Biochemistry (Moscow), Vol. 70, No. 9, 2005, pp. 955-971. Translated from Biokhimiya, Vol. 70, No. 9, 2005, pp. 1157-1176.

REVIEW

The *p53* Gene Family: Control of Cell Proliferation and Developmental Programs

A. A. Morgunkova

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, ul. Vavilova 32, 119991 Moscow, Russia; fax: (7-095) 135-1405; E-mail: morgunkova@yandex.ru

Received September 17, 2004

Abstract—For a quarter of a century the gene *p53* has attracted close attention of scientists who deal with problems of carcinogenesis and maintenance of genetic stability. Multicellular organisms on our planet owe their rich evolution in many respects to the ability of this gene to protect cells from oncogenic transformation and harmful changes in DNA. A relatively recent discovery of structural *p53* homologs, the genes *p63* and *p73*, which seem to have more ancient roots, has roused keen interest in their function. Do they carry out oncosuppressor functions in partnership with *p53* or do they possess their own specific functions? This review analyzes data on p53, p63, and p73 functional activity at the levels of the molecule, cell, and whole organism with the accent on examination of specific p63/p73 targets indicating a unique role of these genes in control of developmental processes.

Key words: p53, p63, p73, carcinogenesis, genetic stability, development, differentiation, regeneration, transcription, differential expression

About 30 gene suppressors of tumor growth are now known [1]. Among them, the gene *p53* plays the leading role in defense of the organism from malignant transformation of cells. This gene maintains genome integrity and stability in multicellular organisms by elimination of cells whose DNA is damaged by various stressing agents, such as IR and UV irradiation, toxic substances, activated oncogenes, free radicals, hypoxia, and viral infection [2]. It has been found on various branches of the phylogenetic tree, from humans to nematodes and fruit-flies [3, 4].

Unlike other anti-oncogenes affiliated to families, *p53* was long thought to be unique in the genome. It was surprising why a gene whose functions are so important for survival is so alone and has no assistant genes. The situation changed in 1997-1998, when proteins highly homologous to p53 were found by chance in human, murine, and rat cells. Afterwards, the genes *p63* and *p73* encoding these proteins were found and arranged into one family with *p53* on the basis of their structural homology [5-7].

STRUCTURES OF p53 FAMILY GENES AND PROTEINS AND IDENTIFICATION OF THEIR FUNCTIONS ON KNOCK-OUT MICE

The genes *p53*, *p63*, and *p73* have similar exon–intron structures (Fig. 1b). Preceding the first nontranslated exon lays a promoter, a DNA region responsible for binding with an enzyme initiating gene transcription. Unlike *p53*, both *p63* and *p73* have a second alternative promoter in the third intron. The use of alternative promoters results in two protein isoforms differing in N-terminal amino acid sequences: TA and ΔN (Fig. 1, a and b). TA (transactivation) isoforms contain and ΔN isoforms do not contain a transactivation domain of the protein, which switches on the transcription of target genes.

Unlike *p53*, an alternative splicing is characteristic of *p63* and *p73* in which individual potential exons are eliminated together with introns. As a result, a variety of p63 and p73 isoforms are produced that differ in their C- rather than N-ends: α, β, γ, δ, ε, and ξ (Fig. 1, a and b). So, because of alternative promoters and alternative

0006-2979/05/7009-0955

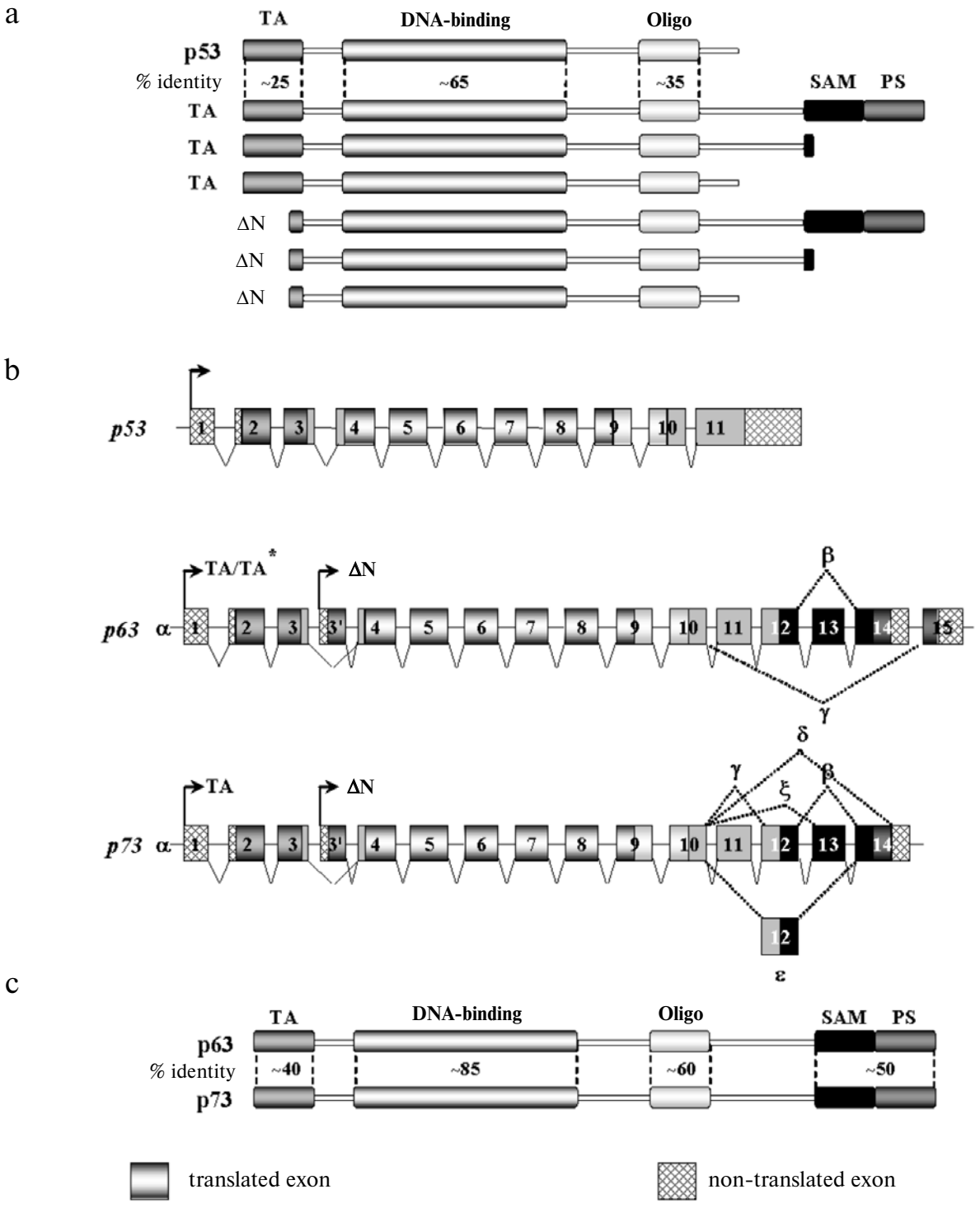

Fig. 1. Structures of p53 family genes and proteins. a) Comparison of domain structures of p53 protein and major isoforms of p63/p73; b) exon-intron structure of *p53/p63/p73*; c) identity of p63 and p73 amino acid sequences. TA, transactivation domain; Oligo, oligomerization domain; SAM, "sterile alpha motif" domain; PS, "post-SAM" domain. The outer promoter produces TA isoforms (TA*, isoform subclass with additional 39 amino acid residues on the N-terminus), and the inner promoter produces ΔN isoforms. Alternative splicing produces the C-terminal isoforms α, β, γ, etc. Exons of *p53/p63/p73* have similar lengths and are interrupted by introns in homologous positions to ensure homology of protein domains. However, the length of introns greatly varies. The first intron is of enormous length, about half of the overall gene (~11 of 20 kb in *p53* and ~30 of 60 kb in *p63/p73*); the third intron of *p63/p73* is also large (~15 kb).

splicing the genes *p63* and *p73* generate multiple proteins, whereas *p53* only encodes a single protein [8, 9].

The proteins p53, p63, and p73 have several common functional domains: transactivation, DNA-binding, and oligomerization domain (Fig. 1, a and c). The oligomerization domain binds the protein globules into tetramer, the DNA-binding domain binds tetramer with target gene, and transactivation domain (if it exists) attracts protein factors initiating transcription (that is, switching on the gene) to the promoter of this gene. All α-isoforms of p63 and p73 also contain the SAM-domain (sterile alpha motif) with unclear functions, which is absent in p53 and all other isoforms of p63/p73. This domain is often found in proteins playing an important role in developmental processes; it serves there for formation of protein–protein contacts. The SAM-domain of p63/p73 may in someway inhibit the transactivation abilities of its carriers, because TA α-isoforms virtually cannot switch on transcription of p53-dependent genes [10-12].

When the "anatomy" of p63 and p73 was elucidated, a question arose as to their function: are they, like p53, oncosuppressors or do they play another role? Oncosuppressor functions of p53 were decisively demonstrated as early as 1992, when knocked-out mice were obtained with completely inactivated (knocked-out) *p53* gene [13]. Since p53 plays an important role in cell cycle control (this was already known at that time), it was amazing that the knocked-out mice were born normal. However, monitoring of their further development demonstrated that they barely reach the age of nine months. They died from spontaneously arising malignant tumors. This fact unequivocally indicated that p53 is not involved in developmental processes, but is indispensable for the body protection from tumorigenic transformation of cells. These data are consistent with observations of patients with hereditary Li–Fraumeni syndrome, in which one of two *p53* gene copies is inactive in all cells. Yet at a young age these patients are highly predisposed to spontaneous progression of various cancer forms. Cancer arises from a cell that has lost p53 functions because of somatic mutation.

Since gene knock-out proved successful for identification of gene functions on the level of the whole organism, it was applied to investigations of p63 and p73. In 1999, two independent groups simultaneously reported their attempts to select mice with destroyed gene *p63* [14, 15]. The results were identical: in both cases newborn knocked-out pups died within the first day from general dehydration. They had severe developmental defects of both ectoderm derivatives and those mesodermal elements, which are ectodermally stimulated during embryogenesis. The condition of the limbs and skin was strikingly affected (Fig. 2). The hind feet were completely absent, and the fore feet were terminated on the level of the humeri. Instead of epidermis covering the body,

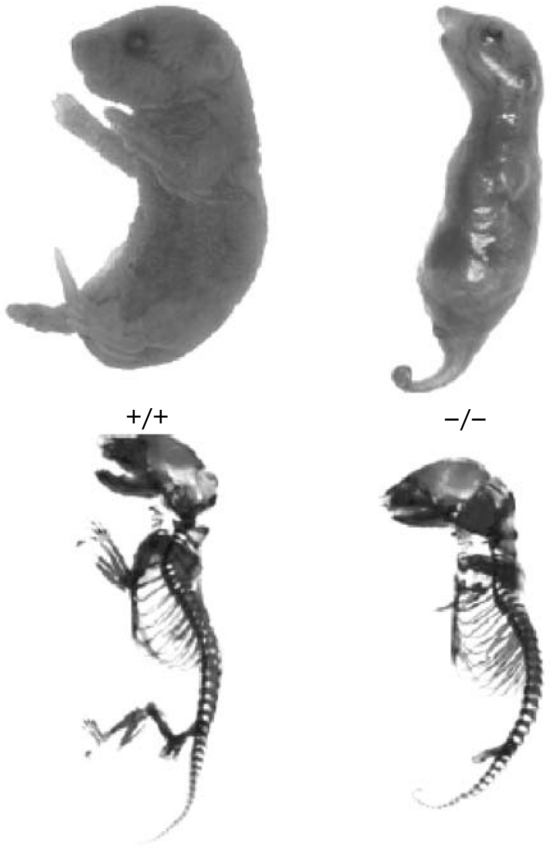

Fig. 2. Normal newborn mice (+/+) and mice with *p63* gene knock-out (–/–). Knocked-out mice have severe developmental defects of limbs and skin [14].

sporadic islets of disorganized epithelial cells lay directly over the bare connective tissue, that is, the pups virtually lacked skin. Hair follicles and teeth and mammary, sweat, lacrimal, and sebaceous glands, that is, skin derivatives developing from the same stem epithelial cells which form epidermis, were also absent. The condition of many internal organs was abnormal because of unformed multilayer epithelium. The head lacked eyelids and vibrissas, and the jaws and palate were underdeveloped.

For some reason, the *p63* knock-out caused fatal, and in fact lethal outcome of embryo development. It was soon found that in normal mice the protein p63 (ΔNp63α) begins to work on the ninth day of the embryo's life. First appearing in oral ectoderm, branchial arches, and tail and limb buds, this protein is then detected in all ectoderm. It achieves highest concentration in a special zone of limb buds, the apical ectodermal ridge (AER), which is necessary for limb growth. Knock-out of *p63* completely inhibited AER production and sharply changed expression pattern of several genes working in limb buds: some genes (*Fgf8* and *Msx-1*) were not switched on, whereas others (*Lmx-1*) lost expression

asymmetry. And not only ectodermal genes (*Fgf8*) were affected, but also some genes functioning in mesoderm (*Msx-1*, *Lmx-1*) in which p63 never appears. Hence, disappearance of p63 eliminates the signal flow from ectoderm to mesenchyme.

A formidable epidermis condition of knocked-out mice was of particular interest. Directed studies of this phenomenon showed that *p63* maintains the process of self-renewal in the population of epithelial stem cells. Both formation and continuous regeneration of multilayer epithelia including epidermis require asymmetric division of stem cells localized in the *stratum basale*: one daughter cell becomes differentiate and ascends to upper layers, whereas another remains immortal (Fig. 3). The absence of epidermis may result from either depletion of stem cell pool or disability of primal ectoderm to differentiate. The second variant is less probable, because the markers of mature terminally-differentiated epidermal cells, such as loricrin, filaggrin, and involucrin, are found in the epithelial cell clusters dispersed about the dermis of knock-out mice. Nonetheless, some keratins, which work in stratification of primitive ectoderm, do not work in these clusters. So, it is possible that the absence of AER, epidermis, and multilayer epithelium of internal organs is the consequence of the same cause.

As in the history of p53 studies, the human naturally simulated analog of mouse *p63* knock-out was found. It was a spectrum of hereditary EEC syndromes (ectrodactyly, ectodermal dysplasia, and facial clefts), in which the dose of *p63* gene was twofold decreased because of mutation in one allele [16, 17]. The patients have symptoms resembling developmental defects in knocked-out mice. Clinical symptoms vary in a broad range including ectrodactyly (finger absence or symphysis), ectodermal dysplasia (anomalies in development of ectoderm derivatives, such as epidermis, hair, nails, and mammary and cutaneous glands), and facial, arm, and foot clefts with formation of cleft palate, harelip, or "lobster claw" (Fig. 4).

One year after the studies on *p63* knock-out were reported, a strain of mice was selected that lacked gene *p73* [18]. These mice compared with wild-type mice were small but had no visible malformation (Fig. 5a). Eighty percent of the newborn mice died within three weeks from massive gastrointestinal and intracranial hemorrhages, and the others survived and, unlike the mice with *p53* knock-out, were not prone to any neoformation.

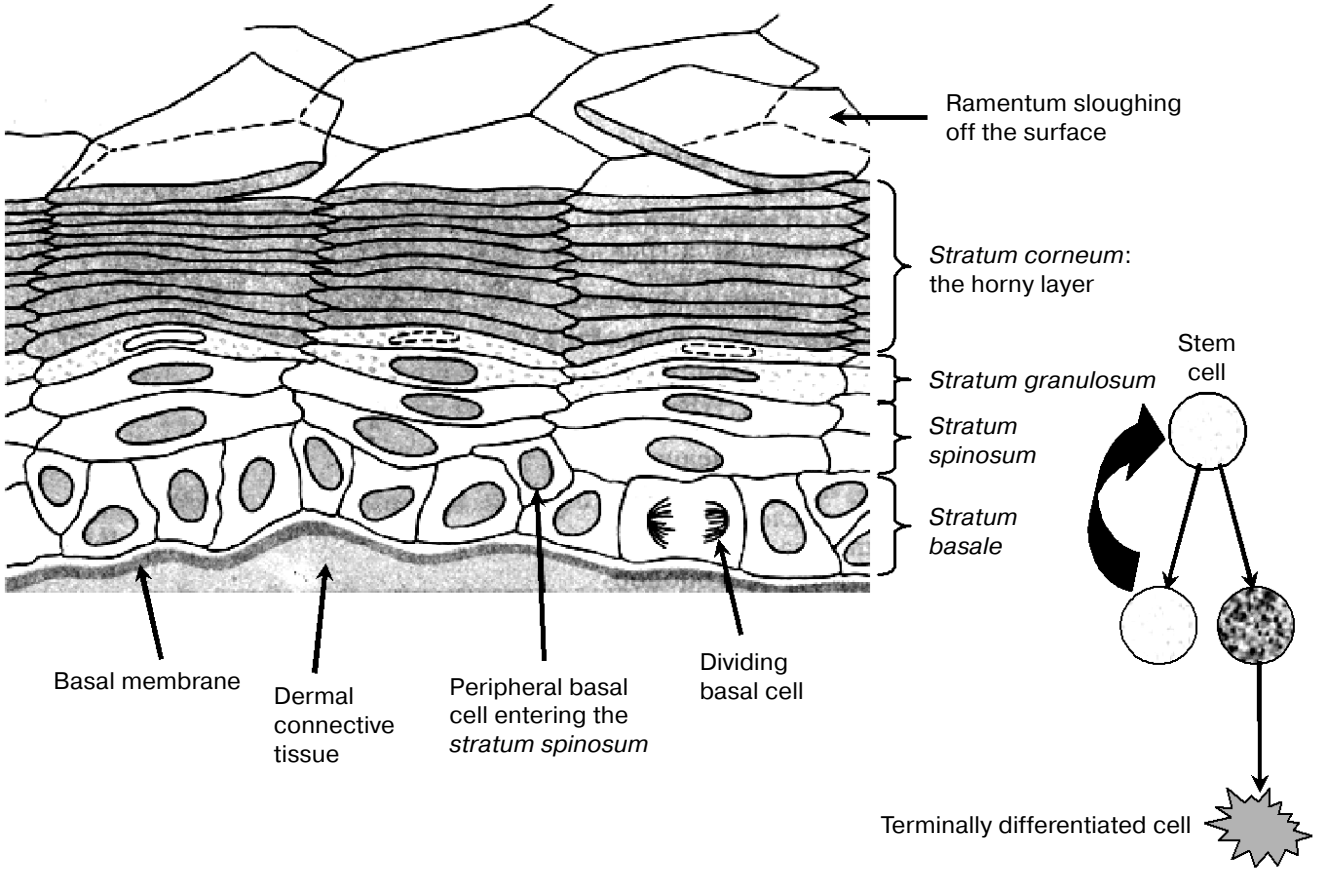

Fig. 3. Structure of epidermis and asymmetric stem cell division.

They suffered from gastrointestinal diseases, chronic otitis, rhinitis, blepharitis, and conjunctivitis, which resemble inadequate inflammatory reaction of hypersensitive epithelium. In these inflammation zones, high level of p73 was found in healthy mice.

Anatomical examination demonstrated significant anomalies of the central nervous system. They were hydrocephaly, that is, excess of cerebrospinal liquor in cerebral cavities, and anomalous development of hippocampus, cerebellum, and some cortical areas. Hydrocephaly often progressed to great enlargement of lateral ventricles with cortical compression and intracranial hemorrhages (Fig. 5, b and c). It could result from either hypersecretion or reduced absorption of liquor. These processes are realized in brain cavities by glial and epithelial cells of vascular plexus, which line the ventricles and normally contain great amounts of p73.

A very unexpected effect of *p73* knock-out in mice was a change in their sexual behavior and reproduction. The knocked-out males demonstrated no interest in healthy females and did not mate, and the knocked-out females, which were bred with wild-type males, did not become pregnant. However, these mice had no defects in reproductive organs. When in healthy mice an extremely high level of p73 was found in neural epithelium of Jacobson's organ, an olfactory structure necessary for detection of pheromones, it was proposed that p73 is involved in pheromone perception. This proposal might explain (at least in part) the described sexual anomalies. It was soon demonstrated that both the pheromone receptors V1R//V2R and the olfactory cell adhesion molecule (O-CAM) are not produced in the knocked-out mice.

Thus, despite structural homology and common family affiliation, the genes *p53*, *p63*, and *p73* show amaz-

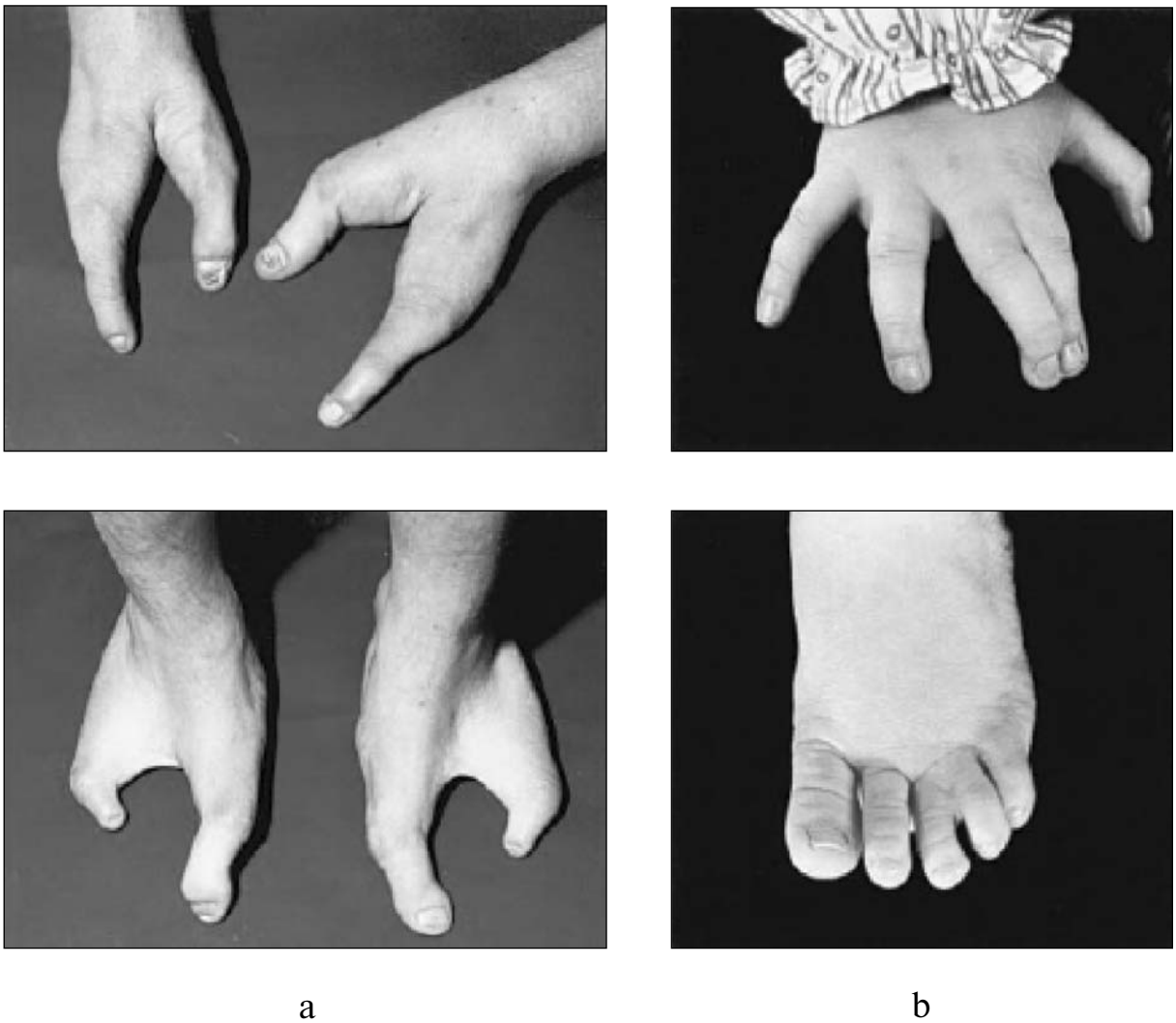

Fig. 4. Effect of mutations in one allele of the human gene *p63* (*p63*+/–). Typical defects of limb development in patients with EEC-syndrome: arm and foot clefts ("lobster claws") (a) and syndactyly (b) [16].

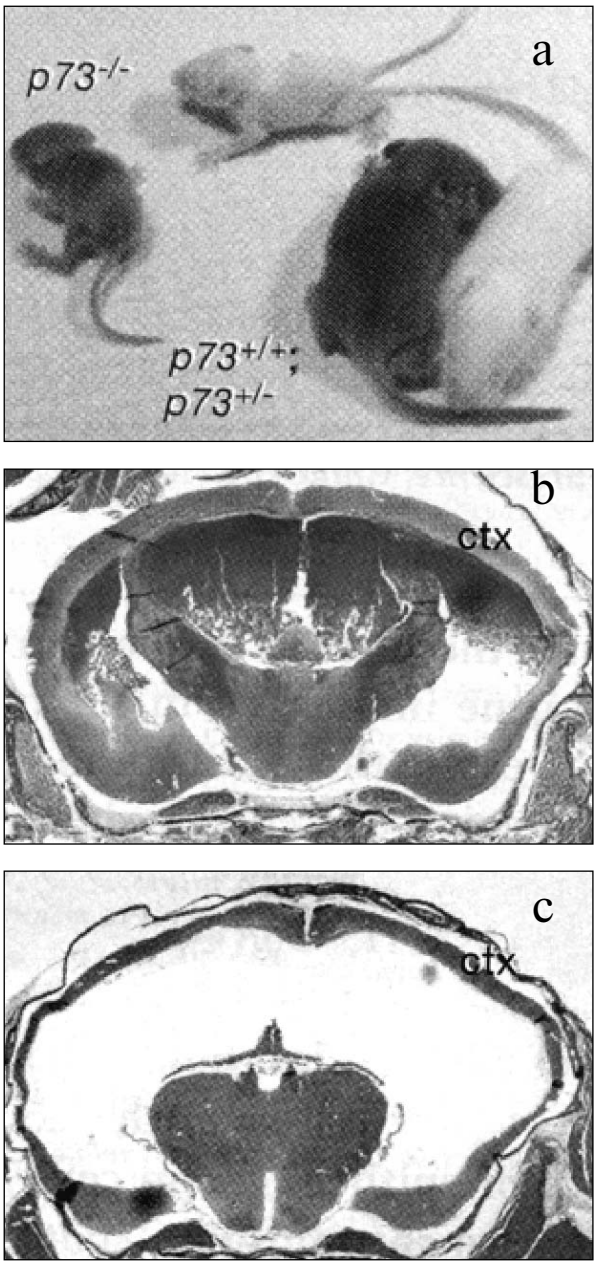

Fig. 5. Effects of *p73* gene knock-out. a) Comparative sizes of ten-day-old pups with different genotypes; b, c) histological brain sections of *p73–/–* mice demonstrating ventricle dilatation (hydrocephalus) with cortical (ctx) compression [18].

ing functional difference on the whole body level. The chief function of *p53* is body protection from cancer, whereas *p63* and *p73* participate in processes of differentiation and development, especially in maintenance of population of stem epithelial cells and in neurogenesis. So, the problem arose of decoding the molecular mechanisms underlying so expressed functional divergence of these three genes.

FUNCTIONS OF *p53*, *p63*, AND *p73* ON CELLULAR AND MOLECULAR LEVELS

General Principles of p53 Functioning in the Cell

It is known that protein p53 continuously monitors conditions of all cells of the body. In genotoxic stress, it either interrupts the cell cycle while DNA reparation continues or switches on apoptosis, the program of cell death. These stress responses occur because p53 works as a transcription factor that can turn on and off a branched network of target genes and due to physical interactions of p53 with other proteins [19, 20].

Regulation of *p53*. Activity of *p53* is preferably controlled on the level of protein. Although the gene is continuously transcribed in all body cells, the protein only lives for 5-20 min and does not achieve high concentration in healthy cells. The lifetime of p53 is controlled by negative feedback by the gene *mdm2*: p53 switches on *mdm2*, whose protein product binds to the transactivation domain of p53 to block its function and facilitate its destruction. The situation changes under stress, because the sites of p53 and mdm2 that are responsible for the protein binding are phosphorylated by protein kinases (ATM and ATR). As a result, the complex p53/mdm2 dissociates, and p53 escapes degradation and accumulates in the cell, so that its amount becomes sufficient for cell cycle arrest and initiation of apoptosis. When oncogenes are activated, p53 is stabilized via interaction with protein ARF, which protects p53 from mdm2 [21, 22].

Following protein kinases, histone acetyl transferases (p300/CBP, pCAF) modifying the C-end of p53 are involved under stress. This facilitates histone acetylation providing a transition of target gene chromatin into the state allowing its contact with the transcription apparatus, that is, causes activation of p53-dependent genes. Acetylation also suppresses p53 escape from the nucleus into the cytoplasm, thus increasing p53 concentration in the cell nucleus [23].

Functions of p53 on the molecular level: switching on/off genes and stress signal transmission via contacts with proteins. p53 recognizes its transcription targets by their specific nucleotide sequence, a responsive element that influences a promoter activity. This element consists of two inverted pentamer repeats divided by a small interval: 5′-PuPuPuC(A/T)(T/A)GPuPuPu – N(0-13) – PuPuPuC(A/T)(T/A)GPuPuPu-3′ [24]. Protein p53 interacts with this element via its DNA-binding domain. This domain is the most vulnerable region of p53, its “Achilles’ heel”. This is because several “hot spots” are localized inside the domain, whose mutations completely disrupt the contact of p53 with DNA of target genes, so that switching on these genes becomes impossible [25, 26]. These mutations of *p53* are often found in tumors. Affinity of responsive elements to the DNA-binding

domain highly varies from gene to gene, thus influencing efficiency of gene switching on.

Gene p53 not only turns on genes, but also turns off some genes by either binding (via histone acetylases and deacetylases) transcription factors necessary for their activation, or by using other mechanisms. Direct contacts of p53 with protein kinases (c-Abl, JNK, and LKB1) or with other proteins also play important roles in stress signal transmission.

Functions of p53 on the cell level: cell cycle arrest and turning on programmed cell death. The cell cycle consists of mitosis and interphase, which in turn includes G1, S, and G2 phases. It is governed by complexes of cyclins with cyclin-dependent protein kinases (CDK), in which cyclin is a regulatory subunit and cyclin-dependent kinase is a catalytic one. In G1 the complexes cyclin D-CDK4/6 and E-CDK2 are functioning, whose main substrate is the protein Rb. Non-phosphorylated Rb binds and eliminates from the cycle the transcription factor E2F, which is necessary for activation of S-phase genes. When CDK phosphorylates Rb, E2F is released, and the cell goes forward in the cycle. The G1 phase is determinative for further destiny of the cell: if it contains damaged DNA, it is arrested. Protein p53 decides to stop the cell or permit it to go into the next division. To arrest the cell, p53 turns on the gene $p21^{Waf1/Cip1}$, whose protein product binds and inhibits cyclin–CDK complexes. As a result, non-phosphorylated Rb holds E2F, and the cycle stops. p53 exerts similar control in S and G2/M phases. The genes *GADD45*, *PA26*, *14-3-3-σ*, and the gene encoding cyclin *G* are also activated by p53 for arrest, but *p21* exceeds them in strength [20, 27].

An alternative reaction of p53 to stress is switching on the program of apoptosis. The suicidal pathway begins from double mitochondrial membranes, between which cytochrome *c* is encased. Cytochrome *c* release into cytoplasm is the beginning of an irreversible reaction chain ending in cell death. The released cytochrome *c* stimulates formation of the complex between Apaf-1 (apoptosis protease-activating factor 1) and caspase 9 (cysteine acid-aspartic protease), which initiates a caspase cascade, that is, the sequence of enzymatic reactions in which activated caspase "*a*" cleaves procaspase "*b*", thus activating it, and the activated caspase "*b*" cleaves procaspase "*c*", and so on. All caspases cut proteins at aspartic acid residues. Since one molecule of enzyme can process very many of its substrate molecules, the process goes rapidly as an avalanche. The final caspases-killers cleave all cellular proteins, and one of them activates the deoxyribonuclease CAD, which cuts DNA. Different events, such as disappearance of necessary growth factors, death receptor activation on the surface of cytoplasmic membrane, and appearance of ROS (reactive oxygen species) in the cell, can lead to critical changes in mitochondrial membranes. A pathway from receptors of death can pass via procaspase 8, which initiates the caspase cascade without mitochondria taking part in it [28].

Protein p53 uses different strategies initiating cell death mechanisms. It activates transcription of genes, whose protein products are localized in mitochondria, and either assists the cytochrome *c* release (*BAX*, *PUMA*, *NOXA*, *p53AIP*) or generates oxidative stress with ROS destroying mitochondrial membranes (*PIG3*, *REDD1*, *p85*). It also turns on the killer pathway of apoptosis by activation of death receptor genes (*KILLER/DR5*, *FAS/APO-1/CD95*, *PIDD*) and transportation of killer receptors from Golgi apparatus to the cytoplasmic membrane. It directly decreases the apoptotic threshold of the cell by activation of gene *Apaf1*. Protein p53 induces apoptosis not only by activation of pathways that lead to death, but also by suppression of mechanisms facilitating cell survival. In particular, p53 turns off the anti-apoptotic gene *Bcl2*. In all appearances, p53-dependent apoptosis is a result of the combined effect of several genes and proteins interacting with p53 [29].

Genes *p63* and *p73*

Differential expression and regulation. When *p53* is compared with *p63/p73*, the first conspicuous fact is the amazing contrast between their patterns of spatiotemporal expression. Gene *p53* functions at all times and in all places and continuously supervises all body cells, whereas *p63* and *p73* only function in separate tissues and organs during certain periods of development. This is possibly connected with fundamental difference in regulation of their transcription. It was already shown that *p53* and *p73* are governed by different combinations of transcription factors (Fig. 6a) [30, 31]. Tissue-specificity of *p63/p73* expression suggests that these genes can never compensate the oncosuppressor functions of *p53*, even if they possess these functions.

Expression patterns of *p63* and *p73* are complicated by multiplicity of their isoforms. As a rule, only one of them is highly active in the cell at one time [32]. All the isoforms bind p53-responsive element, although not so effectively as does p53. So, TA-p63/p73 can assist p53 in its work, or, alternatively, ΔN-p63/p73, which lack the transactivation domain, can suppress its functions: they occupy the binding site without turning on p53-dependent genes. Unlike p53, p63/p73 in tumors are not deteriorated by mutations. Instead, clones are selected in cancer that are characterized by intense expression of ΔN-isoforms, which stimulate proliferation of the cells and give them selective advantages [33, 34].

Differential expression of TA/ΔN-isoforms is determined by various sets of regulatory elements of alternative promoters. The binding site for p53 is only localized before the internal promoter (Fig. 6b). So a negative feedback loop is formed: TA-proteins turn on the ΔN-pro-

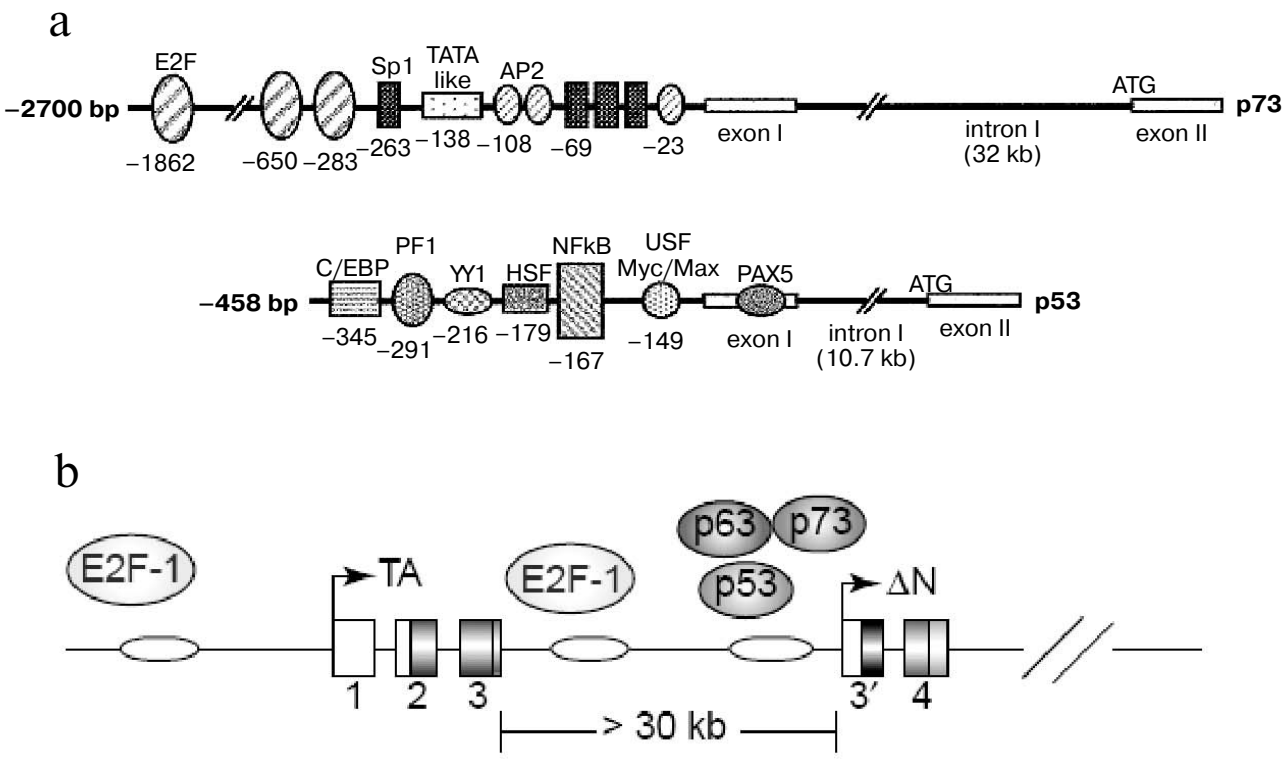

Fig. 6. Regulation of p53, p63, and p73 functioning. a) Difference in structures of regulatory sites of *p53* and *p73* promoters. The binding sites for various transcription factors localized before the first exon are shown [31]. b) Differential control over the work of TA- and ΔN-promoters of the gene *p73*. A p53-responsive element is only before the ΔN-promoter [8].

moter, in turn ΔN-proteins make TA-isoforms functionally inactive either competing with them for responsive element or forming inactive complexes via the oligomerization domain [8, 35].

Differential expression of C-terminal isoforms (α, β, γ, etc.) is under the control of tissue-specific mechanisms of alternative RNA splicing. Splicing instability, which is often observed in cancer, results in different C-terminal variants of p63/p73 in different areas of the same tumor. It is unclear what the meaning of C-terminal variability is because only the α-isoform has a decided difference from others in its SAM-domain. It is possible that the true goal of various modifications of p63/p73 "tails" is elimination of the SAM-domain in some ways.

The difference in regulation of p53 and p63/p73 is also manifested on the level of protein. Both the concentration and stability of p63/p73 are independent of mdm2 and ARF. The proteins rarely react on stress: they do it only in certain cell types in response to certain stimuli. In particular, p73 is sometimes stabilized to evoke apoptosis in response to UV irradiation, cisplatin, and taxol. p63 is known to react to ultraviolet: exposure of keratinocytes leads to increase in the levels of TA-p63 and p53 and decrease in the level of ΔN-p63 followed by cell death [8, 36].

Causes of p63 and p73 activation. One gets the impression that, unlike p53, stress is not the main trigger for both p63 and p73. So, what does lead to their switching on? Because in knocked-out mice the developmental processes were shown to be strongly dependent on the functions of these two genes, one could suppose them to be sensitive to signals of growth and differentiation. Some attempts at studies have already been made in this field. Effects of *p73* in nervous tissue and effects of *p63* in epithelial tissue were primarily examined, because just there the maximum activities of these genes were previously found.

The study of developing sympathetic neurons has shown that in these cells α- and β-isoforms of ΔN-p73 are produced abundantly, which protect the cells from p53-dependent apoptosis and plainly react to NGF (nerve growth factor): expression of ΔN-p73 dramatically increases after addition of NGF and ceases after its removal. NGF plays a momentous role in nervous system development: neurons divide and form outgrowths in its presence and in its absence they die. It has been proved that injection of ΔN-p73 into neurons blocks apoptosis induced by removal of NGF [37]. It was demonstrated in other study that in neurons p73 reacts not only to nerve growth factor, but also to retinoic acid, a derivative of

vitamin A [38]. In embryogenesis this acid plays a role of morphogen, that is, the substance forming a gradient of concentration along the body, which is recognized by the cells and, by switching on/off various gene networks, predisposes the cells to embrace one or another developmental pathway [39, 40]. Morphogen furnishes each cell with positional information ("memory" about its own destination and position in the body), thus regularizing formation of spatial structures, such as limb anatomy. Retinoic acid and other vitamin A derivatives (retinoids) are also important for organization and sometimes regeneration of many tissues in adults [41]. There are data indicating that in brain development retinoic acid stimulates differentiation of both neurons and glia and expedites regeneration of nerves damaged *in vitro*. Experiments on neuroblastoma have shown that undifferentiated neurons, when treated with retinoic acid, began to differentiate with production of molecular markers of nervous system and growth of axons. In this context, the cells begin to express actively the β-isoform of TA-p73, whereas the level of p53 remains unchanged. Interestingly, the differentiation can begin without inducer: it is enough to put TA-p73 into the cells [38]. One can get the impression that factors conducive to cell growth and division switch on transcription of *ΔN-p73*, and factors stimulating differentiation switch on *TA-p73*.

A similar correlation is found in epithelial tissue: the level of ΔN-p63 decreases, whereas the level of TA-p63 increases or remains unchanged in epidermal keratinocytes in the course of their differentiation. The highest concentration of α-isoform of ΔN-p63 is detected in the basal layer of epidermis, where undifferentiated stem cells lie. As step-by-step differentiation of keratinocytes occurs with their gradual migration to the skin surface, the concentration of ΔN-p63 decreases. It is known that division of epidermal cells is stimulated by growth factors, whereas their differentiation is stimulated by removal of these factors or by calcium ions, with retinoic acid influencing both processes. Experiments suggest that under stimulation of human keratinocyte differentiation by removal of epidermal growth factor or by calcium ions the intracellular levels of mRNA and protein ΔN-p63 decreases 5- and 50-fold, respectively. In the same time, 4-fold increment is observed in concentration of mRNA of involucrin (a marker of epidermis differentiation). Simultaneous addition of retinoic acid reverses the process: keratinocytes begin to divide and de-differentiate, and the concentration of ΔN-p63 increases and reaches the initial level. Retinoic acid finely tunes a balance between proliferation and differentiation of epidermal cells. It maintains their high proliferative potential in basal layer with high level of ΔN-p63, and does not disturb differentiation in peripheral layers, that is, provides regeneration of epidermis [42, 43].

It is the concentration of retinoids that apparently determines whether they will induce differentiation or, alternatively, de-differentiation and intense proliferation. A proper gradient of retinoic acid concentration is essential for limb formation [44, 45]. In limb buds of vertebrates, it is actively synthesized by a small group of cells localized in the vicinity of the apical ectodermal ridge. As it diffuses through the cell layers, its concentration drops. In cells, the acid is bound by specific nuclear receptors, which are activated and interact with certain DNA sites to alter expression of various genes. The effect of retinoic acid depends on its concentration; however, this effect changes not gradually, but stepwise, because the cell has several grades of response to the continuously changing signal. This leads to appearance of abrupt qualitative difference between the cells lying along the diffusion gradient of morphogen and formation of complex spatial structures. It is obvious that different concentrations of the same morphogen can differently program the cells. Interestingly, *p63* gene knock-out in mice and deficiency of vitamin A, the precursor of retinoic acid, in embryogenesis have very similar consequences, particularly, abnormal limb development and clefts of lip and palate [46]. This suggests that *p63* might represent a direct target for retinoic acid, and it is *p63* via which the acid works as morphogen both in embryogenesis and in limb regeneration in the adult organism.

It has been demonstrated on the newt model that limb regeneration directly depends on the gene *msx-1*, the very gene which ceases to turn on in limb buds of *p63*-knocked-out mice. This gene contains a homeobox (a sequence of 180 bp found in all genes controlling formation of spatial organization of the body) and encodes a protein-suppressor of transcription. Cell destruction on limb amputation activates enzymes initiating local tissue degradation with release of various cells (fibroblasts, chondrocytes, miofibrillae, Schwann cells, etc.), which de-differentiate into precursor cells to form regeneration blastema, a knob consisting of disorderly mass of these cells at the end of the stump. The lost limb is restored from the regeneration blastema with probable involvement of a morphogen, because the blastema of a limb contains both retinoic acid and its receptors. The ability of terminally differentiated cells surrounding the wound to de-differentiation is a prerequisite to regeneration of any lost organ. As it was found, it is the gene *msx-1* that maintains de-differentiated cellular phenotype in amphibia. It is active in early regeneration blastema of newts, and, when injected into myoblasts (precursors of muscle cells), prevents them from differentiation into myofibrillae (mature muscle cells). In developing limbs of murine embryos, the border between differentiated and undifferentiated cells can be determined by expression level of *msx-1*. It is remarkable that expression of *msx-1 in vitro* together with added growth factors de-differentiate even murine myofibrillas, although mammals cannot regenerate the lost body parts [47, 48]. Since *msx-1* ceases to turn on after *p63* knock-out, one can suppose an

existence of the following fragment of signaling pathway: retinoic acid (or some another signal to regeneration)–*p63*–messenger–*msx-1*. Protein p63 possibly reacts to signals stimulating regeneration and activates the genes responsible for de-differentiation of various tissues. It seems that mechanisms providing regeneration and morphogenesis are similar and p63-dependent.

Thus, the spectrum of stimuli activating the function of p63 and p73 is obviously cardinally different from that commonly stabilizing p53 (table). It is a remarkable general tendency: growth signals switch on ΔN-isoforms and switch off (or do not influence) TA-isoforms of p63/p73, whereas differentiation signals (and those inhibiting proliferation or initiating apoptosis), on the contrary, switch on TA-isoforms and switch off ΔN-isoforms. Speaking in images, both *p63* and *p73* are some kind of "salt-and-pepper integration". Depending on the situation, they may exercise opposite effects on the cell—from de-differentiation and active proliferation to terminal differentiation and growth trapping (or apoptosis). Since the control over proliferation and apoptosis is the task of protein p53, here emerges the substantive question: whether p63 and p73 have their own molecular targets or they are mere p53 partners—its assistants or antagonists depending on what N-terminal form, TA or ΔN, they are represented in? Really, in some cases ΔN-isoform stimulates proliferation just by suppression of p53 functions in the cell, whereas TA-isoform terminates the cell cycle or kills the cell as it assists p53 to switch on transcription of p53-dependent genes. At least in a situation of overproduction both TA-p63 and TA-p73 switch on classic targets of p53: $p21^{Waf1/Cip1}$, *mdm2*, *GADD45*, *14-3-3σ*, *cycline-G*, *IGF-BP3*, *BAX*, and *PIG3*. TA-p73 switches on *14-3-3σ* and *GADD45* even more effectively than does p53, although in other cases affinity of p63 and p73 to p53-responsive elements of these genes is appreciably lower than the affinity of p53. The degree of transactivation of these genes somewhat varies among different C-terminal isoforms of p63/p73, and SAM-containing α-isoforms are virtually non-functional in this respect. Nevertheless, application of novel technologies of the search for regulated genes shows that p63 and p73 activities in the cell are by no means limited by their functional interactions with p53: both proteins have their own transcription targets, which are weakly if not activated by other p53-family members. These are unique molecular targets that are of particular interest for identification of p63/p73 functions on the cellular level and for understanding of the purpose and mechanisms of their action in the course of development.

Signals switching on *p53*, *p63*, and *p73*

Gene	Response to stress signals*	Response to signals of differentiation and development**
p53	+	–
p63	? (+/–)	+
p73	? (+/–)	+

* May spontaneously appear in any cell of the body at any time.

** Regularly vary in space (in different tissues and body parts and also in cells with different positional values) and in time (in the course of development of the organism).

Specific targets of p63 and p73. The first candidates for the role of specific targets of protein p73 (after detection of *pheromone receptors V1R/V2R* in knocked-out mice and the *olfactory cell adhesion molecule O-CAM*) were determined from the above-described experiments on induction of neuronal differentiation by either retinoic acid or direct injection of TA-p73 into neuroblastoma cells. They are *neurofilaments* forming the cytoskeleton of axons and dendrites and the *neural cell adhesion molecule N-CAM*, which is expressed on the surface of neural cells to connect them via homophilic N-CAM/N-CAM contacts. These molecular markers of nervous system always appeared in cells after addition of retinoid or switching on TA-p73. Activity of the *N-CAM* gene promoter increased 3-fold after addition of retinoic acid, 12.5-fold after injection of TA-p73, and 28-fold after combined treatment with retinoic acid and TA-p73 (p53 did not activate *N-CAM* in these cells). When the inhibitory ΔN-isoform of p73 was simultaneously injected into cells, the promoter completely discontinued its work even at high doses of retinoid. This suggests that TA-p73 either directly or indirectly (via some messenger) turns on transcription of *N-CAM* and that retinoic acid initiates differentiation of neurons via induction of TA-p73 [38]. Thus, the first evidence has appeared that the role of p73 in the development of the nervous system is not limited by its protection against p53-dependent apoptosis by ΔN-isoforms of p73.

Another candidate, the protein *aquaporin 3* (*AQP3*), is one of 10 members of the aquaporin family, which plays a role of transmembrane transporter of water and glycerol [49]. It was found to be a target for p73 by the method of subtractive hybridization after injection of TA-p73β into cells [50]. The gene *AQP3* is a direct transcription target of p73, because its promoter contains p53-responsive element possessing high affinity to p73 and very low affinity to p53. Transcription of *AQP3* is intensely induced by TA-p73β, much less by TA-p73α, and is scarcely turned on via p53. Aquaporin 3 is localized on the inner side of the cytoplasmic membrane, across which it transports molecules of water and glycerol. It is remarkable that, like *p73*, *AQP3* is intensely expressed by both ependymocytes (a type of glial cells) lining the bottom of brain ventricles and epithelial cells of vascular plexuses of brain cisterns. Both cell types are involved in maintenance of balance between secretion and reabsorption of brain liquor. This fact allows the suggestion that p73 regulates the homeostasis of cerebrospinal liquid by induction of AQP3, and

disbalance of liquor homeostasis leading to severe hydrocephaly in p73-knocked-out mice occurs, since the gene *AQP3* desists from actuation because of the lack of *p73*. Unfortunately, the relationship between AQP3 and p63 has not yet been analyzed; nonetheless, there is just a chance that p63 will also be an inducer of aquaporin. It is already known that AQP3 together with p63 actively participates in regulation of keratinocyte functions, whose differentiation is accompanied by decrease in expression of AQP3 [51]. Moreover, *AQP3*-deficient mice suffer from various skin defects (xerosis with reduced contents of glycerol, inability of skin for water retention and wound healing, etc.) partially overlapping those observed in p63-knocked-out embryos. The notably trenchant argument supporting a hypothesis on relationship between p63 and AQP3 is the fact that newborn p63-knocked-out mice die apparently from severe dehydration of cells and the whole body.

A vast amount of data on p63/p73 targets comes from the method of cDNA microarrays: cell line is transformed by cDNA of the gene of interest, then cellular mRNA is hybridized with array of thousands of known cDNAs to determine the expression of what genes increases or decreases. This approach has already allowed identification of at least three unique targets of p73: *the genes of α_1-antitrypsin*, *CaN19*, and *JAG2*. All three genes are direct transcription targets of TA-p73: the former two have a p73-binding site in the first intron, and *JAG2* in the promoter [52].

α_1-Antitrypsin or *serpin A1* is the major inhibitor of serine proteinases (enzymes destructing proteins), which regulate various physiological processes, such as inflammation, coagulation, fibrinolysis, complement activation, angiogenesis, neoplasia, and apoptosis [53, 54]. The loss of inhibitory activity of serpins leads to imbalance between proteinases and their inhibitors, which is particularly results in hypersensitivity of the immune system, lung emphysema, mental disorders, thromboses, hepatic cirrhosis, etc. α_1-Antitrypsin has a relatively broad spectrum of activity: it inhibits trypsin, chymotrypsin, thrombin, plasmin, kallikrein, elastase, and collagenase. It is responsible for 90% of the antiprotease activity in blood plasma, in which one of its main physiological functions is inhibition of neutrophilic elastases, markers of inflammation and/or infection. Elastases, among other proteolytic enzymes, are accumulated in lysosomes of neutrophils phagocytizing and destroying bacteria and surrounding tissues to develop an inflammatory reaction. Elastases hydrolyze the fibrillar protein elastin, the major component of elastic fibrillae of connective tissue.

A clear correlation exists between α_1-antitrypsin deficiency and intensity of inflammatory response in several tissues. In particular, lung emphysema appears as a result of exposure of aeriferous ducts to proteolytic activity of elastases because of local (often hereditary) deficiency of α_1-antitrypsin. This allows the hypothesis that multiple and diversified inflammatory reactions observed in p73-knocked-out mice also result from p73-dependent deficiency of α_1-antitrypsin and the destructive action of proteases.

It is possible that α_1-antitrypsin plays another role as well. There are interesting data on an important role of serpins in embryonal development. Elevated expression of one of them (pNiXa) was found on oocytes and embryos of *Xenopus* [55]. Five serpins were found in the tunicate *Polyandrocarpa*, whose intense production begins with budding [56]. These serpins inhibit proteinases (trypsin and elastase), which were found to facilitate de-differentiation of forming bud tissues. In mice, a knock-out of one of the serpins, maspin, is lethal, because it impairs the processes of embryogenesis at very early stages of blastocyst development due to suppression of normal formation of cell contacts [57]. This leads to appearance of unorganized endodermal cell mass instead of normal stratified structure. Functions of this serpin are tightly associated with processes of cell adhesion. α_1-Antitrypsin is also expressed in normal mice on differentiation of ectoderm. Serpins seem conducive to cell adhesion with initiation of cell–cell interactions, and their deficiency facilitates cell dissociation, de-differentiation, migration, metastasis, and angiogenesis.

Thus, α_1-antitrypsin as a direct target of TA-p73 not only protects the body against inadequate inflammatory reactions connected with elastase activities, but also possibly regulates proteolysis and cell adhesion in the course of development. It cannot be ruled out that the disturbance of neurogenesis in p73-knocked-out mice is connected with absence of induction of serpin A1, the more so as serpin deficiency is often observed in dementia.

CaN19, or *S100A2*, is a member of S100-family of proteins binding calcium ions [58, 59]. This family has 19 members, which are differently expressed in various tissues. The proteins of this family are involved in Ca^{2+}-dependent (and sometimes Cu^{2+}- or Zn^{2+}-dependent) regulation of intracellular processes, such as enzymatic activity, proliferation, differentiation, cytoskeleton and membrane formation, intracellular homeostasis of calcium, inflammation, and protection from oxidative damage. Some members of S100-family are released into intercellular space, where they have either trophic or toxic effect depending on their concentration, act as chemoattractants for leucocytes, modulate cell proliferation, or regulate activity of macrophages. Some representatives of S100-proteins take part in establishment of cell–cell contacts. As a rule, S100-protein becomes active after binding with calcium. This is accompanied by its conformational rearrangement with opening of binding sites for target proteins. CaN19 binds not only calcium, but also zinc.

CaN19 was first isolated from human keratinocytes as a candidate for regulator of growth and differentiation. It remains unknown what exactly it does in the cell. In

particular, it is actively expressed in basal epidermal cells and epithelial cells of sebaceous glands and hair follicles. CaN19 demonstrates a contradictory behavior in tumor cells, in particular, it often ceases to work in tumors of glandular genesis, whereas in tumors of epidermal origin it is hyperactive. So, the initial hypothesis on oncosuppressor functions of CaN19 seems to be incorrect. In keratinocytes, epidermal growth factor (EGF) stimulates expression of CaN19 on the transcription level, and in skin injury the protein quickly accumulates in the cells of the hyperplastic zone surrounding the wound. So, it is probable that CaN19 is involved in processes or epidermis regeneration. As a mediator of regeneration, it might be simultaneously connected both with regenerative hypoplasia and regenerative differentiation (it was already mentioned that calcium stimulates differentiation of keratinocytes).

It is completely unclear in what measure p73 is involved in these processes. It is more probable that it is p63 that is responsible for switching on *CaN19* in keratinocytes, because its activity is high in these cells. It was found recently that p63 does activate the *CaN19* promoter due to binding with the same responsive element that the protein p73 binds with, although with less affinity. Surprisingly, ΔN- more than TA-isoform of p63 is responsible for activation of the promoter in this case [60]. As shown, ΔN-p63 many times elevates expression of CaN19. One can offer two explanations for this fact: either the second byway transactivation domain of p63 (TA2) localized nearer to the C-end of the protein is responsible for transactivation of the promoter or a messenger protein rather than ΔN-p63 itself activated by ΔN-isoform turns on transcription of *CaN19* [12]. It remains unclear what the effects of switching on *CaN19* via p73 are and in what tissues they are most expressed. Since several members of S100-family take part in nervous system development and functioning and p73 is well expressed just in neural tissue, one can suppose that effects of its interaction with *CaN19* should be found in nervous cells.

JAG2 (Jagged 2), a transmembrane protein, is one of main ligands of the evolutionally ancient *Notch*-receptor [61]. The gene *Notch* was first found in drosophila. Partial loss of its function leads to roughness of the wing edge, and its complete switching-out is lethal. It is accompanied by development of "neurogenic" phenotype, when embryonic cells destined normally to be epidermal turn into neural cells. A gene with the same properties was identified in nematodes (*LIN-12*) and then in various organisms including humans [62]. This gene encodes a transmembrane receptor with large extracellular and intracellular domains. The extracellular domain contains 36 tandem EGF-like repeats and three cysteine-rich Notch/LIN12 repeats. In vertebrates this domain interacts with two ligand types: Jagged (JAG1 and JAG2) and Delta, which, like does Notch, intersect the cytoplasmic membrane of the cell. A contact between the extracellular domain of Notch-receptor of one cell and its Jagged/Delta-ligand of a neighboring cell induces proteolytic release of the intracellular domain of Notch-receptor which turns on transcription of a series of genes involved in modulation of both cell proliferation and differentiation.

A major effect of this interaction between adjacent cells is that they choose their destiny at the time of the contact: originally equal cells become different after activation of Notch-pathway. In particular, it is the activation of Notch in ectodermal precursors of epithelial and nervous cells that determines the moment of differentiation of cells into epidermal and neuronal lines. The Notch-dependent mechanism of predisposition of cell fates is based on Notch-realized amplification of an insignificant initial difference in receptor and ligand concentration on membranes of adjacent cells. This amplification seems to occur by the positive feedback loop principle: the Notch protein elevates expression of the gene *Notch* in the same cell, and the protein Jagged/Delta elevates expression of the gene *Jagged/Delta*. As a result, one cell begins to express only the receptor, whereas another one expresses only the ligand. Destinies of these cells diverge, because only one of them turns on Notch-dependent genes.

In some cases initially weak difference in receptor and ligand concentrations, which is thereafter amplified via Notch, only accidentally appears between identical cells, whereas in other cases it occurs by the action of some intra- or extracellular factors (as precursor cells compared by these factors are not equal). An example of intracellular factors is the protein Numb, which is asymmetrically distributed between precursor cells of sensory organs after each of their divisions. Numb suppresses the Notch activity, so only one of two neighboring cells expressing both Notch-receptor and its ligand will respond to the receptor stimulation. An example of an extracellular factor is gradient of a signal expanding from the equator of imaginal disks of drosophila and elevating expression of Delta: expression of Notch-receptor and its ligand and, hence, the fate of cells depend on their distance from the source of signal. Whether or not, the interactions of neighboring cells via Notch-Jagged/Delta lead to predetermination of cell destiny with segregation of individual cell lines from the cluster of identical precursor cells, which is absolutely necessary both for tissue formation in multicellular organism and establishment of morphological borders demarcating cell fields (dorsoventral, mediolateral, and rostrocaudal borders of developing primordial organs, border surfaces between somites, etc.). The gene *Notch* reacts to chemical asymmetry of the body, which is determined by retinoic acid and extracellular calcium gradients and by spreading "waves" of protein products of "cyclic" genes (turning on periodically at certain time intervals), and translates it into asymmetric gene expression [63-65].

Thus, Notch is one of the universal instruments of organism development from single cell to complex multicellular 3-D structure. Inactivation of *Notch* is lethal for an organism, and impairments of distinct elements of Notch-pathway severely affect developmental processes. This particularly concerns mutations of *Jagged* genes. The functional deficiency of JAG1 in humans results in Alagille syndrome with abnormal development of heart, lungs, skeleton, eyes, face, and other body parts and organs [66]. Partial deficiency of JAG-2 in mice is accompanied by developmental defects of limbs and craniofacial structures, which closely resemble EEC-defects characteristic of p63-deficiency in patients with ectrodactyly, ectodermal dysplasia, and lip and palate clefts. Transcription of the gene *JAG2* is launched by both p73 and p63, which recognize the same binding site in its promoter region.

Using the method of chromatin immunoprecipitation, it was found that the second intron of the gene *JAG1* also contains "p53"-responsive elements (four copies). This element possesses high affinity to p63; however, p73 also seems to interact with them because both the proteins activate the gene. The protein p53 itself cannot influence *JAG1* and *JAG2* functions, because its affinity to their "p53"-responsive elements is very weak. Although p63/p73 turn on both the genes, p63 probably "specializes" in activation of *JAG1*, whereas p73 mainly activates *JAG2*. It has been already reported that p63 is directly involved in Notch-pathway activation [67]. This was demonstrated by mutual cultivation of two different cell lines on one dish. When p63 was inserted into one line (Saos2), the cells of this line began to express JAG1, whereas the cells of another line (Jurkat) expressed Notch-receptor and mRNA of the gene *Hes1*, whose transcription is launched via Notch. All the above-mentioned facts provide evidence for direct involvement of p63 and p73 in fundamental development processes and their possible participation in diverging of neuronal and epithelial pathways of ectoderm development and formation of multicellular organism from a fertilized ovum.

A directed search for specific gene targets of p63 and p73, which are distinct from those of p53, is now in progress. Experiments on induction of keratinocyte differentiation by calcium ions have demonstrated that TA-p63 turns on the genes encoding *involucrin*, *loricrin*, *transglutaminase 1*, and several *keratins*. These proteins are characteristic markers of differentiated epidermis and play specific functions in its different layers. In particular, involucrin provides a cross-linking of plasma membranes in cornifying ramenta. It is yet unknown whether p63 activates directly or indirectly these genes. There is an activity gradient along TA-p63, TA-p73, and p53: TA-p63 possesses maximum and p53 the minimum ability to turn on their transcription [36, 68].

DNA microarrays revealed that transcription of most p63-dependent genes varies in 2-3 times (5 times maximum). Interestingly, these genes include the *aldehyde dehydrogenase 6* gene, controlling one of the stages of vitamin A conversion into retinoic acid. However, five genes stand out against this background by enormous change in their expression. TA-p63 reduces transcription of genes encoding *interleukin 8* (*IL8*) and *beta-thromboglobulin* (*BTG-like*) 58- and 50-fold, respectively, whereas ΔN-p63 elevates transcription of genes encoding *heat shock proteins* (*Hsp70* and *Hsp70B*) and *ketohexokinase* (*KHK*) 15.8-, 28-, and 52-fold, respectively [69]. Obviously, these great alterations in expression of these genes cannot be accidental. Neither p53 nor p73 influence their work.

The TA- and ΔN-isoforms of protein p63 exercise opposite effects on expression of *IL8* and *BTG* genes: ΔN-p63 activates their function by a mechanism that is still unclear. Although the two genes belong to different families, they show significant resemblance, both structural and functional [70-73]. Initially their activities were identified in blood cells: IL8 in neutrophils, lymphocytes, and monocytes, and BTG in platelets. Both the proteins are chemokines (that is, stimulate chemotaxis, a directed cell motion along a gradient of concentration), and IL8 is a cytokine (cell growth stimulator) as well.

When a blood vessel is damaged, BTG is released from platelets into surrounding space with dual effect on the wound. First, it counteracts the synthesis of prostacyclin by endotheliocytes (lining the inner surface of vessels), which both maintains thromboresistance of the vessel wall in absence of lesion and suppresses platelet aggregation. In doing this, BTG provides clot formation and repair of the vessel. In the second place, it acts as chemoattractant for fibroblasts which quickly begin to migrate to the wound and repair the damaged tissue.

IL8 is actively synthesized in traumas by vessel endothelium and causes activation of leucocytes, in the first instance neutrophils, and their migration to the wound, in which they exterminate bacteria and damaged cells. Moreover, IL8 dramatically increases adhesion of leucocytes to endothelium, thus enabling them to get out the blood flow and penetrate into tissues. IL8 is involved both in inflammatory and reparative processes: it provides chemotaxis of fibroblasts, keratinocytes, and endothelial cells to the locus of injury and stimulates proliferation of these cells as well. Fibroblasts produce molecules of extracellular matrix to "granulate" the wound, keratinocytes repair epidermis, and endotheliocytes form blood vessels. IL8 is expressed not only by various leucocyte types, but also by fibroblasts and epi- and endothelial cells, and its receptors are found on multiple cell types. It is one of the risk factors for cancer, because it elevates proliferative and especially migratory ability of transformed cells and their migration across vessel endothelium, which facilitates metastasis. Moreover, it stimulates neoangiogenesis, that is, emergence of new blood vessels into tumors. In cancer cells, the level of IL8 elevates with

their de-differentiation and increase in aggressiveness [74, 75].

The protein p63 sharply changes (TA decreases, whereas ΔN increases) the levels of BTG and IL8. This proves that p63 can alter (via these proteins) behavior of not only the cells expressing it, but also cells comprising surrounding tissues and blood, which is of particular importance for regeneration and development. ΔN-p63 seems to stimulate their proliferation and migration, whereas TA-p63 terminates these reactions at the moment when regeneration passes from "accumulation" of required cells to terminal differentiation and cessation of growth. In particular, such cooperation of isoforms might occur on vessel formation or restoration: when the process is completed, the vessel endothelium should lose sensitivity to platelets (otherwise clots will be continuously formed). Suppression of both BTG and IL8 on actuation of TA-p63 restores both the level of prostacyclin and thromboresistance and stops unnecessary migration and division of cells to provide their differentiation. The fact that ΔN-p63 is often intensively expressed in cancer can be explained by means that IL8, which is a target for ΔN-p63, gives selective advantage to cancer cells in migration and proliferation, and stimulates blood supply to tumors. The *IL8* transcription decreases up to 60-fold after cell administration with TA-p63, which is strong indicator for the application of TA-p63 to gene therapy of cancer.

Heat shock proteins Hsp70 and *Hsp70B* work in the cell primarily as molecular chaperones: they dissolve and refold denatured or irregularly folded proteins. If the cell cannot for a long time solve the problem of protein folding, the program of apoptosis is initiated. The portion of damaged proteins dramatically increases under heat shock or other cell stresses, so the stress is accompanied by increased production of chaperones. Among them, some proteins provide induction of apoptosis, whereas some other proteins inhibit apoptosis, thus assisting the cell to survive under lethal conditions. The proteins Hsp70 and Hsp70B belong to the group of protein inhibitors of cell death [76-78]. Their activity elevates the apoptotic threshold of the cell and may be conducive to carcinogenesis. Overproduction of these proteins is often found in malignant tumors. The chaperones of the Hsp70 family are also critical for normal passing of the processes of embryonic development: they are active from the very early stages of embryogenesis and are necessary for normal sperm maturation [79, 80]. Because the ΔN-isoform of p63 many times elevates transcription of the genes *Hsp70* and *Hsp70B*, it thereby highly increases both viability of the cells and intensity of protein folding. Although this enhanced expression of chaperones is proper under normal physiological conditions (in particular, in embryogenesis) and essential for fast cell mass accumulation and differentiation, it is dangerous under lethal conditions (such as oxidative stress and oncogene-induced cell transformation), because it results in abnormal lack of susceptibility of affected cells to signals of death.

Ketohexokinase (or *fructokinase*) is an enzyme catalyzing the first step of fructose assimilation: the transfer of a phosphate group from ATP to fructose to produce fructose-1-phosphate and ADP [81]. Fructokinase possesses very high affinity to its substrate but cannot metabolize glucose. It has been found in great amounts in human liver, pancreas, kidney, and intestine; however, its basal level seems to be maintained in all cells of the body. This enzyme uniformly acts in different branches of the evolutionary tree, from very primitive prokaryotes. It was found in extremely halophilic (living in very salt habitats) archaebacterium *Haloarcula vallismortis* [82]. In liver cells, fructokinase is 4-fold more active than glucokinase. This is apparently because fructose is assimilated faster than glucose [83].

Investigations of metabolic changes in cancer cells have been concentrated on glucose for a long time. Naturally, the accelerated proliferation of cells requires intensification of metabolic processes accompanied by the release of large amounts of energy, and oxygen deficiency conditions surrounding transformed cells deep in the tumor which is not reached by capillaries stimulates processes of anoxic glucose metabolism, that is, glycolysis. The intensive consumption of glucose and its glycolytic cleavage are well-known features of cancer cells. However, fructose metabolism through the glycolytic pathway occurs much faster than that of glucose. This is due to higher activity of fructokinase as well as because fructose escapes one very slow stage of the glucose cleavage (catalyzed by phosphofructokinase). Therefore, fructose in comparison with glucose substantially accelerates metabolic processes. In mammals, fructose is actively secreted into the blood circulatory system of the fetus; it is present in amniotic and seminal fluids. This is evidence for its important role in embryogenesis, when energy requirement of cells is drastically elevated, as in carcinogenesis. There are evidences for mito- and angiogenic properties of fructose: fructose-1-phosphate stimulates proliferation in neuronal precursors, and the treatment of endothelial cells with the vessel endothelium growth factor VEGF inducing the growth of blood vessels is accompanied by the elevation of fructokinase gene transcription in endotheliocytes [84, 85].

Thus, is seems that the rapidly dividing cells take energetic advantage from the switch of sugar metabolic pathways to the way of fructose cleavage, and additional energy sources become accessible. Since just the fructokinase is responsible for this "switch" being a catalyst for the first stage on this pathway, its activation can be considered as actuation of mechanisms of "second wind" during rapid cell growth. When ΔN-p63 becomes active, the fructokinase gene expression increases 50-fold and more, thus evidencing for linkage between ΔN-isoforms and mechanisms accelerating metabolic and proliferative processes.

It is obvious now that ΔN-isoforms facilitate cell proliferation due to inactivation of p53 as well as due to their own unique activity [86]. Direct confirmation of this fact was found from studies on p73 functions *in vitro*: the treatment of normal human fibroblasts with ΔN-p73β after removal of growth factors induces their p53-independent division. This is because ΔN-p73 in some way influences cyclin-dependent kinases phosphorylating the Rb protein resulting in its hyperphosphorylation [87]. Another p53-independent link between ΔN-isoforms and cyclin-dependent kinases was found from microarrays: ΔN-p63 3.5-fold elevates transcription of genes encoding serine-threonine kinases *PCTAIRE2* and *PCTAIRE3* homologous to the yeast kinase cdc2 (cell division cycle) and its analog in higher eukaryotes, CDK1 [69]. Great amounts of PCTAIRE proteins are found in both neurons and spermatozoa [88, 89]. Their functions remain unclear, but they were named because of the substitution of serine by cysteine in the conservative sequence PSTAIRE, through which all cyclin-dependent kinases interact with cyclin. It has been demonstrated using microarrays that despite opposite effects of ΔN- and TA-isoforms some gene targets are specific for either ΔN- or TA-variants of p63/p73.

What can be inferred from known functions of the p63 and p73 targets identified to date? Although the search for specific targets is only begun, already known facts are clearly indicative of the "vital aims" of p63/p73 which are not characteristic of p53. To generalize from the above facts, one can set several opposite cellular effects of TA- and ΔN-isoforms of p63/p73, respectively: *cell cycle arrest – induction of proliferation; apoptosis – insusceptibility to death signals; differentiation – de-differentiation; divergence of developmental pathways of adjacent cells – maintenance of cell uniformity; adhesion (cell–cell and cell–substrate conjunction) – suppression of adhesion (cell autonomy); inhibition of both chemotaxis and migration – stimulation of chemotaxis and cell motility; possible stabilization of metabolism (TA-function is unknown) – acceleration of metabolism.*

From this list of functional activities, only the cell cycle arrest and induction of apoptosis are typical for p53, wherein it is without a rival: neither TA-p63 nor TA-p73 can equal this. Although p53, p63, and p73 act as transcription factors with similar DNA-binding domains, promoter specificity of target genes and activity of ΔN-isoforms result in great differences in spheres of their influences on the cell genome. Overlapping effects of p53, p63, and p73 observed in some experiments *in vitro* result apparently only from protein overproduction causing activity that is not typical for this protein under natural conditions. It should be noted again that p63 and p73 cannot completely compensate the functions of p53 at least because they are switched on in response to the growth and differentiation signals, but not stress, and are working in distinct tissues only. However, all cells in the organism need protection from cancerous transformation. For this protection a protein is necessary, which is working ubiquitously and finely sensing the stress condition. Only p53 corresponds to these criteria. And participation of p63 and p73 in proliferation and apoptosis control may be either p53-dependent or independent. Possibly, they are targeted to their own aims connected with execution of organism development programs rather than with protection from genetic instability.

Some differences between the actions of p53, p63, and p73 at the molecular level result in significant discrepancies in their actions on the cell and yet more contrast their effects at the level of whole organism, which is clearly seen on knock-out mice. At the level of the organism, p63 and p73 control embryonal and post-embryonal development including morpho- and organogenesis, regeneration of organs and reparation of wounds, growth of blood vessels and neuron outgrowths, immunity and homeostasis of a number of tissues, and even complex behavioral responses in sexual behavior. All these processes need fine and flexible coordination of the work of large amounts of cell masses that must be governed by social needs of the whole organism and rearrange their activities in agreement with its necessities at the given moment. At some developmental stages, the cells require more autonomy with loss of cell–cell contacts, impetuous growth, de-differentiation, or active migration into other bodily parts. In other situations the cells must be arrested (or even die for formation of required structure), choose their "destiny" and differentiate, stop moving and form cell–cell contacts, that is, become static and highly specialized members of a society called the "multicellular organism". The situations can be met when both processes must go in parallel (for instance, when regeneration occurs, it is necessary to coordinate the processes of de-differentiation and hyperplasia with differentiation and inhibition of proliferation). It is easy to see that p63 and p73 with their competing TA- and ΔN-isoforms are ideally suited for the role of regulators of processes like these. It is apparent also that TA-isoforms support social behavior of cells, and ΔN support individualistic or even aggressive, more typical for single-cell organisms or cancerous cells. Nevertheless, since nature has selected ΔN-isoforms, they are indispensable for life despite their potential danger. It is not inconceivable that a cell can return to the "springhead" of its development with their help, when the organism still exists as undifferentiated zygote and/or blastomeres.

Thus, a gradient apparently exists along the functions of p53 family members directed from the support of developmental programs by p63 and p73 proteins to the support of genetic stability of the organism preferentially by p53 protein. Most probably, the second function appeared secondarily in evolution, after the multicellular organisms have firmly settled the Earth and began a strug-

gle for existence. Then organisms with stable genome effectively countering cancerous transformation acquired certain advantages, because their life was more prolonged and progeny were more numerous. Before p53 appeared, TA-p63 or TA-p73 possibly fulfilled its functions in greatly weaken form. When some successful modification of *p63* or *p73* gene happened generating the gene named "*p53*", a protein appeared with special purpose to protect the cell from cancer transformation. This led to a great advance in evolution of the mutant organism and "fixation" of *p53* gene in evolutionary tree with subsequent divergence of functions among all three members of the family.

REFERENCES

1. Futreal, P. A., Kasprzyk, A., Birnney, E., Mullikin, J. C., Wooster, R., and Stratton, M. R. (2001) *Nature*, **409**, 850-855.
2. Giaccia, A. J., and Kastan, M. B. (1998) *Genes Dev.*, **12**, 2973-2983.
3. Derry, W. B., Putzke, A. P., and Rothman, J. H. (2001) *Science*, **294**, 591-595.
4. Ollmann, M., Young, L. M., Como, C. J., Karim, F., Belvin, M., Robertson, S., Whittaker, K., Demsky, M., Fisher, W. W., Buchman, A., Duyk, G., Friedman, L., Prives, C., and Kopczynski, C. (2000) *Cell*, **101**, 91-101.
5. Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D., and McKeon, F. (1998) *Mol. Cell.*, **2**, 305-316.
6. Little, N. A., and Jochemsen, A. G. (2002) *IJBCB*, **34**, 6-9.
7. Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J.-M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. (1997) *Cell*, **90**, 809-819.
8. Yang, A., Kaghad, M., Caput, D., and McKeon, F. (2002) *Trends Genet.*, **18**, 90-95.
9. Irwin, M. S., and Kaelin, W. G. (2001) *Cell Growth Differ.*, **12**, 337-349.
10. Thanos, C. D., and Bowie, J. U. (1999) *Protein Sci.*, **8**, 1708-1710.
11. Schultz, J., Ponting, C. P., Hofmann, K., and Bork, P. (1997) *Protein Sci.*, **6**, 249-253.
12. Ghioni, P., Bolognese, F., Duijf, P. H. G., Bokhoven, H., Mantovani, R., and Guerrini, L. (2002) *Mol. Cell. Biol.*, **22**, 8659-8668.
13. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992) *Nature*, **356**, 215-221.
14. Mills, A. A., Zheng, B., Wang, X., Vogel, H., Roop, D. R., and Bradley, A. (1999) *Nature*, **398**, 708-713.
15. Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., Tabin, C., Sharpe, A., Caput, D., Crum, C., and McKeon, F. (1999) *Nature*, **398**, 714-718.
16. Celli, J., Duijf, P., Hamel, B. C. J., Bamshad, M., Kramer, B., Smits, A. P. T., Newbury-Ecob, R., Hennekam, R. C. M., Buggenhout, G. V., Haeringen, A., Woods, C. G., Essen, A. J., Waal, R., Vriend, G., Haber, D. A., Yang, A., McKeon, F., Brunner, H. G., and Bokhoven, H. (1999) *Cell*, **99**, 143-153.
17. McGrath, J. A., Duijf, P. H., Doetsch, V., Irvine, A. D., Waal, R., Vanmolkot, K. R., Wessagowit, V., Kelly, A., Atherton, D. J., Griffiths, W. A., Orlow, S. J., van Haeringen, A., Ausems, M. G., Yang, A., McKeon, F., Bamshad, M. A., Brunner, H. G., Hamel, B. C., and Bokhoven, H. (2001) *Hum. Mol. Genet.*, **10**, 221-229.
18. Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikker, P., Sharpe, A., McKeon, F., and Caput, D. (2000) *Nature*, **404**, 99-102.
19. Chumakov, P. M. (2000) *Biochemistry (Moscow)*, **65**, 28-40.
20. May, P., and May, E. (1999) *Oncogene*, **18**, 7621-7636.
21. Michael, D., and Oren, M. (2002) *Curr. Opin. Genet. Dev.*, **12**, 53-59.
22. Ashcroft, M., and Vousden, K. H. (1999) *Oncogene*, **18**, 7637-7643.
23. Ryan, K. M., Phillips, A. C., and Vousden, K. H. (2001) *Curr. Opin. Cell Biol.*, **13**, 332-337.
24. El-Deiry, W. S., Kern, S. E., Pitenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) *Nat. Genet.*, **1**, 45-49.
25. Cho, Y. J., Gorina, S., Jeffrey, P. D., and Pavletich, N. P. (1994) *Science*, **256**, 346-355.
26. Sigal, A., and Rotter, V. (2000) *Cancer Res.*, **60**, 6788-6793.
27. Kopnin, B. P. (2000) *Biochemistry (Moscow)*, **65**, 2-27.
28. Dragovich, T., Rudin, C. M., and Thompson, C. B. (1998) *Oncogene*, **17**, 3207-3213.
29. Schuler, M., and Green, D. R. (2001) *Biochem. Soc. Trans.*, **29**, 684-688.
30. Ding, Y., Inoue, T., Kamiyama, A. J., Tamura, Y., Ohtani-Fujita, N., Igata, E., and Sakai, T. (1999) *DNA Res.*, **6**, 347-351.
31. Strano, S., Rossi, M., Fontemaggi, G., Munarriz, E., Soddu, S., Sacchi, A., and Blandino, G. (2001) *FEBS Lett.*, **490**, 163-170.
32. Ishimoto, O., Kawahara, C., Enjo, K., Obinata, M., Nukiwa, T., and Ikawa, S. (2002) *Cancer Res.*, **62**, 636-641.
33. Irwin, M. S., and Kaelin, W. G., Jr. (2001) *Apoptosis*, **6**, 17-29.
34. Benard, J., Douc-Rasy, S., and Ahomadegbe, J. C. (2003) *Hum. Mutat.*, **21**, 182-191.
35. Nakagawa, T., Takahashi, M., Ozaki, T., Watanabe, K., Todo, S., Mizuguchi, H., Hayakawa, T., and Nakagawara, A. (2002) *Mol. Cell. Biol.*, **22**, 2575-2585.
36. Levrero, M., Laurenzi, V., Cotanzo, A., Sabatini, S., Gong, J., Wang, J. Y. J., and Melino, G. (2000) *J. Cell Sci.*, **113**, 1661-1670.
37. Pozniak, C. D., Radinovic, S., Yang, A., McKeon, F., Kaplan, D. R., and Miller, F. D. (2000) *Science*, **289**, 304-306.
38. Laurenzi, V., Raschella, G., Barcaroli, D., Annicchiarico-Petruzzelli, M., Ranalli, M., Catani, M. V., Tanno, B., Costanzo, A., Levrero, M., and Melino, G. (2000) *J. Biol. Chem.*, **275**, 15226-15231.
39. Kerszberg, M. (1999) *Semin. Cell Dev. Biol.*, **10**, 297-302.
40. Tabata, T., and Takei, Y. (2004) *Development*, **131**, 703-712.
41. Maden, M., and Hind, M. (2003) *Dev. Dynamics*, **226**, 237-244.
42. Parsa, R., Yang, A., McKeon, F., and Green, H. (1999) *J. Invest. Dermatol.*, **113**, 1099-1105.

43. Bamberger, C., Pollet, D., and Schmale, H. (2002) *J. Invest. Dermatol.*, **118**, 133-138.
44. Maden, M. (2002) *Int. J. Dev. Biol.*, **46**, 883-886.
45. Yashiro, K., Zhao, X., Uehara, M., Yamashita, K., Nishijima, M., Nishino, J., Saijoh, Y., Sakai, Y., and Hamada, H. (2004) *Dev. Cell*, **6**, 411-422.
46. Morriss-Kay, G. M., and Ward, S. J. (1999) *Int. Rev. Cytol.*, **188**, 73-31.
47. Odelberg, S. J., Kollhoff, A., and Keating, M. T. (2000) *Cell*, **103**, 1099-1109.
48. Stocum, D. L. (2004) *Curr. Top. Microbiol. Immunol.*, **280**, 1-70.
49. Ishibashi, K., Sasaki, S., Fushimi, K., Uchida, S., Kuwahara, M., Saito, H., Furukawa, T., Nakajima, K., Yamaguchi, Y., Gojobori, T., and Marumo, F. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 6269-6273.
50. Zheng, X., and Chen, X. (2001) *FEBS Lett.*, **489**, 4-7.
51. Zheng, X., and Bollinger, B. W. (2003) *J. Invest. Dermatol.*, **121**, 1487-1495.
52. Fontemaggi, G., Kele, I., Amariglio, N., Rechavi, G., Krishnamurthy, J., Strano, S., Sacchi, A., Givol, D., and Blandino, G. (2002) *J. Biol. Chem.*, **277**, 43359-43368.
53. Morgan, K., and Kalsheker, N. A. (1997) *IJBCB*, **29**, 1501-1511.
54. Janciauskiene, S. (2001) *Biochim. Biophys. Acta*, **1535**, 221-235.
55. Sunderman, F. W., Jr., Varghese, A. H., Kroftova, O. S., Grbac-Ivankovic, S., Kotyza, J., Datta, A. K., Davis, M., Bal, W., and Kasprzak, K. S. (1996) *Reprod. Dev.*, **44**, 507-524.
56. Kawamura, K., Hayata, D., Fujiwara, S., and Yubisui, T. (1998) *J. Biochem. (Tokyo)*, **124**, 1004-1012.
57. Gao, F., Shi, H. Y., Daughty, C., Cella, N., and Zhang, M. (2004) *Development*, **131**, 1479-1489.
58. Xia, L., Stoll, S. W., Liebert, M., Ethier, S. P., Carey, T., Esclamado, R., Carroll, W., Johnson, T. M., and Elder, J. T. (1997) *Cancer Res.*, **57**, 3055-3062.
59. Feng, G., Xu, X., Youssef, E. M., and Lotan, R. (2001) *Cancer Res.*, **61**, 7999-8004.
60. Hibi, K., Fujitake, S., Takase, T., Kodera, Y., Ito, K., Akiyama, S., Shirane, M., and Nakao, A. (2003) *Clin. Cancer Res.*, **9**, 4282-4285.
61. Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999) *Science*, **284**, 770-776.
62. Greenwald, I. (1998) *Genes Dev.*, **12**, 1751-1762.
63. Freemantle, S. J., Kerley, J. S., Olsen, S. L., Gross, R. H., and Spinella, M. J. (2002) *Oncogene*, **21**, 2880-2889.
64. Raya, A., Kawakami, Y., Rodriguez-Esteban, C., Ibanes, M., Rasskin-Gutman, D., Rodriguez-Leon, J., Buscher, D., Feijo, J. A., and Izpisua-Belmonte, J. C. (2004) *Nature*, **427**, 121-128.
65. Freitas, C., Rodrigues, S., Charrier, J. B., Teillet, M. A., and Palmeirim, I. (2001) *Development*, **128**, 5139-5147.
66. Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J., Costa, T., Pierpont, M. E., Rand, E. B., Piccoli, D. A., Hood, L., and Spinner, N. B. (1997) *Nat. Genet.*, **16**, 243-251.
67. Sasaki, Y., Ishida, S., Morimoto, I., Yamashita, T., Kojima, T., Kihara, C., Tanaka, T., Imai, K., Nakamura, Y., and Tokino, T. (2002) *J. Biol. Chem.*, **277**, 719-724.
68. Laurenzi, V., Rossi, A., Terrinoni, A., Barcaroli, D., Levrero, M., Costanzo, A., Knight, R. A., Guerrieri, P., and Melino, G. (2000) *Biochem. Biophys. Res. Commun.*, **273**, 342-346.
69. Wu, G., Nomoto, S., Hoque, M. O., Dracheva, T., Osada, M., Lee, C. R., Dong, S. M., Guo, Z., Benoi, N., Cohen, Y., Rechthand, P., Califano, J., Moon, C., Ratovitski, E., Jen, J., Sidransky, D., and Trink, B. (2003) *Cancer Res.*, **63**, 2351-2357.
70. Zhang, W., and Chen, H. (2002) *Sheng Wu Yi Xue Gong Cheng Xue Za Zhi.*, **19**, 697-702.
71. Xie, K. (2001) *Cytokine Growth Factor Rev.*, **12**, 375-391.
72. Damme, J., Rampart, M., Conings, R., Decock, B., Osselaer, N., Willems, J., and Billiau, A. (1990) *Eur. J. Immunol.*, **20**, 2113-2118.
73. Resmi, K. R., and Krishnan, L. K. (2002) *Thromb. Res.*, **106**, 229-236.
74. Ivarsson, K., Ekerydh, A., Fyhr, I. M., Janson, P. O., and Brannstrom, M. (2000) *Acta Obstet. Gynecol. Scand.*, **79**, 777-784.
75. Lin, Y., Huang, R., Chen, L., Li, S., Shi, Q., Jordan, C., and Huang, R. P. (2004) *Int. J. Cancer*, **109**, 507-515.
76. Garrido, C., Gurbuxani, S., Ravagnan, L., and Kroemer, G. (2001) *Biochem. Biophys. Res. Commun.*, **286**, 433-442.
77. Mosser, D. D., and Morimoto, R. I. (2004) *Oncogene*, **23**, 2907-2918.
78. Beere, H. M., Wolf, B. B., Cain, K., Mosser, D. D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R. I., Cohen, G. M., and Green, D. R. (2000) *Nat. Cell. Biol.*, **2**, 469-475.
79. Luft, J. C., and Dix, D. J. (1999) *Cell Stress Chaperones*, **4**, 162-170.
80. Eddy, E. M. (1999) *Rev. Reprod.*, **4**, 23-30.
81. Hayward, B. E., and Bonthron, D. T. (1998) *Eur. J. Biochem.*, **257**, 85-91.
82. Rangaswamy, V., and Altekar, W. (1994) *J. Bacteriol.*, **176**, 5505-5512.
83. Heinz, F., Lamprecht, W., and Kirsch, J. (1968) *J. Clin. Invest.*, **47**, 1826-1832.
84. Nielsen, F. C., and Gammeltoft, S. (1990) *FEBS Lett.*, **262**, 142-144.
85. Wary, K. W., Thakker, G. D., Humtsoe, J. O., and Yang, J. (2003) *Mol. Cancer*, **2**, 25-36.
86. Kartasheva, N. N., Lenz-Bauer, C., Hartmann, O., Schafer, H., Eilers, M., and Dobbelstein, M. (2003) *Oncogene*, **22**, 8246-8254.
87. Stiewe, T., Stanelle, J., Theseling, C. C., Pollmeier, B., Beitzinger, M., and Putzer, M. (2003) *J. Biol. Chem.*, **278**, 14230-14236.
88. Besset, V., Rhee, K., and Wolgemuth, D. J. (1999) *Cell Growth Differ.*, **10**, 173-181.
89. Herskovits, A. Z., and Davies, P. (2004) *Gene*, **328**, 59-67.