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HACAT AS A MODEL FOR KERATINOCYTES TRANSFORMATION

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Contents

Part I

Abstract

Mutations of the tumor suppressor gene *p53* occur in more than 50% of human malignancies and lead to the loss of suppressor activity. Moreover frequently p53 mutants gain novel, oncogenic properties by transcriptional activation of the genes, involved in cellular proliferation, cell survival and angiogenesis. Today's challenge is to understand mechanisms underlying the gain-of-function of mutant p53 proteins.

By the example of KLF4 promoter we demonstrate that mutant p53 can carries out its gain-of-function by interaction with another p53 family member, transcription factor p63. In the first report we provide strong evidence that KLF4 is negatively controlled by p63 in normal skin in the presence of physiological levels of wild type p53 (wtp53) and that this regulation is subverted by oncogenic mutations of p53, establishing a direct link between these TFs, commonly overexpressed in squamous cell carcinoma (SCC).

These results inspired us to investigate the mechanisms of mutant p53 gain-of-function on the genome scale. In the second manuscript we demonstrate that mutant p53 HaCaT alleles are pro-growth and mutp53 have thousands of binding sites in the human genome; they affect gene expression profoundly, both by binding with p63 to consensus elements and by being tethered by other TFs to their locations.

Although 2 mutant p53 alleles are definitely gain-of-function in the HaCaT, they are not sufficient to render these cells malignant. Based on the sphere-forming assay we developed novel model for the tumorigenic conversion of the HaCaT cells: while immortalized keratinocytes display transformed phenotype *in vitro* and not tumorigenic upon injection into nude mice, HaCaT-derived spheres give rise to the SCC *in vivo*. Thus, this simple model can be

useful for the future studies of the genetic and phenotypic signatures during SCC initiation and development.

There is an indication that mutant p53 can execute its gain-of-function via interaction with NF-Y transcription factor. Ongoing study is devoted to the investigation of NF-YA subunit role in the processes of cellular proliferation and apoptosis in the cells with different p53 status.

State of the Art

1. The skin

The smooth surface of the human skin suggests simplicity, but it embodies one of the most complex and metabolically active organs, which is designed to interact and cope with the environment. The skin is the largest organ system of the body which consists of epithelial layer (epidermis), a connective tissue layer (dermis) and an adipose layer (hypodermis). As a physical barrier it prevents water loss and resists mechanical, chemical and microbial attacks. It also functions to regulate temperature, produce hormones and vitamins, such as Vitamin D, and responds to environmental factors including ultraviolet radiation.

1.1. The keratinocytes and the epidermis

The outer layer, the epidermis, is a terminally differentiated, stratified squamous epithelium that forms protective covering of the skin. The major cell, making up 95% of the total, is the keratinocyte, which moves progressively from attachment to the epidermal basement membrane towards the skin surface, forming several well-defined layers during its transit (Eckert, 1989; Nemes and Steinert, 1999).

Keratinocytes arise from stem cells in the basal layer and progressively mature through the spinous and granular layers until they reach the corneal layer and are finally shed into the environment. Thus, in the normal epidermis, there is a tightly controlled balance between proliferation and desquamation that results in a complete renewal or turnover approximately every 28 days. On its journey to the skin surface, the keratinocyte undergoes a complex program of terminal differentiation, also called keratinization. This cellular program is accompanied by highly regulated and marked changes in gene expression, cellular architecture and enzyme activity. Early steps in terminal differentiation

of cells arising from the basal keratinocyte result in a permanent loss of growth potential, or clonogenicity known as commitment and the subsequent sequential expression of differentiation markers. Among the major products of epidermal keratinization are the differentiated keratin intermediate filaments which are essential for maintaining structural integrity of the epidermis (Fuchs, 1990, 1993; Fuchs and Byrne, 1994).

1.2. Epidermal growth and differentiation

Only the basal layer of epidermal cells has the capacity for DNA synthesis and mitosis. Under an as yet unidentified trigger of terminal differentiation, a basal cell will begin its journey to the skin surface. In transit, it undergoes a series of morphological and biochemical changes that culminate in the production of dead, flattened, enucleated squames, which are shed from the surface, and continually replaced by inner cells differentiating outward.

The process of epidermal growth and differentiation has been subdivided into four parts. Basal cells are distinguished by intracellular cytoskeleton composed of a relatively dispersed, but extensive, network of keratin filaments. These filaments are made of two distinct keratin proteins: keratin 5 and Keratin 14 (Nelson and Sun, 1983).

The four to eight layers of suprabasal spinous cells are postmitotic, but metabolically active. These cells devote much of their protein synthesizing machinery to manufacturing two new keratins, keratin 1 and keratin 10, forming cytoskeletal filaments that aggregate into thin bundles (Eichner et al., 1986). In addition, spinous cells make glutamine and lysine-rich envelope proteins, such as involucrin, which are deposited on the inner surface of the plasma membrane of each cell (Rice and Green, 1979).

As spinous cells reach the granular layer, they stop generating keratin and envelope proteins, and make their final fitting in protein synthesis, including production of filaggrin, a histidine-rich basic protein which may be involved in

the bundling of single filaments into larger, macrofibrillar cables (Fleckman et al., 1985). This process of increased filament packing is thought to enable the keratin filaments to be among the few survivors of the massive destructive phase which soon occurs. Loricrin, a recently described and major component of the cornified envelope is also synthesized at this late stage (Mehrel et al., 1990). As each differentiating cell becomes permeable, a calcium inflow activates epidermal transglutaminase, which then catalyzes the formation bonds, thereby biochemically cross-linking the envelope proteins into a cage (Rice and Green, 1979). As other lytic enzymes are released, all signs of metabolic activity terminate, and the resulting flattened squames are filled with macrofibrils of keratin filaments.

The stratum corneum, composed of terminally differentiated keratinocytes sealed together by lipids, is an impermeable, insoluble, and highly protective fortress, which keeps microorganisms out and essential bodily fluids in. This two compartment system of cellular proteins held together by lipids is called the "bricks and mortar"– construct (Nemes and Steinert, 1999).

1.3. Spontaneously immortalized HaCaT keratinocytes

Fusenig and colleagues established the first and so far unique spontaneously immortalized keratinocyte cell line derived from normal adult human skin. This cell line was termed HaCaT to indicate that it was developed from human adult skin keratinocytes during prolonged cultivation at a reduced Ca^{2+} concentration and elevated temperature (Boukamp et al., 1988).

1.3.1. Genetic alterations

The spontaneous immortalization of the HaCaT cell upon long-term cultivation of the normal human adult keratinocytes occurred as a multistep process, each stage of which was characterized by a number of genetic changes (Boukamp et al., 1988; Boukamp et al., 1997). The first genetic alterations in the

HaCaT population detected by means of cytogenetic assay were 3 translocations leading to the loss of one copy of chromosome arms 3p, 4p, and 9p as well as gain of 9q (Boukamp et al., 1988). A quantity of senescence genes is located on these chromosomes and thus their loss could have contributed considerably to the immortalization.

During continued culture of HaCaT cells additional chromosomal changes could be discovered, such a polyploidization (72-88 chromosomes) (Boukamp et al., 1988). However, although propagated for more than 300 population doublings and more than 6 years, HaCaT cells maintained a constant balance of genetic material and remained non-tumorigenic in contrast to virally transformed keratinocytes (Boukamp et al., 1997). Distinct numerical and structural karyotypic alterations during the HaCaT immortalization process are present in the Table1 and Fig. 1.

Passage no.	Numerical distribuition (% of metaphases)		
	Diploid (46)	Hypodiploid (38-45)	Hepotetraploid (72-88)
2	10	90	0
5	0	67	33
11	0	58	42
17	0	0	100
33	0	0	100
50	0	0	100

Table 1. Chromosomal changes of HaCaT cells during adaptation to autonomous growth *in vitro* (Boukamp et al., 1988).

1.3.2. Telomeres and telomerase

Another mechanism may involve increased activity of the enzyme telomerase that was found to be associated with immortalization of HaCaT cells (Härle-Bachor and Boukamp, 1996). Telomerase is a specialized cellular

enzyme complex with reverse transcriptase activity that maintains stable telomere length. In normal cells, continuous shortening of the telomeres occurs as cells approach cellular senescence. In contrast, HaCaT cells exhibit significantly increased telomerase activity resulting in largely maintained telomere length. These observations indicate that telomerase may play an important role in the immortalization process of these cells.

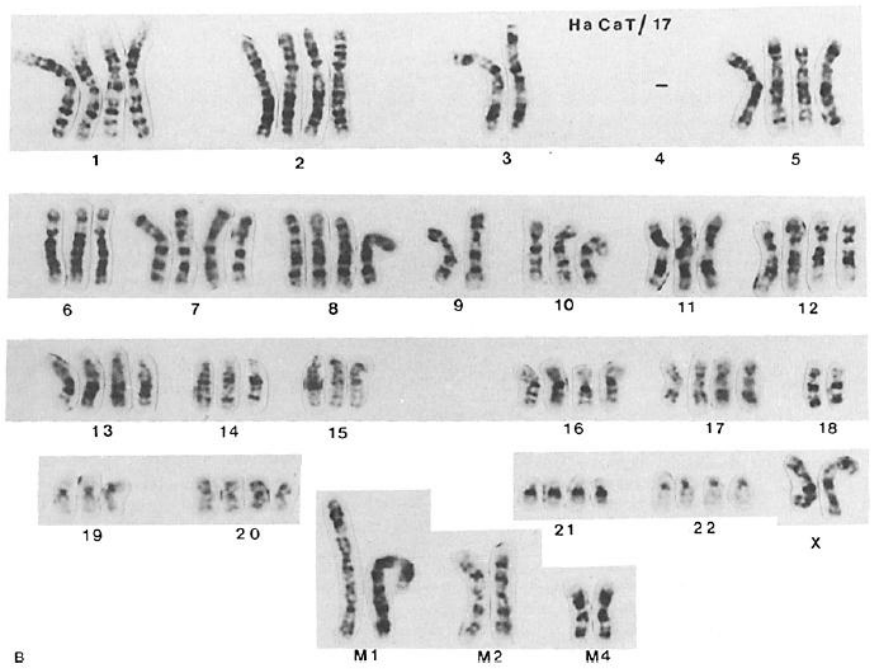

Fig. 1. G-banded karyotype of the HaCaT cells at the passage 17 revealed hypotetraploidy (mode of 82).

1.3.3. Phenotypic changes in the immortalized HaCaT keratinocytes

As expected, HaCaT cells displayed increasing growth potential *in vitro* both at high and clonal cell density (Boukamp et al., 1988; Boukamp et al., 1997). In the soft agar assay HaCaT cells demonstrated anchorage-independent growth that was first visible at passage 18 but at a very low frequency and the efficiency of the growth in soft agar was always low and remained at maximum of 4,5% at high passages. Great increase in the proliferative activity was observed upon transition from the passage 37 to the passage 283 HaCaT cells:

the population doubling time dropped from 23 to 16 hours (Boukamp et al., 1997)

In immortalized by simian virus 40 T-antigen keratinocytes the loss of differentiation was observed, but it's not the case of the HaCaT keratinocytes: even as monolayers, they maintained the capacity to express differentiation markers of the epidermis, such as different keratins (Ryle et al., 1989). Moreover, HaCaT cell differentiation is still regulated by calcium and as differentiation of normal epidermal keratinocytes (Breitkreutz et al., 1993). This differentiation capacity is even more obvious when HaCaT cells are grown as surface transplants on nude mice (Boukamp et al., 1990; Breitkreutz et al., 1991; Breitkreutz et al., 1998). Under these conditions, HaCaT cells form multilayered keratinizing epithelia with a remarkably high degree of organization and differentiation.

Finally, when injected subcutaneously onto nude mice, HaCaT cells do not neither give rise to the tumors nor demonstrate invasive growth behavior up to the highest passages examined (passage 311) (Boukamp et al., 1997). They form epithelial cysts in which cell proliferation stops completely and irreversibly within a few weeks (Boukamp et al., 1988). The unique properties of HaCaT cells, a permanent and stably non-tumorigenic keratinocyte cell line with largely preserved differentiation capacity, made this cell line a paradigm for human epidermal keratinocytes used in more than 600 laboratories worldwide.

1.3.4. Tumorigenic conversion of the HaCaT cells

Human cells demonstrate a remarkable resistance to transformation by oncogenes and chemical agents, however, once immortalized, human cells can be relatively easily transformed into neoplastic cells with these agents (Rhim et al., 1990).

Human immortalized HaCaT keratinocytes were used as an experimental *in vitro* model for studying the mechanisms of human epidermal multistep carcinogenesis (Boukamp et al., 1988; Boukamp et al., 1997; Boukamp et al., 1990; Fusenig and Boukamp, 1998). Immortalization of a cell is considered an early and essential step in the transformation process *in vitro* (Fusenig and Boukamp, 1998). Immortalized cells have morphological alterations, chromosome abnormalities, unlimited growth potential - all properties not found in any cell of the human body except for cancer cells.

The oncogenic conversion of the HaCaT cells can be induced by introduction of additional genetic alterations, e.g. transfection of the *ras* oncogene (Boukamp et al., 1990), by exposing HaCaT cells to culture stress such as growth over many passages in serum-free medium (Hill et al., 1991) and propagation of HaCaT cells at elevated temperature (40 ºC) (Fusenig and Boukamp, 1998). Furthermore the microenvironment was shown to play a significant role in tumorigenic transformation of HaCaT cells. When platelet-derived growth factor B (PDGF-B) was overexpressed in HaCaT cells grafted to mice the cells became tumorigenic (Skobe and Fusenig, 1998). HaCaT cells do not express PDGF-receptor and it was concluded that PDGF expressed by HaCaT is sufficient to activate stromal cells to produce growth factors which in turn stimulate HaCaT proliferation.

1.3.5. Heterogeneity within human epidermal and other cells

Pioneering work of Barrandon and Green revealed 3 clonal types of epidermal keratinocytes with profoundly different proliferative capacity: holoclone has the greatest growth potential and forms large rapidly growing colonies; fewer than 5% of the colonies abort and terminally differentiate. The growth potential of paraclones is limited by 15 generations and then the cells abort and terminally differentiate. The meroclone contains a mixture of the cells of both types and represent transitional stage between paraclone and holoclone

(Barrandon and Green, 1987). Recent studies demonstrated that clonogenic holoclones are bona fide multipotent epidermal stem cells (Barrandon 2005).

The heterogeneity of the cellular composition was demonstrated not just for the normal tissues but also for most human tumors (Heppner et al., 1978). Even if the mechanisms underlying the development of tumor heterogeneity still remain poorly understood, during the past several years the cancer stem cell (CSC) hypothesis for the tumor initiation and progression gained strong support in the cancer research community. CSC model assumes that cancers are maintained and propagated by a small population of the cells within a tumor that possess main stem cell characteristics. The existence of the CSC was first demonstrated in the context of human leukemia (Lapidot et al., 1994), but recently the CSC subset was identified also in the solid tumors, including pancreas (Hermann et al., 2007), prostate (Patrawala et al., 2006), lung (Eramo et al., 2008), colon cancer (Ricci-Vitiani et al., 2007).

Interestingly, also within established malignant cell lines a small population of the cells with self-renewal characteristics and ability to give rise to the differentiated progeny was found (Kondo et al., 2004; Patrawala et al., 2005; Szotek et al., 2006).

2. Tumor suppressor p53

The p53 tumor suppressor is considered to be one of the most important proteins in preventing cancer. The first reports described p53 as protein able to interact with SV-40 T antigen (Lane and Crawford, 1979; Linzer et al., 1979). The protein was prevalent in many transformed cell lines, unlike normal cells where its expression was undetectable (Lane and Crawford, 1979; Rotter et al., 1980), when ectopically expressed p53 contributed to the cellular transformation and there was a great correlation between p53 protein levels and transformation. For these reasons ironically p53 was classified initially as protein, associated with transformation and proto-oncogene. Luckily Levine and colleagues 10

years realized that in all previous reports the mutant forms of p53 were implied and their landmark manuscript demonstrated that wild type p53 (wtp53) actually inhibits cellular transformation (Finlay et al., 1989). Thus, wtp53 was established as a bona fide tumor suppressor rather than an oncogene.

Several factors underscore the importance of p53 in tumor suppression: (1) p53 is the most frequently mutated gene identified in human cancer (50% of all cancers); (2) Li-Fraumeni syndrome is a genetic disease often attributed to a germline mutation in p53 (Malkin et al., 1990); people affected by this syndrome usually develop malignant tumours by early adulthood, upon mutation or deletion of the normal p53 allele; and (3) mice that lack p53 develop normally, but are predisposed to developing lymphoma and a broad spectrum of other cancers, and usually die from their malignancies within 3–6 months of age (Jacks et al., 1994).

2.1. The p53 gene product

Human p53 is a 53 kDa nuclear phosphoprotein, encoded by a 20-Kb gene containing 11 exons and 10 introns (Lamb and Crawford, 1986). This gene belongs to a highly conserved gene family containing at least two other members, p63 and p73, which possess some of the same properties as p53 (Prives and Hall, 1999).

wtp53 protein is composed of 393 amino acids and is commonly divided into three functional domains (Fig. 2): the N-terminus contains an acidic domain (amino acids 1–42) that interacts with components of the transcriptional machinery, such as TBP and TAFs and a proline-rich region with multiple copies of the PXXP sequence (residues 61-94, where P is proline and X - any amino acid). Acidic domain was demonstrated to be crucial for the p53 transcriptional activity (Lu and Levine, 1995) and proline-rich domain is required for apoptosis (Walker and Levine, 1996) and is involved in the p53 negative regulation: this region region plays a role in p53 stability regulated by

MDM2, wherein p53 becomes more susceptible to degradation by MDM2 if this region is deleted (Sakamuro et al., 1997). The central core domain (AA 102-292) contains domain for specific DNA binding and recognizes at least two repeats of the DNA consensus sequence 50-PuPuPuC(A/T)-(T/A)GPyPyPy-30 (Levine, 1997). The oligomerization domain of wtp53 (AA 324–355) participates in the formation of p53 tetramers (dimers of dimers) and this form typically binds DNA in a sequence-specific manner (Wang et al., 1993). The C-terminus of p53 (AA 311–393) includes a nuclear localization sequence and exhibits nonspecific DNA binding (Wu et al., 1995). The role of these interactions is still not well understood. The C-terminus also functions as a negative regulatory domain, perhaps in coupling with DNA-binding domain, to maintain p53 in an inactive form till posttranslational modifications don't active the protein.

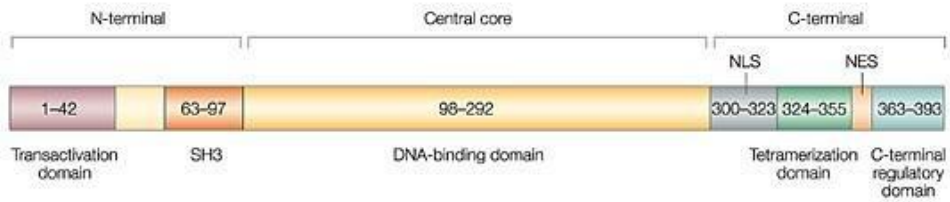

Figure 2. p53 protein structure. The 53 kDa nuclear phosphoprotein p53, of 393 amino acids, comprises several domains, including an acidic N-terminal region containing the transactivation domain, a core containing the sequence-specific DNA-binding domain and a complex C-terminal domain with multiple functions (Bode and Dong, 2004).

2.2. The physiological functions of p53

p53 protein is a tumor suppressor and transcription factor, that plays central role in prevention of the inappropriate cell growth and maintenance of the genome integrity following genotoxic stress (Vogelstein et al., 2000; Vousden and Lu, 2002). After DNA damage (ionizing radiation, UV radiation, cytotoxic drugs treatment, viral infection), heat shock, hypoxia, and oncogene

overexpression, p53 protein undergo post-translational modifications, this leads to overall increase of protein level and as a consequence to the p53 activation (Vogelstein et al., 2000; Vousden and Lu, 2002). The ability of p53 to execute a role of tumor suppressor is attributed to its ability to act as sequence-specific transcription factor that regulates expression of numerous genes (Farmer et al., 1992). Genes activated by wtp53 are functionally diverse and belong to different signalling pathways such as cell-cycle checkpoints, cell survival, apoptosis, and senescence (Hofseth et al., 2004).

Activation of p53 primarily results in cell cycle arrest, presumably to allow for DNA repair before replication or mitosis. A mechanism by which p53 blocks cell cycle progression is through the transcriptional activation of $p21^{WAF1/CIP1}$ (Prives and Hall, 1999). In normal cells p21 inhibits cyclin-dependent kinases, thereby preventing phosphorylation (inactivation) of Rb, which consequently arrests cells in the G1/S phase. Gadd45a is also induced by p53 in response to DNA damage (Kastan et al., 1992). The Gadd45 gene product interacts with various cell cycle-related proteins such as Cdc2 and PCNA and contributes to DNA repair and G2/M cell cycle arrest (Smith et al., 1994). Gadd45a-deficient mice display increased genomic instability and radiation-induced carcinogenesis, similar to p53-deficient mice (Hollander et al., 1999). Thus, Gadd45 participates together with p53 in maintenance of genomic stability.

In some cell types, activation of p53 results in apoptosis rather than cell cycle arrest. Activation of the cell death program by p53 is not clear, but a number p53 target genes are well established pro-apoptotic inducers. One such gene is *bax*, and overexpression of Bax protein is sufficient to induce cell death (Miyashita and Reed, 1995). The insulin-like growth factor binding protein 3 (IGF-BP3) is also regulated by p53 and IGF-BP3 may activate apoptosis by selectively blocking mitogen-activated survival signalling pathways (Levine, 1997). Other p53-regulated genes that may contribute to apoptosis include

KILLER/DR5 and FAS/APO1 (both membrane receptors in the TNFR superfamily) (Owen-Schaub et al., 1995) and Noxa, ei24/PIG8 and Puma (Yu et al., 2001).

It's well known that wtp53 is also able to repress a number of cellular promoters. For example, expression of the survival factor Bcl-2 is suppressed by wtp53 (Miyashita et al., 1994) and loss of this regulation by p53 mutation or inactivation may lead to an up-regulation of Bcl-2 expression and an impaired apoptotic response to genotoxic damage. Expression of the c-myc proto-oncogene is also repressed by wtp53 (Moberg et al., 1992) and elevated levels of c-Myc promote cell cycle progression (Baudino and Cleveland, 2001). As a result, impaired regulation of c-Myc expression through p53 mutation may lead to improper signalling and cell growth, thereby contributing to tumor formation. Thus, p53-mediated transcriptional repression represents another important biological activity, apart from transactivation, by which p53 can negatively regulate cell growth.

2.3. Transcription factor p63

Transcription factor p63 is a member of p53 family that plays a central role in the development of stratified epithelium such as epidermis, breast and prostate. p63 expresses multiple protein isoforms: p63 can be expressed from two different promoters that gives rise to TA- and ΔNp63 isoforms. p63 exhibits 60% homology with p53 in DNA-binding domain and it is 37% identical to p53 in the oligomerization domain (Barbieri et al., 2006). At the C-terminus of p63 α, β, γ isoforms can be produced creating another level of p63 protein diversity. α isoform possess a Sterile alpha-motif (SAM) that is involved in protein-protein interactions (Schultz et al., 1997). TAp63 isoforms could transactivate a reporter gene through a canonical p53 responsive element as well as induce apoptosis (Osada et al., 1998; Yang et al., 1998). Tap63α is the most potent transactivator of the p63 isoforms (Yang et al., 1998). On the contrary, ΔNp63

displays dominant-negative effect on the p53-mediated transcriptional activation (Yang et al., 1998). ΔNp63 is expressed in the proliferative, basal compartment of epithelia, and it's specifically expressed in epidermal stem cells that possess the highest proliferative potential (Westfall et al., 2003). Numerous studies have shown that ΔNp63α is predominant, if not the only one isoform, expressed in epithelial cells (Mills et al., 1999; Yang et al., 1999). ΔNp63α isoform is overexpressed in several epithelial cancers and was detected to have oncogenic potential (Hibi et al., 2000).

p63 deficient mice demonstrate severe developmental problems, for example, the lack of stratified squamous epithelia (Mills et al., 1999; Yang et al., 1999). In the absence of epidermal barrier the mice dehydrate and die shortly after birth. In addition p63-/- mice have major defects in limb development (Osada et al., 1998; Yang et al., 1998). Mutations of p63 in humans also results in numerous developmental defects: malformation of hands and feet, epidermal dysplasia (EEC syndrome) (Roelfsema and Cobben, 1996).

2.4. Mutations of p53

Mutations in the p53 gene are the most frequent mutations observed in human cancers (Hollstein et al., 1991). The unique feature of p53 compared to other tumor suppressor genes is its mode of inactivation: while most tumor suppressor genes are inactivated by mutations leading to absence of protein synthesis (or production of a truncated product), more than 80% of p53 alterations are missense mutations that lead to the synthesis of a stable full-length protein (Soussi and Béroud, 2001) (Fig.3). This selection is believed to be necessary for both a dominant negative activity on wtp53 in the heterozygous cells, and for a gain-of-function that transforms mutant p53 into a dominant oncogene.

An important feature of the p53 protein is the extreme fragility of the DNA binding domain (residues 90–300) (Milner, 1995), as more than 200 of the

393 residues have been found to be modified. Most p53 mutations are localized in the DNA binding domain of the protein (residues 100–300) with 80% of p53 mutations located on exons 5–8 (residues 126–306) (Soussi and Béroud, 2001). Of the mutations in this domain, about 30% fall within 6 "hotspot" residues (residues R175, G245, R248, R249, R273, and R282) and are frequent in almost all types of cancer (Cho et al., 1994). Codon distribution of p53 mutations is represented in the Fig.4.

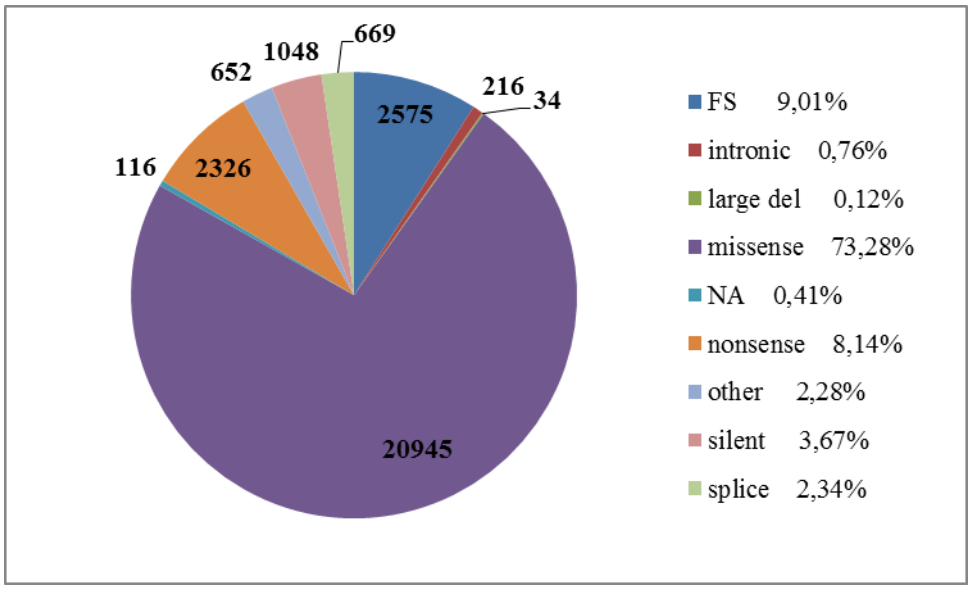

Figure 3. Distribution of p53 mutation type. Somatic mutations – mutations effect (N=28581) (Petitjean et al., 2007). FS –frameshift mutation.

All p53 mutations can be divided into 2 classes on basis of p53 structural differences: mutations that change p53 folding and mutations in the residues involved in DNA recognition (Gannon et al., 1990). Class I mutations, exemplified by mutants at codon 248 (7.6% in the p53 database, http://p53.free.fr/), affect amino acids directly involved in the protein–DNA interaction (Cho et al., 1994). They have a wild-type conformation and they do not bind to the chaperone hsp70 (Ory et al., 1994). Class II mutations, exemplified by the R175H mutant (4.9% in the database), have an altered

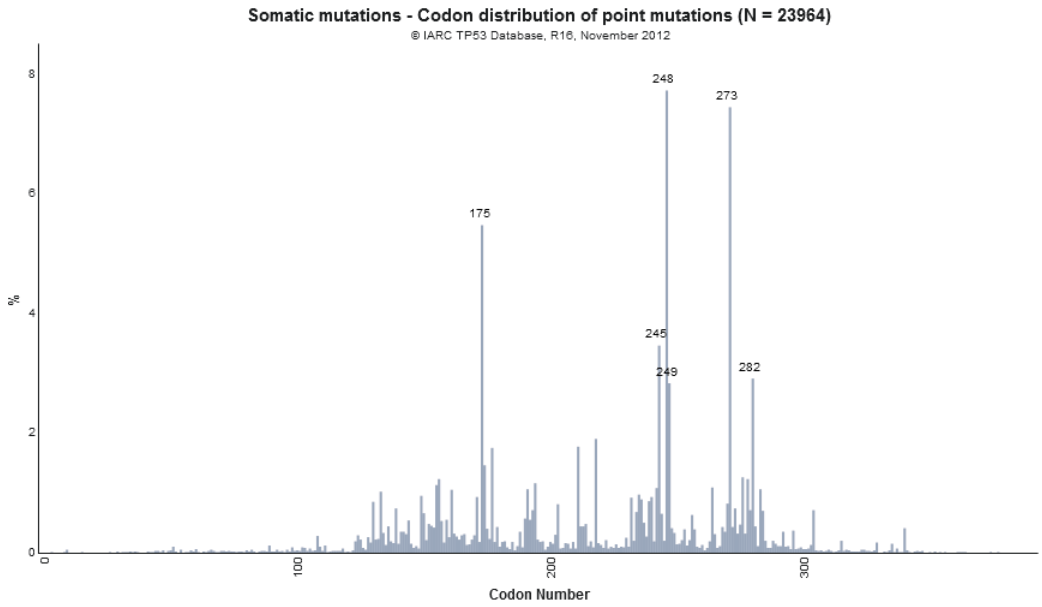

Figure 4. Codon distribution of TP53 mutations (Petitjean et al., 2007).

conformation with intense binding to hsp70. These mutations are associated with a more severe phenotype in vitro than class I mutations (Ory et al., 1994). Such heterogeneity can also lie in the nature of the resulting residue. Mutant R273H has a wild type conformation whereas mutant R273P is denatured (Ory et al., 1994).

2.5. Biological effect of mutation

The concept that mutant p53 acquires some novel, tumor-promoting properties, was established more than 2 decades ago (Dittmer et al., 1993; Sigal and Rotter, 2000). Several studies on genetically modified mice, harbouring some of the most frequently observed in cancers p53 mutations provide a strong evidence of mutant p53 gain-of-function: in contrast to p53 heterozygous or null mice ($p53^{+/-}$ or $p53^{-/-}$) mice with one p53 allele mutated developed diverse tumor spectrum – in addition to lymphomas more carcinomas and sarcomas were observed. The following tumours demonstrated enhanced resistance to chemotherapy and genomic instability (Lang et al., 2004; Olive et al., 2004). In

cell culture models mutant p53 was proved to participate in different pathways: mutant p53 was able to promote invasion, migration, angiogenesis, proliferation and survival.

Different models have been proposed to explain the contribution of mutant p53 to tumor progression (Fig.5): 1) loss of wtp53 tumor suppressor activity; 2) dominant-negative inhibition of wtp53 function, and possibly p53 family members (p73/p63), through oligomerization with mutant p53 in heterozygous cells; and 3) gain-of-function which confers a selective growth advantage to cells expressing mutant p53.

2.6. Dominant-negative interference with p53 family members

One of the hypotheses to explain mutant p53 pro-oncogenic activity assumes that some mutant p53 proteins can interact with and downregulate the transcriptionally active forms of p53 homologous, p63 and p73, leading to reduced apoptotic response and chemioresistance in tumor cells.

p63 and p73 genes are members of p53 family that share significant homology and whose products can function as sequence-specific transcriptional activators (Jost et al., 1997; Kaghad et al., 1997; Osada et al., 1998). It has been demonstrated that ectopic expression of p73 and p63 can transactivate endogenous targets of p53, such as the cell cycle inhibitor p21 (Jost et al., 1997; Kaghad et al., 1997; Pinhasi-Kimhi et al., 1986), as well as p21-containing promoters (Jost et al., 1997; Pinhasi-Kimhi et al., 1986; Yang et al., 1998). Di Como et al. have shown that p73 can activate other p53 target promoters (Di Como et al., 1999) such as the proapoptotic bax gene (Miyashita and Reed, 1995), IGF-BP3 (Buckbinder et al., 1995) and cyclin G (Okamoto and Prives, 1999).

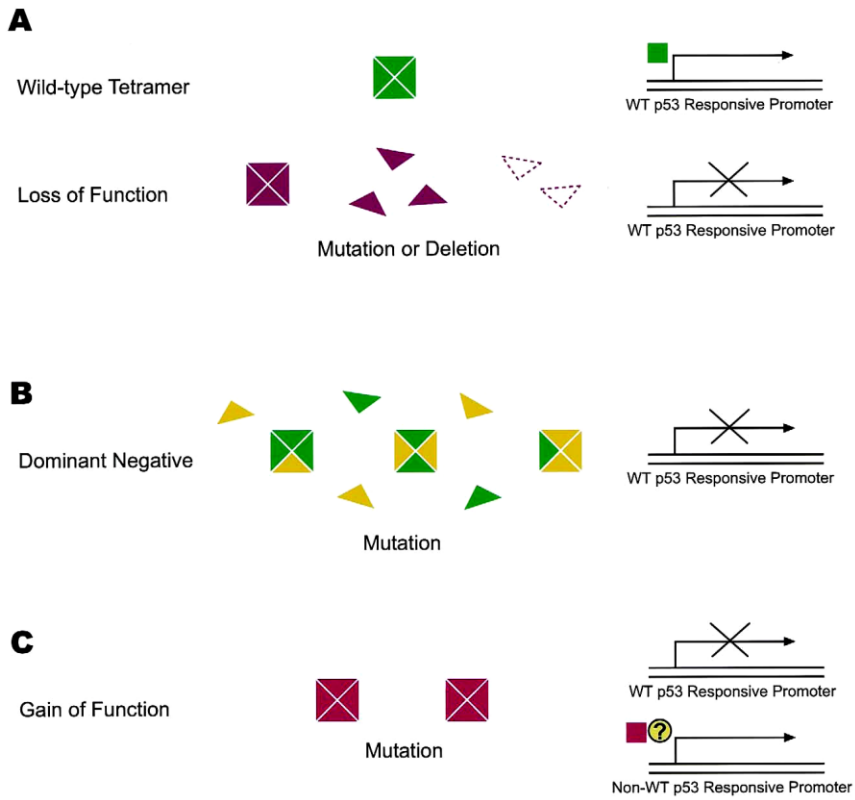

Figure 5. Proposed mechanisms for the role of p53 mutations in tumorigenesis. (A) Loss-of-function. The wtp53 tetramer (green) is transcriptionally active in response to cellular stresses and induces the expression of downstream target genes, such as $p21^{Cip1}$. An inactivating mutation (purple) or deletion of p53 (purple dotted line) results in a complete loss-of-function that eliminates the p53-mediated stress response. (B) Dominant-negative. Some p53 mutants (orange) oligomerize with wtp53 (green) (C) Gain-of-function. Other p53 mutants (fuchsia) possess new functions not shared by wtp53. These are referred to as gain-of-function mutants, which do not transactivate normal p53 target genes; rather, these mutants transactivate a different subset of genes, such as MDR1 and c-myc (Cadwell and Zambetti, 2001).

One of the cellular functions of p53 is to induce apoptosis in response to genotoxic stress, such as damaged DNA (Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997). Similarly, it has been found that overexpression of both p73 and p63 can inhibit cell growth by inducing apoptosis (Jost et al., 1997; Osada et al., 1998; Zhu et al., 1998).

Despite the reports mentioned above the role of p63 and p73 in tumor suppression is still unclear. p63 and p73 knockout mice display defects in development rather than increased tumorigenesis: transgenic $p73^{-/-}$ mice harbour developmental problems in their nervous and immune systems (Yang et al., 2000) and $p63^{-/-}$ mice present severe defects in limb and skin development (Yang et al., 1999). Furthermore, p63/p73 mutations are not commonly found in human tumours: only 3 missense p73 mutations have been found among almost 1000 tumours screened. Multiple studies demonstrate that in different tumour types there is an overexpression of p73 (Kovalev et al., 1998; Sunahara et al., 1998; Yokomizo et al., 1999; Zaika et al., 1999). More recently the overexpression has been shown also for ΔNp63 isoform (Crook et al., 2000; Park et al., 2000; Yamaguchi et al., 2000). Moreover endogenous p73 can be stabilized upon treatment with DNA-damaging agents and its inactivation results in enhanced chemoresistence (Bergamaschi et al., 2003; Irwin et al., 2003). Although less studied, endogenous p63 can determine chemotherapeutic efficiency (Gressner et al., 2005). Finally, in one genetic background mice heterozygous for p63 and p73 can develop tumours (Flores et al., 2005).

It has been shown that a number of p53 mutants are able to associate with p73 and p63 in co-immunoprecipitation assays when they are either expressed ectopically or endogenously (Bergamaschi et al., 2003; Di Como et al., 1999; Gaiddon et al., 2001; Irwin et al., 2003; Marin et al., 2000; Strano et al., 2000). Both “conformation” mutants (e.g. R175 and G245) and “contact” p53 mutants (e.g. R248, R273, R283) have been reported to associate with p63/p73. Furthermore, purified mutant p53 and p63/p73 can bind to each other, strongly indicating that this interaction is direct (Gaiddon et al., 2001; Strano et al., 2002).

Even if the structural basis of interaction between mutant p53 and its homologues is still not well understood, there is a strong evidence that p53 mutants can decrease transcriptional activity of p73 and p63 and their ability to

induce growth suppression and apoptosis (Bergamaschi et al., 2003; Di Como et al., 1999; Gaiddon et al., 2001; Marin et al., 2000; Strano et al., 2000). Importantly, these studies indicate good correlation between mutant p53 binding efficiency and inactivation of p73/p63. Since mutant p53 interacts with p73/p63 DNA-binding domain (Strano et al., 2002), it's possible that p73 and p63 are no longer able to recognize and bind its target genes. Moreover it has been demonstrated in ChiP experiments that p63 recruitment to the target genes as p21, bax was impaired in the presence of endogenous or ectopic mutant p53 (Strano et al., 2002).

Taking into account all these studies we can put forward a hypothesis that mutant p53 can contribute to the cellular chemoresistance by inhibiting endogenous p73 and p63 (Bergamaschi et al., 2003; Irwin et al., 2003). Particularly, human tumours that express endogenously or ectopically mutant forms of p53 capable to bind p73 are more resistant to chemotherapy. It's worth mentioning that cells harbouring p53 mutants that have a greater potential to bind p73 are more resistant to chemotherapeutic drugs. Mutually, downregulation of mutant p53 by introduction of small-interfering RNA enhances the cellular response to drug-induced cell death.

In summary, results obtained from cell culture, mouse models and clinical correlation have implied that some mutants of p53 have gained novel function by inactivation of p63/p73.

2.7. Mechanisms of mutant p53 gain-of-function

Several models have been proposed to explain mutant p53 gain-of-function: 1) altered DNA binding by mutant p53; 2, 3) changes in interaction of mutant p53 with other protein, for example, transcriptional factors; 4) or with proteins that are not directly involved in the regulation of gene expression.

As it was mentioned above most p53 mutants possess amino acid substitution in the DNA-binding domain and don't affect N-terminal

transactivation domain. From this point of view it's reasonable to contemplate that mutation in the DNA-binding domain would rather attenuate than eliminate completely DNA binding. It's apparent that mutant p53 still possesses DNA binding activity even if the ability to recognize classical wtp53 response elements is impaired (Ludwig et al., 1996; Thukral et al., 1995). The presence of mutant p53 bound to DNA allows to put forward a hypothesis that mutant p53 can recognize unique mutant p53 responsive element, which allows it to function as oncogenic factor (Strano et al., 2007; Weisz et al.). However, a consensus for mutant p53 binding still hasn't been recognized (Donzelli et al., 2008; Vaughan et al., 2012).

Another transcriptional activity of mutant p53 consists in interaction with other transcription factors that leads to altered regulation of the target genes (Bargonetti et al., 1997; Sampath et al., 2001; Stambolsky et al., 2010). In some cases, mutant p53 can enhance the transcription by forming complex with transcriptional partner factor, cofactors and other proteins. In other cases the picture can be more complex, for example, in the case of interaction with NF-Y mutant p53 is recruited to its transcription regulatory complex in response to DNA damage and it deregulates the cell cycle checkpoint (Fig. 2b) (Di Agostino et al., 2006; Liu et al., 2011). In the same way following genotoxic stress DNA topoisomerase 2-binding protein 1 (TopBP1) interacts with p53 mutants and NF-YA and promotes mutant p53 and p300 recruitment to NF-Y target gene promoters with following activation of the NF-Y target genes activation (Liu et al., 2011).

On the other hand the interaction with mutant p53 can be inhibitory. Involvement of p53 family members as p63 and p73 – is one of the best examples confirming this model (Gaiddon et al., 2001; Strano et al., 2002). Several reports demonstrated that mutant p53 binds to p63 and p73 and restrains its binding to DNA (Girardini et al., 2011; Liu et al., 2011). In our recent work we provide a proof that mutant p53 is actually hijacked to DNA by p63 and

albeit p63 binding at the classical p63 responsive elements to some other sites, consequently changing the regulation of its target genes expression (Martynova et al., 2012). The main obstacle to get inside mutant p53 and p63/p73 interaction is an existence of various, functionally diverse isoforms of p53 family members: TA isoforms of p63 and p73 (containing the full-length sequence) and the ΔN forms (lacking the N-terminus). All these isoforms are transcriptionally active, but each regulates a different group of genes. Intriguingly, although mutant p53 inhibits the transcriptional activity of the TA isoforms, in some systems the mutant p53–p63 interaction was found to enhance — rather than repress — the expression of some p63-regulated genes (Neilsen et al., 2011). This may reflect a different effect of mutant p53 on TA and ΔNp63, and indicates that, as is also the case for vitamin D receptor (VDR) and the SP1 transcription factor, mutant p53 might act as both an activator and repressor of p63 function (Bargonetti et al., 1997; Gualberto and Baldwin, 1995; Stambolsky et al., 2010).

Another "dark side" of mutant p53 is that it also binds and modulates the function of proteins that are not directly involved in transcription. For instance, p53 gain-of-function mutants promote tumorigenesis by a novel mechanism involving active disruption of critical DNA damage-response pathways: p53 mutants interact with the nuclease Mre11 and suppress the binding of the Mre11-Rad50-NBS1 complex to DNA double-stranded breaks, leading to impaired ATM activation and as consequence, impaired homologous recombination (Liu et al., 2010; Song et al., 2007). Furthermore, the structural mutant p53 proteins interact with BTG2, a cell cycle regulator, preventing it from de-activating H-Ras — with the potential for a number of oncogenic outcomes (Solomon et al., 2012).

It has been also demonstrated that mutant p53 gain-of-function can also contribute to genomic instability and this effect is mediated by topoisomerase 1 (Top1), which maintains topology of DNA. Whereas wtp53 both promotes and counteracts Top1 function, mutant p53 has specifically lost the negative

regulation of Top1, resulting in hyper-recombination and genomic instability (Restle et al., 2008).

Since mutant p53 is expressed at high levels in human tumors it makes this protein extremely attractive target for the anticancer drug development. Ongoing studies are focused on destabilization or inactivation of mutant p53, restoration of wtp53 functions of the mutants or inhibition of mutant p53 aggregation with other proteins. Several molecules have been already unraveled and they are promising for the clinical application in the future. The main obstacle to develop anticancer agents targeting mutant p53 is that different p53 mutants are not functionally identical and therapy should be designed based on the type of p53 mutation present in the cancer. This problem represents a challenge for investigators and opens a wide field for the future research work.

2.8. UV-induced p53 mutations in HaCaT cells

The p53 gene is mutated in a large number of tumors, including squamous cell carcinomas and basal cell carcinomas of the skin (Brash et al., 1991; Brash et al., 1996). Interestingly, in skin carcinomas and also in premalignant lesions on sun-exposed sites, the p53 mutations are mostly C to T base transitions, including about 10% CC → TT double base changes, which are characteristic consequences of UV-induced pyrimidine dimers in DNA (Nakazawa et al., 1994). This is in accordance with epidemiologic data that clearly demonstrated that UV is one of the most important carcinogenic agents for inducing skin tumors. Mutations in the p53 gene are considered early if not initial events in the development of skin cancer (Ziegler et al., 1994).

In accordance with the data obtained from skin carcinomas, HaCaT cells exhibited mutations in both alleles of the p53 gene, as do a number of skin carcinomas (Brash et al., 1996). Interestingly, the p53 mutations in the HaCaT cell line were identified as a C → T transition at codon 179 and a CC → TT base change at codons 281 and 282 (Lehman et al., 1993). Because these

changes are in typical UV hot spots (Brash et al., 1996) and the biopsy sample from which the primary keratinocyte cultures were obtained was from a sun-exposed area, these p53 mutations probably occurred in the patient and subsequently caused the immortalization of the HaCaT cells. However, the mutations in the p53 gene might not have been solely responsible for the immortalization of HaCaT cells, because so far transfection of mutant p53 into human keratinocytes has failed to induce immortality. Hence, other genetic changes leading to the loss of senescence genes may be required for completion of this process. As mentioned above, the immortalized phenotype developed during prolonged cultivation of the keratinocytes in primary culture, and this cultivation was initially performed at an elevated temperature of 38.5°C. Higher culture temperatures induce increased rates of chromosomal breaks and translocations as well as a higher proliferative activity in keratinocytes and fibroblasts (Boukamp et al., 1988; Marczynska et al., 1980). Thus, elevated temperatures may also have contributed to an increased rate of genetic alterations during the process of HaCaT cell immortalization.

In view of these findings, it can be hypothesized that the UV-induced p53 mutations in situ, in combination with stressful culture conditions that led to increased genetic alterations, caused the immortalization of the HaCaT cells. In skin cancer in humans, UV-induced p53 mutations are probably an initial event caused by excessive UV irradiation. This, however, is usually combined with sunburn, which increases skin temperature, which may in turn increase the number of genetic alterations. The postulated etiology of HaCaT cells may thus be representative for the early mechanism of skin carcinoma induction. From these data and the considerations outlined above, it can be concluded that the HaCaT cell line is indeed a biologically meaningful *in vitro* model for studying human skin carcinogenesis.

3. Nuclear Factor Y (NF-Y)

The CCAAT box is one of the most common *cis* elements present in 30% of all eukaryotic promoters (Bucher, 1990). A number of CCAAT-binding proteins have been identified and nuclear factor for Y box (NF-Y) is the most ubiquitous and specific acting as a key proximal promoter factor in the transcriptional regulation of different eukaryotic genes. For NF-Y binding all 5 nucleotides are and there is a strong preference for specific flanking sequences. Genes that harbor NF-Y sites include constitutive, inducible, and cell-cycle-dependent genes, but the mechanism of regulating the expression of these different genes cannot simply be due to NF-Y DNA binding. It is likely that additional factors will be involved in the action of NF-Y. Indeed, recent studies from several laboratories have suggested that NF-Y interacts, either functionally or physically, with other transcription factors or nuclear proteins both *in vitro* and *in vivo* (Framson and Bornstein, 1993; van Ginkel et al., 1997; Zwicker et al., 1995).

NF-Y is a heteromeric protein composed of three subunits, NF-YA, NF-YB and NF-YC, all indispensable for DNA binding (Sinha et al., 1995). Each of the three subunits possesses highly conserved domains. Association of NF-YB with NF-YC is necessary for NF-YA binding and sequence-specific DNA interactions (Sinha et al., 1995). Both NF-YB and NF-YC conserved domains contain putative histone fold motifs (Baxevanis et al., 1995). This motif, common to all core histones, enables histones to dimerize with companion subunits; this motif is responsible for formation of the histone octamer. Recent experiments indicate that this 65 amino acid long motif is necessary for subunit interactions and DNA binding (Sinha et al., 1996).

NF-Y was initially identified as the factor associating with the so-called Y-box, a conserved element of major histocompatibility complex class II promoters (Dorn et al., 1987; Hooft van Huijsduijnen et al., 1990). This transcription factor is involved in the activation of numerous other eukaryotic

promoters such as the type I collagen, albumin, globin, thrombospondin 1, and human thymidine kinase gene promoters (Chodosh et al., 1988; Delvoye et al., 1993; Framson and Bornstein, 1993; Mantovani et al., 1992; Raymondjean et al., 1988). NF-Y is specifically required for genes regulated during the cell-cycle and inducible by external stimuli: essentially all G_2/M genes, for example, are dependent on NF-Y (Elkon et al., 2003; Hu et al., 2006). NF-Y is generally important to recruit neighbouring TFs and, in some systems, PolII, before induction of transcription (Kabe et al., 2005). Consistent with the widespread activity, inactivation of the NF-YA gene in mice is embryonic lethal at a very early stage of development (Bhattacharya et al., 2003).

3.1. Role of NF-Y in cellular proliferation and apoptosis

The NF-YA isoform of the NF-Y transcription factor is believed to be the limiting and regulatory subunit of the trimer, tightly regulated by post-translational modifications (Dolfini et al., 2012a). Expression of the protein is modulated during the cell cycle (Bolognese et al., 1999) and its inactivation leads to downregulation of the cell-cycle genes in differentiated muscle cells (Farina et al., 1999; Gurtner et al., 2003). Moreover NF-Y was shown to be a crucial TF, required for moues ES cell proliferation (Grskovic et al., 2007).

Previous studies aimed at understanding the biological role of NF-Y took advantage of a loss of function approach, such as expression of dominant-negative NF-YA mutants and conditional deletion of the mouse NF-YA gene. When a dominant-negative NF-YA mutant that interacts with -YB/YC but does not bind DNA is expressed in mouse fibroblasts, retardation of cell growth is observed {Hu, 2000}. In MEFs conditional inactivation of NF-YA results in a block in cell proliferation and inhibition of S phase or DNA synthesis, which is followed by induction of apoptosis (Bhattacharya et al., 2003). Taken together, these studies demonstrate that binding of NF-Y to cellular promoters is essential for cell proliferation.

Apoptosis and proliferation are intimately connected. A tight association between regulation of cell cycle and apoptosis was demonstrated for c-myc, E2F1, and cyclins. These genes may induce cell proliferation, cell-cycle arrest, or cell death, with the different outcomes depending on cell type, cellular environment, and genetic background (Vermeulen et al., 2003). Intriguingly, many of these genes are NF-Y transcriptional targets (Di Agostino et al., 2006; Elkon et al., 2003; Farina et al., 1999; Manni et al., 2001).

Less is known by the moment about involvement of NF-Y in the process of programmed cell death. Hughes et al. demonstrated that NF-Y actively participates in the regulation of the Bim expression that is well known mediator of apoptosis in many cell types. Their results indicate that NF-Y cooperates with FOXO3a to recruit CBP/p300 to the Bim promoter to form a stable multi-protein/DNA complex that activates Bim transcription after survival factor withdrawal (Hughes et al., 2011).

Furthermore, it was demonstrated that NF-Y interacts with endogenous Fas/APO1 promoter and these cooperation is enhanced upon DNA damage (Morachis et al., 2010). Moreover overexpression of the NF-Y complex can stimulate basal Fas/APO1 transcription *in vivo*. Thus, NF-Y positively regulates Fas/APO1 expression.

Several studies implicated a relationship between p53 and NF-Y in which p53 interacts directly with NF-Y to repress various cell cycle genes (Imbriano et al., 2005). Interestingly, Morachis et al. provide a proof that NF-Y knockdown by siRNA causes apoptosis while activating many p53 target genes (Morachis et al., 2010). They analyzed RNA levels in NF-Y knockdown cells and observed activation of Fas/APO1, p21, and PUMA genes. This is consistent with previous studies (Benatti et al., 2008).

Recent reports underline a role of NF-YA subunit in the processes of apoptosis, although a role of this protein the programmed cell death is not well understood yet. Numerous efforts to study the role of NF-YA in the cellular

proliferation and apoptosis by overexpression experiments were non successful neither in our lab nor in other research groups. Gurtner et al. failed to obtain stable clones overexpressing NF-YA, since in MEFs unrestrained NF-Y activity promotes apoptosis depending on E2F1 induction and wtp53 activation. On the other hand also inactivation of the NF-YA leads to a remarkable apoptotic response (Benatti et al., 2011).

3.2. Different NF-YA isoforms

Due to alternative splicing event subunit NF-YA exists in human and mouse cells in short (NF-YAs) and long (NF-YAl) isoforms (Li et al., 1992a; Li et al., 1992b). The long isoform carries the full complement of the gene sequence. In short isoform 84 nucleotides are missing; the reading frame is maintained, so that the protein encoded by "short form" mRNAs is shorter by 28 amino acids. Expression of different NF-YA isoforms was demonstrated to be tissue-specific: the long form is more prevalent in the brain, liver, lung, and in fibroblast and teratocarcinoma cells. Conversely, the short form predominates in thymus and spleen and in cells of the B lymphoid lineage (Li et al., 1992a).

Nowadays there is a growing body of evidence about functional duality of NF-YA isoforms: the first indication that NF-YA isoforms are functionally diverse comes from the study of Cystathionine-β-synthase promoter (Ge et al., 2002). NF-YAs demonstrated significantly less synergistic transactivation of the CBS-1b promoter with Sp1 than NF-YAl. Another report provides strong evidence that NF-YAs is a potent cellular regulator of mouse hematopoietic stem cell self-renewal (Dolfini et al., 2012a). Report of our laboratory describes NF-YAs as a crucial TF, involved in the processes of mouse embryonic stem cell (ESC) renewal: NF-YA was demonstrated to activate directly key stem cells genes and, and to promote in the indirect way the association of NANOG to a large part of its regulated ESCs targets (Dolfini et al., 2012b).

4.3. NF-Y and mutant p53 interaction

Even if the mechanisms of mutp53 gain-of-function are still not yet well defined, several models have been arisen. The model of direct transcriptional regulation proposes that mutant p53 proteins, most of which are not supposed to bind DNA directly, may selectively regulate the expression of downstream targets by interacting with other factors that tether p53 to the promoter/regulatory regions of these genes. Di Agostino et al. provide strong experimental support for this model (Di Agostino et al., 2006). The authors demonstrate that three different mutant p53 (p53His175, p53His273, p53His273/Ser309) interact physically with the heterotrimeric transcription factor NF-Y *in vivo* and these mutant p53/NF-Y complexes modulate the expression of key NF-Y-regulated cell cycle genes after adriamycin treatment. It has been already reported that upon DNA damage the expression of some cycle-related NF-Y targets is repressed by wtp53: wtp53 forms a complex with NF-Y on CCAAT box-containing promoters, and upon DNA damage this complex recruits histone deacetylases (HDACs) and releases histone acetyltransferases (HATs), coinciding with the repression of key cell cycle (Imbriano et al., 2005; Manni et al., 2001). Strikingly, Di Agostino et al. show that mutant p53/NF-Y complexes have the opposite effect on transcription to wtp53/NF-Y complexes: at the transcriptional level the expression of NF-Y target genes, involved in cell cycle control (cyclin A, cyclin B1, cyclin B2, cdk1, cdc2).

Mutant p53/NF-Y/HDAC1 complexes were found in the promoters of NF-Y target genes and the presence of these complexes was independent of DNA damage. Association of mutant p53 with these promoters, dependent on the presence of NF-Y and CCAAT box integrity, was increased after adriamycin treatment, and the p300 HAT was then recruited in a manner that requires mutant p53. The switch between HDAC1 and p300 was accompanied by increased acetylation and reduced methylation of neighboring histones on the

cyclin B2 and cdk1 promoters. In contrast, wt p53 interacts with HDAC1 upon DNA damage to repress NF-Y target genes.

Downregulation of the regulatory subunit NF-YA or mutant p53 led to impaired activation of the NF-Y target genes and reduced S phase accumulation after adriamycin treatment. Thus, the ability of mutant p53 to interact with NF-Y and control important cell cycle regulatory genes represents new oncogenic gain-of function activity of mutant protein and specific recruitment of chromatin-modifying enzymes is responsible for this oncogenic potential (Fig.6).

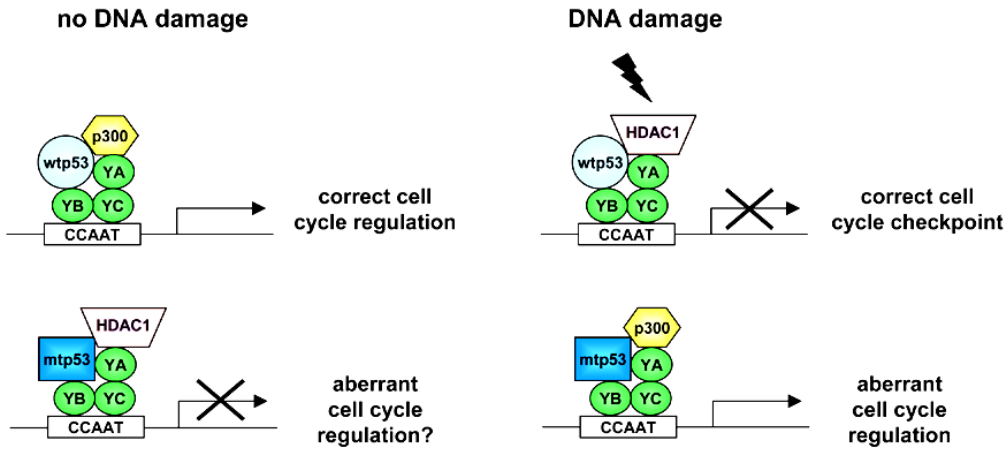

Figure 6. Transcriptional activities underlying mutant p53 gain-of-function (Peart and Prives, 2006). Model proposed by Di Agostino et al. (2006) in which the transcriptional regulation of NF-Y target genes by wt and mutant p53 are opposite following DNA damage due to the recruitment of opposing chromatin-modifying enzymes, conferring oncogenic "gain-of-function" properties to mutant p53 proteins.

Remarkably, this gain-of-function activity seemed to be independent of the particular type of p53 mutation since different p53 mutations (p53His175, p53His273, p53Gly281) have been tested. In this paper the authors used both a DNA contact point mutant and a conformational mutant but it would be

interesting to confirm the universality of their observations by testing a wider spectrum of p53 mutations.

In summary, Di Agostino's report provide mechanistic insights into mutant p53 gain of function activity by interaction with NF-Y and facilitation of NF-Y proliferative target genes transactivation.

Aims of the Project

1) The p63 protein is the "master regulator" of epidermal development, working with other proteins to closely coordinate the expression of genes that control cell growth and differentiation. Our lab has been involved in the identification of p63 targets in human keratinocytes, either by expression profiling or by ChiP on ChiP analysis (Testoni et al., 2006; Viganò et al., 2006). Klf4 is a transcription factor required for establishing the barrier function of the skin. Immunocytochemical analysis and expression data in mouse and human demonstrate strong evidence that p63 and KLF4 protein are localized in the different regions of the skin – while p63 is restricted to the basal cells, KLF4 is normally expressed in the granular and subcorneum strata. Thus, the project was dedicated to the study of possible KLF4 regulation by p63 in the normal keratinocytes and immortalized HaCaT keratinocytes that harbour 2 mutant alleles of p53.

Results of the first part of the research demonstrate, that p63 is a repressor of KLF4 in the normal keratinocytes and this regulation is altered in the presence of mutant p53 in the HaCaT cells – upon these cellular conditions mutant p53 shifts p63 from repressive to activating sites at the KLF4 promoter, thereby, rendering p63 as an activator rather than repressor of KLF4 expression. Thus, we show that H179Y and R282Q p53 mutants possess gain-of-function activity (Publication 1).

2) These results inspired us to investigate mechanisms of mutant p53 gain-of-function. Our preliminary data demonstrated that inactivation of mutant p53 in the human HaCaT keratinocytes leads to significant decrease in cell growth rates. Therefore, to understand the role of mutant p53 in cellular proliferation we have to perform ChiP-Seq experiments to discover mutant p53 whole-genome DNA binding. Study of KLF4 promoter revealed mutant p53 as a p63 assistant in its programme of transcription regulation and analysis of mutant

p53 binding to DNA can shed light on other transcription factors partners. Moreover, the question whether mutant p53 binds DNA directly or by interaction with other proteins, is still open. To solve this and other problems Chip-Seq experiment with following bioinformatics analysis should be performed (Publication 2).

3) At the previous stage of research we discovered that mutant p53 alleles of the HaCaT keratinocytes confer growth advantages to the cells, i.e. possess gain-of-function activity. Although mutations of p53 were shown to contribute to tumor initiation, promotion, aggressiveness and metastasis, in the case of human immortalized keratinocytes it's evident that this genetic alteration is not sufficient to render the cells malignant. Therefore, this part of the project was devoted to the development of the experimental model for the tumorigenic conversion of transformed but non-tumorigenic HaCaT cells. Sphere-forming assay is extensively used for the isolation and propagation of the cancer stem cells from the tumor bulk, so we decided to apply the same protocol for isolation of the HaCaT subpopulation that can resemble somehow tumor-initiating cells (Manuscript in preparation).

4) Although the molecular mechanisms are still unclear, there is no doubt that interaction of p53 with NF-Y contributes remarkably to the cell cycle regulation and apoptosis upon DNA damage. Previous reports investigated the consequences of NF-YB inactivation and by the moment little is known about NF-YA role in the processes of p53-mediated apoptosis and cell cycle progression. This can be explained partially by the fact, that till now all experiments on NF-YA overexpression and/or inactivation were not successfully and no one report provide evidence about existence of the stable clones overexpressing NF-YA. This negative result didn't discourage us and we decided to perform overexpression experiments in the different cell lines, taking into account that success of the experiment can depend on the p53 status. For

this purpose we employed wt p53, null p53, mutant p53 and p53 inactivated by the large T antigen cells.

Main Results

1) Mutant p53 subverts p63 control over KLF4 expression in keratinocytes

p63 protein is a p53 homolog and TF that plays a key role in the skin development (Dai and Segre, 2004). In two independent genetic experiments on p63 deficient mice it was demonstrated that these mice lack stratified epidermis, producing a disorganized singlelayered surface epithelium that was negative for epidermal markers such as keratin K5 and K14 (Mills et al., 1999; Yang et al., 1999). Although the interpretation of the p63 mechanisms is still controversial, there is a strong experimental support that p63 is an epidermal master regulator.

Transcription factor Klf4 is expressed mainly in the suprabasal layers of the epidermis and it's a distinct marker of terminal differentiation. Klf4 is necessary for the establishment of the functional permeability barrier since KLF4 inactivation results in a loss of barrier and ectopic expression of KLF4 in the basal layer of skin promotes barrier acquisition in a dose-dependent manner (Jaubert et al., 2003; Segre et al., 1999). Immunohistochemical stainings of the mouse and human skin clearly demonstrate that expression of p63 and KLF4 proteins is restricted to different regions of the skin: while p63 is expressed in the nuclei of epidermal basal cells (Di Como et al., 2002; Reis-Filho et al., 2002), KLF4 is most highly expressed in the post-mitotic, differentiating epithelial cells of the skin (Garrett-Sinha et al., 1996).

Our data demonstrate that KLF4 expression is increased upon *in vitro* differentiation – as p63 decreased. Differentiation of neonatal human keratinocytes was induced by addition of $CaCl_2$ in medium and the success of the process was controlled by monitoring the levels of involucrin expression, which is well-known marker of keratinocytes terminal differentiation (Watt, 1983), and p21, whose induction was observed in several *in vitro* terminal differentiation systems (Missero et al., 1996), as well as in differentiating tissues *in vivo* (el-Deiry et al., 1995; Parker et al., 1995). During differentiation KLF4

increases at both protein and mRNA level with the highest levels after 10 days. In parallel, ΔNp63α is progressively reduced. Immunofluorescence stainings confirmed increase of KLF4 expression, concomitant with p63 decrease, in neonatal keratinocytes and also in normal human adult skin KLF4 expression was observed at the low in the basal layer of the skin, increasing in subcorneum stratum with maximum at the upper layers, opposite to p63. Thus, KLF4 is activated upon keratinocytes differentiation *in vitro* and is mostly present in the cells with low levels of p63.

At the next stage of research we provide evidence that p63 represses KLF4 in normal keratinocytes. Inactivation of p63 by siRNA, that recognize all 6 isoforms, leads to up-regulation of KLF4 at both protein and mRNA levels in neonatal and adult human keratinocytes. *In silico* analysis of KLF4 promoter revealed 9 putative p63/p53 binding sites and chromatin precipitation experiment showed p63 binding to (-2225), (-1784), (+467) regions and no p53 binding. As positive control for the ChiP experiment we used C40 enhancer of p63 promoter that was demonstrated to be strongly bound by p63 *in vitro* (Antonini et al., 2006). All together these results show that p63 directly represses KLF4 transcription by binding to discrete upstream regions in the promoter.

Intriguingly, in the human immortalized HaCaT keratinocytes, that harbour two mutant alleles of p53, p63 does actually activate KLF4 expression: inactivation of p63 by introduction of siRNA leads to downregulation of KLF4 at both protein and mRNA levels and overexpression causes an increase of KLF4 protein and mRNA levels. To make sure that this effect was achieved by transcriptional stimulation of KLF4 promoter by p63 we performed transfection of the construct, containing luciferase reported under KLF4 promoter, together with control siRNA and p63 siRNA: activation of KLF4 promoter was reduced upon p63 inactivation. Furthermore, transfection of the same construct with a

ΔNp63α vector caused strong upregulation of KLF4 promoter that indicates the transcriptional regulation of KLF4 promoter by p63.

In addition, in ChiP experiment we observed p63 binding to KLF4 promoter, but in the regions, different from those, observed in the neonatal keratinocytes. Moreover, in one region, containing p53/p63 responsive element, we also detected p53 bound together with p63 and this interaction wasn't observed in normal keratinocytes.

HaCaT cells harbour mutations of p53 genes in both alleles: C→T transition at codon 179 (His →Tyr) and a CC→TT base change at codons 281 and 282 (Arg→ Trp) were identified (Lehman et al., 1993). Both mutations are located in DNA-binding domain and these changes are well-known UV hot spots (Brash et al., 1996). While in normal basal keratinocytes p53 is expressed at extremely low level (Pablos et al., 1999), mutant p53 is abundantly present in the HaCaT cells due to prolonged half-life compared with that of the wtp53 protein (Lehman et al., 1993).

We hypothesized that inversed regulation of the KLF4 promoter by p63 could be a consequence of the mutant p53 action, especially after observation that mutant p53 was bound together with p63 on the KLF4 promoter. To clarify the role of mutant p53 in the regulation of KLF4 promoter we inactivated it by introduction of siRNA and observed reduction of KLF4 expression at the protein and mRNA levels. It worth mentioning that expression of p63 was unchanged upon these conditions and was always at the high level. This can indicate that KLF4 decrease wasn't a secondary effect of the p63 protein level. Importantly, expression of the H179Y and R282Q p53 mutants in normal keratinocytes was sufficient to activate of endogenous KLF4 protein.

Strong affinity of mutant p53 to p63 protein was proven in co-immunoprecipitation assay in the HaCaT cells (Gaiddon et al., 2001). To elucidate whether these proteins collaborate on some discrete regions on KLF4 promoter, we performed Chip and re-Chip experiment, using antibodies against

p53 and p63. Both mutant p53 and p63 were bound at the (-400) region on the KLF4 promoter. Altogether, these experiments indicate that p53 mutants have a direct positive effect on KLF4 expression, together with p63, by binding to a core promoter region of KLF4.

Up to 90% of cutaneous SCC lesions have UV-induced signature mutations, in the p53 gene (Brash et al., 1991), resulting in accumulation of the p53 protein. Similarly, ΔNp63α was demonstrated to be expressed at the high levels in HNSCC (Choi et al., 2002; Crook et al., 2000). The role of KLF4 in different cancers is rather controversial, since it can act as a tumor suppressor gene or as an oncogene (Rowland et al., 2005). Tumor suppressor role of KLF4 was observed in in human colon and gastric cancers (Wei et al., 2005; Wei et al., 2006; Zhao et al., 2004), esophagus (Wang et al., 2002), lung (Hu et al., 2009), bladder cancers (Ohnishi et al., 2003) and T-cell leukemia (Yasunaga et al., 2004). Conversely, KLF4 can function as a transforming oncogene. KLF4-transformed rat kidney epithelial cells demonstrate morphologic transformation and increased tumorigenicity in athymic mice (Foster et al., 1999). In human HNSCC and breast cancer, increased KLF4 expression has been reported (Foster et al., 2000; Foster et al., 1999). Moreover, KLF4 expression has been demonstrated to be a poor prognostic factor for early breast cancer and skin cancer (Chen et al., 2008; Pandya et al., 2004) corroborating its oncogenic roles.

Analysis of expression levels in a panel of different skin tumours by immunohistochemical stainings revealed robust p53 and KLF4 expression in the most of SCCs, whereas the expression of p63 was already high. Especially noteworthy is an observation that in the major part of the SCC investigated KLF4 was overexpressed together with p53. In conclusion, these data support the notion that an overexpressed, mutant p53 is involved in the positive regulation of KLF4 in human SCC.

In summary, in this report we present evidence that KLF4 is negatively controlled by p63 in normal skin in the presence of physiological levels of wild-

type p53, and that this regulation is subverted by oncogenic mutations of p53, establishing a direct link between these TFs, commonly overexpressed in SCCs. Because KLF4 overexpression cooperates in reprogramming differentiated cells, our findings have potential consequences for the mechanisms of formation of skin carcinomas.

2) Gain-of-function p53 mutants have widespread genomic locations partially overlapping with p63

All previous data encouraged us to investigate *in vivo* whole-genome DNA binding by mutant p53 and its functional activity. For this purpose we inactivated p53 in HaCaT cells by lentiviral transduction of the vector, expressing shp53 following puromycine selection and scramble shRNA as negative control. Recently, at least 12 different p53 isoforms were identified and data obtained from animal and cellular models indicate that p53 isoforms regulate the cell fate in response to developmental defects and cell damages by differentially regulating gene expression (Marcel et al., 2011). For this reason for inactivation experiment we used shRNA that targets DNA-binding domain of p53 to aim all possible isoforms of p53.

Clones were readily selected, pooled and inactivation of p53 was controlled by qPRC and western blot. While mutant p53 mRNA level was significantly decreased and the protein level was essentially abolished, p63 mRNA was decreased modestly. Remarkably, we never managed to inactivate p63 by shRNA introduction in the HaCaT cells, likely because the role of p63 in the growth and survival of these cells (unpublished data).

With the clones obtained we have performed the experiments to understand the behavior of the p53-inactivated HaCaT cells in response to DNA damage. After UVB irradiation we observed a modest variation in cell cycle progression: increase in subG1 and G1 cells, and a decrease in G2/M in cells lacking mutp53. The degree of apoptosis, as measured by TUNEL assays, PARP

activation and Caspase 8 cleavage was lower in the absence of mutp53, but still present. Thus, other mechanisms compensate for the lack of mutp53 to drive a DNA-damage response.

The most notable difference, however, was the growth rate, as curves were flatter in HaCaT deprived of mutp53. These data confirm that mutp53 are indeed gain-of-function alleles contributing to cellular growth. Thus, even if mutp53 was found to be non-functional regarding induction of apoptosis and cell cycle arrest, there is no doubt that it affects significantly the growth properties of the cells.

To investigate the possible mechanism of mutant p53 gain-of-function, we performed analysis of DNA binding by p63 and mutp53 by ChIP-Seq experiment, using DO1 monoclonal antibody, recognizing the N-terminal domain of p53, and a polyclonal antibody against p63. We identified 7135 peaks for p53 and 3421 for p63 in HaCaT cells, defined as areas with a significant enrichment in the IP with respect to the corresponding genomic region of Input DNA controls run in parallel. We analyzed the locations and we found out that 1591 mutp53 and 907 p63 sites reside in promoters, and 3697 and 1400, respectively, in the body of RefSeq genes. What's important is that notorious p63 targets such as p21, Mdm2 were among the positive targets. We proceeded with validation of the ChiP-Seq data and performed ChiP experiment, using Ab7 polyclonal antibody against p53 and 4A4 monoclonal p63 antibody and some of the positive regions were monitored by qPCR: with the exception of FANCi (1 out of 13), the other targets were enriched. We noticed that some targets scoring positive only for p63 in ChIP-Seq were also somewhat enriched with Ab7, suggesting that we might be underscoring the overlap of the two TFs, possibly because DO1 is unable to pick up the shorter p53 isoforms present in HaCaT cells (Marcel et al., 2011). Finally, p63 targets previously identified as functionally important, such as KLF4, Notch1, TP63, DLX3/4 and JAG2, among others, scored positive for p63 and mutp53.

We therefore felt confident to analyze the ChIP-Seq locations and found the expected overrepresentation in promoters, from -5000 to +1000 of the Transcriptional Start Sites: 1591 mutp53 and 907 p63 sites reside in promoters, and 3697 and 1400, respectively, in the body of RefSeq genes. We characterized the overlap between positive peaks and found out that 19% of p63 peaks overlap with mutp53 in the promoter (175 out of 907) and 17% in the body of genes (240 out of 1400).

Next, we evaluated the enrichment of TFBS in the $p63^{+}$, $mutp53^{+}$ and $p63/mutp53^{+}$ peaks with a width of 150 bp from the center with the Pscan software (Zambelli et al., 2009), using as background a set of 10.0000 sequences of the same size, chosen at random from genomic regions annotated either as "promoter" or "enhancer". It is apparent that a consensus p53/p63 responsive element is at the top of the list in $p63^{+}$ and $p63/mutp53^{+}$ peaks, but not in $mutp53^{+}$, where sites of other TFs predominate: E boxes, variously termed NHLH1, Myf, Mycn, USF1, MYC-MAX, MAXESR (nuclear receptors), AP2. This is an indication that p63, either alone or with mutp53, recognizes its own site, whereas mutp53, in the absence of p63, binds DNA through sequences recognized by other TFs.

We then used the Weeder software (Pavesi et al., 2004) to perform *de novo* motif discovery in the peaks of the three cohorts, in promoters, genes or elsewhere: a TGGGCATGTC sequence clearly emerged in $p63^{+}$, containing a perfect p53/p63 consensus with additional information on the flankings; a similar sequence, lacking the CC at the 3' end, is recovered in $p63/mutp53^{+}$ locations; in $mutp53^{+}$ peaks, instead, the variety of underlying sequences prevented the emergence of a clear consensus by *de novo* analysis, confirming the underlying presence of several unrelated TFBS.

A large number of genomic locations of p63 have been recently discovered in primary keratinocytes using the same antibody employed in our study (Kouwenhoven et al., 2010). We analyzed the data of primary human

keratinocytes and HaCaT cells and found that a substantial number (50%) of HaCaT locations are missing in primary keratinocytes (PHK). We assessed the number of mutp53 peaks in the two populations and found some skewing: 222 were in the PHK common sites, and 367 in the HaCaT-only cohort, suggesting that the presence of mutp53 alters p63 binding to a subset of sites bound in normal keratinocytes.

Gene Ontology analysis retrieved terms such as organ morphogenesis, tissue and epidermal development and positive regulation of transcription in $p63^+$ devoid of mutp53; the same terms were present in the larger $p63^+$ cohort, with the addition of Wnt signaling and induction of apoptosis. Terms related to signal transduction and cell cycle were prevalent in $mutp53^+$ genes. Specifically, the mutp53/$p53^+$ sites were enriched in terms of Wnt signalling and other metabolic terms in the molecular function analysis, such as actin binding, Tyrosine Kinase and GTPase activity. In addition to previously characterized targets families of targets worth mentioning are Wnt genes -Wnt4, Wnt7a, Wnt9a, Wnt10aand Retinoic Acids Receptors, RARα, RARγ and RXRα. Both p63 and mutp53 are present at multiple locations of the large cluster of keratin genes on chromosome 17. From this set of data we conclude that p63 binding in HaCaT is different from PHK, in part due to the presence of mutp53.

Next, we performed profiling analysis of mutp53 inactivated HaCaT cells. A large number of genes were up -1649- or down -1644- regulated, by using a relatively stringent cut-off ratio of 1.5-fold. We characterized the overlap between positive peaks, and found that 19% of p63 peaks overlap with mutp53 in the promoter (175 out of 907) and 17% in the body of genes (240 out of 1400). We validated the profiling results by qRCR: essentially all genes changed expression according to expectations. The overlap between the mutp53 locations and gene expression analysis is 15%. GO categorization identified cell cycle, as well as DNA and RNA metabolisms and response to DNA damage as robustly enriched in the upregulated cohorts, in the downregulated genes, p-

values were less significant with sterol biosynthesis and keratinocyte differentiation being somewhat enriched.

Our work addresses a debated topic concerning the mechanisms of action of gain-of-function mutant p53 and p63. We found that mutp53 HaCaT alleles are pro-growth and mutp53 have thousands of binding sites in the human genome; they affect gene expression profoundly, both by binding with p63 to consensus elements and by being tethered by other TFs to their locations.

3) Spheres from HaCaT: a model for skin cancerogenesis

In the previous stage of research project we established that 2 mutant alleles of p53 (H179Y and R282Q) in the HaCaT immortalized keratinocytes are pro-growth and display gain-of-function activity (Martynova et al., 2012). Although p53 is the key player in the cellular program of tumor suppression and it's mutated in more than 50% of the human tumors (Vogelstein et al., 2000), this genetic alteration of the p53 gene is not enough to render this cells tumorigenic – HaCaT cells are immortalized human keratinocytes that don't give rise to the tumors upon injection into immunocompromised mice (Boukamp et al., 1988). As it was mentioned above, tumorigenic conversion of the HaCaT cells can be achieved by transfection of *ras* oncogene (Boukamp et al., 1990), upon prolonged cultivation in serum-free medium (Hill et al., 1991), and at elevated temperature (Boukamp et al., 1997), by repeated subcloning with forced proliferation (Fusenig and Boukamp, 1998) and by activation of the stromal environment (Skobe and Fusenig, 1998). We decided to established novel, simply model for the HaCaT oncogenic conversion that can be useful for the future studies of the genetic and phenotypic changes upon skin tumor development.

Currently there are 2 models of tumor development: the clonal evolution model suggests that within a population of tumor cells a natural selection occurs that favors cells that have acquired (e.g., through additional mutations) the most

aggressive phenotype (Nowell, 1976). Essentially, this hypothesis assumes that all cells within a tumor hold an equal potential to maintain and advance the tumor to metastasis. Unlike clonal evolution hypothesis, the model of cancer stem cells (CSC) postulates that malignant tumors are initiated and maintained by a small population of cells within a tumor, and these cells possess properties similar to normal adult stem cells - the ability to self-renew and generate differentiated progeny (Wicha et al., 2006).

Existence of the minor subpopulation with enhanced tumorigenic potential was demonstrated also for the established cancer cell lines: rat C6 glioma (Kondo et al., 2004), U373 glioma and MCF7 breast cancer cells (Patrawala et al., 2005), different breast cancer cell lines (Charafe-Jauffret et al., 2009), HT29 and SW1222 colorectal cancer cell lines (Yeung and Mortensen, 2009). The heterogeneity of the cellular composition also was found within culture of human keratinocytes: colony-forming epidermal cells possess 3 clonal types with different proliferating potential (Barrandon and Green, 1987). One of them, holoclone, had the greatest reproductive capacity and holoclone-forming cells are multipotent stem cells (Claudinot et al., 2005).

Nowadays there 3 different strategies for isolation and propagation of the stem-cell like cells from normal and tumor tissues: establishment culture, the magnetic cell sorting (MACS) and the fluorescence-activated cell sorting (FACS) technologies. Sphere-forming assay originally was established for the isolation of the stem cells from central nervous system (Reynolds and Weiss, 1992) and currently is widely used for the CSC selection from brain (Lee et al., 2006), breast (Dontu et al., 2003), colon (Ricci-Vitiani et al., 2007), prostate (Sullivan et al., 2010), ovarian cancer (Zhang et al., 2008) and melanoma (Fang et al., 2005).

In the current study to examine the heterogeneity within the culture of human keratinocytes and existence of tumor-initiating cells we applied sphere-forming assay: HaCaT cells were plated at the low density (<10 000 cells/6

well) in specific medium into ultralow adhesion flasks. The serum-free medium is supplemented with several factors, in particular, bFGF and EGF. After 3-4 days in these culture conditions HaCaT keratinocytes were able to form nonadherent, multicellular aggregates that we termed keratospheres. It's well known that growth and survival of the epithelial cells depend drastically on cell-cell and cell-matrix interactions. Disruption of the substrate adhesions in normal epithelial cells leads rapidly to programmed cell death (Frisch and Screaton, 2001), so epithelial cells in culture once detached from the tissue culture surface normally undergo anoikis. In defined medium keratospheres could be serially passaged for more than 55 passages (more than 1 year in culture) (Part III, Fig.1a). Interestingly, we couldn't obtain keratosphere culture in ultra-low attachment plastic in conventional DMEM for more than 4 passages. Thus, the components of the defined medium are essential for the sphere propagation.

Comparison of the growth characteristics of the keratospheres and its parental HaCaT cell line by trypan blue exclusion method demonstrates that spheres-forming cells grow significantly slower than parental HaCaT cells (Part III, Fig.1b).

The analysis of cell growth characteristics demonstrated that keratospheres in comparison to parental HaCaT cells contain infrequently dividing cells with enhanced capacity to self-renewal (Part III, Fig. 3). This description somehow resembles epidermal stem cells, as Potten et al. argued that under normal conditions *in vivo*, epidermal stem cells are believed to divide infrequently and to have a long cell cycle time (Potten and Morris, 1988). We would like also to emphasize that with increase in keratosphere passage number we could observe morphologic changes of the colonies and its number: larger colonies could be observed for high passage keratospheres and also more colonies were formed for this cells.

Phenotypic characterization of the cells revealed heterogeneity within keratospheres (Part III, Fig.2): floating spheres showed widely diffuse staining

for p63, KRT1, KLF4, which represents differentiated keratinocytes, but interestingly, the cells with enhanced expression for the main basal keratinocyte markers (α6-integrin and KRT14) are located on the perimeter of the spheres, thus indicating that 1) keratinocytes are heterogeneous aggregates, containing both differentiated and undifferentiated cells 2) certain orientation for the keratosphere growth exists: growth vector is directed from the external part of the sphere to the central part, as keratospheres grow from outside to inside.

But the most significant alteration we observed is an acquisition of the keratospheres a capacity to grow in the soft agar in comparison to the parental HaCaT keratinocytes, i.e. the finding that keratospheres became anchorage-independent (Part III, Fig. 4a). HaCaT keratinocytes commonly demonstrate rather inefficient growth in soft agar (Boukamp et al., 1988), but keratospheres showed enhanced growth upon these conditions. The internal control of the experiment was also included: HaCaT cells growing in soft agar in the keratosphere medium (HaCaT + MEGM) didn't demonstrate transformed phenotype in this experiment. To establish whether the components of defined medium could cause the tumorigenic conversion of the immortalized keratinocytes, HaCaT cells were grown in the defined serum-free medium in the tissue culture flasks for 4 passages and then soft agar assay was performed again: as present in the Fig. 4a (Part III) even in this case cells still remained non-tumorigenic since after 4 passages in the keratosphere medium cells failed to form the colonies in the soft agar.

Deserving attention the fact that keratospheres acquired the tumorigenic potential not immediately but after growing for some passages (we observed firstly colonies in soft agar after 8 passages in the culture) (Part III, Fig.4b). As in the case of colony-forming assay in this kind of experiment we also observed the morphological difference of the colonies obtained: higher was the number passage of keratospheres, bigger colonies we could observe.

It's well known that there is reasonably good correlation between *in vitro* transformation and *in vivo* carcinogenesis, but it's not necessarily the case of the skin carcinoma cells (Boukamp et al., 1985). Xenotransplantation into immuno-compromised mice was performed *in vivo* assay to demonstrate tumorigenicity of keratospheres. Although immortalized HaCaT cell line exhibited a transformed phenotype *in vitro,* cells were not tumorigenic after subcutaneous injection: in the original manuscript P. Boukamp (Boukamp et al., 1988) demonstrated that HaCaT cells after subcutaneous injection onto athymic nude mice developed large encapsulated cysts often filled with horny squames. On the contrary, keratospheres of the high passage developed well differentiated squamous cell carcinoma (Part III, Fig. 5). Thus, in this study we demonstrate that performing sphere-forming assay of HaCaT keratinocytes leads to the oncogenic conversion of the immortalized keratinocytes to the tumorigenic phenotype.

50% of the head and neck squamous cell carcinoma (HNSCC) cases harbor mutations in the p53 tumor suppressor gene (Poeta et al., 2007). In HNSCC mutation renders p53 inactive and this state of mutant p53 is associated with tumor progression and decreased overall survival (Poeta et al., 2007). HaCaT cells harbor both alleles of the p53 gene mutated (Lehman et al., 1993) and identified C→T transition at codon in position 179 and CC→TT base changes at codons 281 and 282 are well-known UV hot spot mutations. It's supposed that biopsy for the HaCaT cell line establishment was obtained from the sun-exposed area and these mutations have been already occurred in the patient. Mutation of the p53 gene can be one of the reasons for the immortalization of the human keratinocytes and it's possible that absence of functional p53 protein can lead to the genomic instability and facilitate oncogenic conversion of the HaCaT keratinocytes to the tumorigenic keratospheres.

We focused on analysis of gene expression patterns in HaCaT cells versus SCC developed in mice. Gene expression profiling (GEP) provided us the list of genes with altered expression, which are associated with cell cycle regulation, differentiation, cell division, epidermis development (Part III, Fig.6b). Injection of the keratospheres into immune-compromised mice led to the formation of well-differentiated SCC, and GEP analysis confirmed the overexpression the classical keratinocyte differentiation markers – KRT10, KRT1, Filaggrin, Loricrin, TGM5 (Part III, Fig. 6a, b). During differentiation process keratinocytes withdraw from the cell cycle and in Gene Ontology analysis category "cell cycle" is present in the list of the down-regulated genes with high p-value.

Interestingly, we found members of the matrix metaloprotease family (MMP-1, MMP-3, MMP10), Laminin-γ2 (LAMC2), KRT17, IFI6 , PLAU, among highly up-regulated genes and KRT4, MAL, SCEL are significantly down-regulated in SCC, as it has been already demonstrated in numerous studies (Yu et al., 2008; Ziober et al., 2006). It's well documented that Wnt-pathway is frequently impaired in colorectal, thyroid, hepatocellular, melanoma cancers (Polakis, 2012), but contribution of this pathway in the HNSCC is still unclear. However there are indications that Wnt pathway is also altered in oral cancers. Leethanakul et al. discovered that most HNSCC overexpress members of this signaling pathway: several wnt receptors, and their downstream targets, dishevelled and β-catenin are highly expressed in comparison with normal tissue (Leethanakul et al., 2000). The overview of the dysregulated signaling pathways, involved in the acquisition of the oncogenic characteristics by HaCaT keratinocytes, revealed up-regulated Wnt pathway. This result underlines a potential relevance of Wnt pathway in HNSCC.

Several reports indicate the hedgehog-signalling pathway is involved in the proliferation of the SCC (Koike et al., 2002; Schneider et al., 2011). Our

analysis showed elevated pathway activation in the examined malignant tissue compared to the control samples.

Yu et al performed a genomic meta-analysis of 41 HNSCC gene expression profiles and the list of most frequently misregulated genes arised (Lallemant et al., 2010; Yu et al., 2008). Interestingly, the comparison of our microarray data (HaCaT vs. Tumor) revealed 17 genes out of 25 (68%) intersecting with the genes of the list, comparing HNSCC versus normal mucosa (Part III, Suppl.1). 14 out of 25 (56%) - 3 down-regulated and 11 up-regulated genes demonstrated similar behavior upon tumorigenic transition from HaCaT cells to SCC in our experiment. It's worth mentioning the presence of matrix metalloproteinase-1 (MMP1), ITGA6 from integrin signalling pathway, extracellular matrix protein 1 (ECM1), keratin 13 (KRT13), KRT17, KRT4 in the list of highly reported genes. Laminin-γ2 (LAMC2) is frequently overexpressed in HNSCC (Patel et al., 2002) and in the future studies it should be considered as predictor of the SCC development. These putative transcriptional biomarkers after validation on patients can be useful for the clinical diagnostics in the future.

Upon cultivation of the human immortalized, non-tumorigenic keratinocytes we observed oncogenic transition to the keratospheres that were able to form SCC in the nude mice. Molecular mechanisms of such transition are still unclear, but 2 models can be hypothesized: 1) prolonged passaging of the HaCaT cells in sphere-defined medium leads to the specific selection of the cells with enhanced tumorigenic potential from the heterogeneous culture; 2) culturing in the defined medium in the form of non-adherent clusters results in up/down regulation of the certain pathways that leads to the tumorigenic conversion. Although we have to perform future experiments to understand the mechanisms that underlie oncogenic transition for keratospheres, such model seems to be simple and attractive for investigation of the molecular signatures of the SCC.

4) Role of NF-YA in the processes of cellular proliferation and apoptosis

The NF-Y complex is a key player in the regulation of cell proliferation, supporting basal transcription of numerous cell cycle genes (Kabe et al., 2005; Wasner et al., 2003). Additionally, NF-Y plays a pivotal role in the DNA-damage response, mediating the p53-dependent repression of G2/M genes (Imbriano et al., 2005). Moreover the role of NF-Y in the process of the programmed cell death has recently emerged: Gurtner et al. demonstrated that unrestrained NF-Y activity promotes apoptosis depending on E2F1 induction and wtp53 activation (Gurtner et al., 2010). It's worth mentioning that in the inactivation experiments of single NF-Y subunit (NF-YA or NF-YB) was perceived by cells in the different way, activating diverse cell cycle blocks and signalling pathways (Benatti et al., 2011). The importance of NF-Y is further underscored by the early embryonic lethality of an NF-YA mouse knockout model due to defects in cell proliferation and extensive apoptosis (Bhattacharya et al., 2003).

The genome-wide analysis of NF-Y binding in K562, GM12878 and HeLa S3 tumor cell lines within ENCODE project found that NF-Y preferentially associates with genes involved in the inter-related p53 and TRAIL apoptotic pathways (Fleming et al., 2013). This observation reinforces the notion of a direct and indirect NF-Y/p53 interplay, with opposing functional consequences depending on the p53 status of the cell, i.e. proliferation or apoptosis (Imbriano et al., 2012).

While performing experiments on NF-YA inactivation/overexpression we and other research groups experienced difficulties to obtain stable clones with NF-YA overexpressed/silenced. Gurtner et al. explained this result by the fact that when NF-YA is overexpressed in the wtp53 cellular context, cells undergo apoptosis (Gurtner et al., 2010).

Preliminary data of our collaborators (Imbriano, Mantovani, manuscript in preparation) indicate that transient inactivation of the NF-YA subunit leads to the diverse outcomes in the cells with different p53 status: while cells with wtp53 (or wtp53 with reduced stability due to enhanced ubiquitination by virally encoded E6 protein, resulting in its accelerated degradation –case of HeLa cells) upon knock-down of NF-YA undergo apoptosis, cells harbouring mutant p53 alleles or p53 null cells demonstrate enhanced resistance to the programmed cell death.

We took a decision to perform an opposite experiment and overexpress NF-YA subunit. Since we have already seen in the inactivation experiments the importance of the p53 status, diverse cell lines were chose: wtp53 (HT1080 fibrosarcoma, HCT116 wt colon carcinoma, HepG2 human liver hepatocellular, MEF mouse embryonic fibroblasts), p53 null (PC3 human prostate adenocarcinoma, HCT116 p53 -/- colon carcinoma), mutant p53 (T98G human glioblastoma, HaCaT human immortalized keratinocytes, A431 epidermoid carcinoma), HEK293T (human embryonic kidney cells, transformed by expression of the large T antigen from SV40 virus).

We cloned the long and short forms of NF-YA cDNA from the plasmid vectors, kindly provided by Li et al. (Li et al., 1992a) into pSin-EF2-Sox2-Pur lentiviral vector (Addgene, #16577) via restriction digest with EcoRI and BamHI enzymes. Also dominant negative mutant forms of both NF-YA isoforms were cloned in the same vector backbone. This mutation is a triple amino acid substitution in the DNA binding domain that impairs its ability to bind DNA. It is still able to interact with a NF-YB/-YC dimer, but the resulting trimer is inactive in terms of CCAAT recognition (Mantovani et al., 1994).

For the production of lentiviral particles transient transfection of 293T cells with a 2nd generation packaging system was applied (lentiviral vector, containing transgene, packaging vector pCMV-dR8.2- dvpr, an envelope vector pCMV-VSVG). The lentiviral infection was performed by double spinoculation

of the cells (1h centrifugation, 2000 rpm, with a 5h interval) in the pure viral supernatant in the presence of 2 μg/ml polybrene. 72 h after infection puromycine (Sigma, USA) was added in the medium for the selection (the right concentration of the antibiotic was established before for each cell line). Empty pSin-EF2 -Pur vector served as a negative control. The efficiency of the lentiviral transduction was controlled by additional infection of the cells with GFP lentivirus. After 1-2 weeks in the selection medium stable clones were pooled and the expression level of the transgene was monitored by Western blot.

By the moment lentiviral transduction of 9 different cell lines (+293T cells – work in progress) was performed and the results are present in the Figure 7 (Part II). First of all we controlled p53 status in the following cell lines: wtp53 is known to be expressed at the low levels in the normal cells due to relatively short half-life (~10–20 min) and mutant p53 is characterized by a prolonged half-life compared with wtp53 protein (Cadwell and Zambetti, 2001). In accordance with IARC TP53 Database (http://p53.iarc.fr/) PC3, HCT116 p53 -/- are p53 null; HT1080, HCT116 wtp53, HepG2, MEF have wtp53; T98G, A431, HaCaT harbour mutant p53. Verification of the different NF-YA isoforms in these cell lines demonstrate that HepG2, A431, HCT116 wtp53, HCT116 p53 -/- express short isoform, while T98G and HT1080 have long NF-YA isoform and PC3 express both forms.

All lentiviral transduction experiments were repeated three times. The results of the overexpression experiments are present in the Figure 8 and summarized in the Table in the Figure 7 (Part III). First of all, it's intriguing that in contrast to the previous experiments we finally succeeded to obtain stable clones from some cell lines. We can assume that failure of the preceding experiments was due to the low number of the cell lines examined. You can easily see that overexpression experiment was successful just in 5 cell lines out of 10. Furthermore, it's interesting that not all isoforms could be overexpressed: it's obvious that NF-YA short isoform (YA1) has some advantages comparing

to the long isoform (YA13) and can be expressed easier in the following cell lines.

At the moment we can't draw any conclusion about connection between p53 status and NF-YA overexpression, since we obtain stable clones for wtp53, mutant p53, p53 null cells. Moreover this effect can be cell specific.

On-going experiments are focused on characterization of the stable clones: first of all we compared the growth characteristics of the cells. There is growing body of evidence that short and long NF-YA isoform are functionally diverse (See 4.2. "State of Art"). Evaluation of cellular proliferation using the MTT assay clearly demonstrate that NF-YAs confers growth advantage in comparison to the cells, expressing empty vector (HCT116 wt, U2OS) and also in comparison to the cells overexpressing NF-YAl isoform (HaCaT, HT1080). The same effect of the short NF-YA isoform on the cell proliferation has been already observed in our laboratory in the experiments on the mES (Dolfini et al., 2012b).

Thus, at this stage of research project we established several cell lines overexpressing different isoforms of NF-YA and some of the NF-YA mutants. Future experiments will be performed to understand the behaviour of the cells upon DNA damage, serum starvation, wound healing. Since HaCaT cells represent well characterized model for the keratinocyte differentiation stable HaCaT clones will be checked for the ability to undergo normal differentiation by Ca^{2+} induction.

Conclusions and Future Prospects

In the first part of research we established the link between 3 transcription factors: p63, KLF4 and mutant p53. Based on the results achieved we put forward a hypothesis that mutant p53 hijacks p63 from repressive to activating sites on the KLF4 promoter, radically changing KLF4 regulation at the transcription level. These results are even more intriguing in the light of Takahashi's landmark discovery that KLF4 is one of the transcription factors, indispensable for the reprogramming of the differentiated cells into iPS cells(Takahashi and Yamanaka, 2006). We and other research groups demonstrated that KLF4 is present at the high levels in SCC, where ΔNp63α and mutant p53 are already overexpressed (Deyoung and Ellisen, 2007). Since we discovered the mechanisms by which KLF4 is maintained at the high levels in the SCC, in the future we have a new field of research open to get inside the genesis of the cancer. A group of transcription factors is capable to reprogram differentiated cells into iPS cells, so the current hypothesis that tumours are derived from stem cells should be revised, as reprogramming of already partially differentiated cells, in the context of various genetic alterations, could also lead to tumor formation. Presence of the mutant p53 in the human keratinocytes donates to the cells growth advantages by targeting a gene with reprogramming capacity. The mechanistic details of the KLF4 role in normal keratinocytes versus SCC will be understood once its targets are unravelled by genome-wide approaches.

The results obtained for the regulation of KLF4 expression by p63 and mutant p53 inspired us to investigate the whole-genome mutant p53 binding in human keratinocytes. We found out that mutant p53 can alter profoundly gene expression by binding not just to p63 but to other transcription factors. The future experiments can be focused on identification of other mutant p53 partners that recruit mutant p53 to DNA. Moreover, since it's already known that not all

p53 mutants are functionally equal, it would be also interesting to study gain-of-function in the different cellular context – in the cells harbouring different p53 mutants. Since it's already well documented that different p63 and p73 isoforms have divergent biological properties, it would have sense to examine the mutant p53 interaction with p63 and p73 in the context of p63, p73 isoforms diversity.

We provided clear evidence that mutant p53 alleles of the HaCaT cells possess gain-of-function activity and confer enhanced proliferative potential to the cells. However this genetic alteration is not sufficient to render these cells oncogenic. In the 3rd part of the project we described a model for the skin cancerogenesis based on the sphere forming assay and HaCaT keratinocytes: growing cells in the floating spheres in defined serum-free medium leads to the formation of the keratospheres, that in comparison to the parental HaCaT cells display transformed phenotype and moreover give rise to the SCC when injected into nude mice. Microarray analysis revealed numerous genes whose expression is changed upon transition from the HaCaT cells to the keratospheres and finally to the tumour sample. More detailed analysis of the data can discover candidates for the SCC biomarkers that should be verified on the patients. Thus, sphere-forming assay of the HaCaT keratinocytes represents a simple, relatively inexpensive, not time-consuming model for study of the phenotypic and genetic alterations that occur upon initiation and development of SCC.

Transcription factor NF-Y plays a crucial role in the control of cell cycle progression and actively participate in the response of the cell to DNA damage via invitation with p53. Recent reports also underlie the importance of the NF-Y in the process of the programmed cell death. On-going project is dedicated to the investigation of NF-YA subunit function in the processes of cellular proliferation and apoptosis. By the moment several stable clones overexpressing different isoforms of NF-YA were obtained and future experiment will be focused on the characterizations of these clones in terms of response to DNA damage, serum starvation, wound healing and differentiation.

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Part II

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ORIGINAL ARTICLE

Mutant p53 subverts p63 control over KLF4 expression in keratinocytes

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Genetic experiments established that p63 is crucial for the development and maintenance of pluri-stratified epithelia and KLF4 for the barrier function of the skin. KLF4 is one of the factors that reprogram differentiated cells to iPS. We investigated the relationship between p63 and KLF4 using RNA interference, overexpression, chromatin immunoprecipitation and transient transfections with reporter constructs. We find that p63 directly represses KLF4 in normal keratinocytes (KCs) by binding to upstream promoter sites. Unlike p63, KLF4 levels are high in the upper layers of human skin and increase upon differentiation of KCs *in vitro*. In HaCaT KCs, which harbor two mutant alleles of p53, inactivation of p63 and of mutant p53 leads to KLF4 repression. p63 and p53 mutants are bound to sites in the KLF4 core promoter. Importantly, expression of the H179Y and R282Q p53 mutants in primary KCs is sufficient to activate endogenous KLF4. Finally, immunohistochemical analysis of tissue arrays confirms increased coexpression of KLF4 and mutant p53 in squamous cell carcinomas. Our data indicate that suppression of KLF4 is part of the growth-promoting strategy of p63 in the lower layers of normal epidermis, and that tumor-predisposing p53 mutations hijack p63 to a different location on the promoter, turning it into an activator of this reprogramming factor.
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Keywords: KLF4; p63; mutant p53; keratinocytes

Introduction

p63 belongs to the p53 and p73 family of transcription factors (TFs) that share a conserved DNA-binding domain and similar DNA target sequences in promoters and enhancers of eukaryotic genes. Genetic and biochemical work clarified that p53 directs the DNA-damage response by impinging on cell-cycle control and pro-apoptotic pathways (Aylon and Oren, 2007; Riley *et al.*, 2008). p63, on the other hand, is involved in the development and maintenance of pluri-stratified epithelia, including skin (Koster and Roop, 2004; McKeon, 2004). Several p63 proteins have been described, resulting from two distinct promoters, TAp63 and ΔNp63, and from alternative mRNA splicing at the 3′ end of the gene. The major isoform present in the skin, ΔNp63α, is essential for the development of ectoderm and stratification. Several human syndromes showing abnormalities in limbs, skin and epithelial annexes are caused by missense mutations in the *p63* gene (Rinne *et al.*, 2006). p63 is crucial for the activation of the epithelial-cell adhesion program (Carroll *et al.*, 2006; Barbieri *et al.*, 2006; reviewed in Carroll *et al.*, 2007) and it has a major role in maintaining the proliferative potential of stem cells (Senoo *et al.*, 2007; Su *et al.*, 2009). Mice lacking p63 die soon after birth with severe defects in limb and craniofacial development and absence of skin (Mills *et al.*, 1999; Yang *et al.*, 1999). Unlike p53, p63 is not mutated; yet it is overexpressed in many epithelial tumors, notably squamous cell carcinomas (SCCs) (Deyoung and Ellisen, 2007).

Another TF of which ablation leads to severe skin defects is KLF4: KO mice die 24 h post-birth because of lack of barrier function and consequent liquid loss (Segre *et al.*, 1999). Mice have impaired late differentiation of outer skin layers and perturbed cornified envelop formation. In contrast, overexpression of KLF4 in the skin accelerates the formation of the epidermal barrier (Jaubert *et al.*, 2003). The role extends to other multilayered epithelia, as conditional deletion in the cornea leads to epithelial fragility (Swamynathan *et al.*, 2007). KLF4 belongs to a large family of Kruppel-like activators with zinc-finger DNA-binding domains, recognizing the common GC boxes found in many, if not most, promoters (Kaczynski *et al.*, 2003). Unlike other members of the family, expression is confined to specific tissues, such as the gut, skin and thymic epithelia (Garrett-Sinha *et al.*, 1996; Shields *et al.*, 1996; Conkright *et al.*, 1999).

In general, KLF4 is usually present in well-differentiated, non-proliferating cells, where it serves the role

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of growth suppressor and is activated strongly in fibroblasts arrested by serum withdrawal (reviewed in Ghaleb *et al*., 2005). Profiling experiments have shown that KLF4 activates genes that inhibit proliferation, and represses genes that promote it (Chen *et al*., 2003; Whitney *et al*., 2006; Swamynathan *et al*., 2008). However, studies on KLF4 in tumors suggest that its role is extremely complex and variegated. In the intestine, genetic and expression profiling data indicate that it indeed functions as a tumor suppressor, often inactivated by epigenetic mechanisms in carcinomas (reviewed by Wei *et al*., 2006). On the other hand, KLF4 has emerged in profiling experiments as a gene overexpressed in breast and skin cancers, and overexpression in immortalized rat kidney cells results in hyperplasia and dysplasia (Foster *et al*., 1999, 2000; Huang *et al*., 2005; Chen *et al*., 2008). Transgenic mice in which KLF4 was expressed in the basal layer of the epidermis showed hyperplasia/dysplasia and, in the context of a p53 hemizygous background, these mice developed subcutaneous sarcomas (Foster *et al*., 2005). Moreover, KLF4 overrides Ras^{V12}-induced senescence and induces transformation in primary fibroblasts (Rowland *et al*., 2005).

Links between p53 and KLF4 surfaced in many studies. Cell-cycle block by DNA-damaging agents activates KLF4 expression, but apoptotic doses decrease it (Yoon *et al*., 2003). Interestingly, maintaining elevated levels of KLF4 prevents apoptosis mediated by p53 (Ghaleb *et al*., 2007), and elimination of KLF4 induction leads to apoptosis in cells treated with non-apoptotic doses of DNA-damaging agents (Zhou *et al*., 2009). KLF4 activates CDKIs, p21, p27 and p57 (Chen *et al*., 2001, 2003; Wei *et al*., 2006; Swamynathan *et al*., 2008); in particular, it cooperates with p53 in activating p21 (Zhang *et al*., 2000). KLF4 represses p53 transcription by binding to GC boxes in the p53 promoter: under these conditions, the levels of p21 and Cyclin D1 are crucial for deciding as to whether KLF4 exerts a tumor suppressive–high p21 or oncogenic–high Cyclin D1 activity (Rowland *et al*., 2005).

In addition to mediating a G1/S block, KLF4 prevents cells to enter mitosis with damaged DNA (Yoon *et al*., 2005), and indeed it binds to and represses the Cyclin B2 promoter directly (Yoon and Yang, 2004). Finally, and most importantly, in combination with OCT4, SOX2 and Nanog, KLF4 reprograms a wide range of differentiated cells into iPS, a state resembling very closely, if not identical to, totipotent embryonic stem cells (Takahashi and Yamanaka, 2006). Thus, overexpression of this TF has profound and lasting consequences on the expression of growth-promoting genes. This process is influenced by the p53 status (Hong *et al*., 2009; Kawamura *et al*., 2009; Mariòn *et al*., 2009; Utikal *et al*., 2009).

Our lab has been involved in the identification of p63 targets in human KCs, either by expression profiling or by ChIP (chromatin immunoprecipitation) on chip analysis (Viganò *et al*., 2006; Testoni *et al*., 2006). In this report, we investigated the possible regulation between p63 and KLF4 in primary and immortalized human KCs harboring mutant alleles of p53.

Results

KLF4 is a transcriptional target of p63

We inactivated p63 with a specific siRNA capable of recognizing all p63 transcripts (Testoni *et al*., 2006) and analyzed KLF4 expression, at both the mRNA and protein levels. As shown in Figure 1, efficient down-regulation of ΔNp63α, the most abundant isoform expressed in KCs, leads to KLF4 protein increase both in neonatal (nKCs) and in adult (aKCs) keratinocytes; mRNA levels are significantly higher only in nKCs (Figures 1a and b). The different behavior of nKCs versus aKCs is likely due to the different origin of the cells and their differentiation state; in fact, the basal levels of KLF4 are higher in aKCs (Figure 1c). As controls, we assayed the p63 targets p21 (CDKN1A) and KLF5: the former was decreased, as expected, whereas the latter was increased in nKCs.

We performed an *in silico* analysis of the KLF4 promoter region and located putative p53/p63 and KLF4 binding sites, which are conserved in other mammalian species (Figure 1d). We then performed ChIP with p53, p63 and KLF4 antibodies in aKCs; Figure 1e shows that regions D (−2225), E (−1784) and I (+467) are bound by p63 and KLF4 *in vivo*, whereas areas containing other potential sites are not. p53 is not bound to any of the analyzed regions. The C40 region on the p63 locus served as a positive control for p63 binding (Pozzi *et al*., 2009). Additional ChIP results are given in Supplementary 1. Taken together, these results show that p63 directly represses KLF4 transcription in primary KCs by binding to discrete upstream sequences in the promoter.

KLF4 is regulated during differentiation of the skin

We analyzed the expression of KLF4 in nKCs induced to differentiate *in vitro* by addition of $CaCl_2$ to the medium. Figure 2a shows that KLF4 increases already after 3 days, as detected by western blot, and further increases at later time points. In parallel, ΔNp63α is progressively reduced, as expected. The KLF4 mRNA is also upregulated, moderately at early time points and showing the highest levels after 10 days, as revealed by quantitative reverse transcriptase–PCR (qRT–PCR) analysis (Figure 2b). KLF5, also a putative p63 target (NC and RM, unpublished), is upregulated during this process (Figure 2b). KC differentiation was monitored by measuring mRNA levels of Involucrin and p21, which were reported to increase (Figure 2a, right panels), while ΔNp63α and two previously identified targets activated by p63, SMAD7 and SMURF2, significantly decreased (Pozzi *et al*., 2009). We also monitored KLF4 expression by immunofluorescence staining with p63 and KLF4 antibodies. These experiments confirmed the increase of KLF4 expression, concomitant to a decrease of p63 (Figure 2c, left panels). Furthermore, while in undifferentiated KCs KLF4 is expressed at low levels only in the nuclei, at 5 days it appears also in the cytoplasm, and at 10 days it is distributed almost equally in the nucleus and the cytoplasm. Immunostaining with the KRT1 marker

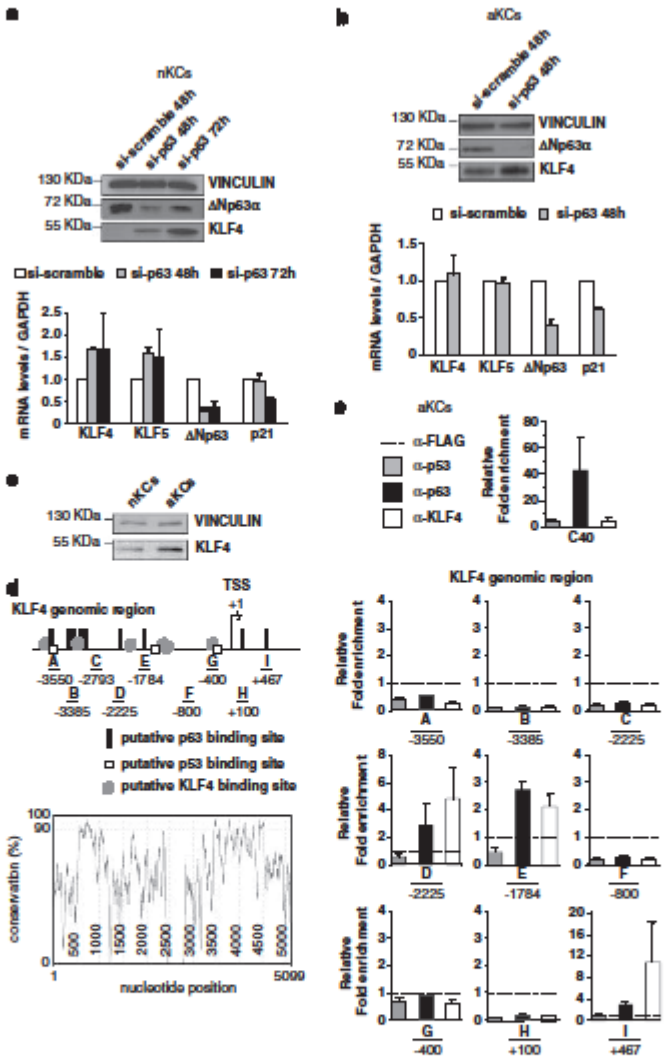

Figure 1 p63 represses KLF4 in normal KCs. (**a**) Inactivation of p63 by siRNA. nKCs were transfected with control scramble or p63 siRNA oligos for the indicated time points. Extracts and RNA were prepared and analyzed by western blot (upper panel) with the indicated antibodies, or qRT–PCR with primers for KLF4, KLF5, ΔNp63 and p21. (**b**) Same as a, except that aKCs were transfected. (**c**) Comparison between endogenous levels of KLF4 protein in nKCs and aKCs. (**d**) Schematic representation of the KLF4 promoter with putative p53/p63/KLF4 binding sites and position of primers used to amplify ChIPped DNA. (**e**). ChIP results with anti-Flag, anti-p53, anti-p63 and anti-KLF4 antibodies in aKCs after qPCR. Enrichment on the C40 enhancer (positive control for p63) is shown in the upper panel. Fold enrichment for each TF represents mean and s.d. of ChIPs obtained with two different antibodies (either monoclonal or polyclonal antibody for p53/p63 and two different polyclonal antibodies for KLF4).

provides the control for differentiation (Figure 2c, right panels).

We analyzed KLF4 expression in human normal adult skin by confocal immunostaining. Supplementary 2 shows that KLF4 expression is mostly nuclear, lower in the basal layer and higher in the subcorneum, contrary to p63, where KLF4 is absent in the upper layers. Interestingly, costaining with p63 reveals that

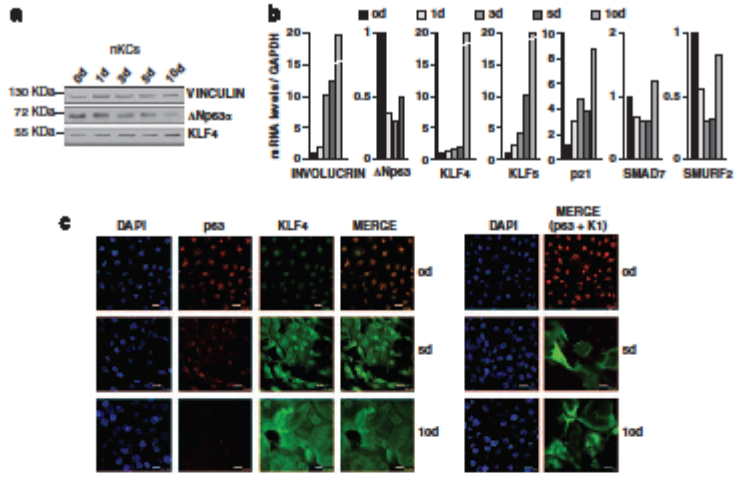

Figure 2 KLF4 increases during KC differentiation. (a) Western blot analysis of nhKCs induced to differentiate by addition of calcium for the indicated number of days. Results with p63, KLF4 and Vinculin antibodies are shown. (b) RNA was extracted at different time points and expression of the indicated genes quantified by qRT–PCR analysis. (c) Immunofluorescence analysis of p63 and KLF4 expression during KC differentiation. DAPI staining is included to identify nuclei. In the right panels, the KRT1 marker of differentiation was used in double staining with p63 to monitor the differentiation process. Scale bars (20 μm) are depicted in white.

some cells in the basal and suprabasal layers coexpress the two proteins (indicated by the white arrows in the merge panel). The KLF4 data are similar to those reported for mouse epidermis (Segre *et al*., 1999; Jaubert *et al*., 2003; Foster *et al*., 2005). Therefore, KLF4 is activated during keratinocyte differentiation *in vitro* and is mostly present in cells with low or non-existent levels of p63 in human skin.

KLF4 is differentially regulated in HaCaT cells

We decided to validate the results obtained above in HaCaT cells, an immortalized but not transformed KC cell line, by inactivating p63 by siRNA: the high KLF4 levels were downregulated at both protein and mRNA levels (Figure 3a). In contrast, overexpression of ΔNp63α caused a further increase in KLF4 protein and mRNA levels (Figure 3b). To ascertain whether this was a transcriptional effect, a luciferase reporter driven by the KLF4 promoter (Dang *et al*., 2002) was transfected in HaCaT cells together with scramble or p63 siRNAs: the activity was reduced upon inactivation of p63 (Figure 3c, left panel). On the other hand, the opposite was observed when the same construct was co-transfected with a ΔNp63α vector (Figure 3c, right panel), indeed indicating a transcriptional regulation of p63 on the KLF4 promoter.

Finally, ChIP experiments in HaCaT cells confirmed direct *in vivo* binding of p53, p63 and KLF4 to the KLF4 promoter: note, however, that p63 binding was mostly mapped to regions B (−3385), G (−400) and I (+467), which is different from that observed for primary KCs (compare Figures 1e and 3d).

Furthermore, we detected the presence of p53, together with p63, on region G (−400), which contains p53/p63 motifs but is not bound *in vivo* by p53/p63 in primary KCs.

HaCaT cells harbor two mutant p53 alleles, carrying a histine to tyrosine mutation at position 179, and an arginine to tryptophan mutation at position 282 (Datto *et al*., 1995). Both mutations are within the DNA-binding domain of p53 and typical of skin tumors (Pfeifer and Besaratinia, 2009). Unlike p53 in normal KCs, of which levels are very low, mutant p53 is expressed at high levels, as in HaCaT cells, and retains the ability to functionally interact with the DNA (Stambolsky *et al*., 2010).

We reasoned that the opposite regulation observed in HaCaT could be due to the presence of the p53 mutants, specifically impinging on p63-mediated regulation of KLF4 through the G (−400) region. We therefore inactivated p53 in HaCaT by transfecting siRNA: Figure 4a shows a reduction of the endogenous mutant p53; the levels of KLF4, and p53 mRNA, as measured by qRT-PCR, were substantially reduced. Importantly, the p63 mRNA and protein levels remained substantially high, indicating that the reduction of KLF4 was not secondary to an effect on p63 levels.

To further prove that p53 mutants are involved in KLF4 control, we overexpressed p53 H179Y and p53 R282Q, either alone or in combination, in neonatal and adult primary KCs, and analyzed KLF4 expression. As shown in Figure 4b, both mRNA and protein levels are increased after transient transfection of the p53 mutants; specifically, their combination yielded very strong over-expression of KLF4 in aKCs.

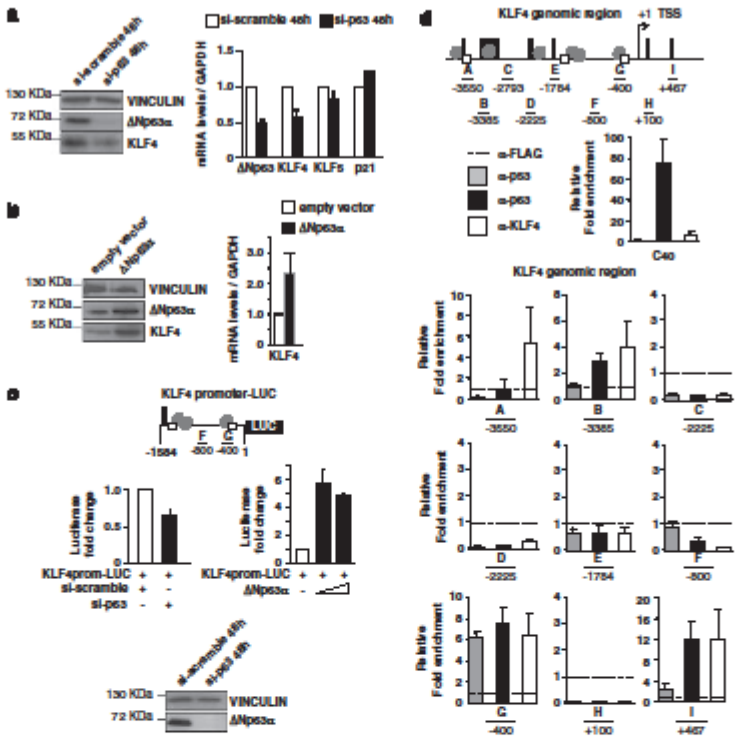

Figure 3 p63 activates KLF4 expression in HaCaT cells. (**a**) Functional interference of p63 in HaCaT immortalized keratinocytes. Cells were transfected with scramble or siRNA for p63; extracts were prepared and checked in western blots with the indicated antibodies (left panel) and mRNA expression of the indicated genes was analyzed by qRT–PCR (right panel). (**b**) Overexpression of ΔNp63α in HaCaT cells; western blot analysis of nuclear extracts (left panel) and qRT–PCR of KLF4 mRNA (right panel). (**c**) The Luciferase construct driven by the KLF4 promoter (−1584/+1) is depicted in the upper panel. The construct was transfected in HaCaT cells together with scramble or p63 siRNA oligos and Luciferase monitored (left panel). p63 inactivation was monitored by western blot (bottom panel). In the right panel, the same construct was co-transfected with an empty vector or with a vector expressing ΔNp63α. (**d**) Schematic representation of the KLF4 promoter, with putative p53/p63/KLF4 binding sites (from JASPAR and from Ortt and Sinha, 2006) and position of primers, is depicted in the upper panel. ChIP results (qPCR) obtained with anti-Flag, anti-p53, anti-p63 and anti-KLF4 antibodies are shown in the lower panel. Enrichment on the C40 enhancer (positive control for p63) is also shown. Fold enrichment for each TF represents mean and s.d. of ChIPs obtained with two different antibodies (either monoclonal or polyclonal antibody for p53/p63 and two different polyclonal antibodies for KLF4).

It was previously shown that mutant p53 has a higher affinity for p63, and heteromeric complexes can be detected, notably in HaCaT cells (Gaiddon *et al*., 2001). Therefore, to demonstrate that mutant p53 and p63 interact at specific sites on the KLF4 promoter, we performed ChIP and re-ChIP experiments with antibodies against p53 and p63. Although endogenous p53 mutants and p63 are similarly enriched on the same KLF4 promoter region G (−400) when considered separately (ChIP and re-ChIP with the same antibody), only ChIP with the p53 antibody followed by re-ChIP with that for p63 is able to enrich for the two TFs (Figure 4c, bottom panel).

Altogether, these experiments indicate that p53 mutants have a direct positive effect on KLF4 expression, together with p63, by binding to a core promoter region of KLF4.

KLF4, p63 and p53 in skin tumors

The majority of SCCs of the skin have missense mutations in the *TP53* gene, resulting in increased levels of p53 proteins (Pfeifer and Besaratinia, 2009, and references therein); similarly, KLF4 and ΔNp63α have been reported to be upregulated in SCC. We decided to analyze KLF4 expression in a panel of different skin tumors by immunohistochemistry: the results are shown in Supplementary Table 1 and indicate that indeed most of the SCCs do have robust expression of KLF4 and p53, whereas the expression of p63, already high, was

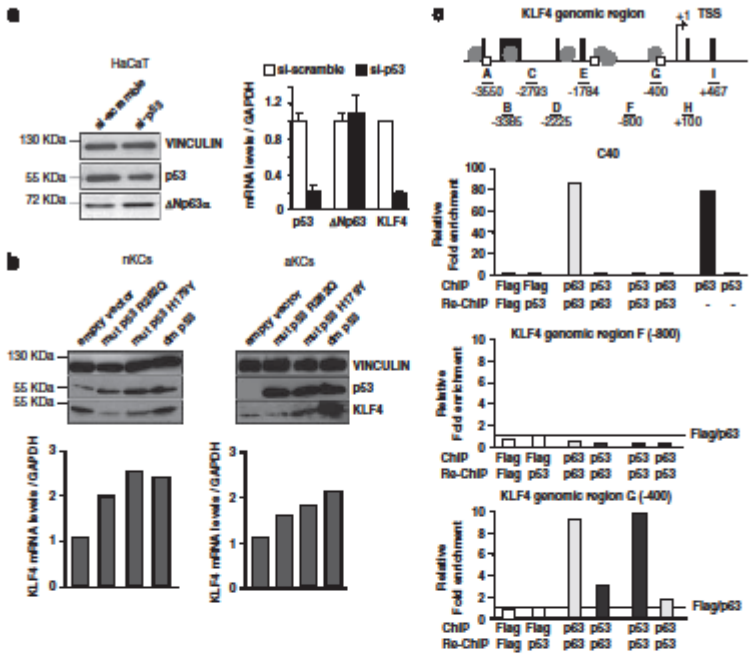

Figure 4 p53 mutants activate KLF4 expression directly. (**a**) Left panel shows western blot analysis of p53 and p63 after inactivation of p53 by siRNA in HaCaT cells. Right panel shows qRT–PCR analysis of KLF4, p53 and p63 expression after inactivation of mutant p53 in HaCaT cells. (**b**) nhKCs (left panels) or aKCs (right panels) were transfected with an empty vector or one expressing the indicated p53 mutants. Nuclear extracts were prepared and controlled in western blots with the indicated antibodies (upper panels). The levels of KLF4 mRNA were evaluated by qRT–PCR after normalization for GAPDH mRNA. (**c**) Schematic representation of the KLF4 promoter, putative p53/p63/KLF4 binding sites, and primer position in the upper panel. ChIP and re-ChIP qPCR analysis of p53 (DO1 monoclonal antibody) and p63 (polyclonal antibody) binding to the KLF4 promoter region in HaCaT cells. Amplicon G (−400) is positive for mutant p53, p63 and p53–p63 DNA binding; amplicon F (−800) is representative of promoter regions negative for mutant p53 and p63; the C40 control region is positive for p63 binding only.

not overly increased. Out of 20 SCCs, 15 showed overexpression rated 3, where 0 is no expression and 3 is maximum positivity of KLF4: in some cases, KLF4 showed nuclear staining, whereas in others expression was mostly in the cytoplasm; this finding has been reported before (Chen *et al*., 2008). As for p53, 8/20 SCCs had staining at level 3 and 4/20 at level 2: the figure of 60% of SCCs overexpressing p53 is confirmatory of previous studies. Note that constitutive overexpression of normal p53 in such tumors has not been reported, although overexpression is invariably associated with p53 mutations. In all but two cases—SCC9 and SCC10—whenever p53 levels were high, KLF4 levels were also high. In SCC17, the levels of KLF4 were unusually low, and p53 was indeed undetectable. Finally, there was no association of expression between p53 and KLF4 in other skin tumors. Figure 5 shows the immunohistochemical staining of representative SCC cases and comparison with normal skin. In none of the normal skin samples did we notice p53 signals above the background. In conclusion, these data support the notion that an overexpressed, mutated p53 is involved in the positive regulation of KLF4 in human SCC.

Discussion

In this report, we present evidence that KLF4 is negatively controlled by p63 in normal skin in the presence of physiological levels of wild-type p53, and that this regulation is subverted by oncogenic mutations of p53, establishing a direct link between these TFs, commonly overexpressed in SCCs. Because KLF4 overexpression cooperates in reprogramming differentiated cells, our findings have potential consequences for the mechanisms of formation of skin carcinomas.

p63 and KLF4 in normal skin

Genetic evidence clarified that p63 and KLF4 are TFs that have a crucial role in the epidermis (reviewed by Dai and Segre, 2004). Expression data in mouse and

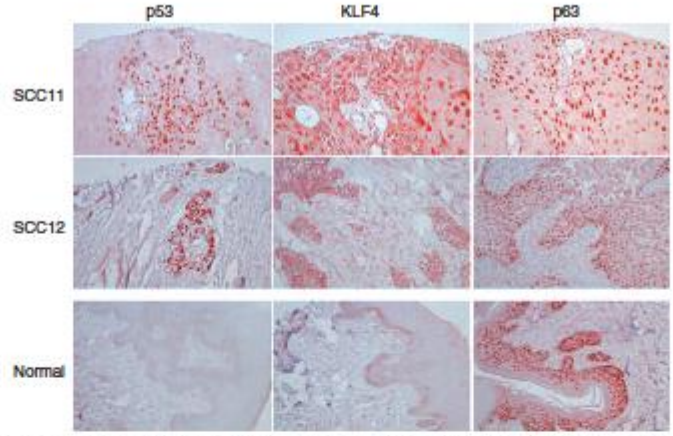

Figure 5 KLF4 is overexpressed in SCCs together with p53. Immunohistochemistry of p53, KLF4 and p63 with two representative human SCCs, SCC11 and SCC12, is shown in Supplementary Table 1. Staining of normal human skin is shown as control.

humans have clearly shown that p63 and KLF4 mark two different territories in the skin: the first exclusively—in mice—or mostly—in humans—confined to the basal layer, whereas KLF4 is high in the granular and subcorneum strata. Our data showing that KLF4 expression is increased upon *in vitro* differentiation—as p63 decreases—and that p63 represses KLF4 transcription are in line with the expression data; this effect is more pronounced in adult KCs compared with neonatal, and it is directly exerted by binding to upstream and downstream sites in the KLF4 promoter region. In general, the analysis of p63 targets derived either by expression profiling or by location analysis (reviewed by Perez and Pietenpol, 2007; Viganò and Mantovani, 2007) has lent support to the idea that the TF is important in determining the proliferative potential of skin basal KCs (Senoo *et al*., 2007; Su *et al*., 2009): among activated targets, in particular, there are pro-growth genes, whereas TFs involved in differentiation, such as c-Jun, C/EBPδ and HBP1, are repressed. Pathways at the crossroad of these decisions, such as Notch, Wnt and SHH, are also controlled. KLF4, therefore, joins an increasingly long list of regulators that are kept at bay in the lower skin layers by p63's direct negative control. Transcriptional profiling has been performed by overexpressing KLF4 in the colon cancer cell line RKO (Chen *et al*., 2003) and in an elegant system of corneal cells derived from KLF4-conditional null mice (Swamynathan *et al*., 2008): this latter study, in particular, reported notorious targets of p63, such as c-Jun and PDGFβ-RII, among genes upregulated by KLF4, and FGF-R2, Id2 and C/EBPδ, among the repressed ones. The latter is particularly interesting, as it was placed upstream of p63 in the regulation of stem cells of the corneal limbus (Barbaro *et al*., 2007), and targets of C/EBPδ, such as Desmocollin 3 and other genes coding for desmosomal proteins (Smith *et al*., 2004), are also targets of KLF4. Therefore, there seems to be an overlap in functions and it will now be important to establish the network of KLF4-regulated genes in the skin.

Forced expression of KLF4 in the basal layer of the skin has been experimentally tested in two transgenic mice models: inducible expression of KLF4 was driven by the Keratin 5 and Keratin 14 promoters, reaching somewhat different conclusions. One study showed that mouse skin undergoes decreased proliferation and anticipated differentiation, which is in line with the anti-growth, pro-differentiation aspect of KLF4 (Jaubert *et al*., 2003). On the other hand, Foster *et al*. (2005) reported repeated cycles of hyperplasia and dysplasia in the skin, eventually leading to development of SCC *in situ*. The origin of the discrepancies between these studies is unclear; interestingly, however, the Foster *et al*. study also reported a genetic interaction with p53 in a p53 hemizygous mouse background. This is an important finding, as it established dysregulation of KLF4 as a predisposing factor for human skin cancer.

p53, p63 and KLF4 in skin cancer

The two most widespread non-melanoma cancers of epithelial origin in the skin are basal cell carcinomas (BCCs) and SCCs: the former is locally destructive, but it rarely metastasizes, while SCC cells have high metastatic potential (Tsai and Tsao, 2004). The risk of developing SCC is directly related to UV exposure and susceptibility to sunburn. Mutations in p53 are widespread in SCC, clearly a predisposing factor, and indeed these tumors carry UVB 'signature' mutations. Importantly, patches of cells overexpressing mutant p53 proteins are detected in the interfollicular epidermis before skin tumors arise, and these 'p53 patches' are

believed to be the precursors of SCCs (de Gruijl and Rebel, 2008). The HaCaT cells analyzed here are immortalized and non-transformed KCs with mutant p53 alleles—R282Q and H179Y—and the levels of the p53 proteins are extremely high. They are not a model for skin cancer, as they are able to differentiate *in vitro*, unable to form colony assays in soft agar and do not generate tumors in nude mice *in vivo* (Boukamp *et al*., 1997; EM and RM, unpublished). However, the p53 missense mutations harbored by HaCaT alleles are indeed UVB signature mutations (Pfeifer and Besaratinia, 2009, and references therein).

Two apparently contradictory results blur our vision as to the role of p53 in skin cancer: mice deficient for *TP53* develop tumors resembling SCC after UV irradiation (Ziegler *et al*., 1994; Li *et al*., 1998; Jiang *et al*., 1999), whereas Li–Fraumeni syndrome patients, who have a germline mutation in *TP53*, are not reported to be at increased risk for SCC (Malkin *et al*., 1990). These findings should be put in the perspective of the growing body of evidence showing that p53 missense mutants not only lose normal p53 functions, but are also often pro-active in tumor formation (reviewed by Donzelli *et al*., 2008; Brosh and Rotter, 2009). Indeed, a set of gain-of-function mechanisms resulting from p53 mutations have been documented. Mutant p53 inactivation in HaCaT, overexpression in normal KC ChIPs and re-ChIPs clearly show that p53 mutations commonly found in SCC subvert the p63 negative regulation of KLF4, identifying a new pathway of co-regulation between p63 and mutant p53.

There are important mechanistic points that need to be addressed. The first is the interplay between p53 and p63/p73: although there is little evidence of heterotetrameric formation of p53–p63 dimers (Davison *et al*., 1999), several reports have indicated that p63/p73 have an increased affinity for p53 missense mutants (Di Como *et al*., 1999; Strano *et al*., 2000; Gaiddon *et al*., 2001). Specifically, p63 was reported to associate with the p53 mutants of HaCaT cells (Gaiddon *et al*., 2001). The second is the finding that mutant p53 is associated with DNA *in vivo* in our ChIP assays. This is not the first report detecting binding of a potentially DNA-binding defective p53 mutant to DNA: binding could be tethered by NF-Y to CCAAT boxes in growth-promoting genes (Di Agostino *et al*., 2006). This is not likely the case with the KLF4 promoter, which lacks any visible CCAAT box; rather, concomitant with the expression of the mutant p53 proteins, there is a relocation of p63 binding to the core promoter, in an area where canonical p53/p63 sites are present. Re-ChIP analysis is consistent with this. Finally, transient transfections with reporter assays devoid of upstream negative, but containing the positive core promoter sites, reproduced the positive effect of ΔNp63α on KLF4 transcription in HaCaT. Thus, we favor a scenario in which mutant p53 hijacks p63 from repressive to activating sites, either imparting novel DNA-binding or trans-activating features, or cooperating with additional neighboring TFs.

Interest in KLF4 has been recently spurred by the finding that it is one of the key TFs leading to reprogramming of differentiated cells into iPS (Takahashi and Yamanaka, 2006). At the same time, high levels of KLF4 are found in SCC, which often have high levels of ΔNp63α (Deyoung and Ellisen, 2007) and mutant p53. A direct link between these three TFs is now established and this could have far-reaching effects in terms of our understanding of the genesis of skin tumors. In fact, the discovery that overexpression of a handful of TFs reprograms differentiated cells should generate a re-evaluation of the current hypothesis that tumors are derived from stem cells, as reprogramming of already partially differentiated cells, in the context of various genetic alterations, could also lead to tumor formation. UVB-derived p53 mutations confer gain-of-function properties to KCs, including direct targeting of a gene with reprogramming capacity, resulting in alteration of the growth-controlling capacity. The mechanistic details of the KLF4 role in normal KCs vs SCC will be understood once its targets are unveiled by genome-wide approaches.

Materials and methods

Cell culture and transfections

HaCaT cells and first passage primary human adult keratinocytes (haKCs), derived from healthy individuals (breast skin biopsies), were grown and handled as previously described (Viganò *et al*., 2006). Maintenance, differentiation and transfection of primary human neonatal keratinocytes (hnKCs; CellNtec, CH) were conducted as previously described (Pozzi *et al*., 2009). For knockdown experiments, 50 nM siRNA oligonucleotides, with a scrambled sequence (Ambion, Foster City, CA, USA), or targeting the central DNA-binding domain of p63 (Testoni *et al*., 2006) or against p53 (5′-GACUCCAGUGGUAAUCUACTT-3′), were used. For overexpression experiments, we used pcDNA3 vector, pcDNA3-ΔNp63α, pcDNA3-p53 wild type, pCR259-p53R282Q and pCR259-p53H179Y (Kato *et al*., 2003). The KLF4 promoter-LUC was kindly provided by Vincent W Yang (Emory University, Atlanta, GA, USA).

Western blot analysis and antibodies

Total or nuclear lysates were separated on 10% SDS–PAGE, transferred onto a nitrocellulose membrane, and probed using the following antibodies: 4A4 monoclonal anti-p63 (Santa Cruz, Santa Cruz, CA, USA), DO1 monoclonal anti-p53 (Genespin, I, Milan, Italy), polyclonal anti-KLF4 (Genespin, I) and monoclonal anti-Vinculin (Sigma, St Louis, MO, USA).

Immunofluorescence analysis

Immunofluorescence analysis of frozen human adult-thigh skin sections and cultured cells was performed as previously described (Viganò *et al*., 2006) with anti-p63, anti-KLF4 (Genespin, I) and anti-KRT1 (Covance, Princeton, NJ, USA) antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Confocal images were obtained using a Leica TCS SP2 AOBS microscope (CIMAINA, University of Milano, Milano, Italy) with an × 40 objective.

Chromatin immunoprecipitation

ChIP analysis was carried out as previously described (Testoni *et al*., 2006), using the following antibodies: 4A4 monoclonal

(Santa Cruz) and polyclonal anti-p63 (Genespin, I), DO1 monoclonal and polyclonal anti-p53 (Genespin), polyclonal anti-KLF4 (Santa Cruz; Genespin, I) and anti-Flag (Sigma). ChIP and re-ChIP experiments were performed as previously described (Furlan-Magaril *et al*., 2009) with DO1 monoclonal anti-p53 and polyclonal anti-p63 antibodies (Genespin, I). Location of potential p63/p53/KLF4 binding sites on the KLF4 promoter was performed by *in silico* analysis (Motif and ConSite; cutoff settings: 80–85%) applying single transcription factor specific criteria (Ortt and Sinha, 2006). Primer pairs are listed in Supplementary 3. Fold enrichment for each TF was calculated as previously described (Pozzi *et al*., 2009).

Immunohistochemistry and skin tumor tissue arrays

Paraffin sections of thickness 14 μm were deparaffinized and then blocked for 60 min in PBST (0.1% Triton X100 in PBS) with 2.5% donkey serum, 2.5% goat serum, 0.5% cold-water fish gelatin and 0.5% BSA. Primary antibodies were applied for 16 h at 4 °C and secondary antibodies were applied for 1 h at room temperature (RT). DAPI nuclei counterstaining was used. Tissue samples were fixed in buffered formalin, dehydrated, embedded in paraffin wax and sectioned. After deparaffinizing and rehydrating, each tissue section was immersed in citrate buffer, boiled 3 times for 5 min in a pressure cooker and washed with TBS buffer. Each section was placed on a Dako cytomation automated immunostainer and incubated with the specific antibody at RT for 45 min, washed with TBS pH 7.6 and incubated with biotinylated goat-anti-mouse-anti-rabbit immunoglobulins (Dako REAL, K5005, Dako, Dn) at RT for 30 min. After incubation with the secondary antibody and a new wash with TBS pH 7.6, sections were incubated with streptavidin conjugated to alkaline phosphatase at RT for 30 min A red chromogen solution was prepared as indicated by Dako REAL datasheet. Each section was counterstained in Mayer's hematoxylin solution and coverslipped.

Skin cancer tissue array (Z7020093, BioChain, Hayward, CA USA), including 48 cases in duplicates from surgical resection of normal, benign and cancerous tissue of the skin and subcutaneous tissues, were fixed in 10% neutral buffered formalin for 24 h and processed using identical SOPs. Sections were picked onto Superfrost Plus or Apes-coated Superfrost slides (Bio Optica, Milan, Italy).

Conflict of interest

The authors declare no conflict of interest.

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Gain-of-function p53 mutants have widespread genomic locations partially overlapping with p63.

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ABSTRACT:

p53 and p63 are transcription factors -TFs- playing master roles in the DNA-damage response and in the development and maintenance of pluristratified epithelia, respectively. p53 mutations are common in epithelial tumors and HaCaT keratinocytes harbor two p53 alleles -H179Y and R282Q- with gain-of-function (GOF) activity. Indeed, functional inactivation of mutp53 affects the growth rate of HaCaT. We investigated the strategy of mutp53, by performing ChIP-Seq experiments of mutp53 and p63 and analyzed the transcriptome after mutp53 inactivation. Mutp53 bind to 7135 locations *in vivo*, with a robust overlap with p63. *De novo* motifs discovery recovered a p53/p63RE with high information content in sites bound by p63 and mutp53/p63, but not by mutp53 alone: these sites are rather enriched in elements of other TFs. The HaCaT p63 locations are only partially overlapping with those of normal keratinocytes; importantly, and enriched in mutp53 sites which delineate a functionally different group of target genes. Our data favour a model whereby mutp53 GOF mutants act both by tethering growth-controlling TFs and highjacking p63 to new locations.

INTRODUCTION

p53/p63/p73 are a family of transcription factors -TFs- that share a conserved DNA-binding domain and a similar DNA target sequence in promoters and enhancers [1-4]. p63 is involved in the development and maintenance of the skin and of pluristratified epithelia. The major isoform present in the skin -DeltaNp63alpha- is essential for development of ectoderm and stratification, through activation of the epithelial cell adhesion program, and it plays a major role in maintaining the proliferative potential of stem cells [3]. p63 is overexpressed in many epithelial tumors, notably Squamous Cell Carcinomas, SCC [4]. The risk of developing SCC is directly related to UV exposure and mutations in p53 are clearly a predisposing factor: indeed UVB "signature" mutations were reported [5]. HaCaT cells are immortalized, non tumorigenic keratinocytes with mutant p53 alleles, R282Q and H179Y [6], which are typical UVB signatures [7]. In this system, the p53 pathway is functional in response to UVB irradiation, in terms of cell cycle block and induction of apoptosis [8, 9]. These cells also express large amounts of DeltaNp63alpha.

Abolition of the p53 powerful tumor suppression functions is an important step in cancer progression, and the location of hotspot mutations in residues important for DNA-binding provided a conceptual framework pointing at a loss-of-function mechanism. However, there is now strong genetic evidence that some p53 missense mutants are pro-active in tumor progression and metastasis formation [Reviewed in 10, 11]. The issue of the interplay between mutp53 and p63/p73 is quite relevant, because genetic experiments suggest a complex role of p63 isoforms in transformation [4], and p53 missense mutants, including those produced by HaCaT alleles, have an increased affinity for p63/p73 [12-14, reviewed in 15]. In addition, the three family members are linked through a microRNAs-based circuit [16]. Two gain-of-function -GOF- mechanisms have been suggested: the first posits that an excess of mutp53 interferes with p63/p73 function,

by inhibiting DNA-binding following a stimulus, or forming inactive aggregates [17]. In the second, mutp53 do reach specific DNA targets, either through protein-protein interactions with other TFs, such as NF-Y, E2F1, NF-KB and VDR [18-21], or *via* p63-guided interactions [22, 23]. Note that "indirect" promoter recruitment of a TF by interactions with another TF bound to its element was first described for the Estrogen Receptor on Fos/Jun sites two decades ago [24], and further detailed for many other TFs ever since. The relative "weight" of the two scenarios is unclear. To examine this, we decided to explore mutp53 *in vivo* DNA-binding and functional activities, and relate it to p63 locations in HaCaT cells.

RESULTS

Mutp53 proteins are highly expressed in HaCaT cells [15], as it often the case with other missense mutp53 alleles: we inactivated the two alleles by stably expressing an shRNA under puromycin selection, in parallel with a control scramble shRNA. The p53 shRNA targets the DNA-binding domain, hence it is aimed at all possible isoforms of p53 [25]. Clones were selected, pooled and mRNA and protein levels of mutp53 and p63 controlled by qRT-PCR and Western blot: Fig. 1A shows that mutp53 mRNA levels are substantially decreased, the protein levels essentially abolished (Fig. 1B). p63 mRNA was modestly decreased. Note that a similar inactivation experiment attempted with p63 shRNAs repeatedly failed to yield colonies, likely because of the key role of p63 in HaCaT survival and growth (EM, RM, unpublished). We studied a few parameters of mutp53-expressing and non expressing HaCaT, before and after UVB irradiation. As shown in Fig. 1C, we noticed a modest variation in cell cycle progression: increase in subG1 and G1 cells, and a decrease in G2/M in mutp53-depleted cells. The degree of apoptosis, as measured by TUNEL assays (Fig. 1D), PARP activation and Caspase 8 cleavage (Fig. 1E) was lower in the absence of mutp53, but still present. Thus other mechanisms compensate for the lack of mutp53 to drive a DNA-damage response. The most notable difference, however, was the growth rate, as curves were flatter in HaCaT deprived of mutp53 (Fig. 1F). These data confirm that mutp53 are indeed GOF alleles contributing to cellular growth.

To investigate the GOF mechanism, we performed analysis of p63 and mutp53 binding to genomic locations by ChIP-Seq experiments, using the DO1 monoclonal, recognizing the N-terminal domain of p53, and a polyclonal against p63. We identified 7135 peaks of mutp53 and 3421 of p63 in HaCaT cells, defined as areas with a significant enrichment in the IP with respect to the corresponding genomic region of Input DNA controls run in parallel (see Methods). The lists of locations and transcription Units are in Supplementary Tables 1 and 2, respectively. Notorious p63 targets such as the p21 and MDM2 promoters were among the positives (See below). We validated the data by using different antibodies, the Ab7 p53 polyclonal, which recognizes all p53 isoforms, and anti-63 4A4 monoclonal (Supplementary Fig. 1). Some of the positive regions in ChIP-Seq were monitored by qPCR: with the exception of FANCi, the other targets were enriched. We noticed that some targets scoring positive only for p63 in ChIP-Seq were also somewhat enriched with Ab7, suggesting that we might be underscoring the overlap of the two TFs, possibly because DO1 is unable to pick up the shorter p53 isoforms present in HaCaT cells [25]. Finally, p63 targets previously identified as functionally important, such as KLF4, Notch1, TP63, DLX3/4 and JAG2, among others, scored positive for p63 and mutp53 (Supplementary Fig. 2).

We therefore felt confident to analyze the ChIP-Seq locations and found the expected overrepresentation in promoters, from -5000 to +1000 of the Transcriptional Start Sites (TSS): Fig. 2A shows that 1591 mutp53 and 907 p63 sites reside in promoters, and 3697 and 1400, respectively, in the body of RefSeq genes. We characterized the overlap between positive peaks, and found that 19% of p63 peaks overlap with mutp53 in the promoter (175 out of 907) and 17% in the body of genes (240 out of 1400).

Next, we evaluated the enrichment of TFBS in the p63$^+$, mutp53$^+$ and p63/mutp53$^+$ peaks with a width of 150 bp from the center with the Pscan software [26] (Fig. 2B), using as background a set of 10.0000 sequences of the same size, chosen at random from genomic regions annotated either as "promoter" or "enhancer". It is apparent that a consensus p53/p63RE is at the top of the list in p63$^+$ and p63/mutp53$^+$ peaks, but not in mutp53$^+$, where sites of other TFs predominate: E boxes -variously termed NHLH1, Myf, Mycn, USF1, MYC-MAX, MAX-ESR (nuclear receptors), AP2. This is an indication that p63, either alone or with mutp53, recognizes its own site, whereas mutp53, in the absence of p63, binds DNA through sequences recognized by other TFs. We then used the Weeder software [27] to perform *de novo* motif discovery in the peaks of the three cohorts, in promoters, genes or elsewhere: Fig. 2C shows that a TGGGCATGTC sequence clearly emerged in p63$^+$, containing a perfect p53/p63 consensus (underlined), with additional information on the flankings; a similar sequence, lacking the CC at the 3' end, is recovered in p63/mutp53$^+$ locations; in mutp53$^+$ peaks, instead, the variety of underlying sequences prevented the emergence of a clear consensus by *de novo* analysis, confirming the underlying presence of several unrelated TFBS.

A large number of genomic locations of p63 were recently reported in primary keratinocytes -PHK- using the same antibody employed here [28]. We analyzed the data of PHK and HaCaT and found that a substantial number -50%- of HaCaT locations are missing in primary keratinocytes (Fig. 3). We assessed the number

of mutp53 peaks in the two populations and found some skewing: 222 were in the PHK common sites, and 367 in the HaCaT-only cohort, suggesting that the presence of mutp53 alters p63 binding to a subset of sites bound in normal keratinocytes. Gene Ontology analysis retrieved terms such as *organ morphogenesis*, *tissue and epidermic development* and *positive regulation of transcription* in p63⁺ devoid of mutp53 (Fig. 3); the same terms were present in the larger p63⁺ cohort, with the addition of *Wnt signaling* and *induction of apoptosis*. Terms related

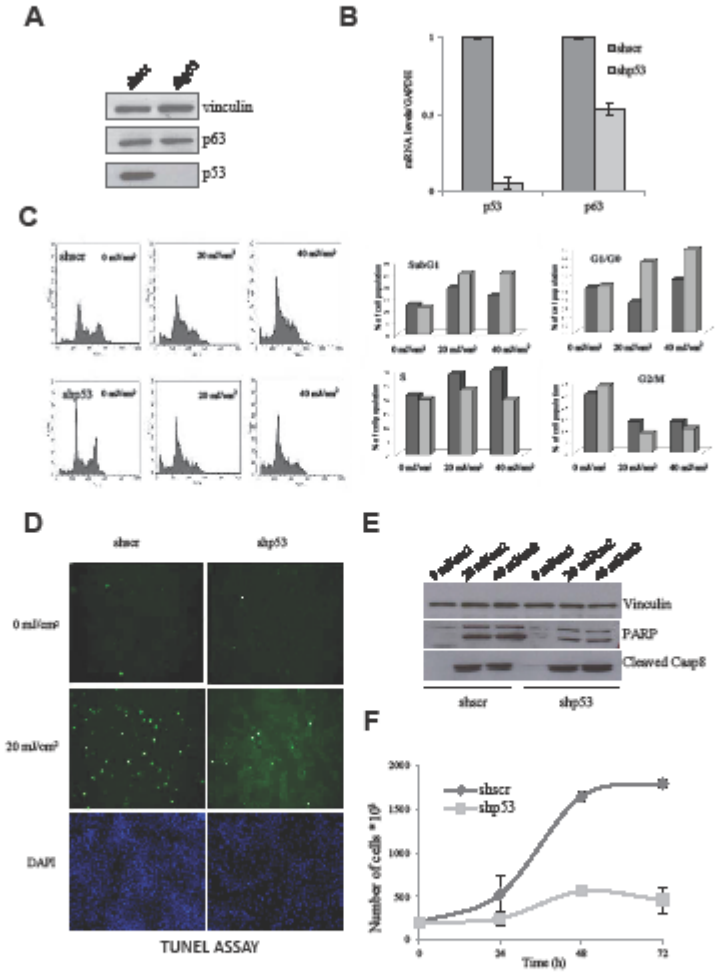
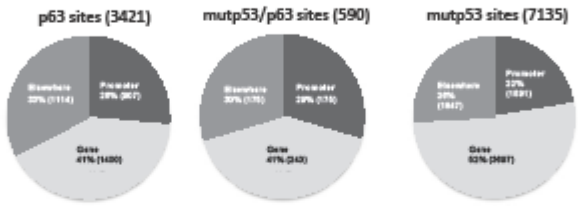


Figure 1: Effect of functional inactivation of mutp53 in HaCaT cells. (A) mutp53 was inactivated by transduction of the HaCaT cells by lentiviral vector expressing shp53 and vector expressing scramble shRNA served as a negative control. After puromycine selection clones were selected, pooled and protein levels of mutp53 and p63 were analyzed by Western blot. Vinculin was used as a loading control. (B) mRNA levels of mutp53 and p63 were subsequently controlled by qPCR. Normalization of cDNA templates was achieved by GAPDH quantification. (C) Inactivation of mutp53 in HaCaT cells leads to the modest variation of cell cycle progression. The medium of HaCaT cells was replaced by PBS and the cells were exposed to 20 mJ/cm² and 40 mJ/cm² concentration of UVB light. After UVB treatment PBS was replaced to growth medium and after 12h of incubation at the standard conditions cells were harvested and cell cycle progression was analyzed by FACS. PBS-treated cells without UVB treatment served as negative control. (D) mutp53 deprived HaCaT cells are less sensitive to the apoptosis. shp53 and shscramble HaCaT cells were treated with UVB irradiation and degree of apoptosis was measured by TUNEL assay (E) as well as PARP activation and Caspase 8 cleavage were controlled by Western blot. (F) mutp53 affects significantly the growth properties of the cells. Cell growth rates of shp53 and shscramble HaCaT were compared by direct counting of viable cells.

to *signal transduction* and *cell cycle* were prevalent in mutp53+ genes (Fig. 3). Specifically, the mutp53/p53+ sites were enriched in terms of *Wnt signalling* and other metabolic terms in the molecular function analysis, such as *actin binding, Tyrosine Kinase* and *GTPase activity*. In addition to previously characterized targets (Supplementary Figure 3a, b), families of targets worth mentioning are Wnt genes -Wnt4, Wnt7a, Wnt9a, Wnt10a- and Retinoic Acids Receptors, RARα, RARγ and RXRα (Supplementary Fig. 3c, d). Both p63 and mutp53 are present at multiple locations of the large cluster of keratin genes on chromosome 17, particularly in a conserved region at 3', overlapping with positive epigenetic marks (Supplementary Fig. 3e). In general, the p63 and mutp53 locations overlap with those of histone post-translational modifications (Supplementary Fig. 3): although firm conclusions are difficult to make because of the difference in the cellular contexts of the ChIP-seq profiles, this is a further indication that the sites identified here are functionally relevant. From this set of data, we conclude that p63 binding in HaCaT is different from PHK, in part due to the coresidency of mutp53, and that the latter recognizes a large set of functionally distinct groups of genes independently from p63.

Next, we performed profiling analysis of mutp53-inactivated HaCaT cells. A large number of genes were up -1649- or down -1644- regulated, by using a relatively stringent cut-off *ratio* of 1.5-fold (Supplementary Table

A



	p63	Mutp53/p63	mutp53
Promoters (-2000 +1000)	782	134	1123
Genes	2142	338	4074

B

p63 promoters

TF	Matrix id	P-value
TP53	MA0106	1.73E-80
TFAP2A	MA0003	2.34E-71
NHLH1	MA0048	6.13E-62
Myf	MA0055	2.10E-61
Arnt-Ahr	MA0006	9.83E-58
Mycn	MA0104	6.76E-48
USF1	MA0093	1.82E-47
Pax5	MA0014	4.40E-36
MafB	MA0117	1.40E-34
ESR1	MA0112	5.66E-34

mutp63/p63 promoters

TF	Matrix id	P-value
TP53	MA0106	0.00E+00
Myf	MA0055	2.59E-222
Hox11-CTF1	MA0119	9.23E-104
MYC-MAX	MA0059	6.33E-85
USF1	MA0093	7.74E-77
MAX	MA0058	1.03E-70
Mycn	MA0104	1.03E-69
HAND1-TCF3	MA0092	3.34E-66
TEAD	MA0090	2.62E-59

mutp63 promoters

TF	Matrix id	P-value
Myf	MA0055	1.20E-84
NHLH1	MA0048	1.27E-73
USF1	MA0093	1.15E-61
Mycn	MA0104	1.90E-56
TFAP2A	MA0003	2.12E-54
MYC-MAX	MA0059	5.16E-49
ESR1	MA0112	2.42E-36
Pax5	MA0014	1.35E-34
MAX	MA0058	1.88E-34
MafB	MA0117	3.07E-33

C

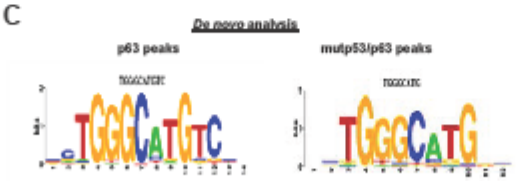


Figure 2: ChIP-Seq analysis of p63 and mutant p53 binding in HaCaT keratinocytes. (A) Distribution of the site position for mutp53 and p63 as well as the overlap between p63 and mutp53 positive peaks are present (UCSC genes http://genome.ucsc.edu/). Also the number of promoters or genes, positive for at least one peak of mutp53 or p63 or both is indicated. (B) Evaluation of TFBS enrichment in the of p63+, mutp53+ and p63/mutp53+ promoters using Pscan software. (C) Analysis of p63+ and p63/mutp53+ binding site sequences by *de novo* motif discovery performed using the Weeder tool (26).

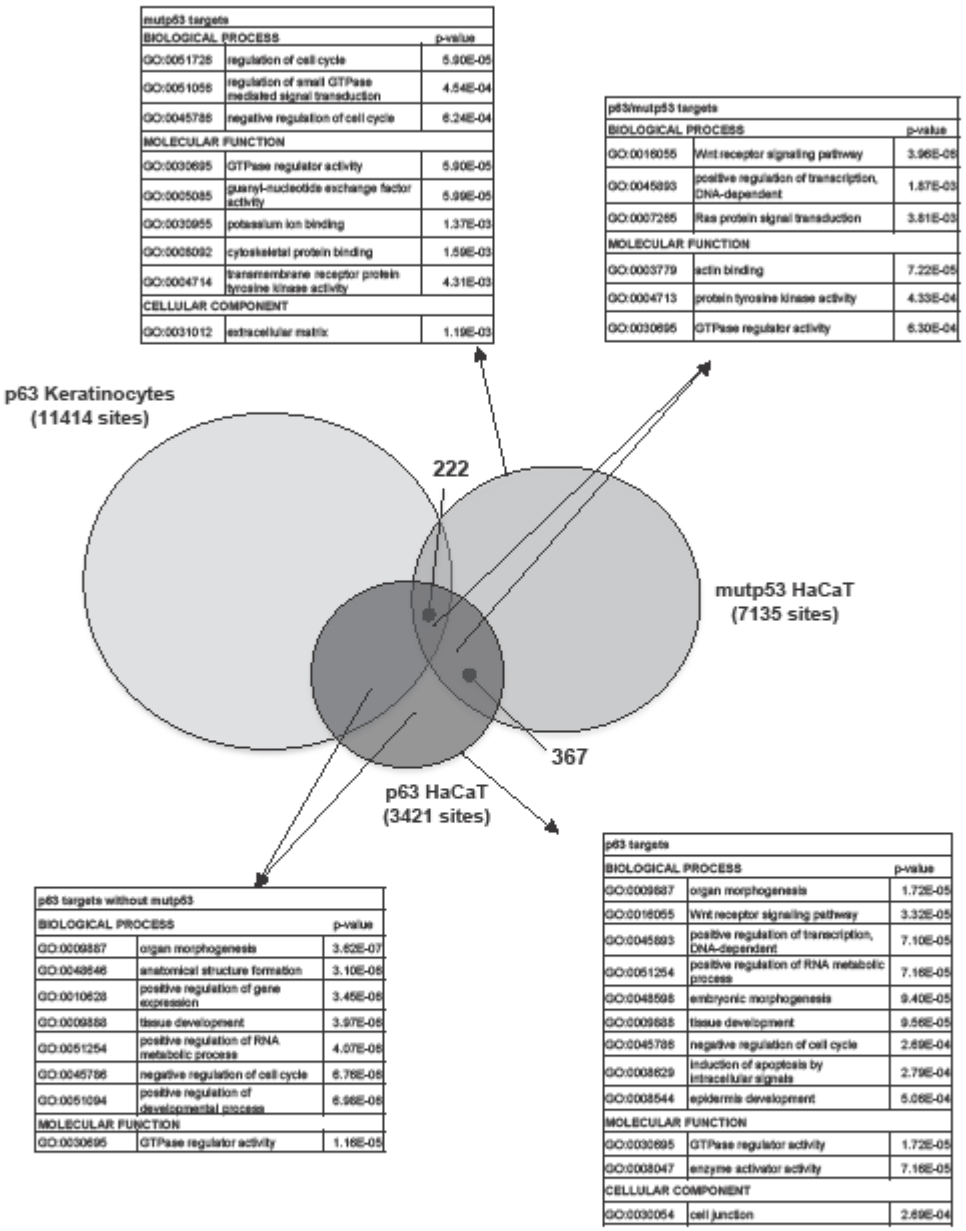
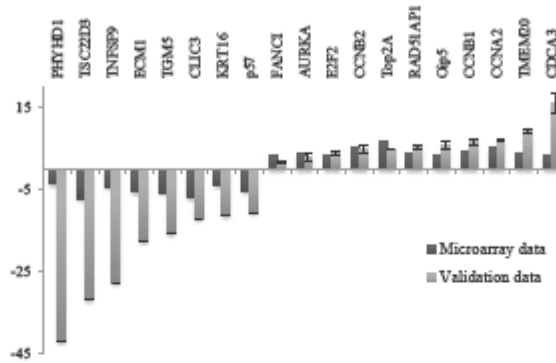


Figure 3: p63 and mutant p53 binding in normal and HaCaT keratinocytes. The comparison of p63 and mutp53 DNA binding sites in HaCaT cells and p63 DNA binding sites in PHK. 222 sites were common for p63, mutp53 in HaCaT and p63 in PHK, but the comparison also revealed other 367 sites that were shared by p63 and mutp53 just in the HaCaT cells. Gene Ontology analysis for $p63^{+}$, $mutp53^{+}$, $p63^{+}/mutp53^{+}$ $p63^{+}/mutp53^{-}$ in HaCaT keratinocytes is present.

3). A quick inspection of the genes identified known p53 targets, such as CDC20, Aurora KinaseA and B, Chek1, Topo IIα, CyclinB1 and B2, CyclinA, CDC2, p57/Kip2. G2/M genes are normally repressed upon DNA-damage in cells harboring wt p53, and regulated in an opposite way by indirect recruitment by mutp53 [29, 30]. We validated the profiling results by qRT-PCR (Fig. 4A): essentially all genes changed expression according to expectations; the degree of variation was greater in qRT-PCRs with respect to profiling data, which is a common finding, indicating

A



B

upregulated genes		
BIOLOGICAL PROCESS		p-value
GO:0022402	cell cycle process	2,28E-27
GO:0006259	DNA metabolic process	1,88E-16
GO:0006396	RNA processing	6,46E-13
GO:0006260	DNA replication	1,08E-12
GO:0034470	ncRNA processing	2,01E-11
GO:0051340	regulation of ligase activity	1,13E-10
GO:0009389	tRNA metabolic process	1,82E-09
GO:0006974	response to DNA damage stimulus	3,16E-09
GO:0042254	ribosome biogenesis	3,67E-08
CELLULAR COMPONENT		
GO:0005694	chromosome	9,88E-18
GO:0005759	mitochondrial matrix	4,23E-17
GO:0005819	spindle	1,28E-13
GO:0005657	replication fork	1,13E-08

downregulated genes		
BIOLOGICAL PROCESS		p-value
GO:0016126	sterol biosynthetic process	4,88E-08
GO:0007243	protein kinase cascade	2,81E-08
GO:0030216	keratinocyte differentiation	5,25E-08

C

Mutp53 activated	
cell cycle	CCNF, CDK4, EIF4G2, PSMA2, PSMB7, PSMC3, AURKA, TFDP1, NOLC1, TUBB3, NCAPD3, ANAPC13, SAC3D1, NCAPG2, TIPIN, CDT1, CDCA3
DNA metabolism	DNMT1, MCM4, PRKDC, RFC4, TOP2A, GMNN, TIPIN, FANCI, MCM10, DMAP1, CDT1, LOC731751
RNA processing	HNRNPM, NOLC1, NOL5A, FAR82, U2AF2, EXOSC2, LSM5, LSM4, TRUB2, CRNKL1, TRMT6, DHX36, DDX54, PUS1

Mutp53 repressed	
sterol biosynthesis	CEL ,CYP1B1, IDI1, LCAT, MVD, SREBF1, STAT6A, CH25H, STARD3, OSBP2
protein kinase	ADRB2, ECM1, LIF, LTBR, MAP3K11, GADD45B, STAT5A, TNFRSF1A, TRAF2, DAPK2, LATS2, CARD10, WNK2
regulation of transferase	ADRB2, CCND2, SFN, MAP3K11, GADD45B, PDGFB, TRAF2, LATS2, CARD10, SERINC2

Figure 4: Profiling of mutp53 inactivated HaCaT cells. (A) Validation of microarray data by qPCR. (B) GO analysis: for the up-regulated genes cell cycle, as well as DNA and RNA metabolisms and response to DNA-damage categories were retrieved with significant p-value, while for down-regulated genes sterol biosynthesis and keratinocyte differentiation categories were obtained even if with the less significant p-value. (C) Examples of the genes that are up-regulated and down-regulated by mutp53 removal subdivided regarding their belonging to the specified GO category.

that we underscored the effect of mutp53 removal in our profiling analysis. The overlap between the mutp53 locations and gene expression analysis is robust -15%- but not absolute (DD, RM, unpublished). Some genes might be indirectly affected by mutp53 removal, and some of the targets are oblivious of its removal, at least in growing HaCaT cells. GO categorization identified *cell cycle*, as well as *DNA* and *RNA metabolisms* and *response to DNA-damage* as robustly enriched in the upregulated cohorts (Figure 4B); in the downregulated genes, p values were less significant with *sterol biosynthesis* and *keratinocyte differentiation* being somewhat enriched. Examples of genes of up and down-regulated categories are shown in Figure 4C.

DISCUSSION

Our work addresses a debated topic concerning the mechanisms of action of GOF mutant p53 and p63. We found that (i) mutp53 HaCaT alleles are pro-growth and mutp53 have thousands of binding sites in the human genome;(ii) they affect gene expression profoundly, both by binding with p63 to consensus elements and by being tethered by other TFs to their locations.

Mice deficient for *TP53* develop tumors resembling SCC after UV irradiation, and other models harboring mutp53 alleles have aggressive features in their epithelial tumors, including increased capacity to metastatize. Hence the hypothesis that certain mutations are GOF has visibly gained ground [10,11]. Specific mutations of p53 alleles are a hallmark of SCC in humans [7]: although HaCaT cells are not tumorigenic *in vivo*, they harbour two alleles that are routinely found in SCC, in addition to large amount of a fully functional ΔNp63α, the most abundant isoform found in human keratinocytes: stable p63 inactivation in HaCaT, in fact, was impossible, presumably because it is required for cellular growth. Instead, inactivation of mutp53 led to suboptimal growth rates and a large change in gene expression, as shown in previous experiments [31], making the system suitable to study p63 in the presence of high amounts of mutp53.

Mechanistically, two models were proposed: (i) mutp53 sequesters tumor suppressors, including p63, in inactive complexes, or it inhibits its DNA-binding capacity [15, 17]; (ii) mutp53 acts as a *bona fide* transcription factor with a "deviant" specificity, through unrelated TFs, or the related p63/p73 [18-22]. Widespread inhibition of p63 DNA binding by p53 DNA-binding mutants seems to be ruled out by our experiments: >3400 p63 locations are retrieved in a cellular context with very high levels of tumor-type mutp53, up to 20-fold excess with respect to p63 [15]. The same conclusion is reached by analyzing gene expression profilings of overexpressed mutp53 in p53 null cells: Neilsen et al. find binding of mutp53 to 6 overexpressed genes [23]. Thus, at the heart of the mutp53 strategy there are interactions with p63, and with other TFs. Interestingly, the p63 locations are partially different from the ones found in normal keratinocytes [28], with >1700 "new" sites, showing an enrichment of mutp53 coresidency. In summary, the two previous scenarios for mutp53 GOF function, both involving tethering to DNA regulatory elements either with p63 (or p73), or *via* other TFs, are operational (Fig. 5). We attempted to "measure" the two mechanisms, and the second appears to be prevalent, but one needs to be very cautious and aware of the bias related to the antibodies used in the analysis: this is particularly relevant for mutp53, since DO1 is oblivious of the mut53 short isoforms very recently described by the lab of JC Bourdon in HaCaT [25]. The picture is therefore likely to be more complex.

The most abundant TFBS scored in the p63 peaks is indeed the p53/p63 RE, as found in Pscan analysis and in the stringent *de novo* motif discovery by Weeder, confirming that DNA-binding is direct through sequence-specific contacts and that p63 is functional. Our Weeder-derived logo incorporates the core central tetranucleotide, CANG from Chip on chip analysis of p63 and p73 [32, 33] and CNTG from SELEX [34]; it does provide additional information in the flankings, and the retrieved decamer is,

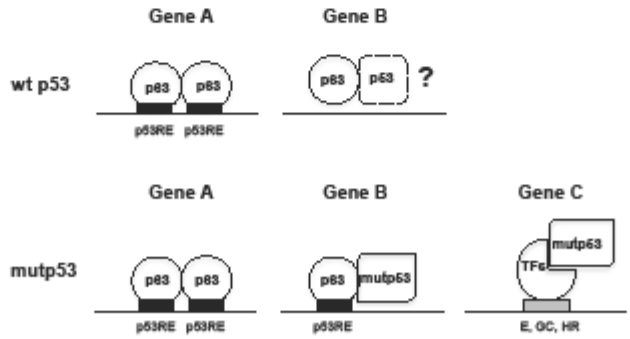


Figure 5: Models of p63 and mutp53 binding to different classes of promoters.

to the best of our knowledge, the most detailed p63/p53 matrix characterized so far. In the mutp53/p63 *loci*, the p53/p63 logo is also obvious, both in Pscan and *de novo* motif discovery; the precision of the ChIP-Seq technique allows us to conclude that the two TFs, which are bound within 50 bp, share the same DNA motif. If anything, tandem elements with variable spacing are enriched in the p63, but not mutp53/p63 cohorts: in the latters, it is unclear whether mutp53 contacts with DNA have a different specificity, or lack it. In general, these data are consistent with a model whereby heteromers, formed by the observed increased affinity of mutp53 for p63 [12-14], and/or higher protein concentrations, are steered to DNA by the p63 sequence-specific capacity. Another aspect that has not been investigated so far is the reciprocal interplay between p63 and wt p53 after DNA-damage in normal cells (Fig. 5): one could imagine, in fact, that the binding of p63 might serve as a "guide" for an activated p53 to find its locations, or at least some of them, during the stress response.

As to tethering of mutp53 *via* unrelated TFs, the lack of a single, clearly recognizeable logo with *de novo* motif analysis strongly indicates that multiple TFs are involved. Even allowing a high degree of freedom, so that "non canonical" sites could be scored [35], we could not come up with any enriched motif. Among the TFBS previously reported to be guiding mutp53 binding, the NF-Y, E2F and NFkB sites [18-20] are statistically enriched in the profiling data (Supplementary Fig. 4). In the ChIP-Seq dataset, we do find the Estrogen Receptor, whose reciprocal interplay with mutp53 is well documented in other epithelial contexts, for example in breast cancer cells [36]. Note that HaCaT do not express ERs (EM, RM, unpublished), but an identical DNA motif is shared with other nuclear receptors, including VDR, recently shown to be enriched in ChIP on chip analysis of SKbR3 cells carrying the -175 p53 GOF mutant [21]. Therefore, our data suggest that mutp53 targets nuclear receptors. GC boxes, E-boxes, AP2 predominate in ChIP-Seq and profilings. E boxes are recognized by a plethora of HLH B-Zip proteins, some of which are known to play a role in epithelial cancer, including in the skin [37]. GC boxes are often overrepresented in many such studies, not least because promoters are embedded in CpG islands: this box is targeted by zinc fingers TFs belonging to the large Sp1 and KLFs family, numbering over 20 members [38]. We have recently detailed that one of these -KLF4- is targeted by mutp53, through p63 [22]. A large body of genetic, biochemical and histopathological evidence points to disregulation of KLF4, KLF5 and KLF6 as important in the progression of epithelial tumors. The lack of previously identified motifs -NFkB, NF-Y, E2F- in the ChIP-Seq dataset is not surprising, considering that the mutant p53 alleles and the cellular context used here are different: this raises the possibility that the set of TFs tethering mutp53 might be specific for mutp53 alleles and/or for a particular cell-type, whether in immortalized or transformed conditions.

As a whole, the p63 preferred GO categories are a variation of *morphogenesis, tissue* and *development* themes, as expected from a master ectodermal regulator; clustering of the normal keratinocytes and HaCaT p63 locations tells a similar story, with targets in the *RNA metabolism* and *transcription* category, which we previously reported. Mutp53, however, visibly changed the configuration, since the mutp53*, as well as the common mutp53/p63*, are shifted toward and enrichment of signaling, cell-cycle regulation and metabolic terms. As for single pathways, those that were previously pinpointed -FGF-R, EGF-R, Wnt, Notch- are confirmed. At least one previously unappreciated group of genes emerged: RARα, RARγ and RXR genes are targeted at multiple locations both by p63 and mutp53. Interestingly, genetic experiments in mice using a dominant negative RARα expressed in basal keratinocytes *via* the K14 promoter caused inhibition of endogenous RARα and RARγ with greatly diminished p63 levels: this led to a dramatic skin phenotype very similar to the one found in p63 KO mice [39]. Given the general anti-proliferative properties of RARs, including in the skin [40], the intersection of p63/mutp53 with RARs is certainly worth more exploration in the future, particularly in tumors.

In summary, our data support the idea that p53 mutants do affect growth by altering gene expression *via* specific binding to discrete DNA elements, in promoters and elsewhere, either through p63, or selected classes of TFs. Furthermore, the normal p63 regulome is altered, and mutp53 partially diverts p63 activity to locations not normally seen in normal keratinocytes. Our work should be extended to different cellular contexts, in tumorigenic cells carrying different GOF mutp53 and splicing isoforms of p63 as well as the related p73.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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MATERIALS AND METHODS

Cells and Infections

Human HaCat keratinocytes were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine and 100 U penicillin/streptomycin. The growth characteristics were obtained by counting the cells in the hemacytometer at the indicated time points. For UVB irradiation, 80% confluent HaCaT cells was replaced by PBS and the cells were exposed to various concentrations (20mJ/cm$_2$, 40mJ/cm$_2$) of UVB light emitted by MacroVue UV-20 (Hoefer, USA) with the peak at 320 nm. After removal of PBS the growth medium was added and the cells were incubated for 12 h at normal conditions and then harvested with cell scraper including those floating in medium. PBS-treated cells without UVB treatment served as negative control.

For the cell cycle analysis 10^6 cells were washed once in PBS and then fixed with 70% cold ethanol. After this one wash in 1% BSA in PBS was performed and the cells were stained for 30' in 1ml of PI solution (20µg/ml Propidium Iodide, 10µg/ml Rnase solution in PBS). The data were acquired by FACS within 2 h after staining. TUNEL assay for detection of apoptosis was performed according to the manufacturer's protocol (Roche Applied Science, Germany). The cells were fixed for 1h in 4% Paraformaldehyde in PBS, rinsed with PBS and permeabilised for 2' in 0,1% Triton X-100 in 0,1% sodium citrate. After 2 washes with PBS cells were stained with 50 µl of TUNEL reaction mixture and then with DAPI and the cells were immediately analysed under a fluorescence microscope.

For the lentivirus production one day before 293T cells were split at the density of 5*106 cells per plate using 20 ml of DMEM medium supplemented with 10% FBS. For the 3 plasmid system the following DNA mix was prepared: 20µg lentiviral vector, 10µg VSVG, 15µg Δ3.8. In the tube with DNA, 400µl of 1,25 MCaCl$_2$ and 1,5ml of H_2O were added; 2ml of 2X HBS (280mM NaCl, 50mM Hepes, 1,5mM Na_2HPO_4, pH 6,95) were added. The transfection mixture was kept 12h after which the medium was changed. After 36h, the viral supernatant was harvested and filtered through a 0,45µm filter. The lentiviral infection of HaCaT cells were performed by double spinoculation of 70% confluent cells (1h centrifugation, 2000 rpm, with a 5h interval) in the presence of 2 µg/ml polybrene. 72 h after infection puromycine (Sigma, USA) was added in the medium for selection. For p53 knockdown in the HaCaT cells, the shp53 pLKO.1 puro plasmid (Addgene, USA) was used and shscramble pLKO.1 puro plasmid was used as control. After 3 weeks in the selection medium, stable clones were pooled.

Western blot analysis was perfomed according to standard procedures with whole cell extracts with DO1 anti-p53, anti-p63 (Genespin, Italy), anti-vinculin (Sigma, USA), anti-cleaved Caspase 8 (Cell Signaling Technology, USA) and anti-PARP (Santa Cruz, USA) antibodies.

ChIP and ChIP-Seq

ChIP was carried out as previously described (21). In brief, about 5 mg of chromatin (equivalent to 18 x 150 mm dishes with cells at 80% confluence) were used in IP experiments, with either mouse monoclonal anti-p53 (DO1), or rabbit polyclonal anti-p63 antibodies (Genespin, I). Each chromatin set was divided into 10 aliquots, which in turn were independently IPed using 10µg of the appropriate antibody. In parallel, 500 µg of chromatin were IPed with 10 g of mouse monoclonal anti-Flag antibody (Sigma) as a control. ChIP-enriched and their Unbound fractions were recovered and subject to crosslink reversal, proteinase K digestion, phenol/chloroform extraction, DNA precipitation and quantitation. Single ChIP-enriched DNA samples were then tested by qPCR to assess enrichment on known targets of either p53 and/or p63. p63 enrichment (mean ± SD) on Myoneurin promoter and C40 enhancer were 2.76 ± 0.46 and 49.88 ± 18.61, respectively; p53 enrichment on LEF1 upstream region were 1.74 ± 0.34 and 7.05 ± 2.12. ChIP-enriched DNAs, as well as half of the corresponding Unbound DNAs, were pooled together, precipitated and quantitated. 50 ng of each ChIP-enriched or Unbound DNA were then converted into a library suitable for high-throughput sequencing using an Illumina Genomic Analyzer following the manufacturer's istructions. Before sequencing, amplified ChIP-samples were tested in parallel to amplified Unbound, pre-amplification ChIP-enriched, and pre-amplification Unbound DNAs, to score for enrichment. Sequence reads were mapped to the masked human genome sequence (assembly GRCh37, retrieved from the UCSC genome browser database (38) using the Seqmap tool (39). Matched was performed by allowing at most two mismatches at any position of the reads. Trimming unmapped reads at the 3'end led to marginal improvements in the number of mapped reads and this step was therefore skipped. Only reads mapping to a unique position on the genome were considered for further analysis. This resulted in about 10 million uniquely mapping reads for each of the two mutp53 experiments (IP and input) and in about 4 million for each of the two p63 experiments. In each experiment, uniquely mapped reads were then extended by 300 bps along the 5'->3' direction. This produced, for each ChIP or input sample, a base pair by base pair coverage map of the genome, that is, giving for each base pair the number of extended sequence reads that contained it. Only base pairs covered by reads mapping on both strands were considered valid for further analysis. Enrichment was then calculated in

each valid base pair by comparing, for each IP experiment, the coverage in the experiment to the coverage in the respective input used as expected value, and computing an enrichment p-value with a negative binomial distribution. Enriched regions were then defined as regions consisting of consecutive base pairs characterized by calculated p-values smaller than 0.01 and not interrupted by a gap of 100 bps or more non valid or with a p-value greater than 0.01. The p-value associated with each of the enriched regions was defined as the minimum p-value among the base pairs belonging to the region. Regions shorter than 150 bps were then discarded regardless of the p-value. The p-value associated with the remaining regions was then used to compute the false discovery rate (FDR) with the Benjamini-Hochberg correction. This resulted in 7136 regions for p53 and 3422 for p63 with FDR lower than 0.01.

To further validate the predicted regions we applied the MACS tool (40) to the same datasets, with default parameters. About 95% of our predicted regions in both mutp53 and p63 experiments were also found as significantly enriched by MACS at p-value 10^{-6} (roughly corresponding to our False Discovery Rate of 0.01).

RNA Profiling

Total RNA was extracted using RNeasy Mini kit (Qiagen, D) according to the manufacturer's protocol. For qPCR analysis, 1μg of RNA was reverse-trascribed using Reverse Transcription System (Promega, USA). The expression level for each gene was normalized with GAPDH. The list of the primers used for qPCR is shown in Supplementary 4. For profiling, RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). 500ng of total RNA was synthesized to biotinylated cRNA using the Illumina RNA Amplification Kit (Ambion, USA). 750ng cRNA was hybridized for 18h to HumanHT12 v. 3.0 Expression BeadChips (Illumina USA) according to the protocol provided by the manufacturer. Hybridized chips were washed and stained with streptavidin-conjugated Cy3 (GE Healthcare, USA). BeadChips were dried and scanned with an Illumina BeadArray Reader (Illumina Inc.) and analyzed with the Illumina BeadStudio v. 3.1.3.0 software. The quantile normalization algorithm was applied on the data set to correct systematic errors. Background was subtracted. For differential expression analysis, three technical replicates of each sample were grouped together and genes with a detection of p-value <0.01, corresponding to a false-positive rate of 1%, were considered as detected. Differently expressed genes were selected with Diff Score cutoff set at ±30, corresponding to a P-value of 0.001.

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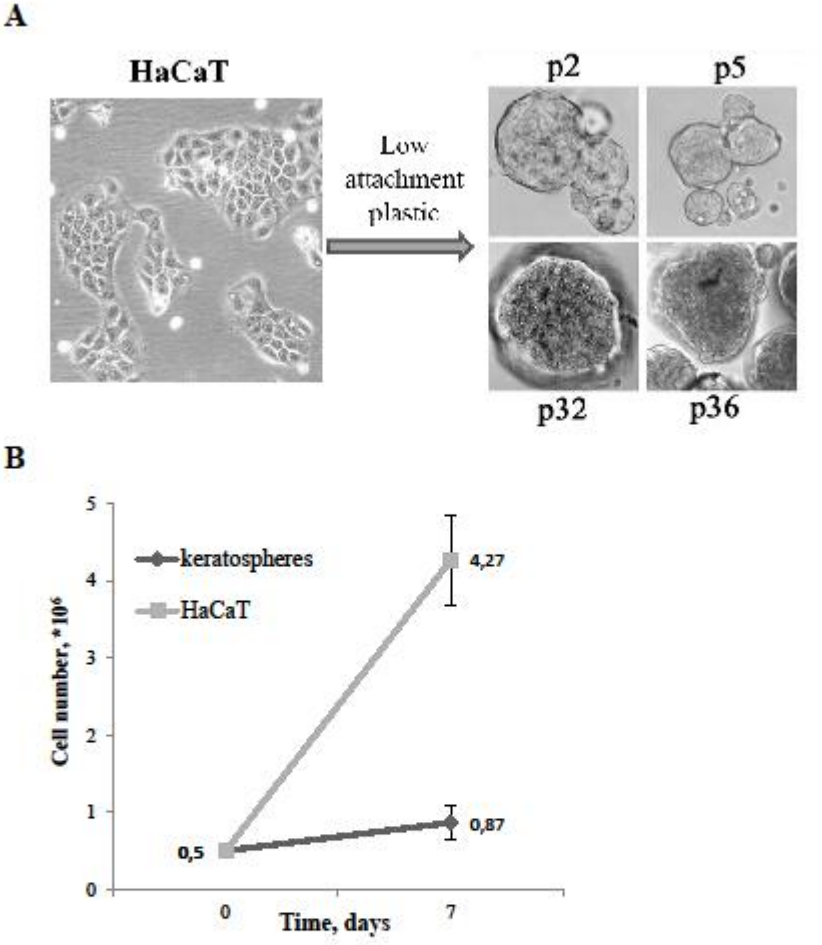
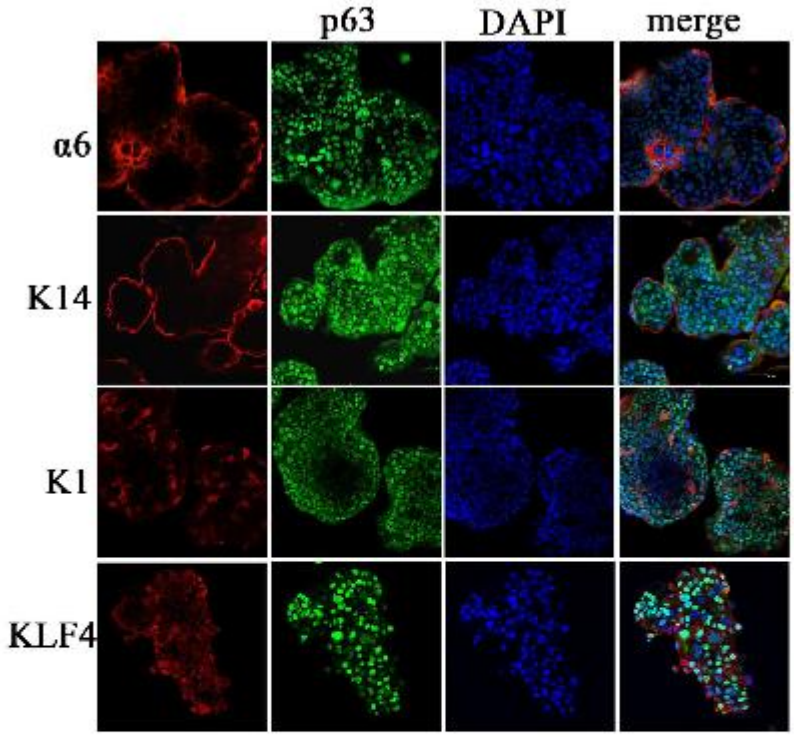
Part III



Martynova et. al Figure 1



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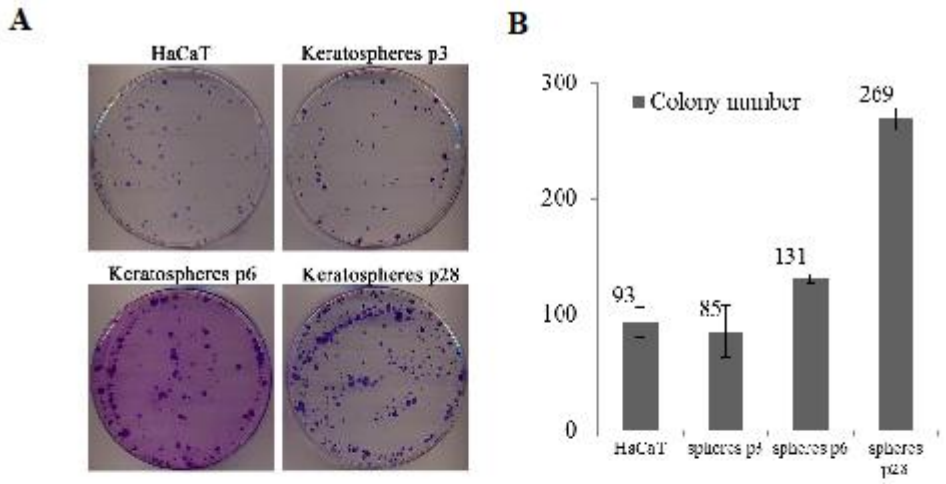
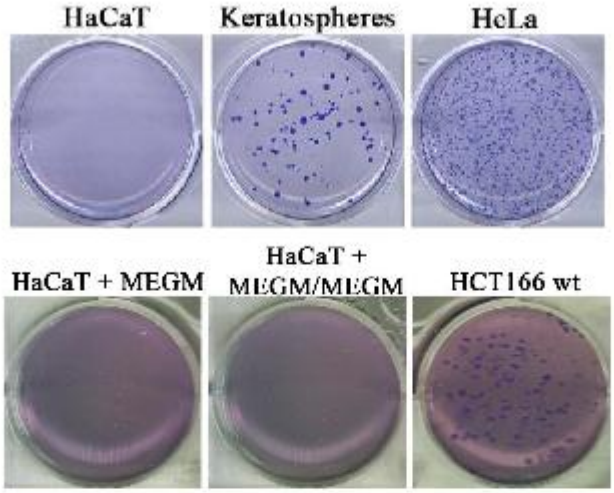
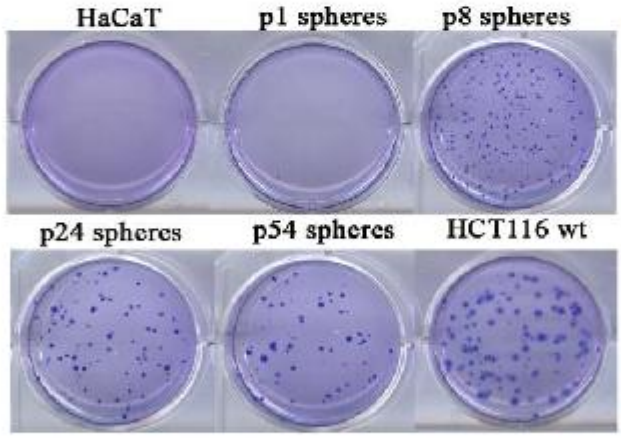


Martynova et. al Figure 3

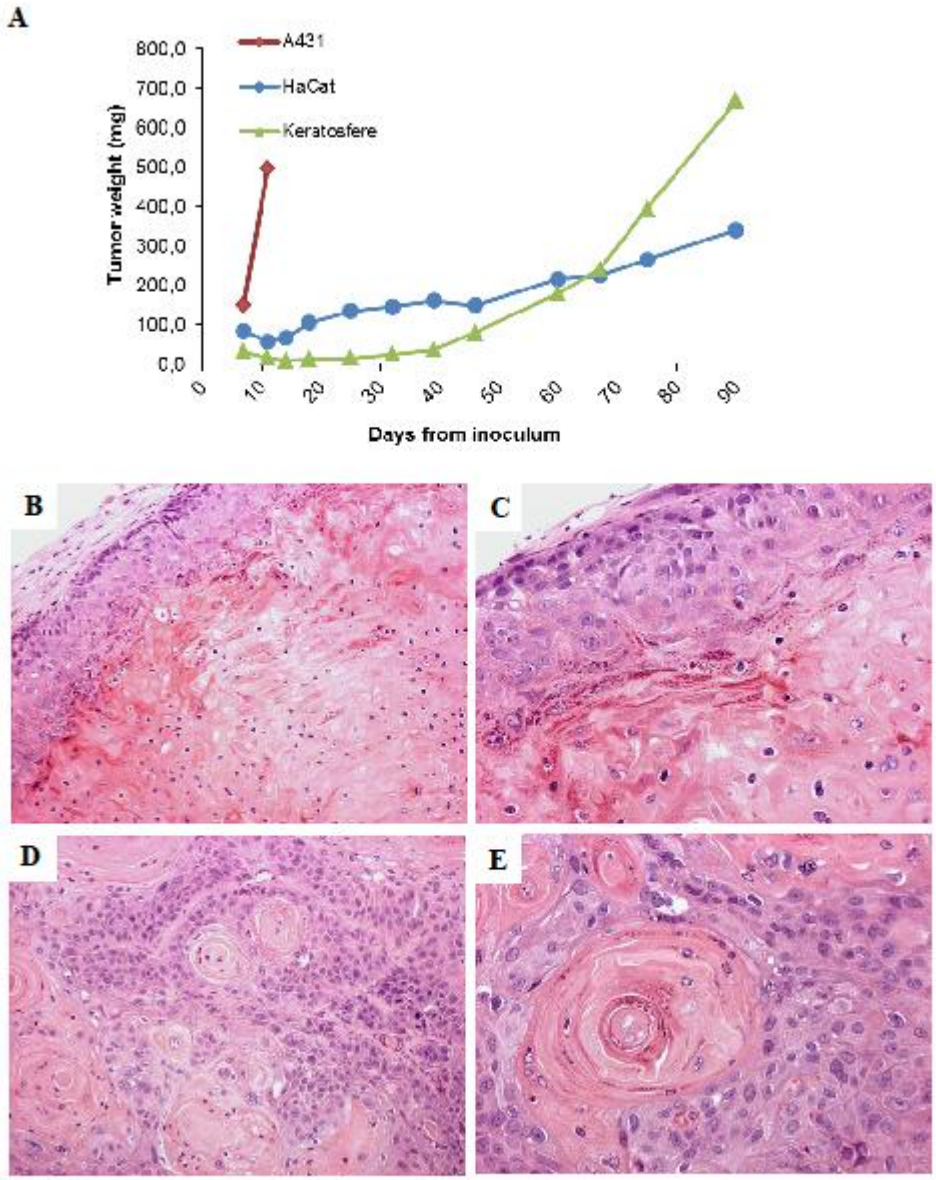
A



B

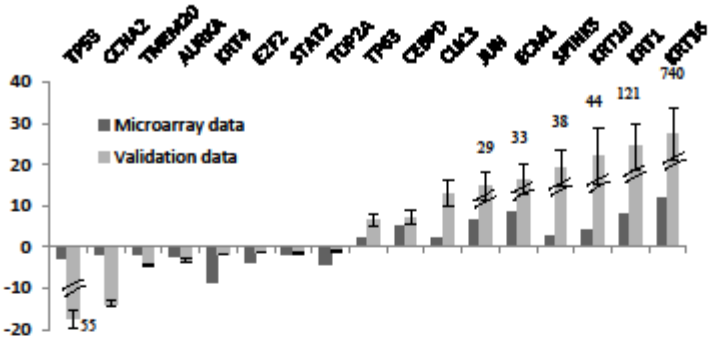


Martynova et. al Figure 4



Martynova et. al Figure 5

A



B

UP

GO.ID	GO.term	p.val
GO:0008544	epidermis development	2,62E-29
GO:0007398	ectoderm development	4,00E-29
GO:0031424	keratinization	1,52E-24
GO:0009888	tissue development	7,17E-22
GO:0009913	epidermal cell differentiation	1,25E-18
GO:0048730	epidermis morphogenesis	5,32E-17
GO:0048729	tissue morphogenesis	1,25E-15
GO:0030216	keratinocyte differentiation	7,68E-13
GO:0001533	cornified envelope	1,24E-12

DOWN

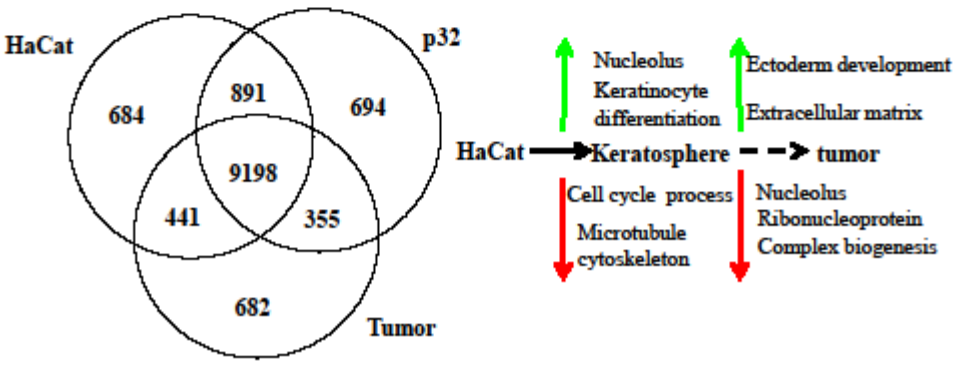
GO.ID	GO.term	p.val
GO:0000278	mitotic cell cycle	4,30E-11
GO:0019752	carboxylic acid metabolic process	1,31E-10
GO:0016614	oxidoreductase activity	1,35E-10
GO:0006082	organic acid metabolic process	1,60E-10
GO:0016616	oxidoreductase activity	2,07E-10
GO:0048037	cofactor binding	4,61E-10
GO:0051301	cell division	1,09E-08
GO:0007346	regulation of mitotic cell cycle	1,54E-08
GO:0022402	cell cycle process	2,05E-08

C

		p-value
hsa01430	Cell Communication	1,11E-11
hsa04310	Wnt signaling pathway	1,12E-06
hsa04360	Axon guidance	6,27E-06
hsa04340	Hedgehog signaling pathway	9,82E-06

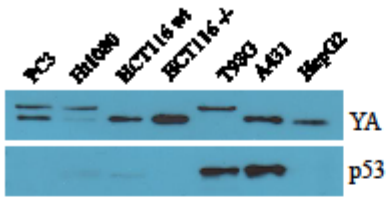
		p-value
hsa00280	Valine, leucine degradation	6,81E-11
hsa00640	Propanoate metabolism	4,69E-08
hsa00071	Fatty acid metabolism	4,62E-06
hsa04110	Cell cycle	6,28E-06

D



Martynova et. al Figure 6

NF-YA and p53 in different cell lines



	PC3	Ht1080	HCT116 wt	HCT116 p53 -/-	T98G	A431	HepG2	HaCaT	293T	MEF
YA status	? S/L	L	S	S	L	S	S	S	s	L
p53 status	null	wt	wt	null	mutp53	mutp53	wt	mutp53	wt+SV40	wt
Clones:										
Empty	-	+	+	+	-	-	-	+	work in progress	-
YA1	-	+	+	+	-	-	-	+		-
YA1M	-	+	-	+	-	-	-	-		-
YA13	-	+	-	+	-	-	-	+		-
YA13M	+	+	-	+	-	-	-	-		-

Figure 7

Stable clones overexpressing different YA isoforms

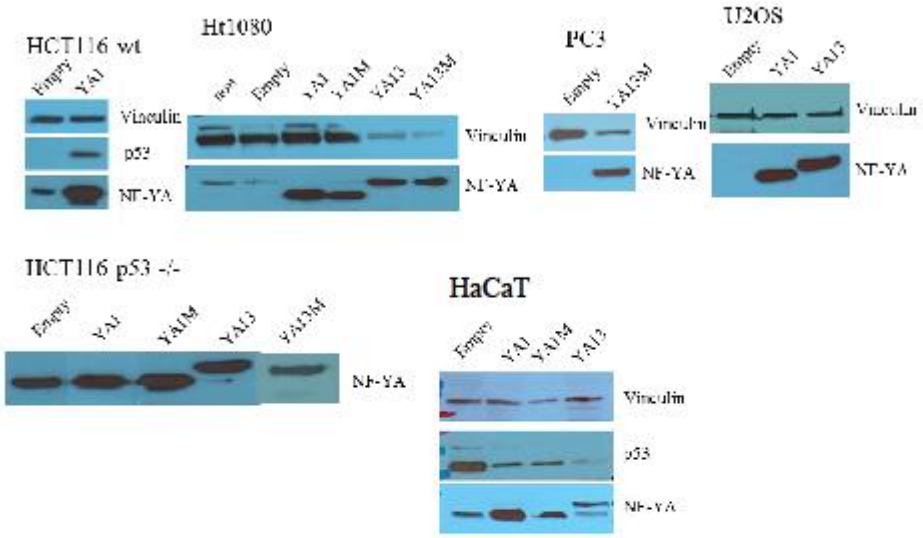


Figure 8

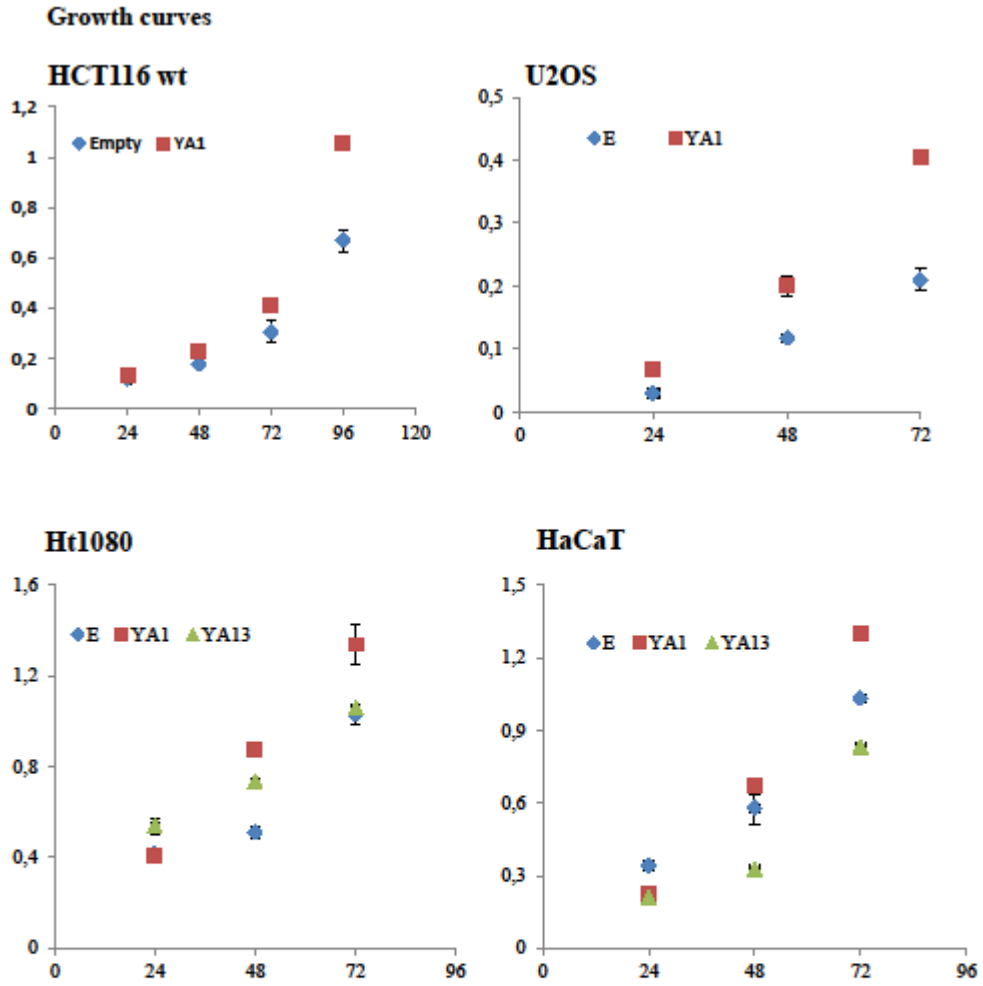

Growth curves
HCT116 wt
Empty
YA1
1,2
1
0,8
0,6
0,4
0,2
0
0
24
48
72
96
120
U2OS
E
YA1
0,5
0,4
0,3
0,2
0,1
0
0
24
48
72
Ht1080
E
YA1
YA13
1,6
1,2
0,8
0,4
0
0
24
48
72
96
HaCaT
E
YA1
YA13
1,5
1,2
0,9
0,6
0,3
0
0
24
48
72
96

Figure 9

Gene	№ of articles reporting the gene	HNSCC vs. normal mucosa	Xenograft vs. HaCaT
KRT4	18	-	←
KRT5	16	-	OD
PLAU	15	+	←
FN1	14	+	NP
MAL	14	-	←
MMP1	13	+	←
COL1A2	13	+	NP
SPARC	13	+	←
POSTN	12	+	NP
IFI6	12	+	←
TGM3	12	-	NP
SPP1	11	+	NP
ITGA6	11	+	←
KRT13	11	-	←
EMP1	11	-	NC
ECM1	11	-	OD
TNC	10	+	←
MPP10	9	+	←
MMP3	9	+	←
MMP12	9	+	OD
LAMC2	9	+	←
IL8	9	+	NP
KRT17	9	+	←
COL5A2	9	+	NP
COL4A1	9	+	←

NP - not present, NC - no change, OD - change in opposite direction, in bold -match

Figure legends

Figure 1: Development of keratospheres (A) Schematic representation of the keratospheres culture establishment: HaCaT cells form anchorage-independent spheres in defined serum-free medium supplemented with bFGF and EGF. Phase contrast photographs of the keratospheres derived from human HaCaT keratinocytes at the passages 2, 6, 32, 36; ×60 objective; (b) Comparison of growth rates for keratospheres and parental HaCaT cells by direct counting of the viable cells.

Figure 2: Phenotypic characterization of the HaCaT-derived spheres. Immunostaining of intact keratospheres analyzed by confocal laser scanning microscopy. Photographs represent positive immunostainings for p63, Klf4, α6-intergrin, CK1, CK14. DAPI staining was included to identify nuclei.

Figure 3: Keratosphere reproductive capacity. (A) Comparison of *in vitro* clonogenecity of the parental HaCaT keratinocytes and keratospheres. 1000 single cells of each cell line were plated at 100 mm tissue-culture Petri dish and cultured for 2 weeks. At the end of experiment cells were stained with Giemsa, photographed, and analyzed for their proliferation efficiency. (B) Quantitative representation of the colony-forming assay, performed in triplicate.

Figure 4: Keratosphere anchorage-independent growth. (A) HaCaT-derived keratospheres demonstrate anchorage-independent growth in the soft agar. 10 000 cells of the parental HaCaT cells, dissociated keratospheres were seeded in the 6 wells in 4 replicates and propagated in two-layer agar-agarose system for 30 days. Afterwards colonies were stained with crystal violet and photographed. Cervical carcinoma HeLa cells were added as positive control. HaCaT-MEGM represents HaCAT cells grown in soft agar experiment in keratosphere medium,

HaCaT-MEGM/MEGM – HaCaT cells, grown for 4 passages in tissue culture flasks in keratosphere medium, and put in soft agar experiment in the same medium (D) Dynamics of the tumorigenic phenotype acquisition by keratospheres: soft agar assay performed with keratospheres at the different passages (p1, p8, p24, p54) reveled that keratospheres don't acquire the ability to the anchorage-independent growth immediately but after some passages in the culture.

Figure 5: Growth behavior of the keratospheres *in vivo*. (A) Tumor growth and survival curve in mice. 10^7 of the HaCaT cells and dissociated keratospheres were injected subcutaneously into immunocompromised mice. The mice were monitored twice a week and tumor weight was calculated. As positive control A431 epidermoid carcinoma cells were added in the experiment and mice from this group in 6 days developed macroscopic SCCs and were sacrificed. HaCaT cells and keratospheres developed slow-growing neoplasms and it took 3 months to obtain macroscopic tumors. (B-E) Histological analysis of the formed neoplasms. (B) Sample HaCaT, keratinizing cyst, 100X; (C) Sample HaCaT, keratinizing cyst, close up on keratohyalin granules, 200X; (D) Sample keratosphere: squamous cell carcinoma, 100X. (E) Sample keratosphere: epithelial pearl with keratohyalin granules, 200X. The pyogranuloma observed in this sample is a common finding in keratin-producing lesions where keratin is acts as a foreign body.

Figure 6: Gene expression profiling of keratospheres. (A) Validation of the microarray HaCaT vs. tumour data by qPCR; (B) GO analysis for the up-regulated genes retrieved epidermis development, keratinization, tissue development, epidermal cell differentiation with significant p-value and cell cycle process, mitotic cell cycle, cell division for the down-regulated genes; (C) Pathway analysis of microarray data revealed cell communication, Wnt and

Hedgehog signaling pathways upregulated and fatty acid metabolism, valine, leucine degradation, cell cycle downregulated upon tumorigenic conversion of the HaCaT keratinocytes to the keratospheres; (D) Global gene expression changes in parental HaCaT cells, keratospheres and tumor samples. Microarray data analysis demonstrate step-by step gene expression changes during transition from immortalized nontumorigenic HaCaT to the transformed keratospheres (at the passage 32) and following gene expression alterations upon SCC formation in the mice from .keratospheres. On the scheme the number of the overlapping genes for each stage is represented as well as appropriate GO categorization is present.

Figure 7: p53 status and NF-YA isoforms in the different cell lines. To verify p53 status and presence of different NF-YA isoforms 30 μg of total extracts were analyzed by Western blot, using DO1 (α-p53) antibody (GeneSpin, Italy) and Mab1 (α-YA) antibody (homemade). In the Table below data about p53 status, NF-YA isoforms and results of the overexpression experiment are summarized.

Figure 8: Stable clones overexpressing different NF-YA isoforms and mutants of different isoforms. To obtain stable clones different cell lines were transduced with lentivitus, encoding NF-YA short and long isoforms and mutants of NF-YA isoforms. Cells infected with lentivirus expressing empty vector served as a negative control. 48h post-transduction, we evaluated the efficiency of the infection by green fluorescence of the GFP-infected cells by fluorescent microscopy. 72h after infection cells were subjected to the puromycine selection and 1-2 weeks later cells were pooled, harvested and overexpression of the transgene was detected by Western blot.

Figure 9: Analysis of the growth characteristics of the stable clones. Growth rates of the stable clones overexpressing NF-YA were evaluated by MTT assay.

Suppliment 1: Comparison of the microarray data (HaCaT vs. Tumour) revealed 17 genes out of 25 (68%) intersecting with the genes of the list, comparing HNSCC versus normal mucosa (Yu et al., 2008).