

# Erk1/2 MAP kinases are required for epidermal G2/M progression

Phillip A. Dumesic,<sup>1,2,3</sup> Florence A. Scholl,<sup>1,2,3</sup> Deborah I. Barragan,<sup>1,2,3</sup> and Paul A. Khavari<sup>1,2,3</sup>

<sup>1</sup>Veterans Affairs Palo Alto Health Care System, Palo Alto, CA 94304

<sup>2</sup>Program in Epithelial Biology and <sup>3</sup>Cancer Biology Program, Stanford University School of Medicine, Stanford, CA 94305

**E**rk1/2 mitogen-activated protein kinases (MAPKs) are often hyperactivated in human cancers, where they affect multiple processes, including proliferation. However, the effects of Erk1/2 loss in normal epithelial tissue, the setting of most extracellular signal-regulated kinase (Erk)-associated neoplasms, are unknown. In epidermis, loss of Erk1 or Erk2 individually has no effect, whereas simultaneous Erk1/2 depletion inhibits cell division, demonstrating that these MAPKs are necessary for normal tissue self-renewal. Growth inhibition caused by

Erk1/2 loss is rescued by reintroducing Erk2, but not by activating Erk effectors that promote G1 cell cycle progression. Unlike fibroblasts, in which Erk1/2 loss decreases cyclin D1 expression and induces G1/S arrest, Erk1/2 loss in epithelial cells reduces cyclin B1 and c-Fos expression and induces G2/M arrest while disrupting a gene regulatory network centered on cyclin B1-Cdc2. Thus, the cell cycle stages at which Erk1/2 activity is required vary by cell type, with Erk1/2 functioning in epithelial cells to enable progression through G2/M.

## Introduction

The Erk1/2, JNK, p38, Erk3/4, Erk5, Erk6, and Erk7/8 cascades are among the major MAPK pathways in mammals. The Erk1/2 MAPK pathway is a kinase cascade that includes Raf MAPK kinase kinases (Raf1, B-Raf, and A-Raf), MAPK/extracellular signal-regulated kinase (Erk; Mek) MAPK kinases (Mek1 and Mek2), and Erk MAPKs (Erk1 and Erk2 [Erk1/2]). Together, these proteins translate extracellular cues into changes in gene expression and protein activity (Chambard et al., 2007). In this pathway, Ras GTPases are activated by receptor engagement, causing recruitment of Raf to the cell membrane for phosphorylation (Shaul and Seger, 2007). Activated Raf then phosphorylates Mek1/2, which in turn activates Erk1/2, leading to the phosphorylation of >160 known nuclear and cytosolic Erk1/2 substrates (Yoon and Seger, 2006). Erk1/2 exert pleiotropic effects, which are perturbed in many disease states, including human cancers, of which 30% show Erk hyperactivation (Hoshino et al., 1999).

Analysis of the Erk1/2 MAPK cascade is complicated by the existence of various isoforms possessing both overlapping and unique functions. However, recently, the effect of genetic abrogation of an entire level of the MAPK cascade was determined

by the generation of skin-specific *MEK1/2* double knockout mice (Scholl et al., 2007). Whereas mice lacking either *MEK1* or *MEK2* alone were normal, double knockout mice died shortly after birth from dehydration secondary to epidermal hypoplasia and hypoproliferation. Inducible knockout of *MEK1/2* in adult mouse skin similarly led to epidermal hypoplasia, hypoproliferation, and apoptosis, establishing the Erk1/2 MAPK cascade as a prerequisite for tissue development and homeostasis and highlighting the importance of identifying the downstream effectors important for this activity.

The only known substrates of Mek1/2 are the serine-threonine kinases Erk1 and Erk2, also known as p44 MAPK and p42 MAPK, respectively (Kohno and Pouyssegur, 2006). Erk1 and Erk2 share 83% amino acid sequence identity and have been traditionally considered functionally redundant (Pouyssegur and Lenormand, 2003). However, recent evidence has revealed multiple scaffolding proteins such as MP1 that sequester the individual Erk isoforms preferentially, suggesting unique roles for the isoforms (Brahma and Dalby, 2007). Indeed, *ERK1* knockout mice are viable, whereas *ERK2* knockout mice die during embryogenesis and display defects in trophoblast formation and

Correspondence to Paul A. Khavari: khavari@stanford.edu

Abbreviations used in this paper: CA, constitutively active; Erk, extracellular signal-regulated kinase; GO, gene ontology; PI, propidium iodide; Mek, MAPK/Erk kinase; Rsk, ribosomal S6 kinase; WT, wild type.

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mesoderm differentiation (Hatano et al., 2003; Saba-El-Leil et al., 2003; Yao et al., 2003). Further studies in ovarian cells, fibroblasts, hepatocytes, and thymocytes have identified differential roles for Erk1 and Erk2 in the control of cell survival and proliferation, emphasizing the importance of cell type in the analysis of Erk function (Fischer et al., 2005; Zeng et al., 2005; Vantaggiato et al., 2006; Fremin et al., 2007).

Among the biological processes influenced by Erk1/2 is cell cycle progression, a mechanism best studied in nonepithelial cell lines. For example, in mesenchymal fibroblasts, Erk1/2 were first identified as a result of their activation by mitogens, with Erk activity throughout G1 phase being required for G1/S-phase cell cycle progression (Meloche and Pouyssegur, 2007). In this context, Erk1/2 influence G1/S transition in myriad ways, including up-regulation and stabilization of cyclin D1, up-regulation of p21 expression with attendant stabilization of cyclin D–Cdk4 complexes, and down-regulation of antiproliferative genes, including Sox6 and Jun1 (Lavoie et al., 1996; Cheng et al., 1999; Yamamoto et al., 2006). Roles for Erk signaling in later stages of the cell cycle such as G2/M progression are controversial, with discrepancies regarding Erk1/2 activity during this stage and the observation that pharmacologic Mek inhibition causes off-target effects that perturb G2/M progression (Roberts et al., 2002; Liu et al., 2004; Shinohara et al., 2006; Kim et al., 2008). Moreover, although most Erk1/2 cell cycle effects have been studied in fibroblast cell lines, Erk1/2 function in epithelial tissues, which are a major site of Erk activation in neoplasia, is less well understood.

In the self-renewing epithelia of the epidermis, Erk1/2 MAPK signaling appears to promote proliferation of basal layer stem cells and to suppress normal differentiation of suprabasal layer cells. Activation of Harvey Ras, Raf1, or Mek1 in mouse and human skin induces epidermal hyperplasia marked by hyperproliferation and reduced differentiation (Tarutani et al., 2003; Scholl et al., 2004). Moreover, most spontaneous epidermal squamous cell carcinomas, which are marked by characteristics of basal layer cells, including integrin expression and proclivity toward proliferation over differentiation, exhibit Ras induction and MAPK cascade hyperactivity (Dajee et al., 2003). Mouse experiments showing that expression of dominant-negative Ras in basal layer skin causes hypoplasia and lethality, whereas Ras blockade in suprabasal keratinocytes has no effect, further support a specific role for Erk1/2 MAPK signaling in the mitotically active basal compartment (Dajee et al., 2002). However, the downstream mechanisms by which the Erk1/2 MAPK cascade sustains proliferation in epithelial cells remain unclear.

In this study, we investigate the roles of Erk1 and Erk2 in human epidermis. We report that depletion of Erk1 or Erk2 alone does not disrupt epidermal proliferation or differentiation, whereas simultaneous depletion of Erk1 and Erk2 induces hypoplasia and hypoproliferation without disrupting differentiation. Effects of Erk1/2 loss are not rescued by activation of the Erk1/2 effectors Rsk1 or cyclin D1, suggesting action of alternate Erk1/2 targets. Indeed, Erk1/2-depleted epidermal cells exhibit a G2/M cell cycle arrest with decreased expression of the transcription factor c-Fos and its target gene cyclin B1. These effects are not observed in fibroblasts, which display G1/S arrest with decreased cyclin D1 expression upon Erk1/2 depletion. Our findings establish differential roles

for Erk1/2 in distinct cell types and identify these MAPKs as required regulators of G2/M progression in the epidermis.

## Results

### Erk1/2 are required for epidermal proliferation

To investigate the roles of Erk1 and Erk2 in epidermal homeostasis, we used RNAi to deplete Erk1, Erk2, or both in human epidermis *in vivo*. Primary human keratinocytes were electroporated with siRNA targeting *ERK1* or *ERK2*, which depleted expression of these proteins either individually or together (Fig. 1 A). To generate skin lacking Erk1/2, siRNA-treated keratinocytes were seeded onto devitalized human dermis and then grafted onto immunodeficient mice (Scholl et al., 2007). By 6 d after grafting, keratinocytes treated with scrambled control siRNA had generated full thickness epidermis marked by stratification and differentiation analogous to that of normal human skin. Immunofluorescence staining with a species-specific antibody against human desmoglein 3 confirmed that the analyzed tissue was of human origin, and antibodies against keratin 10 and transglutaminase 1 revealed proper suprabasal localization of these differentiation markers. Furthermore,  $\beta_1$  integrin, a marker normally expressed by keratinocytes contiguous to the basement membrane, and Ki-67, a marker of cell proliferation, were confined to the basal layer, as in normal human skin. Keratin 5, a marker of basal layer keratinocytes, was expressed throughout the epidermis, as previously observed in regenerated tissue at this time point (Fig. 1 C; Scholl et al., 2007).

Epidermis depleted of either Erk1 or Erk2 individually was indistinguishable from control epidermis, whereas epidermis lacking both Erk1 and Erk2 was markedly hypoplastic, with 68% fewer basal layer cells than control skin ( $P < 0.01$ ). Immunohistochemical staining confirmed that Erk1/2 protein was depleted in Erk1/2 double knockdown epidermis (Fig. S1 A). Cells in Erk1/2-depleted epidermis were not only fewer in number than in control skin but also less likely to be undergoing mitosis, with a mitotic index significantly lower than that of control skin (17 vs. 53%; Fig. 1 B). Although thin, Erk1/2 double knockdown epidermis maintained normal suprabasal expression of differentiation markers, including transglutaminase 1 and keratin 10, along with morphologically normal stratum corneum (Fig. 1 C). In addition, apoptosis was not detected in Erk1/2 double knockdown epidermis nor was it observed in skin treated with any other siRNA, as assessed by TUNEL assay (Fig. S1 B). Thus, whereas epidermal proliferation proceeds normally in the absence of either Erk1 or Erk2, depletion of both gene products together leads to hypoplasia and hypoproliferation without affecting differentiation or cell viability.

To confirm that the impacts of Erk1/2 loss were specific to the loss of Erk1 and Erk2 rather than an off-target effect, we tested whether a constitutively active (CA) Erk2 (Erk2-CA) construct could rescue the phenotype of combined Erk1/2 depletion. Keratinocytes were infected with retroviral vectors encoding Erk2-CA or, as a control, LacZ; 2 d later, the cells were electroporated with scrambled siRNA or Erk1/2 siRNA and used to generate human skin grafts (Scholl et al., 2007). The Erk2-CA construct contains two mismatches within the Erk2 siRNA-binding site and therefore was not efficiently silenced by Erk1/2 siRNA (Fig. 2 A).

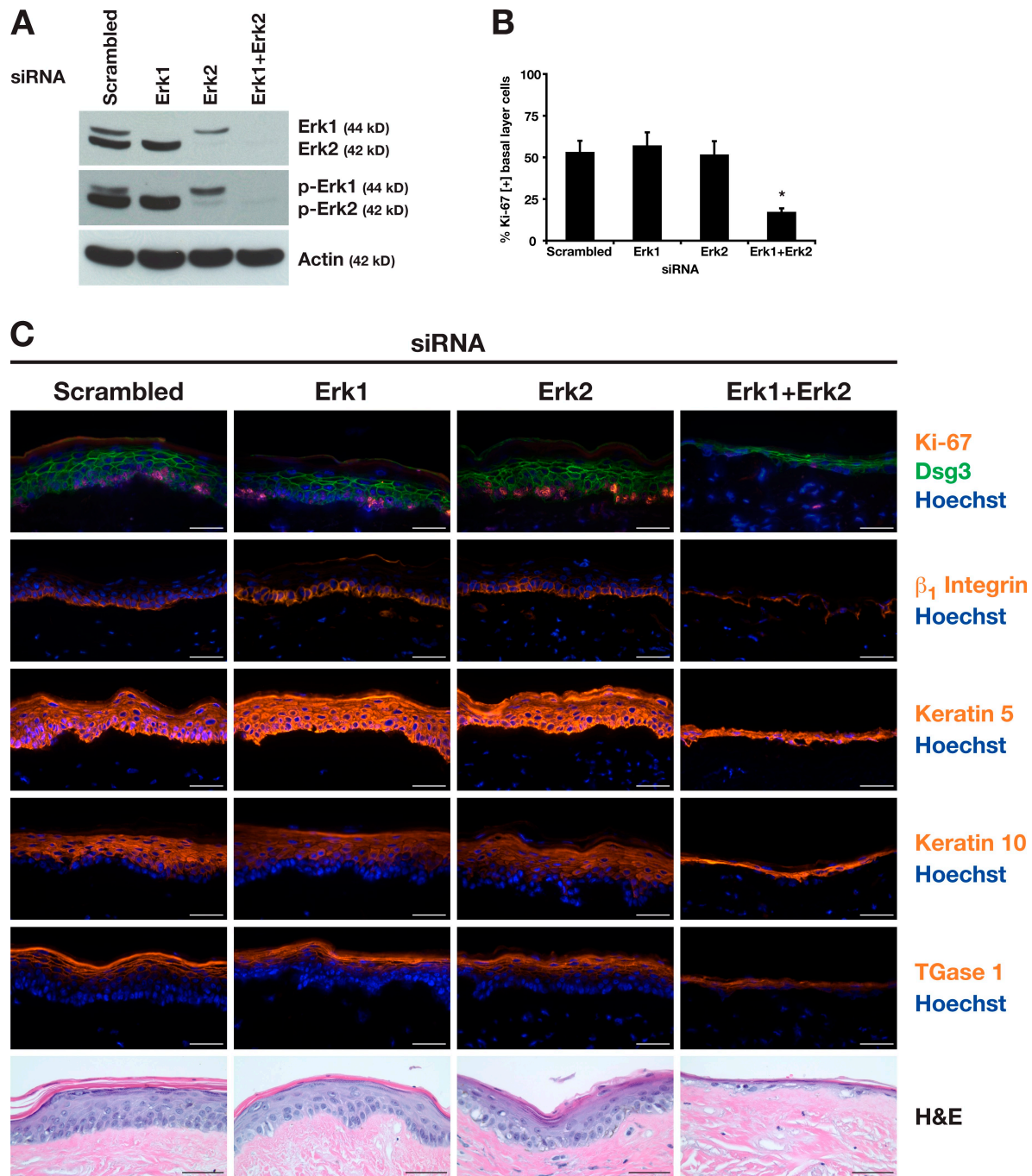


Figure 1. **Combined Erk1/2 depletion inhibits proliferation in human epidermal tissue.** (A) Immunoblot of keratinocyte extracts 4 d after electroporation with the indicated siRNA stained with the antibodies indicated on the right. (B) Mitotic indices of skin grafts comprising keratinocytes treated with the indicated siRNA oligomers.  $n = 3-5$  grafts per genotype; mean  $\pm$  SEM; \*,  $P < 0.01$  versus control. (C) Human skin tissue engineered with the indicated siRNAs, harvested 6 d after grafting, and stained with species-specific antibody against human desmoglein 3 (Dsg3; green), Ki-67,  $\beta_1$  integrin, keratin 5, keratin 10, and transglutaminase 1 (TGase1; orange) and the nuclear counterstain Hoechst 33342 (blue). Bars, 50  $\mu$ m.

Whereas LacZ expression did not affect Erk1/2 knockdown or its associated tissue phenotype, expression of Erk2-CA fully reversed the hypoplasia and hypoproliferation characteristic of Erk1/2 depletion (Fig. 2, B and C). In a separate experiment, overexpression of wild-type (WT) rat Erk2 (Erk2-WT) was also able to fully rescue the effects of Erk1/2 knockdown, confirming that this phenotype is specifically caused by the loss of MAPK cascade signaling components (Fig. S2, A-C).

#### Erk1/2 loss is not rescued by activation of Rsk1 or G1/S cell cycle regulators

Erk MAPK signaling is highly pleiotropic, with >160 Erk1/2 substrates identified (Yoon and Seger, 2006). One major subset of effectors is the ribosomal S6 kinase (Rsk) family of kinases, which regulate cell growth (via phosphorylation of polyribosomal proteins) as well as progression through multiple stages of the cell cycle (via phosphorylation of Myt1 and AP-1 transcription



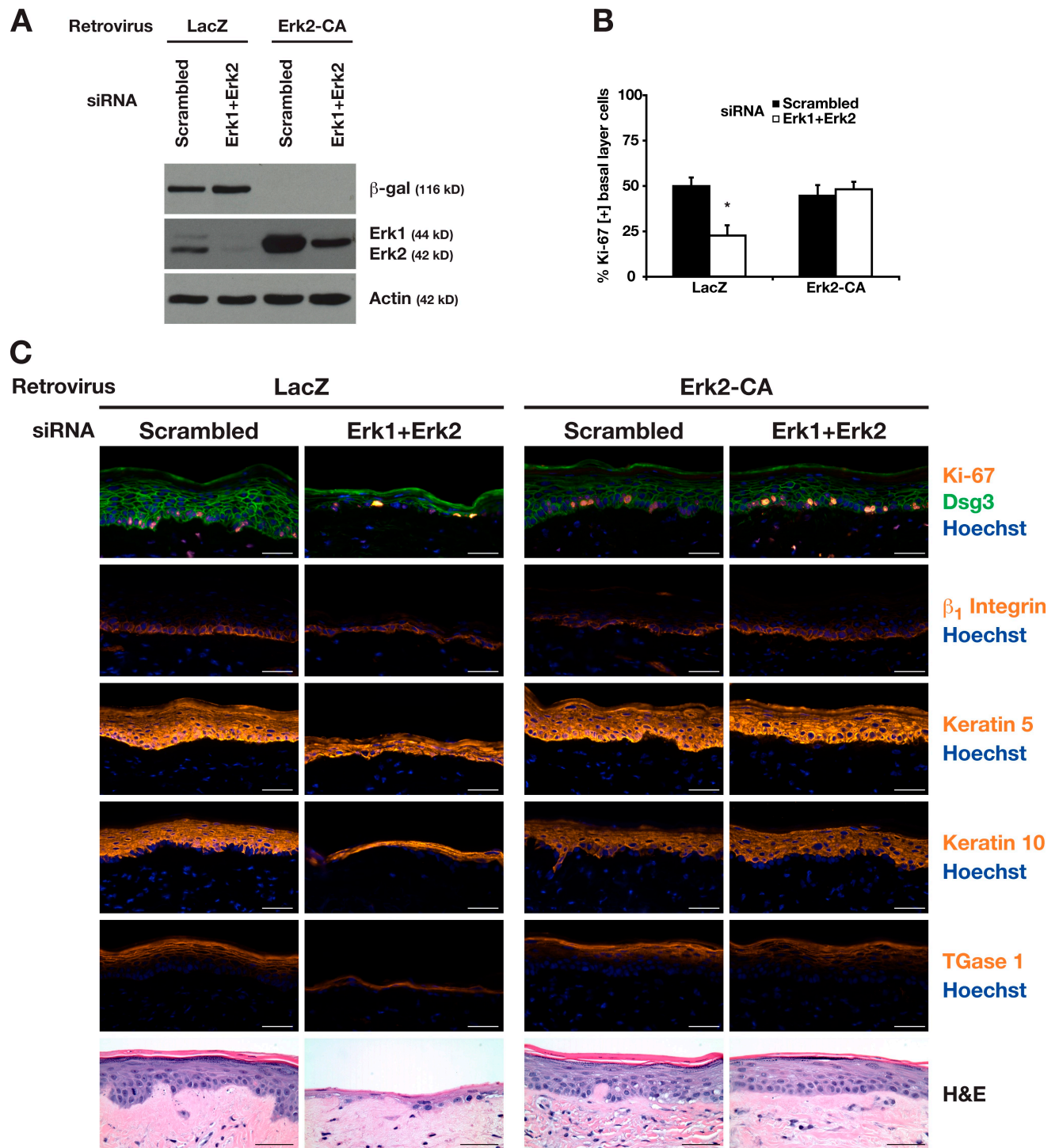
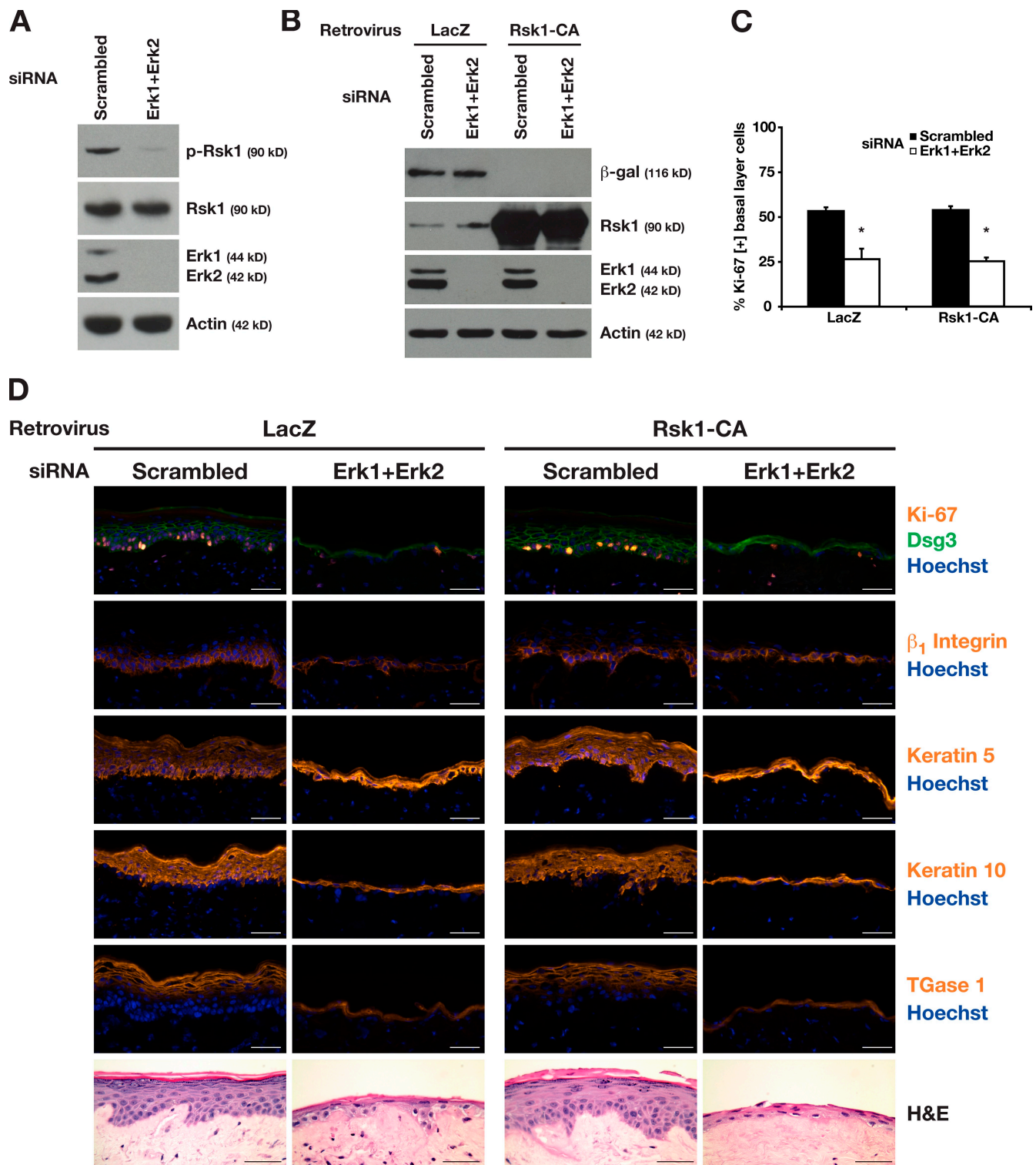


Figure 2. **Active Erk2 rescues the effects of Erk1/2 depletion in human skin.** (A) Immunoblot of keratinocyte extracts after transduction by the indicated retroviral vectors and electroporation with the indicated siRNAs stained with the antibodies listed on the right. (B) Mitotic indices of skin grafts comprising keratinocytes treated with the indicated siRNA and retroviral vectors.  $n = 3$  grafts per genotype; mean  $\pm$  SEM; \*,  $P < 0.01$  versus control. (C) Human skin tissue comprising keratinocytes treated with the indicated siRNA and retroviral vectors, harvested 6 d after grafting, and stained with the antibodies listed on the right (orange or green) as well as the nuclear counterstain Hoechst 33342 (blue). Bars, 50  $\mu$ m.

factors; Frodin and Gammeltoft, 1999). Immunoblotting showed that Erk1/2 depletion led to reduced Rsk1 activation in keratinocytes, confirming that Rsk is a substrate of Erk1/2 in human epidermis (Fig. 3 A). To determine whether Rsk activation could rescue the epidermal effects of combined Erk1/2

loss, we expressed CA Rsk1 (Rsk1-CA) in the context of combined Erk1/2 knockdown (Fig. 3 B). Keratinocytes were infected with retroviral vectors encoding LacZ or Rsk1-CA, after which they were treated with either a scrambled control siRNA or siRNA against *ERK1/2* and used to generate skin grafts.





**Figure 3. Active Rsk1 does not rescue the effects of epidermal Erk1/2 depletion.** (A) Immunoblot of keratinocyte extracts 4 d after electroporation with the indicated siRNA stained with the antibodies listed on the right. (B) Immunoblot of keratinocyte extracts after transduction by the indicated retroviral vectors and electroporation with the indicated siRNAs stained with the antibodies listed on the right. (C) Mitotic indices of skin grafts comprising keratinocytes treated with the indicated siRNA and retroviral vectors.  $n = 3-4$  grafts per genotype; mean  $\pm$  SEM; \*,  $P < 0.01$  versus control. (D) Human skin tissue comprising keratinocytes treated with the indicated siRNA and retroviral vectors, harvested 6 d after grafting, and stained with the antibodies listed on the right (orange or green) as well as the nuclear counterstain Hoechst 33342 (blue). Bars, 50  $\mu$ m.

Erk1/2-depleted epidermis expressing Rsk1-CA showed a phenotype indistinguishable from that induced by Erk1/2 depletion in LacZ-expressing keratinocytes: namely, hypoproliferation,

hypoplasia, and normal differentiation (Fig. 3, C and D). Thus, Rsk1, a single effector arm of MAPK signaling, is insufficient to rescue the epidermal impacts of Erk1/2 loss.

We next investigated whether the proliferation defect of Erk1/2-depleted keratinocytes could be rescued by promoting cell cycle progression via core cell cycle machinery. In fibroblasts, Erk1/2 MAPK signaling promotes G1/S progression by up-regulating cyclin D1 expression: in cultured fibroblasts, cyclin D1 overexpression rescues G1 arrest induced by pharmacologic MAPK inhibition (Villanueva et al., 2007). To determine whether cyclin D1 overexpression could rescue epidermal hypoplasia caused by Erk1/2 depletion, keratinocytes transduced with retroviral vectors encoding either LacZ or cyclin D1 were treated with scrambled siRNA or siRNA against *ERK1/2* and used to generate skin grafts (Fig. 4 A). Whereas cyclin D1-expressing cells treated with scrambled siRNA formed normal skin by 6 d after grafting, cyclin D1-expressing cells treated with siRNA against *ERK1/2* formed hypoplastic skin that was indistinguishable from skin comprising LacZ-expressing, Erk1/2-depleted keratinocytes (Fig. 4, B and C).

Erk1/2 contribute to G1/S progression not only by increasing transcription of G1/S regulators but also by posttranslational regulation, including promotion of cyclin D1-Cdk4 complex formation (Cheng et al., 1998). The loss of these latter Erk1/2 functions may have rendered cyclin D1 overexpression unable to rescue the effects of Erk1/2 knockdown in keratinocytes. Therefore, to rule out G1/S arrest as the primary cause of hypoproliferation in Erk1/2-depleted skin, we attempted to rescue the effect of Erk1/2 knockdown by coexpression of cyclin D1 and Cdk4-C24, a CA point mutant of Cdk4. This combination of genes provides a robust method for bypassing G1/S restraints in human keratinocytes, including G1 blockade induced by oncogenic Ras (Lazarov et al., 2002). Nevertheless, Erk1/2 depletion in skin overexpressing cyclin D1 and Cdk4-C24 led to hypoplasia indistinguishable from that induced by Erk1/2 depletion in control skin expressing LacZ, suggesting that the principal effects of Erk1/2 depletion in human skin are on a cell cycle stage other than G1/S (Fig. S2, D and E).

#### **Erk1/2 loss induces cell cycle arrest in G2/M in epithelial cells and G1/S in fibroblasts**

Failure of cyclin D1 and Cdk4-C24 overexpression to rescue the cell cycle defect of Erk1/2-depleted keratinocytes suggested that this defect involves cell cycle stages other than the G1/S transition. Therefore, we investigated the proliferation and cell cycle profile of Erk1/2-depleted keratinocytes in vitro. During the 7 d after siRNA electroporation, knockdown of Erk1 had no effect on keratinocyte proliferation, whereas knockdown of Erk2 slightly reduced cell division as compared with keratinocytes treated with scrambled siRNA. However, simultaneous depletion of Erk1/2 caused a 77% decrease in keratinocyte number, which is analogous to its in vivo effects (Fig. 5 A). A second pair of siRNA oligomers targeting different regions of *ERK1* and *ERK2* (Erk1 + Erk2 [b]) similarly reduced keratinocyte proliferation in vitro (Fig. 5, B and C). Consistent with these findings, propidium iodide (PI) staining revealed that Erk1/2 depletion caused a significant decrease in the proportion of keratinocytes in S phase as compared with control (25.9 vs. 39.9%), whereas Erk2 depletion caused a minor reduction in S-phase cells (33.6%), and Erk1 depletion had no effect (39.9%; Fig. 5 D).

Surprisingly, keratinocytes lacking both Erk1 and Erk2 were markedly enriched in cells with a 4-N DNA content (33.7 vs. 19.9%), indicating that Erk1/2 loss leads to arrest in the G2/M phase of the cell cycle.

Because these results differed from the previously published G1 arrest observed with Erk1/2 blockade in mesenchymal cells (Meloche and Pouyssegur, 2007), we investigated the effect of Erk1/2 depletion on the cell cycle of primary human skin fibroblasts. Erk1/2 siRNA robustly reduced Erk1/2 protein expression (Fig. 5 E) and reduced cell proliferation as compared with fibroblasts treated with control siRNA (not depicted). Combined Erk1/2 depletion decreased the proportion of cells in S phase and increased the proportion of cells in G1 phase, as compared with control cells, indicating G1-phase arrest (Fig. 5 F). Consistent with a previous study (Villanueva et al., 2007), this G1 arrest was associated with decreased cyclin D1 expression (Fig. 5 G) and could be rescued by overexpressing cyclin D1 and Cdk4-C24 (Fig. S3 A). In contrast, Erk1/2 knockdown in keratinocytes was not associated with decreased cyclin D1 expression (Fig. 4 A), and overexpression of cyclin D1 and Cdk4-C24 had no effect on the cell cycle blockade induced by Erk1/2 depletion (Fig. S3 B). Importantly, both cell types express similar levels of cyclin D1 before Erk1/2 knockdown when compared directly (Fig. S3 C). Thus, Erk1/2 affect cell cycle progression in a cell type-specific manner, with Erk1/2 loss causing G1 and G2/M arrest in fibroblasts and keratinocytes, respectively.

#### **Erk1/2 loss in epithelial cells alters expression of a G2/M regulatory network**

To investigate changes in gene expression associated with Erk1/2 loss in epidermal cells, we undertook gene expression profiling on cells treated with either scrambled control siRNA or each of two unique pairs of siRNA oligomers targeting *ERK1* and *ERK2*. To determine the core set of genes regulated by Erk1/2 signaling and to eliminate gene changes that represent off-target siRNA effects, we isolated genes that met the following criteria: (a) expression level significantly altered (analysis of variance,  $P < 0.01$ ) by both pairs of Erk1/2 siRNAs, as compared with control cells, and (b) expression level altered in the same direction by both pairs of Erk1/2 siRNAs, as compared with control cells. These criteria isolated 1,205 genes, which were subsequently sorted by hierarchical clustering on expression level (Fig. 6 A). As expected, down-regulated genes included *ERK1* and *ERK2*, as well as genes that agreed with previously reported effects of pharmacologic MAPK inhibition (Fig. 6 A; Yamamoto et al., 2006; Gazel et al., 2008).

Gene ontology (GO) term analysis revealed that the top seven most enriched biological process GO terms in this set of 1,205 genes were all related to cell cycle progression (Fig. 6 B). Importantly, these terms included three mitosis-specific terms, which is consistent with the G2/M arrest observed in Erk1/2-depleted keratinocytes. No G1/S-specific GO terms were significantly enriched in this gene set, and examining expression of core G1/S regulators, including cyclin D1 and Cdk4, revealed no change with Erk1/2 knockdown, indicating that the primary effects of Erk1/2 knockdown in keratinocytes were specific to the G2/M cell cycle stages (Fig. S4 A).

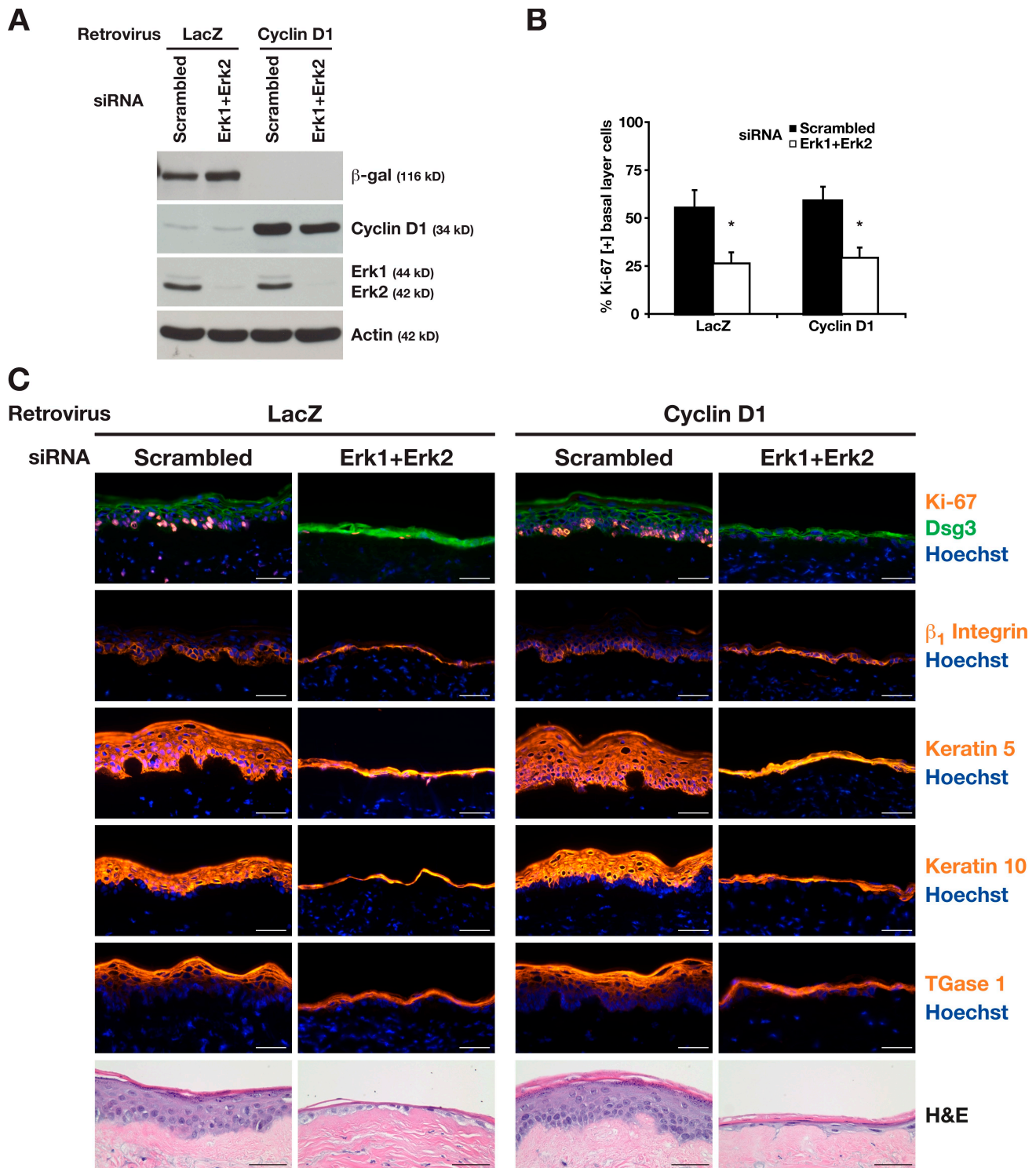
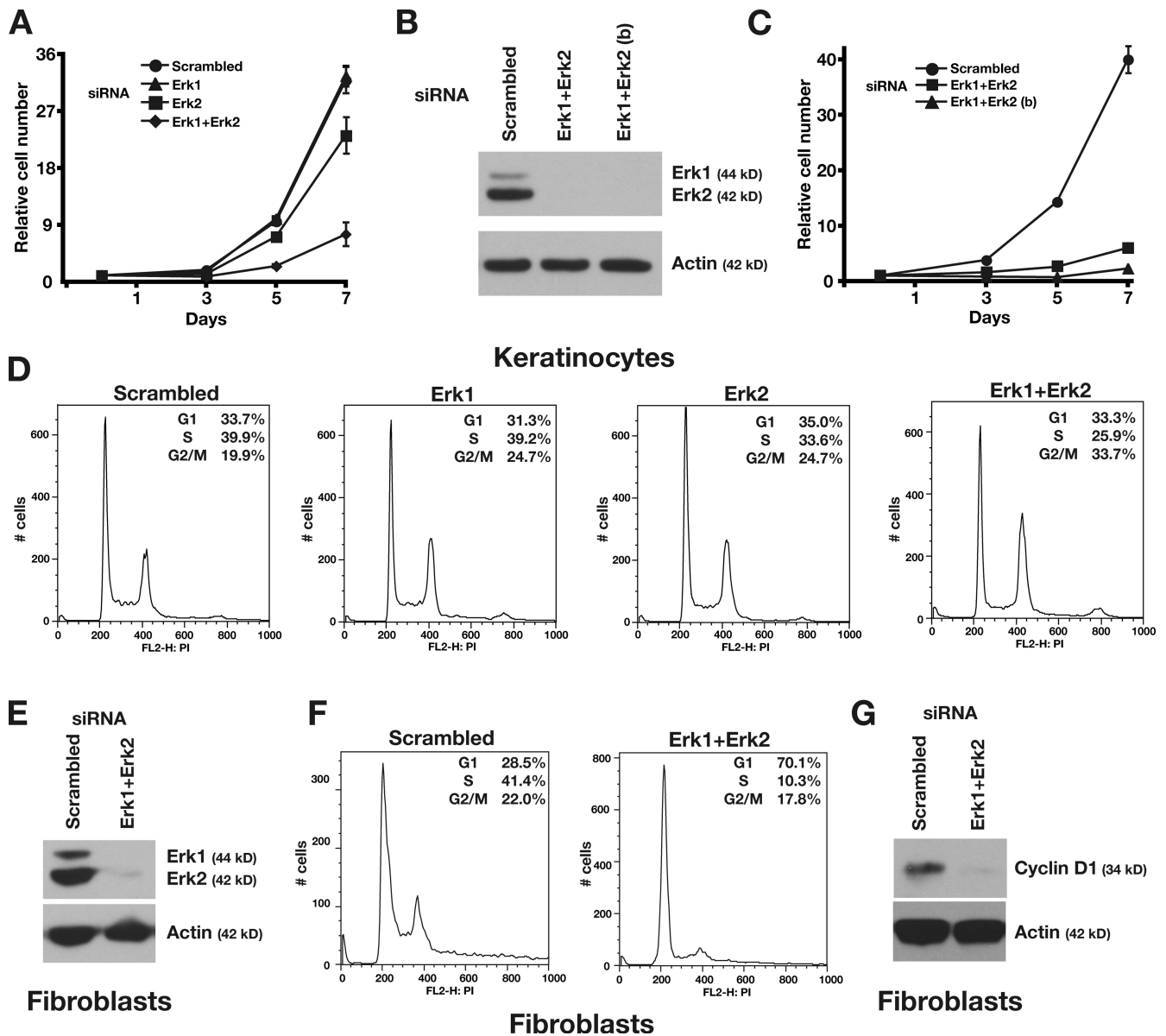


Figure 4. **Cyclin D1 does not rescue the effects of epidermal Erk1/2 depletion.** (A) Immunoblot of keratinocyte extracts after transduction by the indicated retroviral vectors and electroporation with the indicated siRNA stained with the antibodies listed on the right. (B) Mitotic indices of skin grafts comprising keratinocytes treated with the indicated siRNA and retroviral vectors, harvested 6 d after grafting, and stained with the antibodies listed on the right (orange or green) as well as the nuclear counterstain Hoechst 33342 (blue). Bars, 50  $\mu$ m.

To validate the functional importance of a G2/M regulator identified as misexpressed upon Erk1/2 knockdown, we further examined Cdc25C, a phosphatase that promotes G2/M progression by activating the G2/M cyclin-dependent kinase Cdc2.

Quantitative RT-PCR confirmed that Cdc25C is expressed at 38% of its WT level when Erk1/2 are depleted (Fig. S4 A). To determine whether the decreased expression of Cdc25C could have led to the G2/M cell cycle arrest observed in Erk1/2-depleted cells,





**Figure 5. Erk1/2 depletion leads to G2/M arrest in epithelial cells but not in fibroblasts.** (A) Proliferation kinetics of keratinocytes after electroporation with the indicated siRNA.  $n = 3$  per siRNA group per time point; mean  $\pm$  SEM. (B) Immunoblot of cultured keratinocyte extracts 4 d after electroporation with the indicated siRNA, using antibodies listed on the right. (C) Proliferation kinetics of keratinocytes after electroporation with the indicated siRNA.  $n = 3$  per siRNA group per time point; mean  $\pm$  SEM. (D) Histograms of PI fluorescence in keratinocytes 4 d after electroporation with the indicated siRNA. (E) Immunoblot of fibroblast extracts 4 d after electroporation with the indicated siRNA, using antibodies listed on the right. (F) Histograms of PI fluorescence in fibroblasts 4 d after electroporation with indicated siRNA. (G) Immunoblot of fibroblast extracts 4 d after electroporation with the indicated siRNA, using the antibodies listed on the right.

we examined the effect of Cdc25C depletion. siRNA targeting *CDC25C* reduced *CDC25C* mRNA expression to 31% of its WT level and induced a G2/M cell cycle arrest in cultured keratinocytes (unpublished data). In vivo experiments revealed that Cdc25C knockdown causes epidermal hypoplasia similar to that observed upon Erk1/2 depletion, indicating that Cdc25C down-regulation at levels associated with Erk1/2 depletion are sufficient to disrupt epidermal cell cycle progression (Fig. S5, A and B).

We next assessed whether activation of Cdc25C is sufficient to rescue the effects of Erk1/2 depletion in human skin. Because Cdc25C is activated transcriptionally and posttranslationally by Erk1/2, we attempted to rescue the effects of Erk1/2

depletion by expressing a CA form of Cdc25C (Cdc25C-CA) in which the residues phosphorylated by Erk1/2 are replaced by aspartic acid (Wang et al., 2007). Regenerated human skin expressing Cdc25C-CA and treated with siRNA against *ERK1/2* showed hypoplasia and hypoproliferation identical to that of control skin treated with Erk1/2 siRNA, demonstrating that Cdc25C activity alone cannot rescue the effects of Erk1/2 knockdown on epidermal homeostasis (Fig. S5, C–E).

The inability of Cdc25C activation to rescue the effects of Erk1/2 loss suggested that Erk1/2 likely control G2/M progression via multiple different targets. Indeed, gene network analysis demonstrated that of the 1,205 genes differentially expressed

in Erk1/2-depleted keratinocytes, 31 encode direct regulators or effectors of the core G2/M cyclin-dependent kinase Cdc2 (Fig. 6 C). Strikingly, of the posttranslational regulators of Cdc2, the Cdc2 activators Cdc25A and CCNB1 are both down-regulated, whereas the Cdc2 inhibitors BTG2, p53, GADD45, Wee1, ErbB2, MCL1, CDKN3, and CDKN1B are all up-regulated, indicating that gene changes observed in Erk1/2-depleted keratinocytes may broadly contribute to G2/M arrest. Consistent with this, overexpression of cyclin B1 was insufficient to rescue the proliferation defect of keratinocytes lacking Erk1/2, confirming that Erk1/2 activity promotes G2/M progression via a broad network of independent mechanisms that cannot be recapitulated by activation of any single Erk1/2 effector (Fig. S3 F).

### **Erk1/2 regulate c-Fos to sustain cyclin B1 expression in keratinocytes but not in fibroblasts**

The finding that G2/M regulatory molecules such as cyclin B1 are repressed upon Erk1/2 depletion in keratinocytes was surprising given our observation that Erk1/2 knockdown in fibroblasts does not induce substantial G2/M arrest, even when the G1 arrest induced by Erk1/2 depletion is rescued by cyclin D1 and Cdk4-C24 overexpression (Fig. S3 A). Therefore, we investigated whether cyclin B1 expression is suppressed in fibroblasts upon Erk1/2 knockdown, as it is in keratinocytes. Immunoblotting revealed that, whereas cyclin B1 protein levels are dramatically reduced upon Erk1/2 knockdown in keratinocytes, Erk1/2 loss has no effect on cyclin B1 levels in either WT fibroblasts or rescued fibroblasts overexpressing cyclin D1 and Cdk4-C24 (Fig. 6 D). This finding, together with the observation that cyclin D1 is repressed upon Erk1/2 knockdown in fibroblasts but not in keratinocytes (Figs. 4 A and 5 G), confirms that Erk1/2 regulate cyclin expression in a cell type-specific manner.

To identify Erk1/2 effectors whose differential regulation may explain cell type-specific MAPK regulation of cyclin expression, we examined expression of four canonical Erk1/2 effectors after Erk1/2 depletion in WT keratinocytes, WT fibroblasts, or rescued fibroblasts. Whereas Erk1/2 loss had similar effects on c-Jun and Elk1 mRNA expression in all cell types, Erk1/2 loss decreased c-Myc expression solely in fibroblasts and decreased c-Fos expression solely in keratinocytes (Fig. 6, E and F; and Fig. S4 B). Erk1/2 loss decreased c-Fos expression similarly in WT keratinocytes and keratinocytes expressing cyclin D1 and Cdk4-C24, suggesting that expression of these genes did not alter c-Fos regulation in rescued fibroblasts (Fig. S3 D). The 75% decrease in c-Fos expression in Erk1/2 knockdown keratinocytes was associated with a significant decrease in AP-1 reporter activity ( $P < 0.05$ ; Fig. S3 E). In contrast, AP-1 reporter activity in WT and rescued fibroblasts was increased upon Erk1/2 knockdown. Overall, c-Fos expression correlated with AP-1 reporter activity: keratinocytes had the lowest level of c-Fos expression upon Erk1/2 knockdown, as well as the lowest level of AP-1 activity, whereas rescued fibroblasts had the highest level of c-Fos expression upon Erk1/2 knockdown, as well as the highest level of AP-1 activity.

The finding that Erk1/2 activity sustains c-Fos expression uniquely in keratinocytes was striking in light of previous work suggesting that c-Fos expression is correlated with cyclin B1

expression in human endometrial cancers and that Fos binds to and positively regulates the cyclin B promoter in *Drosophila melanogaster* (Bamberger et al., 2001; Hyun et al., 2006). To determine whether c-Fos regulates cyclin B1 expression in human cells, as well as the functional importance of this regulation, we measured cyclin B1 expression and cell cycle progression in keratinocytes and fibroblasts after depletion of c-Fos using siRNA. In both cell types, c-Fos loss decreased cyclin B1 expression and led to G2/M arrest (Fig. 6, G–I). Importantly, the magnitude of G2/M arrest induced by c-Fos knockdown in keratinocytes was similar to that induced by Erk1/2 knockdown, confirming that c-Fos is a major effector of Erk1/2 signaling in this context. Thus, the AP-1 family member c-Fos is required for normal cyclin B1 expression in human cells, and differential regulation of c-Fos by Erk1/2 in keratinocytes versus fibroblasts may contribute to the difference in cyclin regulation and cell cycle progression between these cell types.

## **Discussion**

Using regenerated human skin, we investigated the roles of Erk1 and Erk2 in regulating epidermal growth and differentiation. Depletion of either protein individually caused no abnormal phenotype, whereas combined knockdown of Erk1/2 induced epidermal hypoplasia and hypoproliferation without disrupting differentiation. Erk1/2-depleted keratinocytes exhibited G2/M cell cycle arrest along with deregulation of core G2/M transition controllers, including cyclin B1. These findings contrast with the G1 arrest and cyclin D1 dysregulation observed in Erk1/2-depleted fibroblasts, indicating that control of cell cycle progression by Erk1/2 MAPKs is cell type specific.

### **Functional redundancy of Erk1 and Erk2**

Our findings suggest that the Erk1/2 isoforms possess largely overlapping roles in human skin. Erk1 is approximately twice as prevalent as Erk2 in normal human skin, but phospho-Erk2 is at least as prevalent as phospho-Erk1 in many epithelial tumors, suggesting that Erk2 is preferentially activated in this hyperproliferative state and may be a more potent mitogen (Pelech, 2006). Moreover, *ERK1* knockout in fibroblasts increases Erk2 activation and cell proliferation, and overexpression of WT Erk1 suppresses Erk2 activation and reduces Ras-induced cell proliferation (Vantaggiato et al., 2006). Together, these findings suggest that Erk1 and Erk2 compete for activation by Mek, with Erk2 being more active than Erk1, corresponding with our observation that Erk2 knockdown induces a slight proliferation defect (Vantaggiato et al., 2006). However, importantly, Erk1 knockdown did not increase proliferation of cultured keratinocytes, nor did knockdown of either protein affect the mitotic index of human epidermis in vivo. Assessing the in vivo effects of Erk1 or Erk2 knockdown in the setting of constitutive MAPK cascade activation and quantifying Erk substrate activation in the absence of either Erk1 or Erk2 may be required to detect subtle functional differences between these isoforms.

*ERK1* knockout mice exhibit hyperplastic epidermis and focal cutaneous lesions associated with *Escherichia coli* infection, although the phenotype of this animal model is complicated

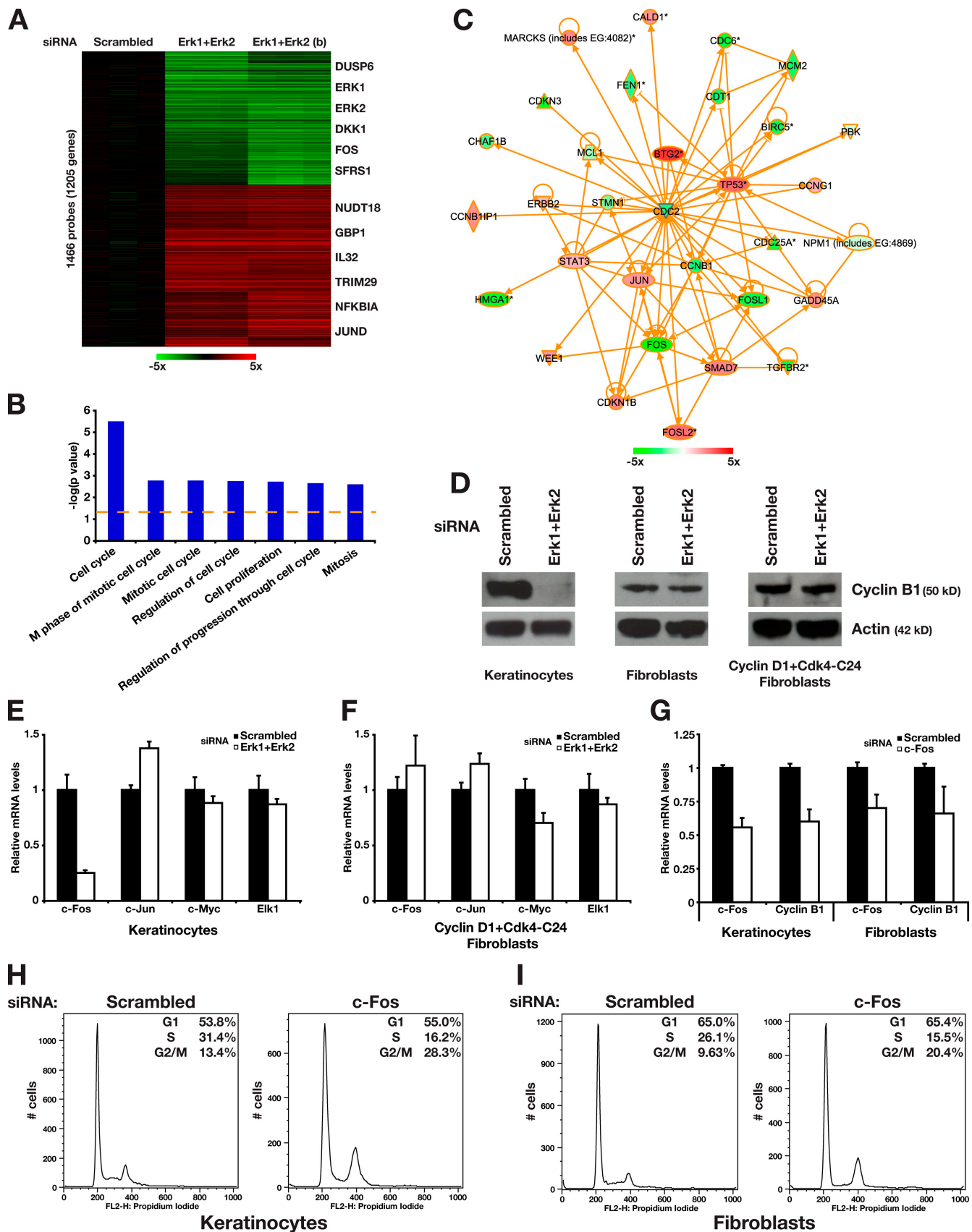


Figure 6. **Differential regulation of c-Fos by Erk1/2 in keratinocytes and fibroblasts contributes to the distinct cell cycle effects of Erk1/2 knockdown in these cell types.** (A) Hierarchical clustering of gene expression in keratinocytes treated with the indicated siRNA. Three replicates of each siRNA group are shown. Selected genes are labeled on the right. (B) Benjamini-corrected p-values associated with the top seven biological process GO terms most enriched in the 1,205 genes identified in A. The dotted line indicates  $P = 0.05$ . (C) Map of direct effectors and regulators of Cdc2 that appeared among the 1,205



by Erk1 depletion from all tissues and the resulting disruption in thymocyte development (Pages et al., 1999; Bourcier et al., 2006). In our experiments, Erk1-depleted skin grafted onto severe combined immunodeficiency mice and maintained under sterile conditions did not display abnormal epidermal homeostasis, suggesting that the epidermal hyperplasia of *ERK1* knockout mice might be secondary to altered immune function. Alternatively, our experiments assessing Erk1 knockdown over 1 wk may not have detected subtle proliferation changes. In support of this, cyclin D1 overexpression under our experimental conditions caused mild hyperplasia rather than the pronounced hyperplasia noted in human skin grafts maintained for 4 wk (Lazarov et al., 2002).

### **Erk1/2 requirement for epidermal cell cycle progression**

The phenotype of combined Erk1/2 knockdown in human skin mimics the phenotype of skin-specific *MEK1/2* double knockout mice, as well as the phenotype of combined *Mek1/2* knockdown in human skin (Scholl et al., 2007). The similarity of these phenotypes supports the current belief that Erk1/2 are the only effectors of *Mek1/2* and suggests that these phenotypes represent the effect of complete ablation of MAPK cascade signaling. Both models were marked by hypoplasia and hypoproliferation despite proper expression of basal layer and early differentiation markers. However, terminal differentiation markers such as loricrin and filaggrin appeared underexpressed in Erk1/2-depleted epidermis, and future studies will be required to assess the long-term effects of Erk1/2 loss on epidermal differentiation.

Although an Erk2 construct resistant to Erk2 siRNA reversed the effect of Erk1/2 depletion, neither a CA Rsk1 construct nor cyclin D1 overexpression was sufficient to rescue the Erk1/2 depletion phenotype. As a direct effector of Erk1/2, Rsk1 has pleiotropic roles in the regulation of cell cycle, protein synthesis, cell growth, and differentiation (Silverman et al., 2004). It promotes G1-phase progression via activation of immediate early gene products and drives G2/M-phase progression via inhibition of Myt1, a kinase that inhibits Cdc2 (Liu et al., 1999; Murphy et al., 2002). Because Rsk1 activation was unable to rescue the proliferation defect of Erk1/2-depleted keratinocytes, we also attempted to rescue this defect by altering the core cell cycle machinery itself via overexpression of cyclin D1 and Cdk4-C24. Although this gene combination induces hyperactivity of the core G1/S cyclin-dependent kinase complex and has been previously shown to overcome G1 arrest in human keratinocytes (Lazarov et al., 2002, 2003), we observed no rescue of Erk1/2 depletion-induced hypoplasia and hypoproliferation, suggesting that Erk actions in this cell type are not limited to the G1/S cell cycle stages. Consistent with this, cell cycle analysis revealed a G2/M arrest in Erk1/2-depleted keratinocytes, contrasting

with the G1 blockade observed by others upon pharmacologic Erk inhibition in fibroblasts that we have reproduced using siRNA against Erk1/2 (Meloche and Pouyssegur, 2007; Villanueva et al., 2007). This discrepancy does not represent a general resistance of fibroblasts to G2/M-phase arrest because siRNA-mediated depletion of a G2/M-phase regulator, Cdc25C, arrested these cells in G2/M phase (unpublished data). These findings support previous observations of delayed G2/M kinetics upon pharmacologic MAPK inhibition in NIH3T3 cells and HeLa cells (Wright et al., 1999; Liu et al., 2004) and imply a substantial role for Erk1/2 signaling at multiple points in the cell cycle.

The finding of G2/M arrest upon Erk1/2 blockade in unsynchronized primary keratinocytes supports previous findings in synchronized cell lines (Wright et al., 1999) and emphasizes the importance of identifying specific Erk substrates responsible for this activity. To that end, our global gene expression analysis of Erk1/2-depleted keratinocytes showed substantial misexpression of G2/M regulators, indicating that broad changes in gene expression rather than dysregulation of a single effector likely govern the observed G2/M arrest. More specifically, the core G2/M regulators, cyclin B1 and cdc2, whose integrated activity is essential for mitotic progression, are significantly down-regulated along with Cdc25A and Plk1, which are positive regulators of the Cdc2–cyclin B1 complex (Fig. 6 C). Simultaneously, negative regulators of the Cdc2–cyclin B1 complex, including p53, GADD45A, and Wee1, are up-regulated with Erk1/2 depletion, indicating additional routes by which Erk depletion may cause G2/M arrest.

The magnitudes of these gene expression changes are relevant to normal progression through G2/M phase. For instance, siRNA-mediated depletion of Cdc25C, which was similar in extent to that caused by Erk1/2 depletion, caused G2/M arrest in cultured keratinocytes and disrupted epidermal homeostasis in vivo (Fig. S5). However, given that Erk1/2 affects a broad network of G2/M regulators, it is unlikely that activating a single Erk1/2 effector could rescue the effects of Erk1/2 loss in human skin. Theoretically, activating the mitosis-promoting factor complex itself (by overexpressing cyclin B1 and a CA mutant of Cdc2) could bypass the need for proper expression of G2/M regulators and rescue the G2/M arrest observed upon Erk1/2 knockdown. However, we find that coexpression of these genes induces apoptosis in human keratinocytes at early time points that preclude analysis of cell cycle effects (unpublished data), which agrees with previous results in other cell types and challenges further attempts to rescue the G2/M blockade induced by Erk1/2 loss (Niida et al., 2005).

Because the cell cycle defects induced by Erk1/2 loss in fibroblasts can largely be corrected by cyclin D1 and Cdk4-C24 overexpression, it is likely that Erk1/2 affect G2/M regulators differently in this cell type. Indeed, we find that fibroblasts, unlike

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genes identified in A. Symbols are color-coded according to gene expression levels in Erk1/2-depleted cells as compared with control. (D) Immunoblot of keratinocyte, fibroblast, or cyclin D1 + Cdk4-C24-rescued fibroblast extracts 4 d after treatment with the indicated siRNA, using the antibodies listed on the right. (E and F) mRNA expression levels of Erk1/2 effectors in keratinocytes (E) or rescued fibroblasts (F) treated with Erk1/2 or scrambled siRNA, as measured by quantitative RT-PCR.  $n = 3$  per group; mean  $\pm$  SEM. (G) mRNA expression levels of c-Fos and cyclin B1 in keratinocytes and fibroblasts treated with c-Fos or scrambled siRNA, as measured by quantitative RT-PCR.  $n = 3$  per group; mean  $\pm$  SEM. (H and I) Histograms of PI fluorescence in keratinocytes (H) or fibroblasts (I) 4 d after electroporation with the indicated siRNA.

keratinocytes, down-regulate cyclin D1 but not cyclin B1 upon Erk1/2 loss. Our experiments indicate that differential regulation of c-Fos may explain this finding, as Erk1/2 are required for c-Fos expression and AP-1 activity in keratinocytes but not fibroblasts. Furthermore, with the two AP-1-binding sites in the *Drosophila CycB* promoter being conserved in the human genome (Hyun et al., 2006), we find that c-Fos is required for normal cyclin B1 expression and G2/M-phase progression in both human keratinocytes and fibroblasts.

In summary, although Erk1 and Erk2 demonstrate functional redundancy in human epidermis in vivo, combined depletion of Erk1/2 disrupts epidermal homeostasis, indicating that Erk1/2 signaling is absolutely required in this setting. Tissue lacking Erk1/2 is markedly hypoplastic and hypoproliferative. These defects are not rescued by activation of a pleiotropic Erk1/2 effector, Rsk1, or by activation of the core G1/S cyclin-dependent kinase complex, indicating that Erk1/2 use alternate mechanisms to control cell cycle progression in this setting. Indeed, Erk1/2 depletion in keratinocytes is marked by G2/M arrest and dysregulated expression of a gene regulatory network, including the Cdc2-cyclin B1 complex and many of its regulators. In contrast, Erk1/2 depletion in fibroblasts induces G1 arrest that can be suppressed by expressing cyclin D1 and Cdk4-C24, indicating that the mechanisms by which Erk1/2 regulate cell cycle progression vary by cell type. In this regard, we found that expression of c-Fos, which controls cyclin B1 levels and G2/M progression in both keratinocytes and fibroblasts, is Erk1/2 dependent only in keratinocytes. These findings indicate that Erk1/2 act in the epidermis as an essential, functionally redundant regulator of homeostasis that sustains basal cell proliferation by promoting G2/M progression.

## Materials and methods

### RNAi and gene transfer in regenerated human skin

Multiple independent sets of siSTABLE siRNA oligomers against *ERK1*, *ERK2*, *CDC25C*, and *FOS*, as well as an siSTABLE scrambled control oligomer, were synthesized by Thermo Fisher Scientific. These oligomers were delivered to primary human foreskin keratinocytes by electroporation using a Nucleofector II (Amax). In each nucleofection,  $10^6$  keratinocytes were electroporated with 1.4 nmol control oligomer, 0.7 nmol Erk1 oligomer and 0.7 nmol control oligomer, 0.7 nmol Erk2 oligomer and 0.7 nmol control oligomer, or 0.7 nmol Erk1 oligomer and 0.7 nmol Erk2 oligomer. To generate a human skin graft,  $3.5 \times 10^5$  siRNA-treated keratinocytes were seeded onto a 2-cm<sup>2</sup> piece of devitalized human dermis and cultured in keratinocyte growth medium for 2 d, at which point the dermis piece was grafted onto a 6- to 8-wk-old female CB-17 severe combined immunodeficiency mouse as previously described (Choate et al., 1996; Scholl et al., 2007). Skin grafts were harvested 6 d after surgery. All animal protocols were approved by the Stanford University Animal Care and Use Committee.

For rescue experiments, keratinocytes or primary human dermal fibroblasts were transduced 2 d before siRNA electroporation with LZRS-LacZ, LZRS-Erk2-CA, LZRS-Erk2-WT, LZRS-Rsk1-CA, LZRS-cyclin D1, LZRS-Cdk4-C24, or LZRS-Cdc25C-CA. LacZ, Erk2-CA, cyclin D1, and Cdk4-C24 retroviral vectors were prepared as described previously (Kinsella and Nolan, 1996; Lazarov et al., 2002; Scholl et al., 2007), whereas the Erk2-WT, Rsk1-CA, and Cdc25C-CA vectors were constructed by cloning of the coding sequence of each gene into pENTR1A, from which it was transferred into the LZRS vector by an LR Clonase reaction (Gateway Technologies; Emrick et al., 2001; Silverman et al., 2004). The plasmid used to generate the Rsk1-CA vector was provided by J. Maller (University of Colorado Health Science Center, Denver, CO), and the plasmid used to generate the Erk2-CA vector was provided by N. Ahn (University of Colorado, Boulder, CO). Viral packaging was performed in 293T Phoenix cells

as previously described (Kinsella and Nolan, 1996). Cells were infected for 1 h in the presence of 5 µg/ml polybrene and concomitantly centrifuged at 300 g and 32°C. Keratinocytes were infected once, whereas fibroblasts were infected twice, 12 h apart. To confirm gene knockdown and transduction efficiency, infected cells were cultured in keratinocyte serum-free medium (Invitrogen) until 4 d after siRNA electroporation, at which point they were harvested for immunoblot analysis.

### Protein expression analysis

Keratinocyte and fibroblast cell lysis and immunoblotting were performed as described previously (Scholl et al., 2007). Primary antibodies used included rabbit anti-p44/p42 MAPK (1:1,000; Cell Signaling Technology), rabbit anti-phospho-p44/p42 MAPK (1:1,000; Cell Signaling Technology), rabbit anti-β-galactosidase (1:20,000 MP Biomedicals), rabbit anti-Rsk1 (1:200; Millipore), rabbit anti-phospho-573-Rsk1 (1:1,000; Cell Signaling Technology), mouse anti-cyclin D1 (1:1,000, BD), rabbit anti-Cdc25C (1:1,000; Cell Signaling Technology), mouse anti-cyclin B1 (1:1,000; Santa Cruz Biotechnology, Inc.), and mouse anti-β-actin (1:5,000, Sigma-Aldrich). Secondary antibodies used were horseradish peroxidase-conjugated donkey anti-rabbit IgG and sheep anti-mouse IgG (1:20,000; GE Healthcare). Supersignal Pico and Supersignal Dura (Thermo Fisher Scientific) were used as chemiluminescence reagents, and film (XAR Biomax; Kodak) was used for detection. Multiple proteins were detected on the same membrane by incubating membranes in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris at pH 6.8) for 20 min at 55°C and then restaining them.

### Immunostaining, immunohistochemistry, and histology

For immunostaining, human skin tissue was frozen in Optimal Cutting Temperature compound (Sakura Finetek) and subsequently cut into 7-µm cryosections and fixed in -20°C acetone for 10 min. For blocking, sections were incubated for 1 h with 10% horse serum in PBS; next, sections were incubated for 1 h with primary antibody and 2% horse serum in PBS, after which they were washed with PBS. Finally, sections were incubated for 1 h with secondary antibody, 2% horse serum, and 2 mg/ml Hoechst 33342 nuclear dye (Invitrogen) in PBS, after which they were washed with PBS and mounted in Prolong Gold mounting medium (Invitrogen). Primary antibodies used included mouse anti-human desmoglein 3 (1:200; Invitrogen), rabbit anti-human Ki-67 (1:100; NeoMarkers), mouse anti-β<sub>1</sub> integrin (1:100; Santa Cruz Biotechnology, Inc.), rabbit anti-mouse keratin 5 (1:1,000; Covance), rabbit anti-mouse keratin 10 (1:500; Covance), and rabbit anti-transglutaminase 1 (1:100; Biomedical Technologies, Inc.). Donkey anti-rabbit Cy3 IgG (1:100; Jackson ImmunoResearch Laboratories), goat anti-mouse Alexa Fluor 555 (1:500, Invitrogen), and goat anti-mouse Alexa Fluor 488 (1:500; Invitrogen) were used as secondary antibodies.

For immunohistochemistry and histology, human skin grafts were fixed in 10% neutral buffered formalin (Sigma-Aldrich) and embedded in paraffin, from which 5-µm sections were cut and stained with hematoxylin and eosin (H&E) or by immunohistochemistry according to standard methods. Permeabilization for antigen retrieval was achieved by microwaving samples in Antigen Unmasking Solution (Vector Laboratories), after which the sections were stained with rabbit anti-p44/p42 total MAPK 137F5 (1:250; Cell Signaling Technology) as primary antibody and biotinylated horse anti-rabbit IgG as secondary antibody (RTU Vectastain Universal Elite ABC kit; Vector Laboratories). Staining and development were performed using the Elite ABC Reagent (Vector Laboratories) and liquid DAB<sup>+</sup> substrate chromogen system (Dako).

For TUNEL apoptosis staining, 7-µm cryosections were fixed in room temperature 4% paraformaldehyde for 20 min followed by 30 min in PBS and 2 min in 100% ethanol. Subsequently, TUNEL enzymatic labeling was performed using the In situ Cell Death Detection kit, TMR red (Roche).

### Cell proliferation assay, cell cycle analysis, and AP-1 reporter assay

To monitor keratinocyte proliferation, cells cultured in keratinocyte serum-free medium were electroporated with siRNA and, 1 d later, plated into 6-cm dishes at a density of  $4 \times 10^4$  cells per plate. Total cell number per plate was determined 3, 5, and 7 d later by trypsinizing each plate's keratinocytes and counting them in a hemocytometer (Hausser Scientific). For cell cycle analysis, keratinocytes were cultured in keratinocyte serum-free medium, and primary human dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum; each medium contained 100 U/ml penicillin and 100 µg/ml streptomycin. 4 d after siRNA electroporation, cells were harvested, and PI staining was performed as previously described (Truong et al., 2006). Watson model cell cycle analysis was performed using FlowJo 8.4.5 software (Tree Star, Inc.).

To measure the effect of Erk1/2 loss on AP-1 activity,  $10^6$  keratinocytes or fibroblasts were electroporated with 1,400 nmol siRNA, 3  $\mu$ g pAP-1-Luc (Agilent Technologies), and 60 ng CMV-RU Renilla plasmid. 2 d after electroporation, 30 ng/ml TPA (12-O-tetradecanoylphorbol-13-acetate) was added to the cell media to stimulate AP-1 activity. 1 d later, the cells were harvested using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was normalized to Renilla activity; the resulting ratios were normalized to control samples.

#### mRNA expression analysis

Microarray analysis was performed on primary human keratinocytes harvested 4 d after siRNA electroporation. Cells were lysed in TRIZOL reagent (Invitrogen), and RNA was subsequently isolated from the aqueous phase using an RNeasy kit (QIAGEN). The Stanford Protein and Nucleic Acid Facility performed cDNA amplification, labeling, and hybridization to HG-U133A2.0 microarray chips (Affymetrix). Data were analyzed using GeneSpring GX (Agilent Technologies) and Ingenuity Pathways Analysis 6 software (Ingenuity Systems).

For quantitative RT-PCR, RNA was isolated using TRIZOL and an RNeasy kit as described in the previous paragraph; it was then treated with RNaseOUT and DNase I (Invitrogen). Reactions were performed using an Mx3000P (Agilent Technologies) instrument and the Brilliant SYBR Green QRT-PCR Master Mix kit (Agilent Technologies). 100 ng RNA was amplified per reaction in the presence of 100 nM of each primer (Fig. S4 C). Each reaction was normalized to levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA. Microarray data are publicly available in the Gene Expression Omnibus under accession no. GSE15417.

#### Statistics

In Erk1/2 knockdown experiments, mitotic indices were compared by analysis of variance, and subsequent post hoc analyses were made using Tukey's test. In rescue experiments, Student's *t* test was used to compare mitotic indices.

#### Image acquisition and manipulation

Immunostained sections were viewed using a microscope (100M Axiovert; Carl Zeiss, Inc.) with a 40 $\times$  Plan-Neofluar  $\infty/0.75$  objective (Carl Zeiss, Inc.), and micrographs were captured using a camera (AxioCam; Carl Zeiss, Inc.) and Openlab 5.0.1 software (PerkinElmer). H&E-stained sections were viewed using a microscope (DM LB; Leica) with a 40 $\times$  N Plan  $\infty/0.65$  objective (Leica). Images were captured using a camera (SPOT Insight QE; Diagnostic Instruments, Inc.) and SPOT 4.5.9.12 software (Diagnostic Instruments, Inc.). All images were captured at room temperature. Photoshop CS3 (Adobe) was used to adjust image brightness and contrast.

#### Online supplemental material

Fig. S1 shows Erk1/2 expression and TUNEL staining in regenerated human epidermis treated with Erk1/2 siRNA. Fig. S2 shows that expression of WT Erk2 but not expression of cyclin D1 and CA Cdk4 is sufficient to rescue the in vivo effects of epidermal Erk1/2 knockdown. Fig. S3 shows that expression of cyclin D1 and CA Cdk4 suppresses the cell cycle defect caused by Erk1/2 depletion in fibroblasts but not in keratinocytes and shows the effect of Erk1/2 knockdown on AP-1 reporter activity in keratinocytes and fibroblasts. Fig. S4 illustrates the effect of Erk1/2 knockdown on expression of G2/M-phase regulators and transcription factors in keratinocytes and fibroblasts and lists the quantitative RT-PCR primers used in this study. Fig. S5 shows that depletion of the Erk1/2 effector Cdc25C is sufficient to induce epidermal hypoplasia, whereas overexpression of CA Cdc25C does not rescue the effects of epidermal Erk1/2 knockdown in vivo. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200804038/DC1>.

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