

## Report

# Regulation of the Cyclin-Dependent Kinase Inhibitor p57<sup>Kip2</sup> Expression by p63

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## KEY WORDS

p63, p57<sup>Kip2</sup>, development

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## ABSTRACT

The cyclin-dependent kinase (CDK) inhibitor p57<sup>Kip2</sup> is a negative regulator of cell proliferation, binding to a variety of cyclin-CDK complexes and inhibiting their kinase activities. The p57<sup>Kip2</sup> gene was recognized as a target gene for p73 $\beta$ , one member of the p53 family. In spite of this, the phenotypes of p73 and p57<sup>Kip2</sup> knockout mice do not resemble each other while there is a phenotypic overlap between the p57<sup>Kip2</sup> null mice, the p63 null mice and patients affected by p63 associated syndromes, suggesting that p57<sup>Kip2</sup> could be indeed a downstream target of p63. By ChIP we determined that in the HaCaT cell line the  $\Delta$ Np63 $\alpha$  protein is associated to three different regions of the p57<sup>Kip2</sup> gene.  $\Delta$ Np63 can activate both the endogenous p57<sup>Kip2</sup> gene and a reporter vector containing a -2191 promoter fragment of the p57<sup>Kip2</sup> gene. Natural p63 mutants, associated to the AEC syndrome, show a partial or complete lack of transactivation potential of the p57<sup>Kip2</sup> promoter, while three other natural p63 mutants, associated to the EEC, LMS and SHFM-4 syndromes, were less affected. These data suggests that p63 play an important role in the regulation of p57<sup>Kip2</sup> expression and that this regulation is subverted in AEC p63 mutants.

## INTRODUCTION

Progression of the cell cycle is promoted by cyclin dependent kinases (CDKs). The activity of cyclin-CDK complexes is regulated by various mechanisms, including association of the kinase subunit with the regulatory cyclin subunit, phosphorylation-dephosphorylation of the kinase subunit, and association of the complex with CDK inhibitors (CKIs).<sup>1,2</sup> To date, seven CKIs have been identified in mammals, and these proteins are classified into two families on the basis of their amino acid sequence similarity and putative targets.<sup>3,4</sup> The Cip or Kip family comprises p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>, each of which possesses a conserved domain, termed the CDK binding-inhibitory domain, at its NH<sub>2</sub> terminus. The Ink4 family consists of p16<sup>Ink4A</sup>, p15<sup>Ink4B</sup>, p18<sup>Ink4C</sup> and p19<sup>Ink4D</sup>, and its members each contains four tandem repeats of an ankyrin motif. Whereas members of the Ink4 family inhibit the activity of CDK4 or CDK6 specifically, members of the Cip-Kip family inhibit a broad spectrum of cyclin-CDK complexes.

p57<sup>Kip2</sup> is ubiquitously expressed in the whole embryo and its signal decreases during development.<sup>5,6</sup> The human p57<sup>Kip2</sup> gene is located on chromosome 11p15.5, a site of frequent loss of heterozygosity in several human cancers.<sup>7,8</sup> Furthermore, rearrangements at 11p15 are apparent in individuals with Beckwith-Wiedemann syndrome, which is characterized by numerous developmental abnormalities as well as a 1,000-fold increase in the risk of childhood tumors.<sup>9-11</sup> Mice lacking p57<sup>Kip2</sup> die immediately after birth as a result of dyspnea caused by severe cleft palate. They also show abdominal muscle defects and skeletal abnormalities due to defects in endochondral ossification.<sup>12-14</sup> The p57<sup>Kip2</sup> gene was recently isolated as a target of the p73 $\beta$  transcription factor, one member of the p53 family.<sup>15</sup> It is interesting to note however that p73 null mice show several developmental defects including hydrocephalus, hippocampal dysgenesis, inflammatory and neuronal defects,<sup>16</sup> but none of these defects resembles the phenotype of p57<sup>Kip2</sup> null mice as it should be the case if the two genes are linked to the same regulatory pathway or belong to a common signaling cascade. Instead, the phenotype of the p57<sup>Kip2</sup> null mice is similar to the knockout phenotypes of p63, the other member of the p53 family:<sup>17,18</sup> p63 null mice have severe cranio facial defects, skin and limbs deficiencies and patients affected by syndromes associated to p63 mutations have cleft palate, limbs deformities and ectodermal dysplasia.<sup>19,20</sup> To date, several mutations in the p63 gene have been identified

associated to several distinct human developmental syndromes, characterized by common features such as limb abnormalities, ectodermal dysplasia, and facial clefts.<sup>19</sup> These syndromes are: the Ectrodactyly-Ectodermal dysplasia facial Clefts (EEC), the Ankyloblepharon-Ectodermal dysplasia-Clefting (AEC), the Limb-Mammary Syndrome (LMS), the Acro-Dermato-Ungual-Lacrimal-Tooth (ADULT), and non syndromic Split-Hand/split-Foot Malformation-4 (SHFM-4). The distribution of mutations over the various p63 protein domains, and the structural and functional implications of these mutations establish a clear genotype-phenotype correlation.<sup>20</sup>

The similarities between the p57<sup>Kip2</sup> and p63 null mice suggested us the possibility that the two genes might be linked to the same regulatory pathway or belong to a common signaling cascade with p63 possibly regulating p57<sup>Kip2</sup> expression. Here we report that the  $\Delta$ Np63 $\alpha$  isoform is bound to the p57<sup>Kip2</sup> promoter in vivo and that a 2 Kb p57<sup>Kip2</sup> promoter fragment is activated by the  $\Delta$ Np63s and repressed by the TAp63s isoforms in different cell lines. Moreover, natural p63 mutations, in particular the AEC mutation (Fig. 1), were indeed altered in their capacity to regulate p57<sup>Kip2</sup> transcription reinforcing the hypothesis that these two genes might be linked to a common regulatory pathway.

## MATERIALS AND METHODS

**Cell culture and transfection.** The human osteosarcoma Saos-2 cell line was maintained in RPMI 1640 medium and 10% fetal calf serum. The murine mesenchymal stem cell line C3H10T(1/2), and the human osteosarcoma U2OS cell line were maintained in Dulbecco's modified Eagle's medium (D-MEM) and 10% fetal calf serum. For transfection, 80000 cells were seeded into 24-well multiplates and on the next day transfected with Lipofectamine 2000 (Invitrogen) under the condition suggested by the manufacturer. The total amount of transfected DNA (1  $\mu$ g) was kept constant using empty vector when necessary. Thirty six hours later, cells were lysed and assayed for luciferase activity. The human keratinocyte HaCat cell line was maintained in D-MEM medium supplemented with 10% fetal calf serum. HaCat cells were transfected with Fugene (Roche) under the condition suggested by the manufacturer.

**Plasmids.** The p63 plasmids were kindly provided by Dr. H. Van Bokhoven.<sup>19</sup> The -2191Kip2, -1550Kip2 and -595Kip2 promoter constructs were kindly provided by Dr. O. Dellatre.<sup>21</sup> The -1091Kip2 promoter construct was kindly provided by Dr. K. Vousden.<sup>15</sup> The -2091Kip2, -1891Kip2, -1791Kip2 and -1664Kip2 constructs were generated by PCR: different oligonucleotide pairs were synthesized in order to obtain progressive deletion of the p57Kip2 promoter. The sequences of the oligonucleotides were as follows:

-2091Kip2 forward:  
TTCGGAGCTCGGTAAAGTGGAAAAATAAAGTA,  
-1891Kip2 forward:  
TTCGGAGCTCTGGAGTGTGGGTATCCGGG,  
-1791Kip2 forward:  
TTCGGAGCTCACTTTGCCTCTTTCTGCCC,  
-1664Kip2 forward:  
TTCGGAGCTCGCGGGAAATTTGTAAATTGTGC.  
The reverse primer for all reactions was  
HpaI: GAGACAGGGCGTTAACCACA.

All oligonucleotides contain adequate restriction sites (underlined) to allow further cloning steps: the amplification products were purified and cloned in the pCR 2.1-TOPO vector under the conditions suggested by the manufacturer (Promega). The cloned fragments were digested with SacI and HpaI, purified and ligated in the -2191Kip2 vector, partially digested with SacI and HpaI.

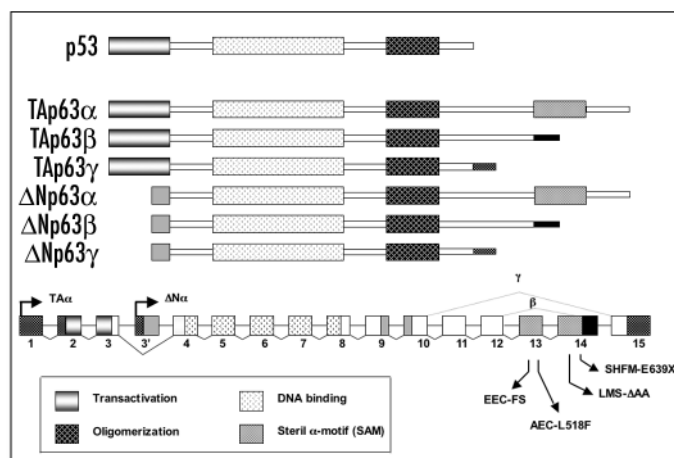


Figure 1 Schematic representation of p63 structure. Intron-exon structure of the p53 and p63 genes, showing the p63 transcriptional start sites and the alternative splicing at the 3' end that gives rise to the  $\alpha$ ,  $\beta$  and  $\gamma$  isoforms. The changes in the reading frame within exons 14 and 15 occurring in the  $\beta$  and  $\gamma$  isoforms are indicated. The positions of the four natural p63 mutations associated to the EEC, AEC, LMS and SHFM-4 syndromes are indicated. All these mutations affect only the  $\alpha$  isoforms.

**Immunoblot analysis.** Thirty-six hours after transfection cells were lysed in 60  $\mu$ l of loading buffer (2% sodium dodecyl sulfate, 30% glycerol, 300  $\mu$ M  $\beta$ -mercaptoethanol, 100 mM Tris-HCl pH 6.8): 15  $\mu$ l of total extracts were separated on a SDS-10% polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher and Schuell). The blots were incubated with the p57<sup>Kip2</sup> C-20 antibody (Santa Cruz Biotechnology) and developed according to the manufacturer's instructions (Super Signal, Pierce).

**Chromatin immunoprecipitations.** Exponentially growing HaCat cells were washed in PBS, incubated for ten minutes with 1% formaldehyde and quenched with Glycine 0.1 M. Cells were sonicated and chromatin fragments of an average length of 1 Kb recovered by centrifugation. Immunoprecipitations were performed with ProtG-Sepharose (KPL, USA), blocked twice at 4°C with 1  $\mu$ g/ml salmon sperm DNA sheared at 500 bp length and 1  $\mu$ g/ml BSA, for two hours and overnight. Chromatin was precleared by adding ProtG-Sepharose for two hours at 4°C, aliquoted and incubated with 3–5  $\mu$ g of the NF-YB purified rabbit polyclonal and p63 antibodies overnight at 4°C. DNA was released by incubating samples for five hours at 65°C, phenol-extracted and ethanol precipitated. Semi quantitative PCRs were performed with the following primers:

-2500 forward: AGGGTATGTAACGCCCCACTGT  
-2500 reverse: TGTCAGGAACGCTGTACACCAC  
-1600 forward: TGATGGCAGCTACACCCCTCTGA  
-1600 reverse: CTTGGCTCTTGATGGTGAACC  
core prom for: CCCGCCTGCAGACAAAGGAG  
core prom rev: CGGCCGCGATTAGCATAATGTAG  
+1000 forward: CTCCTCCCGTGGCATTAAAGG  
+1000 reverse: CGCAATGTGCTGTGTAAGCATT  
+5000 forward: TGGTGCATACAAAGGTGGTTGG  
+5000 reverse: CTAGGGAGGAGGGAGGGATGAA

To produce the anti-p63 antibodies, we cloned TAp63 $\alpha$  (Aminoacids 47-611) and SAM-p63, containing the SAM domain (Aminoacids 508-611) into the PET32 $\beta$  prokaryotic expression vector (Novagen, USA). Recombinant TA-p63 was used to immunize two rabbits. The sera were affinity-purified over two different columns derivatized with the TAp63 and SAM-p63 proteins, respectively. We obtained purified antibodies against (1) the whole protein and (2) specific against the alpha isoforms. The antibodies were checked in Western blots on all p63 isoforms overexpressed in eukaryotic cells. The SAM-p63 abs were indeed >10 enriched for anti-TA and  $\Delta$ Np63 $\alpha$  antibodies (not shown).

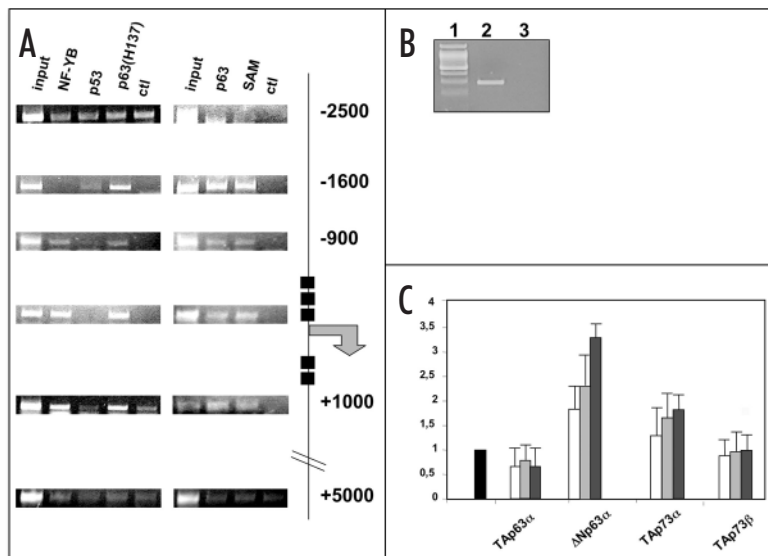


Figure 2. ChIP analysis of the p57<sup>Kip2</sup> promoter. (A) HaCat chromatin was immunoprecipitated with the indicated antibodies (anti-YB, anti-p63 from Santa Cruz and two anti-p63 rabbit antibodies against the whole p63 and against the SAM domain). Several amplicons were used in semi-quantitative PCR amplifications. The -1600 and +1000 regions are highly positives with the three anti-p63 antibodies; the core promoter with the SC-H137. Black boxes: NF-Y binding sites. (B) RT-PCR analysis of endogenous p57<sup>Kip2</sup> expression in HaCat cells: (lane 1, molecular weight marker; lane 2, p57<sup>Kip2</sup>; lane 3 negative control, no DNA). (C) Transcriptional activation of a -2191bp p57<sup>Kip2</sup> reporter plasmid by p63 and p73. HaCat cells were transfected with the -2191bp p57<sup>Kip2</sup> reporter plasmid (0.2 μg) (black bar). Different quantities of expression plasmids for p63 and p73 isoforms were cotransfected (0.1, 0.3, 0.5 μg white, light grey and dark grey bars respectively). Cells were lysed after 36 hours and luciferase activity was determined. The basal activity of the reporter plasmids was set to 1. Data are presented as fold activation/repression relative to the sample without effector. Each histogram bar represents the mean of three independent transfection duplicates. Standard deviation are indicated.

**RNA analysis.** For RNA analysis, 800,000 Saos-2 and U2OS cells were seeded in 100 mm plates. The next day 5 μg of p63 plasmids were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer conditions. Total RNA was prepared with TRIzol reagent (Sigma). For reverse transcription (RT)-PCR detection of gene expression, 3 μg of total RNA was reverse-transcribed using Super Script II (Invitrogen). Total RNA was extracted from growing HaCaT cells with the RNAeasy kit (Qiagen). Three micrograms were reverse transcribed using the Super Script II (Invitrogen).

The following oligonucleotides were used for gene amplification:

The oligonucleotides for the p57<sup>Kip2</sup> PCR were described,<sup>22</sup>  
 forward: TCGCTGCCCGCGTTTGCGCA  
 reverse: CCGAGTCGGTGTCCTTCCG

GAPDH

forward: GTCTCCATCTTCATATGGTAA  
 reverse: CCACCTTCTTGATGTCATCAT

p63

α isoforms:

forward: GTCTCCATCTTCATATGGTAA  
 reverse: CACACTGACTGTAGAGGCA

β isoforms:

forward: AAACGTACAGGCAGCAGCA  
 reverse: CTTGCCAAATCCTGACAATGCTGC

γ isoforms:

forward: GAGGATAGCATCAGAAAACAGCAAG  
 reverse: CTCCACAAGCTCATTCCTGAAGC.

## RESULTS

ΔNp63α interacts *in vivo* with three different regions of the p57<sup>Kip2</sup> gene. A prerequisite for activity of a transcription factor on a putative target gene is the finding that it is bound to relevant sequences *in vivo*. The chromatin immunoprecipitation technique (ChIP) is capable to determine whether this is the case for p63 in the p57<sup>Kip2</sup> gene locus. We used the human keratinocytes HaCaT cell line for chromatin preparation and three different anti-p63 antibodies. As controls, we used anti-YB and anti-p53 antibodies. Note that HaCat cells express almost exclusively the ΔNp63α isoform, as well as two p53 alleles mutated in the DNA-binding domain.<sup>23</sup> We scanned the p57<sup>Kip2</sup> locus with different primers. As shown in Figure 2A, of the six amplicons used, three are strongly positive for p63: -1600, the core promoter at -200 and +1000. The two latter are also positive for NF-Y, a result well expected given the presence of consensus CCAAT boxes in the area. None of the amplicons was positive for p53, confirming the specificity of our ChIP procedure. The -2500 and +5000 regions were negative for all antibodies. The -900 amplicon did show enrichment in both NF-Y and p63 IPs, probably because of the length of our chromatin (1 Kb) that allowed some lateral amplification. However, this was far less pronounced than for the -1600 and core promoter amplicons.

In order to verify whether the association of ΔNp63α with the p57<sup>Kip2</sup> gene was functional in these cells, total RNA was extracted from HaCat cells and used in RT-PCR reactions with oligonucleotides specific for the p57<sup>Kip2</sup> transcript: as shown in Figure 2B the p57<sup>Kip2</sup> mRNA is indeed expressed in HaCat cells.

Therefore, in growing HaCat cells, p63 is functionally associated *in vivo* to the p57<sup>Kip2</sup> gene at three different positions.

**A -2191 bp fragment of the p57<sup>Kip2</sup> promoter is regulated by p63 isoforms.** The ChIP experiments prompted us to tackle a functional analysis of the p57<sup>Kip2</sup> promoter, taking into account two facts: (1) a fragment of 1 kb of the p57<sup>Kip2</sup> promoter was shown to be not responsive to p73 and p53 cotransfection;<sup>15</sup> (2) p63 binds to -1.6 Kb from the start site. We therefore employed a larger fragment of the p57<sup>Kip2</sup> promoter, up to -2191 bp, in transient transfection experiment in the HaCat cell line where p57<sup>Kip2</sup> is expressed endogenously: as shown in Figure 2C, transfection of serial doses of the Tap63α and Tap73β marginally affected the expression of the p57<sup>Kip2</sup> promoter fragment while transfection of Tap73α and to a greater extent ΔNp63α induced its expression. We next performed cotransfection experiments of the -2191 p57<sup>Kip2</sup> promoter with all p63 isoforms, p53, Tap73α and Tap73β in the U2OS human osteosarcoma cell line, expressing p53 but not p63, to verify whether members of the p53 family could regulate its expression. As shown in Figure 3A, in the U2OS cells the -2191 p57<sup>Kip2</sup> promoter activity was marginally affected by cotransfection of serial doses of Tap63α, Tap63β, Tap63γ, p53 and Tap73β expression plasmids; on the other hand, cotransfection of Tap73α led to an increase of the basal activity of the -2191 promoter construct and all ΔNp63 isoforms similarly transactivate this promoter, with the ΔNα isoform being the strongest transactivator (10 fold). We repeated the same experiments in the Saos-2 cell line, that express no p53 and no p63, and very similar results were obtained (data not shown).

In the murine mesenchymal cell line C3H10T(1/2), in which p57<sup>Kip2</sup> expression has been studied,<sup>24</sup> a completely different scenario was obtained: cotransfection of the ΔNp63 isoforms and Tap73α did not have any effect on the -2191p57Kip2 promoter activity, while p53, Tap73β and all Tap63 isoforms had a strong repressive effect on this promoter fragment (Fig. 3B).

These data indicate that the ΔNp63 isoforms and p73α activated the -2191 p57<sup>Kip2</sup> promoter in the U2OS cell line, while the Tap63 isoforms had a strong repressive effect on the -2191 promoter in the C3H10T(1/2) cells and this activity was shared by p53 and Tap73β.

**The -1600 region of the p57<sup>Kip2</sup> promoter is necessary for p63 action.** In order to identify the region of the p57<sup>Kip2</sup> promoter responsible for the



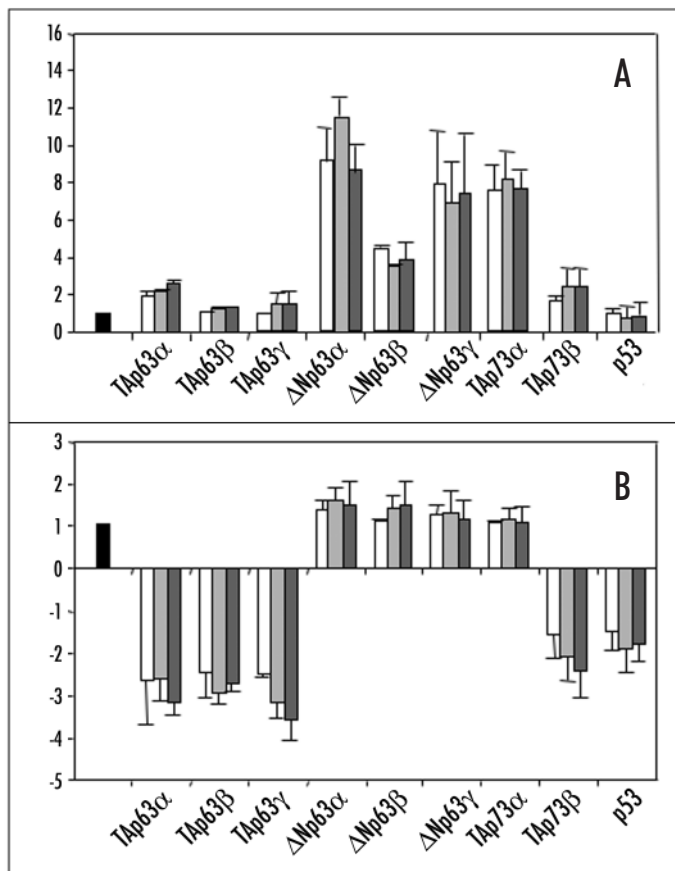


Figure 3. The p57<sup>Kip2</sup> promoter is regulated by p63 isoforms. (A) Transcriptional activation of a -2191bp p57<sup>Kip2</sup> reporter plasmid by p63, p73 and p53. U2OS cells were transfected with the -2191bp p57<sup>Kip2</sup> reporter plasmid (0.2 μg) (black bar). Different quantities of expression plasmids for p63 isoforms, p73 and p53 were cotransfected (0.1, 0.3, 0.5 μg white, light grey and dark grey bars respectively). Cells were lysed after 36 hours and luciferase activity was determined. The basal activity of the reporter plasmids was set to 1. Data are presented as fold activation/repression relative to the sample without effector. Each histogram bar represents the mean of three independent transfection duplicates. Standard deviation are indicated. (B) Transcriptional repression of a -2191bp p57<sup>Kip2</sup> reporter plasmid by p63, p73 and p53. C3H10T1/2 cells were transfected with the -2191bp p57<sup>Kip2</sup> reporter plasmid (0.2 μg) (black bar). Different quantities of expression plasmids for p63 isoforms, p73 and p53 were cotransfected (0.1, 0.3, 0.5 μg) (white, light grey and dark grey bars respectively). Cells were lysed after 36 h and luciferase activity was determined. The basal activity of the reporter plasmids was set to 1. Data are presented as fold activation/repression relative to the sample without effector. Each histogram bar represents the mean of three independent transfection duplicates. Standard deviation are indicated.

observed regulation by ΔNp63 and TAp63 isoforms, we employed deletion constructs of the p57<sup>Kip2</sup> promoter<sup>15,21</sup> in cotransfection experiments with the ΔNp63α isoform in the U2OS cell line and with the TAp63α isoform in the C3H10T1/2 cell line. The results clearly showed that deletion of a region encompassing -2191 and -1550 bp of the p57<sup>Kip2</sup> promoter resulted in substantial loss of both activation and repression by the ΔNp63α and TAp63α isoforms respectively (Fig. 4A and 4B, compare the -2191 and the -1550 constructs): the -1550 bp construct retained however a residual two fold activation and repression in response to ΔNp63α and TAp63α cotransfection, suggesting that two regions of the p57<sup>Kip2</sup> promoter are sensitive to p63 overexpression and this is well in line with the ChIP data that showed p63 binding to the -1600 region and to the proximal promoter region (Fig. 2A). To further narrow the p63 responsive region we generated nested deletion

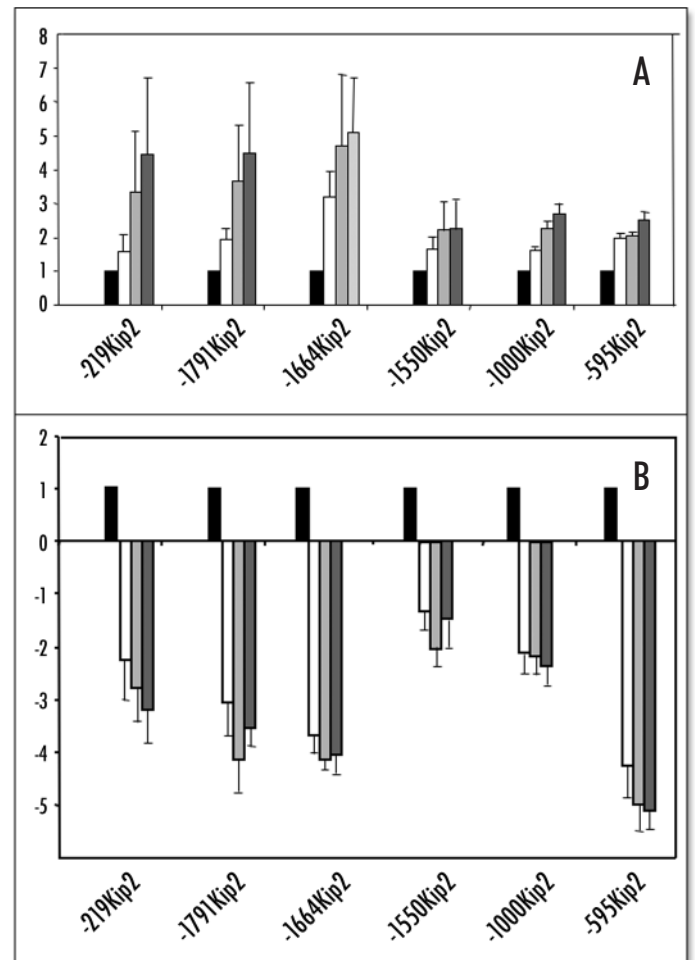


Figure 4. A 100 base pairs region is required for p57<sup>Kip2</sup> regulation by p63. (A) Progressive deletions of the -2191 p57<sup>Kip2</sup> promoter were cotransfected in U2Os cells with different quantities of expression plasmids for ΔNp63α (0.1, 0.3, 0.5 μg) (white, light grey and dark grey bars respectively). The basal activity of the reporter plasmids (0.2 μg) was set to 1 (black bar). Cells were lysed after 36 hours and luciferase activity was determined. Data are presented as fold activation/repression relative to the sample without effector. Each histogram bar represents the mean of three independent transfection duplicates. Standard deviation are indicated. (B) The same constructs as in (A) were cotransfected with TAp63α in C3H10T1/2 cells.

of the -2191/-1550 region by PCR, generating the -1880 (not shown), -1778 and -1664 constructs. Cotransfection of these constructs in the U2Os cell line with ΔNp63α showed that indeed deletion of the region around -1600 (compare the -1664 and -1500 constructs, (Fig. 4A) resulted in a significant loss of activation by ΔNp63α cotransfection. This region was necessary also for repression by the TA isoforms in the the C3H10T1/2 cell line (Fig. 4B).

These results indicate that the same region of the p57<sup>Kip2</sup> promoter around -1600 is responsible for the activation seen in the U2OS cell line by ΔNp63s and for the repression seen with TAp63s in the C3H10T1/2 cell line. Moreover, two regions of the p57<sup>Kip2</sup> promoter are responsive to p63: the -1600 region and the core promoter region.

**The AEC natural p63 mutations subvert p57<sup>Kip2</sup> regulation.** The initial observation of the partial overlap between the p57<sup>Kip2</sup> null mice, the p63 null mice phenotypes and the patients affected by syndromes associated to p63 mutations, prompted us to analyze the activities of natural p63 mutants associated with four different human syndromes on the -2191 p57<sup>Kip2</sup> promoter fragment. Cotransfections experiments of natural p63

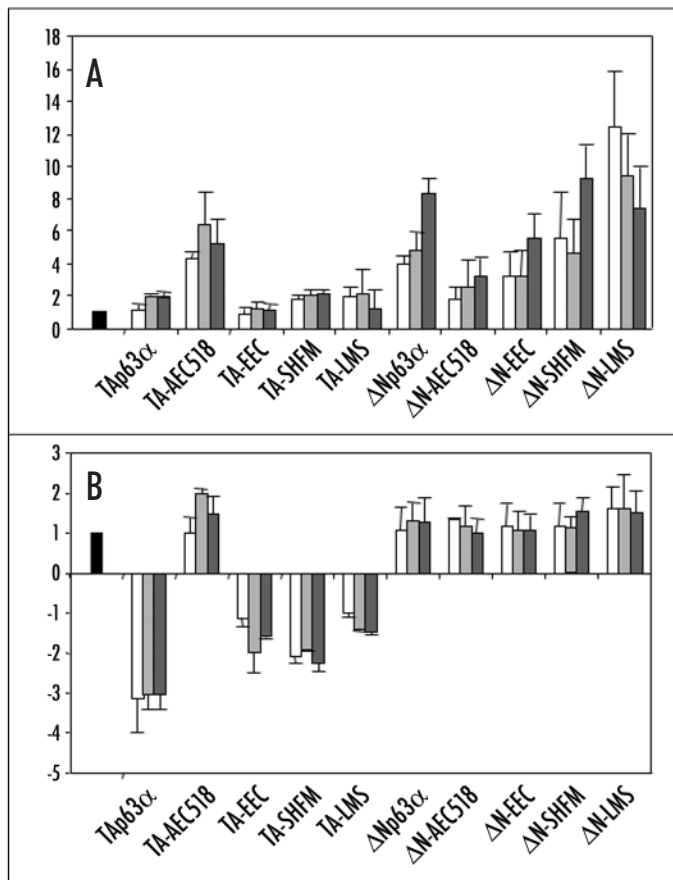


Figure 5. The AEC mutation subvert p63 transcriptional potential. (A) Transcriptional regulation of a -2191bp p57<sup>Kip2</sup> reporter plasmid by AEC, EEC, LMS and SHFM mutations. U2OS cells were transfected with the -2191bp p57<sup>Kip2</sup> reporter plasmid (0.2 μg) (black bar). Different quantity of expression plasmids for p63 mutants were cotransfected (0.1, 0.3, 0.5 μg) (white, light grey and dark grey bars respectively). Cells were lysed after 36 h and luciferase activity was determined. The basal activity of the reporter plasmids was set to 1. Data are presented as fold activation/repression relative to the sample without effector. Each histogram bar represents the mean of three independent transfection duplicates. Standard deviation are indicated. (B) The same constructs as in (A) were cotransfected with TAp63α in C3H10T(1/2) cells.

mutants in the U2OS cell line clearly showed that the ΔN-AEC518 mutant completely lost any transactivation potential while the ΔN-AAA mutant had an increased activation potential: the ΔN-EEC and the ΔN-SHFM behaved as the wild type α isoform (Fig. 5A). The TA mutant isoforms behaved as the wild type TA isoform, having no effect on the basal level of the p57<sup>Kip2</sup> promoter, with the exception of the TA-AEC518 that had a strong activation capacity. In the C3H10T(1/2) cotransfection of the ΔN mutant isoforms did not have effect on the activity of the -2191 promoter construct, behaving like wild type ΔNp63α. On the other hand, of the TA isoforms only the TA-AEC518 isoform was ineffective in repressing the basal activity of the -2191 construct while the other three mutants retained this ability. These data demonstrate that p57<sup>Kip2</sup> regulation is profoundly altered in the AEC p63 mutant.

**The endogenous p57<sup>Kip2</sup> gene is activated by ΔNp63 overexpression.** Our data indicated that members of the p63 family regulate the expression of a p57<sup>Kip2</sup> promoter fragment when overexpressed and that some of the p63 mutants analysed were impaired in this activity. In order to verify whether the endogenous p57<sup>Kip2</sup> gene was subject to the same regulation as the promoter construct used, we transiently transfected the U2Os and Saos-2 cell lines with wild type and mutated ΔNp63 isoforms. The day after

transfection, total RNA was extracted and used in RT-PCR reactions with oligonucleotides specific for the p57<sup>Kip2</sup> transcript. As shown in Figure 6A and 6B, the p57<sup>Kip2</sup> was activated in response to ΔNp63α, ΔNp63β and ΔNp63Δγ overexpression (lanes 2, 3 and 4). The ΔN-EEC, ΔN-SHFM and ΔN-ΔAap63 mutants were still able to activate p57<sup>Kip2</sup> gene expression (lanes 6, 7 and 8) while the ΔN-AEC518 mutant completely failed to do so (lanes 5). In accordance with these data, the endogenous p57<sup>Kip2</sup> protein levels were increased upon ΔNp63α transfection (Fig. 6C). These data indicate that the ΔNp63 isoforms transactivate endogenous p57<sup>Kip2</sup> gene expression and this activity is lost in the AEC natural p63 mutant.

## DISCUSSION

Precise control of cell cycle progression is believed to be critical for normal development, while oncogenesis may be a direct result of its disturbance. Cell cycle progression is regulated predominantly by a series of serine/threonine kinases, the cyclin-dependent kinases (CDKs). The activities of the CDKs are controlled by a group of molecules that inhibit CDK activity, the CDK inhibitors (CKIs). It is widely believed that precise regulation of CKI expression is critical for appropriate development and that breakdown of this regulatory mechanism is deeply involved in oncogenesis and abnormal development. Evidence for this has been provided by the observation that some CKI knockout mice show developmental abnormalities and a predisposition to cancer development.

To date, seven CKIs have been identified in mammals and categorized into two families, the Cip/Kip and Ink4 families. The Cip/Kip family is composed of p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup> and is well conserved phylogenetically but, despite the structural and biochemical similarities among the Cip/Kip members, the phenotypes of knockout mice of each Cip/Kip member are surprisingly different and the p57<sup>Kip2</sup> is the only CDK inhibitor to be required for a correct embryogenesis. The p57<sup>Kip2</sup> protein ceases to be expressed in most organs as an embryo completes its development, in contrast to p27<sup>Kip1</sup>, the expression of which remains constant after birth, implying that p57<sup>Kip2</sup> plays an important role predominantly in early organogenesis. Most p57-deficient mice die immediately after birth due to dyspnea resulting from severe cleft palate, abdominal muscle defects and skeletal abnormalities due to bone ossification defects.<sup>12,14</sup> About 10% of the p57-deficient mice survived beyond the weaning period without showing cancer predisposition.

Little is known about p57<sup>Kip2</sup> transcriptional regulation while that of p21<sup>Cip1</sup> has been investigated extensively<sup>25,26</sup> and turned out to be quite complex; similarly, the regulation of p27<sup>Kip1</sup> seems equally articulated.<sup>27</sup>

Here we report that the cyclin-dependent kinase inhibitor p57<sup>Kip2</sup> gene is a target for p63 mediated transcriptional activation and repression and that three regions of the p57<sup>Kip2</sup> gene are bound by p63 in vivo. The ChIP analysis performed with three anti-p63 antibodies determined that the p57<sup>Kip2</sup> gene is bound by the p63 protein in vivo. Interestingly, the scanning analysis suggests that there are three major areas of binding: an upstream region, which is the focus of the functional analysis performed, the core promoter and a downstream region, whose functional importance was not investigated in the present study. Neither the upstream, nor the core promoter contain bona fide p53 binding sites, suggesting that p63 recognizes DNA through different motifs, or that other DNA-binding factors mediate the association.<sup>28</sup>

The upstream binding site has been functionally dissected. Members of the p53 family, p53 and p73β, failed to induce the -2191 bp p57<sup>Kip2</sup> promoter fragment, while p73α and all ΔNp63 isoforms induced its expression both in HaCat and U2Os cells; all TAp63 isoforms did not alter promoter activities in these cells. On the other hand, the TAp63 isoforms, p53 and p73β had a strong repressor activity in C3H10T(1/2) cells where all ΔNp63 and p73α were totally inert. The different behaviour of p63, p53 and p73 in

the four cell lines used can be due to differential expression of still unknown cofactors that can stimulate/repress p53 family members activities. The differential ability of various p63 isoforms to induce target genes expression has been described in previous studies.<sup>29,30</sup> Our attempts to delineate a p63-responsive element in the -2191 promoter region of p57<sup>Kip2</sup> have led to the identification of a 100 bp fragment that, when deleted, cause a drastic reduction of p63 activation in U2Os cells and a concomitant reduction of repression in C3H10T(1/2) cells, suggesting that both activation and repression are mediated by the same 100 bp region. We further dissected this fragment in EMSA experiments with nuclear extracts derived from cells expressing exogenous and endogenous p63 (not shown); all our attempts to evidenziate p63 binding to this region failed. It is well possible that the conditions employed for binding are not optimal for p63 binding but we rather think that that other DNA-binding factors mediate the association.<sup>28</sup>

A second p63 site was detected in the core promoter region. Here, again, no p53 element is found. We note that there are multiple CCAAT boxes in the promoter; CCAAT boxes are recognized and activated by the NF-Y trimer. NF-Y was recently shown to be required for the association of p53 to promoters of G<sub>2</sub>/M controlling genes in mouse NIH3T3, in the absence of p53-binding sites.<sup>31</sup> Indeed, p57<sup>Kip2</sup> ChIPs positivity for NF-Y is limited to the core promoter and the +1000 region: the presence of multiple CCAAT in the core promoter suggests that indirect binding of p63 through CCAAT-bound NF-Ys is the likeliest possibility to explain p63 positivity in the core promoter. Association of  $\Delta$ Np63 $\alpha$  with multiple CCAAT promoters through direct protein-protein recruitment has been observed (Testoni B, Mantovani R, submitted).

The  $\Delta$ Np63 $\alpha$  is known to behave as a dominant negative factor for p53 transcriptional activity. HaCat keratinocytes contain two mutant p53 alleles;<sup>23</sup> consistent with this, none of these binding sites are recognized by p53 in our study. We and others have shown that this isoform, which is devoid of the N-terminal activation domain, is nevertheless capable to activate p53 targets.<sup>29,32</sup> One important finding from our study is that indeed  $\Delta$ Np63 $\alpha$  is the most potent of activators among the different isoforms on the p57<sup>Kip2</sup> promoter. Together with the ChIP data, this strongly suggests that p57<sup>Kip2</sup> is a physiological target in vivo of this isoform. The TA domain is evidently dispensable for this, and p53 is manifestly not involved. It was important to control this, since p53 mutated in the DNA-binding domain are known to possess a higher affinity for p63 and p73. HaCat keratinocytes contain two mutant p53 alleles;<sup>23</sup> consistent with this, none of these binding sites are recognized by p53 in our study.<sup>33</sup> Thus it is not through heterotetramerization that the p63 presumed tetramer works, equally indicating that activation is not brought by the p53 TA domain. Hence, the additional activation domain identified at the C-terminal of the  $\Delta$ Np63 $\alpha$  is manifestly providing the activation potential.<sup>29</sup> In general, these data strengthen the notion that bona fide targets of this isoform exist that are solely activated, and not repressed.

The AEC p63 mutation profoundly affected p63 transcriptional activity, turning the  $\Delta$ N isoform in a weak activator of the p57<sup>Kip2</sup> promoter and the TA isoform in an activator instead of a repressor. This scenario suggests that during development the expression of the p57<sup>Kip2</sup> gene would be differentially regulated by the same pattern of genes in different cell types. The observation of a clear alteration of p57<sup>Kip2</sup> expression by  $\Delta$ Np63AEC mutants, together with the fact that p57<sup>Kip2</sup> null mice share similar features with AEC patients (cleft lips and palates) suggests that p57<sup>Kip2</sup> may act in the developmental pathways altered in AEC ectodermal dysplasia. We have

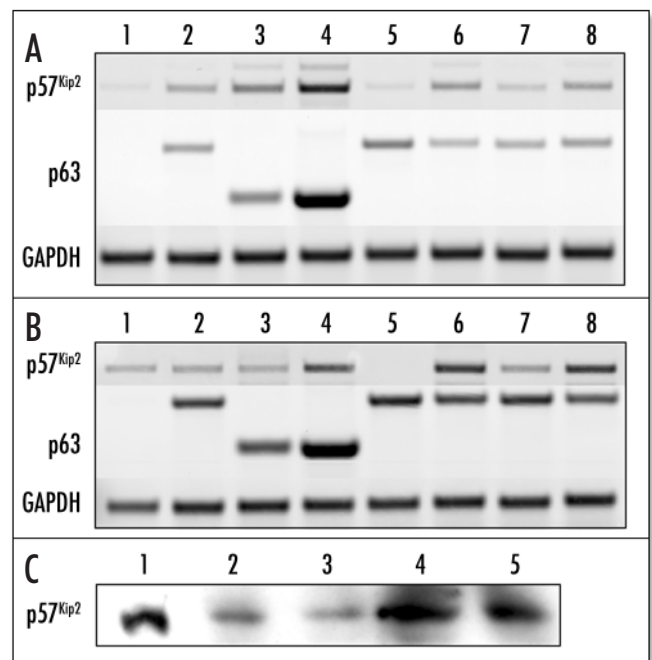


Figure 6. The endogenous p57<sup>Kip2</sup> mRNA and protein are induced upon p63 transfection. RT-PCR analysis of endogenous p57<sup>Kip2</sup> and GAPDH expression in Saos-2 (A) and U2OS (B) cell lines. Cells were transfected with empty vector (lanes 1),  $\Delta$ Np63 $\alpha$  (lanes 2),  $\Delta$ Np63 $\beta$  (lanes 3),  $\Delta$ Np63 $\gamma$  (lanes 4),  $\Delta$ N-AEC518 (lanes 5),  $\Delta$ N-AEC (lanes 6),  $\Delta$ N-SHFM-4 (lanes 7),  $\Delta$ N-LMS (lanes 8); twenty-four hours after transfection mRNA was extracted and levels of endogenous p57<sup>Kip2</sup> and GAPDH and levels of transfected p63 were assessed by RT-PCR. (C) Saos-2 cells were transfected with empty vector (lane 2) with 10 ng of a p57<sup>Kip2</sup> expression plasmid (lane 1) and with increasing amount of the  $\Delta$ Np63 $\alpha$  plasmid (lane 3, 50 ng; lane 4, 100 ng; lane 5, 200 ng). 36 hours after transfection cells were lysed in 60  $\mu$ l of lysis buffer: 15  $\mu$ l of extracts were immunoblotted with p57<sup>Kip2</sup> antibody.

evidences that only the  $\Delta$ Np63 isoforms are expressed during mouse development from E10 through E14 in the limb buds and in the branchial arches (manuscript in preparation) and mounting evidence suggests that the  $\Delta$ Np63 $\alpha$  isoform is predominant in stem cells of multilayered epithelia, in particular keratinocytes.<sup>34,35</sup> Because AEC patients also have dermatological abnormalities,<sup>36</sup> abnormal regulation of p57<sup>Kip2</sup> as suggested by our experiments might contribute to the emergence of the skin phenotype as well.

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