# Supplemental information

# The CDK4/6-EZH2 pathway is a potential therapeutic target for psoriasis

**Anne Müller[1], Antje Dickmanns[2], Claudia Resch[1], Knut Schäkel[3], Stephan Hailfinger[1,4], Matthias Dobbelstein[2], Klaus Schulze-Osthoff[1,5], Daniela Kramer[1,*]**

[1] Interfaculty Institute for Biochemistry, University of Tübingen, 72076 Tübingen, Germany

[2] Institute of Molecular Oncology, Göttingen Center of Molecular Biosciences (GZMB), University of Göttingen, 37077 Göttingen, Germany

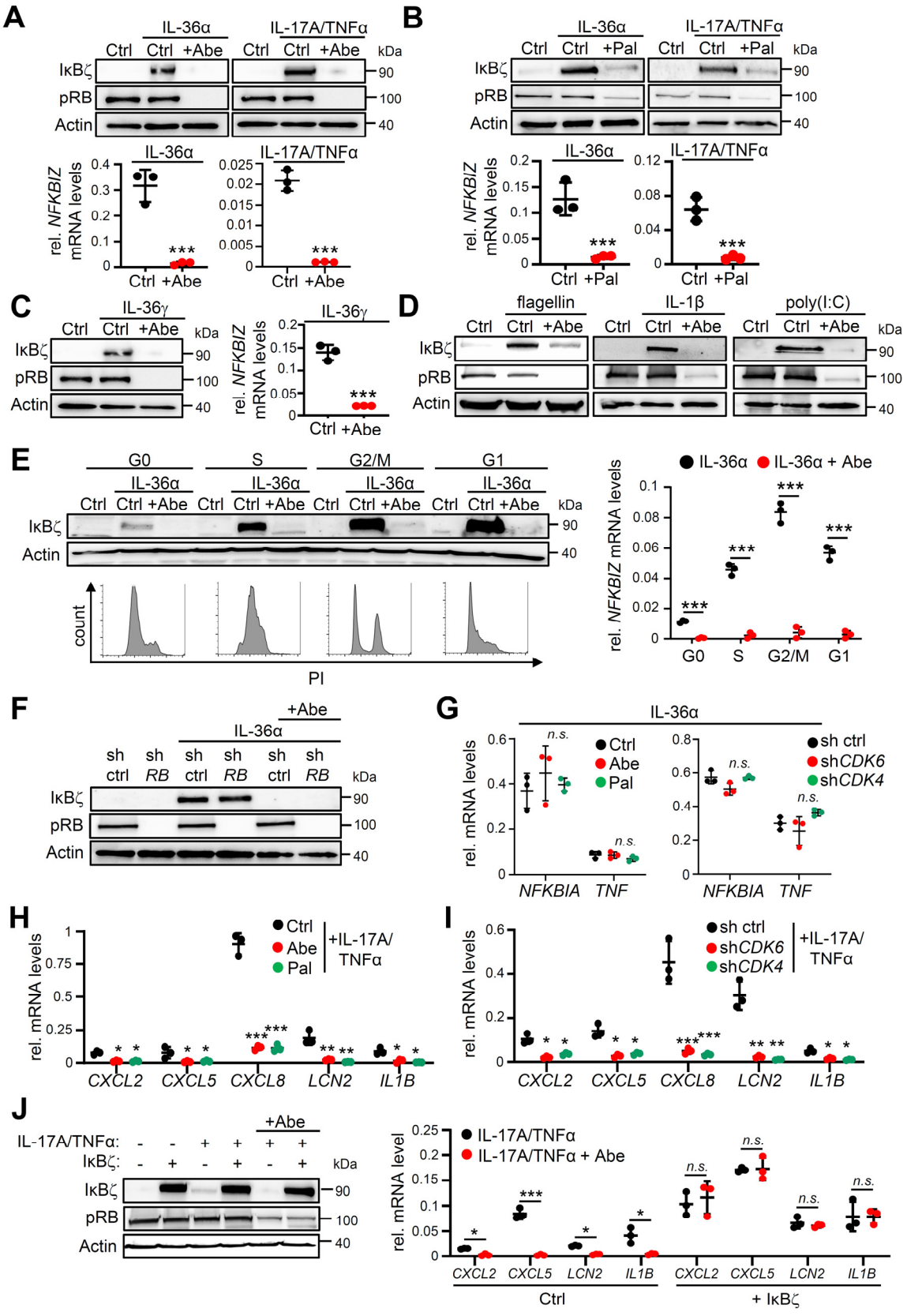
[3] Department of Dermatology, Heidelberg University Hospital, 69120 Heidelberg, Germany

[4] Cluster of Excellence iFIT (EXC 2180) "Image-Guided and Functionally Instructed Tumor Therapies", University of Tübingen, Germany

[5] German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany

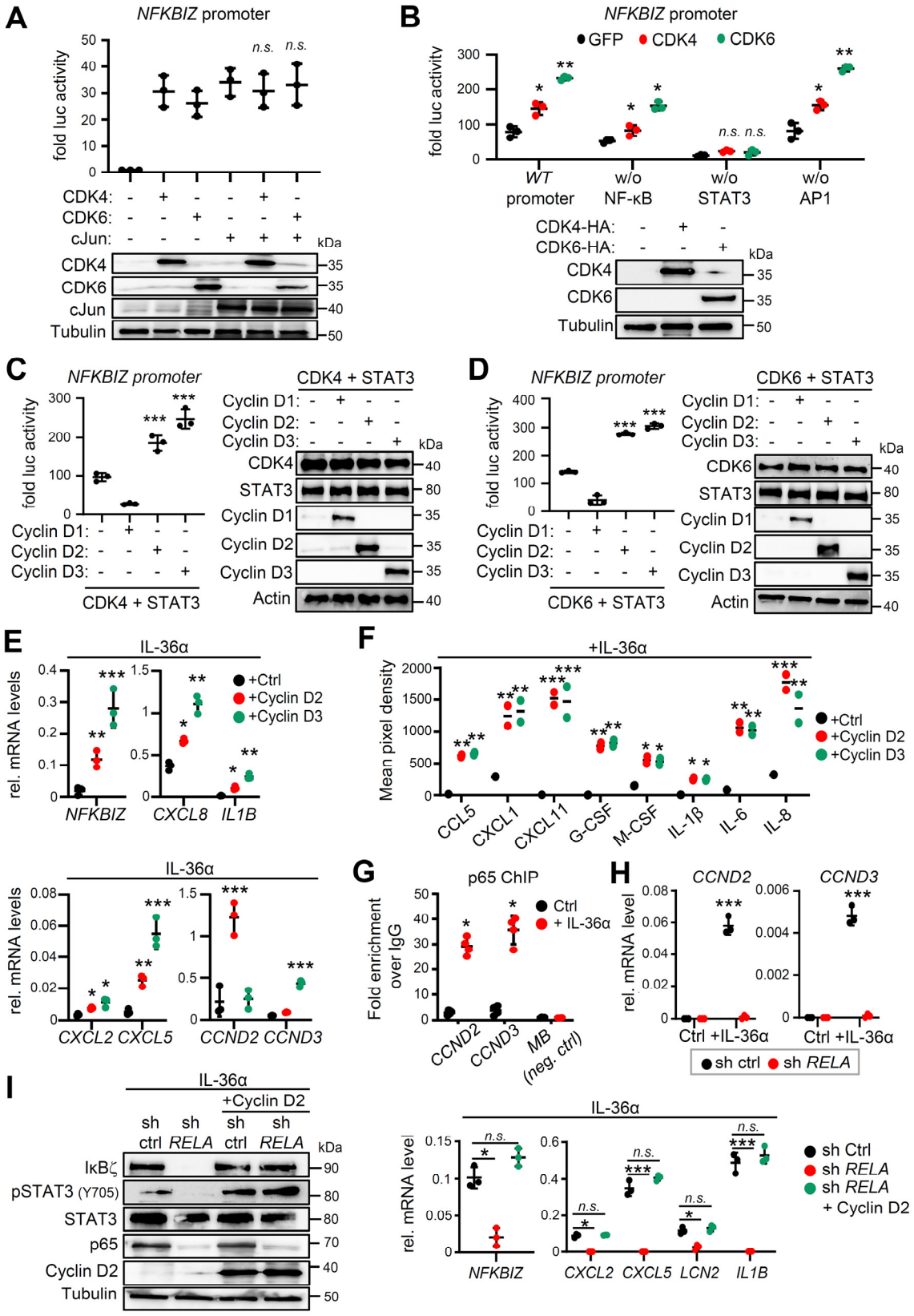
[*] To whom correspondence should be addressed:

Daniela Kramer, Interfaculty Institute for Biochemistry, Hoppe-Seyler-Str.4, 72076 Tübingen, Germany, phone: +49-7071-2974159, email: daniela.kramer@uni-tuebingen.de



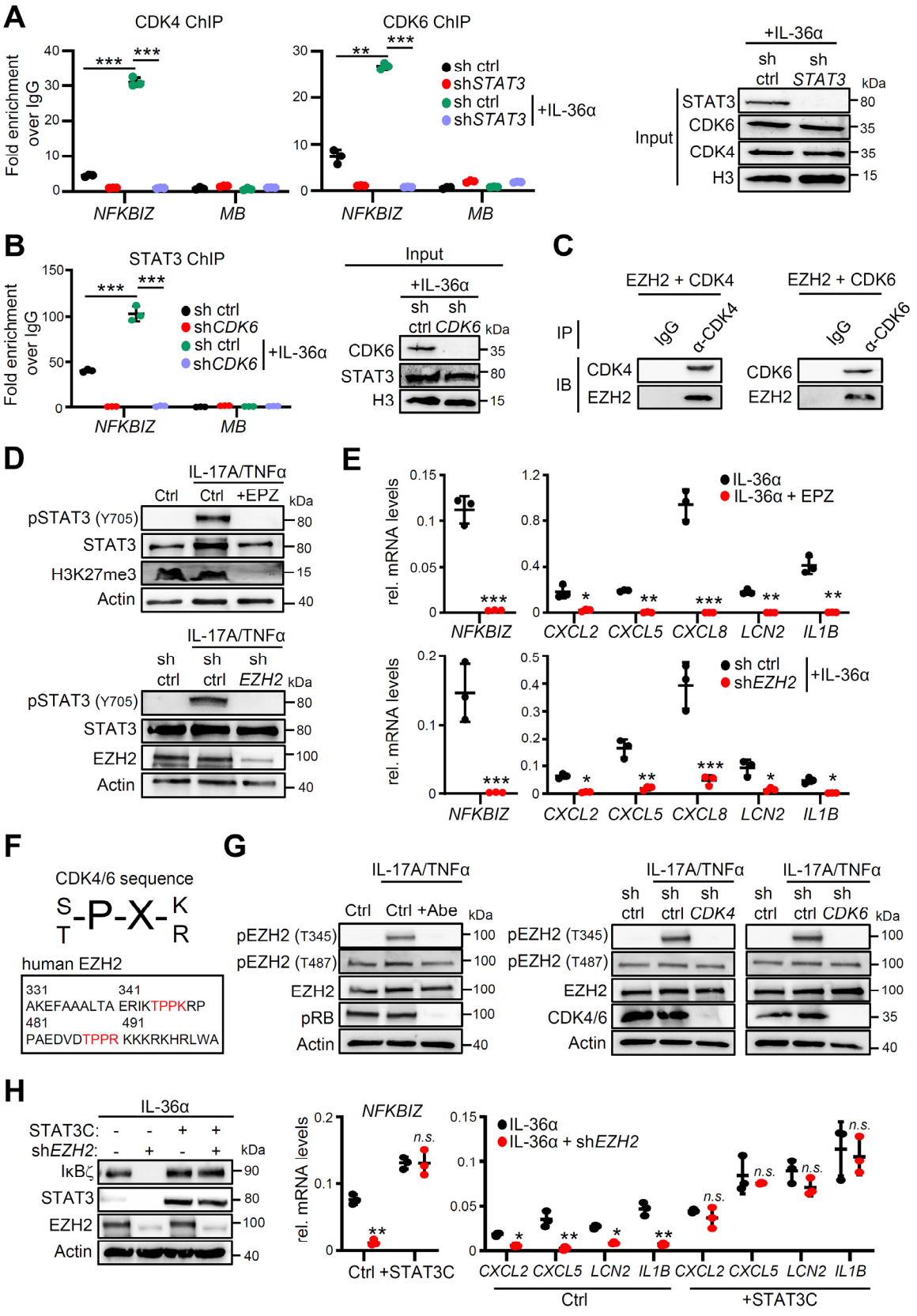

**Supplemental Figure 1. Effects of CDK4/6 inhibition on IL-17A/TNFα-stimulated keratinocytes and analysis of possible cell cycle effects.**

**(A)** IκBζ protein and mRNA (*NFKBIZ*) expression in human primary keratinocytes stimulated for 24 h with IL-36α or IL-17A/TNFα with or without abemaciclib (Abe, added for 2 h). Suppression of Rb phosphorylation controlled effective CDK4/6 inhibition. **(B)** IκBζ expression in human primary keratinocytes stimulated for 1 h with 100 ng/mL IL-36α or 200 ng/mL IL-17A and 10 ng/mL TNFα with or without palbociclib (Pal). **(C + D)** IκBζ expression in primary human keratinocytes treated with IL-36γ (100 ng/mL for 1 h) (C), flagellin, IL-1β (both 100 ng/mL for 1h), or poly(I:C) (10 ng/mL for 4 h) in the presence or absence of abemaciclib (D). **(E)** IκBζ expression in synchronized HaCaT cells. Cells were synchronized by double thymidine block. At different times after release (0 -16 h), cells were stimulated in the different cell cycle phases for 1 h with 100 ng/mL IL-36α in the presence or absence of abemaciclib (Abe). The different cell cycle phases at the time of cell harvesting were controlled by PI staining. **(F)** IκBζ protein levels in *RB*-deficient HaCaT cells treated with IL-36α and abemaciclib. **(G)** Expression of IκBζ-independent genes in CDK4/6 inhibitor-treated and *CDK4/6*-depleted keratinocytes. Stimulation as in (B). **(H + I)** Expression of IκBζ target genes in human primary keratinocytes stimulated for 1 h with 100 ng/mL IL-17A and 10 ng/mL TNFα**,** following CDK4/6 inhibition (H) or shRNAs (I). **(J)** Effect of IκBζ overexpression on CDK4/6-controlled cytokine expression in IL-17A/TNFα-stimulated primary keratinocytes. Treatment as in (B). All analyses: $n = 3 \pm$ SD. Significance was calculated using a 1-way ANOVA for multiple groups and a 2-tailed Student's t-test comparing two groups: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, *n.s.* = not significant.



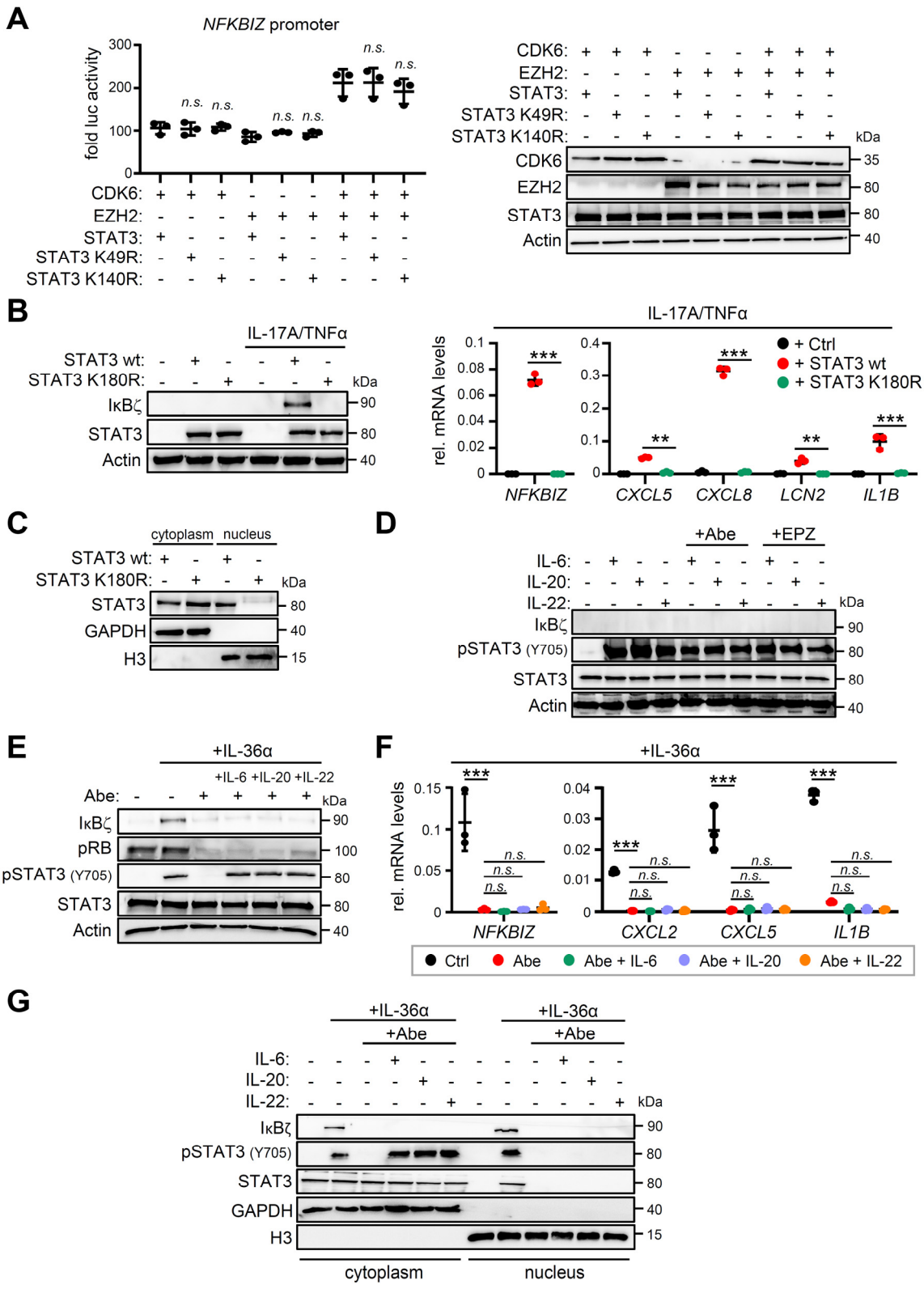

**Supplemental Figure 2. CDK4/6 regulate STAT3-mediated IκBζ induction in a cyclin D2/D3-dependent manner. (A)** *NFKBIZ* promoter activity in HEK293T cells transiently overexpressing CDK4, CDK6 or cJun, alone or in combination. Relative luciferase activity was

normalized to co-transfected Renilla luciferase control. **(B)** Analysis of the *NFKBIZ* promoter in IL-36α-stimulated HaCaT cells using luciferase constructs that harbor deletions of NF-κB, STAT3 or AP1 binding sites. CDK4, CDK6 or GFP as control were transiently co-overexpressed in parallel. Relative luciferase activity was normalized to co-transfected Renilla control. **(C + D)** *NFKBIZ* promoter activity in HEK293T cells transiently overexpressing STAT3 and CDK4 (C) or STAT3 and CDK6 (D)**,** alone or in combination with cyclin D1, cyclin D2 and cyclin D3 overexpression. **(E)** Gene expression in primary human keratinocytes transiently overexpressing cyclin D2 or cyclin D3. Cells were stimulated for 1 h with 100 ng/mL IL-36α. Relative mRNA levels were normalized to *RPL37A*. (Ctrl = cells overexpressing empty control vector). **(F)** Cytokine levels in supernatants of cells, treated for 24 h with IL-36α, similar as in (E). *n* = 2. **(G)** p65 binding to the *CCND2* and *CCND3* locus in primary human keratinocytes. Cells were treated for 5 min with 100 ng/mL IL-36α. **(H)** *CCND2* and *CCND3* mRNA levels in control knockdown (Ctrl) or *RELA* knockdown cells, treated for 15 min with IL-36α, similar as in (E). **(I)** Transient overexpression of cyclin D2 in control or *RELA*-depleted primary human keratinocytes, treated for 1 h with IL-36α, similar as in (E). Significance was calculated using a 1-way ANOVA for multiple groups and a 2-tailed Student's t-test comparing two groups: *p < 0.05; **p < 0.01; ***p < 0.001, *n.s.* = not significant. All analyses: *n* = 3 ± SD.



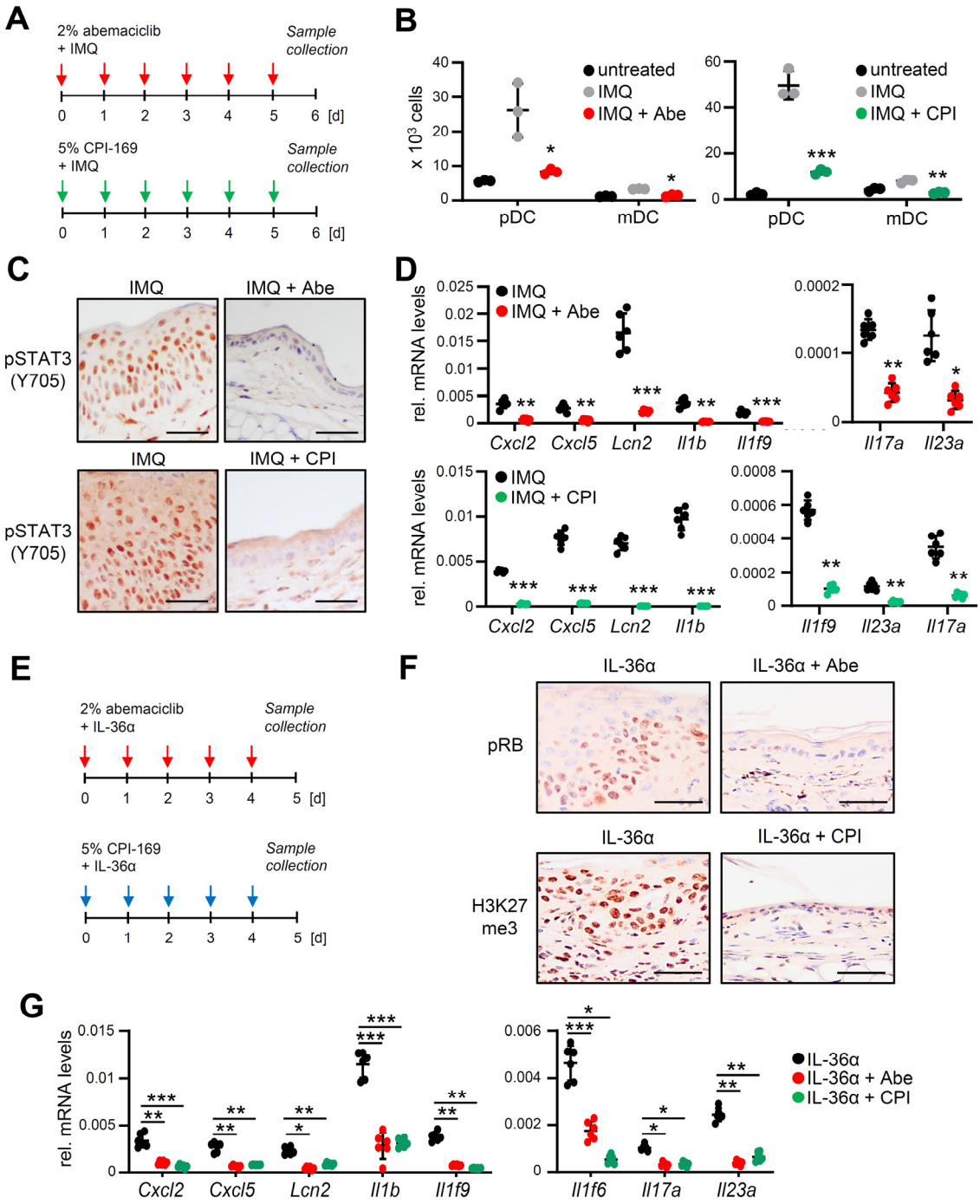

**Supplemental Figure 3. Extended analysis of CDK4/6-mediated phosphorylation of EZH2 at T345 that induces STAT3 activation. (A)** Chromatin immunoprecipitation (ChIP) of CDK4, CDK6 or IgG (control). Control or *STAT3*-deficient HaCaT cells were treated for 30 min

with 100 ng/mL IL-36α. Relative binding was calculated as the fold enrichment over IgG. (*MB* = myoglobulin promoter; internal negative control). Equal CDK4/6 and STAT3 levels were controlled by immunoblot analysis of the ChIP input. **(B)** STAT3 ChIP in IL-36α-stimulated, *CDK6*-deficient cells. Stimulation and analysis as in (A). **(C)** Detection of CDK4/6 interaction with EZH2 in HEK293T cells. EZH2 was transiently overexpressed together with CDK4 or CDK6. CDK4/6-EZH2 complexes were pulled down using a CDK4- or a CDK6-specific antibody or control IgG. **(D)** STAT3 activity was analyzed by immunoblot detection of phosphorylated STAT3 (Y705) in keratinocytes, treated for 1 h with 100 ng/mL IL-17A and 10 ng/mL TNFα in the presence or absence of active EZH2. Detection of H3K27me3 controlled effective EZH2 inhibition (EPZ = EPZ6438 or *EZH2* shRNA knockdown). **(E)** Gene expression in EPZ6438-treated or *EZH2*-depleted human primary keratinocytes (Treatment: 100 ng/mL IL-36α). mRNA levels were normalized to *RPL37A*. **(F)** CDK4/6 substrate sequence and putative CDK phosphorylation sites of human EZH2 (marked in red). **(G)** Analysis of EZH2 activation by immunoblot detection of T345- or T487-phosphorylated EZH2 in IL-17A/TNFα-treated keratinocytes. Cells were treated as in (D), with or without abemaciclib (Abe) or *CDK4/6*-specific shRNAs. **(H)** Analysis of IL-36α-mediated IκBζ induction and target gene expression in *EZH2*-depleted HaCaT cells, which overexpress a hyperactive STAT3 (STAT3C) version. Treatment: 1 h with 100 ng/mL IL-36α. Significance was calculated using a 1-way ANOVA for multiple groups and a 2-tailed Student's t-test comparing two groups: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, *n.s.* = not significant. All analyses: $n = 3 \pm$ SD.



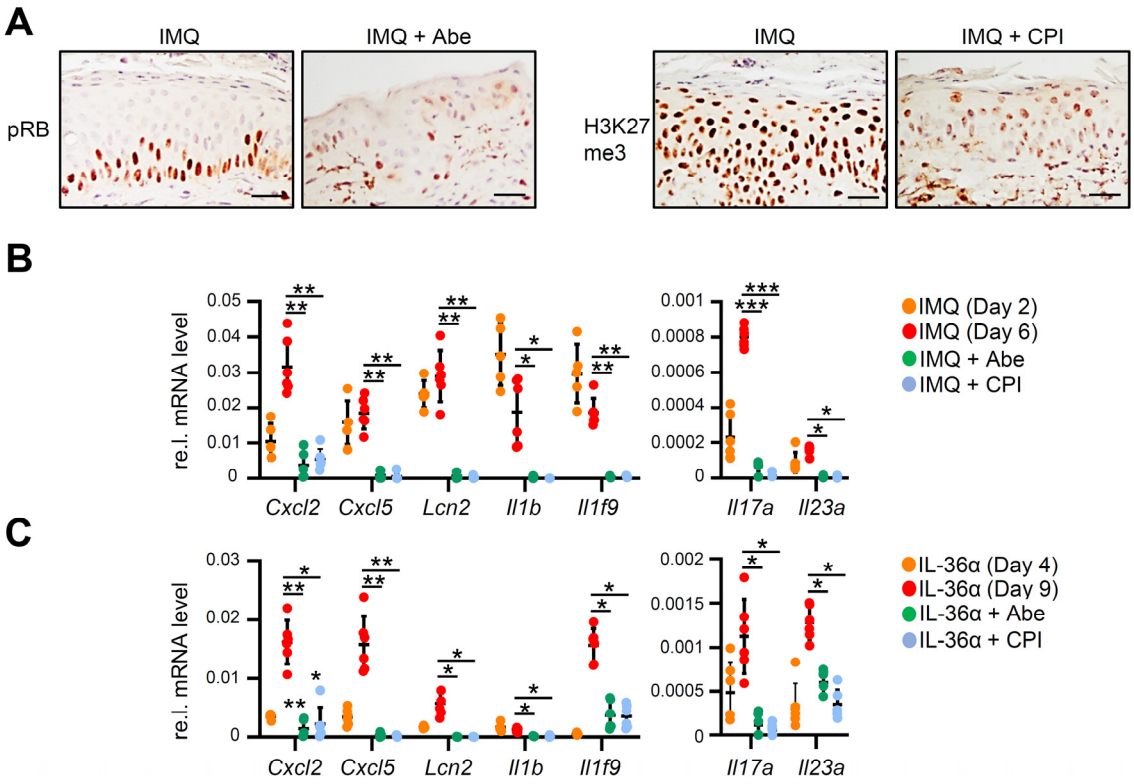

**Supplemental Figure 4. Extended analysis of EZH2-mediated methylation of STAT3 in keratinocytes. (A)** Analysis of *NFKBIZ* promoter activity in HEK293T cells transiently overexpressing CDK6 and EZH2, alone or in combination with wildtype STAT3 or mutant STAT3 K49R and K140R. Relative luciferase activity was normalized to co-transfected Renilla

control. **(B)** Expression of IκBζ and its target genes in *STAT3* KO HaCaT cells, transiently overexpressing wildtype STAT3 or STAT3 K180R. Cells were treated for 1 h with 200 ng/mL IL-17A and 10 ng/mL TNFα. Relative mRNA levels of *NFKBIZ* and its target genes were normalized to *RPL37A*. **(C)** *STAT3* KO HaCaT cells transiently overexpressing wildtype STAT3 or mutant STAT3 (K180R) were stimulated for 1 h with 100 ng/mL IL-36α, followed by nuclear fractionation of the cells and immunoblot analysis. GAPDH and H3 were used as markers for the cytoplasmic and nuclear fraction, respectively. **(D)** Primary human keratinocytes were treated for 1 h with 100 ng/mL IL-6, IL-20 or IL-22 in the presence or absence of abemaciclib (Abe) or EPZ6438 (EPZ). **(E + F)** Primary human keratinocytes were treated for 1 h with 100 ng/mL IL-36α, in the presence or absence of abemaciclib (Abe), IL-6, IL-20 or IL-22, similar as in (D). **(E)** Immunoblot analysis of IκBζ and pSTAT3 (Y705). Actin served as a loading control. **(F)** mRNA levels normalized to *RPL37A*, treated as in (E) **(G)** Nuclear fractionation of primary human keratinocytes treated as in (E). GAPDH and H3 were used as markers for the cytoplasmic and nuclear fraction, respectively. Significance was calculated using a 1-way ANOVA test: *p < 0.05; **p < 0.01; ***p < 0.001, *n.s.* = not significant. All analyses: $n = 3 \pm SD$.




**Supplemental Figure 5. Extended analysis of the in vivo effects of CDK4/6 or EZH2 inhibition on psoriasis induction in the imiquimod (IMQ)- and IL-36 mouse models. (A)** Treatment scheme for induction of IMQ-mediated, psoriasis-like skin inflammation. Mice received daily topical applications of IMQ-containing Aldara cream and abemaciclib (Abe; 10 µL of a 2% solution) or CPI-169 (CPI, 10 µL of a 5% solution). **(B)** Characterization of infiltrating dendritic cell subsets into the ears of IMQ-treated mice by flow cytometry at day 6. Plasmocytoid dendritic cells (pDC) were detected as CD45$^+$, CD11c$^+$, MHC-II$^+$, PDCA-1$^+$,

Siglec-H$^+$, and myeloid derived dendritic cells (mDC) were analyzed as CD45$^+$, CD11c$^+$, MHC-II$^+$, CD172a$^+$. *n* = 6 ears per group ± SEM. **(C)** IHC staining of phosphorylated STAT3 at Y705 (pSTAT3) in the epidermis of treated mice at day 6. Scale bar: 40 μM. **(D)** Gene expression analysis of IκBζ target genes in IMQ-, IMQ/Abe- and IMQ/CPI-treated skin samples at day 6. Relative mRNA expression was normalized to *Actin*. **(E)** IL-36α treatment scheme with topical application of abemaciclib or CPI-169 as in (A). 1 μg murine IL-36α or PBS control was intradermally injected into one ear of the mice for five consecutive days. **(F)** IHC staining in ear skin sections of PBS or IL-36α-treated mice at day 5. pRB and H3K27me3 served as a marker for effective inhibition of CDK4/6 and EZH2, respectively. Scale bar: 40 μM. **(G)** Expression of IκBζ target genes in IL-36α-, IL-36α/Abe- and IL-36α/CPI-treated skin samples of IL-36α-treated mice at day 5. All analyses: *n* = 6 per group ± SEM. Significance was calculated using a 1-way ANOVA test for multiple groups and a 2-tailed Student's t-test comparing two groups: *p < 0.05; **p < 0.01; ***p < 0.001.




**Supplemental Figure 6. Extended analysis of the in vivo effects of CDK4/6 or EZH2 inhibition on already established IMQ- and IL-36-mediated psoriasis-like skin inflammation. (A)** IHC staining of pRB (marker for CDK4/6 inhibition) and H3K27me3 (marker for EZH2 inhibition) at day 6 of IMQ treatment. **(B)** Gene expression analysis of IMQ-treated animals either at day 2 (starting time point of inhibitor treatment, IMQ day 2) or at day 6 (24 h after the last inhibitor application, IMQ day 6). Relative mRNA levels were normalized to *Actin*. **(C)** Gene expression analysis of IL-36α-treated animals either at day 4 (starting time point of inhibitor treatment, IL-36α day 4) or at day 9 (24 h after the last inhibitor application, IL-36α day 9). Relative mRNA levels were normalized to *Actin*. All analyses: *n* = 6 per group ± SEM. Significance was calculated using a 1-way ANOVA test: *p < 0.05; **p < 0.01; ***p < 0.001.

**Supplementary Table S1. Primers used for silent mutagenesis of STAT3.**

| | |
|---|---|
| K49R forward | GACTGGGCATATGCAGCCAGCAGAGAGTCACATGCCACG |
| K49R reverse | CACCAACGTGGCATGTGACTCTCTGCTGGCTGCATATGC |
| K140R forward | CGTAGTGACAGAGAGGCAGCAGATGTTG |
| K140R reverse | GGTTGTAGACGACGGAGAGACAGTGATG |
| K180R forward | AACTACAAAACCCTCAGGAGCCAAGGAGACATGC |
| K180R reverse | GTACAGAGGAACCGAGGACTCCCAAAACATCAAC |

**Supplementary Table S2. Gene expression primer.**

| | |
|---|---|
| Human_CXCL2_forward | TGATTTCACAGTGTGTGGTCAAC |
| Human_CXCL2_reverse | TCTCTGCTCTAACACAGAGGG |
| Human_CXCL5_forward | AGCGCGTTGCGTTTGTTTAC |
| Human_CXCL5_reverse | TGGCGAACACTTGCAGATTAC |
| Human_CXCL8_forward | AAGGTGCAGTTTTGCCAAGG |
| Human_CXCL8_reverse | CCCAGTTTTCCTTGGGGTCC |
| Human_LCN2_forward | AGAGCTACAATGTCACCTCCG |
| Human_LCN2_reverse | TTAATGTTGCCCAGCGTGAAC |
| Human_IL1B_forward | TCAGCCAATCTTCATTGCTCAAG |
| Human_IL1B_reverse | GGTCGGAGATTCGTAGCTGG |
| Human_NFKBIZ_forward | ACACCCACAAACCAACTCTGG |
| Human_NFKBIZ_reverse | TGCTGAACACTGGAGGAAGTC |
| Human_NFKBIA_forward | AAGTGATCCGCCAGGTGAAG |
| Human_NFKBIA_reverse | CTCACAGGCAAGGTGTAGGG |
| Human_TNF_forward | CAAGGACAGCAGAGGACCAG |
| Human_TNF_reverse | CCG GATCATGCTTTCAGTGC |
| Human_RPL37A_forward | AGATGAAGAGACGAGCTGTGG |
| Human_ RPL37A _reverse | CTTTACCGTGACAGCGGAAG |
| Human_CDK4_forward | TCTCGAGGCCAGTCATCCTC |
| Human_CDK4_reverse | GCAGTCCACATATGCAACACC |
| Human_CDK6_forward | GGTACAGAGCACCCGAAGTC |
| Human_CDK6_reverse | CTCCTGGGAGTCCAATCACG |
| Human_EZH2_forward | CTGCTTCCTACATCGTAAGTGC |
| Human_EZH2_reverse | GTGAGAGCAGCAGCAAACTC |
| Human_CCND2_forward | AGCTGTGCATTTACACCGAC |
| Human_CCND2_reverse | CATGCTTGCGGATCAGAGAC |
| Human_CCND3_forward | ACTGGCTCTGTTCGGATGC |
| Human_CCND3_reverse | AGCGCTGCTCCTCACATAC |
| Human_RELA_forward | AGGCTATCAGTCAGCGCATC |

| | |
|---|---|
| Human_RELA_reverse | AGCATTCAGGTCGTAGTCCC |
| Mouse_Cxcl2_forward | CGCCCAGACAGAAGTCATAGC |
| Mouse_Cxcl2_reverse | CTTTGGTTCTTCCGTTGAGGG |
| Mouse_Cxcl5_forward | CCCTACGGTGGAAGTCATAGC |
| Mouse_Cxcl5_reverse | GAACACTGGCCGTTCTTTCC |
| Mouse_Lcn2_forward | AATGTCACCTCCATCCTGGTC |
| Mouse_Lcn2_reverse | ACTGGTTGTAGTCCGTGGTG |
| Mouse_Il1b_forward | AGCTGAAAGCTCTCCACCTC |
| Mouse_Il1b_reverse | GCTTGGGATCCACACTCTCC |
| Mouse_Nfkbiz_forward | AACTCGCCAAGAGACCAGTG |
| Mouse_Nfkbiz_reverse | AGAGCCACTGACTTGGAACG |
| Mouse_Il1f6_forward | GCCTGTTCTGCACAAAGGATG |
| Mouse_Il1f6_reverse | ACAGCGATGAACCAACCAGG |
| Mouse_Il1f9_forward | GTCAGCGTGACTATCCTCCC |
| Mouse_Il1f9_reverse | TGGCTTCATTGGCTCAGGG |
| Mouse_Il23a_forward | CAGCTCTCTCGGAATCTCTGC |
| Mouse_Il23a_reverse | TGTCCTTGAGTCCTTGTGGG |
| Mouse_Il17a_forward | GCCCTCAGACTACCTCAACC |
| Mouse_Il17a_reverse | TTCCCTCCGCATTGACACAG |
| Mouse_Actin_forward | AGGAGTACGATGAGTCCGGC |
| Mouse_Actin_reverse | GGTGTAAAACGCAGCTCAGTA |
| Mouse _Ccnd2_forward | CTCACGTGTGATGCCCTGAC |
| Mouse _ Ccnd2_reverse | TGTTCAGCAGCAGAGCTTCG |
| Mouse _ Ccnd3_forward | TTGCATCTATACGGACCAGGC |
| Mouse _ Ccnd3_reverse | GAGACAGGCGGTGCAGAATC |
| Mouse_Ezh2_forward | ACTGCTTCCTACATCCCTTCC |
| Mouse_Ezh2_reverse | ACGCTCAGCAGTAAGAGCAG |