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Research Article

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Downmodulation or loss-of-function mutations of the gene encoding NOTCH1 are associated with dysfunctional squamous cell differentiation and development of squamous cell carcinoma (SCC) in skin and internal organs. While NOTCH1 receptor activation has been well characterized, little is known about how *NOTCH1* gene transcription is regulated. Using bioinformatics and functional screening approaches, we identified several regulators of the *NOTCH1* gene in keratinocytes, with the transcription factors DLX5 and EGR3 and estrogen receptor  $\beta$  (ER $\beta$ ) directly controlling its expression in differentiation. DLX5 and ERG3 are required for RNA polymerase II (PolII) recruitment to the *NOTCH1* locus, while ER $\beta$  controls *NOTCH1* transcription through RNA PolII pause release. Expression of several identified *NOTCH1* regulators, including ER $\beta$ , is frequently compromised in skin, head and neck, and lung SCCs and SCC-derived cell lines. Furthermore, a keratinocyte ER $\beta$ -dependent program of gene expression is subverted in SCCs from various body sites, and there are consistent differences in mutation and gene-expression signatures of head and neck and lung SCCs in female versus male patients. Experimentally increased ER $\beta$  expression or treatment with ER $\beta$  agonists inhibited proliferation of SCC cells and promoted NOTCH1 expression and squamous differentiation both in vitro and in mouse xenotransplants. Our data identify a link between transcriptional control of *NOTCH1* expression and the estrogen response in keratinocytes, with implications for differentiation therapy of squamous cancer.

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# Multifactorial ER $\beta$ and NOTCH1 control of squamous differentiation and cancer

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**Downmodulation or loss-of-function mutations of the gene encoding NOTCH1 are associated with dysfunctional squamous cell differentiation and development of squamous cell carcinoma (SCC) in skin and internal organs. While NOTCH1 receptor activation has been well characterized, little is known about how NOTCH1 gene transcription is regulated. Using bioinformatics and functional screening approaches, we identified several regulators of the NOTCH1 gene in keratinocytes, with the transcription factors DLX5 and EGR3 and estrogen receptor  $\beta$  (ER $\beta$ ) directly controlling its expression in differentiation. DLX5 and ERG3 are required for RNA polymerase II (PolII) recruitment to the NOTCH1 locus, while ER $\beta$  controls NOTCH1 transcription through RNA PolII pause release. Expression of several identified NOTCH1 regulators, including ER $\beta$ , is frequently compromised in skin, head and neck, and lung SCCs and SCC-derived cell lines. Furthermore, a keratinocyte ER $\beta$ -dependent program of gene expression is subverted in SCCs from various body sites, and there are consistent differences in mutation and gene-expression signatures of head and neck and lung SCCs in female versus male patients. Experimentally increased ER $\beta$  expression or treatment with ER $\beta$  agonists inhibited proliferation of SCC cells and promoted NOTCH1 expression and squamous differentiation both in vitro and in mouse xenotransplants. Our data identify a link between transcriptional control of NOTCH1 expression and the estrogen response in keratinocytes, with implications for differentiation therapy of squamous cancer.**

## Introduction

Squamous cell carcinomas (SCCs) are the most common form of human solid tumors and a major cause of cancer lethality. These highly heterogeneous tumors arise from closely interconnected epithelial cell populations with substantially different self-renewal potential and a highly synchronized program of stratified differentiation. NOTCH signaling plays a pivotal role in diverse developmental, physiological, and pathological processes (1). Among the 4 known NOTCH receptors, NOTCH1 plays the most significant role in squamous cell differentiation (2). Recent whole-genome sequencing studies identified recurrent loss-of-function mutations of the *NOTCH1* gene in head and neck (H/N), cutaneous, lung, and esophageal SCCs (3–7), consistent with the tumor-suppressing function that NOTCH1 activation can play in this tumor type (8). Most attention has been given to its opposite tumor promoting function in other malignancies, such as T cell leukemia (T-ALL) (9) and breast cancer (10). While current drug development attempts are focused on inhibiting NOTCH signaling, it would also be desirable to identify approaches for activation of this pathway for possible differentiation-based therapy of squamous cancer.

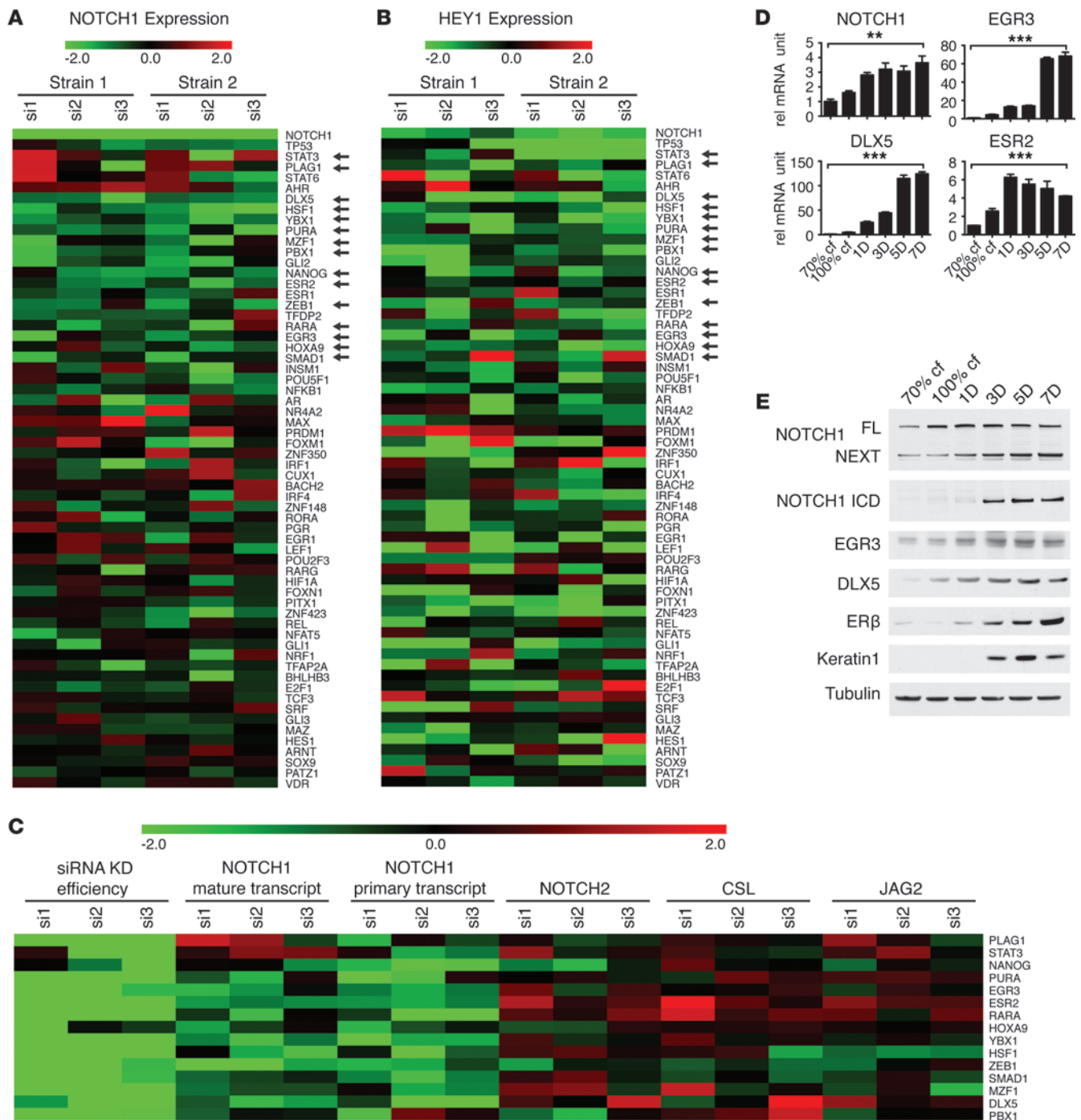
Control of NOTCH1 activity has been highly studied at the level of receptor processing and activation, while surprisingly little is known of direct transcription control of the *NOTCH1* gene (1). We and others have shown that *NOTCH1* is a direct p53 target in keratinocytes and that its downmodulation in keratinocyte-derived tumors can be explained, in part, by mutation or downmodula-

tion of p53 expression (11, 12). In most cells, with the notable exception of T cells (13), transcription of the human *NOTCH1* gene is driven by a single TATA-less “sharp peak” promoter that, in human keratinocytes, is under synergistic negative control of KLF4 and Sp3 (14). A related but more complex mode of regulation has been reported in human esophageal cancer cells, in which *NOTCH1* transcription appears to be under positive KLF5 control as a compensatory mechanism to compromised p53 function (15). A few other transcription factors have been reported to control NOTCH1 transcription in different cell types, including *Ovol2* (16), *FOXN1* (17), *STAT3* (18), *E2A* (19), *NF- $\kappa$ B* (20), and *HIF1 $\alpha$*  (20). While these studies were focused on involvement of individual transcription factors, to the best of our knowledge, no studies have been undertaken to probe into transcriptional control of the *NOTCH1* locus in a more systematic manner.

By a combined bioinformatic and functional screening approach, we have identified 3 direct regulators of the *NOTCH1* gene: *DLX5*, a homeobox protein best known for its role in proximal-distal limb development (21); *EGR3*, an immediate early response gene involved in neuronal plasticity (22); and estrogen receptor  $\beta$  (ER $\beta$ ), whose biological and biochemical functions are much less established than those of its cousin, ER $\alpha$  (23). Altered estrogen signaling is involved in development of a number of cancers, including breast, ovarian, colorectal, prostate, and endometrial cancers, and this pathway has been intensively investigated for pharmacological targeting (24). In breast cancer, there have been various reports on interplay between the NOTCH1 and estrogen/ER $\alpha$  signaling pathways at multiple levels (refs. 25, 26, and refs. therein), but none on NOTCH1 as an ER $\alpha$  transcriptional target. Global gene expression analysis combined with ChIP-seq studies has revealed that

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Figure 1**

Identification of transcription factors that control NOTCH1 expression in HKCs. (A and B) Two different HKC strains were reverse transfected with siRNAs against the indicated set of transcription factor genes, with siRNAs against NOTCH1 and p53 (top 2 lines) as control for effectiveness of the assay. Three different siRNAs per gene were tested (si1–si3), each in triplicate wells. One week after transfection, HKCs were analyzed by RT-qPCR for levels of NOTCH1 and HEY1 expression, with 36β4 for normalization. Results are expressed as heat map of log<sub>2</sub> ratios relative to cells transfected with scrambled siRNA control. Arrows indicate genes selected for further validation. (C) Validation of the above results for the indicated set of genes by reverse transfection of a third independent strain of HKCs, utilizing the same conditions as before. RT-qPCR analysis was used to assess siRNA KD efficiency of each gene and impact on levels of NOTCH1 primary and mature transcripts and other NOTCH pathway components. (D and E) RT-qPCR (D) and immunoblot analysis (E) of expression of the indicated genes in HKCs under proliferative conditions (70% confluence [cf]) and at various time (days [D]) of differentiation induced by high cell density. FL, full length; NEXT, NOTCH extracellular truncation, ICD, intracellular domain. mRNA levels were normalized for 36β4 and presented as fold-changes relative to cells under proliferative conditions. \*\**P* < 0.007; \*\*\**P* < 0.001. Similar results were obtained with analysis of an independent strain of HKCs (Supplemental Figure 2A). For detailed blot information, see complete unedited blots in the supplemental material.



ER $\alpha$  and ER $\beta$  have both common and distinct target genes (27). However, since most ER $\beta$  studies were generated using ectopically expressed protein, characterization of endogenous ER $\beta$  transcriptional function is still missing. Our findings establish that ER $\beta$ , like EGR3 and DLX5, is a direct positive regulator of NOTCH1 expression in keratinocytes and keratinocyte-derived SCC cells. We point to this molecule as a possible therapeutic target for differentiation therapy treatment of SCC. In fact, ER $\beta$  expression and function, linked with NOTCH1-dependent differentiation, are frequently compromised in skin, H/N, and lung SCCs, and increased ER $\beta$  expression or pharmacological treatments with ER $\beta$  agonists can suppress proliferation of SCC cells both in vitro and in vivo, while promoting NOTCH1 expression and differentiation.

## Results

*A transcription factor network involved in control of NOTCH1 gene expression in keratinocytes.* To probe into transcription control of the NOTCH1 gene, we started by examining the chromatin configuration of the human NOTCH1 locus in human primary keratinocytes (HKCs) utilizing whole genome information provided by the ENCODE consortium (<http://genome.ucsc.edu/ENCODE/>). Since insulator elements segregate genomic regulatory units, we focused on the NOTCH1 region delimited by peaks of ChIP sequencing (ChIP-seq) for the insulator protein CTCF. We further focused on chromatin regions of likely regulatory function on the basis of ChIP-seq peaks for modified histones present in enhancers and promoters. Sixty transcription factors with putative binding sites within these regions were selected for functional screening on the basis of their conservation among rat, mouse, and human and/or their epithelial pattern of expression in normal versus pathological conditions (as indicated by EST and cDNA microarray databases) (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI72718DS1).

A custom-made siRNA library was utilized to knock down (KD) expression of these genes in HKCs by reverse transfection. The primary screen was performed twice, with 3 siRNAs per gene, utilizing 2 HKC strains of independent origin. For both screens, cells were collected 1 week after transfection, i.e., under high-density differentiating conditions associated with elevated NOTCH1 expression (11) to capture factors involved in NOTCH1 transcription activation. Successful assay conditions were verified by assessing the impact on NOTCH1 mRNA levels of siRNA-mediated KD of the NOTCH1 gene itself as well p53 as a known positive regulator of NOTCH1 expression (ref. 11 and Figure 1, A and B). In parallel with NOTCH1, we assessed expression of the “canonical target gene” HEY1 as an indicator of endogenous NOTCH activity (11). Fifteen genes with consistent KD-mediated effects on NOTCH1 and HEY1 expression (>1.8-fold up- or downmodulation) in the 2 primary screens were further validated by siRNA KD in a third strain of HKCs (Figure 1C). In parallel with the mature NOTCH1 mRNA, we assessed levels of the primary transcript (by quantitative RT-PCR [RT-qPCR] of the third exon-intron junction) as well as transcripts of other key components of the NOTCH pathway. KD of STAT3, a tumor-promoting gene in keratinocytes (28), and PLAG1, a gene of unknown function in keratinocytes with a versatile role in tumor development (29), caused upregulation of the mature NOTCH1 mRNA and downregulation of the primary transcript, implicating these genes in opposite control mechanisms of NOTCH1 transcription and mRNA stability (Figure 1C). KD of all other genes resulted in a similar downregulation of both mature and primary

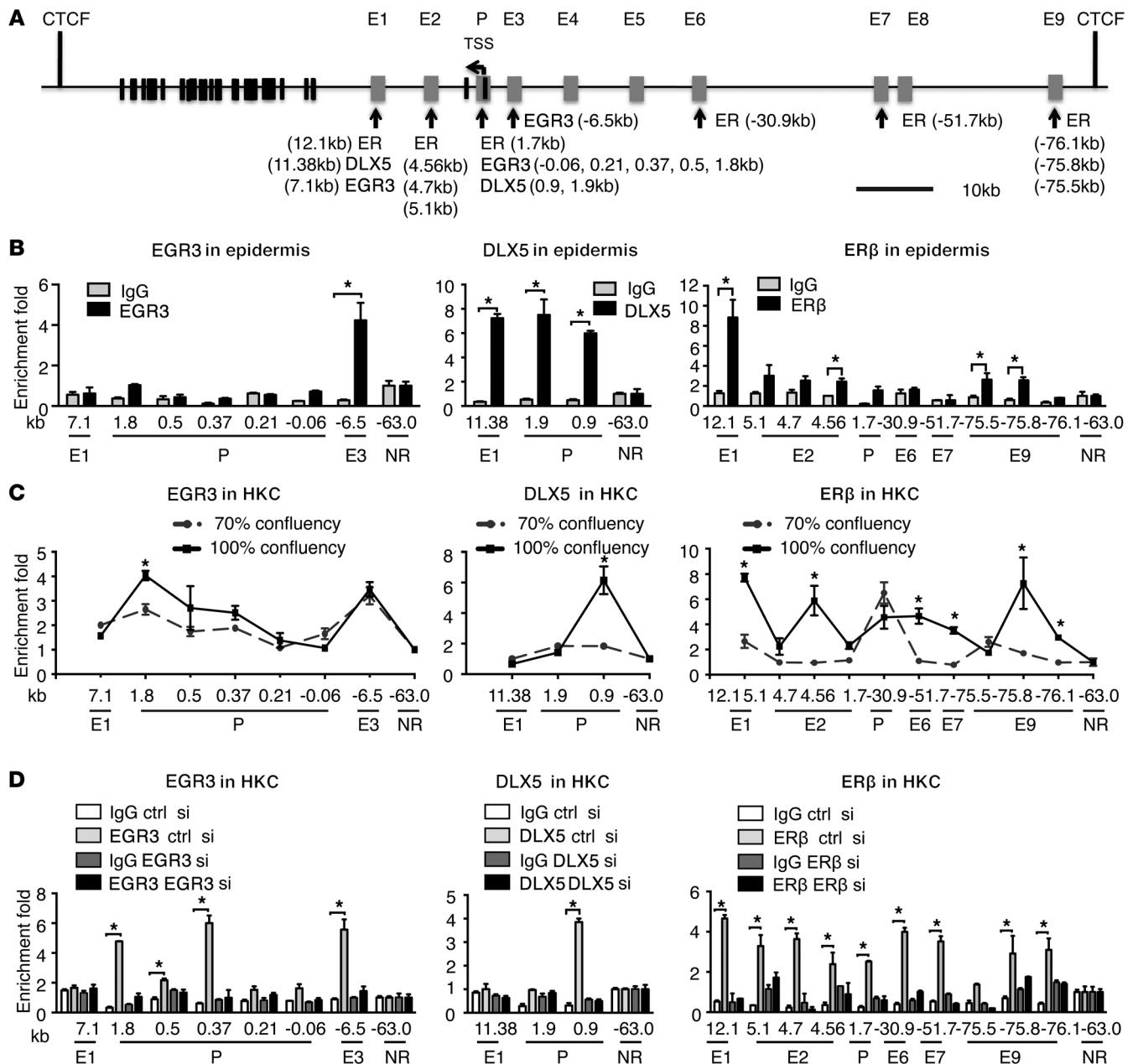
NOTCH1 transcripts, indicating that they function mostly at the level of transcription. Interestingly, expression of other NOTCH-signaling components was differentially affected by silencing of the various genes, in many cases in a manner opposite of that of NOTCH1, indicating that expression of these various components can be genetically dissociated (Figure 1C).

To assess whether NOTCH1 regulators are themselves under NOTCH-signaling control, we evaluated their expression in HKCs upon NOTCH1 KD or activation of the endogenous receptor by coculture with fibroblasts expressing the NOTCH ligand Jagged-2. Expression of most genes did not change consistently under the 2 conditions (Supplemental Figure 1A). Given the positive role of p53 in control of NOTCH1 expression (11, 12), we also examined expression of the identified NOTCH1 regulators in HKCs with p53 KD or stabilization of the endogenous p53 protein by Nutlin-3a treatment. Only ZEB1, which plays a significant role in epithelial-mesenchymal transition in other cellular systems (30), was consistently modulated in both conditions (Supplemental Figure 1B).

*EGR3, DLX5, and ER $\beta$  activate NOTCH1 gene transcription during differentiation.* The transcription factors identified in our screen may fulfill 2 functions, i.e., be required for maintenance of sustained transcription and/or play a more direct positive role in control of the NOTCH1 gene. We reasoned that transcription factors involved in positive control of NOTCH1 expression might be concordantly upregulated with differentiation. Among the 15 NOTCH1 regulators, EGR3, DLX5, and ER $\beta$  (encoded by the ESR2 gene) were consistently upregulated during differentiation (Figure 1, D and E, and Supplemental Figure 2). To assess whether these 3 factors participated directly in transcription control of the NOTCH1 gene, ChIP assays were performed with extracts of total human epidermis. The results showed binding of all 3 factors to the NOTCH1 locus at distinct regulatory regions: EGR3 was detected at a single enhancer region (E3) 6.5 kB upstream of the transcription start site (TSS); DLX5 was found at the promoter region and at a downstream enhancer (E1); and ER $\beta$  was detected at the promoter region (P) as well as at upstream (E9) and downstream (E1, E2) enhancers, with apparently greater binding to the latter (Figure 2B and Supplemental Figure 3A). Similar ChIP assays were performed on HKCs in culture conditions that allowed study of the transition from proliferation to early steps of differentiation. This time-course analysis showed that the binding of these factors in HKCs underwent dynamic change from growing to differentiating conditions, with enhanced binding of the 3 factors to the corresponding regions of the NOTCH1 locus in differentiating HKCs. In these cells, besides the upstream E3 enhancer, EGR3 binding to the NOTCH1 promoter region was also detected. DLX5 was found to bind to the promoter region of the NOTCH1 gene but not to the downstream enhancer. ER $\beta$  was found at multiple regions of the NOTCH1 locus that overlapped to a large extent with those detected in the epidermis, plus additional upstream enhancers (E6, E7) (Figure 2C and Supplemental Figure 3B). Binding specificity of each factor was confirmed by ChIP analysis performed on high-density differentiating HKCs plus/minus KD of EGR3, DLX5, and ER $\beta$  expression (Figure 2D, Supplemental Figure 3C, and Supplemental Figure 4A).

*EGR3 and DLX5 are required for RNA PolIII recruitment to the NOTCH1 locus and ER $\beta$  for RNA PolIII pause release.* For further functional insights, growing versus differentiating keratinocytes were subjected to ChIP with antibodies against RNA polymerase





**Figure 2**

Binding of endogenous EGR3, DLX5, and ER $\beta$  to *NOTCH1* gene locus in human epidermis and HKCs. (A) Schematic representation of *NOTCH1* gene locus. CTCF: insulator elements. Black bars, exons; gray boxes, predicted enhancer (E1–E9) and promoter (p) regions; black arrows, predicted binding regions of DLX5, EGR3, and/or ER $\alpha/\beta$  (nucleotide locations in brackets). (B) ChIP assays of EGR3, DLX5, and ER $\beta$  binding to the corresponding predicted sites of the *NOTCH1* locus in intact human epidermis. All ChIP samples were examined in parallel by PCR amplification of a negative control region (NR) located between enhancers 8 and 9 of the *NOTCH1* locus and devoid of predicted EGR3-, DLX5-, and ER $\beta$ -binding sites. Results are expressed as fold of enrichment for each indicated binding site relative to the negative control region. Statistical significance was determined by unpaired Student's *t* test (\**P* < 0.05). (C) ChIP assays of EGR3, DLX5, and ER $\beta$  binding to the *NOTCH1* locus in HKCs under growing (70% confluency) versus differentiating (100% confluency) conditions. ChIP assays were performed and data analyzed as in **B** (\**P* < 0.05). Enrichment folds in the immunoprecipitates with nonimmune IgGs were in all cases less than 1. (D) ChIP assays of endogenous EGR3, DLX5, and ER $\beta$  binding to the *NOTCH1* locus in differentiating HKCs (100% confluency) with or without individual KD of the 3 genes. Results were analyzed as in **B** (\**P* < 0.05). Results similar to those in **B–D** were obtained with HKCs of independent origin; see also Supplemental Figure 3, A–C.

II (PolII) as well as histone modifications associated with active promoter (H3K4me3 and H3K27ac) and enhancer (H3K4me1 and H3K27ac) regions. PolII binding increased with differentiation in parallel with the active chromatin histone marks, not only

at the promoter and downstream enhancer regions, but also at some upstream enhancers (E3–E6), which may be the result of promoter-enhancer interactions and “chromosome looping” (ref. 31, Figure 3A, and Supplemental Figure 4B).



PolII binding at promoter and enhancer regions was substantially decreased in differentiating HKCs in which *DLX5* and *EGR3* genes were KD (Figure 3B). KD of ER $\beta$  had more complex and unexpected consequences. In HKCs with ER $\beta$  downmodulation, binding of PolII to the downstream transcribed region of the *NOTCH1* gene was, as expected, decreased, while binding to the promoter and 2 of the upstream enhancers (E4, E5) was increased rather than decreased (Figure 3B and Supplemental Figure 4C). This suggests that the presence of ER $\beta$  is required for progression of PolII through the transcribed downstream region and that, in its absence, PolII is still recruited to the gene, but kept in a paused state that is known to also have consequences on “looping” (32).

In genomic regions of active transcription or pausing, elevated PolII is associated with active chromatin configuration, whereas reduction in PolII recruitment leads to nucleosome reassembly and less open configuration (32). Consistent with the suppression of *NOTCH1* gene transcription, H3K4me1 levels at the downstream enhancers (E1 and E2) were decreased in differentiating keratinocytes with *DLX5*, *EGR3*, or ER $\beta$  KD (Figure 3C and Supplemental Figure 4C). Interestingly, at the promoter region, levels of H3K4me3 and H3K27ac, as signs of open configuration, were either decreased (H3K4me3) or unaffected (H3K27ac) in HKCs with *DLX5* and *EGR3* KD, while they were substantially increased in HKCs with ER $\beta$  KD (Figure 3C and Supplemental Figure 4D). Substantially increased H3K27ac was also found at 2 upstream enhancer regions (E4 and E5) in HKCs with ER $\beta$  KD, which paralleled increased PolII binding even at these locations (Figure 3C and Supplemental Figure 4D). Repressive marks, such as H3K9me3 and H3K27me3, were not enriched at any of the regulatory regions (E1–E9) in either control or KD conditions, and levels of other modified histone marks (such as H3K4me1 and H3K9ac) were not consistently altered in the 2 different strains of HKCs that were tested (data not shown).

*DLX5 and ER $\beta$  induce keratinocyte differentiation through a NOTCH-dependent mechanism.* NOTCH signaling plays an important pro-differentiation role in keratinocytes (2). To determine whether *EGR3*, *DLX5*, and ER $\beta$  function as positive determinants of differentiation, several complementary approaches were undertaken. In the first, we found that KD of these genes by lentiviral-mediated shRNA delivery caused, in parallel with decreased NOTCH1 expression, downmodulation of differentiation markers such as keratin 1 and 10, which was rescued to a large extent by exogenous activated NOTCH1 expression (Figure 4, A and B, and Supplemental Figure 5, A–C). The impact on differentiation was further evaluated by assessing behavior of HKCs in skin organotypic cultures in which they underwent a vertical differentiation program closely approximating that occurring in vivo (11). Even under these conditions, *DLX5*, ER $\beta$ , and *EGR3* KD resulted in a significant reduction of NOTCH1 expression, as assessed by immunoblot analysis of proteins recovered from the reconstituted epidermis as well as by immunofluorescence analysis (Figure 4, C–E, and Supplemental Figure 5, D and E). Epidermal reconstitution capability of HKCs was not affected by *EGR3* silencing, while KD of *DLX5* and, to a greater extent, ER $\beta$  resulted in a reduced number of stratified layers and defective cornification and terminal differentiation marker expression (Figure 4D and Supplemental Figure 5, D and E), causing effects similar to those resulting from NOTCH inhibition in keratinocyte 3D cultures (33).

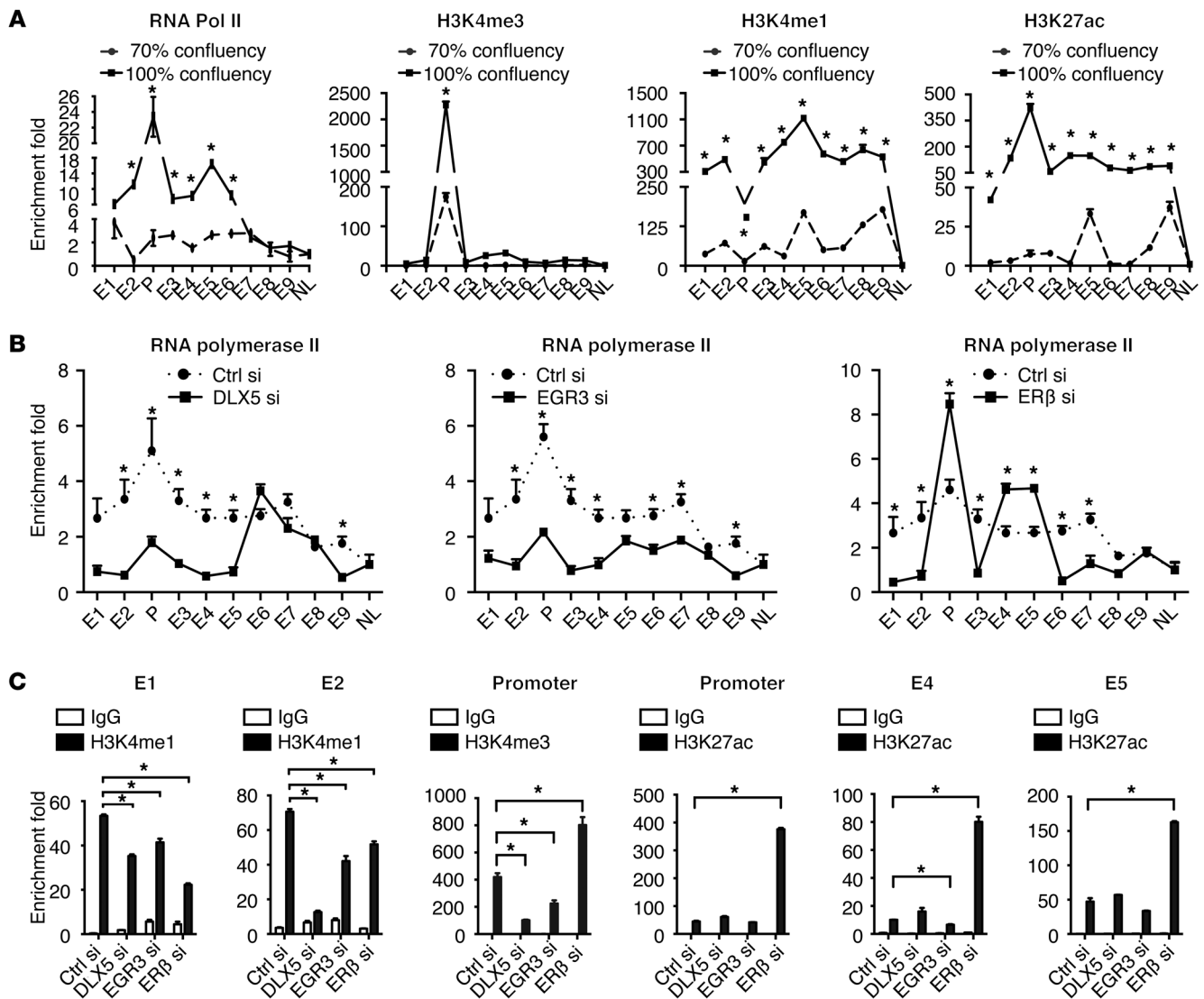
For a complementary gain-of-function approach, we assessed whether increased expression of the *EGR3*, *DLX5*, and ER $\beta$  proteins via retroviral vector transduction to levels comparable to

those found with differentiation was sufficient to induce *NOTCH1* mRNA and protein expression (Figure 5A and Supplemental Figure 6A). Interestingly, immunoblot analysis with antibody against the intracellular activated form of NOTCH1 (NOTCH1 ICD) showed enhanced NOTCH1 activation only in HKCs with increased *DLX5* and ER $\beta$  but not *EGR3* expression (Figure 5A and Supplemental Figure 6B), suggesting that additional posttranscriptional events required for NOTCH1 activation are also induced by the first 2 regulators but not the third. Consistent with these findings, enhanced expression of *DLX5* and ER $\beta$ , but not *EGR3*, led to induction of the canonical NOTCH target *HEY1* as well as the differentiation marker involucrin, which was prevented to a large extent by concomitant inhibition of NOTCH activation by treatment with the  $\gamma$ -secretase inhibitor DAPT or NOTCH1 KD (Figure 5B). Interestingly, increased *EGR3*, *DLX5*, and ER $\beta$  levels had different effects on expression of cell cycle and proliferation marker genes. While increased ER $\beta$  expression did not elicit any changes, elevated *EGR3* and *DLX5* expression downregulated Ki67 and cyclin E2 levels and induced p21<sup>WAF1/CIP1</sup>, consistent with the fact that keratinocyte differentiation and cell-cycle control can be separately controlled (ref. 34 and Figure 5C).

*Deregulated expression of NOTCH1 regulators in SCC of skin, H/N, and lung.* Consistent with its tumor suppressor function, the *NOTCH1* gene is downregulated or mutated in a significant fraction of skin, H/N, and lung SCCs (3, 6, 7, 11). Analysis of gene-expression profiles of these tumors from different data sets confirmed the frequent downmodulation of *NOTCH1*, along with upregulation of genes under negative NOTCH control in keratinocytes, such as p63, integrin  $\alpha_6$ , and integrin  $\beta_4$  (Figure 6A). Expression of “canonical” NOTCH targets of the HES/HEY family was variously modulated, consistent with their capability to crossregulate each other and their regulation by other input signaling pathways (35–38). In particular, *HES1* expression was commonly decreased in skin and H/N SCCs, *HEY1* was downmodulated in the first set of tumors and upregulated in the second, and both *HES1* and *HEY1* were increased in lung SCCs (Figure 6A).

Many transcription factor genes identified in our screen as modulators of *NOTCH1* expression were also deregulated in SCCs, with closer variations in skin and H/N SCCs than in lung SCCs. While *EGR3* was commonly downmodulated in tumors from the 3 body sites, *DLX5* was variably expressed. *ESR2* expression was decreased in skin and lung SCCs, while another *NOTCH1* regulator that is required for ER binding to target DNA, *PBX1* (39), was downmodulated in many skin and H/N SCCs and, more variably, in lung SCCs (Figure 6A). Expression of the *EGR3*, *DLX5*, and ER $\beta$  proteins was found decreased by immunohistochemical analysis of a large set of skin SCCs on tissue arrays (Figure 6B). Decreased expression of these genes was also seen by immunoblot analysis of a number of skin, H/N, and lung SCC cell lines (Figure 6, C and D).

*ER $\beta$  and sex-related gene-expression signatures and mutations in SCCs.* Given the translational potential, for further studies we focused on ER $\beta$ . We assessed at first the global impact of decreased ER $\beta$  signaling on the transcriptional program of primary keratinocytes and to what extent these changes in gene expression overlap with those in clinically occurring SCCs. cDNA microarray analysis of HKCs with and without ER $\beta$  KD confirmed that genes related to epidermal differentiation were downregulated in HKCs with silenced ER $\beta$  expression (Supplemental Table 3). Besides ER-dependent genes, other gene families significantly downmodulated in these cells included genes

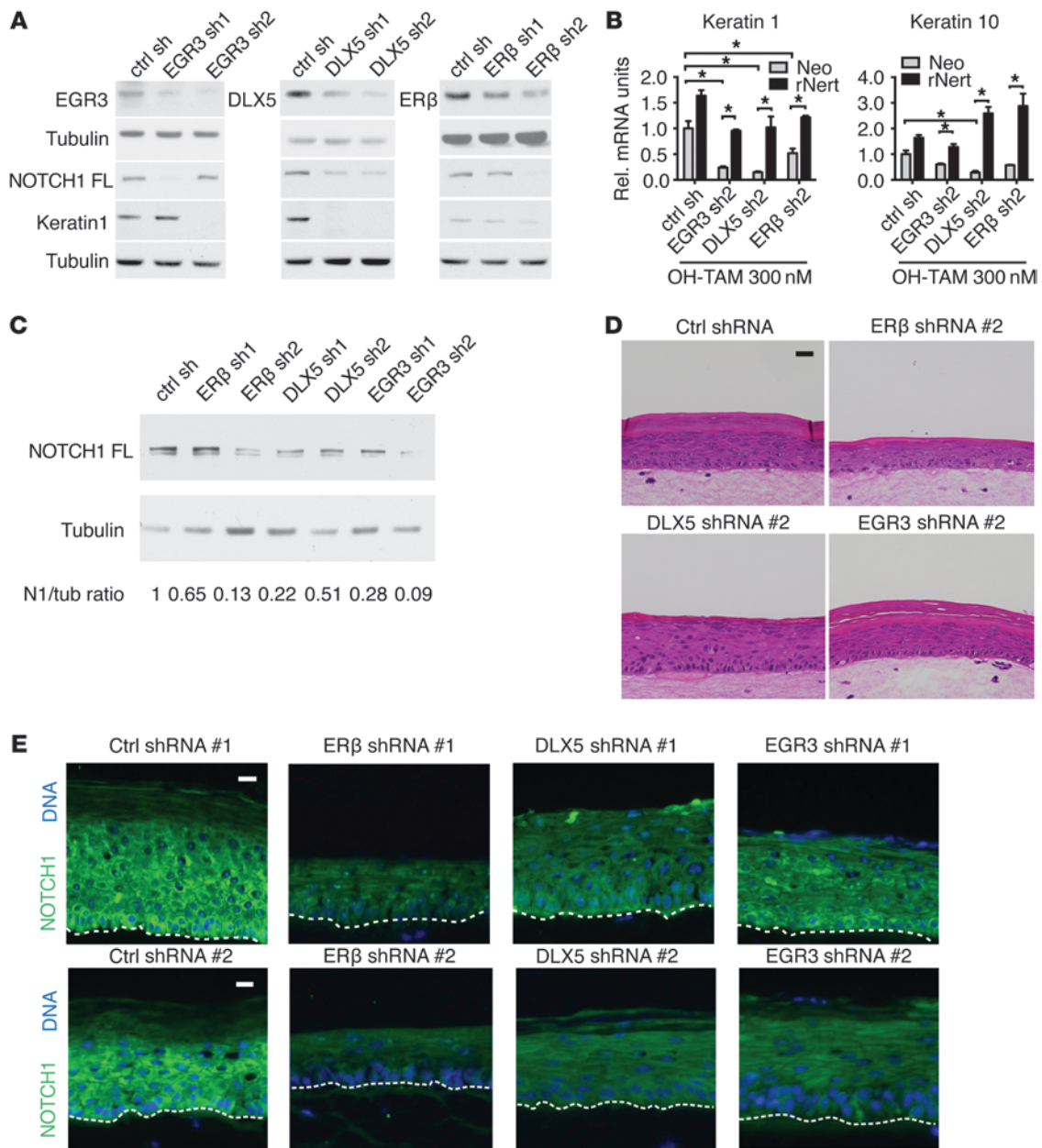


**Figure 3**  
 Essential role of EGR3, DLX5, and ERβ in RNA PolII recruitment to the *NOTCH1* locus and/or pause release. **(A)** ChIP analysis of HKCs under growing versus differentiating conditions for levels of PolII occupancy of the *NOTCH1* locus and associated levels of active histone marks of active promoter (H3K4me3, H3K27ac) and/or enhancer (H3K4me1, H3K27ac) regions. All ChIP samples were examined in parallel by PCR amplification of a negative control locus (NL) in chromosome 4, devoid of active chromatin marks and utilized for similar normalization purposes in previous studies with a number of cell types. Enrichment folds were calculated and plotted as in Figure 2 (\**P* < 0.05). Enrichment folds in the immunoprecipitates with nonimmune IgG were in all cases less than 1. Similar patterns of PolII binding were obtained with HKCs of independent origin; see also Supplemental Figure 4B. **(B)** HKCs transfected with siRNA against EGR3, DLX5, or ERβ versus siRNA controls were processed 96 hours later (at 100% confluency) for ChIP assays of levels of PolII occupancy of the *NOTCH1* locus. For ease of representation, levels of PolII binding in cells with KD of the individual genes are separately shown, utilizing PolII levels in control cells as the same point of reference (\**P* < 0.05). **(C)** HKC samples utilized in **B** were analyzed in parallel by ChIP assays for levels of active histone marks at the *NOTCH1* promoter and enhancer 1, 2, 4, and 5 regions (\**P* < 0.05). Results similar to those in **A–C** were obtained with HKCs of independent origin; see also Supplemental Figure 4D.

involved in stress response and positive regulation of transcription, while other stress-response genes, genes involved in proliferation, wounding, cell migration, and angiogenesis were upregulated (Supplemental Table 3). A substantial fraction of genes within these families were similarly deregulated in gene expression profile studies of lung, H/N, esophageal, and skin SCCs (Figure 7A and Supplemental Table 4). Interestingly, genes in other families were oppositely regulated in the ERβ-silenced HKCs versus clinical SCCs. These include a class of genes

involved in DNA packaging and nucleosome assembly, lipid and estrogen metabolic processes, and a less defined category of genes related to “system development.”

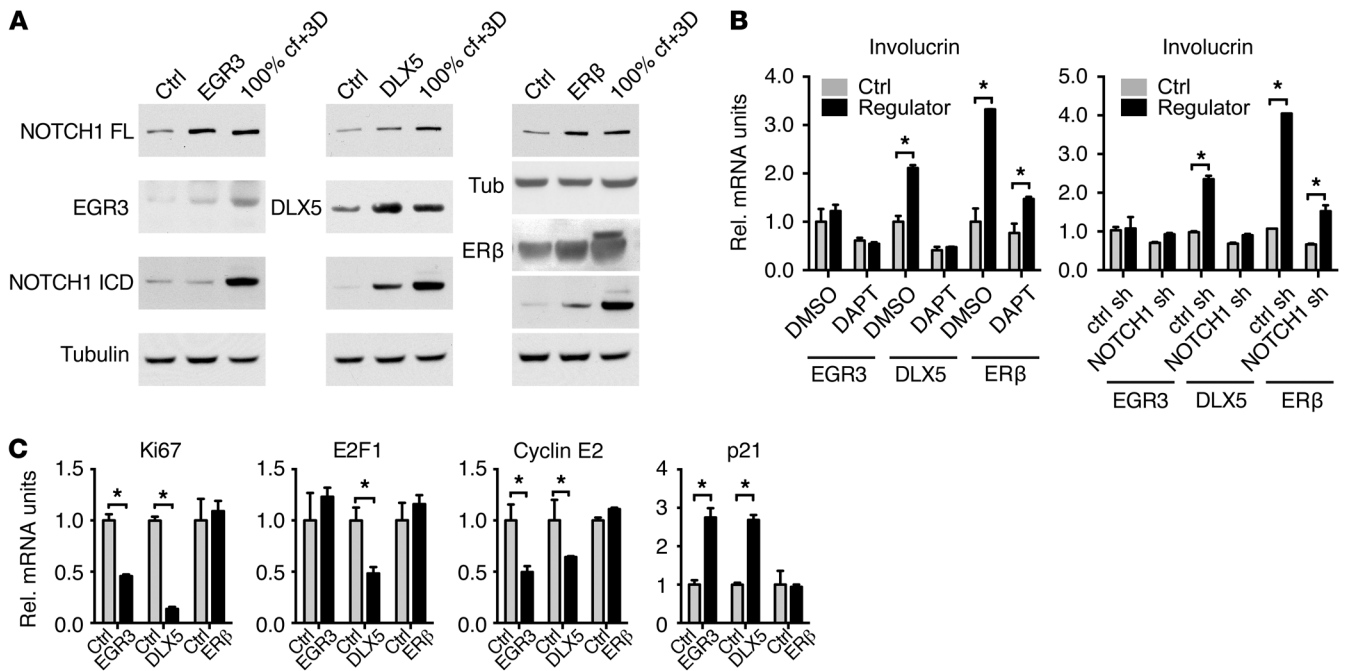
Epidemiologic studies indicate that there is a greater risk of skin, H/N, and lung SCCs in the male versus female populations, which may not be simply due to differences in lifestyle (40). For further insights, we examined the results of next-generation sequencing studies of H/N (The Cancer Genome Atlas, <https://tcga-data.nci.nih.gov/tcga/>; Peter S. Hammerman, personal communication, and



**Figure 4**

Silencing of EGR3, DLX5, and ERβ leads to attenuation of NOTCH1 expression and differentiation. (A) HKCs infected with shRNAs silencing lentiviruses versus control were analyzed 96 hours later by immunoblotting. Gene KD efficiency was assessed by parallel blots of those for NOTCH1 and Keratin 1 expression. Similar results were observed at the *NOTCH1* mRNA level and in another experiment with HKCs of independent origin (Supplemental Figure 5). (B) HKCs infected with lentiviruses as in A were superinfected with retrovirus expressing NOTCH1 intracellular domain fused to the human estrogen receptor (rNert), or vector control (Neo). 24 hours later, cultures were treated with OH-tamoxifen (OH-TAM) for 48 hours for nuclear NOTCH1 intracellular domain translocation. Expression of Keratin genes was determined by RT-qPCR (\**P* < 0.02). Results similar to those were obtained with a second HKC (Supplemental Figure 5C). (C–E) HKCs infected with lentiviruses as in A were grown in duplicate dermal equivalent gels at air-liquid interface for 12 days. The experiment was performed twice. (C) Immunoblot analysis of full-length NOTCH1 expression in reconstituted epidermis with signal quantification (numbers) by densitometric scanning and  $\gamma$ -tubulin normalization. (D) H&E analysis showing defective stratification and cornified layer formation in organotypic cultures with ERβ and DLX5 KD HKCs. Scale bar: 100  $\mu$ m. H&E images of other cultures and immunofluorescence analysis of differentiation marker expression are shown in Supplemental Figure 5D. (E) Immunofluorescence analysis of NOTCH1 expression in the reconstituted epidermis. For each series, image-capture conditions were the same. Scale bars: 50  $\mu$ m. Images are representative of 3 independent fields.





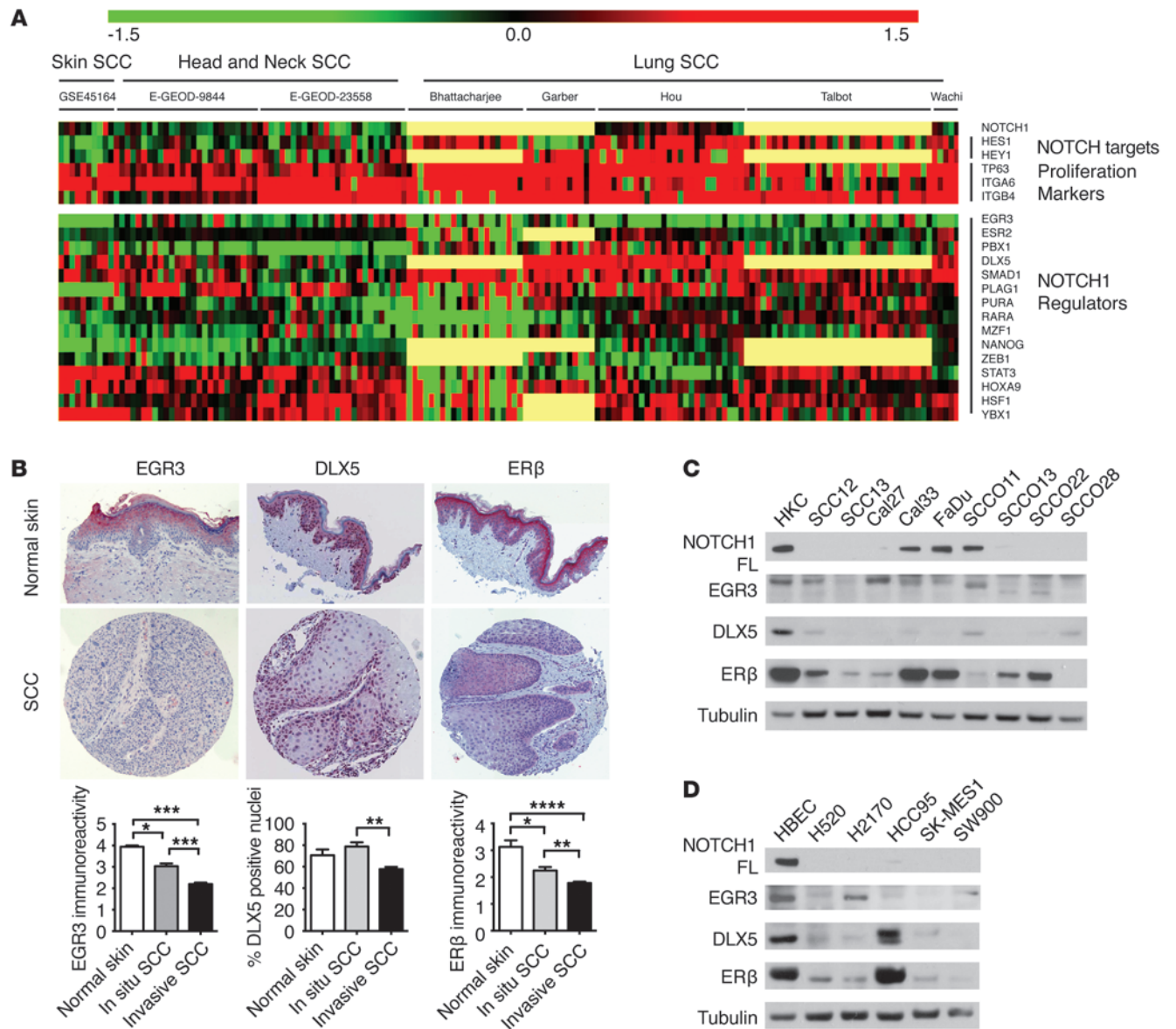
**Figure 5** EGR3, DLX5, and ERβ as regulators of NOTCH1 expression and function in HKCs. **(A)** HKCs under sparse conditions were infected with retroviruses expressing EGR3, DLX5, or ERβ versus empty vector controls as indicated, followed 72 hours later (at approximately 80% confluence) by immunoblot analysis for levels of these proteins as well as the full-length NOTCH1 protein (NOTCH1 FL) and the intracellular activated form (NOTCH1 ICD). HKCs under differentiating conditions (100% confluence for 3 days: 100% cf+3D) were analyzed in parallel as point of reference. Similar results were observed at the RNA level and in other experiments with HKCs of independent origin (Supplemental Figure 6, A and B). **(B)** HKCs with or without infection with EGR3-, DLX5-, and ERβ-expressing retroviruses as in the previous panel were treated with DAPT (10 μM) or DMSO control at 24 hours after infection followed, 48 hours later, by RT-qPCR analysis of involucrin gene expression, with 36β4 for normalization. As an alternative, HKCs stably infected with a shRNA lentivirus against NOTCH1 or empty vector control were superinfected with the EGR3, DLX5, and ERβ expressing retroviruses, followed 72 hours later by RT-qPCR analysis of the same genes. **(C)** HKCs with or without infection with EGR3-, DLX5-, and ERβ-expressing retroviruses as in **A** were analyzed, 72 hours after infection, by RT-qPCR of the indicated genes. \*P < 0.001.

TCGA Network) and lung SCCs (5) that could be divided between female and male patients. Surprisingly, among genes mutated in at least 10% of SCCs, a number exhibited a statistically significant difference in mutation frequency between the 2 sexes (Figure 7B and Supplemental Table 5). Most of these genes have cell regulatory functions that will be interesting to evaluate in the context of estrogen and/or sex-related signaling events. As a complementary approach, we examined whether there may also be differences in the global gene expression profiles of the same set of H/N SCCs analyzed for gene mutations in The Cancer Genome Atlas. A substantial number of genes were differentially expressed in SCCs from patients of the 2 sexes, including, as expected, Y- and X-linked genes (Figure 7C and Supplemental Table 6). Importantly, several genes related to the squamous differentiation program were significantly more expressed in SCCs of female patients, including those coding for desmoglein 1, caspase 14, psoriasin (S100A7), and defensins as well as cornified envelope proteins (Figure 7C).

*ERβ is a positive determinant of NOTCH1 gene expression and function in SCC cells.* To directly assess the impact of increased ERβ signaling in SCC outgrowth, a panel of skin, oral, and lung SCC cell lines was infected with ERβ-expressing versus control viruses. As shown in Figure 8A, proliferation of most cell lines, as assessed by Alamar blue cell density assays, was significantly inhibited as a consequence of ERβ overexpression. Cells with elevated proliferative

potential, as assessed by colony or sphere formation assays, were also significantly reduced (Figure 8, B-E, and Supplemental Figure 7). Increased ERβ expression in many SCC cell lines derived from various body sites was accompanied by upregulation of NOTCH1 and differentiation marker expression (Figure 9A), with induction of the latter being significantly counteracted by DAPT treatment (Figure 9B). To test the in vivo impact of increased ERβ expression, representative cell lines from skin (SCC13), H/N (SCCO13), and lung SCCs (H2170) were assessed by intradermal tumorigenicity assays in immunocompromised mice (41). Tumors formed by cells with increased ERβ expression reached a size similar to that of controls, but with enhanced NOTCH1 expression and differentiation (Figure 9, C and D, and Supplemental Figure 8).

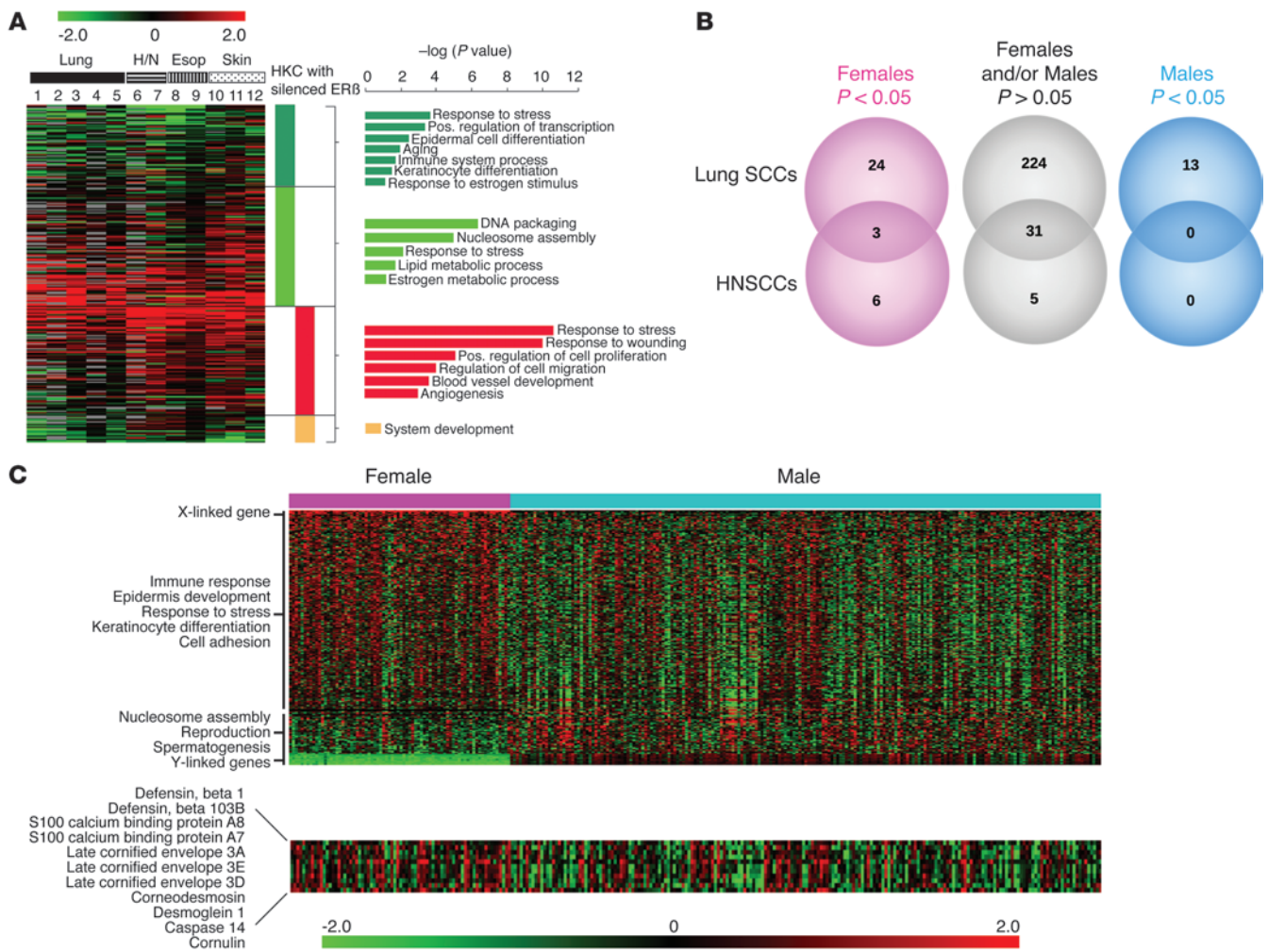
Besides estrogen, other agonists have been developed with elevated specificity for either the ERα or ERβ receptors (42). Use of these molecules could be of substantial translational interest as inducers of squamous cell differentiation and SCC tumor suppression. Treatment of HKCs with either 17β-estradiol (E2) or 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), an ERβ selective agonist (42), resulted in induction of NOTCH1 expression as well as differentiation marker expression (Figure 10A). Little or no induction was observed after treatment with 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), an ERα-selective agonist, while NOTCH1 as well as differentiation marker expression was suppressed by



**Figure 6**  
 Deregulated expression of EGR3, DLX5, and ERβ and other NOTCH1 regulators in cutaneous, H/N, and lung SCCs. **(A)** Heat map of transcriptomic data of the indicated set of genes in a set of skin SCCs versus normal epidermis ( $n = 10/3$ ) analyzed in our own laboratory (GSE45164) and published data sets of H/N and lung SCCs versus normal tissue controls (E-GEOD-9844,  $n = 26/12$ ; E-GEOD-23558,  $n = 27/5$ ) (Bhattacharjee Lung,  $n = 21/17$ ; Garber Lung,  $n = 13/6$ ; Hou Lung,  $n = 27/65$ ; Talbot Lung, 34/28; Wachi Lung,  $n = 5/5$ ). **(B)** Immunohistochemical analysis of EGR3, DLX5, and ERβ expression in tissue arrays of in situ and invasive skin SCCs versus normal skin ( $n = 31, 226, 10, 3$ , respectively). Representative staining is shown along with quantification of percentage of positive nuclei (DLX5) or immunoreactivity (EGR3 and ERβ) as indicated. Error bars represent mean  $\pm$  SEM. Statistical analysis was performed using 1-way ANOVA. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . Original magnification,  $\times 14.4$ . **(C)** Skin (SCC12 and SCC13) and H/N SCC cell lines (Cal27, Cal33, FaDu, SCCO11, SCCO13, SCCO22, and SCCO28) were analyzed with HKCs under growing conditions by immunoblotting. **(D)** Indicated lung SCC cell lines were analyzed in parallel with HBECs for NOTCH1, EGR3, DLX5, and ERβ expression by immunoblotting. For detailed blot information, see complete unedited blots in supplemental material.

treatment with fulvestrant, a complete ER antagonist (42, 43) (Figure 10A). Analysis of SCC gene expression profiles indicated that the *CYP1B1* gene, coding for a hydroxylases of the P450/CYP1 family with a key role in estrogen inactivation and procarcinogenic conversion (44), was frequently overexpressed in SCCs (Figure 7A and Supplemental Table 4). Increased CYP1B1 expression was also found in the panel of SCC cell lines used in this study (Figure 10B).

These cells exhibited different dose sensitivities to DPN treatment, but in all cases, their proliferation and sphere-forming capability were suppressed (Figure 10, C and D), in parallel with induction of NOTCH1 and differentiation marker expression (Figure 10E). As an in vivo test, a cohort of mice was intradermally injected with SCCO13 cells, followed by daily administration of DPN or DMSO vehicle alone. Relative to controls, mice treated with the ERβ-



**Figure 7**

Comparative gene expression and mutation profiles of skin, H/N, and lung SCCs in relation to ER signaling and/or patients' sex. **(A)** Expression profiles of ERβ-controlled genes in clinically occurring SCCs versus normal tissues. Microarray analysis of gene expression in HKCs plus/minus ERβ-KD identified genes under ERβ control (>1.5-fold change; Supplemental Table 3). Expression profiles of these genes were examined in clinically occurring SCCs, utilizing our own and published data sets (Supplemental Table 4: Oncomine). Dark/light green and red columns on the right refer to the set of down- and upmodulated genes in HKCs with ERβ silencing that were found to be concordantly (dark colors) versus discordantly (light colors) regulated in clinically occurring SCCs in parallel with a differential gene family distribution (Supplemental Table 4). **(B)** Numbers of genes with mutations frequencies of 10% or more in lung and H/N SCCs from patients of one or both sexes (Fisher's exact test < 0.05). Overlapping circles show numbers of genes with similar mutation frequency distribution in lung and H/N SCCs. For complete mutation gene list and analysis, see Supplemental Table 5. **(C)** Expression profiles of genes differentially expressed in female versus male H/N patients. Gene families with statistically significant enrichment are indicated on the left (Supplemental Table 6). Genes with a role in squamous differentiation are zoomed in. Log<sub>2</sub> expression values, median centered and divided by SD, are represented.

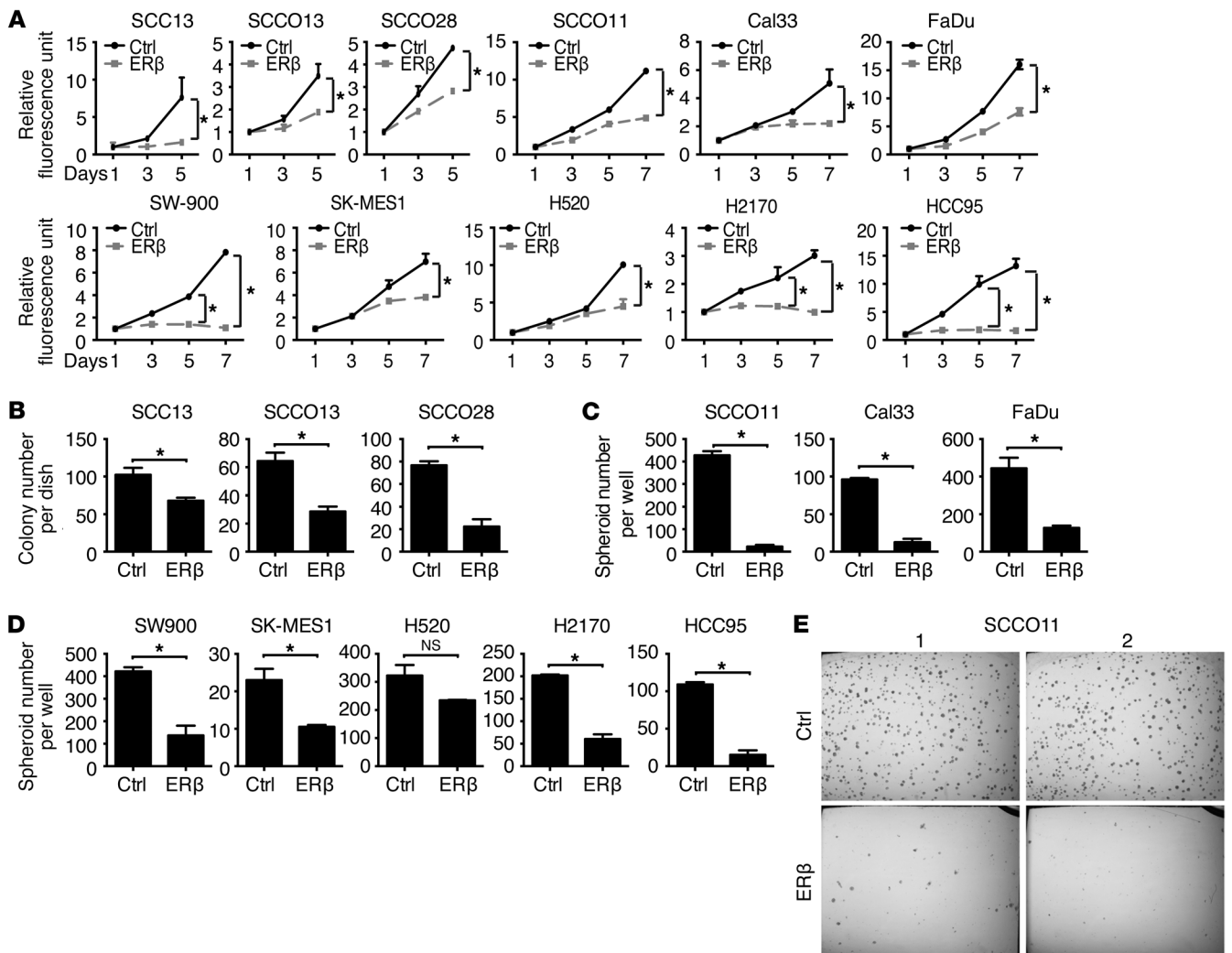
selective agonist exhibited tumors of significantly smaller size and reduced proliferative index (Figure 11A), with increased NOTCH1 and differentiation marker expression (Figure 11, B and C).

**Discussion**

Recent advances in whole-genome analysis provide unique opportunities to probe into coordinate control of gene expression and ensuing biological events. We have identified a transcriptional regulatory network converging on control of NOTCH1 gene transcription in squamous cell differentiation and cancer. Most of the transcription factors are themselves deregulated in skin, oral, and lung SCCs, with some undergoing gene mutations and/or rearrangements (3, 6, 7). Func-

tions of these factors, together with interactions of possible relevance, are summarized in Supplemental Table 2. While genetic and functional evidence indicates that the NOTCH1 gene plays a prevalent role in keratinocyte differentiation and tumor suppression (8), the finding of NOTCH2 and NOTCH3 mutations in SCCs (3–6) point to the possible importance of these genes for the disease. Their expression appears to be under distinct control mechanisms from NOTCH1, as they are oppositely regulated by most of the NOTCH1 regulators that we have identified. For further functional and biochemical studies, we focused on 3 factors that, as discussed here below, play a direct and positive role in NOTCH1 gene transcription in keratinocytes, with ERβ as a possible target of translational significance.





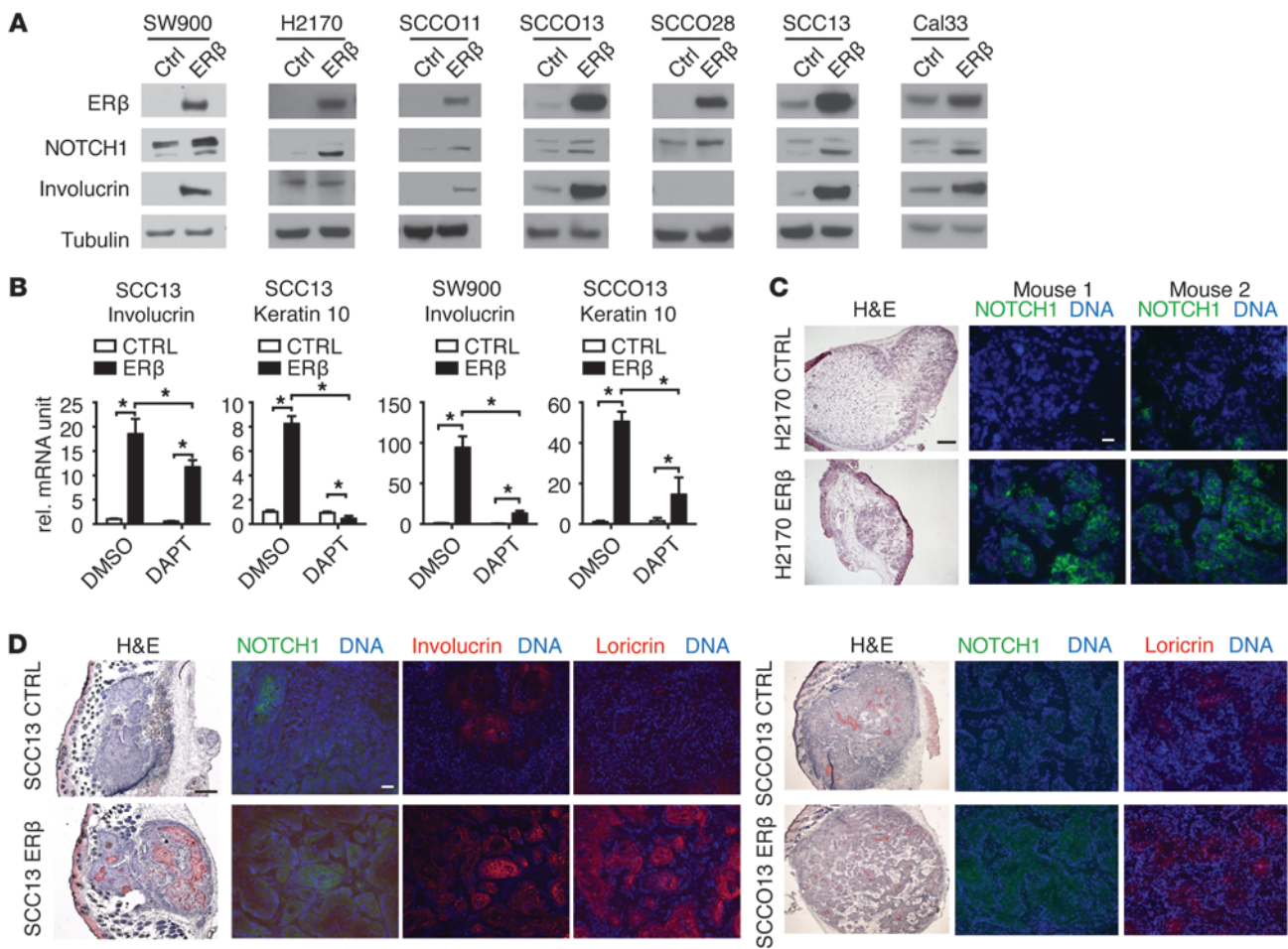
**Figure 8**

Elevated ERβ expression suppresses proliferation of skin, lung, and oral SCC cells. (A) Alamar blue cell density assays. Cell lines derived from skin (SCC13), H/N (Cal33, FaDu, SCCO11, SCCO13, SCCO28), and lung (H520, H2170, HCC95, SK-MES1, SW900) SCCs were infected with either an ERβ-expressing lentivirus (SCC13, SCCO13, and SCCO28) or retrovirus (all other cell lines) versus corresponding empty vector controls, followed, 48 hours later, by G418 selection. Stably infected cells were plated in 96-well plates (2000 cells/well). Alamar blue fluorescence intensity assays were performed in triplicate every 2 days as indicated. Data are presented as mean fold change of fluorescence intensity ± SD over day 1. \*P < 0.05. (B) Clonogenicity assays. Skin (SCC13) and H/N (SCCO13 and SCCO28) SCC cells infected with an ERβ-expressing lentivirus versus empty vector were plated at limited density on triplicate dishes (103 cells/60 mm dish), and colony formation was measured by crystal violet staining 10 days later. \*P < 0.05. (C and D) Spheroid assays. Skin and H/N (C) and lung (D) SCC cells were infected with an ERβ-expressing retrovirus or lentivirus versus empty vector as in A. Stably infected cells were plated in duplicate in Matrigel suspension in 8-well chambers (2000 cells/well). Spheroid numbers were quantified 10 days later by digital acquisition of the whole well images and ImageJ software analysis. \*P < 0.05. (E) Representative images of spheroids formed by SCCO11 cells infected with control versus ERβ-expressing retroviruses. Original magnification, ×2.5. Photographs of spheroids formed by other SCC cells are shown in Supplemental Figure 7B.

EGR3 belongs to a family of 4 highly conserved zinc finger transcription factors, originally identified in the “early response” of cells to growth factor stimulation (45). These proteins bind to the same consensus DNA sequence and can have a significant functional overlap. They have been implicated in many biological processes, including muscle and lymphocyte cell fate determination and neuronal development and plasticity (45). Little is known of functions of these genes in squamous differentiation, with EGR3-deficient mice exhibiting little skin abnormalities, possibly due to functional compensation by other EGR family members (22).

While EGR gene functions have been interconnected with important signaling pathways, such as NF-κB, MAPK, and Calcineurin/NFAT (46), a connection with NOTCH signaling, to our knowledge, has not been reported. We have shown here that EGR3 functions in keratinocytes as a direct positive regulator of NOTCH1 gene expression, with endogenous EGR3 binding to the NOTCH1 locus and being required for RNA PolIII recruitment with differentiation. Exogenous EGR3 expressed at levels similar to those found in differentiation was sufficient for induction of NOTCH1 gene transcription. Interestingly, however, the increased levels of



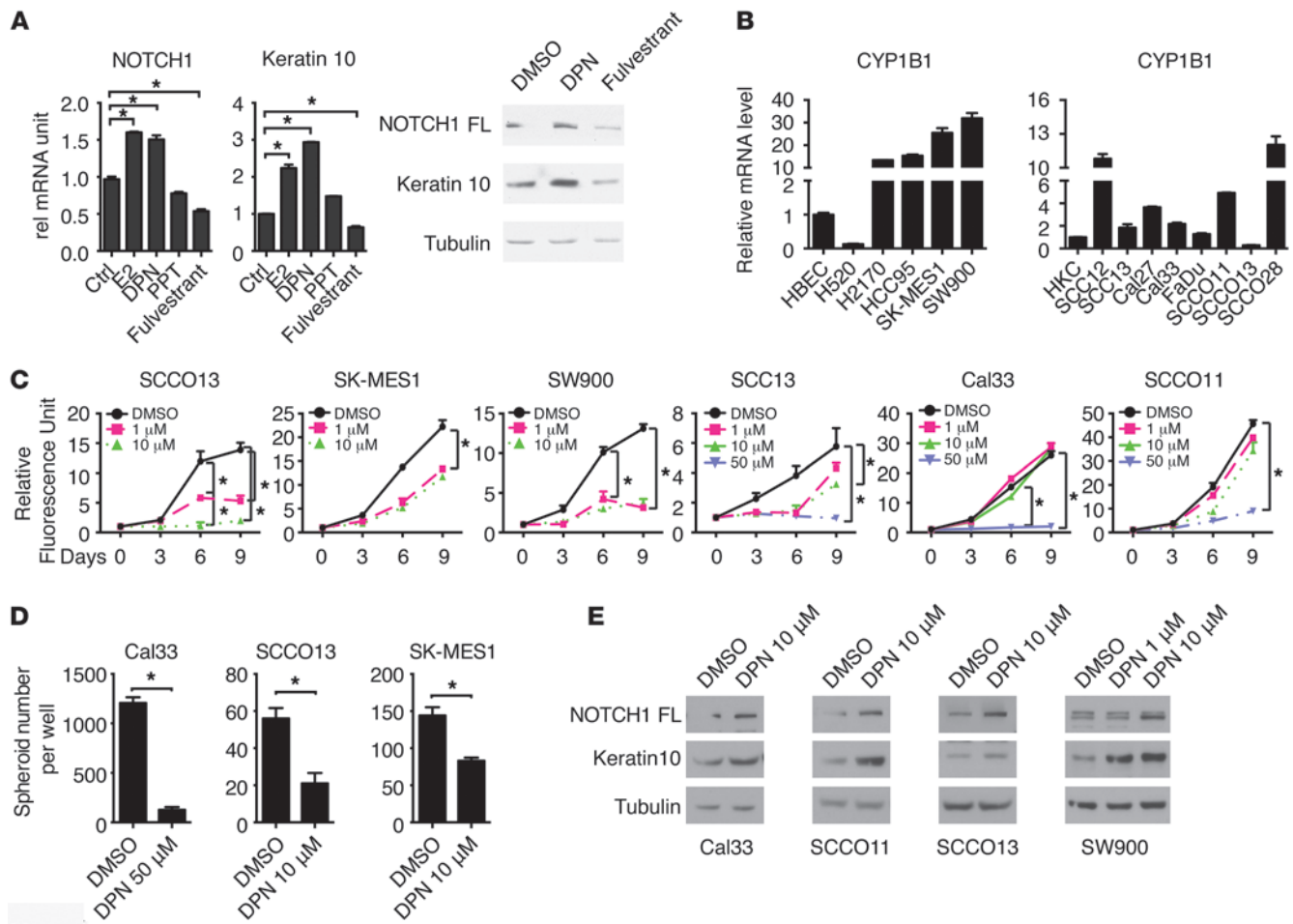


**Figure 9**  
Elevated ERβ expression induces NOTCH1 expression and differentiation. (A) SCC cell lines infected with ERβ-expressing viral vectors versus controls as in Figure 8 were analyzed for expression of the indicated proteins by immunoblotting. (B) SCC13 (skin), SW900 (lung), and SCCO13 (oral) SCC cells infected with ERβ-expressing and control viral vectors were treated 24 hours after infection with DAPT (10 μM) or DMSO control followed, 72 hours later, by RT-qPCR analysis of involucrin and keratin 10 differentiation marker expression. \*P < 0.05. (C) H2170 lung SCC cells infected with ERβ-expressing versus control vectors were injected intradermally in parallel in the right and left suprascapular regions of NOD/SCID mice (n = 5; 1 × 10<sup>6</sup> cells per injection). Animals were sacrificed 1 week later, and tumor samples were processed for H&E and immunofluorescence analysis of NOTCH1 expression. For each tumor pair, images were taken under the same capture conditions and are representative of several independent fields. Black scale bar: 500 μm; white scale bar: 100 μm. (D) SCC13 and SCCO13 cells infected with ERβ-expressing versus control vectors were tested by parallel intradermal injections into mice as in C. Animals were sacrificed 3 weeks later, and tumor samples were processed for H&E and immunofluorescence analysis of NOTCH1 and differentiation marker expression as indicated. Analysis of other tumor pairs is shown in Supplemental Figure 8. Black scale bar: 250 μm; white scale bar: 100 μm.

NOTCH1 mRNA and protein resulting from EGR3 upregulation were not accompanied by a corresponding increase in the proteolytically cleaved activated NOTCH1 protein. It has been recently reported that in keratinocytes, besides its increased expression, NOTCH1 activation depends on the parallel induction of one of its cleaving membrane-bound proteases (47). An interesting possibility is that EGR3 controls expression of NOTCH1, but not of other determinants of NOTCH activation, whose concomitant upregulation may instead account for NOTCH1 activation by DLX5 and ERβ discussed below.

Homeobox genes play a key role in development and cancer (48). Surprisingly, however, a direct connection between this important class of transcriptional regulators and NOTCH signaling has not

been established. DLX5 belongs to the family of Distal-less (DLX) homeobox genes, identified for their role in distal limb development (21). There are 6 family members, with different members having the potential of carrying out the same biological function in different species (21). Dlx5 and Dlx6 have been shown to play an important role downstream of p63 in the apical endodermal ridge and limb development (49). We have found that, in human keratinocytes, DLX5 functions as a direct positive regulator of NOTCH1 expression and exerts a prodifferentiation function that is, in part, NOTCH dependent. In the mouse system, Dlx3 may fulfill a similar function, as premature epidermal differentiation is induced by keratinocyte-specific Dlx3 overexpression (50), while Cre-mediated deletion of the gene results in epidermal hyperplasia



**Figure 10**

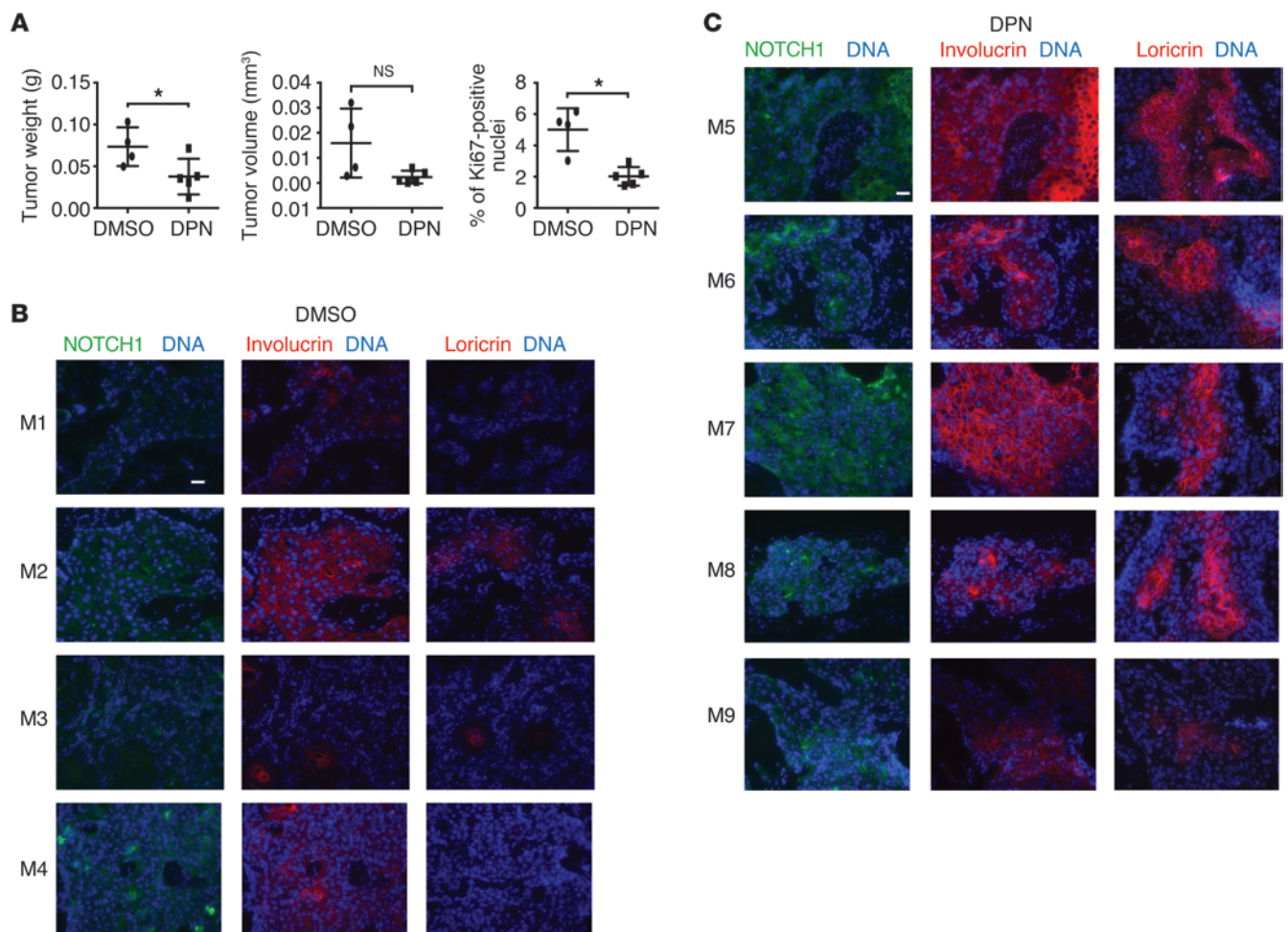
ER $\beta$  agonists induce NOTCH1 and differentiation marker expression in HKCs and SCC cells with concomitantly attenuated proliferation. (A) Differentiating HKCs (100% confluence) were treated with 10 nM estradiol (E2), 100 nM ER $\beta$ -specific agonist (DPN), 100 nM ER $\alpha$ -specific agonist (PPT), 10 nM estrogen receptor panantagonist (fulvestrant) or DMSO control followed, 72 hours later, by RT-qPCR ( $*P < 0.05$ ) and immunoblot analysis of the indicated genes/proteins. For detailed blot information, see complete unedited blots in supplemental material. (B) Expression of CYP1B1 was determined by RT-qPCR in lung and keratinocyte-derived SCC cell lines in parallel with HBECs and HKCs, respectively. (C) Alamar blue density assays of SCC cells were performed in triplicate wells and treated with DPN or DMSO (refreshed every other day). Data are presented as mean fold change of fluorescence intensity  $\pm$  SD ( $*P < 0.05$ ). (D) Indicated SCC cells were plated in duplicate on Matrigel precoated chambers with spheroid number quantification 10 days later by ImageJ analysis of whole-cell images. Data are duplicates  $\pm$  SD ( $*P < 0.05$ ). Photographs and spheroid quantification of other SCC cells are shown in Supplemental Figure 9. (E) Indicated SCC cells were treated with the ER $\beta$ -specific agonist DPN at the indicated doses for 10 days. For detailed blot information, see complete unedited blots in supplemental material.

together with impaired hair follicle differentiation (51). Besides DLX5, another homeobox gene that was shown by our screen to be required for NOTCH1 expression is PBX1, which has previously been implicated in epidermal differentiation and barrier function (52). Of potential relevance for the present studies are findings in other systems that PBX proteins can form heteromeric complexes with DLX proteins (21) and that PBX1 can serve as a “pioneer factor” required for ER function as discussed below (39).

Estrogen receptors play a significant role in human physiology and disease, with a role extending to sex-unrelated organs, such as intestinal and cardiovascular systems (23, 24). The 2 main estrogen receptors, ER $\alpha$  and ER $\beta$ , are encoded by separate genes (*ESR1* and *ESR2*) and exhibit distinct tissue-specific patterns of expression. ER $\beta$  is the form predominantly expressed in human epidermal cells in vivo (53) as well as in culture (our observations). Rela-

tive to ER $\alpha$ , the biochemical function of ER $\beta$  is less established, as it was mostly studied in cells with overexpression of the exogenous protein (27). Like EGR3 and DLX5, we have found that ER $\beta$  is induced and plays a direct positive role in control of NOTCH1 expression in keratinocyte differentiation. Our further biochemical analysis revealed that, while both EGR3 and DLX5 are required for recruitment of RNA PolIII to the *NOTCH1* locus, ER $\beta$  is likely involved in pause release of this enzyme from the TSS. Following establishment of a transcription initiation complex, the onset of elongation is emerging as a highly regulated process for transcription of many genes, especially with developmental and/or signal transduction functions (32). Control of NOTCH1 expression by RNA PolIII pause release has not, to our knowledge, been previously reported. In breast cancer cells, ER $\alpha$  controls transcription through long-range “chromatin looping” (54) and is required for



**Figure 11**

ER $\beta$  agonist treatment delays SCC tumor growth and promotes differentiation. (A–C) SCC013 cells were injected intradermally in the left supra-scapular region of NOD/SCID mice ( $1 \times 10^6$  cells per injection). Forty-eight hours after injection, DPN was injected intraperitoneally into a cohort of mice ( $n = 5$ ) at a dose of 20 mg/kg every day in parallel with another cohort of mice ( $n = 4$ ) injected with DMSO vehicle alone. Animals were sacrificed 10 days later. Tumor weight and volume were measured, followed by determination of Ki67-labeling index by immunofluorescence analysis of histological section (A). \* $P < 0.05$ . Parallel immunofluorescence analysis was used to assess NOTCH1 and differentiation marker expression in all tumor samples, using same image capture conditions (B and C). Scale bar: 100  $\mu$ m.

pause release of *MYB* gene transcription through recruitment of the P-TEFb kinase complex (55). In spite of intensive studies on the interconnection between ER $\alpha$  and NOTCH, direct binding and transcriptional control of the *NOTCH1* gene by ER $\alpha$  has not been seen. Our findings indicate that ER $\beta$  instead carries out this function in keratinocytes and, possibly, beyond.

SCCs are notoriously resistant to conventional and targeted drug treatments, and novel differentiation therapy approaches, alone or in combination, may be of substantial value (56). Besides HKCs, we found that increased ER $\beta$  induces NOTCH1 expression and differentiation also in keratinocyte-derived skin and H/N SCC cells both in vitro and in vivo. Interestingly, ER $\beta$ -induced expression of NOTCH1 and squamous differentiation markers also occur in lung SCC lines, consistent with the capability of bronchial epithelial cells to undergo squamous cell differentiation as a possibly protective reaction against cancer development (57).

The incidence of clinically occurring SCCs in skin, H/N and lung is significantly higher in males than females, with associated pos-

sible differences in survival (58). A likely reason is the higher exposure of the male population to procarcinogenic conditions such as smoking and alcohol abuse. However, estrogen receptor signaling may be a concomitant determining factor in this as well as in other organ diseases unrelated to reproductive function (40). Experimentally, mice with abrogation of estrogen production by ovariectomy or deletion of the ER $\beta$  gene had enhanced sensitivity to endogenous skin tumor development or transplanted skin tumor growth (59, 60). In apparent contrast, estrogen was reported to promote chemically induced lung carcinogenesis (61) and estrogen agonists have been generally reported to enhance proliferation and tumorigenicity of lung cancer cell lines (62). However, chemically induced mouse models reproduce the adenocarcinoma rather than squamous carcinoma form of non-small cell lung cancer (NSCLC) (63), and the tested human cell lines were from lung adenocarcinomas. Underlying their different histological properties, lung adenocarcinomas and squamous carcinomas have substantially different genetic alterations and gene-expression signatures



(63), with loss of function *NOTCH1* gene mutations occurring preferentially in the second type of tumors (5). The possibility that estrogen signaling also plays a tumor-suppressing function in the lung is supported by clinical epidemiological studies showing that, in postmenopausal women, estrogen exposure is associated with reduced risk of NSCLC (64) and nuclear ER $\beta$  expression is a positive prognostic marker for male NSCLC patients (65, 66).

Excitingly, taking advantage of recent next-generation sequencing data, we have uncovered a so-far unsuspected specificity of gene mutations in H/N and lung SCCs of female versus male patients, pointing to a possible molecular basis for differences of the disease between the 2 sexes. Such a possibility is further supported by our finding that SCCs of female and male patients can be discriminated on the basis of their gene expression program, with several squamous differentiation related genes and other gene families being differently expressed (Supplemental Table 6). An attractive possibility raised by further functional studies is that estrogen-dependent control of *NOTCH1* expression and differentiation underlies these sex differences and that, by enhancing the squamous differentiation network that we have identified, estrogen mimetic compounds, in particular, ER $\beta$ -specific agonists, could be used in combination with other treatment modalities of premalignant and malignant lesions.

## Methods

**Cells, tissue samples, and viruses.** HKCs and SCC (SCCO11, SCCO13, SCCO22, and SCCO28) cell lines were obtained and cultured as previously described (11, 41). Oral SCC cells (Cal27, Cal33, FaDu) were provided by Genrich Tolstonog (University Hospital of Lausanne). Human bronchial epithelial cells (HBEC) were purchased from ATCC. Lung SCC cells (H520, H2170, HCC95, SK-MES-1, and SW900) were provided by Peter S. Hammerman (Dana-Farber Cancer Institute, Boston, Massachusetts, USA). SCC samples were obtained at the Department of Dermatology of the University Hospital Zurich from clinical biopsies. Parts not needed for histological diagnosis were further processed with institutional review board approval.

Constructs for retro- and lentiviral production were obtained or made as follows. Retroviral vectors pMSCV-DLX5, pMSCV-EGFP-EGR3, rNERTneo, and Neo were provided by J.R. Testa (Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA), W. Tourtelotte (Northwestern University Feinberg School of Medicine), and U. Just (Christian Albrechts University of Kiel, Kiel, Germany), respectively. pMXs-ESR2 was constructed by cloning the Flag-tagged full-length cDNA of *ESR2* from pCXN2 vector (provided by S. Inoue, University of Tokyo, Tokyo, Japan) into the BamHI/NotI sites of the PMx vector using the following primers: forward, 5'-GATTCCG-GATCCGCCACCATGGACTACAAGGACGACGATGACAAGGATATA-AAAACTACCA-3'; and reverse, 5'-GCTGCTGCGGCCGCTACTGAGACTGTGGGTCTG-3'. Lentiviral vectors of CSII-DLX5, CSII-EGR3, and CSII-ESR2 were constructed by cloning *DLX5*, *EGR3*, and *ESR2* from retroviral vectors into CSII-EF-Rfa-IRES2-Venus vector using Gateway cloning kits (Invitrogen). The following primers were used: DLX5 forward, 5'-CAC-CATGGACTACAAGGACGACGATGACAAGACAGGAGTGTGTTGACAGA-3' and reverse, 5'-CTAATAGAGTGTCCCGGAGGC-3'; EGR3 forward, 5'-CACCATGGACTACAAGGACGACGATGACAAGACCGCAAACCTC-GCCGAG-3' and reverse, 5'-TGAGGCGCAGGTGGTGACCAC-3'; ESR2 forward, 5'-CACCACCATGGACTACAAGGACGACGATGACAAGGATATA-AAAACTACCA-3' and reverse, 5'-CTACTGAGACTGTGGGTCTG-3'. The accuracy of cloned vectors was confirmed by DNA sequencing. The lentiviral shRNA for DLX5 (TRCN0000007448 and TRCN0000007449), EGR3 (TRCN0000013843 and TRCN0000013847), and ESR2 (TRCN0000003325 and TRCN0000003328) was purchased from Openbiosystems.

Clonogenicity and Alamar blue assays were as reported (67). For spheroid assays, 8-well chamber slides were coated with Matrigel (BD Biosciences; 50  $\mu$ l per well) and incubated at 37°C for 20 minutes to allow Matrigel to polymerize. SCC cells were brought into suspension in normal culture medium plus 1% Matrigel (7000 cells/ml) and added in triplicate to the precoated chamber slides (300  $\mu$ l of cell mixture per well). Medium was refreshed every other day. For organotypic cultures, HKCs were infected with shRNA-expressing lentiviruses followed, 48 hours later, by selection for puromycin (2  $\mu$ g/ml) resistance. Selected keratinocyte cultures were reseeded onto collagen gels with embedded J2-3T3 fibroblasts and cultured at the air-liquid interface as previously described (68). After 12 days, epithelial sheets were peeled off the collagen lattice, snap-frozen, and processed for immunoblot analysis, or embedded in tissue-freezing medium for immunohistochemical analysis, or fixed in 10% neutral buffered formalin and embedded in paraffin for standard histology.

**siRNA screen.** A customized library (from Ambion) with 3 individual siRNAs for each selected gene was reversely transfected into HKCs in 384-well plates (30 nM of each siRNA, tested in triplicate wells, in 0.8% HiPerFect (QIAGEN). HiPerFect was diluted in serum free medium (SFM) (Invitrogen) for 5 minutes before mixing with siRNAs diluted in the same medium. The mixture was incubated for 20 minutes at room temperature (RT) and then added to 384-well plates. Plates were spun down at 188 g for 5 minutes, and 5000 HKCs in medium without antibiotics were added to each well robotically. The plates were spun down at 188 g for 5 minutes again and then placed in a tissue culture incubator at 37°C with 5% CO<sub>2</sub>. Twenty-four hours after transfection, the transfection medium was removed and fresh HKC culture medium was added. At this time point, cells in each well were about 90%–100% confluent. Medium was changed every other day. One week after transfection, cells were directly lysed in the wells, followed by RNA and cDNA preparation with a Fast-Lane cell cDNA kit (QIAGEN) following the manufacturer's instructions. siRNAs for DLX5, EGR3, and ER $\beta$  used for ChIP assays were purchased from Ambion/Invitrogen with the following specific sequences. EGR3: 5'-AGAUCACCUCAAGCAAAAAtt-3'; DLX5: 5'-CAGAGAAGGUUUCAGAAGAtt; and ER $\beta$  5'-CCUUACCUGUAAACAGAGAtt-3'.

**RT-qPCR, immunodetection, and ChIP assays.** Conditions for RT-qPCR, immunoblotting, immunofluorescence, and ChIP assays were as previously reported (11, 41). List of gene-specific primers and antibodies is provided in Supplemental Tables 7 and 8, respectively. For immunoblotting, unless otherwise indicated, cells were lysed in SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.2 unit nuclease), and protein separation was done in 15-well 4% to 12% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen). Equal loading controls were done by reprobing the same immunoblots unless otherwise indicated. All assays were done at least twice, with 2 HKC strains or epidermal samples of independent origin.

**Intradermal tumorigenicity assays.** Lentivirally infected SCC13 and SCCO13 cells and retrovirally infected H2170 cells were suspended in Matrigel followed by intradermal injection (1.0  $\times$  10<sup>6</sup> cells in 150  $\mu$ l per injection) into the back skin of 6-week-old NOD/SCID mice (Taconic Farms Inc.) as described (11, 41). To minimize the individual animal variations, cells infected with control and ER $\beta$ -expressing viruses were injected in parallel in the right and left flanks of the same mice. Mice were sacrificed for tissue analysis 3 weeks (SCC13 and SCCO13) or 1 week (H2170) after injection. For ER $\beta$  agonist studies, SCCO13 cells were injected intradermally into NOD/SCID mice as described above. Forty-eight hours later, mice were randomly divided into 2 groups, one receiving DPN (20 mg/kg; DMSO stock solution diluted 1:33 in culture medium) and the other DMSO alone (also diluted 1:33 in culture medium), by daily intra-peritoneal injections for 10 days. Tumor volume was calculated using the following formula: volume = (width)<sup>2</sup>  $\times$  length/2.





**Bioinformatic analysis.** MatInspector software (Genomatix), the Match tool from TRANSFAC, and the UCSC Genome Browser database were used for identification of transcription factors with putative binding sites within the predicted enhancer and promoter sequences of the *NOTCH1* locus as identified by the ENCODE project analysis of HKCs. Our own gene expression microarray database of normal human epidermis and primary skin SCC tumors (GEO GSE45164) and published gene expression array data from Biogps and Genecards were used to “filter” for keratinocyte-expressed transcription factors. For comparative transcriptomic analysis of selected genes in skin and oral SCCs versus normal epidermis and oral mucosa, 2 Affymetrix data sets (our own, GEO GSE45164, for skin SCCs; and ArrayExpress E-GEOD-9844 for oral SCCs) and 1 single-color Agilent oral SCC data set (ArrayExpress E-GEOD-23558) were used. Affymetrix data were processed with the RMA algorithm, while Agilent data were background subtracted (normexp method) and normalized with the quantile functions. Additional skin, oral, and lung SCCs versus normal epidermis, oral mucosa, and lung epithelium data sets were retrieved from the Oncomine RNA array database (<http://www.oncomine.com>). Values of multiple probes targeting the same gene were pooled and averaged, and  $\log_2$  ratios of the individual tumors versus mean values of control tissues were calculated separately for each data set. For determination of the ER-dependent gene expression program, HKCs were transfected with 2 ER $\beta$ -specific siRNAs or scrambled siRNA control followed, 96 hours later, by total RNA preparation and cDNA microarray hybridization (Affymetrix U133A.2.0) and bioinformatics analysis as previously described (41). For comparison of the ER-dependent gene expression program in skin, H/N, esophageal, and lung SCCs, corresponding data sets (Bhattacharjee Lung, Garber Lung, Hou Lung, Talbot Lung, Wachi Lung, Cromer Head-Neck, Ginos Head-Neck, Hu Esophagus, Su Esophagus, Nindl Skin, Riker Melanoma) were retrieved from the Oncomine RNA array database together with our own (GSE45164). For mutation spectrum analysis of H/N and lung SCCs from male versus female patients, data were obtained from the Cancer Genome Atlas and from previously published data sets (5), respectively. For gene expression profiles of H/N SCCs from male versus female patients, data were obtained from the Cancer Genome Atlas. The LIMMA (Linear Models for Microarray Analysis) package was used to identify dif-

ferentially expressed genes in female versus male patients. Functional classification was performed using the functional annotation tool available at the DAVID website (<http://david.abcc.ncifcrf.gov/>).

See complete unedited blots in the supplemental material.

**Statistics.** To assess statistical significance of the results, Prism software 6.0 (GraphPad Software Inc.) unpaired Student's *t* test was used. All real-time RT-PCR samples were tested in triplicate, and error bars represent SD. *P* values of less than 0.05 were considered significant.

**Study approval.** The animal study (protocol #: 2004N000170) was approved by the Subcommittee on Research Animal Care (SRAC), which serves as the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital. The animal study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (NIH publication, 8th edition, Revised 2011).

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