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p21^{WAF1/Cip1} is a negative transcriptional regulator of *Wnt4* expression downstream of Notch1 activation

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In keratinocytes, the cyclin/CDK inhibitor p21^{WAF1/Cip1} is a direct transcriptional target of Notch1 activation; loss of either the *p21* or *Notch1* genes expands stem cell populations and facilitates tumor development. The *Notch1* tumor-suppressor function was associated with down-regulation of Wnt signaling. Here, we show that suppression of Wnt signaling by Notch1 activation is mediated, at least in part, by down-modulation of *Wnts* gene expression. p21 is a negative regulator of *Wnts* transcription downstream of Notch1 activation, independently of effects on the cell cycle. More specifically, expression of the *Wnt4* gene is under negative control of endogenous p21 both in vitro and in vivo. p21 associates with the E2F-1 transcription factor at the *Wnt4* promoter and causes curtailed recruitment of c-Myc and p300, and histone hypoacetylation at this promoter. Thus, p21 acts as a selective negative regulator of transcription and links the Notch and Wnt signaling pathways in keratinocyte growth control.

[*Keywords:* Differentiation; stem cell potential; transcription; chromatin; E2F-1; c-Myc; p300]

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p21^{WAF1/Cip1} was originally identified as a downstream mediator of p53-induced growth arrest, a direct inhibitor of CDK activity (CKI), and a gene whose expression is induced with cellular senescence (Sherr and Roberts 1999). Above and beyond associating with cyclin/CDKs, p21 participates in a number of other specific protein–protein interactions and exerts anti- or proapoptotic functions that are cell type and context dependent. In addition, p21 has the potential of physically associating with transcription factors and coactivators, modulating their function (Dotto 2000). In particular, p21 can bind to the E2F-1 transcription factor, with a consequent inhibition of its activity (Delavaine and La Thangue 1999), and to the N terminus of c-Myc, suppressing c-Myc-dependent transcription by interference with c-Myc–Max association (Kitaura et al. 2000). Moreover, p21 can associate with the coactivator p300 and modulate transcription, depending on the nature of the core promoter (Perkins et al. 1997; Snowden et al. 2000). Recently, inducible overexpression of the p21 protein in a tumor cell line has revealed global changes in gene expression that

may impinge on cell survival (Chang et al. 2000). Little more is known of the biological significance of p21-dependent regulation of gene expression and to what extent it is linked to effects on the cell cycle.

Activation of Notch cell-surface receptors provides a highly conserved mechanism for control of cell-fate determination and differentiation (Artavanis-Tsakonas et al. 1999). The “canonical” pathway of Notch activation involves proteolytic cleavage and translocation of the Notch cytoplasmic domain to the nucleus, where it associates with the DNA-binding protein RBP-J κ (CBF-1, CSL), converting it from a repressor into an activator of transcription (Lai 2002). The best-characterized downstream targets of Notch/RBP-J κ are members of the HES and HERP families of basic helix–loop–helix (bHLH) transcriptional repressors (Iso et al. 2003). In primary keratinocytes, another direct target of Notch/RBP-J κ transcription is p21, which mediates Notch1-induced cell cycle withdrawal (Rangarajan et al. 2001).

Biologically, both Notch1 (Lowell et al. 2000) and p21 (Topley et al. 1999) have been reported to promote the commitment of keratinocyte stem cell populations to differentiation, and loss of either gene substantially enhances susceptibility to *ras*—or chemically induced carcinogenesis (Missero et al. 1996; Topley et al. 1999; Nicolas et al. 2003). Beside p21, Notch signaling affects

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other pathways with significant regulatory functions in keratinocyte growth and differentiation (Lefort and Dotto 2004). In particular, *Wnt* signaling is suppressed by Notch1 activation, and is elevated in keratinocytes and tumors as a consequence of loss of Notch1 function (Nicolas et al. 2003). An increase in *Wnt*/ β -catenin signaling is likely to be biologically significant, as it has been associated with maintenance of keratinocytes in their stem cell compartment (Zhu and Watt 1999) and a number of malignancies, including keratinocyte derived (Gat et al. 1998).

The mechanism for suppression of *Wnt* signaling by Notch1 activation in keratinocytes was not established. Here, we show that Notch1 activation down-regulates this pathway by suppressing *Wnts* gene expression, and that p21 is a key mediator of this negative regulation, at the transcription-chromatin level and separately from effects on the cell cycle.

Results

Activation of Notch signaling negatively regulates Wnts gene expression

We have shown previously that Notch1 activation suppresses *Wnt* signaling in keratinocytes (Nicolas et al. 2003). A possible underlying mechanism could be negative regulation of *Wnt* ligand expression. As an initial test of this possibility, we infected primary mouse keratinocytes with an adenovirus expressing activated Notch1 (Ad-NIC) versus GFP control (Ad-GFP), and measured transcription of specific *Wnt* family members by real-time RT-PCR analysis. As shown in Figure 1A, *Wnt3* and *Wnt4* expression was substantially down-regulated as a consequence of activated Notch1 expression.

To determine whether expression of these *Wnt* genes is also down-modulated by endogenous Notch signaling, we took two complementary approaches. In the first, to reproduce the coincidental up-regulation of Jagged 1/2 expression and Notch1 activation that occurs in differentiating cells of the upper epidermal layers (Rangarajan et al. 2001), we infected keratinocytes with an adenovirus expressing the Jagged-1 ligand. Even in this case, *Wnts* expression was significantly down-regulated (Fig. 1B). As a second approach, we assessed the consequences of deleting the *Notch1* gene. Induction of keratinocyte differentiation by increased extracellular calcium causes activation of the endogenous Notch-signaling pathway (Rangarajan et al. 2001). A substantial suppression of *Wnt3* and *Wnt4* expression was found in control keratinocytes upon induction of differentiation, while this suppression was significantly less in cells with an induced deletion of the *Notch1* gene (Fig. 1C). Consistent with these results, even *in vivo*, *Wnt3* and *Wnt4* expression was significantly higher in the epidermis of mice with a keratinocyte-specific *Notch1* deletion than in the controls (Fig. 1D).

To evaluate the consequences of a more complete suppression of Notch signaling, we examined keratinocytes with an induced deletion of the RBP-J κ gene, a key com-

mon downstream mediator of Notch effects on gene expression. Primary keratinocytes were prepared from mice homozygous for the RBP-J κ gene flanked by loxP sites (Yamamoto et al. 2003), and deletion of this gene was achieved by infection with a Cre-expressing adenovirus (Ad-Cre), as we recently reported (Mammucari et al. 2005). In contrast to control cells, keratinocytes with deletion of the RBP-J κ gene showed no suppression of *Wnt3* and *Wnt4* expression in response to differentiation, with expression of *Wnt4* even increasing at later times (Fig. 1E).

Negative regulation of Wnt signaling by Notch1 activation is mediated by down-modulation of Wnts gene expression

Down-modulation of *Wnt* expression may be required for the normal response of keratinocytes to increased Notch signaling, and more specifically, account for the observed suppression of β -catenin activation. Transgenic mice with keratinocyte-specific *Wnt3* overexpression exhibit an aberrant skin phenotype resulting from aberrant differentiation (Millar et al. 1999). In primary keratinocytes derived from these mice, activated Notch1 failed to induce p21 expression, as it did instead, in cells derived from littermate controls (Fig. 2A). Another consequence of Notch activation is down-modulation of integrins expressed in proliferating keratinocytes of the basal layer (Rangarajan et al. 2001). Activated Notch1 caused the expected suppression of integrin α_6 expression in control keratinocytes, while it caused a slight induction rather than suppression in the *Wnt3* transgenic cells (Fig. 2B). Not all aspects of the Notch response were blocked in these cells. In fact, keratin 1, a differentiation marker of the intermediate epidermal layers controlled by Notch through a RBP-J κ independent mechanism (Rangarajan et al. 2001), was induced by activated Notch1 to an even greater extent in the *Wnt3* overexpressing keratinocytes than in the controls (Fig. 2C).

In parallel with these effects, we assessed whether persistent *Wnt3* expression counteracted suppression of β -catenin activation by Notch signaling. As expected, levels of the unphosphorylated activated form of β -catenin (van Noort et al. 2002) were significantly decreased by activated Notch1 expression in wild-type keratinocytes, while no decrease occurred in the *Wnt3* transgenic cells (Fig. 2D). To assess whether ectopic *Wnt4* expression can similarly counteract the Notch1 effects, primary keratinocytes were infected with a *Wnt4*-transducing or control retroviruses. In the *Wnt4*-overexpressing cells, activated Notch1 failed to reduce levels of β -catenin activation, whereas it reduced them in the control cells (Fig. 2E).

Hes-1 and p21 exert additive suppression of Wnt gene expression

Hes family members are direct targets of Notch/RBP-J κ activation and negatively modulate gene expression (Iso et al. 2003). Nucleotide sequence analysis indicated that

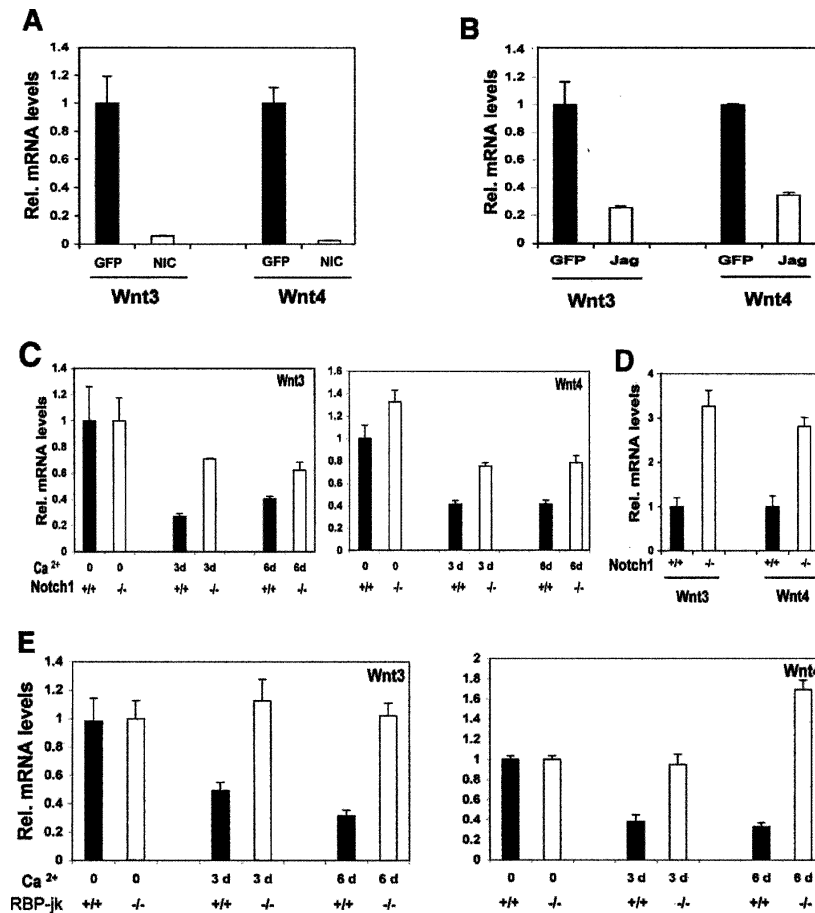


Figure 1. Notch1 activation suppresses *Wnts* gene expression. (A) Down-modulation of *Wnts* gene expression by activated Notch1. Primary mouse keratinocytes were infected with a recombinant adenovirus expressing the constitutive activated form of Notch1 (NIC) or an adenovirus expressing GFP (GFP) for 24 h. mRNA levels for *Wnt3* and *Wnt4* were quantified by real-time RT-PCR. Values are expressed as relative arbitrary units after normalization for GAPDH mRNA levels. (B) Down-modulation of *Wnts* gene expression in response to increased Jagged1 expression. Primary keratinocytes were infected with a recombinant adenovirus expressing full-length Jagged1 (Jag) or the Ad-GFP control (GFP) for 24 h, followed by mRNA quantification of *Wnt3* and *Wnt4* as in A. (C) Differential down-modulation of *Wnts* gene expression upon induction of differentiation of *Notch1*^{+/+} versus *Notch1*^{-/-} keratinocytes. Primary keratinocytes derived from mice with the *Notch1* gene flanked by loxP sites were infected with a Cre-expressing adenovirus to delete the endogenous *Notch1* gene as previously described (Rangarajan et al. 2001). Parallel cultures of the same cells were infected with Ad-GFP as controls. Three days after infection, keratinocytes were induced to differentiate by exposure to elevated extracellular calcium for 3 d (3d) or 6 d (6d). Total RNAs from *Notch1*^{+/+} versus *Notch1*^{-/-} cells were analyzed by real-time RT-PCR for *Wnt3* and *Wnt4* expression as in A and B. (D) Increased *Wnts* expression in the epidermis of mice with an induced deletion of the *Notch1* gene. Total RNA was prepared from the isolated epidermis of

mice with the *Notch1* gene flanked by loxP sites and carrying a keratinocyte-specific Cre transgene versus control Cre-negative littermates (Rangarajan et al. 2001). *Wnt3* and *Wnt4* mRNA levels were quantified by real-time RT-PCR as before. (E) Differential down-modulation of *Wnts* gene expression upon induction of differentiation of *RBP-Jk*^{+/+} versus *RBP-Jk*^{-/-} keratinocytes. Primary keratinocytes derived from mice with the *RBP-Jk* gene flanked by loxP sites were infected with a Cre-expressing adenovirus to delete the endogenous *RBP-Jk* gene as previously described (Mammucari et al. 2005). Parallel cultures of the same cells were infected with Ad-GFP as controls. Three days after infection, keratinocytes were induced to differentiate by exposure to elevated extracellular calcium for 3 d (3d) or 6 d (6d). Total RNAs from *RBP-Jk*^{+/+} versus *RBP-Jk*^{-/-} cells were analyzed by real-time RT-PCR for *Wnt3* and *Wnt4* expression as in A–D.

the promoter regions of the *Wnt3* and *Wnt4* genes contain multiple Hes-1-binding sites. Chromatin immunoprecipitation (ChIP) of keratinocytes infected with a Hes-1-expressing adenovirus (Ad-Hes1) showed that the Hes-1 protein can bind specifically the endogenous *Wnt4* gene at the predicted sites (Supplementary Fig. 1). In parallel with this finding, infection of keratinocytes with the Ad-Hes1 virus resulted in down-modulation of *Wnt3* and *Wnt4* expression, which was, however, less than that caused by activated Notch1 (Fig. 3A). Other Hes family members, such as Hey-1 and Hey-2, are also induced in keratinocytes as a consequence of Notch activation (Mammucari et al. 2005). However, infection of keratinocytes with Hey-1 and Hey-2-expressing adenoviruses caused no suppression of *Wnt3* and *Wnt4* expression (Fig. 3A).

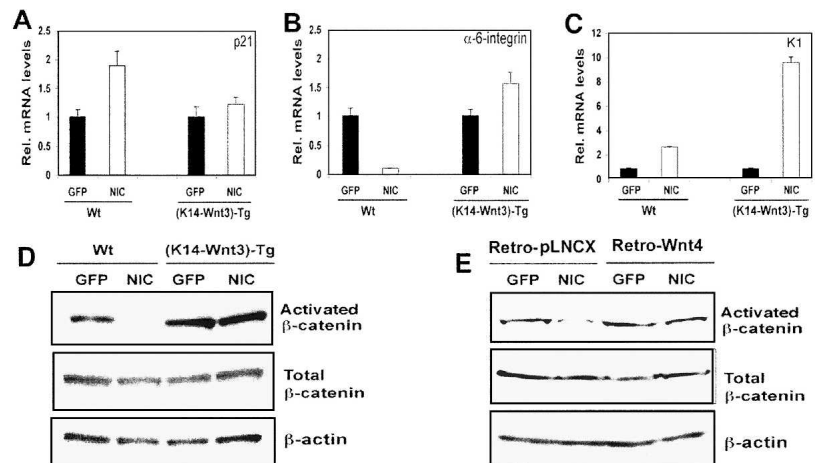
Given that p21 is another direct target of Notch1 activation in keratinocytes (Rangarajan et al. 2001), an attractive possibility was that p21 contributes to down-

modulation of *Wnts* expression. Analysis of keratinocytes infected with an adenovirus expressing the full-length p21 protein (Ad-p21F) versus Ad-GFP control showed that this is indeed the case (Fig. 3A). Moreover, coinfection of keratinocytes with the Ad-Hes1 and Ad-p21F viruses at lower multiplicities showed that concomitantly increased expression of the two proteins had additive effects on down-modulation of *Wnts* expression (Fig. 3B).

To determine whether endogenous p21 is a mediator of down-modulation of *Wnt* expression by activated Notch1, primary keratinocytes derived from *p21*^{-/-} and *p21*^{+/+} mice were infected with the Ad-NIC versus Ad-GFP viruses. As expected, induction of *Hes-1* expression by activated Notch1 occurred to a similar extent in *p21*^{+/+} and *p21*^{-/-} cells (Fig. 3C). In contrast, activated Notch1 expression caused much lesser down-regulation of *Wnt3* and *Wnt4* expression in *p21*^{-/-} than in *p21*^{+/+} keratinocytes (Fig. 3D).

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Figure 2. Notch1 suppresses *Wnt* signaling by down-modulating *Wnts* gene expression. (A–C) Differential regulation of p21, integrin α_6 , and K1 expression by Notch1 in control versus K14-*Wnt3* transgenic keratinocytes. Primary keratinocytes derived from K14-*Wnt3* transgenic mice (Millar et al. 1999) versus transgenic-negative littermate controls were infected with the Ad-NIC (NIC) versus Ad-GFP (GFP) viruses, followed by mRNA quantification of p21, integrin α_6 , and K1 gene, respectively, by real-time RT-PCR. Values are expressed as relative folds of expression, after normalization for GAPDH mRNA levels. (D) Differential down-regulation of activated- β -catenin levels by activated Notch1 in control versus K14-*Wnt3* transgenic keratinocytes. Primary keratinocytes derived from K14-*Wnt3* transgenic mice versus transgenic-negative littermate controls were infected with the Ad-NIC (NIC) versus Ad-GFP (GFP) viruses. Total cell extracts were analyzed by immunoblotting with antibodies specific for the unphosphorylated activated form of β -catenin (van Noort et al. 2002), total- β -catenin or β -actin. (E) Differential down-regulation of activated- β -catenin levels by activated Notch1 in control versus *Wnt4*-overexpressing keratinocytes. Primary keratinocytes were infected with a *Wnt4* transducing (retro-*Wnt4*) versus vector control (retro-pLNCX) retroviruses. Two days after infection, keratinocytes were subjected to selection for G418 resistance, followed by infection with the Ad-NIC (NIC) versus Ad-GFP (GFP) viruses. Total cell extracts were analyzed by immunoblotting for levels of activated β -catenin as in D.



p21 levels increase in cultured primary keratinocytes at early times of calcium-induced differentiation as a consequence of increased Notch1 signaling (Rangarajan et al. 2001). Down-modulation of *Wnt3* expression that accompanies differentiation was found to occur to a substantially lesser extent in keratinocytes lacking the *p21* gene (Fig. 3E). *Wnt4* was already expressed at significantly higher levels in *p21*^{-/-} than in *p21*^{+/+} keratinocytes under basal growing conditions; upon induction of differentiation, *Wnt4* expression was further markedly increased in the *p21*^{-/-} cells, while it decreased in the controls (Fig. 3F). Even in the intact epidermis of *p21*^{-/-} mice in vivo, there was significantly higher *Wnt4* expression than in the control, while no such up-regulation was observed with *Wnt3* (Fig. 3G).

p21 suppresses *Wnt* gene transcription separately from effects on the cell cycle

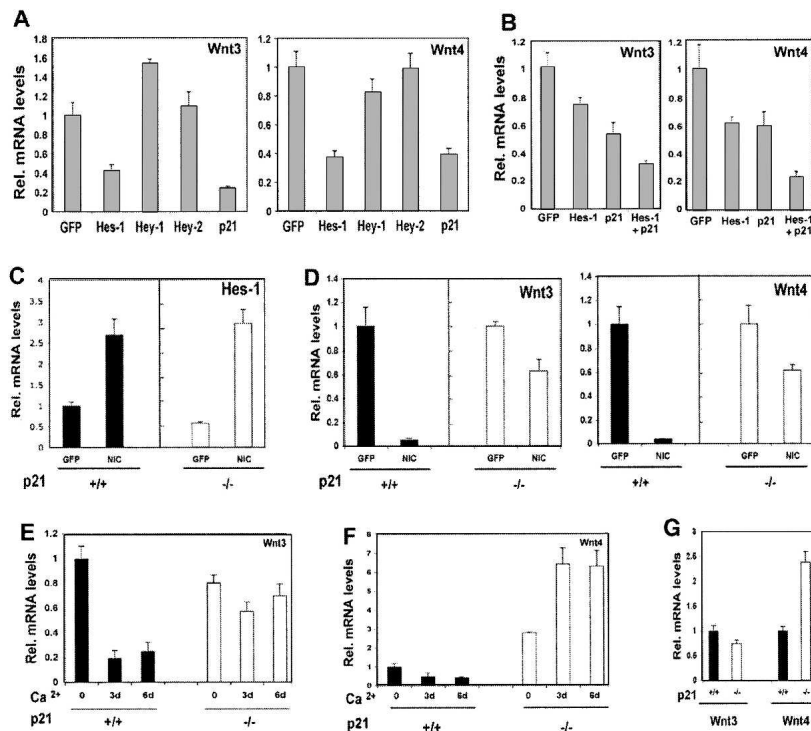
The p21 suppressive effects on *Wnt* gene expression could be an indirect consequence of p21-induced growth arrest. To investigate whether or not this is the case, primary keratinocytes were infected in parallel with adenoviruses expressing the p21 N terminus domain or the unrelated CKIs p16^{Ink4a} or p27^{Kip1}, all of which suppress proliferation without affecting differentiation (Di Cunto et al. 1998). Expression of these proteins caused no down-modulation of *Wnt3* or *Wnt4* expression, in contrast to the suppressive effects exerted by the full-length p21 protein (Fig. 4A).

Suppressive effects similar to those of full-length p21 were also observed after expression of the p21 C terminus domain, which lacks cyclin/CDK binding, but retains the capability to directly modulate proteins involved in DNA replication as well as transcription

(Dotto 2000; data not shown). Consistent with its possible role in transcription, the ability of p21 to down-modulate endogenous *Wnt3* or *Wnt4* expression was counteracted substantially by treatment with a histone deacetylase inhibitor, M334 (a derivative of trichostatin A), with the counteracting effects of this compound being abrogated by concomitant treatment with a specific antagonist, ITSA1 (Fig. 4B; Koeller et al. 2003).

p21 binds to the *Wnt4* promoter in association with E2F-1, causing specific curtailment of *c-Myc* and *p300* at this promoter

While endogenous p21 is required for the effective down-modulation of both *Wnt3* and *Wnt4* expression by Notch1 or differentiation, in the skin of *p21*^{-/-} mice and in primary *p21*^{-/-} keratinocytes under basal conditions, only *Wnt4* is up-regulated (Fig. 3F,G). Thus, for further mechanistic insights, we focused on control of this gene. One proposed mechanism whereby p21 can specifically suppress transcription is through physical association with the transcription factor E2F-1, with E2F-1 providing a bridge to the DNA and the basal transcription apparatus (Delavaine and La Thangue 1999). Computational sequence analysis revealed the presence of two fully conserved E2F-1-binding sites in the TATA box-proximal region of the *Wnt4* promoter (at position -628 and +33 relative to the TATA box) (Fig. 5A). To assess whether the E2F-1 protein indeed binds to this region, ChIP assays were performed with antibodies against this protein. Quantitative real-time PCR showed binding of endogenous E2F-1 protein to the TATA box-proximal region of the *Wnt4* promoter containing the predicted E2F-1-binding sites, while no binding was detected to an upstream region (Fig. 5B). E2F-1 binding to the *Wnt4* pro-



units relative to Ad-GFP-infected control cells. (D) Differential suppression of *Wnts* gene expression by activated Notch1 in $p21^{+/+}$ versus $p21^{-/-}$ keratinocytes. The same samples utilized in C were analyzed for levels of *Wnt3* and *Wnt4* mRNA expression by real-time RT-PCR. Values are expressed as arbitrary units relative, in each case, to Ad-GFP-infected control cells. (E,F) Differential expression of *Wnt3* and *Wnt4* gene expression upon induction of differentiation of $p21^{+/+}$ versus $p21^{-/-}$ keratinocytes. Primary keratinocytes derived from $p21^{+/+}$ and $p21^{-/-}$ mice were either kept under growing conditions or induced to differentiate by exposure to elevated extracellular calcium for 3 d (3d) or 6 d (6d). *Wnts* mRNA levels were quantified by real-time RT-PCR as before. Values are indicated in arbitrary units relative to untreated $p21^{+/+}$ cells. (G) Increased *Wnt4* mRNA expression in the epidermis of $p21^{-/-}$ mice. Total RNA was prepared from the separated epidermis of $p21^{+/+}$ versus $p21^{-/-}$ mice and *Wnt3* and *Wnt4* mRNA levels were quantified as before.

motor was unaffected by increased p21 expression (Fig. 5B), consistent with previous results that p21 does not cause a reduction of E2F-1 DNA-binding activity (Delavaine and La Thangue 1999).

In parallel with the above results, ChIP assays with anti-p21 antibodies showed that this protein binds specifically to the E2F-1-binding region of the *Wnt4* promoter, and not to the upstream region already in cells

Figure 3. p21, like Hes-1, is a negative regulator of *Wnts* gene expression. (A) Hes-1 and $p21^{WAF1/Cip1}$ are negative regulators of *Wnts* gene expression. Primary keratinocytes were infected with adenoviruses expressing the Hes-1, Hey-1, Hey-2, or p21 proteins for 24 h at a multiplicity of 100, followed by mRNA quantification of *Wnt3* and *Wnt4* as before. (B) Additive suppressive effects of Hes-1 and p21 on *Wnts* gene expression. Primary keratinocytes were infected with the adenoviruses expressing Hes-1 (Ad-Hes-1) or p21 (Ad-p21F) viruses either alone or in combination at a multiplicity of 50. Ad-GFP (GFP) was used as a control and added to the Ad-Hes-1 or Ad-p21F viruses when they were used alone, to ensure that cells received, in all cases, the same amount of viral particles (total multiplicity of infection: 100). Quantification of *Wnt3* and *Wnt4* mRNA expression by real-time RT-PCR was carried out as before. (C) Similar induction of Hes-1 expression by activated Notch1 in $p21^{+/+}$ versus $p21^{-/-}$ keratinocytes. Primary keratinocytes derived from $p21^{+/+}$ and $p21^{-/-}$ mice of the same genetic background (Sencar) were infected with the Ad-NIC (NIC) versus Ad-GFP (GFP) viruses. mRNA levels of *Hes-1* were determined by real-time RT-PCR as before. Values are expressed as arbitrary

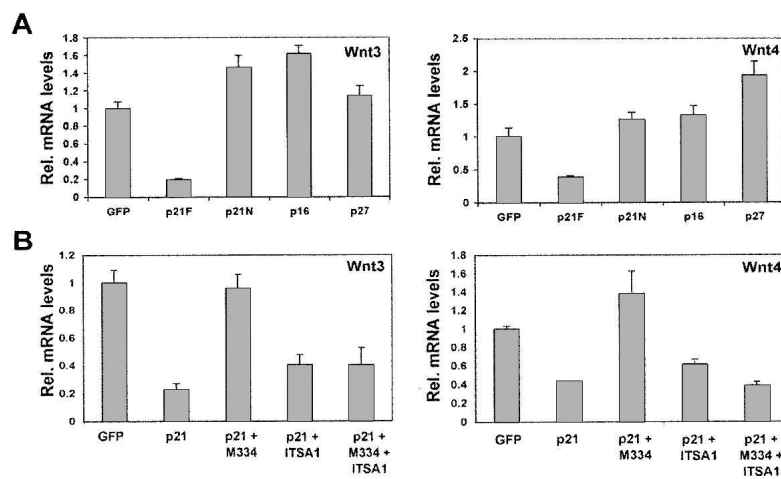
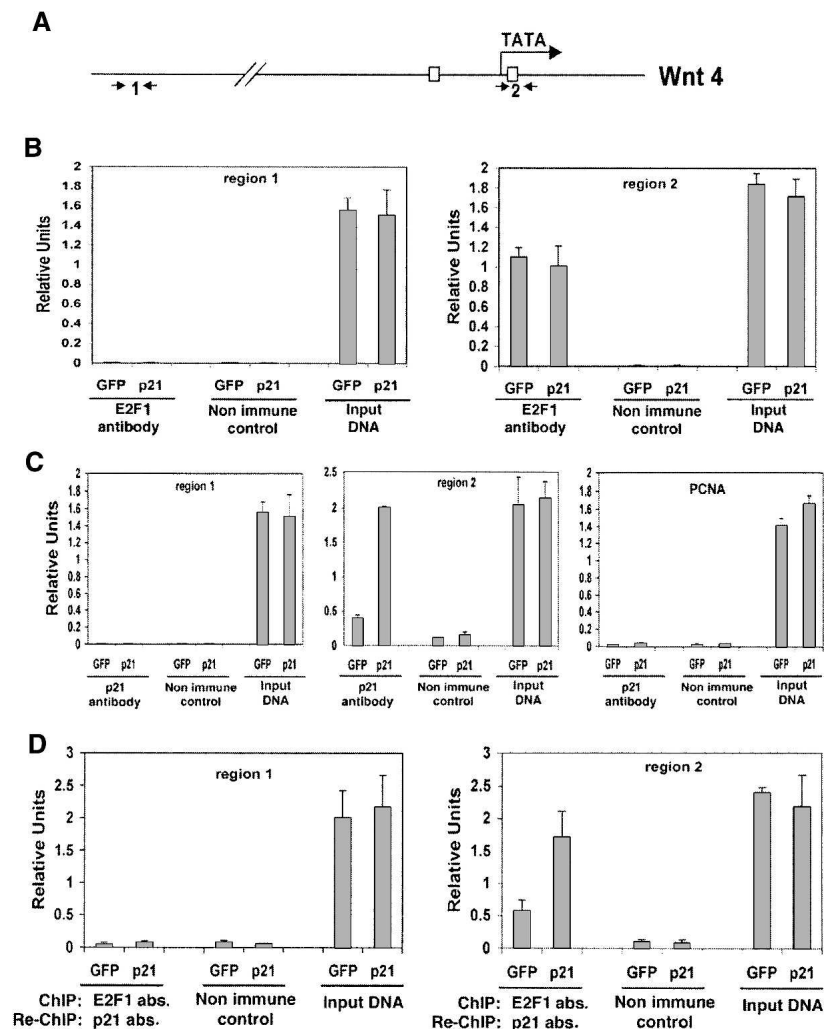


Figure 4. Increased p21 expression suppresses *Wnts* gene expression independently of effects on the cell cycle and at the transcription level. (A) Down-modulation of *Wnts* expression by p21 unlinked from the cell cycle. Primary keratinocytes were infected with adenoviruses expressing GFP (GFP), full-length p21 protein (p21F), the p21 N domain (p21N), p16^{INK4a} (p16), and p27^{Kip1} (p27) for 24 h, followed by *Wnt3* and *Wnt4* mRNA quantification as before. (B) Counteracting effects of histone deacetylase inhibition on *Wnts* suppression by p21. Primary keratinocytes were infected with the Ad-GFP control or the p21-expressing adenovirus (p21F) with or without concomitant treatment with the histone deacetylase inhibitor M334 (5 μ M, an amide analog of Trichostatin A [Jung et al. 1999]) plus/minus an antagonist of this compound, ITSA1 (10 μ M, inhibitor of Trichostatin A 1 [Koeller et al. 2003]). *Wnt3* and *Wnt4* mRNA levels were quantified by real-time RT-PCR as before.

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Figure 5. p21 binds to the endogenous *Wnt4* promoter in association with E2F-1. (A) Map of the *Wnt4* promoter region with indication of the E2F-1-binding sites (open squares) and the position of the oligonucleotide primers utilized for the ChIP analysis described below (nucleotides -6839 to -6718 [1]; nucleotides +21 to +121 [2] relative to TATA box). (B) Specific binding of endogenous E2F-1 to the TATA box-proximal region of the *Wnt4* gene. Primary keratinocytes were infected with the Ad-GFP or Ad-p21F adenoviruses for 24 h. Cells were analyzed by ChIP with antibodies specific for E2F-1 or nonimmune controls, followed by real-time PCR amplification of the indicated regions of the *Wnt4* gene. Chromatin preparations prior to the immunoprecipitation reaction were similarly processed and analyzed as controls for input DNA. (C) Specific binding of p21 to the TATA box-proximal region of the *Wnt4* gene. Primary keratinocytes were infected with the Ad-GFP or Ad-p21F adenoviruses and analyzed by ChIP with antibodies against the p21 protein and corresponding controls as in the previous experiments. Immunoprecipitates were analyzed by real-time PCR for the indicated regions of the *Wnt4* gene as well as for the E2F-1-binding region of the *PCNA* gene (nucleotides -398 to -270 relative to the AUG). (D) Association of the p21 and E2F-1 proteins at the TATA box-proximal region of the *Wnt4* gene. Primary keratinocytes were infected with the Ad-GFP or Ad-p21F adenoviruses and subjected to the ChIP procedure with sequential immunoprecipitation with antibodies against the E2F-1 and p21 proteins or corresponding nonimmune controls. Immunoprecipitates were analyzed by real-time PCR for the indicated regions of the *Wnt4* gene.



under basal conditions, with higher levels of binding in p21-overexpressing cells (Fig. 5C). In contrast to the *Wnt4* promoter, there was no detectable binding of p21 to the promoter for the *PCNA* gene (Fig. 5C), a "classical" E2F-1 target (Polager et al. 2002) whose expression in keratinocytes is unaffected by p21 (our unpublished observations).

To assess a possible physical association between the E2F-1 and p21 proteins at the *Wnt4* promoter, a "Re-ChIP" experiment was performed, whereby cell extracts were sequentially immunoprecipitated with anti-E2F-1 and anti-p21 antibodies, followed by real-time PCR analysis of the recovered DNA. The results were indicative of an E2F-1-p21 complex at the proximal region of the *Wnt4* promoter already in control keratinocytes infected with the Ad-GFP virus and therefore with no exogenous overexpression of p21 (Fig. 5D). A significant increase of this complex was found in p21-overexpressing cells. In contrast, no binding was detected in either control or p21-overexpressing keratinocytes at the *Wnt4* distal region.

Increased levels of p21/E2F-1 binding to the proximal region of the *Wnt4* promoter could result in the concomitant curtailment of positive regulators of transcription such as other transcription factors and/or coactivators. Beside E2F-1-binding sites, the *Wnt4* promoter region contains several fully conserved binding sites (E boxes; at position -1147, +18, +105, relative to the TATA box) for c-Myc (Fig. 6A). ChIP assays with antibodies against this protein indicated that c-Myc does indeed bind to the expected region of the *Wnt4* promoter with no binding further upstream, and that such binding is substantially reduced as a consequence of increased p21 expression (Fig. 6B). Concomitantly, ChIP assays with antibodies against the transcriptional coactivator p300 showed significant down-modulation of binding of this protein to the proximal *Wnt4* promoter region, with little or no effects on the distal region of the same promoter, or the promoter of the unrelated *IGF-1* gene (Fig. 6C).

The above changes could be associated with specific chromatin modifications at the *Wnt4* promoter, ac-

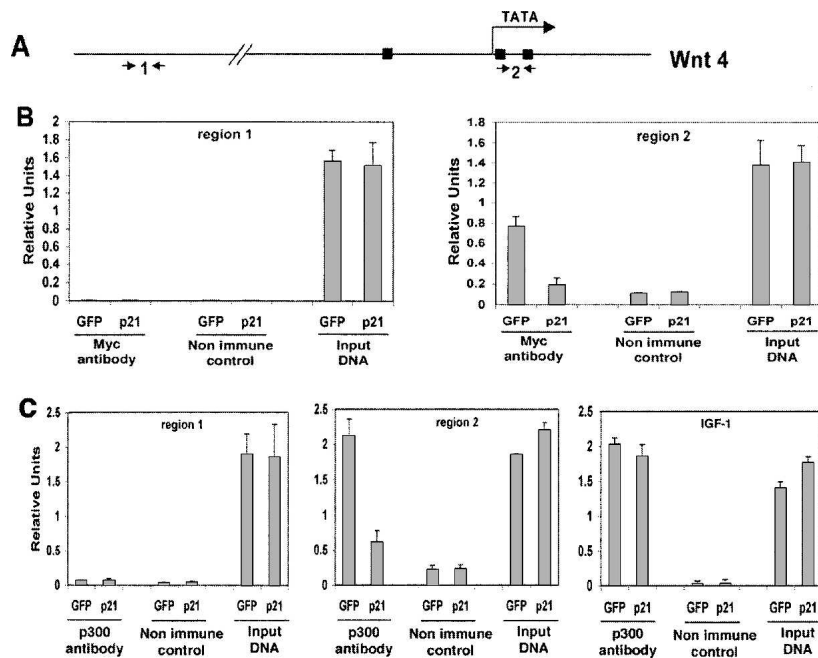


Figure 6. Increased p21 expression curtails recruitment of c-Myc and p300 to the *Wnt4* promoter. (A) Map of the *Wnt4* gene, with indication of the c-Myc-binding sites (solid squares) and the position of the oligonucleotide primers utilized for the ChIP analysis (the same as in Fig. 5A). (B) Decreased recruitment of the c-Myc protein to the TATA box-proximal region of the *Wnt4* gene by increased p21 expression. Primary keratinocytes were infected with the Ad-GFP or p21F adenoviruses for 24 h and analyzed by ChIP with antibodies against the c-Myc protein and corresponding controls as in the previous experiments. Immunoprecipitates were analyzed by real-time PCR for the indicated regions of the *Wnt4* gene. (C) Decreased recruitment of the p300 protein to the TATA box-proximal region of the *Wnt4* gene by increased p21 expression. Primary keratinocytes were infected with the Ad-GFP or Ad-p21F adenoviruses for 24 h and analyzed by ChIP with antibodies against the p300 protein and corresponding controls as in the previous experiments. Immunoprecipitates were analyzed by real-time PCR for the indicated regions of the *Wnt4* gene, as well as for the TATA box-proximal region of the *IGF-1* gene (nucleotides -242 to -141 relative to the AUG).

counting for its decreased transcription. Accordingly, we performed ChIP experiments with antibodies against acetylated histone H4, followed by quantitative real-time PCR for different regions of the *Wnt4* gene (around -6839, -1175, +21, +1329, relative to the TATA box). p21 overexpression caused more than eightfold down-modulation of histone H4 acetylation at the TATA box-proximal region of the *Wnt4* gene (region 2 in Fig. 7A,B), with a more partial reduction in neighboring regions (regions 1a and 3 in Fig. 7A,B) and no reduction, relative to control cells, further upstream (region 1 in Fig. 7A,B).

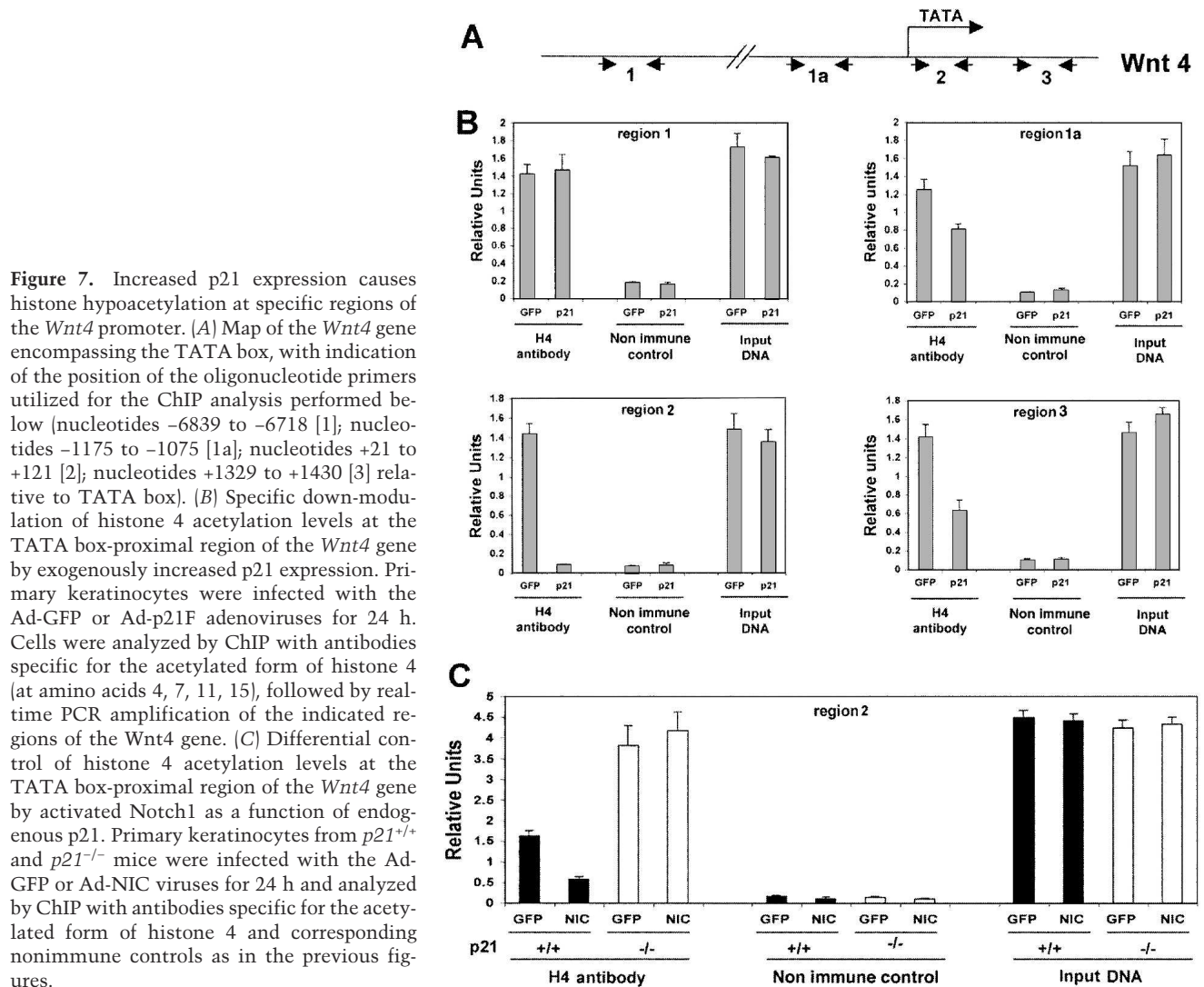
To assess whether endogenous p21 participates in control of the *Wnt4* promoter through a similar mechanism, we performed similar ChIP experiments with primary keratinocytes derived from *p21*^{+/+} versus *p21*^{-/-} mice after infection with the Ad-NIC versus Ad-GFP viruses. Activated Notch1 expression in wild-type keratinocytes caused a significant decrease in acetylated histone H4 levels at the TATA box-proximal region of the *Wnt4* promoter (Fig. 7C). In contrast, in *p21*^{-/-} keratinocytes, levels of acetylated histone H4 were higher than in the *p21*^{+/+} cells and not affected by activated Notch1 expression (Fig. 7C).

Discussion

Keratinocytes provide a well-characterized model system where the complex biological functions of p21^{WAF1/Cip1} have been investigated. Analysis of cells and mice with a disruption of the p21 gene has shown that this molecule is not essential for cell cycle withdrawal of the vast majority of keratinocytes that accom-

panies differentiation (cells that can be equated to transit amplifying keratinocytes exiting the cell cycle) (Missero et al. 1996). Rather, lack of p21 results in an increased number of "putative stem cells," i.e., clonogenic keratinocytes with a broad differentiation potential capable of reconstituting on their own entire hair follicles (Topley et al. 1999). In parallel with these findings, keratinocyte cultures from *p21*^{-/-} mice are more susceptible to *ras* oncogene transformation (Missero et al. 1996; Paramio et al. 2001) and, while apparently normal, the skin of *p21*^{-/-} mice exhibit substantially increased susceptibility to chemically induced carcinogenesis (Philipp et al. 1999; Topley et al. 1999; Weinberg et al. 1999). A role of p21 in control of stem cell populations has also been reported for the hematopoietic system (Cheng et al. 2000). Similarly, the tumor-suppressor function of this molecule goes beyond the skin system, as it affects also mammary and lung cancer development (Adnane et al. 2000; Jackson et al. 2002, 2003).

Notch1 signaling in keratinocytes causes direct positive regulation of p21 expression (Rangarajan et al. 2001) and, like p21, promotes exit from the stem cell compartment (Lowell et al. 2000). However, both Notch and p21 also affect late steps of differentiation (Missero et al. 1996; Rangarajan et al. 2001; Nickoloff et al. 2002), pointing to the possibility of a dynamic equilibrium between self-renewing and more committed populations, which can be controlled at multiple levels, including relative late steps in the terminal differentiation process (Okuyama et al. 2004). Like p21, Notch1 has a tumor-suppressor function in mammalian skin, which has been linked to down-modulation of β -catenin signaling (Nico-



las et al. 2003). We have shown here that the response of keratinocytes to activated Notch1, and more specifically, the decreased levels of β -catenin activation are dependent on down-modulation of *Wnts* gene expression.

Genetic analysis in developmental model systems has pointed to a complex cross-talk between the Notch and Wnt signaling pathways, which can occur at different levels, i.e., extracellularly, by binding of the Wnt ligand to the extracellular domain of Notch (Couso and Martinez Arias 1994), intracellularly, by direct binding of dishevelled to the C-terminal domain of Notch (Axelrod et al. 1996), and, in vertebrate, by Wnt signaling regulating transcription of the Notch ligand Delta-like-1 (Dll1) (Galceran et al. 2004; Hofmann et al. 2004). In the present study, we have shown that Notch activation can, in turn, negatively regulate *Wnt* signaling through down-modulation of *Wnt* gene expression. Many *Wnt* family members exist, which show a very selective pattern of expression at different developmental stages and in various tissues (Logan and Nusse 2004). Surprisingly, little is known about the molecular mechanisms that control ex-

pression of these genes. Our findings establish *Wnt3* and *Wnt4* as targets of transcriptional repression by Notch. Importantly, down-modulation of these genes involves, beside the classical Notch-responsive Hes-1 protein, a novel p21-dependent mechanism, with endogenous p21 playing a preferentially important role in control of *Wnt4* expression.

We have shown that in keratinocytes, p21 functions as a transcriptional regulator that associates physically to the promoter of the *Wnt4* gene. While increased p21 expression suppresses both *Wnt3* and *Wnt4* expression and endogenous p21 is required for the effective down-modulation of both genes by Notch1, in the skin of *p21*^{-/-} mice and in primary *p21*^{-/-} keratinocytes under basal conditions, only *Wnt4* is up-regulated. This is likely a reflection of the fact that, biochemically, we could readily observe association of the endogenous as well as overexpressed p21 protein to the *Wnt4* promoter, while association to the *Wnt3* promoter, if it occurs, is much weaker and harder to demonstrate (our unpublished observations). By ChIP assays, we found that E2F-1 binds

the same region of the *Wnt4* promoter as p21, and that the two proteins can be recovered in association at this promoter. These findings are consistent with an elegant model, whereby E2F-1–p21 association provides a bridging mechanism for bringing p21 to target promoters (Delavaine and La Thangue 1999). Importantly, however, in our cells, p21 binding is specific for the *Wnt4* promoter and does not occur at the promoter of another “classical” E2F-1 target gene such as PCNA, the expression of which is unaffected by increased p21 expression. Concomitantly, p21 binding at the *Wnt4* promoter is linked to curtailed recruitment of c-Myc and p300. By exogenous expression and promoter activity studies, p21 was previously reported to associate with the c-Myc protein suppressing its activity (Kitaura et al. 2000). Our data are consistent with such a mechanism taking place at the *Wnt4* promoter. Even in this case, however, there is an important element of selectivity, in that expression of other classical c-Myc target genes—such as that for ornithine decarboxylase—remains unaffected by p21 expression in keratinocytes (our unpublished observations). Thus, our findings are overall consistent with the emerging crucial role of chromatin configuration and promoter context in control of gene expression (Kadonaga 2004), with a physical and functional interplay between p21 and the specific transcription regulatory apparatus of individual genes such as that for *Wnt4*.

In summary, the common biological function of Notch1 and p21 as negative regulators of keratinocyte self renewal and tumorigenesis can be explained, in part, by one being a mediator of the other in transcriptional suppression of *Wnt* family members with consequent down-modulation of β -catenin signaling. More specifically, p21 is directly involved in transcription regulation of the *Wnt4* target gene, the control of which at the integrated chromatin level, remains an exciting topic for future studies.

Materials and methods

Cell culture and viral infection

Primary mouse keratinocytes were prepared and cultured in minimal essential medium with 4% Chelex-treated fetal calf serum (Hyclone), epidermal growth factor (EGF; 10 ng/mL; BD Biosciences) and 0.05 mM CaCl₂ (low-calcium medium) as previously described (Missero et al. 1996). Keratinocytes differentiation was induced by addition of 2 mM CaCl₂. All adenovirus infections were performed for 1 h in serum and epidermal growth factor-free-low calcium medium as previously described (Di Cunto et al. 1998). Keratinocytes were then incubated in fully supplemented medium for 24 h prior to collection for further analysis. Retroviral infections were carried out as previously described (Missero et al. 1996). Use of adenoviruses expressing activated Notch1 (Rangarajan et al. 2001), full-length and N-terminal p21, p16, and p27 (Di Cunto et al. 1998), Hes-1 (Sriuranpong et al. 2001), Hey-1, and Hey-2 (Mammucari et al. 2005) was previously described. The *Wnt4*-expressing retrovirus was obtained by inserting the mouse *Wnt4* cDNA into the LNCX vector (Palmer et al. 1987).

Analysis of gene expression

Gene expression was compared by quantifying mRNA levels by real-time RT-PCR. For this, total RNA preparations (1–2 μ g) were used in a Reverse Transcriptase reaction with oligonucleotide dT primers, followed by real-time PCR with gene-specific primers (Supplementary Table 1), using an Icyler IQ Real-Time detection System (Bio-Rad) according to the manufacturer's recommendation, with SYBR Green (Applied Biosystems) for detection. Each sample was tested in triplicate, and results were normalized by real-time PCR of the same cDNA with GAPDH primers (Supplementary Table 1).

Antibodies and ChIP assays

Rabbit polyclonal antibodies against E2F-1, p21, c-Myc, and p300 were obtained from Santa Cruz. Anti-active- β -catenin and Anti-tetra-acetylated histone H4 antibodies were obtained from Upstate Biotechnology. Anti-HA and Anti-total- β -catenin antibodies were obtained from Cell Signaling and BD Biosciences, respectively. ChIP analysis was carried out as previously described (Rangarajan et al. 2001). Briefly, $\sim 6 \times 10^6$ primary mouse keratinocytes were fixed with formaldehyde and lysed in SDS lysis buffer (Chromatin immunoprecipitation assay kit; Upstate). DNA in the cross-linked chromatin preparations was fragmented by sonication to an average size of 1 kb. Samples were precleared with salmon sperm DNA/protein A agarose–50% slurry. Antibodies and fresh protein A agarose were added, and incubated overnight at 4°C. Nonimmune controls were performed by incubations of parallel samples with nonimmune IgG/nonimmune serum and/or coated beads alone, with similar background levels being obtained in all cases. Precipitated chromatin complexes were removed from the beads through 30-min incubation with 500 μ L of elution buffer (1% SDS, 0.1 M NaHCO₃). Finally, the protein–DNA cross-links were reversed by an overnight incubation at 65°C and immunoprecipitated DNA was analyzed by real-time PCR. Primers for this analysis are indicated in Supplementary Table 1. For “Re-ChIP” experiments, chromatin complexes immunoprecipitated with anti-E2F-1 antibodies, or nonimmune controls, were eluted by incubation for 30 min at 37°C in 100 μ L of 10 mM DTT. After centrifugation, the supernatant was diluted 20 times with ChIP dilution buffer (Chromatin immunoprecipitation assay kit; Upstate) and immunoprecipitated with antibodies against p21 followed again by the ChIP procedure.

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