b) LINTAD CAR T cells were subcutaneously injected into two sites of the same mice (left and right flanks), where tumor cells (Nalm-6 with constitutive Fluc) had been inoculated 4 days before. Left side was illuminated with blue light for 12 hr (light). Right side was covered with foil (dark). Tumor size was measured by Fluc luminescence reading. All luminescence values were normalized to that of the same tumor on day 3 after tumor inoculation. (c) Same experiment procedures as in b, but uninfected T cells were used instead of LINTAD CAR T cells. Tumor size was measured by Fluc luminescence reading. All luminescence values were normalized to that of the same tumor on day 3 after tumor inoculation. Error bar, SEM (n = 5 mice in each group). Two-tailed Student's t test. Error bar, SEM (n = 5 mice in each group). Two-tailed Student's t test. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

a


Light
Cytoplasm
Nucleus
LexA BS
pMin
Cre
PGK
loxP
ZsGreen
STOP
loxP
CD19CAR

b

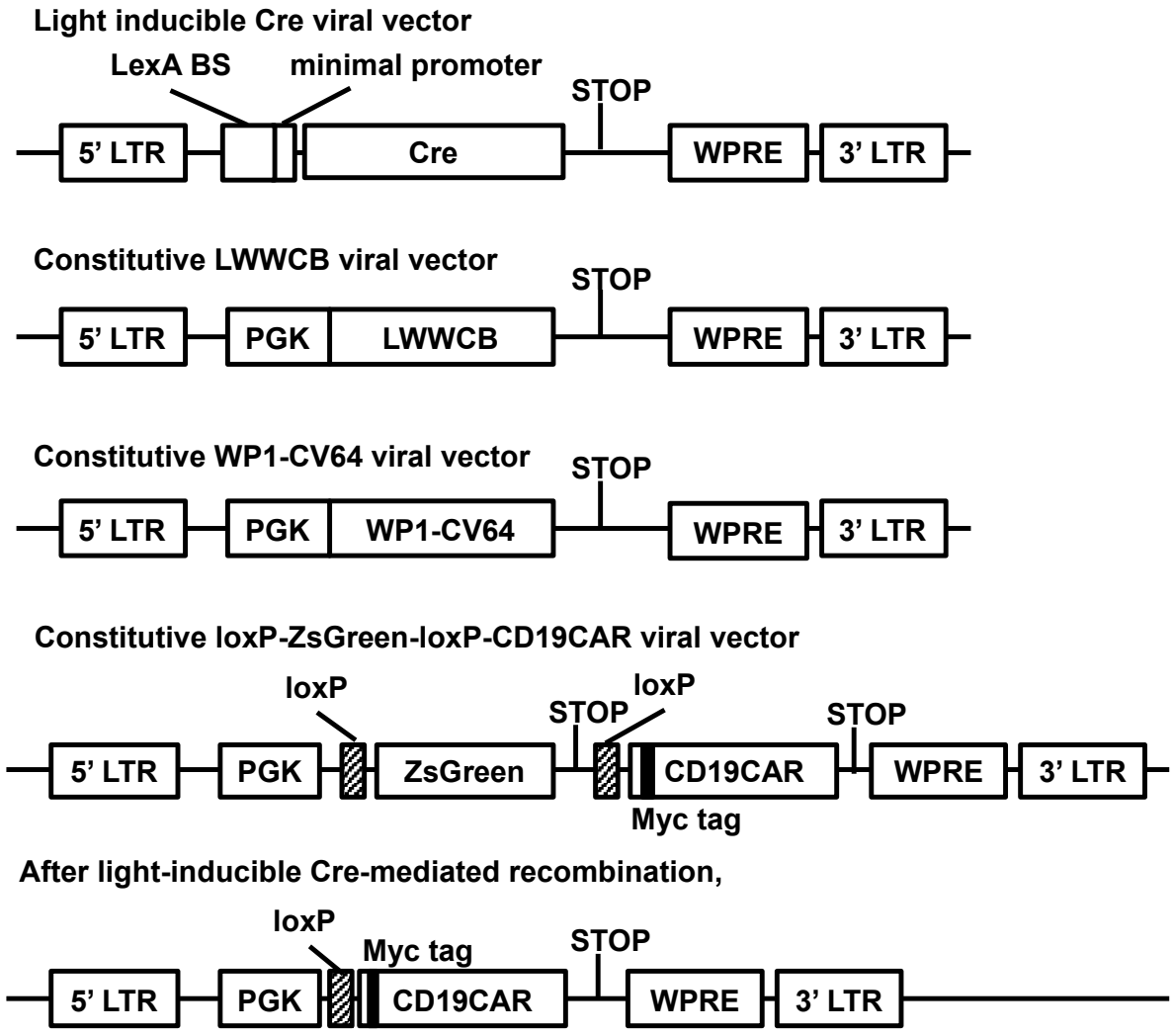

Light inducible Cre viral vector
LexA BS
minimal promoter
STOP
5' LTR
Cre
WPRE
3' LTR
Constitutive LWWCB viral vector
STOP
5' LTR
PGK
LWWCB
WPRE
3' LTR
Constitutive WP1-CV64 viral vector
STOP
5' LTR
PGK
WP1-CV64
WPRE
3' LTR
Constitutive loxP-ZsGreen-loxP-CD19CAR viral vector
loxP
STOP
loxP
STOP
5' LTR
PGK
ZsGreen
CD19CAR
WPRE
3' LTR
Myc tag
After light-inducible Cre-mediated recombination,
loxP
Myc tag
STOP
5' LTR
PGK
CD19CAR
WPRE
3' LTR

Fig. S9. Design and lentiviral vectors used for in vivo cytotoxicity studies. (**a**) Schematics of light-inducible Cre reporter and loxP-ZsGreen-STOP-loxP-CD19CAR cassettes in the cells (LINTAD regulators not shown). Once LINTAD system in the cell is activated by light, Cre can be induced to express and catalyze recombination between loxP sites on the loxP-ZsGreen-STOP-loxP-CD19CAR cassette, thus switching on constitutive CD19CAR expression in the cells. (**b**) Lentiviral vectors for expression of WW-LINTAD (WW-2 in **Fig. S7a**) and light-inducible Cre and loxP-ZsGreen-STOP-loxP-CD19CAR (before and after recombination) cassette. All viral vectors mentioned above are shown.

Supplementary Table

Table S1. Plasmids used in this study.

Supplementary Movies

Movie S1. Light-induced nuclear translocation of LCB of the LINTAD system. HEK 293T cells were transfected with LexA-CIB1-mCherry-biLINuS (LCmB) to track the subcellular localization of LCB before (0 s) and after blue light stimulation (460 nm, 0.5 s/ 30 s, ~10 mW/cm^2).

Movie S2. Light-inducible mNeonGreen expression in HEK 293T cells engineered with LINTAD gene activation system and the light-inducible mNeonGreen reporter. HEK 293 T cells were transfected with LCB, CV and light-inducible mNeonGreen reporter constructs. Blue light stimulation (460 nm, 0.5 s / 30 s, ~10 mW/cm^2) was started at 24 hr after transfection for 24 hr (from 00:00 to 24:00).