HNRNPK maintains epidermal progenitor function through transcription of proliferation genes and degrading differentiation promoting mRNAs

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Maintenance of high-turnover tissues such as the epidermis requires a balance between stem cell proliferation and differentiation. The molecular mechanisms governing this process are an area of investigation. Here we show that HNRNPK, a multifunctional protein, is necessary to prevent premature differentiation and sustains the proliferative capacity of epidermal stem and progenitor cells. To prevent premature differentiation of progenitor cells, HNRNPK is necessary for DDX6 to bind a subset of mRNAs that code for transcription factors that promote differentiation. Upon binding, these mRNAs such as GRHL3, KLF4, and ZNF750 are degraded through the mRNA degradation pathway, which prevents premature differentiation. To sustain the proliferative capacity of the epidermis, HNRNPK is necessary for RNA Polymerase II binding to proliferation/self-renewal genes such as MYC, CYR61, FGFBP1, EGFR, and cyclins to promote their expression. Our study establishes a prominent role for HNRNPK in maintaining adult tissue self-renewal through both transcriptional and post-transcriptional mechanisms.
mammalian epidermis is the outermost layer of the skin, which serves as the initial line of defense to protect our body from environmental and pathogenic factors. The deepest layer of the epidermis, known as the basal layer, contains the undifferentiated stem and progenitor cells. As the cells differentiate they exit out of the cell cycle and migrate upwards towards the surface of the skin and progressively form the more differentiated layers of the epidermis including the suprabasal and granular layers. Terminal differentiation occurs in the stratum corneum where these dead corneocytes eventually gets sloughed off the surface of the skin. Epidermal homeostasis is achieved by the proper balance between self-renewal and differentiation of stem and progenitor cells residing within the basal layer. Perturbations in this delicate balance can lead to skin diseases which can impact up to 20% of the population. A tremendous amount of effort has been focused on defining transcriptional mechanisms that regulate epidermal stem and progenitor cell self-renewal and differentiation. Work by our laboratory and others have shown that transcription and epigenetic factors such as p63, DNMT1, ACTL6A, YAP1, and EZH2 can actively promote epidermal stem and progenitor cell self-renewal while factors such as ZNF750, KLF4, GRHL3, JMJD3, and CEBP alpha/beta are necessary for differentiation. While transcriptional mechanisms that regulate epidermal self-renewal or differentiation have been well described as discussed above, it is not clear whether post-transcriptional mechanisms (non-miRNA) regulate this process. Recently, we found the DEAD-box RNA helicase, RCK/p54 (DDX6) to be necessary for maintaining epidermal stem and progenitor cell function through both the mRNA translation and degradation pathways. DDX6 promotes self-renewal and proliferation while actively suppressing premature differentiation through two distinct mechanisms. First, DDX6 promotes the translation of self-renewal (EZH2, ACTL6A) and proliferation (CDK1, CDK2) transcripts to maintain epidermal self-renewal. The RNA binding protein, YBX1 recruits DDX6 and EIF4E to these self-renewal/proliferation mRNAs through the stem loop structures found in their 3'UTRs. EIF4E is then required for the initiation of translation of these transcripts while DDX6 is necessary for loading of the mRNAs to polysomes. Second, DDX6 binds to mRNAs coding for potent differentiation promoting transcription factors such as KLF4 to promote its degradation in progenitor cells to prevent premature differentiation. DDX6 promotes the degradation of these transcripts by associating with key mediators of the mRNA degradation pathway including EDC3. Currently, it is unclear how DDX6 targets these mRNAs for degradation since YBX1 recruits DDX6 to self-renewal/proliferation transcripts but not differentiation mRNAs such as KLF4.

In an attempt to identify proteins that recruit DDX6 to differentiation promoting mRNAs for degradation, we perform a small RNA interference (RNAi) screen targeting RNA binding proteins that we previously identified by mass spectrometry to associate with DDX6. Through this screen we find heterogeneous nuclear ribonucleoprotein K (HNRNPK) to have a prominent role in epidermal progenitor cell maintenance. HNRNPK was initially discovered as a component of hnRNPs and was overexpressed in a variety of tumors with its oncogenic functions attributed to regulation of EIF4E, C-SRC, and C-MYC. HNRNPK promotes proliferation and prevents differentiation. Keratin 1 (K1), a differentiation specific gene was downregulated by more than 80% as compared to knockdown controls (CTLi) (Fig. 1c and Supplementary Fig. 1f). Similarly, keratin 10 (K10), an epidermal keratinocytes that downregulated by more than 80% as compared to knockdown controls (CTLi) (Fig. 1a, b and Supplementary Fig. 1c, d). There was also an increase in apoptotic cells upon HNRNPK knockdown although it was not statistically significant (Supplementary Fig. 1e). HNRNPK knockdown cells also prematurely differentiated with increased levels of differentiation specific genes many of which have been implicated in skin diseases including KRT1, KRT10, FIP1L1, and LOR (Fig. 1c and Supplementary Fig. 1f). Notably, the mRNAs levels (KLF4, ZNF750, and GRHL3) for genes that code for differentiation promoting transcription factors were also increased (Fig. 1c and Supplementary Fig. 1f).

To determine the impacts of HNRNPK loss in a 3D tissue setting which allows faithful representation of the stratification and gene expression program of human epidermis, CTLi and HNRNPKi cells were used to regenerate human epidermal tissue. Epidermal tissue was harvested at an early timepoint to determine if HNRNPK depletion altered the kinetics of differentiation. Keratin 1 (K1), a differentiation specific cytoskeletal protein, which is normally only expressed beginning in the suprabasal layer was robustly expressed in the basal layer of HNRNPKi tissue suggesting that the stem cell compartment had prematurely differentiated (Fig. 1d, e). Similarly, keratin 10 (K10) and Filaggrin (FLG) were expressed robustly in HNRNPKi tissue whereas expression of these proteins was just starting to appear in control tissue (Fig. 1d, e). Coinciding with the premature...
differentiation of the stem/progenitor cell compartment in HNRNPKi tissue, there was also a loss of the proliferative capacity of the basal layer (Fig. 1d).

HNRNPK maintains epidermal self-renewal in-vivo. To determine whether HNRNPK is acting through non-cell autonomous or cell autonomous mechanisms, as well as whether HNRNPK is necessary for progenitor cell function in-vivo, we used the competition assay we previously developed. To do this, GFP expressing keratinocytes were knocked down with control (CTLi) shRNAs and dsRED cells were knocked down with HNRNPK shRNAs. These cells were mixed at a 1:1 ratio. All genetic modifications such as gene overexpression or knockdown were mediated through retroviral infections and thus are stable in-vivo. To rule out impacts from the fluorescent proteins, dsRED expressing CTLi cells were also mixed with GFP expressing HNRNPKi cells at equal ratios. These different cell
mixtures were used to regenerate human epidermis on immune deficient mice. Initially (Day 3), epidermis generated from GFP-CTLi + dsRED-HNRNPKi cells or GFP-HNRNPKi + dsRED-CTLi cells showed an equal percentage of contribution to the tissue (Fig. 1f, g). In contrast, by 18 days post-grafting, epidermis regenerated from GFP-CTLi mixed with dsRED-HNRNPKi cells were mainly composed of control GFP cells (Fig. 1f, g). Epidermis derived from dsRED-CTLi mixed with GFP-HNRNPKi cells also led to a huge depletion of the GFP expressing HNRNPKi knockedout cells. The few remaining GFP-HNRNPKi cells were all found in the deep differentiated layers of the epidermis (Fig. 1f: white arrowheads). These remaining cells will likely be sloughed off the surface of the skin as they terminally differentiate.

HNRNPK suppresses differentiation and promotes proliferation. RNA-seq analysis was performed on control and HNRNPKi cells to understand the gene expression program that HNRNPK controls. Nine hundred and twenty-two genes (≥2 fold change and p ≤ 0.05, one way Anova) were upregulated in HNRNPKi cells which were enriched in gene ontology (GO) terms such as keratinocyte differentiation, negative regulation of cell proliferation and epidermis development (Fig. 1h, i and Supplementary Data 1). 1311 genes were downregulated with GO terms including mitotic cell cycle phase transition and DNA replication indicating that HNRNPK is necessary for the proliferative capacity of the epidermal cells (Fig. 1h, j and Supplementary Data 1). To determine the extent to which depletion of HNRNPK resembles the differentiation program, the HNRNPK signature was compared to a previously generated RNA-seq data set of genes that changed during calcium mediated epidermal differentiation.33. Nine hundred and fourteen genes were found in the overlap with the vast majority of the genes regulated in the same direction as the differentiation signature (Supplementary Fig. 1g). The overlapped upregulated genes were enriched in GO terms such as keratinocyte differentiation while downregulated genes were enriched in mitotic cell cycle (Supplementary Fig. 1h, i).

HNRNPK degrades differentiation promoting mRNAs. Since HNRNPK has been shown to bind RNA to modulate post-transcriptional gene expression, we performed RNA immunoprecipitations (RIP) under native conditions using a HNRNPK antibody or control IgG on epidermal cell lysates to determine all the transcripts that HNRNPK binds. RNA was purified from the immunoprecipitates and subjected to next generation RNA sequencing (RIP-Seq).36,37 HNRNPK bound to 921 genes (≥4 fold increase over IgG and p ≤ 0.05, one way Anova) which were enriched for GO terms including positive regulation of cell differentiation, polarized epithelial cell differentiation, and regulation of cell proliferation (Fig. 2a, b and Supplementary Data 2). There was also a tight correlation between the replicate HNRNPK RIP-Seq data sets (Supplementary Fig. 1j). To assess whether HNRNPK bound transcripts also changed in expression levels upon HNRNPK depletion, the HNRNPK RIP-Seq dataset was overlapped with the HNRNPK knockdown data. Interestingly, ≥16% (150/921) of the HNRNPK bound genes were differentially regulated upon HNRNPK knockdown which were enriched for GO terms such as epithelial cell differentiation, regulation of cell proliferation, and negative regulation of cyclin-dependent protein kinase activity (Fig. 2c, d). These results suggest that HNRNPK may regulate epithelial growth and differentiation at the post-transcriptional level. Among the genes involved in regulating epithelial cell differentiation and growth that is both bound by HNRNPK and found to be increased in mRNA levels upon knockdown were transcripts coding for differentiation promoting transcription factors including KLF4, ZNF750, and GRHL3, as well as the cell cycle inhibitor P21 (CDKN1A) (Figs. 1h, 2a, c).

To validate the RIP-Seq results, RIP was performed in control and HNRNPKi cells using a HNRNPK antibody or IgG. KLF4, ZNF750, CDKN1A, and GRHL3 mRNAs were found to robustly associate with HNRNPK in control but not in HNRNPKi cells (Fig. 2e, Supplementary Fig. 2a). The transcripts were specifically bound to HNRNPK since binding depended on the presence of HNRNPK in the cells and did not bind transcripts such as GAPDH (Fig. 2e). No binding was detected in the IgG pulldown samples (Fig. 2e, Supplementary Fig. 2a). Since knockdown of HNRNPK led to increases in the mRNA levels of these HNRNPK bound genes, it suggests that HNRNPK may normally be targeting these transcripts for degradation in progenitor cells to prevent premature differentiation and premature cell cycle exit (Fig. 2f). To test this, control and HNRNPKi cells were treated with actinomycin D to determine the half-lives of the mRNAs. Loss of HNRNPK significantly increased the mRNA stability/half-lives of GRHL3, KLF4, and CDKN1A (Fig. 2g, Supplementary Fig. 2b). While not statistically significant, HNRNPK depletion also led to the increased half-life of ZNF750 (Fig. 2g). These results suggest that HNRNPK binds and degrades these transcripts in progenitor cells to prevent premature differentiation.

To determine if HNRNPK may be regulating growth and differentiation through these bound genes, we overlapped our published gene expression signatures of KLF4 and ZNF750 knockdown in differentiated keratinocytes with our HNRNPK gene expression profile. Since we have shown that KLF4 and ZNF750 are required for epidermal differentiation we would
**Fig. 2** HNRNPK binds and degrades mRNAs coding for differentiation-promoting transcription factors to prevent premature differentiation. 

**a** Profiling of HNRNPK-bound transcripts by RNA immunoprecipitation (RNA IP) coupled with deep sequencing (RIP-Seq). Heatmap of 921 genes bound to HNRNPK defined by 4-fold enrichment over IGG and \( p < 0.05 \), one-way ANOVA. \( n = 2 \) independent experiments. 

**b** Gene ontology terms of the 921 genes bound by HNRNPK using Enrichr. 

**c** Venn diagram of overlapped genes between HNRNPKi RNA-Seq and HNRNPK RIP-Seq datasets. 

**d** Gene ontology terms of the 150 overlapped genes. 

**e** RNA IP was performed in CTLi and HNRNPKi cells using an HNRNPK antibody. RT-QPCR was used to determine the levels of binding between HNRNPK and GRHL3, KLF4, ZNF750, GAPDH, or CDKN1A mRNAs in CTLi and HNRNPKi cells. IGG IPs in CTLi and HNRNPKi cells were used as specificity controls. Binding was calculated as a percent of input. 

**f** RT-QPCR for changes in the levels of GRHL3, KLF4, ZNF750, and CDKN1A mRNA expression in HNRNPKi cells. QPCR results were normalized to L32 levels. 

**g** Double knockdown of HNRNPK with GRHL3 or KLF4 was performed and differentiation markers were evaluated by RT-QPCR (\( n = 2 \)). \( n = 3 \) independent experiments performed in Fig. 2 unless otherwise indicated. Mean values are shown with error bars = SD. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), ****\( p < 0.0001 \) (2 way ANOVA followed by Tukey’s multiple comparison test for h, e, 7-test for f, g). Overlap significance in Venn diagrams was determined using hypergeometric distribution p-values (c).
expect the knockdown expression profiles to be counter correlated. One hundred and sixty-six genes were upregulated in HNRNPK knockdown and downregulated in KLF4i cells which were enriched for GO terms such as epidermal cell differentiation and skin development (Supplementary Fig. 3a, b). Three hundred and sixty-six genes were downregulated in HNRNPKi cells which were enriched in GO terms such as positive regulation of cell cycle (Supplementary Fig. 3a, c). Similar results were obtained with the ZNF750 overlap suggesting that these factors may mediate HNRNPK’s impact on growth and differentiation (Supplementary Fig. 3d-f).

To directly determine if the impacts of HNRNPK on epidermal growth and differentiation is mediated through the increased expression of GRHL3, KLF4, or CDKN1A, HNRNPK and each of the genes were simultaneously knocked down and compared to control and HNRNPKi cells (Fig. 2h). The levels of differentiation induced genes LCE3D, FLG, TGM1, and K10 were increased in HNRNPKi cells but were restored similar to control levels in HNRNPK + GRHL3 or HNRNPK + KLF4 double knockdown cells (Fig. 2h). Simultaneous HNRNPK and CDKN1A knockdown had no impact on the premature differentiation phenotype (Supplementary Fig. 2d). These results suggest that HNRNPK prevents premature differentiation of epidermal progenitor cells by binding to and degrading GRHL3 and KLF4 transcripts.

HNRNPK is necessary for DDX6 to bind differentiation mRNAs. We previously demonstrated that DDX6 degrades KLF4 transcripts however it was unclear how DDX6 was targeted to the mRNA. To determine whether HNRNPK recruits the targeted mRNAs to DDX6 or vice versa, RIP experiments were performed. RIP was performed using a DDX6 antibody or IgG in control and HNRNPKi cells. DDX6 was able to bind to KLF4, ZNF750, and GRHL3 transcripts in control but not HNRNPKi cells. DDX6 was also able to bind to ZNF750, and GRHL3 transcripts in control but not HNRNPKi cells (Fig. 2a). Simultaneous HNRNPK and DDX6 double knockdown cells (Fig. 2a). Simultaneous HNRNPK and DDX6 double knockdown had no impact on the premature differentiation phenotype (Supplementary Fig. 2d). These results suggest that HNRNPK prevents premature differentiation of epidermal progenitor cells by binding to and degrading GRHL3 and KLF4 transcripts.

To determine if HNRNPK promotes epidermal growth through suppression of these transcripts, double knockdown experiments were performed and cell number counted 5 days after normalization of the cell number. Again, HNRNPKi cells exhibited dramatic inhibition of proliferation with loss of expression of key proliferation genes such as CCNA2, CDK4, and MKI67. Double knockdown of HNRNPK with GRHL3, KLF4 or CDKN1A resulted in a similar loss of proliferation as HNRNPK knockdown alone and did not restore the proliferation back to control levels (Supplementary Fig. 2d-g). It is intriguing to note that HNRNPK + CDKN1A double knockdown did not correct the proliferation defect even though CDKN1A is a major cell cycle inhibitor that upon knockdown by itself led to ~4 increase in cell numbers as compared to control cells (Supplementary Fig. 2d).

Genome-wide binding sites of HNRNPK. HNRNPK has been reported to be a multi-faced protein which can also bind to DNA and regulate gene expression through transcriptional mechanisms. To explore this possibility, chromatin immunoprecipitation (ChIP) followed by deep sequencing (ChIP-Seq) was performed using a HNRNPK antibody on primary human keratinocytes. MACS2 was used to call 4624 peaks enriched with HNRNPK binding (Supplementary Data 3a). A majority (68%) of the binding was found within genes which includes the 5’ UTR, intron, exon, transcriptional termination site/TTTS, and 3’ UTR (Fig. 4a). The rest of the peaks were within intergenic, promoter, or non-coding regions (Fig. 4a). The peaks mapped back to 2,095 genes which were enriched for GO terms such as positive regulation of telomere maintenance, epidermis development, and positive regulation of mesenchymal cell proliferation (Fig. 4b and Supplementary Data 3a). To gain a better understanding of the distribution of HNRNPK binding across genes, its localization was mapped +/− 5 kb of the transcriptional start site of genes. HNRNPK binding was also compared to histone marks from the ENCODE consortium data set for human keratinocytes. HNRNPK binding was also compared to histone marks from the ENCODE consortium data set for human keratinocytes (Fig. 4c). Interestingly, HNRNPK binding correlated with marks of open chromatin and active transcription including RNA Pol II, H3K4me3, DNase I HSS, and H3K27ac while being depleted from closed/repressive chromatin marks such as H3K27me3 (Fig. 4c). Quantitation of HNRNPK binding +/− 5kb from the TSS also showed that HNRNPK signals were broadly distributed throughout the region which was similar to RNA Pol II binding (Fig. 4d). In contrast, DNase I HSS sites accumulated at the TSS with diminished signal away from the TSS (Fig. 4d). Analysis of all HNRNPK bound peaks also showed HNRNPK correlated the most with RNA Pol II binding (Supplementary Fig. 4).

Since HNRNPK binding was the most similar to RNA Pol II, we performed RNA Pol II ChIP-Seq on primary human keratinocytes in order to directly compare our HNRNPK ChIP-Seq data. RNA Pol II bound to 9485 peaks which mapped back to 3433 genes (Supplementary Data 3b). Interestingly, ~50% (1041/2095) of the genes HNRNPK bound to also had RNA Pol II binding (Fig. 5a). A search for transcription factor binding motifs on the HNRNPK and RNA Pol II co-bound peaks showed enrichment for TEAD2, TCF7L2, LIN54, FOXH1, and BARHL2 (Fig. 5b). The top 2 enriched motifs, TEAD2 and TCF7L2 (also known as TCF4), have been shown to be essential for epidermal stem cell self-renewal (Fig. 5b). The 1041 genes co-bound by HNRNPK and RNA Pol II were also enriched for GO terms such as epidermis development, hemidesmosome assembly, and regulation of keratinocyte proliferation (Fig. 5c). These regulators of keratinocyte proliferation included critical self-renewal and proliferation genes such as MYC, FGFBP1, and CCNA2 (Fig. 5d).
Fig. 3 HNRNPK is necessary for DDX6 to bind differentiation associated mRNAs. a RNA IP was performed in CTLi and HNRNPKi cells using a DDX6 antibody. RT-QPCR was used to determine the levels of binding between DDX6 and differentiation associated mRNAs in the presence or absence of HNRNPK. IGG IPs in CTLi and HNRNPKi cells were used as specificity controls. Binding was calculated as a percent of input. b RNA IP was performed in CTLi and DDX6i cells using a HNRNPK antibody. RT-QPCR was used to determine the levels of binding between HNRNPK and differentiation associated mRNAs in the presence or absence of DDX6. IGG IPs in CTLi and DDX6i cells were used as specificity controls. n = 4. c Western blot analysis of HNRNPK and DDX6 protein levels upon HNRNPK or DDX6 knockdown. d Immunoprecipitations (IPs) were performed using either an HNRNPK or DDX6 antibody or IGG and Western blotted for HNRNPK or DDX6 protein expression. IPs were performed +/- RNase A. Five percent of the cell lysate was used as input. Representative blots are shown. n = 3 independent experiments performed for Fig. 3 unless otherwise indicated. All error bars = SD. ****p < 0.0001, ***p < 0.001 (2 way ANOVA followed by Tukey’s multiple comparison test for a, b).

The proliferation genes are not just due to the differentiation status of the cells but rather a requirement for HNRNPK binding. While HNRNPK is required for RNA Pol II loading onto proliferation genes, it is not clear if active transcription of the proliferation genes is necessary for HNRNPK binding to the genes. To test this, keratinocytes were treated with Actinomycin D to block transcription. As a validation that the Actinomycin D blocked transcription, RNA Pol II CTD phospho Serine 2 (Ser2) ChIP was also performed. RNA Pol II CTD phospho Ser2 recognizes the elongating form of RNA polymerase.

Because half (1,041/2,095) of HNRNPK bound genes had RNA Pol II enrichment, it may suggest that HNRNPK is necessary for RNA Pol II to bind a subset of sites in the genome. In support of this, RNA Pol II can be found in the immunoprecipitate of HNRNPK and vice versa suggesting that these two proteins can associate with each other (Fig. 6a). This association was also dependent on the presence of RNA since RNAse A treatment diminished the interaction (Fig. 6a). To test if HNRNPK is necessary for RNA Pol II binding to HNRNPK bound sites, RNA Pol II ChIP-Seq was performed on HNRNPK knockdown cells. differential was used to determine the differential RNA Pol II peaks between control and knockdown cells. These peaks were mapped back to their nearest genes which accounted for 1,310 genes with diminished/lost RNA Pol II binding upon HNRNPK knockdown while 284 genes gained/increased RNA Pol II binding (Fig. 6b). Incredibly, 36% (373/1041) of the HNRNPK and RNA Pol II co-bound genes lost RNA Pol II binding and resulted in diminished gene expression upon HNRNPK knockdown (Fig. 6b, k and Supplementary Fig. 5a). These genes were enriched for GO terms such as regulation of keratinocyte proliferation suggesting that HNRNPK is necessary for RNA Pol II association with proliferation genes to sustain the proliferative capacity of the epidermis (Fig. 6c). These genes include CCND2, CYR61, EGFRR, ITGB4, PTHLH, MYC, and FGFBP1 where loss of HNRNPK diminishes the ability of RNA Pol II to bind those regions (Fig. 6d–h, Supplementary Data 4). Validation of these results by ChIP-QPCR demonstrate that HNRNPK is necessary for RNA Pol II binding to these genes (Fig. 6i). Loss of RNA Pol II binding also led to the downregulation of each of these genes (Figs. 5g, 6i).

There were 668 genes co-bound by HNRNPK and RNA Pol II that did not significantly lose RNA Pol II binding upon HNRNPK knockdown. To analyze if HNRNPK was impacting RNA Pol II at these sites, the mean density profiles of RNA Pol II binding across the 668 genes were compared between control and HNRNPKi cells. Despite not being significant there was diminished RNA Pol II binding across these 668 genes upon HNRNPK knockdown (Supplementary Fig. 5b). This suggests that HNRNPK presence is required for RNA Pol II binding to HNRNPK bound genes. It is also possible that the loss of RNA Pol II from the proliferation genes is due to the premature differentiation phenotype of HNRNPK knockdown cells rather than a direct requirement for HNRNPK presence in order for RNA Pol II to load onto the genes. To distinguish between these possibilities, HNRNPK and KLF4 double knockdown experiments were performed to prevent the premature differentiation phenotype of HNRNPK knockdown alone (Fig. 2h). In control cells, RNA Pol II bound to each of the proliferation genes whereas in HNRNPK knockdown cells the binding diminished (Supplementary Fig. 5c). In HNRNPK and KLF4 double knockdown cells, the RNA Pol II binding was similar to HNRNPK knockdown cells and significantly reduced compared to controls (Supplementary Fig. 5c). This suggests that loss of RNA Pol II binding to proliferation genes is not just due to the differentiation status of the cells but rather a requirement for HNRNPK binding.

While HNRNPK is required for RNA Pol II loading onto proliferation genes, it is not clear if active transcription of the proliferation genes is necessary for HNRNPK binding to the genes. To test this, keratinocytes were treated +/- with Actinomycin D to block transcription. As a validation that the Actinomycin D blocked transcription, RNA Pol II CTD phospho Serine 2 (Ser2) ChIP was also performed. RNA Pol II CTD phospho Ser2 recognizes the elongating form of RNA polymerase.
Primers were built at the TSS and past the 3' end of each gene. In all 3 proliferation genes tested, Actinomycin D treatment resulted in a dramatic decrease in RNA Pol II phospho Ser2 signal from the 3' end of the gene and a buildup of signal at the TSS (Fig. 7a–c). This is in line with prior reports on how Actinomycin D inhibits transcription by causing the accumulation of CTD phosphorylated RNA Pol II at the TSS43,44. Importantly, blockade of transcription with Actinomycin D had no impact on HNRNPK binding to both the TSS and the 3' end of MYC, EGFR, or CYR61 (Fig. 7a–c). This suggests that active RNA transcription is not required for HNRNPK binding to these proliferation genes and thus HNRNPK association with the genome is not co-transcriptional. Furthermore, there is only an overlap of 184 genes bound by HNRNPK on both the RNA and DNA level with enriched GO terms such as regulation of transcription (Supplementary Fig. 5d, e). These data support a model where HNRNPK's genomic and mRNA targets are for the most part different.

Since HNRNPK is localized throughout the gene body and past the 3' end of genes, it may be possible that it is also regulating other aspects of transcription such as elongation or termination. Transcription elongation is controlled through cyclin T and CDK9 which form P-TEFb to phosphorylate Serine 2 on RNA Pol II CTD to promote elongation. In leukemia cell lines, HNRNPK and CDK9 can associate45. However, in keratinocytes no association between cyclin T or CDK9 could be detected with HNRNPK (Supplementary Fig. 6a, b). HNRNPK associates with the transcription termination factor, XRN2 to play a role in termination of EGR1 in HCT116 cancer cell lines46. In keratinocytes, HNRNPK did not associate with XRN2 (Supplementary Fig. 6c). If HNRNPK regulated transcription elongation then loss of its expression may lead to accumulation of mRNA at the 5' end of regulated genes. Similarly, if HNRNPK regulates transcription termination, then knockdown of HNRNPK may lead to readthrough transcription due to failure to properly terminate transcription as has been reported for loss of gene function for XRN247 and HNRNPK46 in other systems. To determine potential HNRNPK impacts on either transcription elongation or termination, RNA-Seq data for the proliferation genes were analyzed. Notably, none of the proliferation genes had accumulation of transcripts past the 3' end of the genes or accumulation at the 5' end of the genes (Supplementary Fig. 7). In addition, there were no accumulation of RNA Pol II at the 5' or 3' end of HNRNPK targeted proliferation genes upon HNRNPK knockdown (Fig. 6d–h).

**Discussion**

HNRNPK has been extensively studied for its role in tumorigenesis where it can act both as a tumor suppressor or oncogene.
HNRNPK maintains stem and progenitor cell status, we used while inhibiting premature differentiation. To explore how dermal cells where it is necessary to maintain cell proliferation promoting epidermal differentiation 4,5,7. Knockdown of previously published that these factors are absolutely required for such as GRHL3, KLF4, and ZNF750. We and others have pre-

Figure 5: HNRNPK and RNA Pol II co-bind proliferation genes enriched in TEAD2 and TCF4 binding motifs. a Venn diagram showing overlap of genes bound by both HNRNPK and RNA Pol II. b De novo motif search for HNRNPK and RNA Pol II co-bound peaks. c Gene ontology analysis of the 1041 overlapped genes from a, d, e Gene tracks showing HNRNPK (purple) and RNA Pol II (blue) binding to MYC and FGFBP1 genomic regions. The x-axis shows genomic position and y-axis denotes signal strength (RPM, reads per million). Bars over peaks indicate significantly bound peaks with cutoff q-value of 0.05. f ChIP was performed on CTLi (white bar) and HNRNPKi (black bar) cells using a HNRNPK antibody. ChIP was also performed using IGG in CTLi (light gray bar) and HNRNPKi (dark gray bar) cells as a specificity control. HNRNPK binding to each gene was calculated as a percentage of input. n = 2. g RT-QPCR for mRNA expression of genes that are co-bound by HNRNPK and RNA Pol II in CTLi and HNRNPKi cells. QPCR results were normalized to L32 levels. Mean values are shown with error bars = SD. n = 3 independent experiments performed in Fig. 5 unless otherwise indicated **p < 0.01, ****p < 0.0001 (2 way ANOVA followed by Tukey’s multiple comparison test for f, T-test for g)

depending on context.23,48 Understanding the mechanisms for how HNRNPK either promotes or inhibits tumors have been more difficult to address due to the sheer number of cellular processes that it controls. The clear impact of HNRNPK on tumor growth and differentiation suggests that HNRNPK under normal circumstances may be a potent regulator of tissue homeostasis. Progress into this area has been hampered by embryonic-lethal phenotypes in Hnrnpk knockout mouse models.26 Hnrnpk+/− mice showed diminished survival with high penetrance of cancer phenotypes such as myeloproliferation, lymphomas, and hepatocellular carcinomas suggesting that Hnrnpk is necessary for inhibiting proliferation and promoting differentiation in those cell types26.

Interestingly, we find an opposite role for HNRNPK in epidermal cells where it is necessary to maintain cell proliferation while inhibiting premature differentiation. To explore how HNRNPK maintains stem and progenitor cell status, we used RIP-Seq to define the transcripts that HNRNPK directly binds. Notably, HNRNPK bound to 3 of the most important genes known that code for differentiation inducing transcription factors such as GRHL3, KLF4, and ZNF750. We and others have previously published that these factors are absolutely required for promoting epidermal differentiation.4,5,7. Knockdown of HNRNPK led to increased transcript stability and thus increased the mRNA levels of these genes. This suggests that HNRNPK normally functions in stem and progenitor cells to bind and degrade these transcripts to prevent premature differentiation. Supporting this, double knockdown of HNRNPK with KLF4 or GRHL3 can partially reverse the premature differentiation phenotype of just HNRNPKi alone suggesting that these transcription factors are the key factors downstream of HNRNPK that promotes differentiation.

The next question we addressed was how HNRNPK promotes the degradation of differentiation transcripts. Our prior work suggested that the RNA helicase DDX6 which is part of the mRNA degradation complex with enhancer of mRNA-decaping protein 3 (EDC3) binds and degrades mRNAs such as KLF4 to prevent premature differentiation14–16. However it was unclear what was recruiting DDX6/EDC3 to these transcripts since these factors have no inherent RNA binding specificity. Here, we demonstrated that in the absence of HNRNPK, DDX6 could not bind to KLF4, GRHL3, or ZNF750 mRNAs, whereas HNRNPK can still bind in the absence of DDX6. This suggests that HNRNPK is necessary for DDX6 to bind target mRNAs.

Besides its ability to regulate RNA, HNRNPK has also been described to bind DNA. It has been reported as both an activator and repressor of gene expression on the chromatin level. As a repressor, HNRNPK is necessary to recruit the PRC1 complex to Xist targeted regions to mediate chromosomal silencing.49 As an activator, HNRNPK can bind promoter regions to facilitate transcription of genes such as c-myc.30 In leukemic cell lines, HNRNPK is necessary to bring together lineage determining transcription factors with the transcriptional machinery to mediate 5-azacitidine sensitive chromatin structure.45. HNRNPK has also been described to promote transcriptional termination through the XRN2 pathway.46 Since HNRNPK binding to
genomic DNA may potentially regulate genes involved in epidermal self-renewal, we focused on determining its genomic binding sites. HNRNPK binding tended to localize throughout dermal self-renewal, we focused on determining its genomic

**Fig. 6** HNRNPK is necessary for RNA Pol II to bind proliferation genes to maintain the proliferative capacity of epidermal progenitor cells. a Immunoprecipitations (IPs) were performed using either an HNRNPK, RNA POL II antibody or IGG and Western blotted for HNRNPK or RNA Pol II protein expression. IPs were performed +/- RNase A. Five percent of the cell lysate was used as input. Representative blots are shown. n = 3. b Venn diagram showing the 1041 HNRNPK and RNA Pol II co-bound genes that lose RNA Pol II peaks upon HNRNPK depletion (top). The bottom Venn diagram depicts by both HNRNPK and RNA Pol II that lose RNA Pol II peaks upon HNRNPK knockdown. c Gene ontology analysis of the 373 genes bound by both HNRNPK and RNA Pol II that lose RNA Pol II peaks upon HNRNPK knockdown. d-g Gene tracks showing HNRNPK (purple) and RNA Pol II binding to CCND2, CYR61, EGFR, ITGB4, and PTHLH genomic regions. RNA Pol II binding is shown in CTLi (blue) and HNRNPKi (red) cells. The x-axis shows genomic position and y-axis denotes signal strength (RPKM, reads per million). Bars over peaks indicate significantly bound peaks with cutoff q-value of 0.05. ChIP was performed on CTLi (white bar) and HNRNPKi (black bar) cells using a RNA Pol II antibody. ChIP was also performed using IGG as a control in CTLi (light gray bar) and HNRNPKi (dark gray bar) cells. RNA Pol II binding to each gene was calculated as a percentage of input. n = 3. j RT-QPCR for mRNA expression of genes that are co-bound by HNRNPK and RNA Pol II, as well as lose RNA Pol II binding upon HNRNPK depletion. QPCR results were normalized to housekeeping gene (L32). Fold change from CTLi to HNRNPKi is plotted as mean ± SD. n = 3. **p < 0.01, ****p < 0.0001 (2 way ANOVA followed by Tukey’s multiple comparison test for j). k Western blot analysis of RNA Pol II protein levels upon HNRNPK knockdown. Mean values are shown with error bars = SD. n = 3. h–i Western blot analysis of RNA Pol II protein levels upon HNRNPK knockdown. Mean values are shown with error bars = SD. n = 3.

**Immunoprecipitations (IPs) were performed using either an HNRNPK, RNA POL II antibody or IGG and Western blotted for HNRNPK or RNA Pol II protein expression. IPs were performed +/- RNase A. Five percent of the cell lysate was used as input. Representative blots are shown. n = 3.**

**b** Venn diagram showing the 1041 HNRNPK and RNA Pol II co-bound genes that lose RNA Pol II peaks upon HNRNPK depletion (top). The bottom Venn diagram depicts by both HNRNPK and RNA Pol II that lose RNA Pol II peaks upon HNRNPK knockdown. c Gene ontology analysis of the 373 genes bound by both HNRNPK and RNA Pol II that lose RNA Pol II peaks upon HNRNPK knockdown. d-g Gene tracks showing HNRNPK (purple) and RNA Pol II binding to CCND2, CYR61, EGFR, ITGB4, and PTHLH genomic regions. RNA Pol II binding is shown in CTLi (blue) and HNRNPKi (red) cells. The x-axis shows genomic position and y-axis denotes signal strength (RPKM, reads per million). Bars over peaks indicate significantly bound peaks with cutoff q-value of 0.05. ChIP was performed on CTLi (white bar) and HNRNPKi (black bar) cells using a RNA Pol II antibody. ChIP was also performed using IGG as a specificity control in CTLi (light gray bar) and HNRNPKi (dark gray bar) cells. RNA Pol II binding to each gene was calculated as a percentage of input. n = 3. j RT-QPCR for mRNA expression of genes that are co-bound by HNRNPK and RNA Pol II, as well as lose RNA Pol II binding upon HNRNPK depletion. QPCR results were normalized to L32 levels. n = 3. k Western blot analysis of RNA Pol II protein levels upon HNRNPK knockdown. Mean values are shown with error bars = SD. n = 3. **p < 0.01, ****p < 0.0001 (2 way ANOVA followed by Tukey’s multiple comparison test for j, k). Overlap significance in Venn diagrams was determined using hypergeometric distribution p-values (b).
Given the multitude of functions for HNRNPK, it is also possible that HNRNPK regulates other aspects of transcription such as through elongation or termination to control the expression of proliferation genes. HNRNPK has been reported to associate with CDK9 and XRN2 in cancer cells to potentially regulate transcription elongation and termination, respectively.\textsuperscript{45,46} However, we did not find clear evidence for this mode of regulation in keratinocytes. HNRNPK did not associate with either cyclin T or CDK9. HNRNPK depletion also resulted in diminished RNA Pol II binding throughout the entire gene rather than RNA Pol II stalling/backup at the 5' end which would be indicative of alterations in transcription elongation. Similarly, HNRNPK did not associate with XRN2 and there were no detectable alterations in transcription termination.

HNRNPK may also be loading RNA Pol II onto genes through binding enhancers. Twenty-one percent of HNRNPK binding sites are found in intergenic regions and its sites are enriched for YAP1 signaling pathway components which are known to bind enhancers\textsuperscript{31}. This may be an area of future investigation.

HNRNPK's mechanism of function has been difficult to decipher due to its role in numerous cellular processes. To our knowledge, we are the first to comprehensively determine its targets in a high-throughput manner on the RNA and DNA levels, as well as characterize its impacts on adult tissue self-renewal. Furthermore, we have described 2 separate mechanisms by which HNRNPK mediates epidermal self-renewal and prevention of premature differentiation through both mRNA degradation and transcriptional activation (Fig. 7d). It is also interesting to note that our results suggest a previously unknown mode of regulation where HNRNPK is necessary for RNA Pol II to drive the expression of potent regulators of growth and self-renewal.

In summary, our findings describe a novel mechanism for HNRNPK regulated tissue self-renewal through both transcriptional and post-transcriptional mechanisms.

**Methods**

**Cell culture.** Primary human epidermal keratinocytes were derived from neonatal foreskin and cultured in EpiLife medium (ThermoFisher: MEB-1500CA) mixed with human keratinocyte growth supplement (HKGS, ThermoFisher: S0015) and pen/strep.\textsuperscript{11,12,43} Phoenix cells (ATCC CRL-3214) were cultured in DMEM with 10% fetal calf serum.

**Knockdown of genes.** To knockdown HNRNPK stably, retroviruses expressing HNRNPK shRNAs were used. The retroviral constructs (3 µg) were transfected using Lipofectamine 2000 (Life Technologies: 11668027) into amphotrophic phoenix cells. Viral supernatants were collected 48 h post-transfection and used to infect primary human keratinocytes.\textsuperscript{33} Cells were incubated in the viral supernatants and centrifuged at 1000 rpm for 1 h with hexadimethrine bromide (Sigma-Aldrich: H9268). Cells were transduced on two consecutive days. Cells were selected in puromycin 24 h after the last transduction to select for cells stably expressing the shRNAs (the retrovector encodes a puromycin resistance gene). shRNA retroviral constructs were generated by cloning oligos into the pSuper retrovector vector.\textsuperscript{41,42} The shRNA sequence targeting HNRNPK is GGTTTCAGTGCTGATGAAA. The scrambled control shRNA sequence is GATACTGACTACCAAGGAT and cloned into the pSuper retrovector sequence. The scrambled control shRNA sequence is GATACTGACTACCAAGGAT and cloned into the pSuper retrovector sequence.
Organotypic cultures and in vivo competition assays. For the organotypic skin cultures, one million control or HNRNPK knockdown cells were seeded onto devitalized human epidermis to regenerate human epidermis. The HNRNPK transgene was inserted into the New York Firefighters Skin Bank. Dermis seeded cells were raised to the air liquid interface to promote differentiation and stratification. Tissue was harvested 3 days after initial seeding. For the in vivo epidermal progenitor cell competition assay, primary human keratinocytes were first transduced with a retrovirus encoding GFP or dsRED. The GFP or dsRED cells were then infected with retroviruses encoding control (CTL) or HNRNPK (HNRNPK) shRNAs. GFP-CTRL cells were mixed at a 1:1 ratio with dsRED-HNRNPK cells. dsRED-CTRL cells were also mixed with GFP-HNRNPK cells at equal ratios to ensure results were not due to the influence of fluorescence proteins. A total of one million cells were seeded on the devitalized dermis to regenerate human epidermis. The regenerated human epidermis was then grafted into immune compromised mice (NOD SCID Gamma/NSG) purchased from Jackson Labs. Thirteen and eighteen days post-grafting, the human skin grafts were harvested from the mice. The human skin grafts were fixed in 4% paraformaldehyde for 1 h and embedded into OCT compound for sectioning. The contribution of GFP and each from three skin grafts per group per timepoint were imaged and quantified. All animal work was conducted in accordance with UCSD’s IACUC guidelines.

Measurement of mRNA stability and half-life. Control and HNRNPK knockdown cells were transfected with actinomycin D (10 µg/mL) for 0, 0.5, 1, 2, 4, and 6 h to determine the apparent half-life of the mRNAs. RNA was isolated from the samples and RT-qPCR was used to determine the levels of respective mRNAs. Half-lives was calculated using the formula $t_{1/2} = \frac{0.693}{\text{RT-qPCR}}$.

Apopotis assay. Control and HNRNPK knockdown cells were stained with Annexin V conjugated to Alexa Fluor 488 (Life Technologies: A13201) and analyzed using the Guava flow cytometer (Millipore) according to manufacturers instructions.

RNA isolation and RT-qPCR. Total RNA from cells or tissue was extracted using the GeneJET RNA purification kit (Thermo Scientific: K0732) and quantified using a Nanodrop. One µg of total RNA was reverse transcribed using the Maxima cDNA synthesis kit (Thermo Fisher: K1642). Quantitative PCR was performed using the Roche LightCycler 480. The K10 and K14 were used as internal control for normalization. Primer sequences for GAPDH, LCE3D, MV3 and ZNF570 in mRNAs. RNA was isolated from the samples and RT-qPCR was used to determine the levels of respective mRNAs. Half-lives was calculated using the formula $t_{1/2} = \frac{0.693}{\text{RT-qPCR}}$.

RNA immunoprecipitation/high-throughput sequencing (RIP-Seq). Three million control, HNRNPK, or DX5 knockdown cells and 3 µg of each antibody were used for each pulldown experiment. The following antibodies were used for RNA-IP: HNRNPK (Bethyl Laboratories: A300-674A), HDAC3 (Biolegend: NB100–127), and rabbit IgG control (Abcam: ab18413). Cells for the RNA Pol IP were fixed at a nanomolar concentration of 1% formaldehyde. The RIP-Seq libraries were sequenced at the Institute of Genomic Medicine core facility at UCSD. Mean read depth for each RIP-Seq sample was 4.87 million reads.

RESULTS

RNAseq and bioinformatics analysis. Control or cells knocked down for HNRNPK were harvested 6 days after the last infection. Two technical duplicates were obtained for both CTL and HNRNPK and total RNA was isolated using the GeneJET RNA purification kit (Thermo Scientific: K0732) and quantified using a Nanodrop. RNA-seq was performed using the Illumina HiSeq4000 machine at the Institute of Genomic Medicine core facility at UCSD. RNA-seq libraries were prepared with TruSeq RNA Library Prep Kit (Illumina: RS–122–2001) then multiplexed and ~40 million reads per sample were obtained. Reads were aligned to the Gencode v19 transcriptome hg19 using TopHat2 with default settings. Differential expression among samples was calculated using ANOVA from the Partek Genomic Suite (Partek Incorporated). Analysis of the read count distribution indicated that a threshold of ten reads per gene generally separated expressed from unexpressed genes, so all genes with fewer than ten reads were excluded from analysis. Gene lists for significantly upregulated or downregulated genes were created using a hypergeometric test with the Gene Set Enrichment Analysis (GSEA) software (http://software.broadinstitute.org/gsea/index.jsp) with an FDR threshold of < 0.05. Heatmaps for the RNA-seq data were generated using Partek’s Genomic Suite (http://www.partek.com/partek-genomics-suite/).

ChIP-qPCR and ChiP-Seq. Ten million cells and 5 µg of antibody were used for each antibody pulldown experiment for ChIP and ChIP-Seq was performed using the following antibodies: HNRNPK (Bethyl Laboratories: A300-674A), RNA Pol II (Active Motif: 39097), RNA Pol III (DSG, Thermo Fisher 20593, 2 mM DMSO), and mouse IgG (Abcam: ab18413). Cells for the RNA Pol II ChiP-qPCR or ChiP-Seq were fixed at a final concentration of 1% formaldehyde (ThermoFisher 28908). Cells for the HNRNPK ChiP-PCR or ChiP-Seq were fixed in both formaldehyde (1% final concentration) and dioxane/methanol (1:1) for 10 min. ChIP experiments were performed using the SEQUENOM MassARRAY iPLEX platform following the manufacturer's instructions. Rabbit IgG (Millipore: 12–370 and mouse IgG (Abcam: ab18413). Cells for the RNA Pol II ChiP-qPCR or ChiP-Seq were fixed at a final concentration of 1% formaldehyde (ThermoFisher 28908). Cells for the HNRNPK ChiP-PCR or ChiP-Seq were fixed in both formaldehyde (1% final concentration) and dioxane/methanol (1:1) for 10 min. ChIP experiments were performed using the SEQUENOM MassARRAY iPLEX platform following the manufacturer's instructions.
Co-immunoprecipitation experiments. Cells were lysed in a non-denaturing IP buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol). Three micrograms of HNRNPK antibody (Bethyl Laboratories: A300–674A, DDX6 (Novus Biologicals: NB20–192), RNA Pol II (Active motif: 39097), CDK9 (Bethyl Laboratories: A303–493A) or rabbit IgG were complexed with 50 µl of Protein G Dynabeads (Life Technologies: 10004D) at room temperature for 30 min. The antibody conjugated Dynabeads were then incubated with cell lysates on a rotator at 4 °C overnight. The next day, the supernatant was removed and the immunoprecipitated complex was washed with 500 µl of IP wash buffer for a total of 6 times at room temperature. Then 30 µl of RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and 15 µl NuPAGE® LDS Sample Buffer (Life Technologies: NP0008) were used to elute at 70 °C. Immunoprecipitates were loaded onto SDS-PAGE gels for Western blot. For samples with RNAse treatment, after the last wash of IP wash buffer, the immunoprecipitated complexes were then washed with PBS once and then equally dividing into two tubes in 1 mL of PBS. One tube was subjected to 100 µg/ml RNAse A treatment for 1.5 h on ice. The immunoprecipitated complexes with or without RNAse A treatment were washed three times with IP wash buffer before elution and blotted using Western blot11.

Inhibition of transcription with actinomycin D. Proliferating primary human keratinocytes were treated with 5 µg/ml Actinomycin D as described in the “ChIP-qPCR and ChIP-Seq” section.

Statistical analysis. Graph data are presented as mean ± SD. Statistical analyses were performed using GraphPad Prism. Student’s t tests and One-way ANOVA were used to compare between two or more groups, and significant changes were defined as p < 0.05. The number of biological experiments performed is indicated by N in the figure legends.

Scatter plot of ChIP-Seq data comparisons. Replicate ChIP-Seq bam files of HNRNPK and RNA Pol II were merged using Samtools. Bam files of H3K27ac, H3K4me3, H3K36me3, DNase I, and H3K27me3 were downloaded from the ENCODE database. Total count for each sample was obtained by Samtools. The read counts over the HNRNPK peak regions were then obtained using bedtools. RPKM for each peak region was computed using the following formula:

\[
\text{RPKM} = \frac{\text{read count} \times 10^9}{\text{total read count} \times \text{peak length}}
\]

A RPKM cutoff (0.0001 for counts over HNRNPK peaks) was then applied to remove the low count peaks regions followed by log2 transformation. R package ggplot2 was used to plot the scatter plot.

Data availability
The GEO accession number for RNA-Seq, RIP-Seq, and ChIP-Seq data reported in this paper is GSE123257.

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Author contributions
J.L. and G.L.S. conceived of the project, designed the experiment and wrote the paper. J.L., Y.C., [,] and M.T. performed the experiments. Y.W., J.L., X.X., and O.H. provided technical assistance and bioinformatics analysis.

Additional information
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