Runx1 and p21 synergistically limit the extent of hair follicle stem cell quiescence in vivo

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Mechanisms of tissue stem cell (SC) quiescence control are important for normal homeostasis and for preventing cancer. Cyclin-dependent kinase inhibitors (CDKis) are known inhibitors of cell cycle progression. We document CDKis expression in vivo during hair follicle stem cell (HFSC) homeostasis and find p21 (cyclin-dependent kinase inhibitor 1a, Cdkn1a), p57, and p15 up-regulated at quiescence onset. p21 appears important for HFSC timely onset of quiescence. Conversely, we find that Runx1 (runt related transcription factor 1), which is known for promoting HFSC proliferation, represses p21, p27, p57, and p15 transcription in HFSC in vivo. Intriguingly, in cell culture, tumors, and normal homeostasis, Runx1 and p21 interplay modulates proliferation in opposing directions under the different conditions. Unexpectedly, Runx1 and p21 synergistically limit the extent of HFSC quiescence in vivo, which antagonizes the role of p21 as a cell cycle inhibitor. Importantly, we find in cultured keratinocytes that Runx1 and p21 bind to the p15 promoter and synergistically repress p15 mRNA transcription, thereby restraining cell cycle arrest. This documents a surprising ability of a CDki (p21) to act as a direct transcriptional repressor of another CDki (p15). We unveil a robust in vivo mechanism that enforces quiescence of HFSCs, and a context-dependent role of a CDki (p21) to limit quiescence of SCs, potentially by directly down-regulating mRNA levels of (an)other CDKis.

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Here we document the transcriptional regulation of CDKis and their potential control by Runx1 in HFSCs. We use mouse genetics tools to concomitantly target Runx1 and p21 in vivo and examine their interplay in tumors, in cells in culture, and in the normal hair cycle.

Results

Regulation of CDKI Expression During Normal HFSC Homeostasis. To explore CDKis transcriptional control in HFSCs, we documented their mRNA expression profiles in CD34+/α6-integrin⁺ bulge cells isolated from mouse skin by fluorescence-activated cell sorting.

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Next we wondered what regulates transcription of these CDKis throughout the hair cycle. We previously showed that Runx1 protein is expressed in the bulge during anagen and is at low levels at catagen and telogen when HFSCs are quiescent (15). This suggested an inverse correlation of Runx1 protein and CDKi mRNA level in the bulge throughout the hair cycle, making Runx1 a potential repressor of CDKis (Fig. 1E). Indeed, p21 was up-regulated in the bulge when we previously deleted Runx1 at anagen (16). Here we isolated CD34+/α6+ bulge cells from Runx1 KO mice at quiescence [postnatal day (PD) 21] before the onset of the Runx1 phenotype. We observed not only p21, but also p27 (three of three mice) and p57 (two of three mice showed increased expression whereas one showed a similar level compared with WT) mRNAs up-regulated in the Runx1 KO (CD34+/α6+) cells relative to WT (see Fig. 4A). This was consistent with the prolonged quiescence of HFSCs in the Runx1 KO and suggested that Runx1 acts as a repressor for several CDKis in vivo.

Loss of Runx1 in cell culture induced a severe proliferation defect and p21 loss rescued this phenotype (16), suggesting an important genetic interaction between Runx1 and p21 in vitro. To ask whether this interaction works by the direct binding of Runx1 to the p21 promoter, which Runx1 may inhibit, we performed chromatin immunoprecipitation (ChiP) on mouse keratinocytes. This revealed enrichment of Runx1 at a conserved Runx1 binding site in the p21 genetic locus (p21−β5) relative to other regions (H3K27me3) and to Runx1 inducible KO cells (Fig. 1D). In addition, the p21 promoter was enriched with a repressive histone mark, H3K27me3 upon Runx1 overexpression (Fig. S1B and C), likely via recruitment of corepressors of the Polycomb group proteins (22). In conclusion, a number of CDKis, including p21, p27, p57, and p15, are expressed and/or up-regulated at the mRNA level in the bulge at quiescence. Their redundant presence likely contributes to overall bulge cell quiescence and timely cell cycle exit in catagen. Moreover, p21 is likely a direct downstream repressoral target of Runx1 and down-regulation of Runx1 protein in the bulge could result in the up-regulation of p21, followed by cell cycle exit at catagen.

(FACS) at different hair cycle stages. We used HFSCs at proliferation (anagen) or quiescence (catagen/telogen) and performed quantitative real-time (q)PCR. First, p21 mRNA expression was up-regulated more than twofold in the WT quiescent vs. proliferative bulge (Fig. 1B), in agreement with nuclear p21 protein signal in catagen/telogen bulge (Fig. 1C). Nuclear p21 is known to inhibit cell proliferation (21). Intriguingly, p57 and p27 involved in HSC quiescence (4, 5), were up-regulated in the bulge relative to nonbulge (CD34+/α6+) at all hair cycle stages (Fig. 1B), suggesting that they may be responsible for the overall low rates of bulge cell division (10). Moreover, p57 and p15 mRNAs were found at higher levels in the catagen and telogen bulge than in the anagen. p19 was equally expressed and p18 seemed to be more specific to the nonbulge cells, whereas p16 was undetectable in any of the cell fractions tested (Fig. 1B).

Fig. 1. Regulation of p21 and other CDKis expression in normal HFSC homeostasis. (A) HFSC dynamics during normal skin homeostasis. (B) qPCR analysis of CDKis mRNAs in WT [telogen (PD20−21), anagen (PD24−27), and catagen (PD40−42)] bulge (CD34+/α6+) and nonbulge (CD34+/α6−) cells (Fig. S1A). (C) Immunofluorescence staining of paraffin skin sections at anagen (PD27) and catagen (PD39) shows nuclear p21 (green) in quiescence. (D) ChiP-qPCR with Runx1 antibody and primers to Gapdh and different regions of the p21 promoter (Table S1) in WT and Runx1 inducible KO (IKO) keratinocytes (Fig. S1B and C). (E) Model for cell cycle control factors show inverse relation of p21 and other factors with Runx1 protein expression (red) (15).
PD47). Interestingly, all CDKis remained unchanged in p21 KO, suggesting that enough CDKis may be already expressed in the bulge to maintain bulge cell quiescence even in the absence of p21 (Fig. S3F).

A simple Poisson model fitted the WT experimental data (Fig. 2F) (SI Materials and Methods) and indicated approximately three divisions per bulge cell in one hair cycle, as we previously reported (10). Intriguingly, this model did not fit the KO experimental data indicating that the bulge cells do not have a homogeneous response to p21 KO (Fig. 2G and SI Materials and Methods). A mathematical model assuming that only a subpopulation of the bulge cells respond to p21 loss does fit the data (Fig. S2B) and suggests that ∼40% of the KO bulge cells present at PD47 had replicated at least three times more than WT. Collectively, p21 KO bulge cells divide more in vitro and in vivo. Extra divisions affect even the most quiescent bulge cells, but all cells eventually return to quiescence.

To see whether p21 regulates rates of proliferation or timely cell cycle exit during quiescence, we performed short doxy chases at anagen and catagen (Fig. 2B). Bulge cells self-renew symmetrically at anagen to replenish their pool depleted by migration in the preceding phase (13, 25). Analysis of sorted bulge cells showed comparable numbers of bulge cell divisions in WT and p21 KO skin during PD24–27 (rapid self-renewal phase) (Fig. 2H and Fig. S3A) with no significant differences in BrdU+ bulges at PD27 (Fig. 2K), suggesting that p21 did not affect the rates of self-renewal in early anagen. However, by PD35, which marks the beginning of catagen when bulge cells return to quiescence, increased growth of ∼9% (as estimated by mathematical modeling) of p21 KO bulge cells could be detected from FACS and BrdU data at PD35 (Fig. 2J and K and Fig. S3B and D). This increase in p21 KO bulge cell proliferation was more pronounced by PD42 as seen in additional divisions clearly detected in the PD36–42 chase (Fig. 2J and Fig. S3C). Our mathematical modeling indicated that the WT bulge cells reduced their division rates by sevenfold in catagen compared with anagen (SI Text 3), but that the reduction for KO cells was roughly half as much. Both WT and KO bulge cells eventually entered quiescence by telogen as shown by BrdU staining (Fig. 2K, PD47). In summary, whereas WT bulge cells divided approximately three times in anagen and had greatly reduced proliferation after catagen onset, p21 KO bulge cells overall divided approximately one to two more times before entering quiescence, reflecting the existence of a cell fraction with a higher replication rate and/or extended replication period.

Interestingly, the extra bulge cells generated by prolonged proliferation due to p21 loss did not increase the bulge cell pool (Fig. 2E and Fig. S4A) measured by number of CD34+ cells per bulge at second telogen. This is, at least in part, explained by a KO-correlated increase in apoptosis in early catagen. Levels of apoptotic caspase-3+ cells in the p21 KO bulge showed an approximately threefold increase at PD35, the stage when we also detected failure of p21 KO cells to enter quiescence (Fig. 2K and Fig. S3E). This was consistent with the known anti-apoptotic role of p21 (26). Moreover, catagen bulge up-regulated not only pro- but also anti-apoptotic genes (27), likely to protect against excessive bulge cell death upon p21 loss (Fig. S4B). Finally, we did not observe colocalization of BrdU+ and caspase-3+ bulge cells, ruling out the possibility that p21 KO bulge cells die as they enter...
the cell cycle at an unpermitted time. Thus, it appears that some p21 KO bulge cells die to compensate for prolonged proliferation and to keep the bulge cell pool size constant. In conclusion, we found that p21 is important for regulating general rates of HFSCs in vitro whereas in vivo its up-regulation at quiescence, likely driven by down-regulation of Runx1 protein in the bulge, limits the extent of HFSC divisions during homeostasis.

**Runx1/p21 Genetic Interactions Are Context Dependent.** Although p21 KO rescued the proliferation phenotype of keratinocytes in culture (16), it was unclear whether Runx1-driven effects on the cell cycle in vivo or in tumors were mediated by p21. To ask whether prolonged HFSC quiescence in Runx1 single-KO mice is mediated via derepression of p21, we generated Runx1/p21 double-KO (dKO) mice and examined the proliferation onset by skin color change (pink to black). Unexpectedly, HFSCs in the dKO mice not only failed to overcome the prolonged quiescence imposed by Runx1 loss as we predicted, but in fact remained in telogen much longer (Fig. 3 A–C). These in vivo data contrasted strikingly with our previous in vitro study (16) and indicated that in vivo but not in vitro, double KO of a CDKi (p21) and its upstream repressor (Runx1) triggered deep repression of the bulge cell proliferation.

To examine the effect of Runx1 and p21 interaction in tumors we used a two-step carcinogenesis protocol, using DMBA/TPA on single- and double-Runx1/p21 KO mice (Fig. 3D). This generates skin tumors that eventually progress to squamous cell carcinoma (SCC) (28). At least some of these SCCs were previously shown to originate from bulge SCs (29-31). Because carcinogenic treatment can produce different amounts of tumors on different genetic backgrounds (32), we controlled the mating scheme to generate four different genotypes with a fixed ratio of BL6 and CD1 backgrounds for the final experimental mice. This may be responsible for the high error bars that did not allow us to detect significant tumor differences between WT and p21 KO mice, as previously reported (19, 20). Upon DMBA/TPA treatment initiated at the second telogen, Runx1 KO mice, however, had significantly (P < 0.05, weeks 11–20) fewer papillomas compared with WT, as expected. At 15–16 wk of TPA treatment, the dKO mice had significantly more tumors than the Runx1 KO (P < 0.05) but fewer than WT and p21 KO mice (Fig. 3 D–F), suggesting a partial rescue of the tumor impairment phenotype imposed by the Runx1 KO. However, by the 20th week of TPA treatment the differences in tumor burden or fraction of tumor-free mice were not statistically different from those of the Runx1 single-KO mice. These results suggest that p21 repression via Runx1 plays a minor role in promoting skin papillomas and that additional Runx1 targets may have a critical role for tumor heterogeneity of cell cycle in vivo. Moreover, this increase in p15 was also detected at the protein level (Fig. 4A). Interestingly, dKO bulge cells showed an approximately fivefold increase in p15 expression whereas none of the single-KO or the nonbulge cells showed p15 up-regulation in vivo. Moreover, this increase in p15 was also detected at the protein level (Fig. 4C). Interestingly, we noted that p27 and p57, which were somewhat up-regulated in the single Runx1 KO, were expressed at normal levels in dKO bulge cells (Fig. 4A) (Discussion).

Because, unlike dKO bulge cells in vivo, which remain profoundly quiescent, the dKO cells grow normally in culture (16), we hypothesized that the prolonged HFSC quiescence in vivo might be due to compensation by redundant CDKis up-regulated upon concomitant Runx1/p21 loss. Thus, we analyzed CDKi mRNA expression in bulge (CD34+ / keratin+ ) cells from telogen mice of all four genotypes (Fig. 4A). Interestingly, dKO bulge cells showed an approximately fivefold increase in p15 expression whereas none of the single-KO or the nonbulge cells showed p15 up-regulation in vivo. Moreover, this increase in p15 was also detected at the protein level (Fig. 4C). Interestingly, we noted that p27 and p57, which were somewhat up-regulated in the single Runx1 KO, were expressed at normal levels in dKO bulge cells (Fig. 4A) (Discussion).

To test whether p15 alone could block the proliferation of cycling cells in log-phase cultures, we transfected keratinocytes with p15-GFP cDNA and examined the cell cycle profiles relative to those of nontransfected and GFP-myc control transfected cells by FACS. Gating on the GFP+ (transfected) cells showed >50% reduction in S/G2/M phases in both WT and dKO keratinocytes with a concomitant increase in G1/G0 induced by p15 overexpression (Fig. 4D and E and Fig. S5D). On the other hand, shRNA knockdown of p15 showed increased proliferation, which was more prominent in dKO than in WT keratinocytes (Fig. 4F and Fig. S5E). Finally, to explore how Runx1 and p21 may
synergistically repress p15 transcription, we examined their direct binding on the p15 promoter region. p21 was previously shown to bind to E2F binding sites and repress downstream transcription on the Wnt4 (wingless-related MMTV integration site 4) promoter (33). Importantly, we found that Runx1 and p21 both bind to p15 promoter regions at their own respective binding motifs (Fig. 4 G and H), suggesting that they may directly repress p15 transcription.

**Discussion**

Here we probed the mechanism of HFSC quiescence in normal tissue and in stress conditions of tumors and cell culture. We found several CDKis (p21, p57, p27, and p15), known to play overlapping roles in halting the cell cycle (3, 4) transcriptionally up-regulated either in the bulge at all stages or at the onset of HF quiescence. Using pulse-chase H2B-GFP mice (10) we find that p21 represses HFSC rates of proliferation in vitro and limits the HFSC extent of proliferation at catagen onset in vivo, but does not affect HFSC self-renewal rates at anagen. Overall p21 KO bulge cells undergo approximately one to two more divisions at catagen, but increased apoptosis maintains the bulge cell pool at a constant size. Mathematical modeling suggests a temporary escape from quiescence in a fraction of the KO bulge cells (SI Materials and Methods). Interestingly, loss of Dacapo, the *Drosophila* p21 homolog, also leads to an additional round of cell division before developmental arrest (34). The p21 KO bulge cells eventually enter quiescence likely via concerted action of other bulge up-regulated CDKis (p27, p57, and p15).

One of the upstream players in the mechanism of CDKi control in vivo appears to be the transcription factor Runx1, which may repress p21, p27, p15, and p57 mRNA production and is directly bound to the p21 promoter where it promotes H3K27me3 accumulation. We propose that p21 (and possibly p57) derepression due to down-regulation of the Runx1 protein at catagen promotes the timely onset of WT HFSC quiescence and limits the expansion of their pool (this work). Conversely, Runx1 protein expression in the bulge at anagen represses p21 expression and promotes self-renewal (16).

The Runx1/p21 dKO mice display a surprising extension of HFSC quiescence in vivo, whereas keratinocytes in culture grow normally (16). Tumors showed an intermediate effect, suggesting that additional Runx1 targets, of which Stat3 is an important player (31), are at play to promote tumor growth. These data exposed a robust SC control in vivo that seemingly limits the normal SC pool size and enforces quiescence. A potential factor in this mechanism is another CDKi, p15, which is up-regulated normally (16). Tumors showed an intermediate effect, suggesting that additional Runx1 targets, of which Stat3 is an important player (31), are at play to promote tumor growth. These data exposed a robust SC control in vivo that seemingly limits the normal SC pool size and enforces quiescence. A potential factor in this mechanism is another CDKi, p15, which is up-regulated specifically in the bulge only in the Runx1/p21 dKO mice. Our data suggest that when cultured cells are crowded, transcriptional up-regulation of this CDKi (p15) is driven by direct synergistic derepression of its promoter via loss of p21 and Runx1. Previously, p21 and Runx1 were shown capable of acting as transcriptional repressors (14, 33, 35, 36), and we find that they bind to the predicted DNA sites on the p15 promoter. Additionally, p15 expression is able to hamper cell cycle progression of cultured keratinocytes. These data reveal the case of one CDKi as a direct transcriptional repressor of another, to finely tune the extent of quiescence (Fig. 4F).

Because SCs in tissues need not only to be quiescent to protect their genome, but also to activate rapidly when needed, it may be that high steady-state levels of CDKis are undesirable. An intriguing mechanism to keep CDKI levels in check may involve the dual role of some CDKis as CDK inhibitor and transcriptional repressors. Recently p27 was also shown to repress transcription, although direct targeting by p27 of another CDKi did not surface from this study (37). It will be interesting to know how general is the ability of one CDKI to directly repress another, such that when one is inadvertently lost, another is up-regulated to maintain proliferation control. For example, if p15 could repress
p27 and p57, this would explain why the latter two were not expressed in the context of the Runx1/p21 dKO when the level of p15 was high. Clearly there is more to learn about CDKIs beyond their traditional function in CDK inhibition. Testing triple-KO p21/Runx1/p15 mice will begin to address this model in vivo, although additional control layers may further enforce quiescence and possibly complicate the analysis.

Collectively, our study uncovers a complex and robust mechanism in vivo that enforces SC quiescence and a constant SC pool size and is synergistically tempered by Runx1 and, unexpectedly, by its downstream CDK target (p21). Moreover, we unveil a role of a CDKIs (p21) to antagonize proliferation by direct transcriptional repression of another CDKI (p15) in vitro, thereby modulating the strength of cell cycle arrest.

### Materials and Methods

**Computation and Mathematical Modeling.** We infer the proportions of cells that have divided n times by a hierarchical Bayesian analysis that incorporates both the biological variations between mice, the experimental variations that broaden the FACS histograms into Gaussians, and the variations between the extents of H2B-GFP labeling in different mice. In addition, an enhanced method of variational Bayesian Gaussian mixture modeling (38, 39) is developed that can cope with noise in the low-dimensional Gaussian mixture modeling problems as it provides a Bayesian approach to modeling analyses that systematically includes both intramixture and intermixture variations (details in SI Materials and Methods).

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