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EGFR signaling as a negative regulator of Notch1 gene expression : a differentiation/apoptosis control mechanism for proliferating keratinocytes and cancer cells

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Abstract

The Notch1 gene plays an important role in mammalian cell fate decision and tumorigenesis. Upstream control mechanisms for transcription of this gene are still poorly understood. In a chemical genetics screen for small molecule activators of Notch signaling, we identified Epidermal Growth Factor Receptor (EGFR) as a key negative regulator of Notch1 gene expression in primary human keratinocytes, intact epidermis and skin squamous cell carcinomas (SCCs). The underlying mechanism for negative control of the Notch1 gene in the human cells, as well as in a mouse model of EGFR-dependent skin carcinogenesis, involves transcriptional suppression of p53 by the EGFR effector c-Jun. Suppression of Notch signaling in cancer cells counteracts the differentiation inducing effects of EGFR inhibitors, while, at the same time, synergizing with these compounds in induction of apoptosis. Thus, our data reveal a novel role of EGFR signaling in negative regulation of Notch1 gene transcription, of potential relevance for combinatory approaches of cancer therapy.

Keywords

Chemical Genetics; Squamous Cell Carcinoma; Keratinocytes; Notch; EGFR

Cell growth and differentiation are controlled by a complex interplay of signaling pathways functioning in an “integrated”, rather than sequential or parallel fashion. Chemical Genetics is based on the principle of using small molecular weight compounds to abrogate or enhance specific regulatory pathways, providing a powerful approach to analyze complex regulatory systems. In the present study we utilized this approach to probe into the signaling network involved in control of Notch1 gene expression and function in human keratinocytes, skin and tumors.

Notch signaling plays a key role in promoting keratinocyte differentiation and suppressing keratinocyte-derived tumors[1, 2]. Notch receptors, with Notch1 and 2 being the main forms

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expressed in keratinocytes, are processed by a Ca^{2+}-dependent protease in the Golgi prior to transport to the cell membrane. Upon interaction with transmembrane ligands (Jagged1 and 2 or Delta1–4) expressed on the surface of neighboring cells, Notch receptors are activated by consecutive cleavage by an ADAM metalloprotease and a presenilin/γ-secretase complex. The resulting Notch intracellular domain (ICN) translocates to the nucleus where it associates with the DNA binding protein CSL (CBF-1 or RBP-Jκ in mammalian cells), converting it from a repressor into an activator of transcription[3]. Binding of a second ancillary protein, Mastermind-like 1 (MAML1) or related family members, is required for sustained levels of Notch/CSL-dependent transcriptional activation (through recruitment of further transcription co-activators such as p300)[4].

A 51 amino acid peptide (MAM51) corresponding to the amino terminal region (aa 13–74) of the MAML1 protein competes for MAML1 binding to the Notch/CSL complex, thereby preventing downstream transcription[5]. We recently showed that, in primary human keratinocytes, suppression of Notch signaling by this approach results in a lesser commitment to differentiation, expansion of stem cell populations and dramatically increased susceptibility to *ras*-induced oncogenic conversion[2]. Similar effects were observed after pharmacological suppression of endogenous Notch activity by a γ-secretase inhibitor[2]. These findings are likely to be of clinical significance, as Notch1 gene expression and activity are substantially down-modulated in keratinocyte cancer cell lines and tumors, with expression of this gene being under positive p53 control in these cells[2, 6].

EGFR is among the most intensely studied and well understood determinants of epithelial cell proliferation, and EGFR inhibitors have surfaced as an outstanding example of rational-based drug design for tumors[7]. EGFR signaling is likely to function in the keratinocyte proliferative compartment of the epidermis as a “built-in” mechanism to maintain self renewal and, at the same time, suppress differentiation, in contrast to the upper layers where this pathway is down-modulated. In fact, abrogation of EGFR/ERK signaling in proliferating keratinocytes, by either chemical or genetic manipulations, induces differentiation, while sustained activation of this pathway, under conditions where it is normally down modulated, suppresses differentiation[8, 9]. This has potentially important implications for keratinocyte-derived tumors, where EGFR signaling is persistently activated and promotes proliferation[10]. At the biochemical level, little is known on the link between EGFR/ERK pathways and control of differentiation. Here we report a novel role of this pathway in negative regulation of Notch1 gene transcription in both normally proliferating keratinocytes and cancer, which impinges on control of differentiation as well as apoptosis.

Results

Negative regulation of Notch1 gene expression by EGFR/ERK signaling

Little is known of pathways involved in upstream control of Notch1 gene expression and activity in keratinocytes, and mammalian cells in general. To address this issue we undertook a chemical genetics approach. Rather than screening a large collection of unknown chemicals, we chose a library of 489 compounds, approved by the Food and Drug Administration (FDA) and of established target selectivity, using a Luciferase Notch/CSL-responsive reporter as a read-out. The negative regulators of Notch signaling identified by this screen included inhibitors of metalloproteases (MMP)and γ-secretase, which are required for endogenous Notch activation[4], confirming the validity of the assay (suppl. Table I). Statistical analysis of the results pointed to a number of other candidate pathways. In particular, the most significant compounds to induce Notch activity were kinase inhibitors that target components of signaling networks connected with EGFR signaling, which was of special interest, given the relevance of this pathway in keratinocytes and cancer[11, 12].

To validate the findings of our screen, we compared the effects of EGFR inhibition and stimulation on endogenous Notch signaling in human primary keratinocytes. A dose-response of human primary keratinocytes to the EGFR inhibitor AG1478 was determined, on the basis of decreased phosphorylation of the EGFR, ERK1/2, c-Jun and Elk proteins, as well as down-modulation of c-Fos, which is controlled by growth factors more indirectly, through SRF and TCF-dependent transcription, at the level of gene transcription (Supplemental Fig. 1). At the same doses, there was induction of the "canonical" Notch target genes Hes1, Hes5 and Herp1, while, conversely, EGF treatment suppressed expression of these genes (Fig. 1A). In parallel with this effect, Notch1 mRNA levels were increased by EGFR inhibition, while they were down-regulated by EGF treatment (Fig. 1B). Consistent with a transcriptional mechanism, no increase of Notch1 mRNA stability was observed in EGFR inhibitor-treated cells after Actinomycin D treatment (data not shown). The results were confirmed at the protein level, by immunoblotting of AG1478- and EGF-treated keratinocytes with antibodies against total and cytoplasmic activated forms of Notch1 as well as Hes1 (Fig. 1C). Effects similar to those of AG1478 were also elicited by Tarceva, an EGFR inhibitor approved for clinical use[13] (Supplemental Fig. 2). Besides chemical inhibition, up-regulation of Notch1 activity and expression were also observed after knockdown of EGFR expression by transfection of keratinocytes with specific siRNAs (Fig. 1D). Unlike Notch1, Notch2 expression was modulated by EGFR signaling at the mRNA but not protein level (Suppl. Fig. 3A; Fig. 1C), while no consistent changes were found in expression of the Notch ligands Jagged 1 and Delta like 1 (Suppl. Fig. 3B,C).

EGFR suppression is expected to cause growth inhibition and increased apoptosis[7, 14], a fact that we experimentally confirmed, raising the possibility that the induction of Notch1 expression is only an indirect consequence of these events. However, treatment of keratinocytes with TNF-α at pro-apoptotic concentrations had no effects on levels of Notch1 expression, which was also not affected by suppression of keratinocyte growth by TGF-β treatment (Supplemental Fig. 2).

The ERK1/2 kinases and the AP-1 transcription complex function as downstream effectors of EGFR activation[11]. Induction of Notch1 gene expression similar to that caused by EGFR suppression was observed after siRNA-mediated knockdown of the MEK1 and ERK1 genes while, consistent with their proposed distinct function in keratinocytes[15], knockdown of MEK2 or ERK2 had no such effect (Fig. 1E). In contrast to MEK1 and ERK1, no increase of Notch1 expression, or even suppression, was also observed after knock-down and/or pharmacological inhibition of the p38 and JNK kinases, AKT and PKA (Fig. 1E and Supplemental Fig. 2). Induction of Notch1 expression similar to that caused by EGFR and ERK suppression occurred also after knockdown of c-Jun and c-Fos, two key AP-1 family members (Fig. 1F,G). Even in this case, the effects were specific, as they were not observed after knockdown of other AP-1 family members like JunB, Jun D and Fra1, nor of Elk-1, a transcription factor which is activated by EGFR activation though a separate mechanism from AP1[16] (Fig. 1F).

Modulation of Notch1 gene transcription by EGFR signaling through p53

We and others recently showed that the Notch1 gene is a direct transcriptional target of p53 in keratinocytes[2, 6, 17]. Consistent with these previous results, our chemical screen pointed to a p53 inhibitor, pifithrin, as a negative regulator of Notch signaling (suppl. Table I), a finding which we directly confirmed by treating keratinocytes with this compound (data not shown). We therefore surmised that a p53-dependent mechanism may underlie up-regulation of Notch1 expression by EGFR suppression. To test this possibility, p53 expression was suppressed in primary keratinocytes by siRNA knockdown. This resulted in reduced levels of Notch1 expression already under basal conditions and, much more substantially, in response to EGFR knock-down (Fig. 2A). Consistent with a p53-dependent transcriptional control mechanism,

luciferase reporter activity of a 2.4 kbp Notch1 promoter region containing p53 binding sites[2,6] (but not of a shorter region lacking these sites) was induced in HKCs after EGFR inhibition, with such induction being abrogated by p53 knock-down (Fig. 2B).

Endogenous p53 activity, as assessed by expression of well-established target genes, $p21^{WAF1/Cip1}$ and Gadd45α[18], was induced as a consequence of EGFR inhibition (Fig. 2C). There was also a substantial increase of Mdm2, a negative regulator of p53 stability and itself a p53 target gene[18] (Fig. 2D). Consistent with the negative feedback loop between p53 and Mdm2 protein expression, induction of p53 protein expression by AG1478 became much more evident in cells concomitantly treated with Nutlin, an Mdm2 inhibitor[19] (Fig. 2E).

Emerging evidence points to the importance of control of p53 activity by transcription of this gene ([20–22] and refs. therein). Consistent with this possibility, real time RT-PCR analysis showed that p53 mRNA levels were significantly increased as a consequence of EGFR inhibition while, conversely, were reduced by EGF treatment (Fig. 2F). Previous work with mouse embryonic fibroblasts indicated that the p53 gene can be a direct target of c-Jun-mediated transcriptional suppression[23]. Consistent with this mechanism, chromatin immunoprecipitation experiments showed that the endogenous c-Jun protein binds to a predicted AP-1 binding region of the p53 promoter in control keratinocytes, while such binding is abrogated in EGFR-inhibitor-treated cells (Fig. 2G). In functional luciferase reporter assays, activity of the p53 promoter was suppressed by increased c-Jun expression, while it was induced by siRNA-mediated c-Jun knockdown (Fig. 2H), with a similar effect on endogenous p53 gene transcription (Fig. 2I). The role of p53 in mediating control of Notch1 expression was demonstrated by the fact that induction of Notch1 expression by c-Jun knock-down was blocked by the concomitant down-modulation of p53 expression (Fig. 2J).

EGFR-p53-Notch control of differentiation in primary keratinocytes and intact skin

EGFR signaling provides a break to differentiation, while increased Notch activity promotes this process[1]. Real time RT-PCR as well as immunoblot analysis showed that down-modulation of EGFR signaling, by either transfection with siRNAs against EGFR or AG1478 treatment, induced expression of several terminal differentiation markers in keratinocytes, including Keratin1 and 10 and Involucrin (supplemental Fig. 4 and data not shown). To assess whether induction of differentiation by EGFR suppression is due to up-regulation of Notch signaling, primary human keratinocytes were infected with a retroviral vector expressing a 51 amino acid peptide (MAM51) that competes for MAML1 binding to the Notch/CBF-1 complex, thereby preventing downstream transcription[5]. Treatment of MAM51-expressing keratinocytes with AG1478 caused a similar induction of Notch1 expression as control cells; by contrast, induction of Hes1 and differentiation markers was suppressed (Fig. 3A). Chemical inhibitors of γ-secretase activity like DAPT suppress proteolysis-dependent activation of endogenous Notch receptors[24]. As with MAM51 expression, treatment of primary keratinocytes with DAPT counteracted induction of Hes1 and differentiation marker expression caused by EGFR down-modulation (Fig. 3B). Similar counteracting effects were also observed after siRNA-mediated knock-down of Notch1 and p53 expression, indicating that up-regulation of these genes by EGFR inhibition is responsible for the observed induction of differentiation (Fig. 3C). In Drosophila, rather than being involved in direct inhibition of Notch activity and/or expression, EGFR signaling was reported to negatively regulate Groucho, a downstream effector of Notch (as well as other pathways)[25]. Such indirect mechanism does not apply to our system. In fact, EGFR activation exerted no counteracting effects on induction of differentiation by activated Notch1 (Fig. 3D) and differentiation was suppressed rather then induced by Hes1, a mammalian homologue of Groucho (Fig. 3E).

Growth/differentiation control of keratinocytes in culture is likely to differ in significant aspects from control of these cells in intact skin. For further validation of our findings, we

resorted to several complementary approaches. For the first, mice with a GFP reporter for Notch activity[26] were injected with the EGFR inhibitor AG1478. Immunofluorescence analysis showed significantly elevated GFP expression in the epidermis of the AG1478 treated mice versus the control (Fig. 4A). For confirmation and quantification of the results, the epidermis of these mice was separated from the underlying dermis by a brief heat treatment, followed by total RNA preparation and real time RT-PCR analysis. This confirmed increased GFP expression, which paralleled increased expression of the endogenous Notch1, p53 and Keratin 1 genes (Fig. 4B, C). As a second approach, the epidermis of homozygous mice for a hypomorphic EGFR mutation was analyzed, in parallel with heterozygous littermates. Even in this case, decreased EGFR activity was found to result in increased p53 and Notch1 expression (Fig. 4D).

In the human situation, inhibitors of the EGFR pathway are now used in clinical treatment of several types of cancer[7, 13]. Immunofluorescence analysis of skin biopsies from a cohort of melanoma patients treated with the MEK inhibitor AZD6244 in parallel with age and sex matched control showed a significant up-regulation of Notch1 expression (Fig. 4E), which paralleled that of p53 ([27] and data not shown). Similar results were obtained with an organ culture system of freshly excised human skin, using an optimized method that allows maintenance of viable tissue with no sign of degeneration and/or altered differentiation for up to 7 days (our unpublished observations). Immunohistochemical as well as real time RT-PCR analysis indicated that, even in these conditions, EGFR inhibition caused a parallel induction of Notch1 activity and expression, and differentiation in keratinocytes, together with p53 and $p21^{WAF1/Cip1}$ (Fig. 4F, Supplemental Fig. 5). Using this approach, we tested whether the increase in keratinocyte differentiation is Notch dependent. For this, human skin cultures were treated with AG1478 plus/minus DAPT. As shown in Fig. 4G, induction of Keratin 1 and Involucrin expression by EGFR inhibition was counteracted by the concomitant treatment with the Notch/γ-secretase inhibitor, while, as expected, induction of the Notch1 gene itself, or of p53, was unaffected or even increased.

Inhibition of EGFR signaling in cancer cells induces Notch1 gene expression through p53

To assess whether the p53/Notch regulatory loop discovered here applies to conditions where increased EGFR signaling has been causally linked with cancer development, we analyzed transgenic mice expressing a constitutive active form of the EGFR/Ras adaptor protein SOS under control of a keratin 5 promoter (K5-SOS-F). These mice develop spontaneous skin tumors strictly dependent on the presence of functional EGFR[28]. In K5-SOS-F transgenics with a concomitant keratinocyte-specific deletion of the c-*Jun* gene, skin tumor development is impaired, correlating with reduced EGFR expression and increased differentiation[29]. The present results suggested that EGFR/c-Jun regulation of p53 and Notch1 expression may also be involved. In fact, real time RT-PCR analysis showed significantly higher levels of Notch1 and p53 expression in the small tumors formed by K5-SOS-F transgenic mice with epidermal deletion of the *c-Jun* gene ($c\text{-}Jun^{\Delta ep}$ SOS^{+}) relative to tumors formed in K5-SOS-F transgenics with the intact *c-Jun* gene ($c\text{-}Jun^{f/f}$ SOS^{+}) (Fig. 5A). These data were confirmed at the protein level by immunoblot analysis of a separate set of tumors, as well as by immunofluorescence for Notch1 expression (Fig. 5B, C).

To assess whether similar EGFR regulation of Notch1 expression applies to human cancer, keratinocyte-derived SCC cells (SCCO28, SCC12 and SCC13) with wild type p53 (http://www.sanger.ac.uk/genetics/CGP/CellLines/) were treated with EGFR inhibitor. Besides mutations, p53 activity can also be reduced in tumors as a consequence of decreased p53 gene transcription [22, 30–33]. Consistent with this mode of regulation, EGFR inhibition of SCC cells induced expression of the p53 gene as well as of $p21^{WAF1/Cip1}$, indicative of increased p53 activity (Fig. 6A, B). This was paralleled by a substantial increase of Notch1

mRNA and protein levels and differentiation markers (Fig. 6C, D: Supplemental Fig. 6A). As with primary keratinocytes, p53 knockdown experiments showed that even in cancer cells, induction of Notch1 expression by EGFR-inhibition is p53-dependent (Fig. 6E).

Cancer cell lines can differ substantially in their control mechanisms from cells in primary tumors. Therefore, as a further validation of our findings, the same organ culture system described above for intact skin was adapted to the analysis of clinically occurring SCCs freshly excised from patients. The dissected more homogeneous parts of tumors were cut into small pieces of the same size (2×2 mm), and placed into multi-well dishes as for skin organ cultures. In five independent tumors, EGFR inhibition resulted in reduction of c-Fos expression, indicative of EGFR signaling suppression, and concomitant induction of Notch1, p53 and Keratin 1 (Fig. 6F; Supplemental Fig. 6C). In four other tumors no such effects were observed, consistent, in two cases, with resistance of EGFR inhibition (as assessed by no decrease in c-Fos expression) and, in the other two, undetectable p53 expression or activity (data not shown).

Inhibition of Notch signaling in cancer cells suppresses differentiation induced by EGFR suppression while it synergizes for apoptosis

As with primary keratinocytes, even in SCC cells inhibition of EGFR signaling caused up-regulation of differentiation markers expression, through a Notch dependent mechanism (Supplemental Fig. 6A, B). We have recently found that Notch-dependent differentiation of keratinocytes render these cells more resistant to apoptosis[17]. Thus, an attractive possibility was that suppression of Notch signaling, while suppressing the pro-differentiation effects of EGFR inhibitors, may synergize with these compounds in triggering apoptosis. To assess this possibility, SCC cells were treated with DAPT plus-minus EGFR inhibitor. As shown in Fig. 7A, the concomitant treatment led to a substantial increase of apoptosis. These findings were paralleled by a synergistic induction of Bim1 expression (Fig. 7B), a pro-apoptotic Bcl2 family member that has been recently implicated in the response of cancer cells to EGFR inhibitors[34].

To further validate the relevance of these findings for the behavior of cancer *in vivo*, immune-compromised mice were injected with SCC cells expressing the Notch inhibitory MAM51 peptide, in parallel with control cells. After formation of sizable tumors (4 weeks after injections), mice were treated with AG1478 for a week. RT-PCR analysis of tumor RNAs showed substantially higher levels of Hes1 and differentiation marker expression in tumors formed by control than MAM51-expressing cells, while levels of Bim1 were oppositely regulated (Fig. 7C). This was paralleled by a higher apoptotic fraction in tumors with suppressed Notch signaling (Fig. 7D).

Discussion

Loss- and gain-of function experiments in mice as well as work with human keratinocytes have established that EGFR signaling plays a key role in positive control of keratinocyte growth potential and carcinogenesis[10]. A similar role has been found for downstream effectors of this pathway at the level of transcription, like c-Jun[9]. Besides enhancing proliferation, we have shown here that EGFR signaling plays a significant role in suppressing differentiation through negative regulation of Notch1 gene expression and activity. This mechanism is likely to provide a break for the commitment to differentiation of keratinocytes in the basal proliferative compartment of the epidermis as well as in cancer, where EGFR signaling is characteristically elevated[10].

In the upper epidermal layers, EGFR signaling is normally down-modulated and therefore ceases to be relevant. In fact, differentiation of keratinocytes as they migrate to the upper epidermal layers is induced by multiple EGFR-unrelated events including, most notably, loss

of integrin-mediated adhesion to the matrix and establishment of cadherin-dependent cell-cell adhesion[35]. This is consistent with control of the Notch1 gene by EGFR signaling – via p53 - being relevant for the behavior of proliferating keratinocytes in normal skin and cancer, while additional multiple mechanisms are responsible for the increase of Notch signaling in normally differentiating keratinocytes of the upper epidermal layers[1].

The cross-talk between the Notch and EGFR signaling has been well documented in genetic model systems, where these pathways can function in either an antagonistic or synergistic fashion, depending on tissue and developmental context (REF). We have shown here a novel function of EGFR signaling in negative control of Notch1 gene expression, through a mechanism involving transcriptional down-regulation of the p53 gene. Previous studies pointed to NF-κB control of p53 gene expression[36], which could be of relevance to the present situation, as NF-κB activity is induced in keratinocytes with differentiation[37], while its suppression promotes tumor development[38]. However, expression of NF-κB responsive genes, as an indication of endogenous activity, is not induced in keratinocytes by EGFR suppression (data not shown). As an alternative mechanism, we have found that control by AP-1 family members is involved. Among these, c-Jun can either activate or suppress direct target genes[39]. Previous work with mouse embryo fibroblasts has indicated that c-Jun can be a direct negative regulator of p53 gene expression[23]. Consistent with this conclusion, we have found that endogenous c-Jun binds to the p53 promoter in both normal and SCC-derived keratinocytes, and that expression of the p53 gene in these cells is enhanced by c-Jun knock-down. Notch1 expression is also induced by c-Jun knockdown, in a p53-dependent fashion. This concomitant mode of regulation of p53/Notch1 expression by EGFR signaling was further validated by our findings with organ cultures of intact human skin and SCCs, as well as a mouse model of skin cancer formation dependent on EGFR and c-Jun function.

EGFR has become an important target of cancer drug design, and several selective EGFR inhibitors have now been approved for clinical use. Recent data have highlighted the fact that inhibitors directed to critical receptors like EGFR utilize the cell death pathway for inducing tumor regression[34]. However, the exact molecular mechanisms underlying sensitivity and resistance of tumor cells to EGFR inhibition - including the contribution of other integrated pathways like the ones we have identified here - remain to be elucidated[40]. Importantly, suppression of Notch signaling in squamous carcninoma cells counteracts the differentiation inducing effects of EGFR inhibitors, while, at the same time, synergizing with these compounds in induction of apoptosis. Other ongoing studies in the laboratory indicate that the enhancing effects of Notch suppression on apoptosis may extend also to lung cancer cells. This suggests an attractive new avenue of combination approaches for cancer therapy that may enhance the potency of EGFR inhibitory agents on tumors, while at the same time ameliorating their well known toxic effects on the skin which have been attributed, at least in part, to aberrant differentiation[12].

Methods

Cell culture and viruses

Culturing of primary human keratinocytes and SCC 12, 13 and 028 cells, and infection with the MSCV-MAM51[5] and control retrovirus were as previously reported[2]. Adenoviruses for Notch1, Hes1 and GFP and adenoviral infections were previously described[37]. NCI-H1299 and NCI-H1299 were cultured in RPMI medium (Invitrogen) supplemented with 10% bovine serum. Chemical inhibitors, AG1478 (LC Labs), Nutlin (Calbiochem), DAPT (Calbiochem), ERK inhibitory peptide-cell permeable (Calbiochem) were dissolved in DMSO and applied at the indicated concentrations. For knock down experiments, cells were transfected as described[37] with validated stealth siRNAs for human EGFR, p53, and c-Jun in parallel with corresponding Stealth siRNA controls (Invitrogen), or ERK1, ERK2 and Notch1 (GeneGlobe,

Qiagen) and analyzed 48–72 hours after transfection. The Notch1 promoter regions −2472/−1 and −392/−1 (numbered referred to the ATG) were amplified by PCR from human genomic DNA with the primer pairs 5′-CTGCCTCCCGACCTGTAGGAG-3′ and 5′-GCCTCCCCACCGGCTGCCCTC-3′ and 5′-CTCGGGGAGGCGCAAAGGCGG-3′ and 5′-GCCTCCCCACCGGCTGCCCTC-3′ and subcloned into the of pGL4 vector (Promega) using the KpnI/NheI sites. The two promoter regions were inserted into lentivector pTRH4-mCMV-Luc (System Biosciences) at the EcoRI/SpeI sites. Lentiviruses were produced as described before[41]

Quantitative real time RT PCR, chromatin immunoprecipitation and immunodetection techniques

Conditions for real time PCR analysis, chromatin immunoprecipitation Chip), immunoblotting and immunofluorescence were as previously described[2]. The list of gene-specific primers is provided in Supplemental Table II. We used the following antibodies: Notch1 (Santa Cruz, C-20), activated Notch1 (Cell Signaling), Hes1 (Chemicon AB5702), Keratin1 (Babco AF87), Involucrin (Babco PRB140C), EGFR (Cell Signaling), p53 (Cell Signaling), MDM2 (Cell Signaling), Integrin β4 (Santa Cruz), γ-Tubulin (Sigma GTU87), for immunoblotting for mouse proteins: Notch1 (Pharmingen), p53 (Novacastra), c-Jun (Transduction Labs) and Actin (Sigma), c-Jun for Chip assays (Santa Cruz, H79).

Organ cultures

Discarded human skin samples from abdominoplasty procedures were obtained from the Centre Hospitalier - Universitaire Vaudois (Lausanne, Switzerland) under patients' agreement and institutional approval. Skin samples, sterilized in 70% ethanol and cut, after removal of subcutaneous fat, into 1x1cm pieces, were placed in keratinocyte serum-free medium (KSF, GIBCO-BRL) supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE), in 0.25% agar (Sigma). The epidermis was maintained at the air-medium interface. For RNA collection, skin samples were placed in preheated PBS at 60 °C for 45 seconds, then chilled (on ice) in 0.1M PBS for 1 minute, followed by mechanical separation of epidermis and dermis. The epidermis was homogenized in TRI Reagent (Sigma) for RNA preparation. Human SCC samples were obtained as discarded material from Mohs micrographic surgery at Massachusetts General Hospital (Boston, MA) with patients' and institutional approvals. Tumor samples were sterilized in 70% ethanol, cut into pieces of approximately 2×2 mm and placed in semi-solid medium similarly to skin organ cultures.

Tumorigenicity assays

For *in vivo* tumorigenicity assays, control and MAM51 expressing SCCO28 cells were brought into suspension, admixed with Matrigel (BD Biosciences), and injected (5×10^6 cells/injection) subcutaneously in 8 weeks old female athymic nude mice. Four weeks later animals were treated three times (every other day) with AG1478 (1 mg/animal, dissolved in 200 μl 50% DMSO:DMEM) or DMSO vehicle control by i.p. injections. Mice were sacrificed 2 days after the last treatment and tumors processed for RNA preparation and analysis.

TUNEL Assays

Cells were trypsinized, recovered by centrifugation at 300 g and fixed in 2% paraformaldehyde in PBS for 16 h. Permeabilization and enzymatic labeling with TMR red-conjugated-dUTP were performed according to the manufacturer's protocol (Roche, IN). The percentage of cells that incorporated the fluorescence-conjugated dUTP was determined by flow cytometry. TUNEL assay on histological sections was analyzed with fluorescent microscopy and IPLab software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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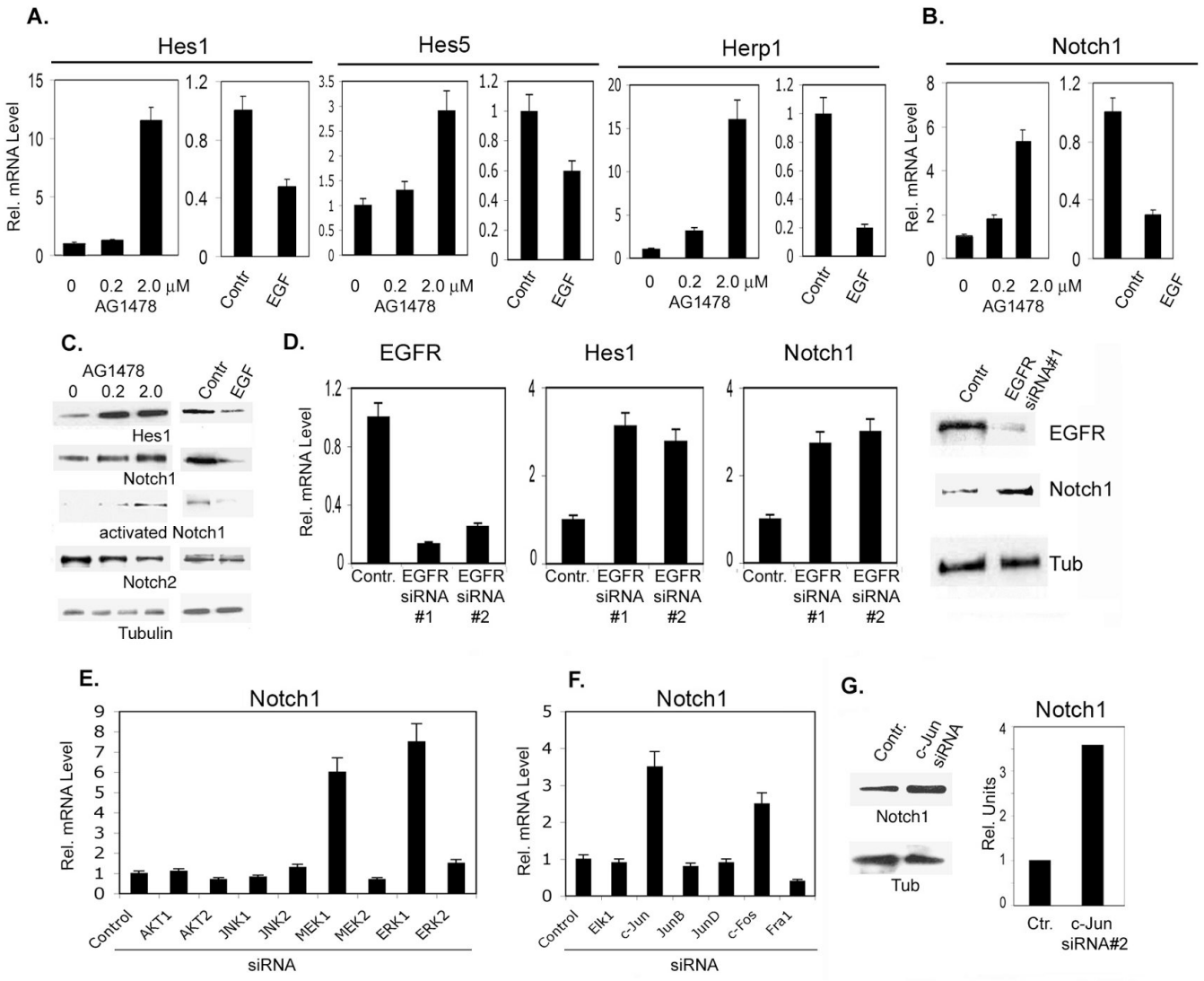

Fig. 1. Negative control of Notch1 activity and expression by EGFR/ERK/AP1 signaling

A and B : Primary human keratinocytes (HKCs) were treated with AG1478 at the indicated concentrations or with recombinant EGF (1.0 ng/ml) for 24 hours. Hes1, Hes5 and Herp1 (CHANGE NAME IN THE FIG. FROM HEY2 TO HERP1) (A) and Notch1 (B) mRNA levels were quantified by real-time RT-PCR. Values are expressed as relative units after internal normalization for 36B4 mRNA levels, with similar results being obtained after normalization for β-actin mRNA. C : HKCs were treated with AG1478 and EGF as in the previous panel, followed by immunoblot analysis for Hes1, total and activated Notch1, Notch2, and γ-Tubulin as equal loading control. D : HKCs were transfected with two different siRNAs specific for EGFR in parallel with scrambled siRNA control. Expression of the EGFR, Hes1 and Notch1 genes was assessed, 48h after transfection, by real time RT-PCR analysis (left panels), and confirmed at the protein level by immunoblotting (right panels). E and F : HKCs were transfected with validated siRNAs for the indicated protein kinase (E) and transcription factor (F) genes, in parallel with scrambled siRNA control. >80% knock down efficiency was obtained for each of these genes, as verified by real time RT-PCR analysis with the corresponding specific primers 48h after transfection (data not shown). Levels of Notch1 mRNA expression were assessed by real time RT-PCR analysis as in the previous panels. For

ERK1, ERK2, c-Jun, c-Fos and ELK1, similar results were obtained after transfection with a second set of specific siRNAs. G : HKCs, transfected with siRNA specific for c-Jun and scrambled siRNA control as in the previous panel, were analyzed by immunoblotting for total Notch1 and γ-Tubulin as equal loading control. Data were quantified by densitometric scanning, using the γ-Tubulin signal for normalization (right panel).

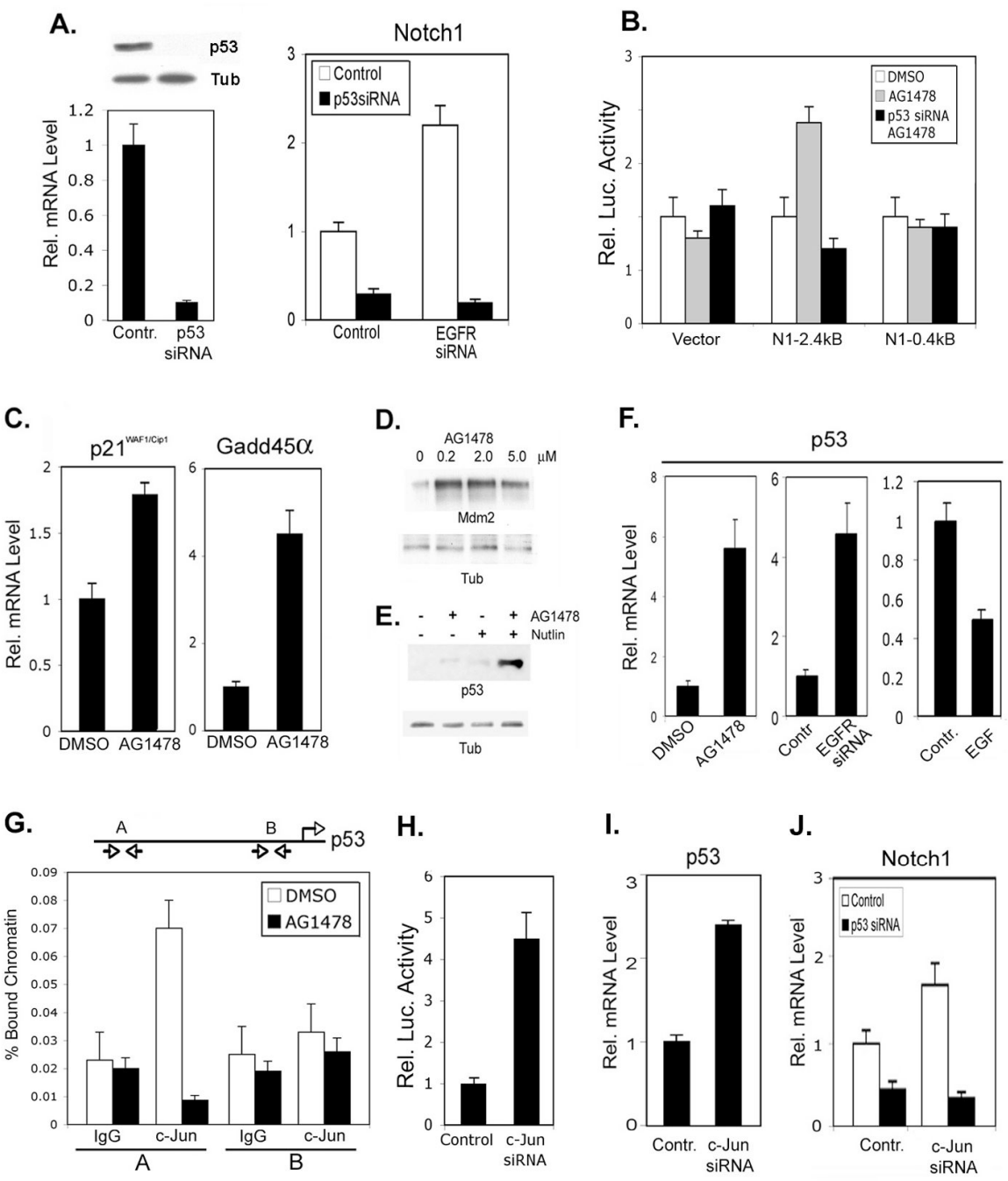

Fig. 2. Suppression of EGFR signaling induces Notch1 expression through p53

A : HKCs were transfected with siRNAs specific for p53 plus/minus siRNAs for EGFR in parallel with scrambled siRNA control. Cells were analyzed 48 hours after transfection. "Knock down" of p53 expression was confirmed by real time RT-PCR and immunoblot analysis (left panels). Hes1 and Notch1 expression was assessed by real time RT-PCR as for the previous experiments. B : HKCs were infected with the lentiviral reporter vectors pTRH4-N1-2.4 (N1-2.4) and pTRH4-N1-0.4 (N1-0.4), carrying an internal luciferase gene driven by either a 2.4 or 0.4 kb region of the human Notch1 promoter (nucleotides −2472 to −1 and −392 to −1 from the initiation codon, respectively), which contains and lacks, respectively, the mapped p53 binding sites[2, 17]. The pTRH4 vector, carrying the luciferase gene devoid of

exogenous promoter, was used as control. HKCs stably infected with these viruses were transfected with siRNAs specific for p53 or scrambled siRNAs, followed by treatment (for 24 hours) with AG1478 (2 μM) or DMSO control, as indicated. For each set of cells, values are expressed as relative luciferase activity after protein normalization. C : HKCs were treated with AG1478 (2 μM) or DMSO control for 24 hours, followed by real time RT-PCR analysis of $p21^{WAF1/Cip1}$ and Gadd45α expression. D : HKCs treated with AG1478 or DMSO as in the previous experiments, were analyzed by immunoblotting with antibodies against Mdm2, and γ-Tubulin as equal loading control. E : HKCs were treated with AG1478 and Nutlin (2 μM) for 24 hours, individually and in combination, followed by immunoblot analysis of p53 and γ-Tubulin expression. F : HKCs were treated with AG1478, transfected with EGFR-specific siRNA, or stimulated with EGF as in the previous experiments, and analyzed, in parallel with the corresponding controls, for levels of p53 mRNA expression by real time RT-PCR. G : HKCs were treated with AG1478 or DMSO control for 24 hours. Cells were then processed for chromatin immunoprecipitation analysis (ChIP) with an antibody against c-Jun and purified rabbit IgG as non-immune control. Real-time PCR of a distinct region of the human p53 gene promoter containing several conserved AP-1 binding sites (around position −2.6 kb, Site A in the map above) was performed along with PCR of a more downstream region devoid of such sites (around position −0.3 kb, Site B). The primers used to amplify these p53 promoter regions are listed in Supplemental Table II. H : cells were co-transfected with the p53n-Luc reporter plasmid[22], carrying a luciferase gene driven by a 2 kb region of the human p53 promoter, together with an expression vector for human c-Jun (c-Jun-pCMV,[42]) or empty vector control (left panel), or with siRNAs against c-Jun and scrambled siRNAs control. Luciferase activity was determined 48h later. For each set of cells, values are expressed as relative luciferase activity after protein normalization. I : HKCs were transfected with siRNAs against c-Jun in parallel with scrambled siRNA control (as in Fig. 1F, G) followed by measurement of p53 expression by real time RT-PCR. J : HKCs were transfected with siRNA against c-Jun either alone or in combination with siRNA against p53, in parallel with scrambled siRNA control. Notch1 expression was determined by real time RT-PCR as before.

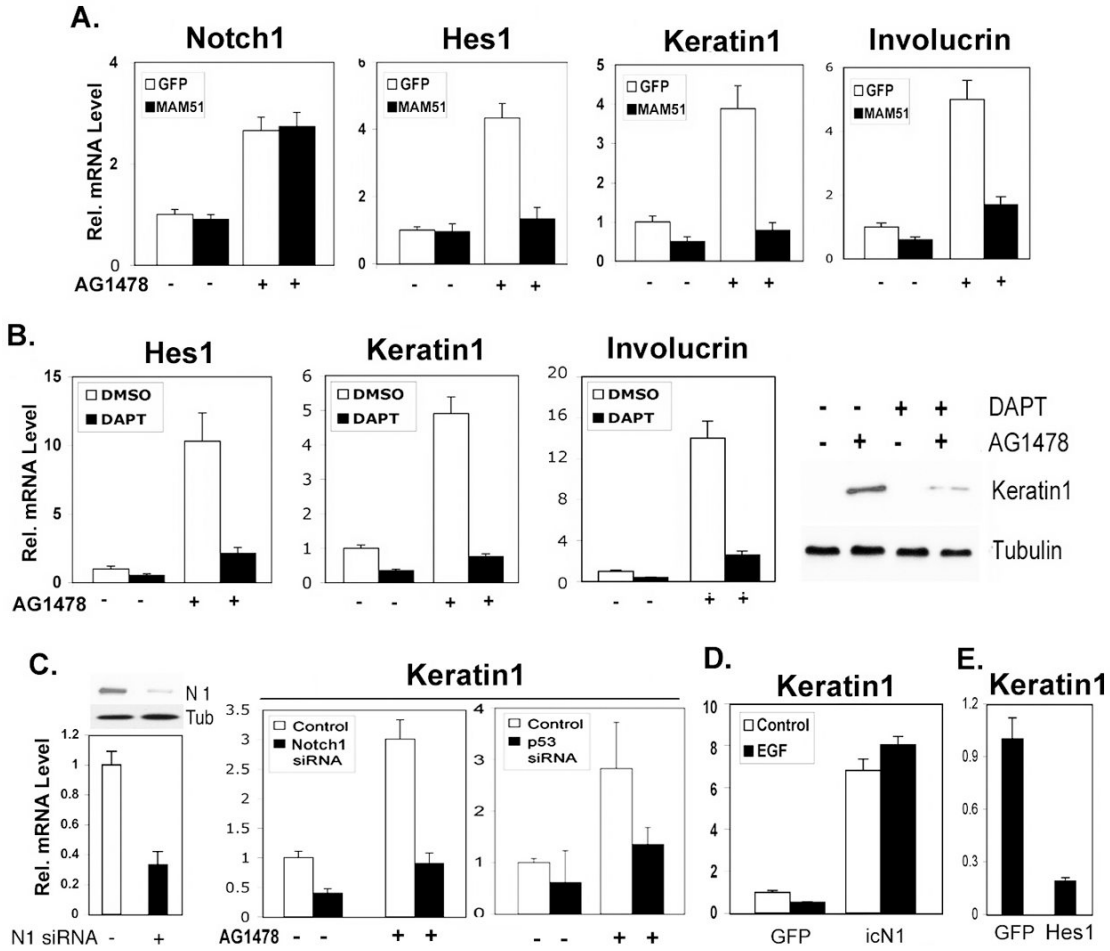

Fig. 3. Down-modulation of EGFR signaling induces keratinocyte differentiation through a Notch dependent mechanism

A : HKCs infected with a retrovirus expressing the dominant negative MAM51 peptide or GFP control (black and white bars, respectively) were treated with AG1478 (2 μM) or DMSO control for 24 hours. Expression of the Notch1, Hes1, Keratin 1 and Involucrin genes was assessed by real time RT-PCR. B : HKCs were treated with AG1478 (2 μM) and DAPT (10 μM) or DMSO control for 24 hours, followed by real time RT-PCR analysis of Hes1, Keratin 1 and Involucrin expression (left panel). Similarly treated cells were also analyzed for Keratin1 protein expression by immunoblot analysis, with γ-tubulin as equal loading control (right panels). C : HKCs were transfected with Notch1 or p53 specific siRNAs in parallel with scrambled siRNA control (black and white bars, respectively) and treated with AG1478 (2 μM) or DMSO followed by RT-PCR for Keratin1. “Knock down” of Notch1 expression was confirmed by real time RT-PCR and immunoblot analysis (left panels). D : HKCs were transduced with adenoviruses expressing activated form of Notch1 or GFP control and 24 h after infection stimulated with EGF (1 ng/ml). Expression of Keratin1 was assayed by RT-PCR analyses. E : HKCs were transduced with adenoviruses expressing Hes1 or GFP control followed by RT-PCR analysis of Keratin1.

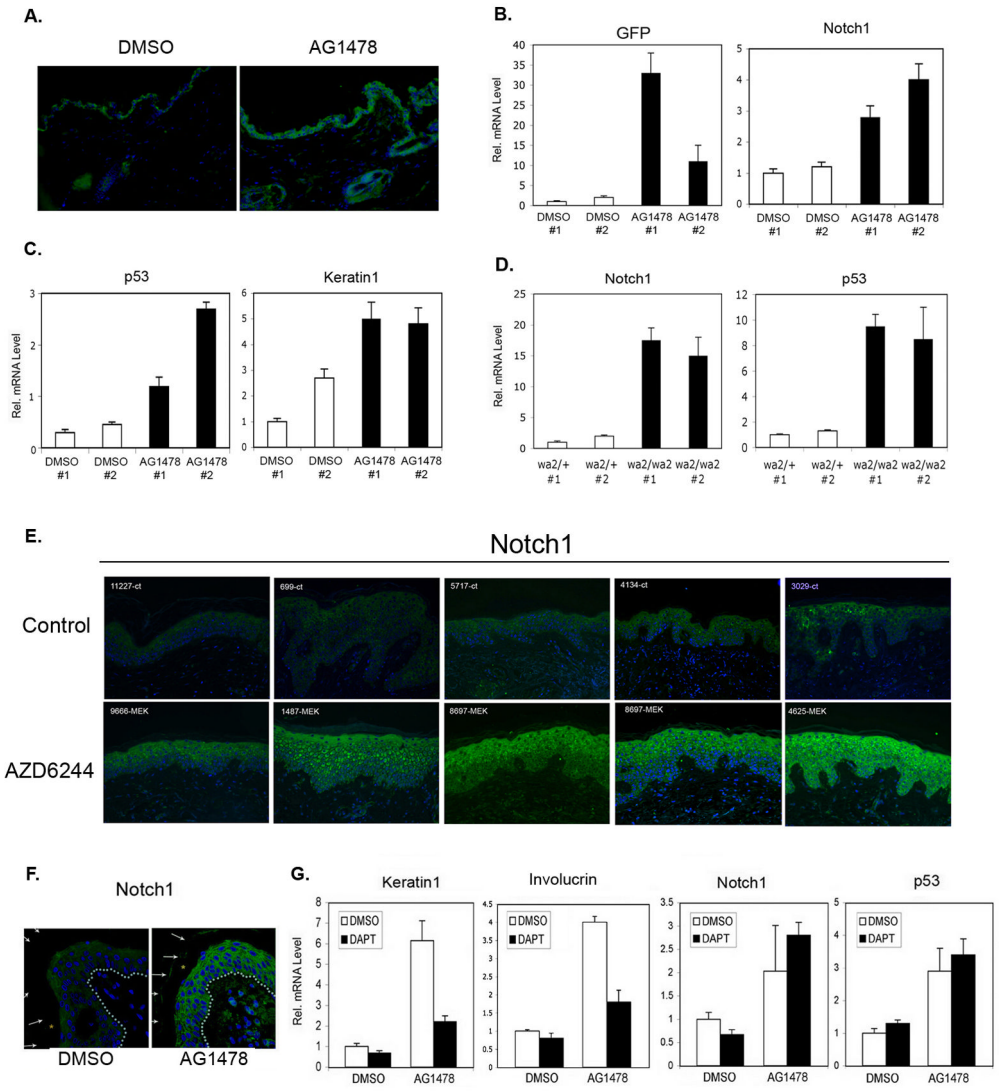

Fig. 4. Suppression of EGFR signaling induces p53 and Notch1 expression in intact mouse and human epidermis

A : Trangenic mice expressing a GFP reporter gene from a Notch/CBF1-responsive promoter[26] were injected intraperitoneally with AG1478 (1 mg, in 100 μl 75% DMSO) or DMSO control every two days. 6 days later, mice were analyzed for GFP expression by immunofluorescence of back skin sections using anti-GFP antibodies. B and C : Back skin of the same mice as in the previous panel was isolated and the epidermis was separated from the underlying dermis by a brief heat treatment. Expression of the GFP transgene, as well as of the endogenous Notch1, p53 and keratin 1 genes was determined by real time RT-PCR, using β-Actin for internal normalization. D : Back skin epidermis of homozygous *wa-2* mice (carrying a point mutation of the EGFR gene resulting in a > 90% fold decrease of EGFR activity,[43] and heterozygous littermates was analyzed in parallel for endogenous p53 and Notch1 expression by RT-PCR as in the previous panel. E : skin biopsies from a cohort of melanoma cancer patients following treatment with the MEK inhibitor AZD6244 (ARRY-142886; ASTRA Zeneca) were analyzed, in parallel with biopsies from age- and gender-matched controls retrieved from the same body region, for expression of Notch1 expression by immunofluorescence analysis with anti-Notch1 antibodies. Same exposure and image capture conditions were utilized for all samples. In parallel with the increased Notch1 expression,

immunostaining with anti-p53 antibodies demonstrated increased levels of this protein[27] and data not shown). (F) : Freshly excised human skin samples were placed in semisolid medium and treated with DMSO control or AG1478 (10 μM) for 24h. Histological sections were analyzed by immunofluorescence with antibodies against the Notch1 protein. Arrows indicate the outermost layer of the epidermis and dotted line the innermost. H&E staining and immunofluorescence of parallel sections with anti-Keratin1 antibodies are shown in supplemental Fig. 5A. B : Freshly excised human skin samples, as in the previous panel, were placed in semisolid medium and treated with DMSO or AG1478 (10 μM) plus/minus DAPT (10 μM) (black and white bars, respectively) for 24h. The epidermis was separated from the underlying dermis by a brief heat treatment (60°C, 45”) followed by total RNA preparation and analysis of mRNA expression of the indicated genes by real time RT-PCR, using β-actin mRNA for normalization.

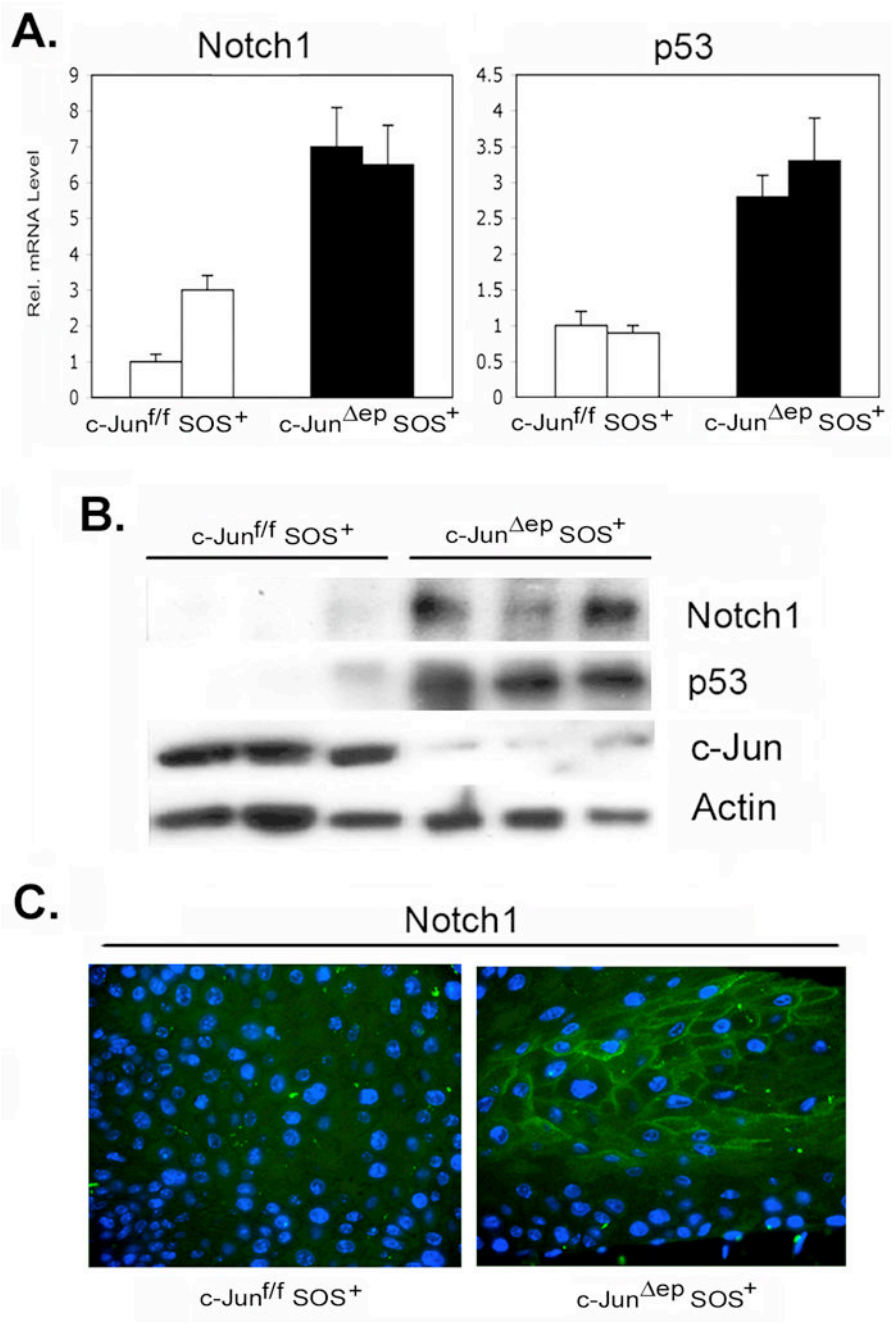

Fig. 5. Differential Notch1 and p53 expression in the EGFR-dependent SOS-mouse skin tumor model plus/minus c-Jun deletion

A : Tumors formed by two K5-SOS-F transgenic mice with an intact c-Jun gene ($Jun^{f/f}$ SOS^{+}) and from two transgenics with a concomitant keratinocyte-specific deletion of the *c-Jun* gene ($c\text{-}Jun^{\Delta ep}$ SOS^{+}) were analyzed by real time RT-PCR for levels of Notch1 and p53 expression (left and right panels, respectively), using β-actin for normalization. B : Extracts from another set of tumors, from three mice per genotype, was analyzed by immunoblotting with antibodies against the indicated proteins. C : Immunofluorescence/confocal analysis with anti-Notch1 antibodies of tumors formed by K5-SOS-F transgenics plus-minus c-Jun deletion. Hoechst staining was used for cell identification.

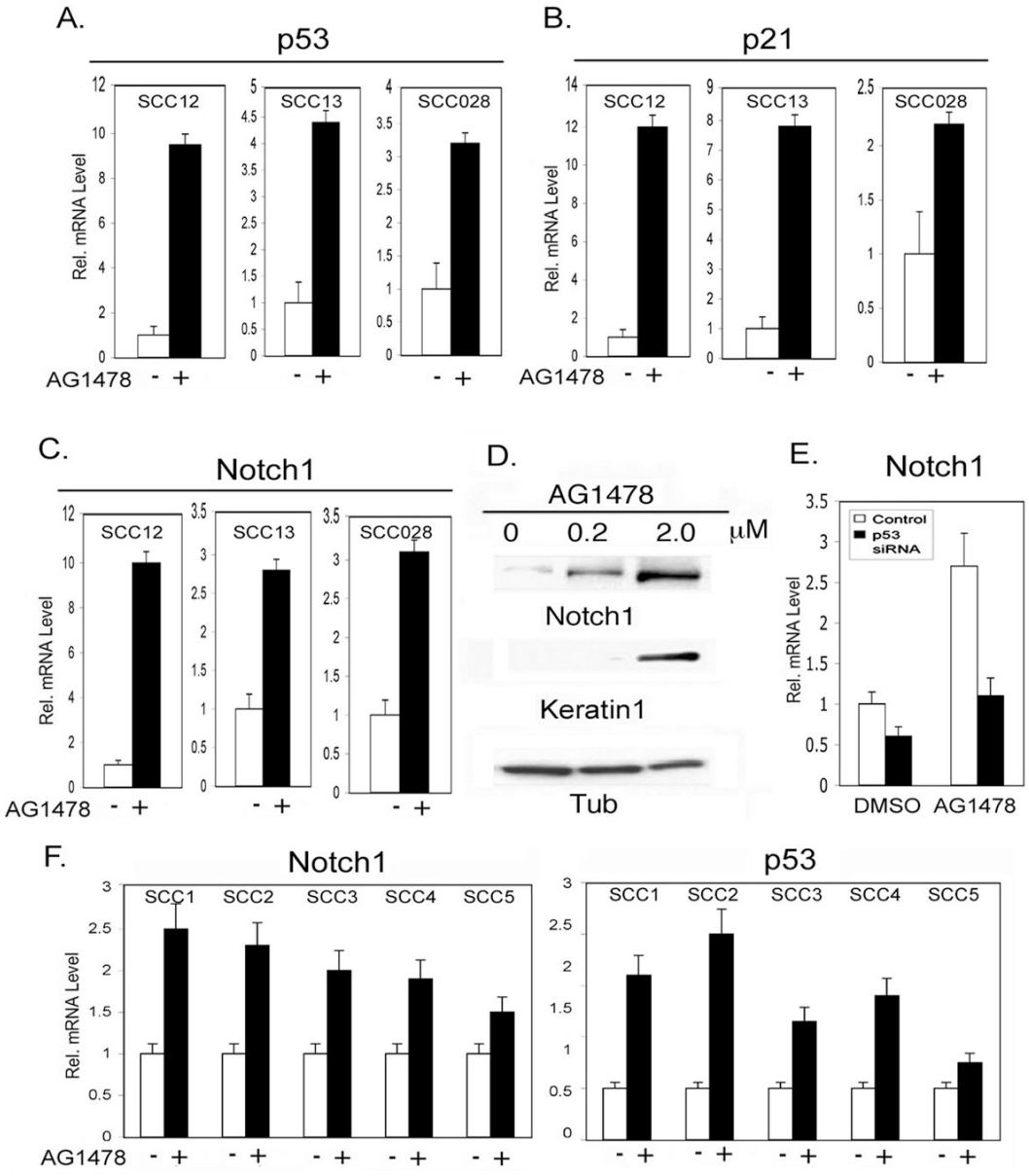

Fig. 6. EGFR-dependent regulation of p53 and Notch in cancer cell lines and human squamous cell carcinomas (SCCs)

A – C: SCC12, SCC13 and SCCO28 cells, treated with AG1478 (2 μM) versus DMSO control for 24 hours, were analyzed for levels of p53 (A), $p21^{WAF1/Cip1}$ (B), and Notch1 (C) expression by real time RT-PCR. D : SCCO28 cells were treated with AG1478 at the indicated concentrations for 24h followed by immunoblot analysis for Notch1 and γ-Tubulin as equal loading control. E : SCCO28 cells were transfected with p53-specific siRNA in parallel with scrambled siRNA control followed, 24 hours later, by treatment with AG1478 or DMSO for 24 hours. Real time RT-PCR was used to determine resulting levels of Notch1 expression, as well as to verify p53 knockdown (not shown). F : Surgically excised squamous cell carcinomas from five different patients (SCC1, 2, 3, 4, 5) were cut into small pieces of similar size, placed in semisolid medium and treated with AG1478 (10 μM) or DMSO control for 24 hours. Real time RT-PCR was used to measure expression of Notch1 and p53. Analysis of c-Fos (as a

measure of response to EGFR inhibition) and keratin 1 expression in these tumors is shown in supplemental Fig. 6C.

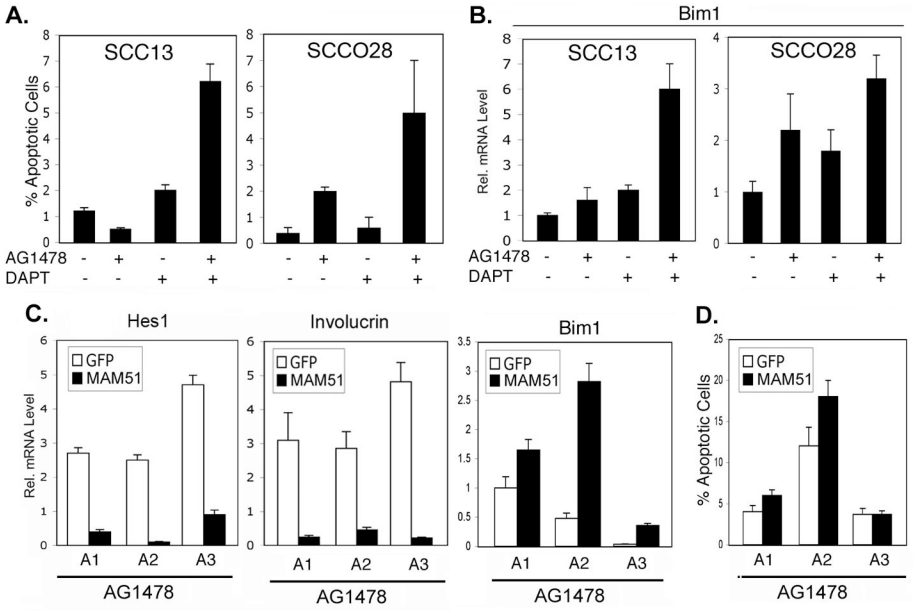

Fig. 7. Enhanced apoptosis in squamous carcinoma cells by concomitant suppression of EGFR and Notch signaling

A : SCC13 and SCCO28 cells were treated with DMSO, AG1478 (5 μM), DAPT (20 μM) alone or in combination for 72 h. Cells were analyzed by TUNEL assays and flow cytometry. Values represent the average percentage of apoptotic cells from 3 independent experiments ±SD. B : SCC13 and SCCO28 cells, treated as in previous panel, were analyzed for expression of the pro-apoptotic gene Bim1 by RT-PCR. C : SCC formation was induced in nude mice by subcutaneous injections of SCCO28 cells expressing MAM51 or GFP control (black and white bars, respectively). To minimize individual animal variations, each mouse was injected in parallel, on the right and left flank respectively, with the two kinds of cells. Four weeks after injections, the tumor bearing mice were treated three times, every other day, with AG1478 (1 mg/animal, mice A1, A2, A3). RNA isolated from tumor tissues were analyzed by real time RT-PCR for expression of the indicated genes. D : Frozen sections from the same surgically excised tumors as in the previous panel were analyzed by TUNEL labeling. The fraction of TUNEL positive cells was determined in four different microscopy fields per tumor, using LPLab software. Average percentage of apoptotic cells ± SD is represented. The statistical significance of the observed differences was calculated by Ttest ($P<0.05$).