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# Itch inhibition regulates chemosensitivity *in vitro*

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**Abstract**

Itch is a member of the HECT family of ubiquitin E3 ligases, and regulates the stability of several proteins involved in response to genotoxic stress. We have previously shown that p73 and p63, two members of the p53 family of tumour suppressors, are targets for Itch-mediated ubiquitylation and degradation. Here, we show that depletion of Itch by RNA interference augments apoptosis upon treatment with chemotherapeutic drugs. We also show that cells with no functional p53 are more sensitive to Itch depletion, highlighting the importance that changes in levels of Itch may play in majority of cancers, where p53 is absent or mutated. Furthermore, reintroduction of Itch in fibroblasts obtained from Itch deficient mice results in reduced cell death upon DNA damage. Overall our findings suggest that inhibition of Itch potentiates the effect of chemotherapeutic drugs revealing the pharmacological potentials of targeting Itch for cancer therapy.


*Keywords:* Itch; p73; p63; p53 Chemosensitivity; p53; E3 ligase; Protein degradation; Proteasome; Drug discovery

p73 and p63 are structurally and functionally similar to the p53 tumour suppressor. They both induce cell cycle arrest and apoptosis and have recently been shown to act as tumour suppressors *in vivo* [1]. Recent work using p63/p73 null mice has clearly demonstrated that both these proteins have tumour suppressor functions independent of p53 [2]. Indeed, p63 and p73 mutant mice are predisposed to aggressive epithelial tumours common in humans (e.g. lung and mammary adenocarcinomas), unlike p53 null mice, which primarily develop thymic lymphomas and sarcomas [2]. Therefore in tumours where p53 is mutated or absent (more than 50% of human cancers) control of the p63/p73 proteins levels may be of critical importance in regulating the sensitivity of the transformed cells to chemotherapeutic agents.

We have shown that the E3 ligase, Itch, is a key player in the control of p63 and p73 protein levels and provides a new mechanism that may play a relevant role in the cellular response to genotoxic stress. Itch belongs to the Nedd4 like family of ubiquitin E3 ligases [3] and is characterized by a modular organization that includes: an N-terminal protein kinase C-related C2 domain, multiple WW domains, and a C-terminal HECT (homologous to the E6-associated protein carboxyl-terminus) Ub-protein ligase domain. Itch deficient mice (Itchy) develop a fatal disease characterized by constant itching of the skin and development of severe inflammatory and immune disorders [4]. At the cellular level, Itch promotes the ubiquitylation and degradation of several transcription factors, such as c-Jun and Jun-B [4,5], Notch [6] and proteins involved in cell death regulation such as c-FLIP [7]. The potential role of Itch in apoptosis is further strengthened by the observation that Itch is downregulated in tumour cell lines upon treatment with DNA damaging agents [9].

*Abbreviations:* HECT, homologous to the E6-associated protein carboxyl-terminus; Ub, ubiquitin; WW, triptophan domain; MEF, mouse embryonic fibroblasts; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SAM, sterile alpha motif; FACS, fluorescent activated cell sorter; siRNA, small interference RNA.

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Itch binds to p63/p73 via the C-terminal PPxY motif, which is absent in the p53 protein [8,9], and as a result Itch can only target p63/p73, but not p53, for poly-ubiquitylation and degradation [8,9]. Consequently the ability of Itch to control the steady state protein levels of crucial regulators of apoptosis such as p63/p73 raises the question of whether inhibition of Itch may be useful in regulating chemosensitivity, particularly in p53 null tumour cells.

In this study, we have analysed the contribution that Itch activity has to induction and execution of apoptosis after chemotherapy-induced DNA damage using both Mouse Embryonic Fibroblasts (MEFs) obtained from Itch wild type (MEFs +/+) and Itch deficient mice (Itchy) (MEFs −/−) as well as knocking down Itch expression in carcinoma cell lines by RNAi. We show that MEFs −/− die more readily than MEFs +/+ and that knockdown of Itch in HeLa cells also increases susceptibility to DNA damage induced apoptosis. These results suggest the potential exploitation of Itch as a target for cancer therapy.

## Materials and methods

*Cell culture and transfections.* H1299 and Saos-2 were grown in RPMI medium (GibcoBRL 31870), whilst HeLa, HEK293T and Cos-1 cells were grown in Dulbecco′s modified Eagle′s medium (GibcoBRL 61965). All media were supplemented with 10% (vol/vol) fetal bovine serum (GibcoBRL), and cells were cultured at 37 ° C in a humidified atmosphere of 5% (vol/vol) CO2 in air. Transient transfections were performed with Lipofectamine 2000 or Calcium Phosphate reagents according to the protocol of the manufacturer (Invitrogen).

*Plasmids.* The pSUPER-ITCH2, pSUPER-ITCH18 and pSUPER-scrambled vectors were generated by insertion in pSUPER vector (OligoEngine) of oligos targeting the following sequences: ITCH 2, AAACATTAAAGTCAAACAATATG, ITCH 18 AAGGAGCAACATC TGGATTAATA, (These sequences are 100% identical both in human and mouse ITCH); and a scrambled shRNA control.

Myc-Itch and Myc-Itch mutant (C830A) plasmids were used as described in [9].

*Western blot.* Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk powder in Tris-buffered saline-0.05% Tween 20 for 1 h. Immunodetection was performed by incubating the membranes with the different primary antibodies diluted in blocking buffer for 2 h at room temperature or overnight at 4 °C. After four washes with Tris-buffered saline-0.05% Tween 20, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase for 1 h. After four washes, blots were developed with ECL Plus detection kit (Amersham), and membranes were exposed to Hyperfilm chemiluminescene film (Amersham). The following antibodies were used: Itch (611199 BD), p73 SAM domain and full-length specific antibodies (Sayan et al., 2005), anti-Actin (C-11; Santa Cruz), anti-Jun-B (sc-46; Santa Cruz), anti-p53 (sc-6243; Santa Cruz).

*Genotoxic stress in primary cells from Itchy mice.* MEFs were isolated from wt and Itchy littermates at E13.5 and cultured for 1 passage. Cells at p2 were then stimulated with different drugs, and samples were collected for protein and apoptosis analysis. Apoptosis was evaluated at 24 by subdiploid peak analysis, AnnexinV staining and loss of mitochondrial membrane potential.

*Effects of shRNA against Itch on response to DNA damage and effect on p73 levels.* Cells were transfected with plasmid, 24 h later cells were replated in 100 mm dishes after further 24 h cells were treated with chemotherapeutic agents and samples were collected 24 h after drug treatment. Samples were collected for protein and FACS analysis.

## Results and discussion

To assess the potential of Itch to modulate apoptosis, we compared the cell death obtained in MEF +/+ and MEF −/− treated with DNA damaging agents. We detected an increased number of hypodiploid cells in MEFs −/− compared to MEFs +/+ after treatment at the highest dose of cisplatin for 24 h (Fig. 1A). Similarly, this dose of cisplatin produced more Annexin V positive cells (Fig. 1B) and a greater loss of mitochondrial membrane potential (Fig. 1C) in MEFs −/− compared to MEFs +/+. Similar results were also observed using MEFs treated with doxorubicin (data not shown).

As a control we assessed the levels of p53 and p73 in both the MEFs +/+ and MEFs −/−. As expected, since p73 is a known Itch target, we detected increased basal protein levels of p73 in MEFs −/− (Fig. 1D). Consistent with the fact that p53 degradation is not regulated by Itch, due to the absence of the PPxY motif in p53, expression of wild type p53 is similar in both sets of MEFs, both at basal levels (Fig. 1D).

p73 is able to induce apoptosis independently of p53 [10–12] in response to a variety of DNA damaging agents [13]. Therefore, as the MEFs +/+ and MEFs −/− both contain p53, which in this instance could play a role in cell death signalling, we tested the role of Itch in DNA-damage induced cell signalling using cell lines that were null for p53 expression in order to evaluate more accurately the contribution of Itch target proteins like p63 and p73. HeLa cells were used as a model as they are p53 null, due to p53 binding to the HPV E6 protein, rendering it non-functional, and over expression of p73 in this cell line sensitises HeLa cells to apoptosis induced by DNA damage agents [14,15]. A number of short hairpin interfering RNAs (siRNA or shRNA) were tested for downregulation of Itch expression in HeLa cells (Fig. 2A). The majority of shRNAs targeted the regions of Itch outside the WW and HECT domains, which are not conserved among the E3 Nedd4-like family members. Itch levels were reduced by shRNAs 2 and 18 in all the p53-null cell lines tested, including HeLa, H1299, Saos-2, 293 T and Cos cells (Fig. 2B). The sequences targeted by these two shRNAs are identical between human and mouse Itch, allowing effective knockdown of Itch in cell lines from both species. When Itch expression is reduced by shRNA in HeLa cells, their sensitivity to doxorubicin induced cell death is enhanced (Fig. 2C). This increase in sensitivity is even greater than the effect observed when comparing MEFs +/+ and MEFs −/−, and can be explained by the absence of a functional p53 in these cells. Similarly, when MEFs −/− have wild type Itch expression restored, their sensitivity to doxorubicin is reduced (Fig. 2D). However, when a catalytic mutant of Itch that cannot ubiquitylate its substrates is transfected into MEFs −/−, doxorubicin sensitivity is unaffected. This implies that the catalytic activity of Itch and its ability to ubiquitylate target proteins for degradation is important for its contribution to cell death.

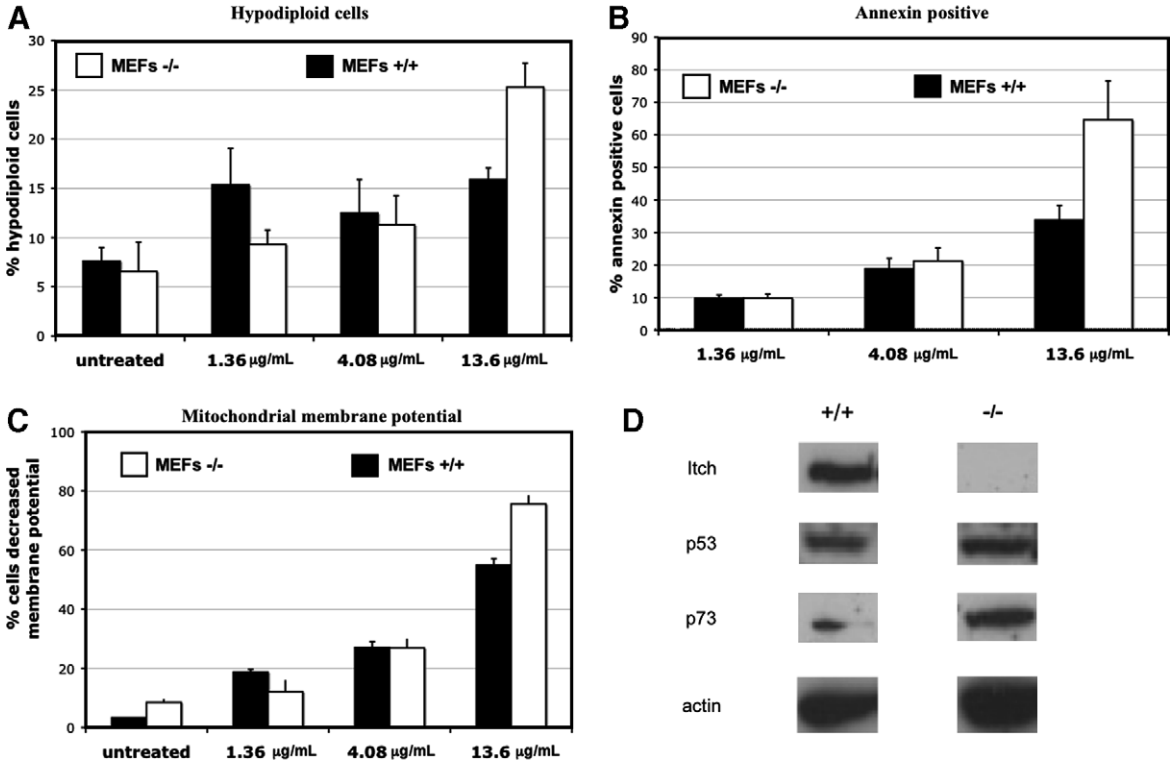


Fig. 1. Induction of apoptosis after 24 h of treatment with the indicated concentration of cisplatin in MEFs +/+ and MEFs −/−. (A) Analysis of hypodiploid cells. (B) Analysis of annexin positive cells after 24 h cisplatin treatment. (C) Analysis of cells that have lost mitochondrial membrane potential after 24 h cisplatin treatment. Black bars indicate MEFs +/+; white bars indicate MEFs −/−. (D) Lysates of MEFs +/+ and MEFs −/− harvested from E13.5 day mice were analysed by Western blot for Itch, p53 and p73 with actin as a loading control.

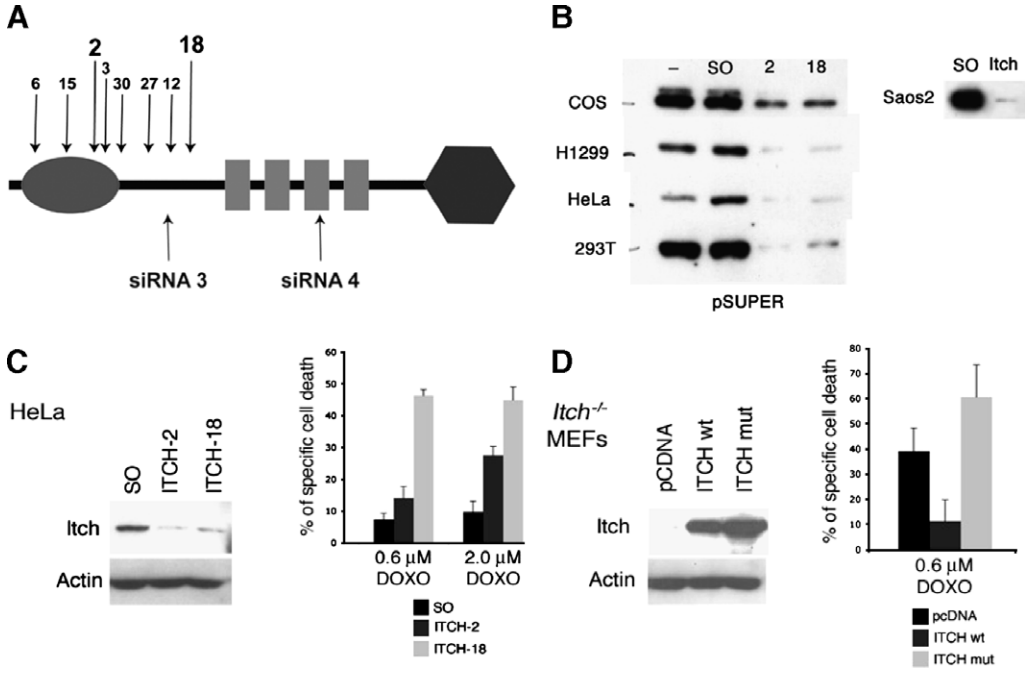


Fig. 2. RNAi of Itch and reintroduction of Itch into Itch −/− cells. (A) Schematic of Itch protein indicating the location of the siRNA constructs. Circle; C2 domain, rectangles; WW domains, hexagon; HECT domain. (B) Knockdown of Itch in HeLa, H1299, 293 T, Cos and Saos-2 cells using two siRNA constructs. (C) Cell death observed in these HeLa cells in which Itch expression has been knocked down by RNAi when treated with the indicated doses doxorubicin. (D) Reintroduction of Itch wild type into MEFs −/−, but not a catalytically inactive mutant, induces chemoresistance to doxorubicin.

In this paper we have shown that the absence of Itch in cells obtained from Itch deficient mice leads to increased basal expression of p73 as well as to an increased number of apoptotic cells after 24 h treatment with DNA damaging agents. Moreover, the role of Itch in blocking apoptosis was more evident when similar experiments were performed in p53 null cell lines where Itch expression was knocked down using RNAi.

Overall these observations suggest that inhibition of the activity of Itch and subsequent accumulation of pro apoptotic substrates is very important in regulating sensitivity to DNA damaging agents use in chemotherapy. In particular the regulation of two targets of Itch, p63 and p73, is of special importance in cancer treatment as these genes are seldom mutated in human tumours and can induce apoptosis by p53-independent pathways. Therefore, inhibition of Itch is even more relevant in tumours that do not express functional p53 (approximately 50% of human cancers) and reveals Itch as a potential pharmacological target in cancer therapy.

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