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

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Abstract

Psoriasis is a frequent inflammatory skin disease characterized by keratinocyte hyperproliferation and a disease-related infiltration of immune cells. Here, we identified a novel pro-inflammatory signaling pathway driven by the cyclin-dependent kinases (CDK) 4 and 6 and the methyltransferase EZH2 as a valid target for psoriasis therapy. Delineation of the pathway revealed that CDK4/6 phosphorylated EZH2 in keratinocytes, thereby triggering a methylation-induced activation of STAT3. Subsequently, active STAT3 resulted in the induction of IkBζ (IkappaBzeta), which is a key pro-inflammatory transcription factor required for cytokine synthesis in psoriasis. Pharmacological or genetic inhibition of CDK4/6 or EZH2 abrogated psoriasis-related pro-inflammatory gene expression by suppressing IkBζ induction in keratinocytes. Importantly, topical application of CDK4/6 or EZH2 inhibitors on the skin was sufficient to fully prevent the development of psoriasis in various mouse models by suppressing STAT3-mediated IkBζ expression. Moreover, we found a hyperactivation of the CDK4/6-EZH2 pathway in human and mouse psoriatic skin lesions. Thus, this study not only identifies a novel psoriasis-relevant pro-inflammatory pathway, but also proposes the repurposing of CDK4/6 or EZH2 inhibitors as a new therapeutic option for psoriasis patients.
Introduction

Psoriasis is a mixed autoimmune and autoinflammatory skin disease, affecting 2-3% of the population worldwide. Psoriatic skin lesions are characterized by keratinocyte hyperproliferation and a massive infiltration of immune cells, such as neutrophils, macrophages and Th17 cells (1). The cytokine families IL-17 and IL-36 have been identified as key factors driving the establishment of psoriatic plaques (2). Therefore, state-of-the-art therapies comprise neutralizing antibodies against IL-17 (3, 4), while IL-36 antagonists are currently tested in clinical trials (5). Although psoriasis therapy with neutralizing antibodies is very effective, disadvantages comprise high costs, difficult application routes, systemic side effects such as upper respiratory tract infections and long-term therapy resistance due to the development of anti-drug antibodies (6, 7). Therefore, effective new therapy approaches against psoriasis are needed.

Previously, IκBζ, encoded by the gene NFKBIZ, has been identified as a key regulator of transcription in psoriasis (8, 9). IκBζ represents an atypical member of the IκB family that is inducibly expressed and then accumulates in the nucleus, leading to the activation or repression of a selective subset of NF-κB target genes (10). Especially in keratinocytes, IL-17A, alone or even more potently in combination with TNFα, as well as IL-36 cytokines, trigger a NF-κB- and STAT3-dependent transcriptional upregulation of IκBζ expression (9). Subsequently, IκBζ induces a subset of IL-36- and IL-17-responsive target genes in keratinocytes, including CXCL2, CXCL5, CXCL8, LCN2, DEFB4 or IL1B, which all have already been implicated in the pathogenesis of psoriasis (2). How IκBζ regulates these downstream target genes remains elusive though. It is assumed that IκBζ recruits epigenetic modifiers, such as TET2 or the SWI/SNF complex, to the promoter sites of its target genes, leading to a change in DNA methylation or nucleosome remodeling (11, 12).

In agreement with its role as a key regulator of psoriasis-related gene expression, IκBζ-deficient mice are completely protected against imiquimod (IMQ) or IL-36-mediated psoriasis-
like skin inflammation (8, 9). Moreover, human psoriatic skin lesions are characterized by an upregulated expression of IκBζ (8, 9). Altogether, these findings validate IκBζ as an attractive new therapeutic target in psoriasis. As IκBζ lacks any enzymatic activity, it is difficult to develop direct IκBζ inhibitors (13). Therefore, small molecule inhibitors blocking the induction or downstream function of IκBζ could represent an alternative strategy for targeting IκBζ in psoriasis.

CDK4 and CDK6, in complex with cyclin D1, cyclin D2 or cyclin D3, represent well known cell-cycle regulating kinases that can phosphorylate RB, leading to the release of E2F transcription factors and G1-S cell cycle transition (14). Consistently, amplification of CDK4 and CDK6 as well as overexpression of cyclin D proteins are frequently observed events in cancer, leading to the excessive proliferation of tumor cells (15, 16). ATP-competitive CDK4/6 inhibitors, such as palbociclib and abemaciclib, have been developed for anti-cancer therapy and were recently approved for treatment of breast cancer patients (17). Interestingly, common side effects of a CDK4/6 inhibitor therapy constitute neutropenia and leukopenia (17, 18). Moreover, it was found that CDK4/6 inhibition modulates immune cell functions in kinase-dependent or independent manners (19-22). Mechanistically, it is assumed that these atypical functions of CDK4 and CDK6 derive from their recently discovered role as cofactors for immune regulatory transcription factors (23-25). Especially CDK6 can co-localize at promoter regions of a subset of NF-κB, STAT3 or AP1 target genes, thereby changing the DNA-binding properties or activity of these transcription factors.

We screened for small-molecule inhibitors of IκBζ action in keratinocytes and identified CDK4/6 inhibitors as potent suppressors of IL-36- and IL-17A/TNFα-mediated IκBζ expression. Mechanistically, CDK4/6 inhibitors suppressed the activity of STAT3, which was identified as a major transcriptional regulator of IκBζ expression in keratinocytes. STAT3 activation was promoted by CDK4/6-mediated phosphorylation of the methyltransferase EZH2, triggering the subsequent methylation of STAT3 and induction of IκBζ expression. Importantly, topical administration of CDK4/6 or EZH2 inhibitors on the skin completely prevented experimental
psoriasis by suppressing STAT3 activation and consequently, IκBζ expression in keratinocytes. Moreover, as cyclin D2, cyclin D3 and EZH2 were found to be overexpressed in human psoriatic skin lesions, we propose repurposing of CDK4/6 and EZH2 inhibitors for topical skin treatment of psoriasis patients.
Results

CDK4/6 inhibitors suppress the expression of IκBζ and IκBζ-dependent, pro-inflammatory genes in IL-36α- and IL-17A/TNFα-stimulated keratinocytes

IκBζ represents an attractive therapeutic target for psoriasis, however, due to a lack of enzyme activity, direct inhibition of IκBζ is not feasible. Key regulators in psoriasis constitute IL-17 and IL-36 family members (26, 27), which predominantly trigger a pro-inflammatory response in keratinocytes that is dependent on IκBζ (8, 9). Thus, we screened for small-molecule inhibitors that are able to block induction of IκBζ expression in response to either IL-36α or IL-17A. Previously, it was shown that IL-17-induced IκBζ expression is strongly increased in combination with TNFα (8, 9). Intriguingly, we found that two CDK4/6 inhibitors, abemaciclib (Figure 1A, Supplemental Figure 1A) and palbociclib (Supplemental Figure 1B), completely blocked IL-36α- or IL-17A/TNFα-mediated induction of IκBζ expression in primary human keratinocytes. Moreover, we observed similar effects in response to IL-36γ, IL-1β or the TLR ligands flagellin and poly(I:C) (Supplemental Figure 1C and D), thereby revealing a conservation of this pathway in keratinocytes.

To explore whether these effects were due to a CDK4/6 inhibitor-mediated G1-cell cycle arrest, we repeated the experiments in synchronized and single cell cycle phase-arrested keratinocytes. IL-36α treatment triggered IκBζ induction largely in all phases of the cell cycle, which was completely suppressed by abemaciclib (Supplemental Figure 1E). Moreover, depletion of RB by RNA interference did not influence IL-36-mediated induction or abemaciclib-mediated suppression of IκBζ (Supplemental Figure 1F), thereby clearly indicating that the effect of CDK4/6 inhibitors on IκBζ expression was independent of their ability to trigger cell cycle arrest. Instead, we revealed that CDK4/6-dependent induction of IκBζ was mediated at the transcriptional level, as palbociclib and abemaciclib treatment abrogated the expression of a luciferase construct harboring the NFKBIZ (IκBζ) promoter in IL-36α-stimulated HaCaT cells (Figure 1B). Interestingly, also shRNA-mediated depletion of CDK4 or CDK6 was sufficient to
suppress IL-36α- or IL-17A/TNFα-dependent expression of \( \text{IkB}\zeta \) in human primary keratinocytes, thereby excluding off-target effects of the applied inhibitors (Figure 1C and D).

Accordingly, \( \text{IkB}\zeta \)-dependent target genes, such as \( \text{CXCL2}, \text{CXCL5} \) or \( \text{CXCL8} \), were strongly downregulated in IL-36α- and CDK4/6 inhibitor-treated keratinocytes (Figure 1E), as well as in CDK4- or CDK6-deficient cells (Figure 1F), whereas other NF-κB-dependent, but \( \text{IkB}\zeta \)-independent genes, such as \( \text{NFKBIA} \) or \( \text{TNF} \), remained unaffected (Supplemental Figure 1G).

Similar effects of pharmacological or shRNA-mediated inhibition of CDK4/6 were obtained in IL-17A and TNFα-stimulated cells (Supplemental Figure 1H and I). CDK4/6 inhibitors have the potential to inhibit CDK9 kinase activity, although much higher concentrations are needed (17). To rule out effects deriving from the suppression of CDK9 activity, we transiently overexpressed CDK4, CDK6 or CDK9 in HaCaT cells and analyzed IL-36α-mediated gene expression. Overexpression of CDK4 and CDK6, but not CDK9 could increase IL-36α-mediated, \( \text{IkB}\zeta \)-dependent target gene expression in keratinocytes, thereby further confirming the specificity of CDK4 and CDK6 in regulating pro-inflammatory target gene expression in keratinocytes (Figure 1G).

We hypothesized that CDK4/6 are not involved in the direct regulation of \( \text{IkB}\zeta \) target gene expression but rather trigger the expression of \( \text{IkB}\zeta \), which in turn induces a secondary, \( \text{IkB}\zeta \)-dependent gene expression in stimulated keratinocytes. To test this hypothesis, we transiently overexpressed \( \text{IkB}\zeta \) in IL-36α- or IL17A/TNFα-stimulated primary human keratinocytes in the presence or absence of abemaciclib (Figure 1H, Supplemental Figure 1J). In this set-up, exogenous overexpression of \( \text{IkB}\zeta \) completely abolished abemaciclib-mediated suppression of \( \text{IkB}\zeta \) target gene expression, thereby validating CDK4/6-mediated transcriptional upregulation of \( \text{IkB}\zeta \) as a pre-requisite for CDK4/6-dependent, pro-inflammatory gene expression in keratinocytes.

**CDK4/6-dependent induction of \( \text{IkB}\zeta \) expression is mediated by STAT3 in a cyclin-dependent manner.**
Beside their known involvement in cell cycle regulation, CDK4 and CDK6 have been described to function as transcriptional cofactors for STAT3, NF-κB or AP-1 (23-25). As we revealed a CDK4/6-dependent induction of IκBζ on the transcriptional level, we next explored the responsible transcription factor. Of note, binding sites for all three transcription factors were previously identified at the **NFKBIZ** promoter region (9). Interestingly, expression of both CDK4 and CDK6 increased the STAT3-mediated induction of **NFKBIZ** promoter activity, whereas no synergistic effects could be observed when CDK4/6 were co-overexpressed with NF-κB p65 or cJun (Figure 2A, Supplemental Figure 2A). In agreement, deletion of the STAT3-binding site abrogated the expression of the **NFKBIZ** luciferase reporter in IL-36α-stimulated, CDK4/6-overexpressing HaCaT cells, whereas deletion of the NF-κB or AP1 motif had only a minor or almost no effect (Supplemental Figure 2B). Finally, transient overexpression of a constitutively active STAT3 mutant (STAT3C) abrogated the inhibitory effects of CDK4/6 inhibition on the induction of IκBζ (Figure 2B) and IκBζ-dependent target gene expression in IL-36α-stimulated primary keratinocytes (Figure 2C), thereby validating STAT3 as the responsible transcription factor for CDK4/6-mediated effects in keratinocytes.

Previous publications reported that CDK6 acts as a cofactor for STAT3, independently of its kinase function (23). Therefore, we tested if a kinase-dead mutant of CDK6 (CDK6 DN) could still synergize with STAT3 in driving the expression of the **NFKBIZ** luciferase reporter construct. Surprisingly, the kinase-dead mutant was not able to cooperate with STAT3 anymore, whereas a hyperactive version of CDK6 (CDK6 S178P) further increased the activity of the **NFKBIZ** promoter in a STAT3-dependent manner (Figure 2D). Accordingly, cyclin D2 and cyclin D3, which associate with CDK4/6 to activate their kinase function (14), synergized with CDK4/6 and STAT3 in activating the **NFKBIZ** luciferase promoter, whereas cyclin D1 failed to do so (Supplemental Figure 2C and D). Moreover, cyclin D2 and cyclin D3 overexpression significantly elevated the expression of **NFKBIZ** and its target genes in IL-36α-stimulated keratinocytes (Supplemental Figure 2E and F).
Cyclin D2 and cyclin D3 levels are transcriptionally regulated by NF-κB (28, 29). Therefore, we hypothesized that IL-36α or IL-17A/TNFα stimulation results in a transient NF-κB-dependent upregulation of cyclin D2/D3, thereby explaining the cooperation of CDK4/6 and STAT3 in triggering IκBζ expression. Indeed, we detected a rapid binding of NF-κB p65 to the promoter regions of \textit{CCND2} and \textit{CCND3} upon stimulation of primary keratinocytes with IL-36α (Supplemental Figure 2G). Consequently, IL-36α stimulation led to increased expression of \textit{CCND2} and \textit{CCND3} in a p65-dependent manner (Supplemental Figure 2H), thus validating a NF-κB-mediated transcriptional upregulation of cyclin D2 and cyclin D3 in stimulated keratinocytes. Although p65 failed to cooperate with CDK4/6 in the induction of the \textit{NFKBIZ} promoter directly (Figure 2A), we hypothesized that NF-κB participates in the induction of IκBζ in keratinocytes by transcriptionally upregulating cyclin D2/D3 levels leading to activation of CDK4/6. In agreement, whereas knockdown of p65/\textit{RELA} abrogated IκBζ expression in IL-36α-stimulated keratinocytes, exogeneous overexpression cyclin D2 could fully restore the expression of \textit{NFKBIZ} and its target genes in IL-36α-stimulated primary keratinocytes (Supplemental Figure 2I). Thus, our data imply that IL-36α and IL-17A/TNFα stimulation of keratinocytes first activates NF-κB, leading to an upregulation of cyclin D2 and D3 levels. Subsequently, CDK4/6 become activated, leading to a STAT3-mediated induction of IκBζ.

**CDK4/6 phosphorylate EZH2 to induce STAT3-mediated IκBζ expression.**

Next, we explored the mechanism of how CDK4/6 regulate STAT3-mediated expression of IκBζ. Of note, in chromatin immunoprecipitation (ChIP) analyses CDK4/6 was found to localize to the \textit{NFKBIZ} promoter region, which depended on the presence of STAT3 (Supplemental Figure 3A). \textit{Vice versa}, knockdown of \textit{CDK6} abrogated the binding of STAT3 at the \textit{NFKBIZ} promoter (Supplemental Figure 3B). We reasoned that this interdependency was due to a CDK4/6-dependent regulation of STAT3 activity in keratinocytes. Accordingly, whereas the putative CDK-dependent phosphorylation site of STAT3 at threonine 727 (T727) remained unaffected (30), phosphorylation of STAT3 at tyrosine 705 (Y705), a pre-requisite for STAT3 activation, was completely absent in abemaciclib-treated or CDK4/6-deficient cells after
stimulation with IL-36α (Figure 3A and B). As CDK4/6 are not able to directly trigger Y705 STAT3 phosphorylation, we assumed that CDK4/6-mediated activation of STAT3 might be exerted through an altered availability or activity of a cofactor needed for STAT3 activation in keratinocytes.

Previously, EZH2, a methyltransferase that directs H3K27me3 in conjunction with the PRC2 complex, was found to be important in the differentiation and function of keratinocytes (31-33). Moreover, it was revealed that EZH2 can methylate STAT3 at lysine 49, 140 or 180, thereby modulating STAT3 activity by affecting the subcellular localization or phosphorylation status of STAT3 at tyrosine 705 (34-36). We hypothesized that CDK4/6 might phosphorylate EZH2 in keratinocytes, thus enabling EZH2-mediated methylation and activation of STAT3. Pulldown assays in HEK293T cells validated an interaction of CDK4 and CDK6 with EZH2 (Supplemental Figure 3C). In agreement, EZH2 inhibition by EPZ6438 or shRNA-mediated depletion of EZH2 inhibited STAT3 activation and induction of IκBζ in IL-36α- or IL-17A/TNFα-stimulated keratinocytes (Figure 3C, Supplemental Figure 3D). Furthermore, pharmacological inhibition or depletion of EZH2 effectively prevented IκBζ-dependent target gene expression in IL-36α-treated keratinocytes (Supplemental Figure 3E). Thus, we hypothesized that CDK4/6 phosphorylates EZH2 in keratinocytes, thereby regulating EZH2-dependent activation of STAT3.

In primary human keratinocytes, expression of EZH2 itself was induced by IL-36α (Figure 3D), in line with its previous identification as an NF-κB-regulated target gene (37). Of note, EZH2 harbors two potential CDK phosphorylation sites at threonine 345 and 487 (Supplemental Figure 3F), which were previously shown to be phosphorylatable by CDK1/2, thereby modifying EZH2 function (38-40). Indeed, phosphorylation of EZH2 at threonine 345 (T345), but not at threonine 487 (T487) was induced in IL-36α- or IL-17A/TNFα-treated keratinocytes, whereas abemaciclib treatment or CDK4/6 depletion completely abrogated this inducible EZH2 phosphorylation (Figure 3D, Supplemental Figure 3G). Moreover, phosphorylated EZH2
(T345) preferentially interacted with STAT3 in HaCaT cells, whereas CDK4/6 inhibition did not only abrogate the phosphorylation of EZH2 but also its interaction with STAT3 (Figure 3E).

These data suggest that CDK4/6-mediated phosphorylation of EZH2 at threonine 345 represents a regulatory switch, leading to the interaction of EZH2 with STAT3 and subsequent STAT3 activation. Accordingly, whereas wildtype EZH2 synergistically induced the expression of the *NFKBIZ* luciferase promoter in cooperation with CDK4/6 and STAT3, an EZH2 mutant lacking the CDK4/6-directed phosphorylation site (EZH2 T345A) abrogated CDK4/6- and STAT3-mediated *NFKBIZ* promoter-driven luciferase expression (Figure 3F). Furthermore, transient expression of a phospho-mimicking EZH2 (T345D) version could override abemaciclib-mediated suppression of IκBζ induction and IκBζ target gene expression in IL-36α-stimulated primary keratinocytes (Figure 3G), whereas transient overexpression of IκBζ abolished the effects of the pharmacological EZH2 inhibitor (Figure 3H). Finally, also STAT3C overexpression could override target gene expression defects in IL-36α-stimulated, *EZH2*-depleted keratinocytes (Supplemental Figure 3H), thereby validating STAT3 as the main target for suppression of gene expression in EZH2 inhibitor-treated keratinocytes. Therefore, we conclude that IL-36α- and IL-17A/TNFα-mediated, CDK4/6-dependent induction of IκBζ expression is mediated by phosphorylation of EZH2 at T345, thereby triggering an EZH2-dependent activation of STAT3 in keratinocytes.

**CDK4/6-phosphorylated EZH2 mediates STAT3 methylation at K180, leading to IκBζ expression in keratinocytes.**

As reported before, EZH2 can methylate STAT3 at lysine 49, 140 or 180, thereby changing its transcription factor function or subcellular localization (34-36). Thus, we immunoprecipitated STAT3 in STAT3- and EZH2-overexpressing HEK293T cells in the presence or absence of abemaciclib, and analyzed the methylation status of STAT3 using a pan-methyl-lysine-specific antibody. Simultaneous overexpression of EZH2 and STAT3 induced methylation of STAT3, as expected, whereas CDK4/6 inhibition abrogated lysine methylation of STAT3 (Figure 4A). Furthermore, lysine methylation of STAT3 was detectable upon co-overexpression of CDK6
and STAT3, whereas pharmacological EZH2 inhibition abrogated STAT3 methylation (Figure 4B). Thus, CDK4/6 might indeed trigger an EZH2-dependent methylation and activation of STAT3.

EZH2-dependent methylation sites of STAT3 at lysine 49, 140 and 180 were previously identified by mass spectrometric analyses in tumor cells (34-36). Thus, we substituted all three lysine methylation sites with arginine residues and tested the STAT3 mutants for their potential to activate NFKBIZ luciferase promoter expression. Whereas mutations of STAT3 at K49 and K140 had no effect on the induction of NFKBIZ promoter expression, alone or in combination with CDK6 and EZH2 (Supplemental Figure 4A), mutation of lysine 180 (STAT3 K180R), abrogated STAT3-mediated NFKBIZ promoter activation (Figure 4C). Thus, we hypothesized that CDK4/6-activated EZH2 methylates STAT3 at lysine 180, which is needed to induce IkBζ expression in stimulated keratinocytes. In agreement, reconstitution of CRISPR/Cas9-generated STAT3 knockout keratinocytes with wildtype STAT3, but not with the STAT3 K180R mutant, fully reconstituted IkBζ expression and IkBζ-mediated target gene induction upon IL-36α or IL-17A/TNFα stimulation (Figure 4D, Supplemental Figure 4B). This correlated with an absence of nuclear translocation of STAT3 K180R in IL-36α-treated keratinocytes (Supplemental Figure 4C), as observed before (34). Accordingly, mutant STAT3 K180R and EZH2 were unable to bind to the NFKBIZ promoter region in IL-36α-stimulated keratinocytes anymore (Figure 4E). Thus, whereas IL-36α stimulation triggered wildtype STAT3 binding to the NFKBIZ promoter region together with EZH2 and CDK4/6, inhibition of CDK4/6 (Abe) or EZH2 (EPZ) abrogated the recruitment of this multi-protein complex (Figure 4F). These results therefore suggest that CDK4/6 phosphorylates EZH2 to induce EZH2-dependent K180 STAT3 methylation, leading to the recruitment of the heteromeric complex to the NFKBIZ promoter and subsequent induction of IkBζ and its target gene expression in keratinocytes.

Finally, we wanted to know if cytokines that activate the classical JAK/STAT3 pathway could override CDK4/6- or EZH2 inhibitor-mediated suppression of STAT3 activation. As revealed before (41-43), stimulation of primary keratinocytes with the cytokines IL-6, IL-20 or IL-22,
which are upregulated in psoriatic lesions, led to the phosphorylation of STAT3 (Supplemental Figure 4D) (41-43). Of note, neither abemaciclib, nor EPZ6438 was able to abrogate STAT3 phosphorylation under these conditions, implying that CDK4/6 and EZH2 specifically control phosphorylation of STAT3 upon stimulation with IL-36α or IL-17A/TNFα. Importantly, even though IL-6, IL-20 or IL-22 could re-establish STAT3 phosphorylation in IL-36α- and abemaciclib-treated keratinocytes, stimulation with these cytokines failed to restore IκBζ and its target gene expression (Supplemental Figure 4E and F), nor was it able to re-establish the nuclear translocation of STAT3 in keratinocytes (Supplemental Figure 4G). This finding implies that CDK4/6-EZH2-mediated methylation of STAT3 is distinguished from the activation of STAT3 by the JAK/STAT pathway.

**Human and murine psoriatic lesions are characterized by overexpression of cyclin D2, cyclin D3 and EZH2.**

Our findings suggest that CDK4/6 mediates the phosphorylation of EZH2 in a cyclin D-dependent manner, leading to STAT3 activation and IκBζ expression. We therefore investigated a potential relevance of this pathway in skin biopsies from psoriasis patients. Human psoriatic lesions, compared to non-psoriatic lesions or unaffected skin, were characterized by an upregulation of \textit{CCND2} and \textit{CCND3} (Figure 5A). In contrast, \textit{CCND1} levels were decreased or remained unaffected in lesional skin biopsies. This is in line with our previous observation (Supplemental Figure 2C and D) that cyclin D1, unlike cyclin D2 and cyclin D3, did not synergize with CDK4/6 and STAT3 in increasing \textit{NFKBIZ} promoter activity or expression of IκBζ and its target genes. In addition, \textit{EZH2} mRNA levels were significantly upregulated in human psoriatic skin lesions (Figure 5B). Immunohistochemistry further demonstrated that, on the protein level, human EZH2, which was only weakly expressed in normal skin, was strongly overexpressed in the basal cell compartment of psoriatic skin lesions, revealing a typical nuclear localization (Figure 5C).
Next, we asked if an upregulation of cyclin D2, cyclin D3 and EZH2 can be also detected in relevant psoriasis mouse models. In the standard model using the TLR7 agonist imiquimod (IMQ), psoriasis-like skin inflammation was triggered by daily application of an IMQ-containing cream on the ears for 6 days, while in a second model daily intradermal injections of IL-36α into the skin of mouse ears were employed for 5 consecutive days. After 6 or 7 days of treatment, not only skin inflammation, but also increased expression of Ccnd2, Ccnd3 and Ezh2 mRNA was detectable in both animal models (Figure 5 D and E). Moreover, increased protein levels of cyclin D2/D3 and EZH2 could be detected in the epidermis of IMQ-treated mouse ears (Figure 5F). Thus, in addition to the previously demonstrated overexpression of IκBζ in psoriasis (8, 9), a hyperactive cyclin D-CDK4/6 pathway and elevated EZH2 expression are evident in murine and human psoriatic skin lesions.

**Topical application of inhibitors targeting CDK4/6 or EZH2 protects against experimental psoriasis in vivo.**

IκBζ is one of the key transcriptional regulators in the pathogenesis of psoriasis (8, 9). Due to our finding that CDK4/6 and EZH2 inhibitors suppressed psoriasis-related, pro-inflammatory gene expression downstream of IL-36α or IL-17A/TNFα, we next investigated the potential of CDK4/6 and EZH2 inhibitors to block experimental psoriasis in vivo. Moreover, we reasoned that topical application of both inhibitors would be sufficient, as the epidermis constitutes the main target for CDK4/6 and EZH2 inhibition. A pre-requisite for efficient take-up of small-molecule inhibitors from the skin are hydrophobicity of these substances. Thus, we selected more hydrophobic inhibitors, such as abemaciclib (for CDK4/6 inhibition) or CPI-169 (44) (for EZH2 inhibition) that are more likely to penetrate the outer skin barrier. Psoriasis-like skin inflammation was induced in the above-mentioned psoriasis model by daily application of an IMQ-containing cream on the ears of wildtype mice for 6 days, before animals were sacrificed and analyzed at day 6 (45). Abemaciclib, CPI-169 and ethanol as vehicle control were daily applied on the ear skin in parallel to IMQ (Supplemental Figure 5A). Whereas IMQ-treated ears
exerted ear thickening, along with keratinocyte hyperproliferation and immune cell infiltration, topical application of abemaciclib or CPI-169 strongly suppressed IMQ-induced, psoriasis-like skin inflammation (Figure 6A and B). Both inhibitors effectively penetrated the skin and inhibited CDK4/6 or EZH2, as detected by loss of pRB (for CDK4/6 inhibition) or H3K27me3 (for EZH2 inhibition) expression in the epidermis of treated mice (Figure 6C). Moreover, abemaciclib treatment significantly suppressed the infiltration of neutrophils, macrophages and T cells in IMQ-treated mice (Figure 6D), while topical application of the EZH2 inhibitor CPI-169 fully abrogated immune cell infiltration upon IMQ treatment (Figure 6E). Of note, also the number of infiltrating plasmacytoid (pDC) and myeloid dendritic cells (mDC) was significantly suppressed by application of both inhibitors (Supplemental Figure 5B). Importantly, whereas IMQ treatment effectively induced IκBζ expression in the skin, along with phosphorylation of EZH2 at T345 and of STAT3 at Y705, topical administration of abemaciclib or CPI-169 completely abrogated these signaling events (Figure 6F, Supplemental Figure 5C). As a positive control, stabilization of the CDK4/6 substrate pRB (46), as well as EZH2-directed H3K27 methylation were strongly reduced in either CDK4/6 or EZH2 inhibitor-treated mouse skin (Figure 6F). Accordingly, expression of IκBζ target genes, such as Cxcl2 and Cxcl5, and DC- and T-cell-derived cytokines, such as Il17a or Il23a, was significantly downregulated in IMQ- and abemaciclib- or IMQ- and CPI-169-treated skin (Supplemental Figure 5D).

Treatment of mice with the TLR7 agonist IMQ represents a standard mouse model for psoriasis (45), however, IMQ activates immune cells in the first instance, rather than an initial keratinocyte-derived pro-inflammatory response, as it is likely to happen in human psoriasis pathogenesis. Thus, we additionally investigated the therapeutic effects of abemaciclib or CPI-169 in an IL-36-triggered psoriasis-like dermatitis mouse model (Supplemental Figure 5E). As previously reported (9, 47), repeated intradermal injections of IL-36α into the skin of mouse ears induced ear swelling, keratinocyte hyperproliferation along with immune cell infiltration (Figure 6G and H). As a control for drug penetration in the skin of IL-36-treated animals, effective inhibition of CDK4/6 and EZH2 methyltransferase activity was controlled by staining
for pRB and H3K27me3, respectively (Supplemental Figure 5F). Similar to the IMQ mouse model, topical application of abemaciclib or CPI-169 effectively blocked keratinocyte hyperproliferation and immune cell infiltration (Figure 6G and H). Moreover, both inhibitors suppressed IL-36-mediated expression of IκBζ, phosphorylation of EZH2 at T345 and activation of STAT3 (pSTAT3 Y705) in the skin of treated mouse ears (Figure 6I). Accordingly, IκBζ target gene expression and key cytokine expression, such as Il17a and Il23a, were effectively blocked as well (Supplemental Figure 5G). Thus, inhibition of CDK4/6 or EZH2 in IMQ- or IL-36-mediated psoriasis-like skin inflammation mouse models effectively prevented psoriasis induction in vivo, by suppressing STAT3-mediated induction of IκBζ expression and IκBζ target gene expression.

**CDK4/6 and EZH2 inhibitors effectively attenuate already established psoriasis-like skin inflammation in vivo.**

As CDK4/6 and EZH2 inhibitors could fully prevent the onset of psoriasis in vivo, we next investigated if inhibition of the CDK4/6-EZH2 pathway also attenuates already established psoriatic disease. We therefore first induced psoriasis-like skin inflammation in mice with IMQ and then topically applied abemaciclib or CPI-169 after day 2 of IMQ treatment (Figure 7A). Already three treatments with IMQ effectively induced ear swelling and keratinocyte hyperproliferation. These psoriasis-like symptoms could be fully reversed by topical application of abemaciclib or CPI-169 starting (Figure 7B and C, Supplemental Figure 6A). Moreover, psoriasis-related, pro-inflammatory gene expression as well as infiltration of neutrophils, macrophages and T cells, which was detectable at the third day of IMQ treatment, were fully resolved by both inhibitors (Supplemental Figure 6B, Figure 7D). Finally, inhibition of CDK4/6 or EZH2 abrogated IκBζ expression as well as phosphorylation of STAT3, as detected by immunoblot analyses of whole skin lysates at day 6 (Figure 7E).
Similar results were obtained by topical application of abemaciclib or CPI-169 on established skin lesions in the IL-36α psoriasis-like mouse model. In this experimental set-up, application of both inhibitors at day 4 effectively resolved IL-36α-induced ear swelling, keratinocyte hyperproliferation and immune cell infiltration as well as psoriasis-associated gene expression, IκBζ expression and activation of STAT3 (Figure 7F-I, Supplemental Figure 6C). Thus, topical application of CDK4/6 and EZH2 inhibitors did not only prevent the onset of experimental skin inflammation, but also resolved already established psoriasis-associated symptoms in IMQ- or IL-36α-treated mice. In view of its increased activity in human psoriatic skin and the results obtained in psoriasis-like mouse models, inhibition of this pathway by topical application of CDK4/6 and EZH2 inhibitors could therefore provide a new therapeutic strategy for the treatment of psoriasis patients.
Discussion

CDK4/6 inhibitors have been developed and approved for the treatment of cancer patients in order to restrain hyperproliferation of tumor cells (17). Recently, it was found that CDK4/6 do not only control cell cycle progression by phosphorylation of RB, but also regulate immune cell differentiation and function (20, 21, 48). In this context, CDK4/6 have been implicated as transcriptional cofactors that activate a subset of NF-κB or STAT3 target genes (23-25). Based on our results in cultured keratinocytes, human skin biopsies and mouse models, we propose to repurpose CDK4/6 inhibitors for psoriasis therapy. Moreover, our results uncovered a new pathway involving CDK4/6-mediated phosphorylation of EZH2 and EZH2-dependent methylation and activation of STAT3, leading to the inducible expression of IκBζ and IκBζ-dependent target genes in keratinocytes. These findings suggest also the use of EZH2 inhibitors to treat psoriasis.

IκBζ, encoded by \textit{NFKBIZ}, constitutes a risk gene for the development of psoriasis (49). Moreover, we recently reported that IκBζ is overexpressed in human psoriatic lesions, whereas global and keratinocyte-specific IκBζ KO mice are completely protected against psoriasis-like skin inflammation in several psoriasis models (8, 9, 50). Mechanistically, IκBζ is transcriptionally induced in keratinocytes by IL-17 and IL-36, which triggers the expression of psoriasis-relevant target genes encoding for selective chemo- and cytokines and antimicrobial proteins. Deficiency of IκBζ therefore prevents the recruitment of neutrophils and monocytes that are needed for skin inflammation (9, 50). Collectively, our data suggest that interfering with IκBζ expression or function in keratinocytes might be a promising strategy for psoriasis therapy. As IκBζ is crucial for both IL-36 and IL-17 signaling, CDK4/6 inhibitors might be applicable for different subtypes of psoriasis.

Unfortunately, based on a lack for enzyme activity, direct pharmacological inhibition of IκBζ function remains difficult (13). We therefore sought to block the transcriptional induction of IκBζ and identified small-molecule inhibitors of CDK4/6 and EZH2 as potent suppressors of IκBζ...
expression in keratinocytes. CDK4/6 have been previously shown to modulate several immune-relevant transcription factors by both kinase-dependent and -independent mechanisms (23-25). In the present study, we clearly demonstrate that STAT3-mediated induction of IκBζ expression is kinase-dependent, as ATP-competitive CDK4/6 inhibitors, such as abemaciclib or palbociclib, abolished IκBζ expression. Consistent with these findings, a hyperactive but not a dominant-negative version of CDK6 increased NFKBIZ promoter activity. Moreover, cyclin D2 and cyclin D3 elevated the expression of NFKBIZ and its target genes, supporting the need for CDK4/6 kinase activity.

Despite the requirement of the kinase activity, the involvement of CDK4/6 could be separated from its classical role in cell cycle regulation and phosphorylation of RB. Thus, depletion of RB failed to restore IκBζ expression upon CDK4/6 inhibition. Moreover, IκBζ expression was principally induced by IL-36 stimulation in all phases of the cell cycle, except for G0-arrested cells that revealed a weaker IκBζ expression. Importantly, although IκBζ expression does not rely on CDK4/6-mediated cell cycle progression, CDK4/6 inhibitors might also have beneficial effects in psoriasis treatment by additionally blocking keratinocyte hyperproliferation, which is a hallmark of psoriasis (2).

In this study, we demonstrate a major role for STAT3 in driving keratinocyte-specific IκBζ expression. IκBζ expression in keratinocytes is predominantly controlled from the proximal promoter 2 of the NFKBIZ locus, containing different transcription factor binding sites than the better investigated distal promoter 1 (9). So far, we have not compared the promoter usage in distinct cell types, but we consider it likely, that the contribution of the individual promoters and STAT3 to IκBζ expression differs among different cell types. Our experiments show that CDK4/6 do not directly phosphorylate STAT3 but EZH2, which induces IκBζ and IκBζ-dependent pro-inflammatory target gene expression in a STAT3-dependent manner. This finding seems surprising at the first glance, since EZH2, as part of the PRC2 complex, is mainly involved in gene repression through trimethylation of H3K27. Recently, however, EZH2 was also found to induce gene expression independently of the PRC2 complex, via interaction with
β-catenin or the SWI/SNF complex (51, 52). CDK4/6 phosphorylated EZH2 at T345, thereby inducing an EZH2-dependent methylation of STAT3 at K180, and subsequent induction of IκBζ expression by STAT3. EZH2 phosphorylation at T345 was previously described to be mediated by CDK1 and CDK2, leading to an EZH2-directed epigenetic silencing of genes during G2 phase (39, 53). Thus, even though CDK-mediated phosphorylation of EZH2 at T345 seems to be conserved, its impact on EZH2 function and the choice of methylation substrates might depend on the specific stimulus or cell cycle phase.

Upon CDK4/6-mediated phosphorylation, EZH2 preferentially interacted with STAT3, resulting in STAT3 K180 methylation and enhanced STAT3 activation. Similar observations were made in glioblastoma, where IL-6-induced STAT3 activation is controlled by EZH2-mediated trimethylation of STAT3 at K180 (34). Thus, phosphorylation of EZH2 might induce a switch in EZH2 function from H3K27 trimethylation and transcriptional repression to non-canonical functions, including STAT3 methylation and gene activation. Whether this gene-activating function of EZH2 requires the PRC2 repressor complex or whether it is PRC2-independent remains to be resolved. In addition to its main function in transcriptional repression, non-PRC functions of EZH2 via direct binding to transcriptional regulators have been reported before. For instance, EZH2 was shown to act as a co-factor for transcription factors, such as the androgen receptor, β-catenin or NF-κB, leading to target gene activation (52, 54, 55). Similar to other non-histone targets, however, the exact molecular events that link STAT3 methylation to STAT3 activation are currently unknown. In agreement with a previous report (34), our data implies that K180 methylation of STAT3 might be needed for the nuclear import of phosphorylated STAT3.

Regardless of the detailed mechanism of EZH2-mediated STAT3 activation, our study has also important clinical implications. Our results suggest that targeting of the CDK4/6-EZH2-STAT3 pathway does not only suppress cytokine-mediated induction of IκBζ and pro-inflammatory target gene expression, but also inhibits immune cell recruitment and skin inflammation. We demonstrate in the IMQ- and IL-36-mediated psoriasis-like mouse models that both CDK4/6
and EZH2 inhibitors completely blocked the development of psoriatic skin lesions. The therapeutic effect of the inhibitors concurred with a suppression of IκBζ expression and a strong inhibition of IκBζ target gene expression, including chemokines (e.g. Cxcl2, Cxcl5), cytokines (e.g. Il1f9, Il1b, Il17a, Il23a) and anti-microbial proteins (e.g. Lcn2). In contrast, genes that were not IκBζ-dependent, such as NFKBIA and TNF, remained unaffected upon CDK4/6 or EZH2 inhibition. These findings further support the view of a rather selective role of IκBζ in the control of immune responses and also indicate that inhibition of IκBζ will be associated with less side effects than a broad inhibition of NF-κB by toxic IKK inhibitors.

In line with previous reports showing an upregulated expression of IκBζ in psoriasis (8, 9), we detected an increased nuclear accumulation of EZH2 and elevated cyclin D2 and D3 levels, both in mouse models of psoriasis and in human psoriatic skin lesions. Previous studies also found that mutations in the STAT3 signaling pathway constitute a risk factor for the development of psoriasis (43), while constitutively active STAT3 characterizes the epidermis of human psoriatic lesions (56). Collectively, this suggests that the CDK4/6-EZH2-STAT3 pathway is hyperactive in psoriatic skin lesions. As inhibition of IκBζ blocks multiple signaling pathways in psoriasis, targeting IκBζ might increase overall therapy responses as well as prevent the development of therapy resistance. Due to the clinical availability of hydrophobic CDK4/6 and EZH2 inhibitors, we propose formulation of these inhibitors for instance in a cream for topical treatment of psoriatic skin lesions. Topical drug administration will also restrict potential side effects and might be especially promising for those patients who have developed resistance to current psoriasis therapies.
**Methods**

**Cell culture and treatment.** HaCaT cells were obtained from Petra Boukamp (57) and maintained in DMEM with 10% FCS and antibiotics. Human primary keratinocytes were freshly isolated from foreskin and maintained in CnT-07S medium with gentamycin (CELLnTEC). Recombinant human IL-36α (6995-IL; aa 6-158), IL-36γ (6835-IL; aa 18-169) and mouse IL-36α (7059-ML; aa 6-160) were purchased from R&D Systems. Recombinant IL-17A (11340174), TNFa (11343013), IL-1β (11340013), IL-6 (11340064), IL-20 (11340203) and IL-22 (11340223) were ordered from Immunotools. Flagellin (vac-fla) and polyI:C (vac-pic) were purchased from Invivogen. In cell culture experiments, all cytokines were used at 100 ng/mL end concentration, except for IL-17A (200 ng/mL) and TNFα (10 ng/mL). Flagellin was applied at 10 ng/mL and poly I:C was added at a final concentration of 100 ng/mL. The following inhibitors were purchased from Selleckchem: Abemaciclib mesylate (LY2835219, S17158), palbociclib isethionate (S1579), EPZ6438 (tazemetostat, S7128) and CPI-169 (S7616). If not otherwise indicated, the inhibitors were used in cell cultures at the following concentrations: abemaciclib (16 µM), palbociclib (50 µM) and EPZ6438 (10 µM). When indicated, cells were starved overnight, prior to cytokine treatment, by removing cell culture supplements from the growth medium.

**Generation of knockdown cells.** Lentiviral particles were produced in HEK293T cells using the second-generation packaging system (pMD2.G, 12259 and psPAX2, 12260, from Addgene). Keratinocytes were transduced in the presence of 8 µg/mL polybrene, packaging plasmids and 5 µg of the respective shRNA construct (all from Dharmacon): pLKO.1-puro (sh ctrl); pLKO.1-TRCN0000009876 (shCDK4); pLKO.1-TRCN0000010473 (shCDK6); pTRIPZ-EZH2 (V2THS63066, shEZH2); pLKO.1-TRCN0000040167 (shRB); pTRIPZ noncoding ctrl (RHS4743); pLKO.1-TRCN0000020840 (shSTAT3); pLKO.1-TRCN0000014683 (shRELA), followed by puromycin selection (1 ng/mL, Invitrogen). For induction of EZH2 knockdown, pTRIPZ ctrl and pTRIPZ-EZH2 expressing cells were treated for 24 h with 2 µg/mL doxycycline (AppliChem) prior to stimulation and harvest of the cells.
Luciferase constructs and reporter assays. Luciferase constructs were generated as described and based on the pInducer20 plasmid (Addgene, 44012) (9). 1*10^4 HEK293T cells were transfected for 24 h using HeBS buffer and CaCl_2 and a mixture of 400 ng firefly luciferase vector and 100 ng TK-Renilla vector. For expression of other proteins, the following concentrations were purchased from Addgene: 70 ng p65 (106453), 200 ng cJun (102758), STAT3 (8706) or EZH2-HA (24230) constructs and 500 ng CDK4-HA (1868), CDK6-HA (1866), CDK6DN (1869), cyclin D1-HA (11181), cyclin D2 (8958) and cyclin D3 (10912). Additionally, CDK6 S178P expression construct was gifted by Michael Kracht, University of Giessen, Germany) (58). For transfection of HaCaT cells, 3*10^5 cells were transfected for 4 h using Lipofectamine 3000 and a mixture of 800 ng firefly luciferase vector, 200 ng TK-Renilla vector and 4 μg expression or control plasmids according to the manufacturer’s instructions (Thermo Fisher, L3000015). 36 h after transfection, luciferase activity was measured with the Dual Luciferase Reporter Assay Kit (E2980, Promega). Expression of the reporter constructs was calculated as the fold induction over unstimulated transfected cells from data of three independent experiments.

Transient overexpression in HEK293T, HaCaT cells and primary keratinocytes. HEK 293T cells (ACC 635, DSMZ Braunschweig, Germany) were transfected using HeBS buffer and CaCl_2. HaCaT cells and primary keratinocytes were transfected with Lipofectamine 3000, according to the manufacturer’s instructions (Thermo Fisher, L3000015). 5 μg expression constructs were incubated with 3*10^5 cells for 4 h. 36-48 h post transfection, cells were harvested and analysed. NFKBIZ (44012), CDK9-HA (28102) and Flag-STAT3C (8722) expression constructs were purchased from Addgene and pINTO-GFP-EZH2 T345A and pINTO-GFP-EZH2 T345D were provided by Danny Reinberg (New York, USA).

Generation of STAT3 mutants. Mutation of STAT3 at K49, K140 and K180 was performed by site-directed mutagenesis of the human STAT3 pcDNA3 construct from Addgene (71447), which was previously cloned into the Strep-tagged backbone (pEXPR-IBA103). Substitution of the amino acid was performed with self-designed primers (Supplemental Table 1) (59).
**CRISPR/Cas9 gene editing of STAT3 KO HaCaT cells.** The CRISPR/Cas9 one vector system was used to generated STAT3 KO HaCaT cells according to the protocol of Shalem et al. (60). The guide RNA against STAT3 (forward: 5’-CACCGACTGCTGGTCAATCTCTCCC-3’, reverse: 5’-AAACGGAGAGATTGACCAGCAGTC-3’) was cloned into the Cas9 containing lentiCRISPRv2 containing Cas9 vector (Addgene, 52961), followed by lentiviral transduction and puromycin selection.

**Synchronization of HaCaT cells.** Synchronization of the cells with a double thymidine block was performed as described (61). After the second thymidine block, cells were released in normal medium. At 0, 4, 10 and 14 h after release, cells were stimulated with IL-36α and/or abemaciclib for 1 h. Propidium iodide staining was performed by flow cytometry (LSRII, Becton Dickinson) to detect the cell cycle phase at the time point of cell harvest.

**Western blot analysis.** Western blot analysis was performed as described (9). The following antibodies were used and purchased from Cell Signaling: anti-IκBζ (9244), anti-pSTAT3 at Tyr705 (9145), anti-pSTAT3 at Ser727 (9134), anti-STAT3 (12640), anti-p65 (8242), anti-EZH2 (5246), anti-pRB (at Ser807/811; 8516), anti-FoxM1 (5436), anti-H3 (4499), anti-CDK4 (12790), anti-CDK6 (13331), anti-CDK9 (2316), anti-cyclin D1 (2978), anti-cyclin D2 (3741), anti-cyclin D3 (2936), anti-c-Jun (9165), anti-H3K27me3 (9733), anti-GAPDH (2118), anti-H3 (9715) and anti-β-actin (3700). Anti-α-Tubulin (T9026) was purchased from Sigma-Aldrich. Anti-β-Gal (sc377257) and anti-GFP (sc9996) were obtained from Santa Cruz Biotechnology. Anti-pEZH2 at T345 (61242) and anti-pEZH2 at T487 (12820) were purchased from Active Motif and anti-pan-methyl-lysine antibody was purchased from Enzo (ADI-KAP-TF121-E). For detection of mouse IκBζ, a self-made rabbit antiserum raised against peptides CSAPGSPGSDSSDFSS and CLHIRSHKQKAGQ was applied (50). Of note, immune blot images may derive from multiple gels. Additional loading and inhibitor controls from each membrane are depicted in the raw data file.

**Chromatin immunoprecipitation (ChIP).** ChIP assays were performed as described (62). After sonification, chromatin was incubated with protein G-coupled Dynabeads (10004D,
Invitrogen) and 2 μg of STAT3 (Thermo Fisher, MA1-13042), CDK4 (Cell Signaling, 12790), CDK6 (Sigma, HPA002637), EZH2 (Diagenode, C15410039), NF-κB p65 (Diagenode, C15310256) or control IgG antibody (Abcam, ab46540) overnight at 4°C. The promoter region of myoglobulin (MB) served as an internal negative control (forward: 5’-CTCTGCTCCTTTGCCACAAC-3’, reverse: 5’-GAGTGCTCTTCGGGTTTCAG-3’). ChIP primers corresponding to the promoter region of NFκBIZ (forward 5’-GCCTTAACTGGCTCTACGC-3’, reverse 5’-CTGGCAAGTCTCGGAAGGAG-3’), CCND2 (forward 5’-GGGAGAGGGAGGAGCTAA-3’, reverse 5’-GAGAGGTGAGGGCAGAGAGA-3’) and CCND3 (forward 5’-GGCAATTACAGCCACATTCC-3’, reverse 5’-GTTGGCAACAGACACTGCTA-3’) were self-designed. Data from two independent experiments is presented as the fold enrichment, calculated over the percentage of input from the IgG control ChIP.

Co-Immunoprecipitation (CoIP). Cells were lysed by mechanical disruption using a dounce homogenizer and standard lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1x Protease inhibitor cocktail, Roche). Subsequently, lysates were sonicated for 5 minutes at high power (Bioruptor, Diagenode), followed by pre-clearing of the lysates with protein A/G PLUS agarose beads (Santa Cruz, sc-2003) for 1 h at 4°C. Pre-cleared lysates were incubated either with antibodies specific for CDK4 (Cell Signaling, 12790), CDK6 (Sigma, HPA002637), EZH2 (Cell Signaling, 5246), STAT3 (MA1-13042, Thermo Fisher,) or β-Gal (sc-19119, Santa Cruz) as an IgG control, overnight at 4°C. For endogenous IPs immune complexes were precipitated with protein A/G PLUS agarose beads and eluted by 6x SDS-PAGE sample buffer.

Cytokine array. Cytokine levels were detected from primary human keratinocytes that had been treated for 24 h with 100 ng/mL IL-36α, using the human cytokine array from R&D Systems (ARY005B) according to the manufacturer’s instructions. Prior to analysis, input lysates were normalized to equal cell numbers. Spot intensity was quantified with the dot blot analyser from ImageJ and normalized to the reference spots. Relative expression levels are represented as mean pixel intensities.
**Gene expression analysis by qPCR.** Gene expression analyses were performed as described (9). Relative gene expression was analysed using self-designed primers ordered at Metabion (Supplemental Table S2). Relative mRNA levels were calculated by normalization to the human reference gene *RPL37A* or the mouse reference gene *Actin* using the 2-ΔΔCt method.

**Mice.** Experiments were conducted in accordance with the German law guidelines of animal care. Ears of female C57BL/6 mice (8-12 weeks old, from Jackson Laboratory) were topically treated for 6 consecutive days with 5 mg Aldara cream (containing 5% imiquimod, 3M Pharmaceuticals) and 10 µL abemaciclib (2% in 10 µL ethanol), 10 µL CPI-169 (5% in 10 µL ethanol) or vehicle control. At day 7, mice were sacrificed and analysed. In the therapeutic mouse model, IMQ was applied first to establish psoriasis-like skin lesions. Then, starting on the third day of IMQ administration (IMQ day 2), IMQ and the inhibitors were added in parallel until mice were sacrificed at day 6 (IMQ day 6). In the IL-36α-mediated psoriasis model, ears of male C57BL/6 mice (8-12 weeks old, from Jackson Laboratory) were treated by intradermal injections of 1 µg murine IL-36α (7059-ML, R&D Systems) or PBS control for 5 consecutive days. For application of abemaciclib (2% in ethanol), CPI-169 (5% in ethanol) or the vehicle control, substances were mixed with Miglyol 812 (Carl Roth) in a ratio of 1:2. Inhibitors were topically applied 6 h prior to intradermal injections of IL-36α or PBS. Mice were sacrificed and analysed at day 6. For treatment of established psoriasis-like skin disease, mice were treated every second day with IL-36α for 8 days, followed by the analysis of the mice at day 9 (IL-36 day 9). Both abemaciclib and CPI-169 were daily applied on the skin, starting from the third IL-36α administration (IL-36 day 4).

**Flow cytometry.** Sample preparation was performed as described (9). The following anti-mouse antibodies from BioLegend were used: anti-CD45 FITC (103107), anti-CD11b Pacific Blue (101223), anti-Ly6G PE (127607), anti-F4/80 APC (123115), anti-CD11c Pacific Blue (117322), anti-MHC-II APC (107613), anti-CD172a PE (144011) and anti-Siglec-H PE (129605). Anti-PDCA-1 APC (17-2092-80) and anti-αβTCR Pacific Blue (HM3628) were
purchased from Invitrogen, and anti-γδTCR APC (17-5711-82) from Sigma. Acquisition was performed with the LSRII flow cytometer (Becton Dickinson) and live, single cells were gated using the FlowJo (Tree Star) software.

**Histology.** Ear sections from mice were fixed in 10% formalin (Carl Roth) and subsequently embedded in paraffin. 5-µm sections were prepared and incubated with the following antibodies from Cell Signaling: pSTAT3 (9145), pRB (8516), H3K27me3 (9733) and EZH2 (5246), cyclin D2 (3741) and cyclin D3 (2936). Antigen retrieval was performed in 1 mM EDTA pH 8.0 for pSTAT3, and 10 mM citrate buffer pH 6.0 + 0.5% Triton X-100 for EZH2, H3K27me3, pRB, cyclin D2, cyclin D3. After incubation with peroxidase-coupled secondary antibodies, sections were stained with DAB substrate.

**Analysis of patient data.** Gene expression data originated from the GEO data set GSE13355 (63, 64). Pre-normalized gene expression values from each sample was directly taken from the GEO profile data set GDS4602. The following reporters were taken for analysis: EZH2: ID 203358_s_at, CCND1: ID 208711_s_at, CCND2: ID 200953_s_at and CCND3: ID 201700_s_at.

**Statistics.** Results from in vivo experiments are represented as the mean ± SEM. Results from cell culture experiments are represented as the mean ± SD. Significance was calculated using a 1-way ANOVA to compare multiple groups and a 2-tailed Student’s t-test was applied when two groups were compared with each other. A P value less than 0.05 was considered to be significant. Significance is depicted as asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, n.s. = not significant).

**Study approval.** All animal experiments were approved by the Regierungspräsidium Tübingen, Germany (IB 4/18G, IB 1/19G). Human psoriasis skin samples came from the Department of Dermatology, Heidelberg University Hospital. Experiments were approved by the ethics committee of the University Hospital Heidelberg. Isolation of primary human
keratinocytes from foreskin was approved by the local ethics committee of the University Hospital Tübingen.
Author contributions

A.M., A.D. and C.R. performed experiments and data analysis. S.H., K.S-O., M.D. and D.K. designed the experiments. K.S. donated human psoriasis skin samples and helped with the analysis. S.H., K.S-O., M.D. and D.K. wrote the manuscript.

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Declaration of interests

References


Figure 1. CDK4/6 regulate the expression of IκBζ and its pro-inflammatory target genes in IL-36α- and IL-17A/TNFα-stimulated keratinocytes.
(A) Human primary keratinocytes were treated for 1 h with 100 ng/mL IL-36α or 200 ng/mL IL-17A and 10 ng/mL TNFα. The CDK4/6 inhibitor abemaciclib (Abe) or an ethanol vehicle control (Ctrl) were added in parallel. Phosphorylation of RB (pRB) served as a control for CDK4/6 inhibition, and actin as a loading control. Relative mRNA levels of IκBζ (NFKBIZ) were normalized to the reference gene RPL37A. (B) Luciferase assay of IκBζ (NFKBIZ) promoter activity in HaCaT cells that were cytokine-stimulated for 24 h in the presence or absence of the CDK4/6 inhibitors abemaciclib or palbociclib (Pal). Relative luciferase (luc) activity was normalized to an internal Renilla luciferase control that was transfected in parallel. Endogenous protein levels were analyzed as input controls by immunoblotting (bottom). (C + D) CDK4 and CDK6 were depleted in primary human keratinocytes by lentiviral transduction of shRNA. Ctrl shRNA- or CDK4/6 shRNA-depleted cells were treated with (C) IL-36α or (D) IL-17A/TNFα, similar as in (A). (E + F) Human primary keratinocytes were stimulated with IL-36α as in (A). (E) Cytokine gene expression in CDK4/6 inhibitor-treated cells. (F) Relative gene expression levels in IL-36α-treated control or CDK4/6-depleted cells. (G) Transient overexpression of CDK4, CDK6 or CDK9 in HaCaT cells, treated for 1 h with 100 ng/mL IL-36α. (H) Cytokine gene expression in IL-36α-treated primary keratinocytes overexpressing IκBζ in the presence or absence of abemaciclib. All analyses: n = 3 ± 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.
Figure 2. STAT3 mediates CDK4/6-dependent IκBζ induction in keratinocytes.

(A) Luciferase assay of the NFKBIZ promoter in HEK293T cells after transient expression of CDK4, CDK6, STAT3 or p65, alone or in combination. The plasmid amounts for STAT3 (200 ng) and p65 (70 ng) were adjusted to achieve similar luciferase activity in the absence of CDK4/6 expression. Overexpression of the HA-tagged CDK4 and CDK6 proteins was detected using a HA-antibody. (B) Primary human keratinocytes with a transient overexpression of hyperactive STAT3 (STAT3C) were treated for 1 h with 100 ng/mL IL-36α and abemaciclib (Abe). Left: NFKBIZ mRNA levels normalized to RPL37A. Right: Immunoblot analysis of STAT3C overexpression and CDK4/6 inhibition. (C) IκBζ target gene expression in STAT3C-overexpressing primary keratinocytes. Treatment as in (B). (D) Luciferase activity assay of the NFKBIZ promoter in HEK293T cells overexpressing STAT3 alone or in combination with wildtype CDK6 (wt), hyperactive CDK6 (S178P) or a kinase-dead CDK6 mutant (CDK6 DN).
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.
Figure 3. CDK4/6 phosphorylate EZH2 to induce STAT3 activation.

(A) STAT3 activity was detected by analyzing the phosphorylation state at tyrosine 705 (Y705) and threonine 727 (T727) of STAT3 in primary human keratinocytes. After overnight starvation,
cells were stimulated for 1 h with IL-36α or IL-17A/TNFα in the presence or absence of abemaciclib (Abe). (B) STAT3 activity in CDK4- and CDK6-depleted keratinocytes. Stimulation as in (A). (C) Immunoblot detection of phosphorylated STAT3 (Y705) in IL-36α-stimulated keratinocytes, in which EZH2 function was suppressed by the EZH2 inhibitor EPZ6438 (EPZ, 10 µM) or shRNA-mediated knockdown. Detection of H3K27me3 controlled effective EZH2 inhibition or depletion. (D) Immunoblot detection of phosphorylated EZH2 at threonine 345 (T345) and threonine 487 (T487) in abemaciclib-treated or CDK4/6-depleted keratinocytes following stimulation with IL-36α. (E) Co-immunoprecipitation of EZH2 and STAT3 in HaCaT cells treated for 30 min with IL-36α in the presence or absence of abemaciclib. An EZH2-specific antibody or IgG were used for pulldown of protein complexes. STAT3 and pEZH2 (T345) were detected by immunoblotting. (F) Luciferase activity assay of the NFKBIZ promoter in HEK293T cells, which transiently overexpress CDK6, wildtype EZH2 (wt), mutant EZH2 (T345A) or STAT3, alone or in combination. Equal protein expression was detected by immunoblotting (bottom). n = 3 ± SD. (G) Gene expression in IL-36α- and abemaciclib-treated, primary keratinocytes following transient expression of a phospho-mimicking EZH2 (T345D) mutant. Left: Input controls. mRNA levels of NFKBIZ and its target genes were normalized to RPL37A (right). n = 3 ± SD. (H) Overexpression of IκBζ overrides the inhibitory effects of EPZ6438 (EPZ) on IL-36α-stimulated gene expression in primary keratinocytes. n = 3 ± 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.
Figure 4. CDK4/6-dependent, EZH2-mediated methylation of STAT3 at lysine 180 induces IκBζ expression in keratinocytes.

(A + B) Detection of methylated STAT3 by co-immunoprecipitation. EZH2 and STAT3 (A) or CDK6 and STAT3 (B) were transiently expressed in HEK293T cells. After 1 h of treatment with (A) abemaciclib (Abe) or (B) EPZ6438 (EPZ) cell lysates were prepared and subjected to co-immunoprecipitation. The results were analyzed by western blotting.
immunoprecipitation using a STAT3-specific antibody or control IgG. (C) *NFKBI*Z promoter-driven luciferase activity in HEK293T cells, transiently expressing CDK6 and EZH2, alone or in combination with wildtype (wt) STAT3 or methylation-defective STAT3 mutant (K180R). $n = 3 \pm SD$. (D) Analysis of $I\kappa B\zeta$ and $I\kappa B\zeta$ target gene expression in STAT3 wt or STAT3 K180R expressing HaCaT cells. STAT3 wt or STAT3 K180R constructs were transiently expressed in STAT3 KO HaCaT cells, followed by stimulation for 1 h with IL-36α. $n = 3 \pm SD$. (E) Chromatin immunoprecipitation (ChIP) of STAT3, EZH2 or IgG control in STAT3 KO HaCaT cells reconstituted with either STAT3 wt or STAT3 K180R after 30 min of stimulation with IL-36α. Fold enrichment at the *NFKBI*Z promoter or at the myoglobin genomic region (MB; as negative control) was calculated relative to the IgG control. $n = 3 \pm SD$. (F) ChIP of STAT3, EZH2, CDK4 and CDK6 in IL-36α-stimulated HaCaT cells stimulated for 30 min with IL-36α. Shown is the fold enrichment over IgG control. $n = 3 \pm SD$. Significance was calculated using a 1-way ANOVA for multiple groups and a 2-tailed Student’s t-test comparing two groups: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. 
Figure 5. Increased expression of cyclin D2, cyclin D3 and EZH2 in human and murine psoriasis.

(A) Expression data from skin biopsies of 64 healthy individuals and 58 psoriasis patients were analyzed from the GEO profile data set GDS4602. Shown are normalized expression values for \textit{CCND1}, \textit{CCND2} and \textit{CCND3}. \textit{EZH2} mRNA (B) and protein levels (C) in human skin samples from healthy and psoriasis patients; retrieved from the same data set as in (A) and (B). Significance was calculated with a 1-way ANOVA test: *p < 0.05; **p < 0.01; ***p < 0.001. Scale bar: 100 µm. (D) Analysis of \textit{Ccnd2}, \textit{Ccnd3} and \textit{Ezh2} mRNA levels in IMQ-treated mice
ears at day 6. Values were normalized to Actin. n = 6 per group ± SEM. (E) Analysis of Ccnd2, Ccnd3 and Ezh2 mRNA levels in IL-36α-treated mice ears at day 5. n = 6 per group ± SEM. Significance was calculated using a 2-tailed Student’s t-test: *p < 0.05; **p < 0.01; ***p < 0.001. (F) IHC staining of EZH2, cyclin D2 and cyclin D3 in untreated (Ctrl) and IMQ-treated mouse ears at day 6. Scale bar: 40 µm.
Figure 6. CDK4/6 and EZH2 inhibition prevents imiquimod (IMQ)- and IL-36-mediated psoriasis-like skin lesions in vivo.
(A) Ear thickness measurements during topical treatment of mice with IMQ with or without abemaciclib (Abe; 10 µL of a 2% solution; top) or the EZH2 inhibitor CPI-169 (CPI, 10 µL of a 5% solution; bottom). n = 6 mice per group ± SEM. (B) H&E staining of untreated (Ctrl), IMQ-, IMQ and Abe- or IMQ and CPI-treated ears. Scale bar: 100 µm. (C) Phospho-RB (pRB) and H3K27me3 staining after 6 days of treatment validated effective CDK4/6 and EZH2 inhibition, respectively. Scale bar: 40 µm. (D) Infiltrating immune cells in mouse ears at day 6 of treatment were quantified as follows: Neutrophils: CD45+, CD11b+, Ly6G+; macrophages: CD45+, CD11b+, F4/80+; T cells: CD45+, CD3+ and αβ-TCR+ or γδ-TCR+. n = 3 mice per group ± SEM. (E) Flow cytometry analysis of IMQ- or IMQ- and CPI-169-treated mouse ears at day 6. (F) Protein levels in untreated (Ctrl) and treated mouse skin tissue at day 6. (G) Ear thickness of IL-36α-treated mice at day 5. Ears of mice were daily treated by intradermal injections with 1 µg IL-36α. Control mice received injections with PBS. Additionally, mice received topical treatment with ethanol as control (Veh), 2% abemaciclib (Abe) or 5% CPI-169 (CPI). n = 6 mice per group ± SEM. (H) H&E staining of PBS- or IL-36α-treated ears at day 5. Scale bar: 100 µm. (I) Immunoblot analysis of IκBζ, EZH2 phosphorylation (pEZH2 T345) and STAT3 activation (pSTAT3 Y705) in treated mouse skin tissue at day 5. pRB and H3K27me3 were analyzed as positive controls for drug action. 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.
Figure 7. CDK4/6 and EZH2 inhibitors attenuate established psoriasis-like skin lesions in vivo.
All analyses were performed with \( n = 6 \) mice per group ± SEM. (A) Treatment scheme for the therapy using the imiquimod (IMQ) mouse model. To explore whether CDK4/6 and EZH2 inhibitors suppress already established psoriasis-like skin inflammation, mice were first treated with imiquimod, followed by the application of 2% abemaciclib or 5% CPI-169 solution starting at the third IMQ application. (B) Ear thickness measurements during treatment. (C) H&E staining of untreated (Ctrl), IMQ-, IMQ and Abe- or IMQ and CPI-treated ears. H&E staining shows the prevalence of psoriasis-like symptoms at IMQ day 2 when the inhibitors were applied for the first time. Scale bar: 100 µm. (D) Quantification of infiltrating immune cells in mouse ears at day 6. Immune cell subpopulations were quantified as in Figure 6D. \( n = 3 \) mice per group ± SEM. (E) Protein levels in untreated (Ctrl) and IMQ-treated mouse skin tissue in the presence or absence of abemaciclib or CPI-169 at day 6. Mice were treated as in (A). FOXM1 and H3K27me3 were analyzed as positive controls for drug action. (F) Treatment scheme in the IL-36-induced psoriasis mouse model. IL-36-mediated psoriasis-like dermatitis was induced by administration of 1 µg IL-36α at every second day. Control mice received PBS. Starting from day 4 of IL-36α injection, ethanol as Vehicle (Veh), 2% abemaciclib or 5% CPI-169 were daily applied by topical administration. (G) Ear thickness measurements during IL-36α treatment. (H) H&E staining of PBS- or IL-36α-treated ears at day 9. Scale bar: 100 µm. (I) Immunoblot analysis in IL-36α-treated mouse skin tissue at day 9. Significance was calculated using a 1-way ANOVA for multiple groups and a 2-tailed Student’s t-test comparing two groups: *\( p < 0.05 \); **\( p < 0.01 \); ***\( p < 0.001 \).