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Review

p63 is a suppressor of tumorigenesis and metastasis interacting with mutant p53

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p53 mutations, occurring in two-thirds of all human cancers, confer a gain of function phenotype, including the ability to form metastasis, the determining feature in the prognosis of most human cancer. This effect seems mediated at least partially by its ability to physically interact with p63, thus affecting a cell invasion pathway, and accordingly, p63 is deregulated in human cancers. In addition, p63, as an 'epithelial organizer', directly impinges on epidermal mesenchimal transition, stemness, senescence, cell death and cell cycle arrest, all determinant in cancer, and thus p63 affects chemosensitivity and chemoresistance. This demonstrates an important role for p63 in cancer development and its progression, and the aim of this review is to set this new evidence that links p63 to metastasis within the context of the long conserved other functions of p63.
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Facts

- p63 containing the transactivation domain (TAp63) and amino-deleted p63 isoforms (ΔNp63) exert distinct (often opposite) functions on stemness, cycle arrest, mobility and invasion (epithelial–mesenchymal transition, EMT) and senescence.
- TAp63 induces cell death and cell cycle arrest with tumor-suppressor features.
- ΔNp63 exerts oncogenic properties and is generally over-expressed in cancer.
- TAp63 and ΔNp63 (and their ratio) regulates chemosensitivity.
- Mutant p53 binds to, and inhibits the activity of TAp63.
- p63 (mainly the TA isoform) suppresses metastasis formation by decreasing mobility and invasion.

Open Questions

- Which isoform is responsible for each distinct function: stemness, cycle arrest, mobility and invasion (EMT), senescence?
- *In vivo* identification of the molecular targets and determinants for each function.
- Relative control, and potential therapeutic manipulation, of the relative expression of TAp63 and ΔNp63 in individual cancers. How are they related to diagnosis and prognosis?

p63 is a member of the p53 family, which also includes p73. The birth of p63 was a dystocial delivery. Although the gene was formally described in 1998,[1] it was originally isolated from rat tissues as *ket* in 1997[2] and the human sequence was reported in 1998 by different groups who variously referred to the encoded protein as p40,[3] p63,[1] p73L[4] or p51A.[5] Fortunately, this confusion of names was soon clarified by adopting the p63 classification, following a paper that provided the correct context to understand its function.[1] The identification of p63, as well as that of p73 a year earlier, was unexpected as it came after 20 years of intensive studies on p53,[6,7] and also because its physiological role is mainly developmental rather than tumor suppressive, despite its striking amino acid identity with both p53 and p73.[8] For recent reviews, see refs 9–13.

Like other members of the p53 family, the *TP63* gene is expressed as multiple isoforms with distinct properties, including a full length and an amino-deleted isoform, named TAp63 and ΔNp63, respectively[14,15] (Figure 1). TAp63 contains a transcription domain and can induce cell cycle arrest and apoptosis,[16] linking this protein to the DNA damage response function that is commonly associated with the p53 family. At the physiological level, TAp63 seems to be expressed predominantly in oocytes, although it has been detected at low levels in other tissues including the epidermis, especially following stress, and functions to protect them from toxic insults;[17,18] consequently, TAp63 has been called the 'guardian of the female germline'.[9] Conversely, ΔNp63, the shorter isoform without the N-terminal TA that is transcribed

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Keywords: p63; p53; p73; cancer; metastasis; tumor suppressor
Abbreviations: TAp63, p63 containing the transactivation domain; ΔNp63, amino-deleted p63 isoform; TA, transactivation domain; DBD, DNA-binding domain; miR, micro-RNA; EMT, epithelial–mesenchymal transition; GSK3β, glycogen synthase kinase 3β; PP2A, protein phosphatase 2A; TGFβ, transforming growth factor-β; SCC, squamous cell carcinoma; HNSCC, head and neck squamous cell carcinomas
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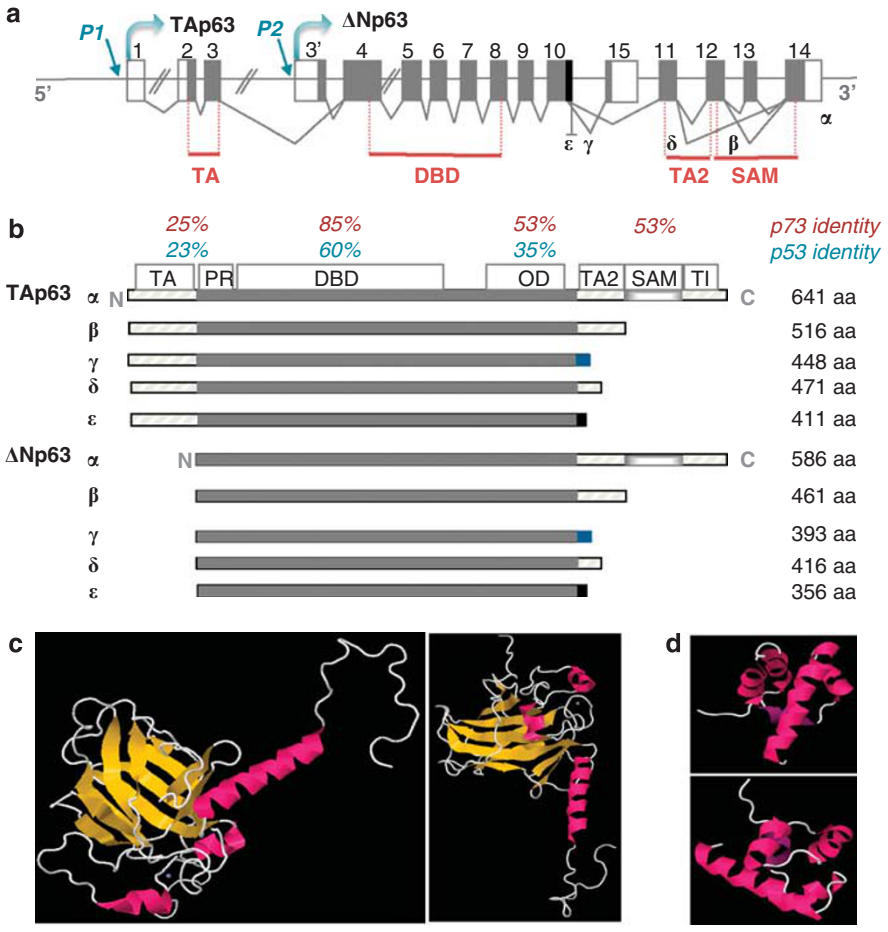

Figure 1 The p63 proteins. The TP63 gene (**a**) codifies several proteins (**b**) thanks to two distinct promoters (P1 and P2) and 3′ alternative splicing. In addition to the full length α isoform, two isoforms have been described: a β isoform (skipping exon 13) and a γ isoform (alternative exon 15, following exon 10, with its stop codon and distinct 3′-UTR. *In silico* analysis predicted the δ isoform (skipping exon 12–13) and the ε isoform (premature termination in intron 10 retaining the 5′-portion of intron 10 with a stop codon). The structure is currently available only for the DBD (MMDB ID: 67838 PDB ID: 2RMN) (**c**) and for the SAM domain (MMDB ID: 30268 PDB ID: 1RG6) (**d**). The principal domains are shown, with their identity with both p73 and p53. TA, transactivation domain (aa 1–64, residues of human TAp63); PR, proline-rich domain; DBD, DNA-binding domain (aa 142–323); OD, oligomerization domain (aa 353–397); TA2, second transactivation domain (aa 410–512); SAM, sterile alpha motif (aa 502–566); TI, transcription inhibitory domain (aa 568–641)

from a separate second promoter, is expressed primarily in the epidermis, and is involved in epithelial development.[19,20] Indeed, the full knockout of p63 is lethal owing to the absence of the epidermis,[19,20] suggesting that the prime developmental role of ΔNp63 is in the formation of the epidermis and its appendages, such as hairs and sebaceous glands.[21–23] This epithelial role by p63, and particularly the underlying molecular mechanisms, has been the subject of animated yet still partially unresolved debate throughout the last decade.

Several major issues are responsible for these controversies. First, as *TP63* is expressed from two distinct promoters, each being able to produce at least five alternative 3′ splicing isoforms, it remains unclear which isoform is responsible for each specific phenotype. Second, it is becoming clear that p63 regulates an impressive array of genes.[24,25] A recent, whole-genome tiled array analysis of ΔNp63 target genes revealed that nearly 5800 gene promoters were directly bound by endogenous p63 in human cells, of which approximately 1000 showed expression changes in response to p63 expression.[26] These target genes include, for example, 200 transcription factors, a large number of adhesion molecules, and a functionally diverse set of signaling molecules. Thus, p63 may be directly affecting nearly 7% of the coding genes in the genome, suggesting highly complex interactions with a large number of pathways. In addition, p63 regulates the expression of a number of non-coding regulatory RNAs such as micro-RNAs (miRs), as well as Dicer,[27] an enzyme essential for miR processing. The presence of so many isoforms, Figure 1, with distinct and extremely powerful transactivation properties, makes the conclusion about function and underlying mechanisms of p63 difficult to elucidate. Third, it is becoming clear that the roles of p63 in development and in adulthood, particularly in adult cancers, are quite distinct. In particular, while the major developmental role seems to be epithelial, the role of p63 in cancer only becomes evident in adulthood.

The aim of this review is therefore to set this new evidence, occasionally with my personal views, that links p63 to tumorigenesis and metastasis within the context of its long conserved other functions.

p63 and 'La Famiglia' p53

p63 is the most recently discovered but the most ancient member of the p53 family[8–15,28] (Figure 1). As indicated above, p63 is transcribed from two promoters, giving rise to proteins that may (TAp63 isoforms) or may not (ΔNp63 isoforms) contain the TA,[1] and alternative splicing at the 3′-end produces additional proteins (α–ε isoforms),[29] although there is no *in vivo* evidence for two latter isoforms. Whereas the TAp63 proteins are capable of transactivation, the ΔNp63 forms can also act in a dominant-negative fashion to counteract the transcriptional activity of the TAp63 isoforms and p53.[8,15] In addition, a comprehensive and systematic investigation of the transcriptional and growth inhibition activities of all the p63 isoforms identified a novel activation domain – TA2 – within exons 11 and 12 and a transcription repression (TI) function encoded in the C-terminus of the longest TAp63α isoform. The importance of this regulatory domain is also evident from the identification of mutations in this TAp63α-C-terminus in human patients.[30] Further investigation of the mechanism of inhibition suggested that the TI domain binds to the N-terminal TA and inhibits the transcriptional activity by masking important amino acids. The active molecule is a tetramer, like p53 and p73. The ability of different isoforms to tetramerize, not only within the p63 members, but also with p53 or p73, drastically expands the complexity of the system.[31–33] p63 protein is targeted for degradation by the E3 ligase ITCH, which belongs to the HECT family.[34] In addition to regulating p63, ITCH also targets p73, JUN, JUNB, Notch and FLIP (also known as CFLAR) for degradation.[35] This predicts not only a drastic effect of ITCH inhibitors, but predicts that ITCH may be an important therapeutic target in cancer.

In marked contrast to p53, *TP63* is not a classical tumor-suppressor gene, but is instead important in embryonic development. The phenotype of patients with a p63 mutation and that of *Trp63*$^{-/-}$ mice has revealed a key role for p63 in development, particularly of epithelia, and these phenotypes are summarized in Table 1. Mice devoid of both copies of *Trp63* are born lacking limbs as well as skin and its appendages, such as hair shafts, follicles and sebaceous glands,[19,20,36,44] see later; in zebrafish also ΔNp63 is required for epidermal development.[46] *Trp63*$^{-/-}$ animals also lack tooth primordia, mammary tissues, prostate, bladder, eyelids and a wide range of other tissues. All of these defects,

Table 1 Mouse models of Trp63

Mouse	Reference	Notes	Phenotype
Trp63$^{-/-}$	Yang *et al.*[19]	Deletion of exons 6–8	Embryonically lethal; no epidermis
Trp63$^{-/-}$ Brdm2	Mills *et al.*[20]	Insertional mutagenesis, resulting in a duplication of exons 5–10 with a stop codon[a]	embryonically lethal; no epidermis
Trp63$^{-/-}$ Brdm3	Mills *et al.*[36]	Insertional mutagenesis, resulting in a duplication of exons 5–7 with a stop codon	Embryonically lethal; no epidermis
Conditional Cre/lox TrTAp63$^{-/-}$	Su *et al.*[27,37]	Conditional cre-lox deletion of TAp63	Normal limb development, decreased hair morphogenesis, ulcerated wounds, decreased wound healing, deregulated stemness and accelerated aging; prone and develop metastatic tumors
Conditional Cre/lox TrTAp63$^{-/-}$	Suh *et al.*[17]	Conditional cre-lox deletion of TAp63	No defects described so far
Conditional Cre/lox TrTAp63$^{-/-}$	Keyes *et al.*[38]	Conditional cre-lox deletion of TAp63	TAp63 essential for Ras-induced senescence; TAp63$^{-/-}$ increases proliferation/oncogenesis
Trp63$^{+/-}$	Flores *et al.*[39]	Trp63$^{-/-}$; 4 backcross into C57BL/6	Higher tumor burden and metastasis compared with p53$^{+/-}$ mice
TrΔNp63$^{-/-}$ knock-down	Koster *et al.*[40]	RU486-inducible transgene expression of inverted exon 3 resulting in dsRNA transcript processed into ΔNp63-specific siRNAs	Skin fragility and erosion with suprabasal defects
K5::ΔNp63α	Candi *et al.*[22]	Transgenic mice under K5 promoter	No major phenotype
Trp63$^{-/-}$::K5::ΔNp63α	Candi *et al.*[22]	K5 transgenic mice genetically complemented in Trp63$^{-/-}$	Embryonically lethal; partial attempt to reconstitute epidermis
K5::TAp63α	Candi *et al.*[22]	Transgenic mice under K5 promoter	No major phenotype
Trp63$^{-/-}$::K5::TAp63α	Candi *et al.*[22]	K5 transgenic mice genetically complemented in Trp63$^{-/-}$	Embryonically lethal; no epidermis
RU486-lung-inducible ΔNp63α	Koster *et al.*[41]	Transgenic mice	No major phenotype
RU486-lung-inducible TAp63α	Koster *et al.*[41]	Transgenic mice	squamous metaplastic lung lesions, K5/K14 expression, with hyperproliferation; epidermal expression, accelerated chemical-induced tumor development and progression, resulting in EMT to spindle cell carcinomas and lung metastases
ΔNp63α and ΔNp63β	Romano *et al.*[42]	Tetracycline-inducible, reverse tetracycline-activator (rtTA) transgenic mice from a tissue-specific promoter	Lung epithelium exhibit squamous metaplasia
Loricrin::ΔNp63α	Liefer *et al.*[43]	ΔNp63-specific transgenic under the loricrin promoter	Normal epidermal development; UV-B challenge exhibit 45% less apoptosis
Trp63R279H	Lo Iacono *et al.*[44]	Knockin for the R279H mutation found in ectodermal dysplasia	Cranofacial and limb defects, ectodermal dysplasia

[a]Owing to used KO methodology these mice are prone to sporadic reversion events in which the wild-type p63 allele is re-created through spontaneous homologous recombination, resulting in patchy normal epidermal differentiation[45]

described in several mouse models (Table 1), have been observed in ectodermal dysplasia patients carrying heterozygous (dominant) p63 mutations.[30] Most commonly, human p63 mutations (basically localized in every domain, but particularly in the DNA-binding domain (DBD), similar to the distribution of mutations described in p53) found in ectodermal dysplasia syndrome patients give rise either to amino acid substitutions in the DBD that abolish p63 DNA-binding ability, or to shifts of the reading frame, which specifically truncate the TAp63α isoform.[13,30] However, recent data have shown that p63 also has an important role in the development and progression of cancers.

p63 Expression in Cancer

p63 has added a new dimension to the role of the p53 family in cancer biology owing to its effects in the DNA damage response and in the regulation of cell cycle arrest,[1,47,48] apoptosis,[16,47,49] stemness[50] and tumorigenesis.[39,51–55] p63 has been detected in several cancers, including pre-cancerous conditions of the oral mucosa,[56] and cancers of the prostate,[57–59] bladder,[60–63] thyroid,[64] lung,[65–70] cervix[71,72] and breast,[73–76] see Table 2. However, the detailed mechanisms of its role in tumorigenesis remain partially obscure at the molecular level and particularly the relative contribution of the distinct pathways (functions) exerted by p63.[81]

Mutations of p63 are extremely rare in human cancers, indicating it is not a canonical tumor suppressor. Most tumors ($>$80% of primary head and neck squamous cell carcinomas (HNSCCs), as well as other squamous cell epithelial malignancies and non-small cell lung cancer) retain p63 expression, where it is often overexpressed and occasionally amplified.[78,80,82,83] (Table 2) Frequently, tumors have simultaneous transcriptional upregulation of both *TAp63* and Δ*Np63* isoforms, with ΔNp63 being predominant at the protein level.[78,80,82] This would be in accordance with the anti-apoptotic and proliferative (oncogenic) effects of the ΔNp63 isoform described above. Moreover, ΔNp63α expression directly correlates with a poor clinical response to cisplatin in HNSCC.[79] DNA damage by chemotherapy in HNSCC causes a decrease in ΔNp63-mediated transcriptional repression by blocking responsive elements sites to TAp63 or sequestering TAp63 in less active hetero-tetramers, together with increased expression of p73, and allows TAp73-mediated cell death.[84] Together, these data suggest that it is not necessarily the levels of individual p53 family members, but rather the ratio between TA (transcriptionally highly active; showing tumor-suppressor functions) and ΔN (transcriptionally less active, and acting as dominant negative over the TA isoforms; showing oncogenic properties) isoforms that determines the biological outcome. Clearly, however, although this may be true for HNSCC and other squamous cell epithelial tumors, this cannot be the whole story in cancers in general as malignant lymphomas seem to preferentially express TAp63.[77,85]

Do Trp63 genetically modified mice develop cancer? The analysis of *Trp63*$^{-/-}$ mice has led to conflicting results with regard to its role in tumorigenesis. Although some *Trp63*$^{+/-}$ mice are cancer-prone,[51] this appears to depend on other factors like for example the genetic background, as *Trp63*$^{+/-}$ mice of a different inbred strain show premature aging but no cancer,[86] even when p53 is compromised.[87] A note of caution is required when using Brdm2 mice,[86] because exon skipping could occasionally result in wanton expression of the p63γ isoform, resulting in a partial knockout.[45] Although these conflicting results appear difficult to reconcile, the ratio between the different p63 isoforms (and also other p53 family members) may in theory cause strain-specific differences. In this respect, p63 and p73 are required for full p53 transactivation of apoptotic transcriptional targets such as Bax but not for cell cycle-related targets such as p21, thus suggesting that the interplay between members of the family may have a big impact on tumorigenesis.[39,49] The strain-specific differences also suggests that genes outside the p53 family may also influence the behavior of p53 family members, adding further complexity to the story. Clearly, however, more work is needed to understand how the different genetic background could influence tumorigenesis.

p63 is a Suppressor of Metastasis

More aggressive, metastatic tumors lose p63 expression, suggesting that p63 loss accelerates tumorigenesis and metastatic spread[61,88] (Figure 2). Correspondingly, disruption of p63 in squamous cell lines results in upregulation of genes associated with increased invasiveness and metastasis in tumors,[62] see also below. This suggests that p63 is a marker of non-invasive epithelial tumors,[72] such as ductal carcinoma *in situ* of the breast or prostatic intraepithelial neoplasia. Indeed, sclerosing adenosis or small foci of dense fibrosis with distortion of the normal acinar architecture, remain p63 positive. This highlights the potential value of p63 as a differential diagnostic marker of tumors with more benign properties.

The fact that a majority of cancers harbor p53 mutations does not necessarily mean that p53 is absent or inactive because p53 mutants may gain new properties not present in the wild-type protein.[92–94] Recently, a novel mechanism has been reported whereby mutant p53 (e.g., 175H; 273H), independently of its function in the response to DNA damage, physically interacts with p63, resulting in the loss of the anti-metastatic properties of p63[89,90] (Figure 2). Distinct mechanisms of mutant p53 (175H; 273H)–p63 interaction have been described. First, transforming growth factor-β (TGFβ)-dependent expression of SMAD2 creates a platform for p53R175H – phosphorylated by NRAS – to interact with p63, creating a ternary complex (of SMAD2-p53R174H-p63).[90] This results in reduced expression of two downstream metastasis-suppressor genes, *BHLHE41* (which encodes SHARP1) and cyclin G2 (*CCNG2*), and patients with low expression of these two genes are at higher risk of metastasis and show reduced survival.[90]

In the second mechanism, in H1299 human cells lacking TGFβ receptors, a direct interaction between mutant p53 (175H; 273H) and p63 seems to occur, resulting in inhibition of p63-mediated regulation of integrin $\alpha5\beta1$ and epidermal growth factor receptor endocytosis cycling.[12,89] It is unclear whether this effect is common to all p53 mutants or confined to a particular subset, but this would be important information to refine the clinical predictive value of p53 genotyping.

Table 2 Major p63 expression and gene changes in cancer

Cancer	Observation	Notes	Reference
Pre-cancerous conditions	p63+ in dysplastic oral mucosa, with loss of E-cadherin	53 Pt; Ab	Das *et al.*[56]
Many cancers	p63 expressed in thymomas, non-Hodgkin's lymphoma, basal cell/squamous cell carcinoma, not in adenocarcinomas of breast/prostate	583 Pt; Ab	Di Como *et al.*[55]
Many cancers	ΔNp63 is the most expressed isoform	26 Pt; Ab	Nylander *et al.*[77]
Bladder cancer	Low p63 associates with higher TNM and low β-catenin; p63 prognostic effect is independent of TNM	75 Pt; Ab	Barbieri *et al.*[62]
Bladder carcinoma	ΔNp63 correlates with high MW cytokeratins in basal cancer, more aggressive with poor prognosis	202 Pt; Ab	Karni-Schmidt *et al.*[63]
Breast cancer	p63+ prognostic factor in ER+ Pt; no correlation with standard parameters	2158 Pt; microarray	Hanker *et al.*[73]
Breast cancer	p63 improves identification of myoepithelial cells	101 Pt; Ab	Aikawa *et al.*[75]
Breast cancer	D2-40+ myoepithelial expression with p63+ defines lymphovascular invasion in high risk Pt	240 Pt;	Gudlaugsson *et al.*[76]
Bladder cancer	Loss of ΔNp63 associate with progression/invasion	160 Pt; Ab (PCR)	Urist *et al.*[61]
Bladder cancer	ΔNp63 associate with invasiveness and upregulation of N-cadherin	7 Pt;	Fukushima *et al.*[60]
Breast cancer	MFG-E8 (ligand of integrin $\alpha v\beta 3$-5) is a p63 target *in vivo*	36 Pt; 10 erbB2+ Pt; PCR	Yang *et al.*[74]
Cervical carcinoma	p63 marker of squamous differentiation; HPV-p63 association	250 Pt; Ab;	Wang *et al.*[72]
Cervical carcinoma	p63 preferentially expressed in immature squamous cells; useful for differential diagnosis in early stages	79 Pt; Ab	Quade *et al.*[71]
Head and neck cancer	All head and neck squamous cell carcinoma p63+; ΔNp63α predominant protein	36 Pt; Ab; PCR	Sniezek *et al.*[78]
Head and neck cancer	ΔNp63α predominant protein and reflects platinum response and favorable outcome	54 Pt; Ab	Zangen *et al.*[79]
Lung squamous cell carcinoma	Increased copy number in 88% SCC, 42% LCC, 11% AC; ΔNp63α predominant protein, associated with better prognosis	217 Pt; Ab; PCR	Massion *et al.*[80]
Lung squamous cell carcinoma	NSCLC differential diagnosis: p63+ in 100% SCC, 10% AC); p63 & cytokeratin5/6 allow accurate classification 77% cases	39 Pt (20 AC, 15 SCC, 4 LCC); Ab	Mukhopadhyay and Katzenstein[66]
Lung squamous cell carcinoma	Correlation podoplanin/CD44/ p63 in a hierarchical manner	162 Pt; Ab	Shimada *et al.*[65]
Lung squamous cell carcinoma	p63 distinguish AC, (−ve) from SCC, (+ve)	425 Pt (200 AC, 225 SCC); microarray	Terry *et al.*[67]
Lung squamous cell carcinoma	Cytokeratin5/7, TTF1, p63 allow accurate classification of all NSCLC cases	103 Pt; Ab	Righi *et al.*[74]
Lung squamous cell carcinoma	ΔNp63 is the isoform that distinguish AC from SCC	17 Pt; Ab	Uramoto *et al.*[68]
Lung squamous cell carcinoma	Higher ΔNp63/TAp63 ratio in NSCLC indicating poor outcome	46 Pt (26 AC, 17 SCC, 3LCC); Ab; PCR	Iacono *et al.*[70]
Lymphoma	TAp63 is the most expressed isoform	45 Pt; Ab; PCR	Iacono *et al.*[70]
Prostatic cancer	p63 marker of early neoplastic lesion (intraepithelial neoplasia *versus* adenocarcinoma)	90 Pt; Ab	Hull *et al.*[57]
Prostate cancer	ΔNp63α more abundant than TAp63; 1 mutation exon 8;	75 Pt; PCR; Ab	Kellogg Parsons *et al.*[59]
Prostate cancer	p63 correlates with proliferation and non-invasion; increased cytoplasmin p63 associates with mortality	298 Pt; Ab	Dhillon *et al.*[58]
Thyroid carcinoma	p63+ in 41% papillary, 29% follicular carcinoma, 67% follicular adenoma	39 Pt; Ab	Tan *et al.*[64]

Abbreviations: Ab, antibody immunostaining; AC, adeno carcinoma; HPV, human papilloma virus; LCC, large cell carcinoma; microarray, mRNA macroarray analysis; NSCLC, non-small cell lung carcinoma; PCR, PCR mRNA detection; Pt, patients cases; SCC, squamous cell carcinoma; TNM, tumor node metastasis classification; + or +ve, positive; − or −ve, negative. Please note that the specificity of the antibody, and the primers used for the PCR, often do not allow a proper discrimination of the p63 isoforms. I deeply apologize, for severe space constraints, for the large number of studies not reported in this indicative table

The molecular interaction between mutant p53 and p63 involves both the formation of hetero-aggregates between the two proteins, and the tetramerization via the oligomerization domain domains to form large aggregates.[95] This suggests an increased propensity of the conformationally mis-structured mutant p53 to aggregate both as homotetramers (p53) and heterotetramers (p63), resulting in a gain of function, Figure 3.

A further way by which TAp63 may influence metastasis is by the direct transactivation of *DICER1* and *miR-130B*, two genes that also inhibit metastasis.[27] Whether this is also inhibited by mutant p53 is currently unknown, but would be expected because mutant p53 seems to repress p63 transcription[89,90] and also DICER1/miR-130B are dependent on p63 transcription to affect metastasis.[27] *DICER1* expression is inhibited by miR-103 and miR-107, thus also conferring migratory properties and empowering metastatic dissemination of cancer cells by inducing an EMT,[91] a signature that is important in metastasis formation,[96] see also the section below on 'Epithelial Organizer'.

Which of these mechanisms is pivotal as a suppressor of metastasis? And how does this reconcile with the notion that TAp63 acts as a tumor suppressor while ΔNp63 as an

oncogene? Possibly these latter effects are more important in tumorigenesis and expansion of the primary, while a migratory effect (mediated by any of the above mechanisms) is involved in invasion and metastasis. These new data,[27,89,90] while revealing a crucial role for p63 as a metastasis suppressor involving an adhesion program, also emphasizes that it is the expression and interaction of the different isoforms of all p53 family members (p53, mutant p53, TAp63, ΔNp63) that contributes to the tumor phenotype. It also suggests new factors that may be used both as predictors of tumor behavior and as novel therapeutic targets.

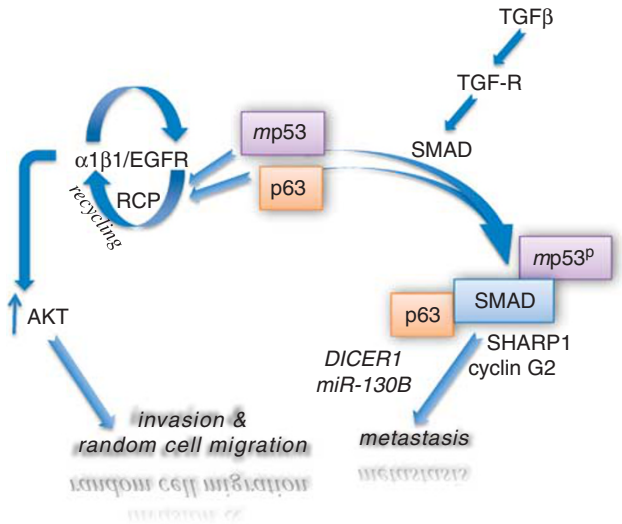

Figure 2 Mutant p53 and p63 as a suppressor of metastasis. Two models showing how mutant p53 gains a novel function through its ability to interact with p63, thus becoming able to affect cell invasion and metastasis. According to REF[89] (left arm) mutant p53 interacts with p63 and regulates integrin recycling in a Rab-coupling protein (RCP)-dependent manner. This increases the intracellular steady-state levels of AKT and thus affects random cell mobility and invasion. According to REF[90] (right arm) transforming growth factor-β (TGFβ) binds its receptor (TGFR), allowing the increase in SMAD2, which is able to bind mutant p53 (mp53) that has been phosphorylated in a NRAS-dependent manner (not shown in the figure). A tripartite complex is then formed, with SMAD2 bridging mutant p53 to p63. This results in the regulation of metastasis through SHARP1 (also known as BHLHE41) and cyclin G2[90] through integrin endocytosis recycling[89] or through *DICER1* and *miR-130B*[27,91]

Clinical Implications

The involvement of p63 in tumorigenesis and metastasis has evident clinical implications.

Response to chemotherapy. The expression of p63 is able to strongly influence the response to chemotherapy.[16,79,97] Indeed, at least *in vitro*, TAp63 directly transactivates the CD95 gene via the p53-binding site in the first intron resulting in upregulation of a functional CD95 death receptor; the same occurs for TNF-R and TRAIL-R death receptor systems.[16] Additionally, TAp63 activates the mitochondrial apoptosis pathway by direct transactivation of proapoptotic Bcl-2 family members like Bax and BCL2L11 as well as RAD9, DAP3 and APAF1.[16] Of clinical relevance is the fact that TAp63 is induced by many chemotherapeutic agents and that inhibiting TAp63 function leads to chemoresistance, at least *in vitro*.[98–100] ΔNp63α is, indeed, able to confer resistance to cisplatin via a transcriptional regulation of AKT1.[101] This is not the sole mechanism, because phosphorylated ΔNp63α can downregulate expression of several miRs, including miR-181a, miR-519a, miR-630 and induce expression of miR-374a, which in turn are targeting several proteins (TP53-S46, HIPK2, ATM, CDKN1A and 1B,

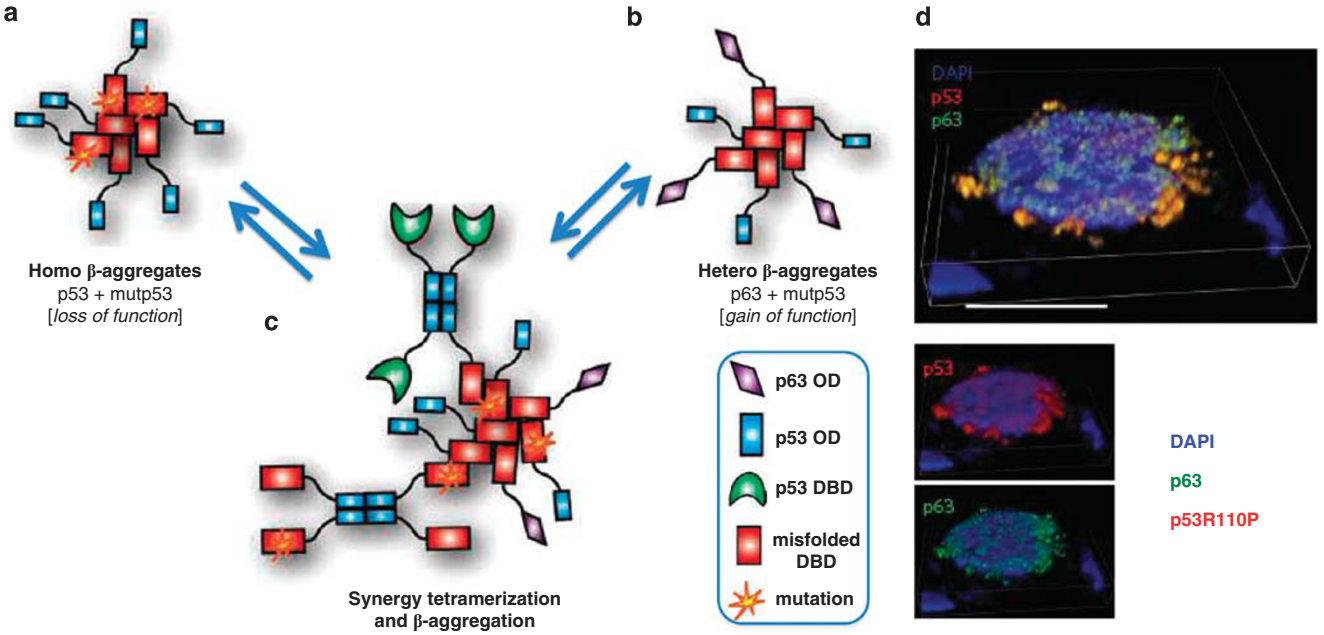

Figure 3 Interactions and aggregations within the p53 family members. Conformationally destabilized mutant p53 forms aggregate with wild type p53 (**a**), acting as a dominant-negative (in addition to competing for the same promoter-binding sites), or with p63 (**b**), causing a gain-of-function effect because of altered p63 transcriptional activity. Interactions through the OD (**c**), recruits additional p63 in the aggregates, augmenting the effect of mutant p53. (**d**) Colocalization of p63 (green) with mutant p53 (red) in the nucleus (blue). DBD, DNA-binding domain; OD, oligomerization domain. Modified with permission from Xu *et al.*[95]

CASP3, PARP1 and 2, DDIT1 and 4, BCL2 and BCL2L2, TP73, YES1 and YAP1) for downregulation, and hence conferring resistance.[102] Therefore, p63 status could be a predictor of chemosensitivity and chemoresistance.

Protecting fertility following chemotherapy. It is, of course, of paramount importance that cancer treatments do not harm normal cells, and in particular the germ cells. This is particularly relevant when administering chemotherapy for pediatric cancers and in women of child-bearing age. Indeed, the activation of the death machinery by chemotherapy and radiotherapy elicits DNA damage in the germinal cells such as in the oocytes, and may result in reproductive failure. The death of germinal cells is mediated by TAp63 and not by p53.[17] Recently, this has been shown to require the kinase activity of ABL acting on specific residues of the TAp63 protein.[18] Consequently, the ABL-specific inhibitor imatinib impairs TAp63 activation and therefore spares oocytes and their function during chemotherapy (Figure 4). Conversely, because several cancers specifically overexpress ΔNp63, in these cases imatinib inhibits ΔNp63[103] and ΔNp73 anti-apoptotic function,[104] thus improving the therapeutic index. The concept of using imatinib as adjuvant chemotherapy to spare oocyte function is quite attractive, although these data require confirmation and a better understanding of the particular DNA damage repair systems in oocytes *versus* the surrounding tissue. The retention of damaged oocytes, with their potential for producing fetal abnormalities, may be less desirable than allowing them to die.

After birth, the primordial oocyte pool lies in a quiescent state for many years until a single oocyte becomes activated, producing a monthly mature oocyte ready for fertilization. During this lengthy quiescent period TAp63, specifically expressed in oocytes, guards and protects the oocyte to allow their proper function. If indeed p63 is crucial for the fertility of the egg,[17,18] preventing the proliferation of non-fertilized eggs, I could also hypothesize that TAp63 may be involved in the aborted 'virgin birth' (that is, the development of a non-fertilized egg) that results in teratomas and teratocarcinomas.

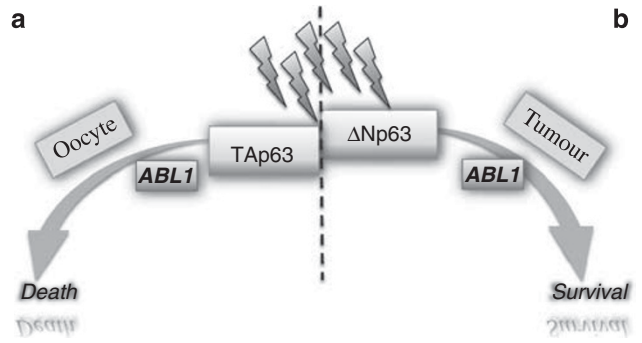

Figure 4 Phosphorylation of p63 regulates death and survival in cancer cells and in oocytes. The TAp63 isoform seems to be specific for oocytes[17] (**a**) while ΔNp63 is highly expressed in cancer cells[103] (**b**). Both isoforms require phosphorylation on specific residues by the ABL kinase to become activated,[18,103] and, following DNA damage, induce death of oocytes and survival of cancer cells. Consequently, ABL inhibition could affect the survival of oocytes (left), while disrupting a relevant survival mechanism in cancer cells (right)

Possible Molecular Mechanisms Involving p63 Function in Cancer

Several mechanisms have been proposed for p63 functions that may have a crucial role in cancer (Figures 5 and 6). However, we have to remember that, since much of the p63 research has been performed in epithelial models, there could be similarities, but also differences when applied in a tumor context.

What is the function of p63 in the epidermis? See Figure 7. The first controversy surrounds which isoform determines the lack of skin development in *Trp63*$^{-/-}$ mice: TAp63[40,41] *versus* ΔNp63.[17] However, work by Thesleff,[21] Melino[22] and Khavari[23] has clearly shown that ΔNp63 is the major (99% of total Trp63 mRNA, as shown by *in situ* hybridization and qRT-PCR) isoform involved in epidermal development. This has also been shown using an embryonic stem cell model *in vitro*.[109] A contribution by TAp63 remains possible, as the message is indeed expressed during various stages of development as well as in adulthood, and *TAp63*$^{-/-}$ mice have a detectable epithelial phenotype with blisters and epidermal ulcers.[37] Moreover, in a different animal model, TAp63 contributes to the maintenance of dermal and epidermal precursors, genomic stability, and organismal longevity;[28,37] mice age prematurely and develop blisters, skin ulcerations, senescence of hair follicle-associated dermal and epidermal cells, and decreased hair morphogenesis.[37] A further controversy has emerged, assigning either a stem cell regulatory role or a differentiation role to p63. Work by McKeon[50] shows that ΔNp63 is required for the maintenance of 'stemness' of stem cells within all stratified epithelia, which includes skin, breast and prostate, among others, and that the phenotype (absence of stratified epithelial) is the consequence of tissue failure owing to depletion of the stem cell compartment. Before its role in adult stem cell regulation, ΔNp63 is essential for the initiation of epithelial commitment and the stratification cycle of the embryonic ectoderm.[110] At this embryonic stage, ΔNp63 switches on an array of genes required for epidermal morphogenesis while repressing those involved in mesodermal lineage commitment.[110]

What is the function of p63 in cancer? In addition to the roles indicated for the epidermis, different hypotheses have been proposed (Figures 5 and 6), and particularly the idea that ΔNp63 maintains not only the normal epithelial stem cell population, but may also be involved in the persistence of cancer stem cells, especially in epithelial tumors.

Here, I will briefly review the different possibilities.

Cell death. TAp63 can transactivate genes involved in different steps of the apoptotic program.[16] TAp63γ accumulates following DNA damage and induces apoptosis via Bax, and cell cycle arrest via p21WAF/CEP1.[43] Interaction of the induced proteins with other p63 isoforms (e.g., ΔNp63) or other members of the p53 family modulates this pro-apoptotic activity,[16,43] a function shared to different extents by the other TAp63 isoforms and by ΔNp63.[47] ΔNp63 does not have pro-apoptotic activity either in the epidermis or elsewhere, but can inhibit the transcriptional activity of TAp63 by competition for the same responsive elements or by sequestration, forming inactive (or differently active)

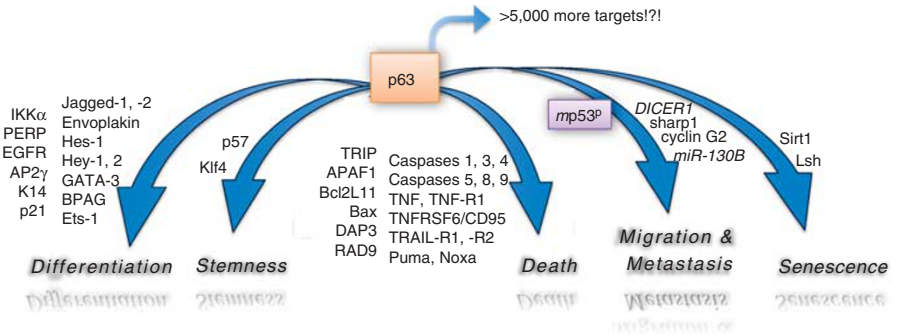

Figure 5 Cancer pathways involving p63. p63 can potentially transactivate several thousand target genes, regulating several biochemical pathways that are relevant to tumorigenesis. Although p63 can transactivate the same targets as p53, indicating a high similarity in the interaction with the responsive element, p63 has indeed distinct targets; as all p63 isoforms share the same DNA-binding domain, there is no significant qualitative difference in the recognition of the responsive element but the composition of the transcription complex seems to be distinct, resulting in different biological effects. First, TAp63 is able to regulate cell death[16] through several distinct mechanisms, depending on the stressor and the tissue involved (*death*). Second, ΔNp63 controls the formation of the epidermis,[22,105] and thus it is implicated in related tumors, acting on specific promoters, some of which are illustrated schematically (*differentiation*). However, a crucial role in cancer seems to be on the proliferation potential of the stem cell compartment,[50] although in this case, the underlying molecular targets are still not fully clear (*stemness*). This activity strongly suggests a corresponding role in cancer stem cells, though this has yet to be formally proven. Furthermore, p63 regulates the senescence pathway both in normal and in cancer cells,[38,106] (*senescence*), via distinct yet unclear targets. Finally, p63, neutralized in cancer cells by binding to mutant p53, regulates an adhesion and epithelial–mesenchymal transition pathway that is crucial for migration, invasion and metastasis[27,89,90] (*migration and metastasis*). The main transcriptional targets directly regulated by p63 are indicated in their current nomenclature

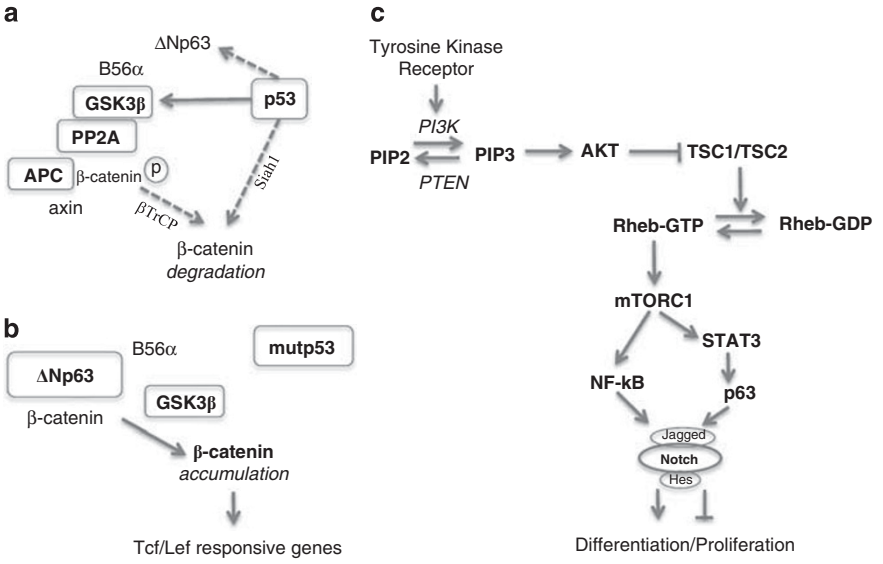

Figure 6 p63 signaling in cancer cells. Opposing effects of ΔNp63 and β-catenin in cancer cells.[107] In normal cells (**a**), p53 facilitates the degradation of ΔNp63 and seems to activate GSK3β, causing degradation of β-catenin. In squamous cell lung cancer (**b**), mutant p53 fails to downregulate ΔNp63, which binds B56α, inhibiting GSKβ and altering the β-catenin APC-binding complex, with accumulation of non-phosphorylated β-catenin.[107] (**c**) STAT3/p63 regulate Notch signaling downstream of TORC1 to affect the molecular switch of differentiation *versus* proliferation.[108] NF-kB counteracts this pathway

hetero-tetramers. As a result of the possibility of multiple signaling interactions between p63 isoforms (not only TAp63 *versus* ΔNp63, but also between the C-terminal isoforms α, β, γ, see above) and between family members (e.g., TAp73, ΔNp73, p53 and Δ133p53), it is likely that the distribution of the expression of different p53 family members and their respective isoforms could contribute to the physiological function of p63 as well as to stress responses, tumorigenesis and chemoresistance. This multifaceted scenario may help to explain why p53 status is not a universal predictor of response to chemotherapy, as TAp63α modulates chemosensitivity, for example, of hepatoma cells.[16,97] TAp63α activates both death receptor- and mitochondria-mediated apoptosis pathways, and both mechanisms are clearly reinforced by concomitant treatment with chemotherapeutic drugs. Of clinical importance, endogenous TAp63α is induced by a variety of chemotherapeutic agents, and blocking TAp63α function leads to enhanced chemoresistance, as discussed above. These data are consistent with p63 participating in p53-mediated DNA damage responses via the regulation of cell death.

DNA damage responses and cell cycle regulation. TAp63, and to some extent also ΔNp63, has a role in the responses to DNA damage and regulation of the cell cycle.[23] TAp63 can directly regulate the expression of

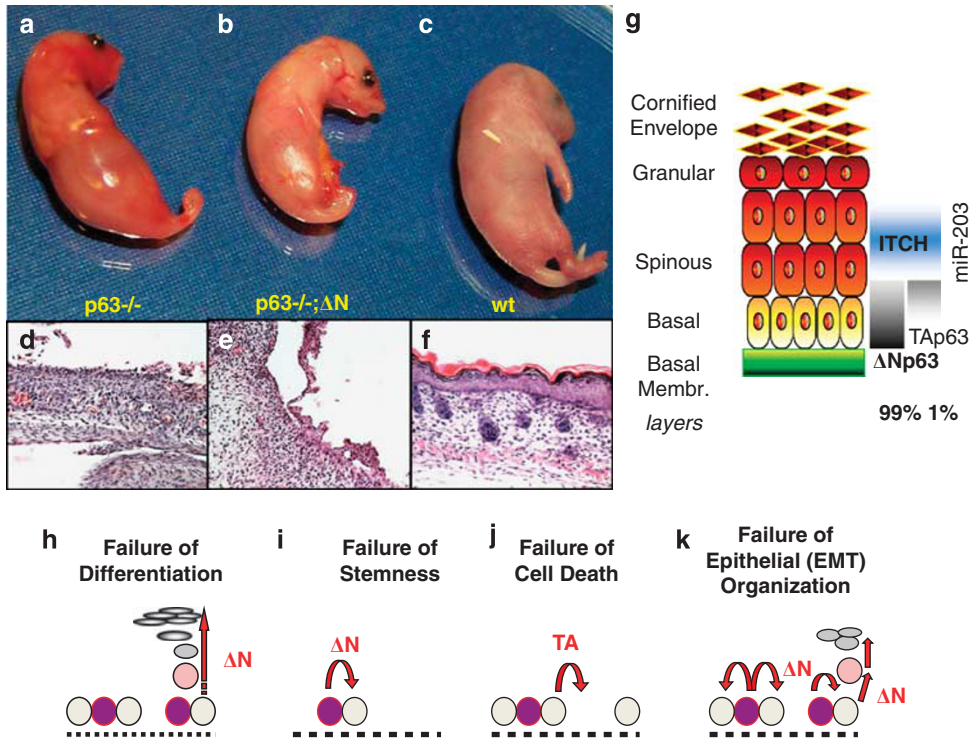

Figure 7 Potential functions for p63 in the epidermis. *Trp63*$^{-/-}$ mice have no skin or limbs (**a**) as compared with wt (**c**), because of the total absence of epidermis (**d** *versus* **f**). This is mainly due to the ΔNp63 isoform, as seen from genetic complementation studies (**b** and **e**). In this study,[22] for example, the p63-null mice were crossed with a transgenic mouse overexpressing TAp63 or ΔNp63 under the control of the promoter of keratin 5, in the basal layer; complementation with ΔNp63 (**b** and **e**), but not with TAp63, partially rescued the phenotype indicating that ΔNp63 is the isoform predominantly responsible for the absence of epidermis in the full knockout mice (**a** and **d**).[22] p63 is expressed in the basal layer of the epidermis (**g**) as the ΔNp63 isoform (99% by PCR in adult mice), and its localization is restricted to the basal layer by the action of the E3 ubiquitin ligase ITCH as well as by miR-203. Modified with permission from.[22] The major role of ΔNp63 during development is, therefore, in the formation of the epidermis through a mechanism not fully clarified at the molecular level. This is important as it could also highlight the potential role of p63 in cancer (Figures 5 and 6). Distinct hypotheses were initially formulated, assigning a crucial role for ΔNp63 in '*differentiation*' (**h**), for example, transactivating K14, IKKα, of pluri-stratified epithelia.[41] Recent data unveiled the role of ΔNp63 in regulating the proliferation potential of the '*stem cell*' (**i**) compartment, although the underlying molecular mechanisms have not been fully clarified yet.[50] In addition to being essential in development, this pathway could also be important in cancer. On the other hand, TAp63 seems to be mainly involved in '*cell death*' (**j**), for example, transactivating Puma, Noxa, CD95, Bax.[16] This, clearly, could be relevant in adult tumorigenesis. TAp63-related cell death seemed to be mainly involved in defense from exogenous stresses (sun burn cells following UV irradiation), while ΔNp63 seemed to affect the physiological differentiation pathway. Finally, very recent data suggest the existence of a further additional pathway, induced by ΔNp63, able to regulate adhesion and EMT as '*epithelial organizer*' (**k**), see main text

cell cycle-related genes such as p21,[111,112] as well as apoptotic genes as mentioned above. TAp63γ is the most active form at transcriptional level and accumulates in a phosphorylated form during DNA damage (by doxorubicin) with a drastically prolonged half-life.[113] This implies a role for TAp63 in DNA damage responses, as for example shown in the ovary, see also the section 'Protecting fertility following chemotherapy'.

Differentiation. Overall the hypothesis of p63 regulating differentiation is supported by solid data, with several transcriptional targets of ΔNp63 being clearly relevant for epidermal differentiation,[110] such as keratins 14 (*KRT14*),[22,114] 5 and 7 (*KRT5, KRT7*)[42] or adhesion molecules,[105] indicating a primary role in the formation of the epidermis. However, islets of fully differentiated cells are present in the *Trp63*$^{-/-}$ mice,[19] indicating that the absence, or reduced, proliferative potential of the stem cell compartment is responsible for the absence of the epidermis in these knockout mice and the few remaining cells are able to differentiate normally. Whether other isoforms such as TAp63 could promote epithelial differentiation is still an interesting open question. Indeed, TAp63-null mice do have an epithelial phenotype presenting as blisters and skin ulcerations as described below.[37] It is conceivable, although not formally proven, that ΔNp63 exerts a role in the differentiation status of cancers, thus affecting the transition toward anaplastic tumors.

Stemness. Work on epidermis[50] and in the thymus[114] highlights a crucial role for ΔNp63 in regulating the proliferative potential of the stem cell compartments in these two tissues.[50,114] In particular, the evidence for a crucial role of ΔNp63 in stem cell maintenance in the epidermis is persuasive.[50] The major effect of ΔNp63 lies in controlling the number of asymmetric doublings of this compartment, and *Trp63*$^{-/-}$ cells have clear evidence of the very few cells – created by asymmetric divisions of stem cells – available to progress to a mature differentiation pathway. The organization of the basal layer requires a tight regulation

of asymmetrical divisions as part of the proliferation phase. Polarity of epithelial cells directs the apical basal and planar axes and has crucial roles in cell–cell adherence, tight junctions and asymmetrical cell division;[115] in fact p63 has been found to affect the polarity of epithelial cells *in vivo* during embryonic development.[115] In particular, ΔNp63 controls the proliferative potential, the clock of the number of divisions, through a yet unknown mechanism.

Recently, p63 has been shown to directly repress KLF4, a major factor that reprograms differentiated cells into iPS by binding to an upstream promoter site.[116] Indeed, the epidermis shows a mutually exclusive expression of ΔNp63 and KLF4. Of interest for this discussion, however, is the evidence that mutant p53 counteracts the action of ΔNp63 on KLF4 expression, suggesting that p63 and KLF4 are not only relevant in the growth-promoting effect of p63 in keratinocytes, but also in the tumor-predisposing p53 mutations which, by hijacking p63, results in KLF4 dysregulation. In addition, TAp63 regulates self-renewal of dermal precursors via p57Kip2.[37]

However, although ΔNp63 is involved in stemness, it is clearly not the only player, as indicated by the following: (i) ΔNp63 expression is found in a significant proportion of the basal layer of mouse or human epidermis[14] (where stem cells should be rare); (ii) all holoclones are positive for ΔNp63 (while only a minority of these epithelial cells, purified *ex vivo*, have proliferative clonal potential *in vitro*; similarly, none of the transit amplifying cells, reviewed by Blanpain and Fuchs[115] should have expressed ΔNp63); (iii) p63 transactivates a huge number of genes not associated with stem cell function, such as adhesion molecules,[105] indicating that several parallel pathways are activated by ΔNp63 in addition to a pure stem cell pathway; (iv) ΔNp63-positive cells do not co-express stem cell markers and the related downstream transcriptional targets genes that are involved in maintaining stem cell function and asymmetric division.

Therefore, despite the strong morphological and functional evidence linking ΔNp63 to stemness,[50] several molecular details still await clarification. Considering the proposed crucial role of cancer stem cells in tumorigenesis, this property of ΔNp63 could be of major relevance in oncology.

Senescence. Solid evidence indicates that, both *in vitro* and *in vivo*, p63 deficiency evokes the tumor-suppressive mechanisms of senescence.[38,86] At the molecular level, TAp63 induces senescence independent of p53, p19ARF and p16INK4A, but requires p21WAF/CIP1 and Rb, and this mechanism inhibits *in vivo* progression of established cancers.[106] On the other hand, ΔNp63 overrides oncogene-induced senescence, driving *in vivo* tumor progression.[117] This effect seems to require the chromatin-remodeling protein Lsh, as a direct transcriptional target of ΔNp63.[117] A further target involving p63 and senescence is Sirt1.[118] This anti-senescence effect of ΔNp63 seems to cooperate with its stemness effect, thus facilitating tumor progression. Again, therefore, TAp63 has a tumor-suppressor function while ΔNp63 manifests oncogenic properties.

Epithelial organizer. Several recent findings[119–121] allow me, here, to propose the hypothesis that p63 is an epithelial organizer, and is involved as a modulator of EMT. This hypothesis would reconcile several apparently discordant functions of ΔNp63 during the development of epithelia, as well as in cancer biology, namely the activation of apparently distinct pathways discussed above: stemness, cytokeratins, adhesion molecules, differentiation proteins and cell cycle regulators. All these pathways are simultaneously required to organize and structure epithelia. The expansion of the epidermal basal layer through the proliferation potential of the transit amplifying cells (reviewed by Blanpain and Fuchs,[115] see below) is implicit in this EMT hypothesis. The role of p63 in stem cell maintenance, as first postulated by McKeon, is thus related to its role in EMT, being one of the fundamental pathways to guarantee the role as an epithelial organizer.

EMT is important in embryogenesis, as well as in invasiveness and metastasis in cancer. According to the proposed hypothesis that p63 is an epithelial organizer, ΔNp63 is a modulator of EMT. Thus, ΔNp63 is downregulated during EMT in cancer and epithelial cells;[119] it is involved in the regulation (by direct transcription) of crucial players of EMT including SNAIL1/2/3, NOTCH and Jagged (Figure 6), which are involved in epithelial asymmetric division and tissue polarity,[115] and involved in adhesion.[105] The expression of ΔNp63 is downregulated during EMT by the signaling molecule SNAIL, and this enhances the invasiveness of squamous cell carcinoma (SCC) cells in parallel with, and independently of, the downregulation of E-cadherin.[119,120] Depletion of ΔNp63α and ΔNp63β induces EMT in breast cell lines;[122] conversely, ΔNp63γ *per se* is able to cause EMT with elevated TGFβ-1, -2, -3 and its downstream effectors Smads2/3/4 via a p63-binding site in intron 1 of TGFβ.[122] These data are compatible with p63 expression in presarcoma lesions *versus* sarcoma.[121] Epithelial cells are motile and can move away from their nearest neighbors, while remaining within the epithelial layer. However, cells do not detach and move away from the epithelial layer under normal conditions. Conversely, mesenchymal cells do not form an organized cell layer, nor do they have the same apical–basolateral organization and polarization of the cell surface molecules and the actin cytoskeleton as epithelial cells. They contact neighboring mesenchymal cells only focally, and are not typically associated with a basal lamina. Thus, this function of p63 might be also relevant in the epidermis, as well as for the above described role in metastasis.

Other pathways. Finally, there is solid evidence of additional signaling pathways involving p63 in cancer, in part indicated above, Figure 6.

Wnt signaling: The effect of p63 on β-catenin, a critical event in cancer that results from Wnt activation, is complex, and it is counterbalanced by p53 itself. Indeed, ΔNp63 associates with the B56α regulatory subunit of protein phosphatase 2A (PP2A) and glycogen synthase kinase 3β (GSK3β), forming a multiprotein APC complex that results in a dramatic inhibition of PP2A-mediated GSK3β reactivation.[107] This inhibitory effect of ΔNp63 on GSK3β mediates a decrease in phosphorylation levels of β-catenin, which induces intranuclear accumulation of β-catenin and activates β-catenin-dependent transcription of genes such as

Tcf/Lef.[107] Conversely, p53, though not mutant p53, leads to a βTrCP-dependent degradation of β-catenin, hence counterbalancing the effect of ΔNp63.

mTOR and Notch signaling: mTOR is a positive regulator of Notch signaling via the induction of the STAT3/p63/Jagged signaling cascade.[108] Here, Notch seems to act a molecular switch between proliferation and differentiation, where high mTOR activity, via high Notch, impairs differentiation in human breast cancer lines.

Hedgehog signaling: Both TA63 and ΔNp63 have been reported to induce the expression of Sonic Hedgehog[123] by direct binding to the promoter; accordingly, p63-null mice show reduced Sonic Hedgehog expression. Conversely, Indian Hedgehog seems to be able to regulate the p63 promoter.[124] A reciprocal regulation between p63 and Indian Hedgehog, involving Gli3, seems to be relevant for the activated stem cells in both stratified epithelia and breast cancer cells.[124]

Conclusions and Perspectives

The ancestor of the p53 family is a p63-like protein that first appears in unicellular flagellates,[125] and radiation into clear p53, p63 and p73 proteins only occurs with the emergence of vertebrates.[125] In organisms such as the precursors of sea anemones, worms and flies, the principal role of this p63 precursor is the preservation of germline genomic integrity, a function that has clearly been conserved through at least 1 billion years of evolutionary time.[9] As the family diversified, so did its functions, with the N-terminally truncated isoform of ΔNp63 having a role in maintenance of the stem cell pool, and potentially, I have argued, in the perpetuation of cancer stem cells.

Although our understanding of the role of p63 isoforms, and in particular of TAp63, remains preliminary, and of their influence on chemosensitivity by TAp63 even more so, it is likely that chemosensitivity may be determined not only by p53, but also by p73- and p63-function, and dominant-negative isoforms could have a crucial role in this scenario. The potential synergistic effects of different p53 family members in the response to chemotherapeutic drugs should be taken into consideration for the development of future anticancer strategies.

The clinical problem of human cancer is metastasis. There is now evidence that, perhaps as a reflection of its role in epithelial development and in stemness, p63 as an 'epithelial organizer' is also important in EMT, an essential prerequisite for invasion and migration of tumor cells.[126] The present day complexity of the p53 family, with multiple isoforms now recognized for each member, allows the possibility of a whole web of inter-family interactions. This results in a highly plastic and subtle modulation of the activity of an individual isoform, and we are now beginning to see, for example, in the interaction between mutant p53 and p63, how this may influence the pathology of cancer.

Here, we highlight the important role for TAp63 and ΔNp63 in tumorigenesis and metastasis, although the underlying molecular mechanisms still require further clarification. Specifically, the issue of the differential effects of the splicing isoforms is basically untouched. Clearly, the identification of specific interactors, acting as modulators, of transcriptional targets, including miRs, and of the degradation mechanisms are still too preliminary to allow a consistent movement to develop drugs that target p63 in cancer.

Conflict of Interest

The author declares no conflict of interest.

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