YAP1/TAZ-TEAD transcriptional networks maintain skin homeostasis by regulating cell proliferation and limiting KLF4 activity

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The Hippo TEAD-transcriptional regulators YAP1 and TAZ are central for cell renewal and cancer growth; however, the specific downstream gene networks involved in their activity are not completely understood. Here we introduce TEADi, a genetically encoded inhibitor of the interaction of YAP1 and TAZ with TEAD, as a tool to characterize the transcriptional networks and biological effects regulated by TEAD transcription factors. Blockage of TEAD activity by TEADi in human keratinocytes and mouse skin leads to reduced proliferation and rapid activation of differentiation programs. Analysis of gene networks affected by TEADi and YAP1/TAZ knockdown identifies KLF4 as a central transcriptional node regulated by YAP1/TAZ-TEAD in keratinocyte differentiation. Moreover, we show that TEAD and KLF4 can regulate the activity of each other, indicating that these factors are part of a transcriptional regulatory loop. Our study establishes TEADi as a resource for studying YAP1/TAZ-TEAD dependent effects.

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The barrier function of the skin is heavily dependent on the balance between self-renewal and differentiation of its basal stem/progenitor cell population. Transitions between stemness and differentiation states are determined by transcriptional and epigenetic changes that shape the genomic landscape of epithelial cells, affecting the differential binding of transcriptional activators and repressors to genes that participate in stem cell-renewal and the initiation of terminal differentiation1–3. YAP1 and its paralog TAZ (WWTR1) are co-transcriptional regulators downstream of the Hippo pathway4–5 that are essential for skin homeostasis and epithelial stem cell maintenance6–9. YAP1 and TAZ are also implicated in skin basal (BCC) and squamous (SCC) cell carcinoma formation10,11. Indeed, this axis has been recently listed as one of the top 10 signaling pathways altered in human cancer12.

The main stem cell regulatory and oncogenic functions of YAP1 and TAZ have been attributed to their interaction with TEAD transcription factors13–15. TEAD transcriptional-networks are a main component of the initiation of the epithelial state and are downregulated during keratinocyte differentiation2, highlighting the relevance of TEADs in epithelial homeostasis. However, studies of the role of YAP1/TAZ in skin homeostasis and cancer involve the knockout of these co-transcriptional activators, impinging not only on TEAD-dependent events, but also potentially affecting a myriad of other transcriptional and signaling components that interact with YAP1 and TAZ16,17. For example, YAP1 and TAZ have been proposed to regulate Wnt signaling by interacting with the destruction complex18 and by sequestering disheveled in the cytoplasm19. The disruption of these numerous cytoplasmic and nuclear pathways makes it difficult to pinpoint exactly the TEAD-specific effects mediated by YAP1 and TAZ, particularly in vivo.

To characterize the precise transcriptional events regulated downstream of YAP1/TAZ-TEAD, we introduce TEADI (TEAD-inhibitor), a genetically encoded fluorescently-tagged inhibitor of the interaction of YAP1 and TAZ with TEAD transcription factors. Here we use TEADI to identify the transcriptional networks regulated by TEAD using as a model human keratinocytes and mouse skin.

**Results**

**Development of TEADI.** To target TEAD transcriptional activity we designed a genetically encoded dominant-negative protein (TEADI) containing TEAD-interacting domains that bind to TEAD and prevent its interaction with co-transcriptional activators. Specific TEAD-interacting domains are present in the Hippo-family members YAP1 and TAZ, and in vestigial-like (VGLL) proteins20,21, and it has been shown that peptides containing these domains are effective in antagonizing YAP1 activity by blocking YAP1 binding to TEAD21,22.

TEADI was constructed using as a starting point the TEAD-binding-domain (TBD) of the VGLL4 protein (TBD VGLL4) and the TEAD binding domain of YAP1 (TBD YAP1), previously shown to cooperatively block the interaction between YAP1 and TEADs as part of the Super-TDU peptide21. This sequence was modified by including a flexible linker sequence and by introducing a Pro98Glu substitution in TBD YAP1 (TBD YAP1*) that improves binding affinity to TEAD20. Genetically-encoded inhibitors give more flexibility to increase in protein size compared with soluble peptide inhibitors, allowing us to include the TEAD interaction domain of TAZ (TBD TAZ)20 to increase inhibition towards TAZ, a bipartite nuclear localization signal (BNPNLs)22 to target the construct, and a green fluorescent protein (GFP) to easily track expression and localization of TEADI. The final construct consists of the following domains separated by linker sequences: GFP-TBD VGLL4-TBD YAP1*-TBD TAZ-BNPNLs (Fig. 1a, see full sequence details in the Methods section). TEADI has a predicted molecular weight of 39 kDa, its expression is easily traceable by fluorescent microscopy and its nuclear localization allows for a specific blockage of TEAD without affecting cytoplasmic functions of YAP1 and TAZ.

Transduction of cells with a plasmid containing TEADI identified that this inhibitor is well expressed and localized to the nucleus (Fig. 1a) and can block basal TEAD-reporter activity as well as YAP1- and TAZ-induced TEAD activity in cells (Fig. 1b). Co-immunoprecipitation experiments showed that TEADI reduces the interaction of YAP1 and TAZ with TEAD, but it does not alter the interaction of these proteins with LATS1, a member of the Hippo pathway (Fig. 1c). Our results indicate that TEADI is a valid genetically encoded YAP1/TAZ-TEAD interaction inhibitor to study TEAD-dependent transcription and biological effects.

**TEAD activity maintains keratinocytes in a progenitor state.** To study the effects of inhibiting TEAD-dependent transcription in keratinocytes we took advantage of immortalized N/TERT2G keratinocytes that show similar epidermal differentiation in 2D culture and 3D organotypic skin models to human primary keratinocytes14,15. N/TERT2G keratinocytes transduced with adenoviruses expressing TEADI (ad-TEADI) showed a significant decrease in proliferation (Fig. 1d) accompanied by a marked increase in the mRNA expression of the differentiation markers keratin 1 (KRT1) and transglutaminase 3 (TGM3), and a reduction of the basal/progenitor markers keratin 5 (KRT5), compared with cells transduced with adenoviruses expressing GFP (ad-GFP) (Fig. 1e). We also observed a significant reduction in the mRNA expression of the YAP1/TAZ reporter genes CYR61 and CTGF (also known as CCN1 and CCN2 respectively, Fig. 1e) and reduced CYR61 protein expression (Supplementary Fig. 1a), indicating that TEADI blocks the activity of YAP1/TAZ-TEAD. TEADI expression also lead to a decrease in the protein expression of the basal markers p63 and KRT5, and an increase in the protein levels of the differentiation markers keratin 10 (KRT10) and involucrin (IVL) (Fig. 1f and Supplementary Fig. 1b, c), as well as a decrease in proliferation as measured by Edu-DNA incorporation and PCNA staining (Supplementary Fig. 1d, e). Blockage of TEAD by TEADI did not result in changes in the expression levels of YAP1 or TAZ (Fig. 1f) or nuclear localization of YAP1 (Supplementary Fig. 1f). Endogenous TEAD protein coimmunoprecipitation experiments in keratinocytes showed that TEADI reduces the interaction of YAP1 and TAZ with TEAD transcription factors (Supplementary Fig. 1g).

We next generated stable N/TERT2G keratinocytes expressing tetracycline-inducible TEADI or GFP as a control by lentiviral transduction and subjected these cells to 3D differentiation in cell culture inserts, which recapitulate the differentiation stages of skin in an in vitro system23. When N/TERT2G keratinocytes were induced to express TEADI, cells showed signs of early differentiation, resulting in thinner epidermal cultures with an aberrant and increased differentiation pattern (Fig. 1g). Immunofluorescence (IF) staining showed reduced expression of the basal marker KRT5 as well as reduced levels of the proliferation marker PCNA in organotypic cultures expressing TEADI (Fig. 1h and Supplementary Fig. 1h). Interestingly, cultures showed increased staining of the differentiation marker KRT10, which also labeled cells in the basal/progenitor layer (Fig. 1i), indicating early activation of differentiation programs. However, cells retained the ability to form a layered epidermis in the absence of TEAD activity (Fig. 1g). Our results indicate that...
TEAD interaction with YAP1/TAZ is necessary to maintain keratinocytes in a basal, undifferentiated state.

TEAD transcriptional networks in keratinocytes. RNA sequencing (RNA-seq) was performed to characterize the transcriptional effects of TEAD inhibition in N/TERT2 keratinocytes transduced with ad-TEADi or control ad-GFP after 12, 24 and 48 h. Differentially regulated genes were considered as having an absolute fold change (|FC|) ≥ 1.5 and a false discovery rate (FDR) adjusted q-value (q) < 0.05. We could not observe any significant differentially-regulated genes at 12 h, probably due to a low expression of TEADi at this time point (Fig. 2a), however the expression of several genes was affected by TEAD inhibition at 24 and 48 h, with a significant overlap of differentially regulated genes (Fig. 2b and Supplementary Data 1). TEADi early response transcripts present in both the 24 and 48 h datasets included several genes involved in epithelial differentiation, including NOTCH3, FOXN1, ZNF750, IVL, CNFN, and DSC2, as well as the YAP1/TAZ response genes CYR61 and CTGF.

Comparing the differentially regulated genes following TEADi expression at 48 h in keratinocytes with those found to be regulated by YAP1 in other studies, we identified a core of well-established genes regulated by YAP1 and TAZ, which included AXL, CTGF, CYR61, and FST shared among the three datasets (Fig. 2c). To further compare the transcriptional consequences of TEAD blockage with those triggered by loss of YAP1 and TAZ, we performed RNA-seq in N/TERT2 keratinocytes with YAP1 and TAZ knockdown by pooled-small interfering RNA (siYAP1/TAZ, Supplementary Fig. 2a and Supplementary Data 2). As expected, siYAP1/TAZ reduced keratinocyte proliferation (Supplementary Fig. 2b) and lead to gene expression changes that produced a larger amount of significant differentially regulated genes than TEADi (Fig. 2d). However, significant overlap was observed between differentially regulated genes in both conditions (Fig. 2d), confirming that common gene networks are
Fig. 2 TEAD transcriptional networks in keratinocytes regulate cell cycle entry and differentiation programs. a Western blot analysis of expression of GFP (Ad-GFP, control) and TEADi (Ad-TEADi) in N/TERT2G cells. b Venn diagram showing the overlap between differentially regulated genes in TEADI vs GFP (q < 0.05, [FC] ≥ 1.5) in N/TERT2G keratinocytes at 24 and 48 h following transduction. c, d Venn diagrams showing the overlap between differentially regulated genes (q < 0.05, [FC] ≥ 1.5) by TEADI expression in N/TERT2G keratinocytes and published YAP1 signatures3,26 (c) or siYAP1/TAZ (d). e Graph indicating the fold change (Log2 FC) of genes related to canonical Wnt/β-catenin signaling (IPA) in the siYAP1/TAZ and TEADi 48 h datasets. Only differentially regulated genes are shown. White color indicates the gene is not differentially regulated in that dataset. The IPA significance p-value (p) of overlap for the Wnt/β-catenin canonical pathway is indicated for each dataset. f IPA activation Z-score for canonical Wnt/β-catenin signaling in the indicated datasets. g Western blot analysis of expression of the indicated markers in N/TERT2G cells. Active β-Catenin refers to non-phospho β-Catenin at Ser45. h Graph indicating the GO biological process terms enriched in upregulated (q < 0.05, FC ≥ 1.5) and downregulated (q < 0.05, FC ≤ −1.5) genes by TEADI expression in N/TERT2G keratinocytes at 48 h and fold change (Log2 FC) of genes related to GO keratocyte differentiation and cell cycle at corresponding times compared with GFP expressing cells. i IPA functional analysis of activation state of upstream transcriptional regulators for genes in b. j Western blot analysis of the expression of cell cycle regulators in N/TERT2G cells transduced with GFP (Ad-GFP, control) or TEADi (Ad-TEADI) for 48 h. k Luminescence cell proliferation assay in N/TERT2G cells transduced with pooled siRNAs for E2F1. l Western blot analysis of the expression of cell cycle regulators and differentiation markers in N/TERT2G cells transduced with pooled siRNAs for E2F1 for 48 h. In k n = 3 biological replicates. Mean ± SD is shown; two-way ANOVA with Sidak’s multiple comparison test. In b, c and d p indicates the p-value of the overlap in Venn diagrams, Fisher’s exact test. Source data are provided as a Source Data file.

To better illustrate the differences between TEADI and YAP1 and TAZ knockdown that can lead to non-overlapping effects we focused on the Wnt/β-catenin pathway. Regulation of the activation of β-catenin has been shown to be independent of TEAD transcription factors and dependent on the direct interaction of YAP1 and TAZ with components of the Wnt signaling pathway.18,19 Analysis of canonical pathways affected by siYAP1/TAZ and TEADI in Ingenuity Pathway Analysis (IPA) indicated that, although under both conditions Wnt/β-catenin signaling gene networks are differentially regulated (Fig. 2e), siYAP1/TAZ leads to a clear increase in the activity of the pathway when compared with TEADI (Fig. 2f). Indeed, several downstream targets of Wnt signaling were activated by siYAP1/TAZ and not TEADI, including AXIN2 and CD44 (Fig. 2e). By utilizing an antibody that recognizes non-phospho active β-Catenin (Ser45)27, we confirmed that siYAP1/TAZ results in a significant increase in the amount of active β-catenin in keratinocytes, while TEADI results in minimal alterations in the levels of active protein (Fig. 2g and Supplementary Fig. 2c). Our results indicate that YAP1/TAZ modulate Wnt signaling through TEAD-dependent and independent events and confirm that TEADI can discern YAP1/TAZ-TEAD specific effects.
We next focused on the TEAD-dependent gene networks in keratinocytes. Gene ontology (GO) analysis of the differentially regulated genes in the TEADi dataset at 48 h identified that TEAD inhibition induces a rapid activation of differentiation and skin development gene networks and a downregulation of genes involved in cell cycle progression and processes (Fig. 2h). TEAD blockage resulted in a rapid increase in the expression levels of differentiation markers including TGM1 and 3 and involucrin (IVL) and reduced the expression levels of several cell cycle regulators, including E2F1 (Fig. 2h). Analysis of gene sets common to TEADi and siYAP1/TAZ, which would constitute the core of YAP1/TAZ-TEAD transcriptional activity, showed similar GO terms related to cell cycle and differentiation (Supplementary Fig. 2d). The overlap in the effects of TEADi and siYAP1/TAZ was validated even when we selected the top genes modified by TEADi and siYAP1/TAZ by applying more stringent threshold levels (|FC| ≥ 2 and q < 0.01, Supplementary Fig. 2e), and indicate that the most significant processes differentially regulated under both conditions are related to adhesion and differentiation (Supplementary Fig. 2e).

Analysis of upstream regulators by IPA in the TEADi 48 h dataset identified that transcriptional networks related to E2F, cyclins, YAP1, and MYC were downregulated, while networks related to cell cycle inhibitors were upregulated (Fig. 2i). Indeed, TEAD inhibition results in a reduction of E2F1 and cyclin D levels (Fig. 2i). However, we were not able to detect differences in p53, p16, apoptosis markers or phospho-Rb levels, (Fig. 2i) and Supplementary Fig. 2f, g), indicating that the inhibition of cell cycle could be a direct result of the transcriptional effect of TEAD on the expression of E2F1 cell cycle regulators. Supporting a direct role of TEAD in controlling E2F transcription, TEADi expression resulted in a reduction in the mRNA levels of several members of the E2F family, including E2F1, 2, 5 and 8 (Supplementary Fig. 2h), and in a decrease in luciferase signal in a reporter containing the human E2F1 promoter (Supplementary Fig. 2i, j). Knockdown of E2F1 (siE2F1) is sufficient to reduce cell growth in keratinocytes (Fig. 2k, l), indicating a critical role for E2F1 regulating cell cycle entry. Interestingly, while reduced levels of E2F1 halted keratinocyte proliferation and reduced the levels of cyclin D expression, it did not result in increased expression of the differentiation marker KRT10 or reduced expression of the basal marker KRT5 (Fig. 2i).

Overall, our results indicate that TEADi can be used to dissect YAP1/TAZ-TEAD specific gene networks and that TEAD controls cell cycle progression in keratinocytes by regulating the levels of E2F1.

**TEAD and KLF4 limit each other activity.** YAP1/TAZ-TEAD complexes can modulate transcription at several levels, including direct promoter regulation, enhancer association, or binding with chromatin regulating proteins. Indicating that TEAD can control epithelial homeostasis indirectly by regulating the expression and chromatin accessibility of other transcription factors affecting central cell renewal and differentiation gene networks.

To dissect global transcriptional networks affected by TEAD-blockage we performed a transcription factor binding site enrichment analysis in the TEADi upregulated and downregulated gene sets (Fig. 3a and Supplementary Fig. 3a–d). We found that only about 19% of the downregulated genes and 25% of the upregulated genes at 48 h present predicted TEAD-binding sites in their promoter regions (Supplementary Fig. 3a, b). Overrepresented conservation transcription factor binding sites in the downregulated gene set included API, TEAD1, Myc and E2F1 (Fig. 3a and Supplementary Fig. 3c), supporting the role of TEAD in facilitating transcription from factors important for keratinocyte self-renewal and proliferation. Interestingly, binding sites in upregulated genes in keratinocytes showed a clear enrichment for KLF4 (Fig. 3a and Supplementary Fig. 3d). While KLF4 is a well-known stem cell factor in embryonic and induced pluripotent stem cells, in keratinocytes is central to the specification of differentiated cells, suggesting that YAP1/TAZ-TEAD could regulate differentiation by limiting the activity of KLF4.

Inhibition of TEAD transcriptional activity by TEADi in keratinocytes resulted in increased nuclear KLF4 expression at 48 h (Fig. 3b) and KLF4 was necessary for the activation of differentiation following TEAD inhibition as measured by KRT10 expression (Fig. 3c). Interestingly, knockdown of KLF4 resulted in increased expression of the YAP1/TAZ-TEAD target CYR61 (Fig. 3c), indicating the possibility of a dual regulation in which TEAD and KLF4 limit each other activity. Supporting this, KLF4 knockdown resulted in increased association between YAP1 and TEAD (Fig. 3d). In contrast, overexpression of KLF4 in keratinocytes lead to an increase differentiation and reduced levels of CYR61 (Fig. 3e). Consistent with KLF4 regulating the transcriptional activity of TEAD, knockdown of KLF4 in HEK293 cells results in a significant increase in TEAD-reporter activity (Fig. 3f).

To confirm the mutual regulation of transcriptional networks under TEAD and KLF4, we performed RNA-seq in N/TERT2G keratinocytes with siKLF4 (Supplementary Data 3). KLF4 knockdown resulted in the upregulation of genes related to cell cycle and division and downregulation of transcripts that participate in epidermis development and biological adhesion (Fig. 3g). IPA analysis of upstream networks affected by siKLF4 indicated an activation of transcriptional targets of MITF, E2F factors and YAP1 (Fig. 3h), confirming that reduction in KLF4 levels leads to an increase transcriptional activity downstream of YAP1. Indeed, differentially regulated genes in siKLF4 showed a significant overlap with genes differentially regulated by TEADi at 48 h and by siYAP1/TAZ (Fig. 3i), further confirming that the transcriptional networks downstream from KLF4 and TEAD-YAP1/TAZ are closely intertwined. Since KLF4 is primarily involved in the specification of differentiated cells, we analyzed the expression of differentiation markers and found that TEADi and siYAP1/TAZ consistently lead to upregulation of key differentiation genes, including IVL, FOXN1, KRT1, and KRT10, while siKLF4 results in downregulation of these transcripts (Fig. 3j). It is worth noting that siYAP1/TAZ leads to an increase in KLF4 mRNA and protein levels and activation of differentiation (Fig. 3j, k), which recapitulates the effect of TEADi but is not further increased by the expression of the TEAD inhibitor (Fig. 3k), confirming that the effects observed by TEADi are indeed mediated by YAP1/TAZ activity.

Our results indicate that the balance of transcriptional activity between KLF4 and YAP1/TAZ-TEAD is critical for the activation of differentiation gene networks, suggesting a mechanism could be in place by which they can directly modulate each other activity. It has been shown that YAP1/TAZ cooperate with KLF4 to promote the differentiation of mouse intestinal cells into goblet cells by direct binding of YAP1/TAZ to KLF4. Co-immunoprecipitation experiments demonstrated that KLF4 interacts with TEAD, YAP1 and TAZ in keratinocytes and that this interaction is increased by TEADi (Fig. 4a). Our results indicated two scenarios for TEAD and KLF4: one in which YAP1/TAZ and TEAD binding to KLF4 can affect the activity of this differentiation factor, an another one in which KLF4 could bind to YAP1/TAZ-TEAD complexes to limit their transcriptional activity. To demonstrate whether KLF4-YAP1/TAZ interaction has a functional consequence on KLF4 activity, we constructed GAL4-DNA binding domain fusion proteins (indicated as GAL4...
with the 329 N-terminal amino acids of KLF4, which harbor the activation and repression domains but excludes the zinc finger domain, and two fragments containing only the KLF4 activation domain (1–156) or only the repressor domain (159–329). Overexpression of wild type YAP1 or a YAPI S94/F95 mutant that does not bind to TEAD, resulted in a marginal potentiation of GAL4-KLF4 1–329 and GAL4-KLF4 1–156 activity, while the construct containing the repressor domain (GAL4-KLF4 159–329) did not induce transcription. This marginal increase in activity, coupled to the fact that keratinocytes can activate KLF4 and differentiation in the absence of YAPI/TAZ, indicates that YAPI/TAZ interaction with KLF4 might not have functional consequences during keratinocyte differentiation. On the other hand, KLF4 was able to reduce YAPI-induced expression of CYRF6 and activation of TEAD transcription, particularly GAL4-KLF4 1–329 and GAL4-KLF4 1–156 (Fig. 4d, e). KLF4 lacking the DNA-binding domain was still able to bind to TEAD, particularly the activation domain of KLF4 (Fig. 4f). Collectively, these results expose a scenario where KLF4 binds YAPI/TAZ-TEAD complexes to limit TEAD transcription.

TEADi disrupts epithelial homeostasis in the mouse skin. To define the role of YAPI/TAZ-TEAD transcription in specific tissues in vivo we developed a tetracycline-inducible TEADi transgenic mouse. We targeted expression of TEADi to the epidermis and its stem cells by breeding our TEADi transgenic line with the 329 N-terminal amino acids of KLF4, which harbor the activation and repression domains but excludes the zinc finger domain, and two fragments containing only the KLF4 activation domain (1–156) or only the repressor domain (159–329). Overexpression of wild type YAP1 or a YAPI S94/F95 mutant that does not bind to TEAD, resulted in a marginal potentiation of GAL4-KLF4 1–329 and GAL4-KLF4 1–156 activity, while the construct containing the repressor domain (GAL4-KLF4 159–329) did not induce transcription (Fig. 4c). This marginal increase in activity, coupled to the fact that keratinocytes can activate KLF4 and differentiation in the absence of YAPI/TAZ (Fig. 3j, k), indicates that YAPI/TAZ interaction with KLF4 might not have functional consequences during keratinocyte differentiation. On the other hand, KLF4 was able to reduce YAPI-induced expression of CYRF6 and activation of TEAD transcription, particularly GAL4-KLF4 1–329 and GAL4-KLF4 1–156 (Fig. 4d, e). KLF4 lacking the DNA-binding domain was still able to bind to TEAD, particularly the activation domain of KLF4 (Fig. 4f). Collectively, these results expose a scenario where KLF4 binds YAPI/TAZ-TEAD complexes to limit TEAD transcription.

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Fig. 4 KLF4 interacts with the YAP1/TAZ-TEAD complex. a Coimmunoprecipitation experiments in N/TERT2G cells transduced with TEADi or GFP. b Schematic representation of GAL4-KLF4 fusion proteins and western blot showing their expression in HEK293 cells. c Transcriptional activity of GAL4 DNA-binding domain fusion proteins measured by luciferase assay by UAS-Luc in HEK293 cells. d Western blot analysis of the expression the indicated markers in HEK293 cells transduced YAPI in combination with the indicated GAL4 fusion proteins for 48 h. e Transcriptional activity of TEAD in HEK293 cells transduced with YAPI and GAL4–KLF4 fusion proteins or GAL4 control. f Coimmunoprecipitation experiments in HEK293 cells transduced with the indicated GAL4–KLF4 proteins and Myc-tagged TEAD, HA-tagged TAZ and FLAG-tagged YAPI. In c n = 4 biological replicates; in e n = 3 biological replicates. Mean ± SD is shown; one-way ANOVA with Dunnett’s multiple comparisons test. Source data are provided as a Source Data file.

Histological analysis showed skin thinning 4 days following TEADi induction, and ulcer formation and areas of reactive thickened epidermis surrounding the wounds in mice after 12 days (Fig. 5b). Quantification of basal cell proliferation by PCNA staining demonstrated that TEADi expression leads to a significant reduction in basal keratinocyte cell proliferation as early as 4 days, and this reduction is sustained after 12 days (Fig. 5c). One possible explanation for the observed phenotype is that reduced proliferation of basal keratinocytes leads to stem/progenitor cell depletion in the epidermis, disrupting skin homeostasis and facilitating wound formation. Indeed, we observed low levels of the basal marker KRT5 at 4 and 12 days following TEADi expression (Fig. 5d), accompanied by an increase in expression of the differentiation markers KRT10 and loricrin (Fig. 5d and Supplementary Fig. 4a). Furthermore, the levels of the basal and stem cell marker p63 were rapidly reduced by TEADi expression in the epidermis (Fig. 5e) and isolated keratinocytes from mice after 4 days of TEADi expression showed reduced clonogenic capacity (Supplementary Fig. 4b), indicating stem/progenitor cell depletion. Aligned with our in vitro results, TEADi also lead to a marked increase in KLF4 expression as early as 4 days after induction and thickened epidermal areas in 12-days mice also showed increased KLF4 expression (Fig. 5f).

Remarkably, despite the observed stem/progenitor cell depletion, mice that developed ulcers after 10–20 days of TEADi induction showed thickened epidermis in several areas surrounding wounds (Fig. 5a and Fig. 6a). These thickened epidermal areas were mostly composed by cells positive for KRT10 and loricrin and negative for the basal markers p63 and KRT5 (Fig. 6a and Supplementary Fig. 4a), indicating that they are differentiated cells. One possible explanation for these thickened and differentiated epidermal areas is that cells transitioning into differentiation with low expression of TEADi can still proliferate. Indeed, we were able to detect proliferating cells in the epidermis of mice at 12 days that present low levels of KRT5 staining (Figs. 5c and 6b). In addition, proliferating areas at 12 days were negative for TEADi expression (Fig. 6c) and we observed a reduction over time of cells expressing detectable levels of TEADi (Fig. 6d), probably caused by the fact that TEADi positive cells rapidly differentiate, leading to a termination of T5rTA expression and, consequently, TEADi downregulation. Of interest, labeling of lymphoid cells and macrophages in the skin with CD45 indicated an increase in inflammatory cell infiltration by 4 days that is further increased by 12 days following TEADi expression (Fig. 6e). Inflammatory cell infiltration is commonly caused by the disruption of epithelial integrity and could be a plausible trigger for the observed keratinocyte activation and thickened epidermal areas at late time points.

Altogether, our results indicate that blockage of TEAD activity in the basal compartment of the skin leads to reduced proliferation and increased differentiation of basal cells, resulting in the depletion of KRT5+/p63+ progenitor/stem cells and disruption of epithelial integrity.

Discussion

The precise study of the transcriptional networks regulated downstream of YAPI and TAZ in somatic stem cells and cancer, particularly in the skin, is hindered by numerous factors. First, knockout of YAPI is sufficient to disrupt stem cell function and skin homeostasis in developing mice, whereas both YAPI and
TAZ have to be downregulated to observe an effect in adult skin, BCC and SCC8,9,11. Consequently, the generation of double knockout mice is required to characterize the effects of YAP1/TAZ in adult tissues. Second, YAP1/TAZ interact with numerous effectors that regulate not only gene transcription but also other processes16–19, indicating that YAP1/TAZ knockout results in countless transcriptional, structural and signaling effects, which are usually cumbersome to dissect. Finally, it has been suggested...
that YAP1 knockout mice could still express shorter isoforms of YAP1 protein, accounting for phenotypic differences in mouse studies\(^3,4\). To overcome these roadblocks, here we use the expression of TEADi, a genetically encoded inhibitor of the interaction of YAP1/TAZ with one of their main downstream effectors, TEAD transcription factors, to dissect the specific regulatory gene expression networks downstream of YAP1/TAZ-TEAD.

We demonstrate that TEAD activity regulates epithelial cell homeostasis at two independent levels (Fig. 7a): by controlling the expression of key factors necessary for cell cycle entry and proliferation, and via a regulatory loop with master regulators of commitment to differentiation. TEAD regulates the transcription of the E2F1 gene and E2F1 knockdown recapitulates the cell cycle blockage produced by TEAD inhibition, indicating that E2F1 is a central regulator for proliferation in keratinocytes. However, E2F1 knockdown is not sufficient to trigger the rapid activation of differentiation programs observed by TEAD expression.

Blockage of YAPI/TAZ binding to TEAD resulted in activation of KLF4 transcriptional networks, a central factor necessary for differentiation commitment\(^30,31\), and we demonstrate that KLF4 and YAPI/TAZ-TEAD transcriptional networks are closely intertwined. Our results suggest that TEAD and KLF4 transcription factors are at the crossroad of a regulatory loop that determines the differentiation state of keratinocytes. KLF4 is downregulated during skin cancer progression and KLF4 knockout mice show increase cancer incidence\(^30\), pointing towards a role of KLF4 regulating both activation of differentiation programs and direct repression of pathways involved in epithelial stem cell maintenance, including YAPI/TAZ-TEAD. Furthermore, given that YAPI/TAZ-TEAD transcriptional networks are the downstream effector of numerous chemical and biomechanical signals, TEAD could serve as the link between KLF4 activity and microenvironmental chemical and mechanical cues. Although we demonstrate that KLF4 can directly bind and regulate the activation of YAPI/TAZ-TEAD complexes, the precise mechanism by which KLF4 acts and by which TEAD regulates KLF4 expression requires further investigation.

Our results also highlight the essential role of TEAD transcriptional regulation to maintain epithelial homeostasis in vivo. TEAD transcription is necessary to keep basal progenitor cell proliferation and renewal, and inhibition of TEAD results in rapid differentiation and barrier disruption, leading to immune cell infiltration. Interestingly, despite the reduction in basal cell proliferation, both human and mouse keratinocytes are able to form a layered epidermis in the presence of TEADi, although with a significant increase in differentiated cells. This phenotype could be the result of heterogeneous levels of TEAD inhibition across basal cells or the expansion of transient amplifying cells that might not require TEAD activity to proliferate.

The peptides used to build TEADi were selected due to their proven specificity towards TEAD binding\(^20\,22\) and our results suggest that the main effects of TEADi are indeed mediated by YAPI/TAZ-TEAD activity. One limitation of our model is that despite the specificity of the TBDs they could cause additional nuclear effects that are not mediated by YAPI/TAZ, particularly the TBD of VGLL4. It has been shown in numerous studies that the main function of VGLL4 in cells is to block the binding of YAPI/TAZ to TEAD transcription factors\(^31,38\,42\), indicating that TBD-VGLL4 should not have any additional effects than to potentiate endogenous VGLL4-mediated TEAD inhibition. In addition, VGLL4 knockout mice have no reported phenotype on skin development\(^42\), suggesting that blockage of VGLL4 in keratinocytes might not have any functional consequences. It is worth noting also that TEADi and YAPI/TAZ knockdown will cause additional non-overlapping effects due to the fact that both are different conditions (Fig. 7b): in TEADi expressing cells, YAP1 and TAZ remain present to interact with other factors; while in siYAP1/TAZ cells, structural interactions of YAP1 and TAZ, including interactions with the β-Catenin destruction complex, are altered, causing additional effects not present in TEADi cells.

Considering that the Hippo pathway constitutes one of the top signaling pathways altered in human cancer\(^12\), disruption of YAPI/TAZ-TEAD complexes has become a main target to suppress oncogenic activity. TEADi could potentially be used to dissect the TEAD-dependent and independent roles of YAPI/TAZ signaling and aid in the discovery of improved targeting strategies for this pathway in cancer and other pathologies. In conclusion, the use of TEADi could become an additional invaluable resource for studying YAPI/TAZ-TEAD dependent transcription, with improved advantages that include rapid and simple inhibition of TEAD transcription and specific blockage of nuclear events mediated by both YAPI and TAZ without affecting structural or cytoplasmic functions of these proteins.

**Methods**

**DNA constructs.** TEADi was cloned using a gBlocks Gene Fragment (Integrated DNA Technologies) coding for the following amino acid sequence downstream of GFP into a pCEFL vector: SVDDHFAKALGDTWLQI corresponds to the human ADGSEFESPKKKRKVE. SVDDHFAKALGDTWLQI corresponds to the human ESFFKEP corresponds to the human TAZ-TEAD binding domain (TBD TAZ)\(^20\), and 8xTEAD-Luc (pGL3b 8xGTIIC-\(*\) corresponds to the human VGLL4-TEAD binding domain (TBD VGLL4)\(^21\). The constructs were a gift from Kunliang Guan (Addgene plasmids #32839, #33057 and #24638 respectively). For the E2F1 promoter reporter, a region of ~1.7 kb upstream of the transcription initiation site of the human E2F1 gene was cloned by PCR from human genomic DNA (Bioline) with the following primers: forward 5′GGCTGGTACACAGGTTCGCTC3′, reverse 5′TTTTGCGGGAAGAAGCC3′; this region was then cloned into the pGL4.21(luc2P/Puro) vector (Promega). GAL4-KLF4 constructs were cloned by PCR of corresponding human KLF4 amino acids downstream of the DNA-binding domain of GAL4 into a pCEFL vector. pCEFL GAL4bd was a gift from Stephen Elledge (Addgene plasmid #44012)\(^43\), 8xTEAD-Luc (pCEFL gBlocks Gene Fragment 8xTEAD binding domain).
from Silvio Gutkind and has been described previously.10 For KLF4 overexpression pMSCV-Flag-hKLF4 was used, a gift from Juan Belmonte (Addgene plasmid #20074).

Cell culture, transfections, and adenoviral transductions. All cells were cultured at 37 °C in the presence of 5% CO₂. HEK293 cells were obtained from AddexBio and Lenti-X™ 293T cells from Takara Bio and cultured in DMEM (Sigma-Aldrich Inc) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich Inc) and antibiotic/antimycotic solution (Sigma-Aldrich Inc). N/TERT-2G keratinocyte cell line24,25 was provided by Ellen H. van den Bogaard (Radboud University Medical Center, Nijmegen, The Netherlands) and James Rheinwald (Brigham and Women’s Hospital, Boston, MA, USA), and cultured in EPILIFE medium (Life Technologies) with Human Keratinocyte Growth Supplement (HKGS, Life Technologies). HEK293 cells and Lenti-X™ 293T were obtained directly from the described company and not further authenticated. N/TERT-2G cells were authenticated by STR profiling with the following results: TH01 8, 9.3; D5S818 11, 12; D13S317 8, 12; D7S820 9, 11; D16S539 10, 11; CSF1PO 10, 12; vWA 16, 18; TPOX 8, 11; Amelogenin X, Y. For siRNA experiments, cells were transfected with the corresponding siRNAs one day after plating and were treated/harvested 48 h after transfection. siRNAs were siGENOME SMARTpool from Dharmacon/Horizon, siE2F1 (M-003259-01-0005), siKLF4 (M-005089-03-0010), siYAP1 (M-012200-00-0005), siTAZ (M-016083-00-0005) and non-targeting control siRNA (D-001206-13). siRNA was transfected at a concentration of 8 pmol cm⁻² using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. For adenoviral transduction, cells were

Fig. 6 TEADi expression in the mouse skin induces wounding and inflammation. a Histology and IF pictures of the skin of a TEADi-expressing mouse showing a wound area and the surrounding epithelium. b Representative IF pictures of the skin of WT or TEADi mice induced for 12 days to show expression of the proliferation marker PCNA and the basal marker KRT5. c Representative IF picture of the skin of TEADi mice induced for 12 days to show expression of the proliferation marker PCNA and GFP. d Representative IF pictures and quantification of the skin of TEADi mice induced for 4 and 12 days to show expression of GFP. e Representative IF pictures and quantification of immune cell infiltration labeled by CD45 staining in the skin of TEADi mice. Inserts show magnification of the highlighted area. Location of the basal membrane is indicated with a white dotted line. In d 4d n = 69 and 12d n = 61; (e) WT n = 107, 4d n = 106 and 12d n = 114 fields from 3 different mice in each condition. Violin plots shows density of data and box plot shows median, 25 and 75% quartile, 95% confidence interval and dots indicating potential outliers; in d two-tailed unpaired t test and in e two-way ANOVA with Dunnett’s multiple comparison test. Source data are provided as a Source Data file.
incubated with a multiplicity of infection (MOI) of 25 with adenov-GFP (control) or adeno-TEADi for the indicated times. TEADi adenoviruses were produced, purified and titered by Vector Biolabs in an adenoviral-Type 5 (ΔE1/ΔE3) backbone with a CMV promoter, for GFP control Ad-CMV-GFP (Vector Biolabs, catalog no. 1060) was used. Lentiviruses and retroviruses were produced in Lenti-X 293T cells with the transfer and packaging plasmids, using TransIT-293 Transfection Reagent (Mirus) according to the manufacturer’s instructions. Supernatant was collected 48 h after transfection and concentrated using Lenti-X Concentrator (Clontech, catalog no. 631232). Viruses resuspended in PBS were used to infect N/TERT-2G keratinocytes after transfection and concentrated using Lenti-X Concentrator (Clontech, catalog no. A4403) and 10 ng/ml keratinocyte growth factor (SIGMA; catalog no. K5316). Cells were centrifuged at 400 × g for 8 min, resuspended in keratinocyte media described above and plated in collagen coated plates.

Gene expression analysis and quantitative PCR. Keratinocyte RNA was isolated and processed using RNasey Plus Mini Kit (Qiagen) according to the manufacturer’s instruction. Cells were lysed using the Precellys lysis kit (Bertin Instruments). mRNA integrity was measured with Agilent TapeStation system and samples with RIN < 8 were not further processed. mRNA expression profiling was performed in the CCR-Sequencing Facility at the NIH. Reads of the samples were trimmed for adapters and low-quality bases using Trimmomatic software before alignment with the reference genome Human—hg19 and the annotated transcripts using STAR. Gene counts were filtered by genes with ≥8 reads and normalized to TMM (Trimmed Mean of M values) and TPM (Transcripts Per Million) using Partek Flow software, version 7.0.18 (Partek Inc). TMM normalized counts were used for differential analysis using PARTEK Flow GSA algorithm (Partek Inc).

One microgram of cDNA was used as template for quantitative polymerase chain reaction with reverse transcription (qRT-PCR) analysis using SensiFAST cDNA Synthesis Kit (Bionime) and SensiFAST SYBR Hi-ROX Kit (Bionime). Samples were analyzed using a 7900HT Fast Real-Time PCR System. Oligonucleotides used for amplification were (Gene, Forward sequence 5′−3′, Reverse sequence 5′−3′): RPLP0, 5′-TGTCTGCTCCCACAATGAAAC-3′, 5′-TCGTCTTTAAACCCTG-3′; KLF4, 5′-ATCTCTGAGATGAACCGGATGATC-3′, 5′-CAGATTG

Fig. 7 TEAD regulation of keratinocyte proliferation and differentiation. a Simplified model summarizing the results from our study. Blockage of TEAD activation by TEADi triggers differentiation and reduced proliferation. In turn, activation of KLF4 can also lead to inhibition of YAP1/TAZ-TEAD transcription. b Comparison between the model of action of TEADi vs siYAP1/TAZ which might explain the lack of complete overlap in gene networks under both conditions. In cells expressing TEADi, YAP1/TAZ can still directly or indirectly regulate other factors that regulate transcription (TFs) and maintain cytoplasmic complexes, conditions that are not reproduced under YAP1/TAZ knockdown. Created with BioRender.
Immunoblot analysis. For Western blot\textsuperscript{44,47}, cells were lysed by sonication at 4 °C in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitor cocktail (Roche, \#583904001) and phosphatase inhibitors (PhosSTOP, Sigma-Aldrich, \#4906837001). Equal amounts of total protein samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Antibodies used were: anti-GAPDH (Cell Signaling; clone no. 14C10; catalogue no. 2118; 1:2000), anti GFP (Cell Signaling; clone no. D5; catalogue no. 2956; 1:2000), anti-HA tag antibody (Cell Signaling; clone no. C29F4; catalogue no. 3472; 1:2000), anti-FLAG tag antibody (Cell Signaling; clone no. 9A10; catalogue no. 8146; 1:1000), anti-Myt tag antibody (Cell Signaling; clone no. 71D10; catalogue no. 2278; 1:1000), YAP1 (Cell Signaling; clone no. D8H1X; catalogue no. 14074; 1:1000), TAZ (Cell Signaling; clone no. V386; catalogue no. 4883; 1:1000), LAT51 (Cell Signaling; clone no. G66B5; catalogue no. 3477; 1:1000), Pan-TEAD (Cell Signaling; clone no. D3F7L; catalogue no. 39692; 1:1000), KRT5 (BioLegend; catalog no. 95901; 1:1000), KRT10 (BioLegend; catalog no. 950401; 1:1000), Invucerin (SantaCruz; catalogue no. sc-21748; 1:500), E2F1 (Cell Signaling; catalogue no. 3742; 1:1000), Cyclin D1 (Cell Signaling; clone no. 92G2; catalogue no. 2978; 1:2000), Cyclin E1 (Cell Signaling; catalogue no. SC421; 1:1000), Total β-Catenin (Cell Signaling; clone no. D19A8; catalogue no. 8480; 1:1000), Active β-Catenin (Cell Signaling; clone no. D2U8Y; catalogue no. 19807; 1:1000), Cleaved-Caspase3 (Cell Signaling; clone no. 5A1E; catalogue no. 9664; 1:1000), Cleaved-PARP (Cell Signaling; clone no. D64E10; catalogue no. 5625; 1:1000). Secondary HRP-conjugated antibodies used were: Pierce peroxidase goat anti-mouse IgG (H + L) (ThermoFisher, catalogue no. 31342; 1:1000), Pierce peroxidase goat anti-rabbit IgG (H + L) (ThermoFisher, catalogue no. 31462; 1:4000), Anti-rat IgG (Cell Signaling catalogue no. 7077; 1:4000), Mouse Anti-rabbit IgG Conformation Specific (Cell Signaling, clone no. L27A9; catalogue no. 5127; 1:4000) and Rabbit Anti-mouse IgG Light Chain Specific (Cell Signaling, clone no. D2-12A; catalogue no. 58882; 1:4000). Secondary antibody incubation was followed by incubation with a ChemiDoc Imaging System (Bio-Rad) with Clarity Western ECL Blotting Substrates (Bio-Rad). Blot images were processed and quantified using ImageLab software v5.2.1 (Bio-Rad). Uncropped scans are available in the Source Data file.

Luciferase assays and immunoprecipitation. Luciferase assays were performed in HEK293 cells. To measure TEAD activity, cells in 12 or 24 well plates were co-transfected with GAL4-KLF4 constructs (0.3 µg cm\textsuperscript{-2}) and reporter vector containing a pTIGHT inducible promoter, a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and a poly(A) sequence, all flanked by the chicken h-globin gene insulator (HS4) sequence to avoid positional effects\textsuperscript{47}. The following reporters: forward 5′-AGGGATTCTGAACAGCATG-3′ (GFP), 5′-GAGGACCTGACCGCCTGA-3′ (β-gal) and the control sequence were inserted into pMD19-T vector (Takara). GFP was isolated by Pmel digestion from vector DNA and purified for microinjection into C57 mice fertilized oocytes. Founder mice were identified for the presence of the transgene by screening genomic DNA from tail biopsies using a PCR reaction with the following primers: forward 5′ GGATTTAGTGAACCGCATG 3′ and reverse 5′ GAGGACACCTGACCGCCTGA 3′ (geline 250 bp). PCR reactions were performed as follow: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final cycle with 5 min of extension at 72 °C. FVB/N mice carrying the cytokeratin 5 promoter in the reverse transgenic (Invitrogen; #MMS-101R) were provided by Kelly K. The following antibodies were provided by Kelly K. All male and female mice were used in the studies and all experiments were conducted using littermate controls. Housing conditions were as follow: temperature set point was 72 ± 4 °F (22.2 ± 2.2 °C), light cycle of 12 h on 6 am to 6 pm and 12 h off, NIH-031 rodent diet. Doxycycline was administered in the food grain-based pellets (Bio-Serv) at 6 g kg\textsuperscript{-1}. Doxycycline treatment was started between weeks 6 and 10 after birth.

Immunofluorescence. For IF in cells, cells were seeded in coverslips or 24 well plates and treated as described. Then cells were washed with ice-cold PBS and fixed with 3% paraformaldehyde in PBS for 10 min at room temperature. After three washes with PBS, cells were permeabilized with TritonX100 0.1% in PBS on ice for 10 min. Fixed cells were primary antibody overnight at 4 °C, washed three times with PBS and then incubation with the secondary antibody for 1.5 h at room temperature. Immunofluorescence analysis of mouse skin was performed on tissue sections embedded in paraffin. Sections were prepared for staining by antigen retrieval in 10 mM Sodium Citrate buffer (pH 6) and washed with bidistilled water 3× for 5 min. Slides were then incubated with the primary antibody overnight at 4 °C, washed three times with PBS and then incubation with the secondary antibody for 1.5 h at room temperature. Sections were mounted in FluorSave Reagent (Millipore, \#345789) with 1× coverslips for staining. Nuclei were stained with Hoechst 33342 (Invitrogen, \#H35703). The following anti-TEAD antibodies were used: GFP (Cell Signaling; clone no. D5.1; catalogue no. 2956; 1:500), GFP Polyclonal Antibody (ThermoFisher; catalogue no. A-6455; 1:500), GFP Polyclonal Antibody Alexa Fluor 488 (ThermoFisher; catalogue no. A-213111; 1:500), Pan-TEAD (Cell Signaling; clone no. D3F7L; catalogue no. 13295; 1:2000), KRT10 (BioLegend; catalogue no. 620100; 1:500), PCNA (Cell Signaling; clone no. PC-10; catalogue no. 567474; 1:4000), KRT5 (BioLegend; catalogue no. 905901 and 905501; 1:2000), p36 (Cell Signaling; catalogue no. 39692S; 1:2000), CYR61 (Cell Signaling; clone no. D4H5; catalogue no. 14479; 1:1000), GAL4 (SantaCruz; catalogue no. SC510; 1:1000), Total β-Catenin (Cell Signaling; clone no. D19A8; catalogue no. 8480; 1:1000), Active β-Catenin (Cell Signaling; clone no. D2U8Y; catalogue no. 19807; 1:1000), Cleaved-Caspase3 (Cell Signaling; clone no. 5A1E; catalogue no. 9664; 1:1000), Cleaved-PARP (Cell Signaling; clone no. D64E10; catalogue no. 5625; 1:1000). Secondary HRP-conjugated antibodies used were: Pierce peroxidase goat anti-mouse IgG (H + L) (ThermoFisher, catalogue no. 31342; 1:1000), Pierce peroxidase goat anti-rabbit IgG (H + L) (ThermoFisher, catalogue no. 31462; 1:4000), Anti-rat IgG (Cell Signaling catalogue no. 7077; 1:4000), Mouse Anti-rabbit IgG Conformation Specific (Cell Signaling, clone no. L27A9; catalogue no. 5127; 1:4000) and Rabbit Anti-mouse IgG Light Chain Specific (Cell Signaling, clone no. D2-12A; catalogue no. 58882; 1:4000). Secondary antibody incubation was followed by incubation with a ChemiDoc Imaging System (Bio-Rad) with Clarity Western ECL Blotting Substrates (Bio-Rad) according to the manufacturer’s instructions. Blot images were processed and quantified using ImageLab software v5.2.1 (Bio-Rad). Uncropped scans are available in the Source Data file.


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Author contributions
R.I.B. initiated the study; Y.Y. and R.I.B. designed the study and experiments; Y.Y., J.P., A.F., and R.I.B. performed experiments; P.A. generated TRE-TEAD4I transgenic mice; Y.Y., J.P., A.F., and R.I.B. analyzed and interpreted data; Z.W., Q.C., and R.I.B. provided administrative, technical, and material support; Y.Y. and R.I.B. prepared the paper.

Competing interests
The authors declare no competing interests.

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