Loss of E2F7 Expression Is an Early Event in Squamous Differentiation and Causes Derepression of the Key Differentiation Activator Sp1

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Squamous differentiation is controlled by key transcription factors such as Sp1 and E2F. We have previously shown that E2F1 can suppress transcription of the differentiation-specific gene, transglutaminase type 1 (*TG1*), by an indirect mechanism mediated by Sp1. Transient transfection of E2F1–E2F6 indicated that E2F-mediated reduction of Sp1 transcription was not responsible for E2F-mediated suppression of squamous differentiation. However, we found that E2F4 and E2F7, but not E2Fs 1, 2, 3, 5, or 6, could suppress the activation of the Sp1 promoter in differentiated keratinocytes (KCs). E2F4-mediated suppression could not be antagonized by E2Fs 1, 2, 3, 5, or 6 and was localized to a region of the human Sp1 promoter spanning −139 to +35 bp. Chromatin immunoprecipitation analysis, as well as transient overexpression and short hairpin RNA knockdown experiments indicate that E2F7 binds to a unique binding site located between −139 and −119 bp of the Sp1 promoter, and knockdown of E2F7 in proliferating KCs leads to a derepression of Sp1 expression and the induction of TG1. In contrast, E2F4 knockdown in proliferating KCs did not alter Sp1 expression. These data indicate that loss of E2F7 during the initiation of differentiation leads to the derepression of Sp1 and subsequent transcription of differentiation-specific genes such as *TG1*.

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INTRODUCTION

Squamous differentiation refers to the process by which proliferative basal keratinocytes (KCs) irreversibly inhibit the expression/activity of genes required for proliferation (for example, *cdc2* or *E2F1*; Jones *et al.*, 1997; Dahler *et al.*, 1998) and start to induce the expression/activity of genes required for differentiation (for example, *Sp1*, transglutaminase type 1 (*TG1*), and *involucrin*, Eckert *et al.*, 1997; Eckert *et al.*, 1998; Wong *et al.*, 2003; Wong *et al.*, 2005). The mechanism by which proliferation and differentiation are regulated is primarily transcriptional, and a number of transcription factors (for example, E2F, AP2, Sp1 and Ets) have been implicated in the control of this process (Eckert *et al.*, 1997; Eckert *et al.*, 1998; Dicker *et al.*, 2000; Wong *et al.*, 2003; Wong *et al.*, 2005).

The Sp1 transcription factor family are key regulators of squamous differentiation, with Sp1, Sp3, and Sp4 all being expressed by human KCs (Apt *et al.*, 1996; Chen *et al.*, 1997; Park and Morasso, 1999; Wong *et al.*, 2005). Sp1 is an activator of squamous differentiation (Apt *et al.*, 1996; Wong *et al.*, 2005), whereas Sp3 inhibits Sp1-induced squamous differentiation (Apt *et al.*, 1996; Phillips *et al.*, 2004). For this reason, it has been speculated that the induction of differentiation is governed, in part, by the ratio between activating Sp1 and inhibitory Sp3. For example, Sp1 is preferentially expressed in differentiated KCs both *in vitro* and *in vivo* (Apt *et al.*, 1996; Wong *et al.*, 2005) and is able to activate the transcription of differentiation-specific genes (Wu *et al.*, 1994; Lee *et al.*, 1996; Banks *et al.*, 1998; Jang and Steinert, 2002). Moreover, transient overexpression of Sp1 in growth-arrested KCs is able to induce the expression of differentiation-specific markers (Wong *et al.*, 2005). In contrast, Sp3 is expressed in undifferentiated KCs (Apt *et al.*, 1996; Wong *et al.*, 2005) and suppresses Sp1-mediated transcription of differentiation-specific genes (Lania *et al.*, 1997). Finally, the Sp1/Sp3 ratio is disrupted in differentiation-resistant squamous cell carcinomas (SCC) *in vitro* and *in vivo*, whereas transient overexpression of Sp1 in growth-arrested SCC cells (induced by E2F inhibition) can induce the expression of squamous differentiation markers in SCC cells

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Abbreviations: ChIP, chromatin immunoprecipitation; HEK, human epidermal keratinocyte; KC, keratinocyte; SCC, squamous cell carcinomas; shRNA, short hairpin RNA; TG1, transglutaminase type 1

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(Wong *et al.*, 2005). These data indicate that the induction of squamous differentiation is associated with an increase in the expression of Sp1 relative to Sp3.

The E2F family of transcription factors comprise E2Fs 1–8 and can be broadly classified as activating (E2F 1, 2, and 3a) or inhibitory (E2Fs 3b–8) E2Fs (Johnson and DeGregori, 2006; DeGregori and Johnson, 2006). Previous studies indicate that E2F activity stimulates proliferation and suppresses squamous differentiation (Pierce *et al.*, 1999; Dicker *et al.*, 2000; Paramio *et al.*, 2000; Wong *et al.*, 2003) in KCs. For example, it has been shown that inhibition of E2F activity results in the suppression of proliferation and sensitizes KCs to subsequent differentiation stimuli, such as Sp1 (Wong *et al.*, 2005). Finally, the E2Fs expressed in differentiated KCs also serve to modulate the expression of differentiation markers (Wong *et al.*, 2003). In particular, we recently showed that the inhibitory E2F, E2F7, was preferentially expressed in proliferative KCs and may be involved in the initiation of squamous differentiation (Endo-Munoz *et al.*, 2009). Thus, E2F regulation of differentiation appears to be of physiological significance and our earlier studies have indicated that the suppression of the *TG1* gene by E2Fs1–5 was indirect in nature and was mediated by Sp1 (Wong *et al.*, 2003). This raises the issue of whether E2F-mediated suppression of squamous differentiation could be mediated by the suppression of Sp1 expression.

RESULTS

Suppression of Sp1 expression is E2F4 isoform specific

Sp1 mRNA and protein are induced when proliferating human epidermal KCs (HEKs) are induced to differentiate by maintaining cells at confluence for 48 hours (Supplementary Figure S1A and B online). The differentiation status of the HEK cultures is confirmed by the decrease in the proliferation-specific gene, *cdc2* (Dahler *et al.*, 1998), and the induction of the differentiation-specific gene, *involucrin* (Crish and Eckert, 2008) (Supplementary Figure S1B online). The differentiation-specific expression of Sp1 is confirmed in human skin, in which the proliferation-specific protein, PCNA, is confined to the basal KCs, whereas involucrin is localized to the suprabasal KCs (Supplementary Figure S1C online). The Sp1 transcription factor displayed a nuclear localization pattern and was more abundant in the suprabasal layers of the epidermis (Supplementary Figure S1C online).

We transiently overexpressed E2Fs 1–6 in differentiated KCs and measured the activity of a co-transfected 1.2-kb fragment of the human Sp1 promoter driving the expression of a luciferase reporter gene. We found that transient overexpression of E2F4 and, to a lesser extent, E2F2 could significantly suppress the SP1-Luc activity, whereas E2Fs 1, 3, 5, and 6 could not (Supplementary Figure S2A online). The lack of an effect on SP1-Luc activity by E2Fs 1, 3, 5, and 6 was not due to the lack of expression, as the same constructs (except E2F6 which does not regulate differentiation, Wong *et al.*, 2004) were all able to suppress differentiation-specific TG1-Luc activity (Supplementary Figure S2B online). Next, we showed that E2F4 is expressed in KC nuclei throughout the epidermis *in vivo* (Supplementary Figure S2C online).

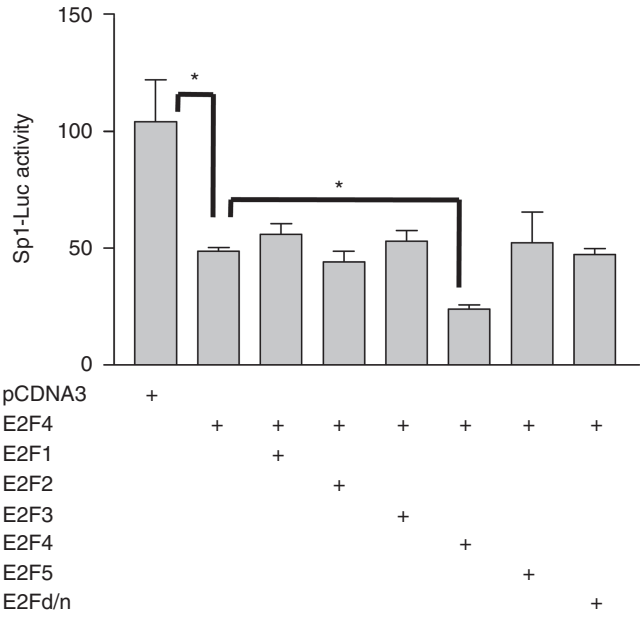

Figure 1. Suppression of the human SP1 promoter is E2F isoform specific. Confluent differentiated keratinocytes (KCs) were co-transfected with the SP1-Luc construct + either 0.3 μg pcDNA3 or the E2F4 expression plasmid + 0.3 μg of one of E2F1–E2F5 expression plasmids or a dominant-negative form of E2F1 construct. All transfections were normalized for transfection efficiency on the basis of β-actin–chloramphenicol acetyl transferase activity. All data are presented as a percentage of the pcDNA3-transfected control and are expressed as mean ± SEM of triplicate determinations from three independent experiments. $*P<0.05$.

We tested whether the ability of E2F4 to suppress Sp1-Luc activity in differentiated KCs could be antagonized by other E2Fs. Differentiated KCs were co-transfected with sufficient E2F4 plasmid (0.3 μg) to repress the Sp1 promoter (Figure 1, lane 2) plus equivalent amounts of expression plasmids (0.3 μg) coding for individual E2Fs 1–5 or a dominant-negative form of E2F1 (Dicker *et al.*, 2000), which codes for the DNA-binding domain and heterodimerization domain of E2F1 (Figure 1, lanes 3–8). E2Fs 1, 2, 3, 5, and dominant-negative form of E2F1 did not antagonize, or add to, E2F4-mediated repression of the Sp1 promoter (Figure 1). In contrast, transient overexpression of E2F4 further suppressed E2F4-mediated SP1 promoter repression (Figure 1). These data indicate that the suppression of the Sp1-Luc promoter construct is E2F4 selective.

E2F4 suppresses Sp1 transcription via a regulatory region in the proximal 139 bp of the Sp1 promoter

We co-transfected a deletion series of the human Sp1 promoter–reporter constructs into differentiated KCs with or without E2F4 (Figure 2). Analysis of the deletion series indicated that a threefold, E2F4-mediated suppression of Sp1 transcription was present between −139 and −89 bp (Figure 2). In contrast, the ability of E2F4 to suppress Sp1 transcription was not present in deletions of −89 bp or less (Figure 2).

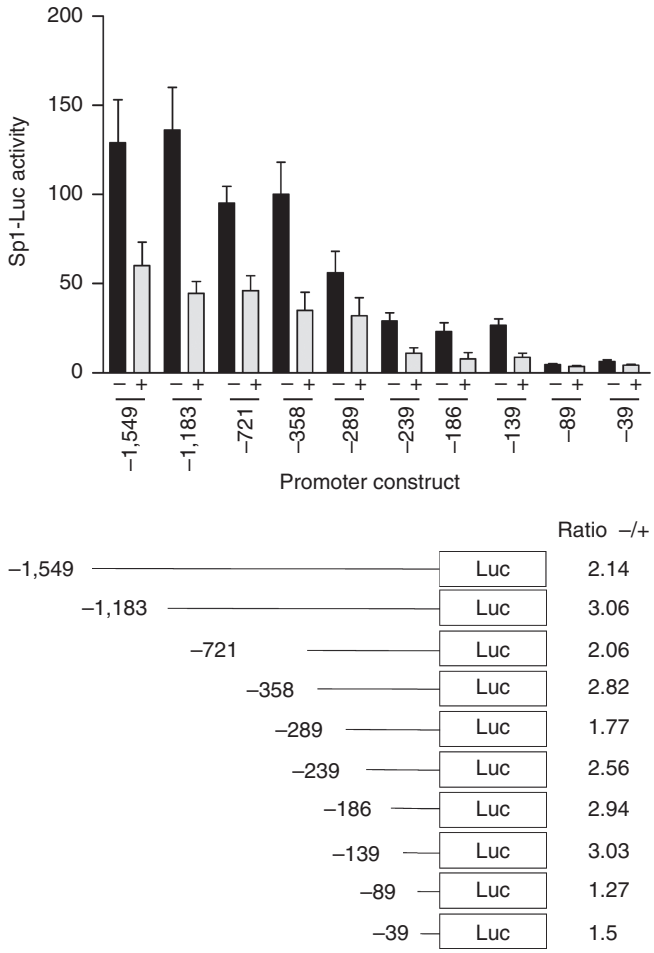

Figure 2. E2F4 suppression of SP1 transcription is mediated via elements between −139 and −89 bp. Confluent differentiated keratinocytes (KCs) were co-transfected with a series of Sp1 promoter fragments linked to a luciferase reporter with either pcDNA3 (−) or E2F4 (+). All transfections were normalized for transfection efficiency on the basis of β-actin–chloramphenicol acetyl transferase activity. All data are presented as percentage of the pcDNA3-transfected control (−358 bp construct) and are expressed as mean ± SEM of triplicate determinations from three independent experiments. The lower panel depicts the same data expressed as a ratio of activity in controls over activity in the presence of co-transfected E2F4.

E2F4 and E2F7 repress Sp1 expression in proliferating KCs

E2F7 is an inhibitory E2F that is expressed selectively in proliferating KCs and is able to modulate differentiation in an E2F1-independent manner (Endo-Munoz *et al.*, 2009). Consistent with this, data presented in Figure 1 indicates that the selective suppression of Sp1 by E2F4 is also E2F1 independent. Thus, we explored the possibility that E2F7 could also suppress the Sp1 promoter and that the suppression of the Sp1 promoter, by E2F, may be more physiologically relevant in the context of the proliferating KC. Transient transfection of either E2F4 or E2F7b (the major E2F7 transcript in undifferentiated KCs) into differentiated KCs resulted in a statistically significant suppression of the full-length Sp1 reporter (Figure 3a). Moreover, co-transfection of E2F4 + E2F7b produced an additive suppression of the Sp1 promoter (Figure 3a). As, E2F7b is selectively expressed in proliferating KCs (Figure 3b), we hypothesized that the suppression of Sp1 by E2F4 and/or E2F7 may have more physiological relevance in the context of proliferative/undifferentiated KCs. We examined the proximal Sp1 promoter region (spanning the −139 to +35 nucleotide region in Figure 2) using a series of antibodies in chromatin immunoprecipitation (ChIP) assays on proliferating and confluent/differentiated KCs (Figure 4a). Figure 4 shows that E2F7 and, to a minor extent, E2F4 and Sp1 bind to the E2F4/7-responsive region of the Sp1 promoter in proliferating KCs. Sp1 has been previously reported to modulate this promoter region in HeLa cells (Nicolas *et al.*, 2003). Consistent with a loss of E2F7 expression in differentiated KCs, we observed a significant loss of E2F7 bound to the Sp1 promoter in differentiated KCs while retaining Sp1 binding (Figure 4a and b). We used the proximal E2F1 promoter region as a positive control, as it has been previously shown to bind E2F1 and E2F7 in proliferation-competent U2OS cells (Di Stefano *et al.*, 2003). E2F1, 4, 7, and Sp1 bound the E2F1 promoter in proliferating KCs, whereas only the suppressive E2F4 was bound to the E2F1 promoter in growth-arrested/differentiated KCs (Figure 4a).

Co-transfection of proliferative KCs with the Sp1-Luc reporter plus E2F4 or E2F7b expression plasmids resulted in the suppression of the Sp1 promoter and Sp1 mRNA expression (Figure 5a and b). Consistent with this, transient overexpression of an E2F7 shRNA construct (Endo-Munoz *et al.*, 2009) derepressed Sp1 promoter activity and Sp1 mRNA, whereas transient overexpression of an E2F4 shRNA construct (D2, 72% knockdown, see Materials and Methods) did not (Figure 5a and b). Similar results for different E2F4 or E2F7 shRNA constructs were obtained (data not shown). These data suggest that, although E2F4 and E2F7 can repress Sp1 promoter activity, only E2F7 appears to do so in a physiologically relevant setting. We examined whether the transient overexpression of E2F7 shRNA would induce differentiation-specific genes, *TG1* or *involucrin*. Transient overexpression of E2F7 shRNA produced a 1.7-fold increase in TG1 mRNA expression, but failed to induce involucrin mRNA expression (Figure 5c). Finally, we tested the ability of the D2 E2F7 shRNA construct to derepress Sp1 transcription in the HaCaT KC cell line (Figure 5d). Similar to the primary cultures of HEKs, E2F7b shRNA derepressed Sp1 transcription in HaCaT cells.

The E2F7-selective repressive site in the Sp1 promoter is, to our knowledge, previously unreported

The E2F7 responsive site within the Sp1 promoter (−139 to −89 bp) did not contain a consensus E2F-binding site, but did contain AP2- and Sp1-binding sites. Gel shifts with competing AP2, SP1, or E2F consensus binding sites and supershift analyses with antibodies against Sp1, Sp3, Sp4, E2F1, E2F4, E2F7, AP2α, AP2β, and AP2γ were all negative (data not shown). Use of competitor double-stranded DNA regions indicated that DNA binding to the −139 to −89 bp region was located between −139 and −119 bp (Figure 6a). Consistent with the E2F7 ChIP data (Figure 4), binding to the −139 to −119 bp region is restricted to proliferating HEKs

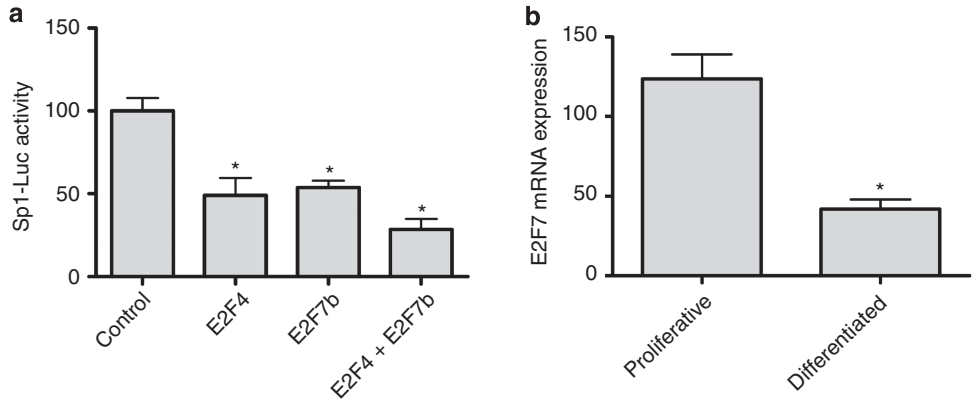

Figure 3. E2F7 suppresses the SP1 promoter. (**a**) Confluent/differentiated keratinocytes (KCs) were co-transfected with the Sp1-Luc construct + either the E2F4 (0.3 + 0.3 μg pcDNA3), E2F7b (0.3 + 0.3 μg pcDNA3), or E2F4 + E2F7b (0.3 μg each) expression constructs. All transfections were normalized for transfection efficiency on the basis of β-actin–chloramphenicol acetyl transferase activity. All data are presented as a percentage of the pcDNA3-transfected control and are expressed as mean ± SEM of triplicate determinations from three independent experiments. (**b**) RNA was harvested from proliferative or confluent/differentiated KCs and E2F7b mRNA expression determined by quantitative RT-PCR. Data presented as mean ± SEM from triplicate determinations from two independent experiments. $*P<0.05$.

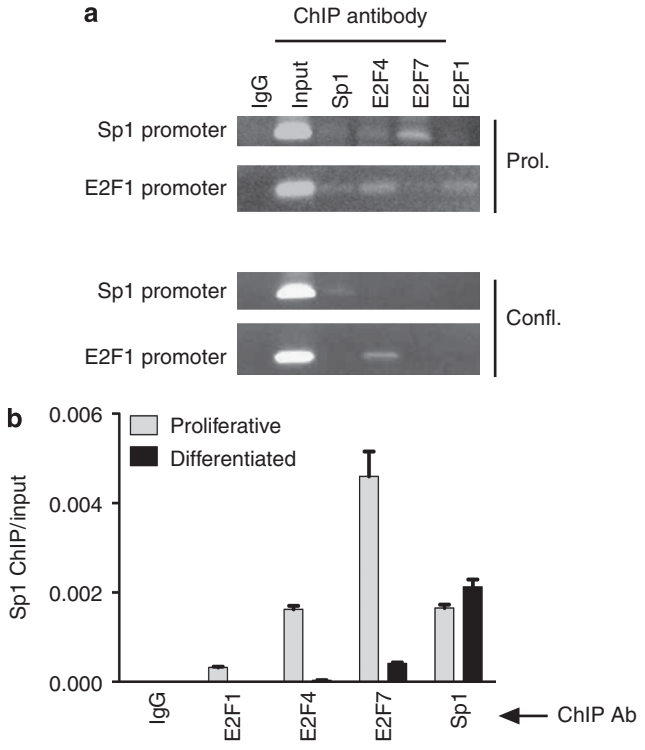

Figure 4. E2F7 is selectively bound to the human Sp1 promoter in proliferative keratinocytes (KCs). (**a**) Extracts were from proliferative (Prol.) or confluent/differentiated (Confl.) human KCs prepared for chromatin immunoprecipitation (ChIP) analysis. Extracts were incubated with antibodies against Sp1, E2F4, E2F7, E2F1, or IgG. ChIP-enriched fractions were then examined for the presence of Sp1 or E2F1 promoter sequences by PCR. In total, 10% of the input extract was used as a positive control. A representative experiment is shown. (**b**) The ratio of Sp1 promoter amplified from the ChIP extracts over the amount of Sp1 promoter amplified from the input is shown for triplicate determinations from two independent experiments. Data shown as mean ± SEM.

(Figure 6b). Mutation scanning of the −139 to −119 bp region revealed that bases −131 and −130, and −125 and −124 were required for DNA binding in this region (Supplementary Figure S3A and B online). Mutating the −131 and 130, and −125 and 124 doublet bases, in the context of Sp1-Luc, resulted in derepression of Sp1-Luc activity compared with wild-type Sp1-Luc activity in proliferating HEKs (Supplementary Figure S3C online).

DISCUSSION

This study identifies the Sp1 promoter as a physiologically relevant and specific target of E2F7. This is supported by several observations. Specifically, E2F7 is preferentially expressed in proliferating KCs and is preferentially localized to the E2F repressive region of the Sp1 promoter in proliferative KCs but not in differentiated KCs. Mutation of specific bases within the E2F7-sensitive region of the Sp1 promoter derepresses Sp1 transcription. Finally, transient overexpression of E2F7 shRNA derepresses Sp1 transcription and expression, resulting in the induction of TG1 transcription. In contrast, although E2F4 clearly displayed selectivity for the Sp1 promoter when overexpressed in differentiated KCs, it is unlikely to be of physiological relevance as E2F4 is expressed at similar levels in proliferative and differentiated KCs *in vitro* (Paramio *et al.*, 2000; Wong *et al.*, 2003) and *in vivo* (Supplementary Figure S2 online). In addition, E2F4 shRNA does not derepress Sp1 transcription or expression in proliferating KCs, nor does it induce differentiation markers. Finally, E2F4 bound poorly to the E2F repressive region of the Sp1 promoter *in vivo* in both proliferative and differentiated KCs.

The mechanisms regulating KC proliferation and the transcriptional induction of differentiation genes have been extensively studied (Eckert *et al.*, 1997; Eckert *et al.*, 1998). In this context, we have previously reported that the initiation of squamous differentiation requires the inhibition of activating E2Fs (Dicker *et al.*, 2000; Paramio *et al.*, 2000; Wong *et al.*, 2003). This results in an irreversible growth arrest and the sensitization of KCs to a subsequent differentiation stimulus, in part delivered by Sp1 activation (Wong *et al.*, 2003; Endo-Munoz *et al.*, 2009). We now report that a key event in the

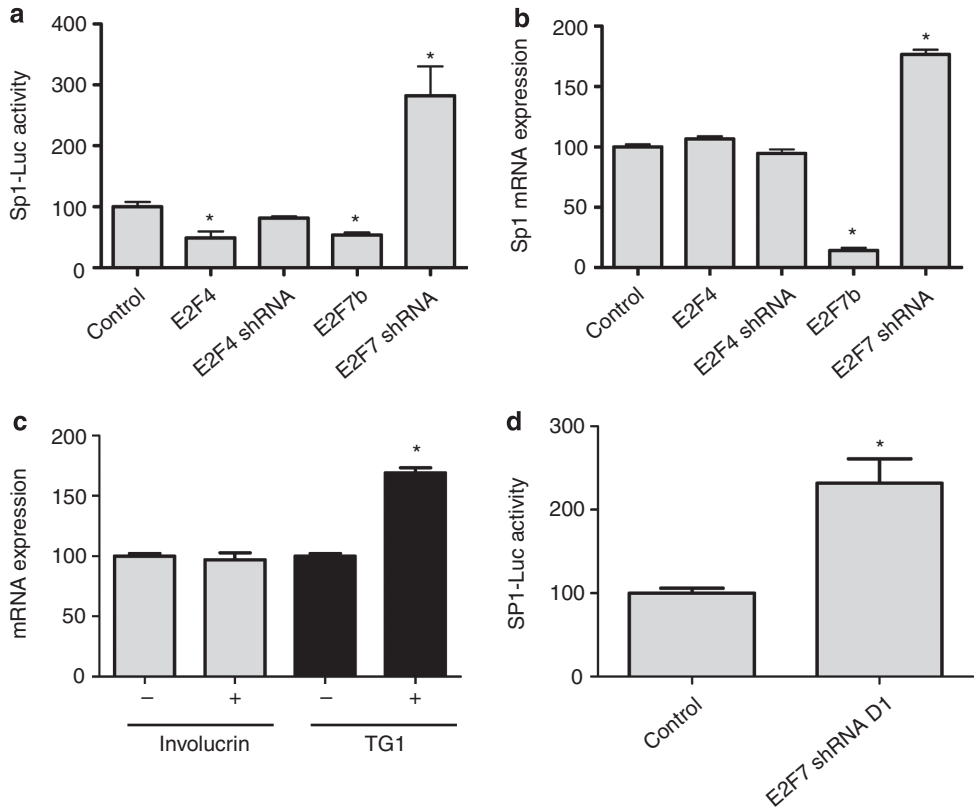

Figure 5. E2F7 is a physiologically relevant repressor of the Sp1 promoter. Proliferating keratinocytes (KCs) were co-transfected with green fluorescent protein (GFP) plasmid combined with pCDNA3 (Control) or an E2F4, E2F7b, E2F4 shRNA, or E2F7 shRNA expression plasmid. (**a**) KCs were also co-transfected with the SP1-Luc construct + β-actin–chloramphenicol acetyl transferase reporters. (**b**) Transfected cells were enriched for GFP positivity by Moflo and assessed for SP1 mRNA, or (**c**) E2F7 shRNA (+) or vector control (−) transfected keratinocytes were assayed for TG1 and involucrin mRNA expression. (**d**) Proliferating HaCaT cells were co-transfected with Sp1-Luc + β-actin Cat + either pcDNA3 (Control) or E2F7 shRNA (D1 construct shown). Sp1-Luc activity was measured and normalized against β-actin–chloramphenicol acetyl transferase activity. Data are expressed as mean percent control ± SEM of triplicate determinations from three experiments. $^*P<0.05$.

initiation of differentiation is the loss of E2F7 expression, which results in the derepression of the differentiation-inducing transcription factor, Sp1 (Supplementary Figure S4 online). Specifically, when KCs receive a stimulus to differentiate (for example, protein kinase C activation or confluence), they inhibit the activity of activating E2Fs (Jones *et al.*, 1997; Paramio *et al.*, 2000) resulting in a growth arrest. As E2F7 expression is transcriptionally regulated by activating E2Fs such as, E2F1 (Di Stefano *et al.*, 2003; de Bruin *et al.*, 2003), the induction of growth arrest is also accompanied by the loss of E2F7 expression, resulting in the derepression of Sp1 expression and the potential activation of elements of the squamous differentiation program (Supplementary Figure S4 online).

The data from this study, and the work of others, would suggest that, although Sp1 is a key differentiation factor, the induction of Sp1 alone may not be sufficient to induce a complete squamous differentiation program. For example, although Sp1 is required for the transcription of a variety of squamous differentiation-specific genes (for example, *TG1* and *involucrin*) the differentiation-specific expression of these genes, in truth, is controlled by the combinatorial action of multiple transcription factors such as Sp1, AP2, Fra-1, POU2F3, FOXQ1, CREBP, Ets, or AP1 (Medvedev *et al.*, 1999; Popa *et al.*, 2004; Crish and Eckert, 2008; Sen *et al.*, 2009). Moreover, derepression of Sp1, following E2F7 knockdown, resulted in an induction of TG1, but not of involucrin, in proliferating human KCs. Thus, Sp1 may be sufficient for the activation of a subset of differentiation genes, but it is not sufficient for the activation of the complete program of squamous differentiation. This is consistent with a recent report in which it was shown that activation of a subset of squamous differentiation genes could be initiated by demethylase-mediated derepression of key differentiation factors such as POU2F3 (Skn-1a) or FOXQ1 (Sen *et al.*, 2009). Our findings combined with those of Sen *et al.* (2009) indicate that derepression of key differentiation-inducing factors by either transcriptional or epigenetic means may be a central regulatory event in the initiation of squamous differentiation.

The suppression of Sp1 transcription by E2F7 may explain why SCCs are insensitive to differentiation stimuli. Similar to most tumors, SCCs have a disrupted E2F:Rb axis characterized by constitutive activation of activating E2Fs, such as E2F1, resulting in the activation of E2F1 transcription (Dicker *et al.*, 2000; Endo-Munoz *et al.*, 2009). This in turn causes an induction of E2F7 transcription (Li *et al.*, 2008; Zalmas *et al.*, 2008; Endo-Munoz *et al.*, 2009). The increase in E2F1 and

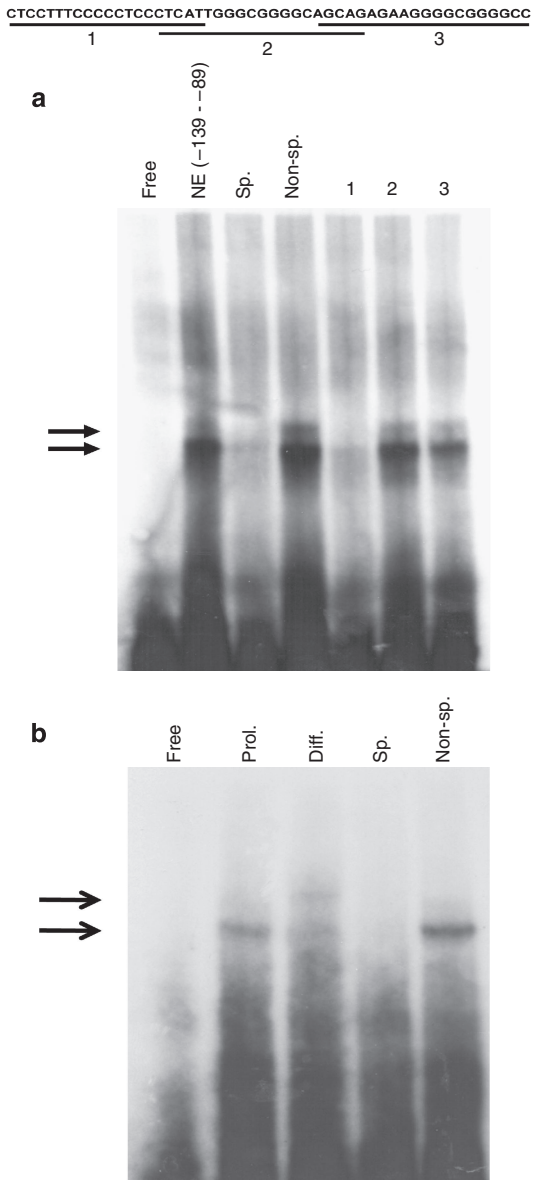

Figure 6. DNA binding to the E2F7 responsive site is restricted to −139 and −119 bp in the human Sp1 promoter. Nuclear extracts (NE) from human epidermal keratinocytes (HEKs) were prepared and incubated with ^{32}P-labeled fragment of double-strand DNA corresponding to the −139 to −89 bp region of the human Sp1 promoter and subjected to electromobility shift assays, as described in Supplementary Figure S3 online. (**a**) Specific DNA-binding complexes are shown (arrows). Competition with double-strand oligonucleotides corresponding to pieces of the −139 to −89 bp region and competition with an unrelated double-strand oligonucleotide (Non-sp) are shown. (**b**) Nuclear extracts from proliferative (Prol.) or confluent/differentiated (Diff.) HEKs were incubated with ^{32}P-labeled probe as in **a**. Representative gel shifts from three independent experiments are shown.

E2F7 expression would be predicted to render the SCCs insensitive to normal differentiation stimuli, leading to the continued repression of the Sp1 promoter by E2F7, even in the presence of a differentiation stimulus. Such a model is consistent with previous reports that SCCs and SCC cell lines are characterized by an unchanging Sp1/Sp3 ratio in response to a differentiation stimulus (Wong *et al.*, 2005). This is also supported by data showing that transient overexpression of Sp1 in SCCs can induce differentiation markers in growth-inhibited SCC cell lines (Wong *et al.*, 2005).

The molecular basis for E2F4 and E2F7 selective repression of the SP1 promoter involves a, to our knowledge, previously unreported, mechanism. In general, E2F family members exhibit similarity in their transcriptional targets (Kel *et al.*, 2001; Muller *et al.*, 2001). However, some reports have emerged showing that various E2F isoforms can bind selective genes (Kel *et al.*, 2001; Muller *et al.*, 2001). In particular, Araki *et al.* (2003) showed that E2F4 selectively bound the 5′-TTTCGCGGCAAA-3′ element within the E2F1 promoter, whereas E2F1 and E2F3 bound the 5′-TTTG GCGCGTAAA-3′ element of the same promoter during cell cycle traverse. However, in the studies of Araki *et al.* (2003) the E2F4-selective and E2F1/3-selective elements still conform to a consensus E2F-binding site (5′-TTTSGGCSMDR-3′; Kel *et al.*, 2001). In this study, we found the SP1 promoter to be E2F7 and E2F4 responsive. However, we were unable to show that E2F1 binds the SP1 promoter or that E2F1, 2, 3, or 5 were able to modulate SP1 promoter activity. This is consistent with the lack of consensus E2F-binding sites (5′-TT TSGGCSMDR-3′; Kel *et al.*, 2001) within the −139 to −89 bp region of the SP1 promoter. As E2F7 is known to bind consensus E2F elements within the E2F1 promoter, yet E2F7, but not E2F1, bound and repressed the Sp1 promoter in KCs, it is clear that the E2F7-responsive region within the SP1 promoter (5′-CTCCTTTC**CC**CCTC**CC**TCAT-3′) is, to our knowledge, previously unreported, and differs to the published E2F consensus sequence (Note: underlined nucleotides refer to identified essential binding elements in Supplementary Figure S3 online). However, although our ChIP analysis indicated that E2F7 binds the Sp1 promoter and our mutation analysis indicated that mutation of specific bases within the region resulted in the derepression of Sp1 transcription, we did not determine whether E2F7 bound the DNA directly or whether E2F7 binding was mediated *via* interacting partner protein(s).

MATERIALS AND METHODS

Tissue culture of epidermal KCs

Isolation and culture of normal HEKs from neonatal foreskins have been described elsewhere (Jones *et al.*, 1997). Briefly, HEK cultures were grown and maintained in low-calcium (0.05 mM Ca^{2+} final concentration) serum-free KC culture medium (Invitrogen, Sydney, NSW, Australia). Culture of HaCat cells was as described before (Dicker *et al.*, 2000). Subconfluent cultures of early passage (passage 2–3) HEKs were collected when proliferative cells were required. HEK cultures maintained at confluence for at least 48 hours were collected when growth-arrested, differentiated KCs were required (Dicker *et al.*, 2000).

Protein isolation and western blotting

Total cellular protein was collected and quantified as described (Brinkmann *et al.*, 2001). Protein (5 μg) was then electrophoresed on either a 10 or 7.5% SDS-PAGE gel and transferred onto polyvinylidene difluoride membrane (Brinkmann *et al.*, 2001). Immunodetection of cdc2 (1:1,000; Ab-1, PC25; Oncogene Science,

Cambridge, MA), involucrin (1:1,000; Sigma-Aldrich, Sydney, NSW, Australia; I-9018), or Sp1 (1:1,000; 07-124; Upstate Biotechnology, Sydney, NSW, Australia) were performed and visualized by standard techniques (Dahler *et al.*, 1998; Serewko *et al.*, 2002; Wong *et al.*, 2005).

ChIP assays

HEKs were trypsinized and ChIP was performed for IgG, E2F1, E2F4, and Sp1, as previously described (Wong *et al.*, 2005). In addition, ChIP assays were also performed using an anti-E2F7 antibody (2 μg per sample, sc-66870, Santa Cruz Biotechnology, Santa Cruz, CA). Primers for the amplification of the human E2F7 (125 bp product) and Sp1 promoters (174 bp product) were as follows: E2F7 forward primer 5′-AGGAACCGCCGCCGTTGTTCCCGT-3′, reverse primer 5′-GCTGCCTGCAAAGTCCCGGCCACT-3′ (Zalmas *et al.*, 2008), Sp1 forward primer 5′-ATATATACGCGTTAGGTTGGGCTTGTGG CG-3′, reverse primer 5′-ATATATCTCGAGTGGCAGCTGAGGGA CAAG-3′. In all instances, an IgG negative control and a positive control (10% of the input cellular extract) were analyzed at the same time. Promoter fragments were amplified for 35 cycles and then run on a 1.8% agarose gel. Quantitation of ChIP data for the Sp1 promoter studies was estimated by comparing the amount of Sp1 promoter PCR product in the ChIP extract relative to the same product present in the ''Input''.

RNA isolation and RT-PCR

The isolation of RNA and the preparation of single-strand cDNA has been described (Walshe *et al.*, 2007). Estimation of actin, E2F4, TG1, involucrin, Sp1, and E2F7 mRNA expression was performed by real-time quantitative PCR (Endo-Munoz *et al.*, 2009; Walshe *et al.*, 2007). The primer sequences for Sp1 and E2F4 are as follows: Sp1 forward 5′-CAAAAAGAAGGAGAGCAAAA-3′, reverse 5′-GATGG CTTGAGTTGTAAAGG-3′, E2F4 quantitative RT-PCR primer targeting p107/p130 binding was purchased from SABiosciences (Frederick, MD) (PPH00362E-200).

Immunohistochemistry

Immunohistochemistry was performed on formaldehyde-fixed paraffin-embedded sections of human skin (Serewko *et al.*, 2002; Wong *et al.*, 2005). Human skin was collected in accordance with the institutional ethics committee (Princess Alexandra Hospital). Immunohistochemical detection of E2F4 (1:500; sc-866, Santa Cruz) used similar methodology as for Sp1 (Wong *et al.*, 2005).

Promoter cloning, transfection, and reporter assays

A fragment comprising −1.549 Kb to +35 bp of the human *Sp1* gene (Nicolas *et al.*, 2001) was generated by amplification from human genomic DNA and directionally cloned into the PGL2 basic promoter construct (Promega, Sydney, NSW, Australia). From this, were generated a series of sequence-verified Sp1 promoter deletions, by PCR amplification, spanning −1549 bp (Sp1-Luc), −1183, −721, −358, −239, −186, −139, −89, and −39 bp. In addition, we introduced a scanning series of two base mutations into the −139 to −119 bp region of the Sp1-Luc construct using a PCR-based method (Finnzymes, no. F-541; Genesearch, Arundel, Queensland, Australia). Transfection protocols, and luciferase and chloramphenicol acetyl transferase reporter assays have been described (Wong *et al.*, 2003). All transfections are normalized for transfection efficiency by reference to co-transfected β-actin–chloramphenicol acetyl transferase promoter activity.

Overexpression, shRNA knockdown, and sorting

Expression plasmids for E2F4 and E2F7b have been described (Wong *et al.*, 2003; Endo-Munoz *et al.*, 2009). E2F7 shRNA expression plasmids and knockdown efficiency have been described previously (Endo-Munoz *et al.*, 2009). shRNA clones against E2F4 and expressing green fluorescent protein were purchased from SABiosciences. shRNA constructs tested (D1–D4) knocked down E2F4 mRNA expression in KCs to 54 ± 4, 28 ± 2, 96 ± 9, and 47 ± 2% of shRNA control-transfected KCs, respectively. In the instance of the E2F4 and E2F7 overexpression plasmids, the KCs were co-transfected with a green fluorescent protein expression plasmid (Smith *et al.*, 2004). Transfected cells were isolated by flow cytometry 48 hours after transfection (Endo-Munoz *et al.*, 2009) based on green fluorescent protein expression.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Apt D, Watts RM, Suske G *et al.* (1996) High Sp1/Sp3 ratios in epithelial cells during epithelial differentiation and cellular transformation correlate with the activation of the HPV-16 promoter. *Virology* 224:281–91

Araki K, Nakajima Y, Eto K *et al.* (2003) Distinct recruitment of E2F family members to specific E2F-binding sites mediates activation and repression of the E2F1 promoter. *Oncogene* 22:7632–41

Banks EB, Crish JF, Welter JF *et al.* (1998) Characterization of human involucrin promoter distal regulatory region transcriptional activator elements-a role for Sp1 and AP1 binding sites. *Biochem J* 331:61–8

Brinkmann H, Dahler AL, Popa C *et al.* (2001) Histone hyperacetylation induced by histone deacetylase inhibitors is not sufficient to cause growth inhibition in human dermal fibroblasts. *J Biol Chem* 276: 22491–9

Chen TT, Wu RL, Castro-Munozledo F *et al.* (1997) Regulation of K3 keratin gene transcription by Sp1 and AP-2 in differentiating rabbit corneal epithelial cells. *Mol Cell Biol* 17:3056–64

Crish JF, Eckert RL (2008) Synergistic activation of human involucrin gene expression by Fra-1 and p300–evidence for the presence of a multiprotein complex. *J Invest Dermatol* 128:530–41

Dahler AL, Jones SJ, Dicker AJ *et al.* (1998) Keratinocyte growth arrest is associated with activation of a transcriptional repressor element in the human cdk1 promoter. *J Cell Physiol* 177:474–82

de Bruin A, Maiti B, Jakoi L *et al.* (2003) Identification and characterization of E2F7, a novel mammalian E2F family member capable of blocking cellular proliferation. *J Biol Chem* 278:42041–9

DeGregori J, Johnson DG (2006) Distinct and overlapping roles for E2F family members in transcription, proliferation and apoptosis. *Curr Mol Med* 6:739–48

Dicker AJ, Popa C, Dahler AL *et al.* (2000) E2F-1 induces proliferation-specific genes and suppresses squamous differentiation-specific genes in human epidermal keratinocytes. *Oncogene* 19:2887–94

Di Stefano L, Jensen MR, Helin K (2003) E2F7, a novel E2F featuring DP-independent repression of a subset of E2F-regulated genes. *EMBO J* 22:6289–98

Eckert RL, Crish JF, Banks EB *et al.* (1997) The epidermis: genes on – genes off. *J Invest Dermatol* 109:501–9

Eckert RL, Crish JF, Robinson NA (1998) The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation. *Physiol Rev* 77:397–424

Endo-Munoz L, Dahler AL, Teakle N *et al.* (2009) E2F7 can regulate proliferation, differentiation, and apoptotic responses in human keratinocytes: implications for cutaneous squamous cell carcinoma formation. *Cancer Res* 69:1800–8

Jang SI, Steinert PM (2002) Loricrin expression in cultured human keratinocytes is controlled by a complex interplay between transcription factors of the Sp1, CREB, AP1, and AP2 families. *J Biol Chem* 277:42268–79

Johnson DG, DeGregori J (2006) Putting the oncogenic and tumor suppressive activities of E2F into context. *Curr Mol Med* 6:731–8

Jones SJ, Dicker AJ, Dahler AL *et al.* (1997) E2F as a regulator of keratinocyte proliferation: implications for skin tumor development. *J Invest Dermatol* 109:187–93

Kel AE, Kel-Margouli OV, Farnham PJ *et al.* (2001) Computer-assisted identification of cell cycle-related genes: new targets for E2F transcription factors. *J Mol Biol* 309:99–120

Lania L, Majello B, DeLuca P (1997) Transcriptional regulation by the Sp family proteins. *Int J Biochem Cell Biol* 29:1313–23

Lee JH, Jang SI, Yang JM *et al.* (1996) The proximal promoter of the human transglutaminase 3 gene. Stratified squamous epithelial-specific expression in cultured cells is mediated by binding of Sp1 and Ets transcription factors to a proximal promoter element. *J Biol Chem* 271:4561–8

Li J, Ran C, Li E *et al.* (2008) Synergistic function of E2F7 and E2F8 is essential for cell survival and embryonic development. *Dev Cell* 14:62–75

Medvedev A, Saunders NA, Chistokhina A *et al.* (1999) Regulation of the transglutaminase I gene. Identification of DNA elements involved in its transcriptional control in tracheobronchial epithelial cells. *J Biol Chem* 274:3887–96

Muller H, Bracken AP, Vernell R *et al.* (2001) E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. *Genes Dev* 15:267–85

Nicolas M, Noe V, Ciudad CJ (2003) Transcriptional regulation of the human Sp1 gene promoter by the specificity protein (Sp) family members nuclear factor Y (NF-Y) and E2F. *Biochem J* 371:265–75

Nicolas M, Noe V, Jensen KB *et al.* (2001) Cloning and characterization of the 5′-flanking region of the human transcription factor Sp1 gene. *J Biol Chem* 276:22126–32

Paramio JM, Segrelles C, Casanova ML *et al.* (2000) Opposite functions for E2F1 and E2F4 in human epidermal keratinocyte differentiation. *J Biol Chem* 275:41219–26

Park GT, Morasso MI (1999) Regulation of the Dlx3 homeobox gene upon differentiation of mouse keratinocytes. *J Biol Chem* 274:3887–96

Phillips MA, Jessen BA, Lu Y *et al.* (2004) A distal region of the human TGM1 promoter is required for expression in transgenic mice and cultured keratinocytes. *BMC Dermatol* 4:2

Pierce AM, Schneider-Broussard R, Gimenez-Conti IB *et al.* (1999) E2F1 has both oncogenic and tumor-suppressive properties in a transgenic model. *Mol Cell Biol* 19:6408–14

Popa C, Dahler AL, Serewko-Auret MM *et al.* (2004) AP-2 transcription factor family member expression, activity, and regulation in human epidermal keratinocytes *in vitro*. *Differentiation* 72:185–97

Sen GL, Webster DE, Barragan DI *et al.* (2009) Control of differentiation in a self-renewing mammalian tissue by the histone demethylase JMJD3. *Genes Dev* 22:1865–70

Serewko MM, Popa C, Dahler AL *et al.* (2002) Alterations in gene expression and activity during squamous cell carcinoma development. *Cancer Res* 62:3759–65

Smith L, Dahler AL, Popa C *et al.* (2004) Modulation of proliferation-specific and differentiation-specific markers in human keratinocytes by SMAD7. *Exp Cell Res* 294:356–65

Walshe J, Serewko-Auret MM, Teakle N *et al.* (2007) Inactivation of glutathione peroxidase activity contributes to UV-induced squamous cell carcinoma formation. *Cancer Res* 67:4751–8

Wong CF, Barnes LM, Dahler AL *et al.* (2003) E2F modulates keratinocyte squamous differentiation: implications for E2F inhibition in squamous cell carcinoma. *J Biol Chem* 278:28516–22

Wong CF, Barnes LM, Dahler AL *et al.* (2005) E2F suppression and Sp1 overexpression are sufficient to induce the differentiation-specific marker, transglutaminase type 1, in a squamous cell carcinoma cell line. *Oncogene* 24:3525–34

Wong CF, Barnes LM, Smith L *et al.* (2004) E2F6: a member of the E2F family that does not modulate squamous differentiation. *Biochem Biophys Res Commum* 324:497–503

Wu RL, Chen TT, Sun TT (1994) Functional importance of an Sp1- and an NFkB-related nuclear protein in a keratinocyte-specific promoter of rabbit K3 keratin gene. *J Biol Chem* 269:28450–9

Zalmas LP, Zhao X, Graham AL *et al.* (2008) DNA-damage response control of E2F7 and E2F8. *EMBO Rep* 9:252–9