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Review

Regulation of $p27^{Kip1}$ and $p57^{Kip2}$ functions by natural polyphenols

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Abstract: In numerous instances, the fate of a single cell not only represents its peculiar outcome but also contributes to the overall status of an organism. In turn, cell division cycle and its control strongly influence cell destiny playing a critical role in targeting it towards a specific phenotype. Several factors participate to the control of growth and among them p27 and p57, two proteins modulating various transitions of cell cycle, appear to play key functions. In this review, the major features of p27 and p57 will be described, focusing in particular on their recently identified roles not directly correlated to cell cycle modulation. Then, their possible role as molecular effectors of polyphenols activities are discussed. Polyphenols represent a large family of natural bioactive molecules that have been demonstrated to play promising protective activities against several human diseases. Their use has also been proposed in association with classical therapies for ameliorating their clinical effects and for diminishing their negative side activities. The importance of p27 and p57 in polyphenol cellular effects will be discussed with the aim of identifying novel therapeutic strategies for the treatment of important human diseases, such as cancers, characterized by an altered control of growth.

Keywords: $p27^{Kip1}$; $p57^{Kip2}$; polyphenols; EGCG, resveratrol

1. Introduction

During the last two decades, polyphenols has gained a huge interest as potential adjuvant cancer drugs and cancer-preventing molecules. In vitro studies underlined that the beneficial proprieties of polyphenols rely in part on their antioxidant activity [1, 2] and in part on their ability to modulate signaling pathways related to cell cycle progression and cell survival [3, 4]. Convergent points of these cellular processes are represented by cyclin-dependent kinase (CDK)s with their modulatory partners, cyclins, and CDK inhibitors (CDKIs). Particularly, CDKI proteins play fundamental roles in tuning the activity of CDK complexes in response to endogenous and exogenous stimuli, including drug treatments. Regarding CDKI classification, two families are recognized: p16INK4a, p15INK4b, p18INK4c, p19INK4d belong to the INK4 (INhibitors of CDK4(6) family, while p21Cip1, p27Kip1 and p57Kip2 (hereinafter p21, p27, p57) are members of the CIP (CDK Interacting Proteins)/KIP (Kinase Inhibitory Proteins) family [5].

For a long time, CIP/Kip protein functions have been mainly related to their ability to bind and inhibit different Cyclin-CDK targets [6-8]. As a matter of facts, these proteins restrain growth during: i) development; ii) differentiation, and iii) response to various cellular stresses and drug treatments. It is to underline that, although apparently redundant, each CIP/Kip member cannot be completely surrogated by its siblings, suggesting specific roles [9]. p21 is mainly, but not only, a p53 effector and is up-regulated when p53 is activated, as is the case, for example, in response to different DNA-damaging conditions [10]. This CDKI mediates cell cycle arrest in G1 and G2 phases to allow DNA repair or to induce apoptosis when the damage cannot be repaired. Alternatively, p53-independent signaling, cell growth factors (like PDGF, FGF and EGF) or epigenetic modulators have been reported to be capable to activate p21 expression [11]. p27 has been mainly considered as a major conduit of specific external stimuli to drive the decision of the cell to enter (frequently

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re-enter) or not the cell cycle and, eventually, to divide [12]. Finally, studies on p57 have underlined the importance of this CDKI in the cell cycle control during embryonic development and maturation [13]. Accordingly, its tissue expression shows a restricted pattern during organogenesis and even more in adult life [14].

However, as always happens in nature, the truth of things appears much more complex. Continuously, pivotal new functions have been ascribed to all CDKIs, being several of them CDK-unrelated. Today, CIP/Kip proteins have been associated to the control of multiple complex processes, such as cell proliferation, apoptosis, autophagy, survival, DNA-damage response, gene transcription and translation, cytoskeleton rearrangement and dynamics, mitotic steps and many other functions [9, 15-17].

2. p27 and p57 protein major features, properties and functions

p27 and p57 (as also p21) share a certain degree of homology in their structure, particularly at their N-terminal region, where a conserved domain is allocated, necessary and sufficient for binding and inhibiting CDK complexes. As shown in Figure 1A, such protein region, defined Kinase Inhibiting Domain (KID), consists of a cyclin-binding subdomain (termed D1) and a CDK-binding subdomain (termed D2) joined by a linker subdomain of about 22-residues (called LH). Recently, by means of in silico studies, an ODD-like motif (Oxygen-Dependent Degradation like motif) has been recognized in their N-terminal domain for a putative interaction with VHL (Von Hippel Lidau) protein, which however needs further investigation [18].

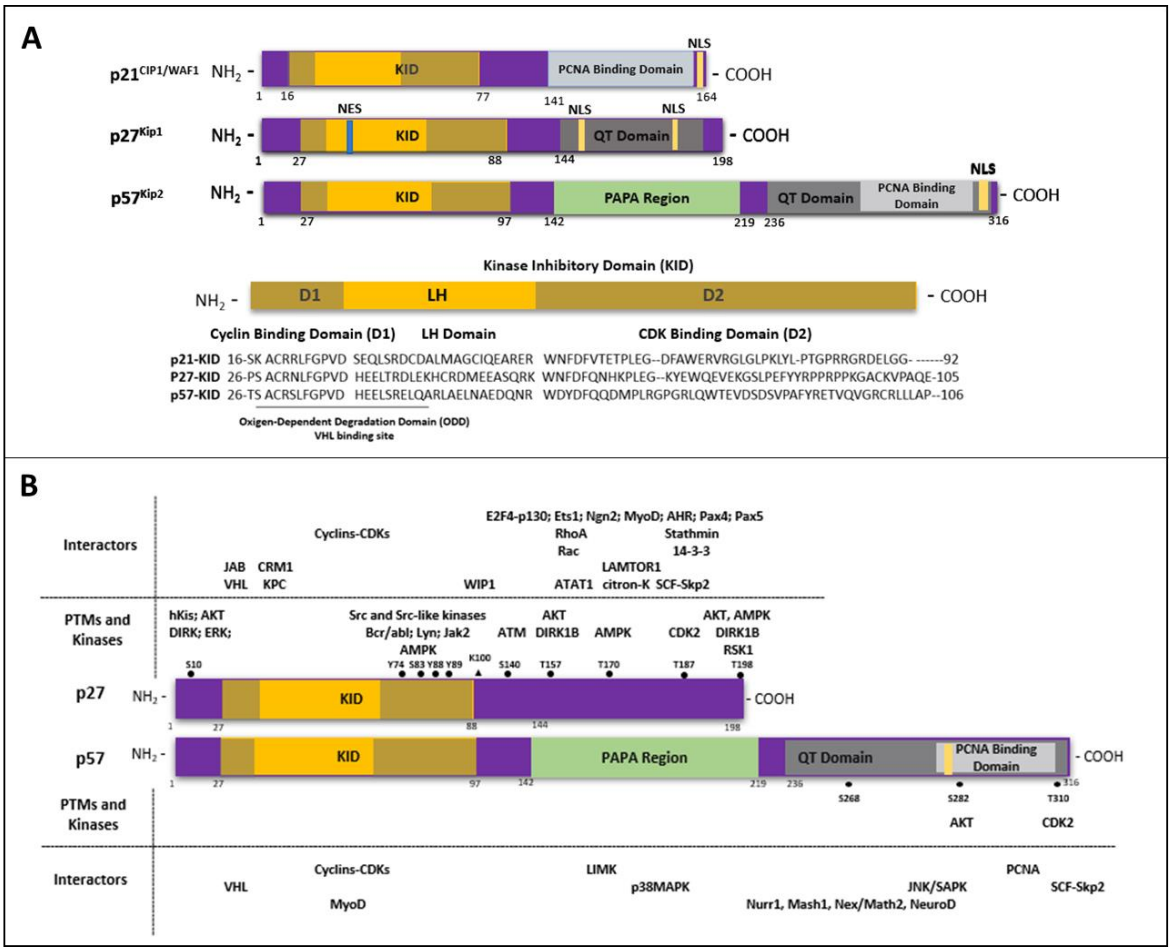

Figure 1. Domain structures of CIP/Kip proteins with the main PTMs, putative kinases and interactors of p27Kip1 and p57Kip2. Panel A: Schematic representation of p21Cip1, p27Kip1 and p57Kip2 protein domain structures. The three siblings share a highly conserved N-terminal domain called Kinase Inhibitory Domain (KID) that includes the cyclin-binding domain (D1) and the

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CDK-binding domain (D2) connected by a linker helix (LH). The Oxigen-Dependent Degradation Domain (ODD), reported for the binding of VHL, partially overlap with the D1 domain. NES (Nuclear Export Signal); NLS (Nuclear Localization Signal). Panel B: Known PTMs, kinases and interactors of p27 and p57. In the panel the domain structures of p27 and p57 are reported. The main sites of phosphorylation for both the proteins are indicated with spots along the sequence. At the site of the phosphorylation are also indicated the putative kinases responsible for the phosphorylations. One acetylated site is reported for p27 (K100) and it is indicated on the protein with a triangle. The interactors of the two proteins, mentioned and described in the text, are also reported in correspondence of their binding site.

Differently, the C-terminal domains (CTDs) of the two proteins vary in length and sequence, exhibiting either similar or different features. Both CTDs show putative nuclear localization signals. Moreover, p27 and p57 C-terms contain a QT domain made by glutamine and threonine repeats involved in protein-protein interaction [19]. Inside the QT domain, p57 displays a Proliferating Cell Nuclear Antigen (PCNA)-binding domain, which is also present in the C-terminal of p21. In addition, it has been reported that the QT box of p57 directly binds the kinase JNK/SAPK (c-Jun NH2-terminal kinase/stress-activated protein kinase), determining its inhibition [20, 21]. p21 is also able to bind and suppress JNK/SAPK activity although via its amino-terminal domain. On the contrary, p27, which contains a QT domain, does not inhibit JNK/SAPK activity. An additional unique domain (made by PAPA, proline-alanine, repeats) is also present in the central portion of p57, upstream of the QT domain, whose functions have not been completely elucidated although it confers peculiar biochemical and biophysical properties to the protein [22].

p27 and p57 are intrinsically disordered proteins (IDPs), having a scarce degree of stable tertiary structures which can be adopted, totally or in part, upon binding to specific interactors [23, 24]. This property, strongly diverging from the classical structure-function paradigm, confers high conformational flexibility to these proteins along with the ability to recognize and interact with a plethora of different partners. In turn, the large plasticity gives to IUP the possibility of exerting a multiplicity of functions. On the other hand, in view of their complex roles, the disruption of p27/p57 homeostasis is associated with numerous diseases, including cancer and neurodegeneration [25, 26].

2.1. p27: a key ambiguous protein with multiple contrasting roles

2.1.1. p27 structure and function

p27 has been first identified in 1994 as a CDKI homolog to p21 [27-29]. Since then, myriad papers reported p27 levels increase in response to anti-mitogenic molecules, differentiation signals, cell-to-cell contact, mitogen-starvation, loss of adhesion to the extracellular matrix and other conditions. Although able to bind and inhibit virtually all cyclin-CDK complexes, p27 seems to certainly inhibit cyclin E(A)/CDK2, particularly at G1/S boundary of the cell cycle, while some degree of ambiguity exists about its activity on cyclin Ds-CDK4(6) [30]. Specifically, the kinase is inhibited in quiescent cells, while in cycling cells p27 participates to the assembly, nuclear import and allosteric activation of Cyclin Ds-CDK4/6 enzymatic complex [31, 32]. The activity of p27 on cyclin B/CDK1 complexes was first evoked by the observation that p27 inhibits the kinase in CDK2-ablated mice. Accordingly, CDK1 activity upregulation has been reported in p27-/- animals [33, 34].

Cdkn1b-/- mice (Cdkn1b is the mouse p27 coding gene) show increased body size, hyperplasia of different organs, retinal dysplasia and neocortex alterations, and female sterility [35, 36]. Moreover, heterozygous Cdkn1b+/- mice show a major susceptibility to develop tumors after chemical carcinogen treatment or irradiation, compared to their littermates, and spontaneously develop pituitary malignancies late in life [37, 38].

Besides CDK-regulative activity, several other canonical and non-canonical functions, not all strictly CDK-dependent [17] have been demonstrated. This is certainly due to the disordered nature

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of p27 [8]. Furthermore, like for other IDPs, post-translational modifications (PTMs), predominantly phosphorylations, contribute in addressing the protein towards specific conformations, affecting stability, subcellular localization and functions. A number of p27 phosphorylation sites have been reported. Only in limited cases, all the involved kinases and their specific biological roles have been clearly identified [39, 40]. The most characterized site of modification is Threonine 187 (T187), which is in vivo and in vitro target of active CDK2- or CDK1-containing complexes [41, 42]. Specifically, T187 phosphorylation induces the formation of a phosphodegron, essential for the recognition of and binding to S-phase kinase-associated protein 2 (Skp2), which represents the substrate-recognition subunit of the E3 ubiquitin ligase complex SCF-Skp2 (SCF, Skp-Cullin-F-box). The Skp2-mediated ubiquitination of p27 also requires Cks1 (CDK subunit 1), which cooperates as a linker protein to the binding between Skp2 and pT187-p27 [43]. The process finally addresses p27 to proteasomal degradation inside the nucleus along S-G2 phases, allowing cell cycle progression [41, 44].

Phosphorylation of tyrosine(s) (Y74, 88, and 89) has been reported as due to non-receptor tyrosine kinases, including Src and Src-like kinases, such as Lyn and Bcr/Abl. The main role of p27 tyrosine phosphorylation is to shift the role of p27 (when bound in the CDK2 catalytic cleft) from kinase inhibitor to CDK substrate, allowing T187 phosphorylation and consequent protein degradation. This pathway therefore removes p27-dependent inhibition of CDK2 and favors cell cycle progression in response to mitogenic signals [45-48].

Serine 10 (S10) phosphorylation represents a quantitatively important p27 PTM. It has been reported not only to stabilize the protein in the nuclear compartment [49-51], but also to allow p27 exit from the nucleus into the cytosol when quiescent cells re-enter the cell cycle from G0 phase [52]. The kinases reported as able to phosphorylate p27 in S10 comprise hKis, Dirk, ERK, AKT, and CDK5 (reviewed in [17, 39]. In post-mitotic neurons, CDK5-dependent S10 phosphorylation stabilizes p27 protein in the cytoplasm, particularly in the perinuclear region, where p27 is in turn able to inhibit RhoA-ROCK pathway, causing the activation of cofilin, a F-actin severing protein. This mechanism results in actin reorganization and neuronal migration [53].

Phosphorylation of threonine (T) 157 and/or 198 has been associated with p27 sequestration in the cytosol. As matter of facts, T157, although not conserved in mouse p27, falls within the Nuclear Localization Sequence in human p27 [54-56]. Reported roles for T198 phosphorylation include assembly of cyclin Ds-CDK4/6 complexes, RhoA-ROCK pathway inhibition upon RhoA binding, stathmin interaction and therefore both actin and microtubule cytoskeleton modification ([17, 57] and reference therein). The kinases identified as responsible for such PTM include AKT, RSK1, which is a downstream effector of PI3K and MAPK, and AMPK (AMP-activated protein kinase). Particularly, AMPK-dependent T198 phosphorylation has been associated to the activation of the autophagy process in response to nutrient restraining condition [58]. Other sites of modification include S140, which is a target of ATM kinase involved in early response to DNA damage [59]. The major site of modifications of p27 and the putatively involved kinases are highlighted in Figure 1B, together with the major p27 interactors.

In summary, as evident from the numerous phosphorylated residues, p27 is a crucial protein on which multiple pathways converge, playing different functions both in the nucleus and in the cytoplasm [17].

In the nucleus, beside its cyclin-CDK inhibition activity, p27 participates in the control of pre-replicative and replicative complex formation and in the regulation of gene expression, due to the ability to interact with specific transcription factors on defined chromatin regions. Interactions have been reported with Ngn2, MyoD, AHR, Pax4, Pax5 ([60] and reference therein). It also binds the acetyltransferase and transcriptional co-activator PCAF that in turn acetylates p27 on K100 modulating the stability of the protein [61]. ChIP on chip experiments performed in quiescent NIH373 cells and MEFs demonstrated that p27 significantly associates with E2F4-p130 complexes and with ETS1 and mainly acts favoring the recruitment of co-repressors such as HDAC1 and

mSIN3A on the promoters of several target genes [62]. Other reports also demonstrated p27 C-terminal domain association to different intergenic chromatin regions, while the KID at the N-terminal domain favors the recruitment on chromatin of specific CDKs, causing in some cases their inhibition. However, the transcriptional effects of p27 appear only partially CDK-dependent, since expression of p27CK- mutants (which do not inhibit CDK activities) are still able to modulate the expression of certain target genes. p27 transcriptional regulation has been also involved in embryonic stem cell differentiation and in the transcriptional repression of SOX2 [63], one of the three Yamanaka factors (Oct4, Klf4, and Sox2) involved in reprogramming pluripotent stem cells (iPSCs) [64]. On the other side, a role of p27 as a prooncogenic transcriptional coregulator of cJun in specific settings of cancer cells with hyper-activated PI3K/AKT has been recently reported [65].

In the nucleus, p27 may also play a role in the DNA damage response (DDR). p27 involvement in the maintenance of genome integrity has been evoked due to the phenotypical response of p27 null and heterozygous mice to chemical carcinogens or γ-irradiation. p27 may act at two different time points in response to DNA double-strand breaks: in a first initiation phase, p27 is directly phosphorylated by ATM in S140 and this event might extend its half-life, allowing p27 to mediate the G1 checkpoint arrest; in a second phase, in condition of persistent exposure to the DNA-damaging agent, p27 might be stabilized by a pathway independent of ATM/ATR activities, relying instead on p38MAPK activation [59]. In turn, through its CDK-inhibitory activity, p27 participates to a G2/M checkpoint arrest [66]. As reported recently, the phosphorylation on S140 is a target of WIP1 (Wildtype p53-Induced Phosphatase-1), whose overexpression and/or mutation have often been associated with oncogenesis [67].

In the cytosol, p27 has been associated to the control of programmed cell death or autophagy, to the modulation of both actin filaments and microtubule (MT) cytoskeleton dynamics, thus impacting both on growth and survival, and on cell movement, invasiveness, and metastatization [17]. Several pieces\ of evidence contribute to clarify the role of p27 in the homeostasis of filaments of actin and in the correct organization of the contractile ring during cytokinesis. This is achieved through Rac, RhoA, and citron kinase involvement [68, 69]. p27 is also able to interact with stathmin, an MT destabilizing factor [70], and PRC1, a protein involved in microtubule cross-linking and central spindle formation, thereby affecting cell shape and motility and proper mitotic division [71]. p27-dependent augmented microtubule stability has also been linked to the control of cell cycle entry in mitogen-stimulated cells, since it favors the endocytic-trafficking of H-Ras and its ubiquitination, causing the reduction of H-Ras–MAPK signaling [72].

Recently, Nguyen group also discovered in mice cortical neurons the involvement of p27 in the stabilization of alpha-tubulin acetyl transferase 1 (alpha-TAT1), the principal enzyme involved in MT post-translational modifications [73], adding important pieces of information to the comprehension of p27 activity on MT cytoskeleton dynamics in vesicles trafficking and neuron migration. In addition, the cytoplasmic CDKI is involved in the enhancement of epithelial-mesenchymal transition, a well-known pro-carcinogenetic process, by inducing Twist1 up-regulation via activation of STAT3 [74]. Cytoplasmic p27 might promote invadopodia turnover and formation of invadosomes, i.e. the active cellular structures playing a fundamental role in the degradation and invasion of the extracellular matrix. Mechanistically, it binds cortactin and facilitates the interaction with (and the phosphorylation by) PAK1, a Rac/CDC42-dependent kinase crucial for cytoskeleton reorganization. [75]. Overall, p27 levels and localization impacts on cell shape and motility, either facilitating invasion and metastatization, or alternatively exerting anti-migratory activities, depending on the cellular context.

p27 has been identified as a pivotal player during metabolic stress. Accordingly, p27 is involved in pathways correlated to autophagy and apoptosis [58]. Under conditions of cellular stress, p27 might thwart apoptosis by its capability of preventing Cdk2 activation as well as to reduce the activity of Bax, a pivotal proapoptotic factor [76, 77]. Other reports point to the pro-apoptotic effects of p27 [78]. Particularly, it has been shown that p27 overexpression in lung

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cancer cell lines induces cell cycle arrest and apoptosis through pRb expression downregulation [79]. Furthermore, in a cohort of patients with oral and oropharyngeal squamous cell carcinomas, the induction of spontaneous apoptosis was higher in cancers expressing p27 than in p27-negative tumors [80], due to the positive correlation to Bax expression.

In 2007, Liang and colleagues have shown that the energy/nutrient sensing kinases LKB1-activated AMPK phosphorylates p27 on S83, T170 and T198, increasing the protein stability and cytosolic localization of p27, with consequent activation of autophagy and reduction of apoptosis [58]. p27 was also required for starvation-induced autophagy in MEFs, underscoring a function of p27 under conditions of scarcity of nutrients. Conversely, when mTOR-raptor complex, a key serine/threonine kinase that stimulates protein synthesis inhibiting autophagy, is active, it enhances p27 phosphorylation on T157 through the activation of SGK1 (Serum and Glucocorticoid-Inducible Kinase 1) and, in turn, favors its sequestration in the cytoplasm causing cell cycle progression [81]. The importance of T198 phosphorylation instead was confirmed by transfection experiments of the phosphomimetic T198D-p27 mutant showing cytosolic protein localization and autophagy activation [58, 82]. Finally, the silencing of CDKN1B affects autophagy caused by serum starvation or glucose withdrawal and induces apoptosis [58].

The mechanistic relationship between p27 and autophagy remains undefined, but cannot be considered a function independent of CDKI activity, since CDK2/CDK4 depletion partially reproduces the effects of p27 on autophagy. Recently, a new mechanism has been proposed. Specifically, under amino acid deprivation, a p27 fraction localizes on lysosomes where it binds LAMTOR1 and, in turn, hampers its capability of activating mTORC1. By this way, p27 might induce autophagy. The finding is confirmed by experiments in p27-/- MEFs that, when deprived of amino acids, show autophagy resistance. In this specific genetic setting and condition, autophagy activation was further inhibited through sequestration in the cytosol of the transcription factor TFEB that controls the expression of genes involved in lysosomal biogenesis and autophagy [83].

In conclusion, the complex and heterogeneous phenotypic effects associated to p27 are often so divergent that they have stimulated the evocative definition, at least in the context of carcinogenesis, of a “Janus” protein with a dual role, i.e. tumor suppressor or tumor promoter. However, based on the continuously identified novel functions of p27, a definition of “many-faced protein” appears more appropriate. This high heterogeneity is probably dependent on two major factors, i.e. its structure and the specific context (cell phenotype, environmental conditions, interacting protein abundance and cell treatments) in which the protein works.

2.1.2. Mechanisms of p27 cellular level regulation

The mechanisms controlling the cellular p27 levels are multiple, namely the regulation of CDKN1B transcription, the control of mRNA translation efficiency and the protein targeted degradation. So far, the major known activators of CDKN1B transcription are members of the FoxO (Forkhead box class O) family of transcription factors [84]. Thus, all the pathways that modulate FoxO activity, including PI3K/AKT, control p27 contents [85, 86]. Intriguingly, factors activating AKT comprise various cytokines and cAMP/PKA pathway. FoxO proteins also promote p27 nuclear localization and reduce the levels of COP9 subunit 5, a protein involved in p27 degradation [46]. Other factor affecting positively p27 transcription include, among others, Sp1, E2F-1, BRCA1, Kruppel-like factor 7 and nuclear hormone receptors. Transcription factors which inactivate p27 promoter comprise c-Myc, Id3, Hes1, Notch/HTRT1, and HDACs [87].

The 5’-untraslated region (UTR) of p27 mRNA has been described as highly structured. It includes an IRES (Internal Ribosome Entry Sequence) and an upstream ORF (uORF) sequence located in a cell cycle regulatory element (CCRE). The IRES probably sustains the translation under conditions of unfavorable growth conditions [88]. The CCRE is formed by a C/G rich sequence and a uORF that, analogously to other uORFs, might codify for a peptide regulating the main p27 coding ORF translation probably during cell division cycle progression. p27 translation of is also

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regulated by different miRNAs (mainly miR221 and miR222) although the precise meaning of this mechanism is not completely understood [89].

As anticipated before, the levels of p27 are controlled mostly by proteasomal degradation. Different mechanisms of p27 proteolysis have been described that occur in different phases of cell cycle division. G1->S transition requires a cytosolic degradation by a ubiquitination mechanism that, apparently, does not require an initial phosphodegron formation. Specifically, it is driven by KIP1 Ubiquitylation Promoting Complex (KPC). On the other hand, the shuttling of p27 from nucleus to cytosol appears to be the initial targeting step for the KIP1-dependent degradation and seems to involve the phosphorylation on S10 and binding to CRM1 [90]. Conversely, during S/G2 phases p27 removal occurs mostly in the nuclear compartment by means of the above described process which involves the sequential phosphorylation on T187, Skp2-dependent ubiquitination and proteasomal degradation [41]. Other reported mechanisms of p27 catabolism require the activity of the protease calpain, as shown by [91] in a model of preadypocyte mitotic clonal expansion.

2.2. p57, an unrevealed protein involved in cell cycle control, cell differentiation, cell death, and senescence

2.2.1. p57 Structure and function

Among the CIP/Kip family proteins, p57 is certainly the less characterized member. This is probably due to its scarce expression in adult organisms, restricted to few tissues [14, 22, 92]. Conversely, it is largely present in embryonal tissues suggesting a central role in differentiation and morphogenesis. Accordingly, the Cdkn1c null mice (Cdkn1c and CDKN1C are the mouse and human gene encoding p57, respectively) are not vital or die soon after birth. The modified animals (or embryos) present developmental defects that partially mimic the human Beckwith-Wiedemann Syndrome phenotype, a rare genetic disease characterized by developmental defects, overgrowth and tumor predisposition. On the contrary, the excess of p57 protein in mice leads to an increase of embryonic lethality and a reduction in the size of the body, suggesting that p57 dosage needs to be finely tuned to ensure a correct morphogenesis [13, 93]. Furthermore, the substitution of CDKN1C with CDKN1B (in other words, introduction of CDKN1B gene at CDKN1C locus leaving the transcriptional control mechanism) cannot completely compensate the role of p57, suggesting peculiar functions of the protein [94]. Various reports suggest a p57 involvement during the quiescence and maintenance of adult stem cells. So far, this has been clearly proved in hematopoietic and neuronal stem cells [95, 96]. The protein also increases during differentiation of skeletal muscle myoblasts, podocytes, keratinocytes and cortical precursor [97, 98].

Additional features clearly differentiate p57 from its siblings. First of all, the genomic localization of CDKN1C (chromosome 11p15.5) is of particular relevance, in that this region is highly imprinted and only the maternal allele is expressed. As consequence, mutations that interest the maternal allele behave as in homozygosity. This finding is certainly important in explaining the phenotypes associated to CDKN1C genetic alterations. A second matter of complexity is that, even and probably more than p27, an extended part of the protein is in an unfolded status with a huge variability, especially in length, among species [99]. Third, as for p27, PTMs might influence the fate of the protein. Up to now, scarce information on p57 PTMs are available. Phosphorylation have been reported for residues S268, S282, T310 (Figure 1B). On the other hand, being the protein an IDP, a clear characterization of its PTMs is mandatory. The most frequently confirmed site of phosphorylation is T310. Phosphorylation on T310 plays a role analogous to that on p27 T187 and is involved in a ubiquitin-proteasome-dependent mechanism of degradation [100]. In brief, phosphorylation on T310 determines the formation of a phosphodegron which functions as a recognition site for Skp2 protein of the E3 ubiquitin ligase SCF complex.

Analyzing the domains of the protein (Figure 1), the amino-terminal region of p57 is not only necessary for the binding of cyclin-CDKs complexes through the KID domain, as described for p27, but it is also involved in numerous interactions during differentiation processes. It can interact with helix-loop-helix transcription factors, such as NeuroD, Nex/Math2, Mash1 for neuronal

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differentiation [101] and Nurr1, an orphan nuclear receptor, particularly important for dopaminergic neuron differentiation [102]. p57 also binds B-Myb, a transcription factor playing a key function during early embryonic development [103]. Several studies have examined the effect of p57 on the stability of MyoD, one of the muscle-specific transcription factors expressed in proliferating myoblasts prior to terminal differentiation [104]. The authors reported two different mechanisms, namely: the inhibition of cyclin E/CDK2 kinase activity and a physical interaction with MyoD, thus promoting MyoD-DNA binding during the myogenic differentiation [105]. Of interest, recently it has reported that p57 is able to interact with VHL, starting from the consideration that, like the other CIP/Kip proteins, shows an ODD-like motif partially overlapping the KID in the N-terminal domain. Even though the functional meaning of the interaction needs to be elucidated, a regulatory connection between the HIF-1α and p53 pathways with the control of the cell cycle is proposed [18].

The central part of the protein is a characteristic that definitely distinguishes p57 from p21 and p27. To be noted, human p57 is a 316-amino-acid protein with a calculated MW of 32177 but migrates at 57 kDa in SDS-PAGE electrophoresis, hence the name p57 (this behavior is not observed for p21 and only at a minor extent for p27). An explanation may lie in the presence of the PAPA region, a sort of hinge made of proline and alanine repeats between the N- and the C-ends of the protein, whose function is still unclear. Few reports suggest that it is important for the modulation of protein-protein interactions. Particularly, this region was reported to be involved in the binding of p57 with LIMK1, an effector of RhoA pathway in the modulation of actin dynamics. In a report, it has been suggested that the overexpression of p57 determined the nuclear localization of LIMK1 with subsequent loss of LIMK1-associated actin stress fibers [106]. Successively, Vlachos and colleagues demonstrated that the interaction of p57 with LIMK1 enhanced the enzymatic activity of the kinase and thereby promoted the stabilization of the actin fibers through the phosphorylation of cofilin, the actin severing protein [107]. In a further study, knockdown of p57 delayed the migration of neurons in the cortical plate during mouse development; however, whether this resulted from the sequestration of LIMK1 was not investigated [108]. This bulk of evidence allows the hypothesis of common mechanistic features of the CIP/Kip proteins, which exert antiproliferative functions in the nucleus, but might participate in even pro-carcinogenetic processes in the cytoplasm through modulation of cytoskeletal dynamics.

The PCNA binding motif, homolog to that of p21, is located at the C-terminal mojety. Even though the affinity of the binding p57-PCNA is lower compared to that with p21, the functional importance of the complex is evident in the IMAGe (OMIM 614732) and Russel Silver (OMIM 180860) syndromes, characterized by undergrowth and development defects, where mutations in this domain have been identified [109].

The C-terminal domain of p57 has also been correlated to the apoptotic process. In normal cell lines, the protein seems to exert an anti-apoptotic function mainly related to the ability of the C-terminal domain of p57 to bind and inhibit JNK activities in a CDK-independent manner [20]. Particularly, in myoblasts the binding of the protein to JNK competes and interferes with the interaction between JNK and c-Jun for the promotion of the JNK/SAPK apoptotic signaling [20]. It is well known that the JNK/SAPK transduction cascade is preferentially activated by a large number of "stresses", including UV radiation, ionizing radiation, ROS (Reactive Oxygen Species) [110]. Through this pathway, the signals proceed towards the JNK-dependent activation of transcription factors that drive apoptotic processes and/or block cell proliferation. In this context, the inhibitory action of p57 could be an important mechanism by which the protein exerts its function on processes such as cell death and differentiation [20, 111].

This evidence is in line with studies performed on miceCdkn1c-/- that reported a high death rate at birth while, in those who survived, alterations of the differentiation programs, along with an increase in cell apoptosis [13, 98]. Thus, the ability of regulating CDKs activities with the N-terminal domain, together with the capacity to bind and to inhibit JNK/SAPK through the C-terminal domain represent important features by which p57 controls cell cycle progression and cell death.

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Comparably to the JNK pathway, also p38 MAPK is activated by a variety of cellular stressors regulating processes from cell cycle checkpoints to cell differentiation and apoptosis [112]. Joaquin and colleagues in 2012 reported the involvement of p57 in cell survival to various stimuli depending on p57 stabilization by p38 phosphorylation. The protein then could exert its CDK2 inhibitory activity, causing cell cycle arrest at G1 phase and allowing cells to respond to the stress condition. It has to be noted that the authors analyzed the phosphorylation of the mouse recombinant p57 protein transfected in HeLa cells, a human cervical cancer cell line, and confirmed the role of p57 in response to stressors in MEFs [103]. Unfortunately, human and mouse p57 proteins have structural differences that limit the extension of results obtained in a mouse model to human. On the opposite, the protein seems to exert pro-apoptotic function in cancer. As a matter of facts, p57 was reported to enhance the staurosporine-induced apoptosis in HeLa cells and this effect was independent of the CDK-inhibitory activity of the protein [113]. The observation was confirmed in 2007 by a study demonstrating that p57 sensitized the cells to proapoptotic agents like cisplatin, etoposide and staurosporine. Intriguingly, the process involves a rapid translocation of p57 into mitochondria followed by the activation of the intrinsic pathway of apoptosis [107]. Additional experiments confirm a role of p57 in apoptosis, although the findings are frequently conflicting and dependent on the cell type and the experimental approach employed. In lung and colon carcinoma cell lines, p57 protects against doxorubicin-dependent cell death [114], while in other models p73 induces apoptosis via p57 increase. Specifically, p63 and p73 are members of the p53 family of transcription factors and they are involved in cell cycle arrest and induction of the apoptosis. In a model of teratocarcinoma cell line, the presence of p73 has been reported as responsible for the induction of p57 and BAX expression, leading to apoptosis [115]. In the same way, silencing of CDKN1C determined the suppression of the apoptosis mediated by p73 followed to cisplatin treatment in lung and colorectal cancer cell lines [116]. Interestingly, we recently reported the involvement of p57 in campthotecin-induced topoisomerase inhibition and consequent DNA Double Strand break damage response [117]. However, depending on the cell model, the induction of p57 by anti-cancer drugs might be seen as a resistance factor to the treatment.

Very few data are reported relatively to a connection between p57 and autophagy. Recently, it has been shown that p57 accumulation decreased autophagy in hepatocarcinoma cells due to EGFR-targeted therapy. The mechanism by which p57 exerts this activity requires the activation the PI3K/AKT/mTOR signaling pathway that sensitize cells to EGFR-inhibitor treatments [118].

p57 has been associated to cellular senescence. Particularly, the protein induces senescence when overexpressed in epithelial cells [119]. In hepatocellular carcinoma Hes1-depleted cells p57 induces cellular senescence [120]. The inducible expression of p57 drives senescence also in astrocytoma cell lines [121]. Interestingly, senescence that traditionally is associated with aging and pathological alterations, recently has been related to embryonic development. In placental tissues, where the expression of p57 is particularly high, the presence of the protein in placental extravillous trophoblasts after the invasion of the decidua has been considered a marker of senescence [122] [184].

2.2.2. Regulation of p57 cellular levels

The expression of p57 changes during development till to be detectable only in a subset of human adult tissues. These pieces of evidence together with the studies on KO mice and on the previously mentioned genetic syndromes underline the role of this CDK regulator in embryogenesis, development and aging.

The main mechanism of p57 abundance control is at transcriptional level to ensure an accurate modulation of p57 dosage (reviewed in [123]). CDKN1C maps in a highly imprinted region with a very complex mechanism of gene transcription regulation [124, 125]. To be noted, the presence of multiple CpG islands upstream and downstream the transcription start site favors the epigenetic control of CDKN1C and the differentially methylated region on parental alleles leads the maternal allele to express the protein [126]. Thus, epigenetic control represents the main mechanism for the

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transcriptional regulation of CDKN1C. For example, during muscle development, the induction of p57 might require the action of MyoD. This latter can bind to a long distance element located in the KvDMR1 imprinting control region determining the release of a chromatin loop and favoring the access to the promoter of CDKN1C [127].

Silencing of p57 expression has also been found in several tumors due to genetic and epigenetic modulation of CDKN1C expression, although post-transcriptional regulation has been reported in some cell lines, as reviewed by Borriello and colleagues [19].

In addition, treatments with demethylating agents (such as 5'-azacytidine) and/or with histone deacetylase inhibitors (HDAC inhibitors, HDACIs) are able to enhance CDKN1C expression through multiple epigenetic and genetic mechanisms [128]. Specifically, treatment with HDACIs determines a chromatin remodeling that makes a minimal region, from -87 to -113 bp, of the CDKN1C promoter available for the binding of the transcription factor Sp1 (Several Stimulatory Protein-1) which positively regulates the expression of p57 in response to HDAC inhibitors [129-131]. However, CDKN1C expression can be also modulated through changing in the activity of several transcription factors. Indeed, the promoter region contains consensus sites for several transcription factors, including EGR1 (Early Growth Response 1), p63, p73 and GRE (glucocorticoid response element) [132, 133]. EGR1 is a positive modulator of CDKN1C transcription [134, 135]. Its action is reduced when it is associated with the chimeric protein PAX3-FOXO1, originated by the common chromosomal translocation characterizing some cases (alveolar subtype) of childhood rhabdomyosarcoma. The constitutively expressed oncogene PAX3-FOXO1, by inhibiting the EGR1 activity, negatively regulates CDKN1C expression and determines an increase in the proliferation of myoblasts [134]. In some studies, the expression of the gene CDKN1C was also associated with the activity of p63 and p73, two proteins that, analogously to their family founder p53, are involved in the response to DNA damage. The observation that p63 knock-out mice exhibit a similar phenotype to Cdkn1c-/-mice suggests that p57 might be a p63 target [136]. Similarly, p73 controls p57 levels by recognition of and binding to a consensus sequence in the promoter of CDKN1C [137]. A GRE has been identified in human and mouse p57 promoter [138]. Glucocorticoids exert antiproliferative effects on numerous cell types, including HeLa cells. Several experimental studies suggest that dexamethasone treatment directly induces transcription of CDKN1C and p57 protein can be considered involved in the glucocorticoid-induced antiproliferative effect [139]. In addition, it has been reported that other transcription factors can regulate p57 expression and might control the CDKI levels: they are ETS (erythroblastosis virus E26 oncogene), a TATA box binding protein (TBP), OCT1 (octamer-binding transcription factor 1), NF1 (neurofibromin 1), HES1 (hairy and enhancer of split 1), Herp2 (Hes-related Repressor Protein 2), which are NOTCH effectors [133].

Finally, besides epigenetic changes and signal-transduction modulation, regulations by microRNA might be an additional mechanism contributing to p57 level control in an ample variety of solid and liquid tumors [140]. Particularly, miR25, miR221 and miR222 were reported as able to target directly CDKN1C RNA in gastric cancer [141] hepatocellular carcinoma (HCC) and human T-cell lymphoblastic lymphomas [142] and miR-92b has been reported as responsible for p57 downregulation in HCC tissues and cell lines, enhancing the tumor radio-resistance to IR-based radiotherapy [143].

3. KIP proteins as molecular effectors of polyphenols

This section will analyze how polyphenols can interfere with p27/p57 regulation. Although the first publication on this topic is dated about 20 years ago, it is of interest to observe the evolution of the field and the attempts of many authors to find functional relationships and specificity between given phenolic compounds and their effects on cell growth mediated by p27/p57.

The following paragraphs are dedicated to those phenolic compounds that largely intercepted the scientists' interest.

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3.1. p27 and polyphenol phenotypic effects

3.1.1. Epigallocatechin-3-gallate (EGCG)

Early studies indicated that EGCG (Figure 2; Table 1) induced G0/G1-phase cell cycle arrest and apoptosis in A431, a human epidermoid carcinoma cell line, in a concentration range of 10-80 μg/ml (about 20-170 μM). This effect coincided with a significant dose- and time-dependent upregulation of p27 and of other cell division cycle inhibitors, such as p21, p16INK4A and p18INK4C, decreased expression of cyclin D1 and inhibition of Cdk2, Cdk4 and Cdk6 kinase activities [144]. A similar effect was evidenced on both androgen-sensitive (LNCaP) and androgen-insensitive (DU145) human prostate carcinoma cells at comparable concentrations (about 10-80 μM). In this case, the block at G1-S transition and the induction of G1 arrest was followed by apoptotic induction [145]. Later, in cervical tumor cells (HeLa, Caski, and SiHa), it was reported that the cell cycle arrest and apoptotic induction triggered by EGCG (10-80 μM) was mediated by a specific EGCG-dependent suppression of EGFR activation, resulting in inhibition of Erk1/2 and AKT enzymatic activities. These events were associated with downstream changes, such as increased p53, p21, p27 levels, reduced Cdk2 kinase activity and cyclin E levels [146]. Evidence have been published on the capability of EGCG to act in combination therapy. BT474 and JIMT-1 human breast cancer cells are both resistant to trastuzumab, the humanized monoclonal antibody against HER2, the oncogene that encodes EGFR type 2, which is overexpressed in about 30% of breast cancers and considered a marker of poor prognosis. In these cell lines, the treatment with EGCG (45–90 μM) inhibited cell growth and induced apoptosis throughout a pathway that included reduced phosphorylation of AKT-Ser473, which freed FOXO3a to translocate into the nucleus where it could activate target genes, including CDKN1B, responsible for cell cycle arrest. FOXO proteins are evolutionarily conserved transcription factors whose inactivation is frequently observed in human cancers [147]. Accordingly, in trastuzumab-resistant breast cancer cell lines, the expression of p27 closely correlated with FOXO3a nuclear accumulation [148].

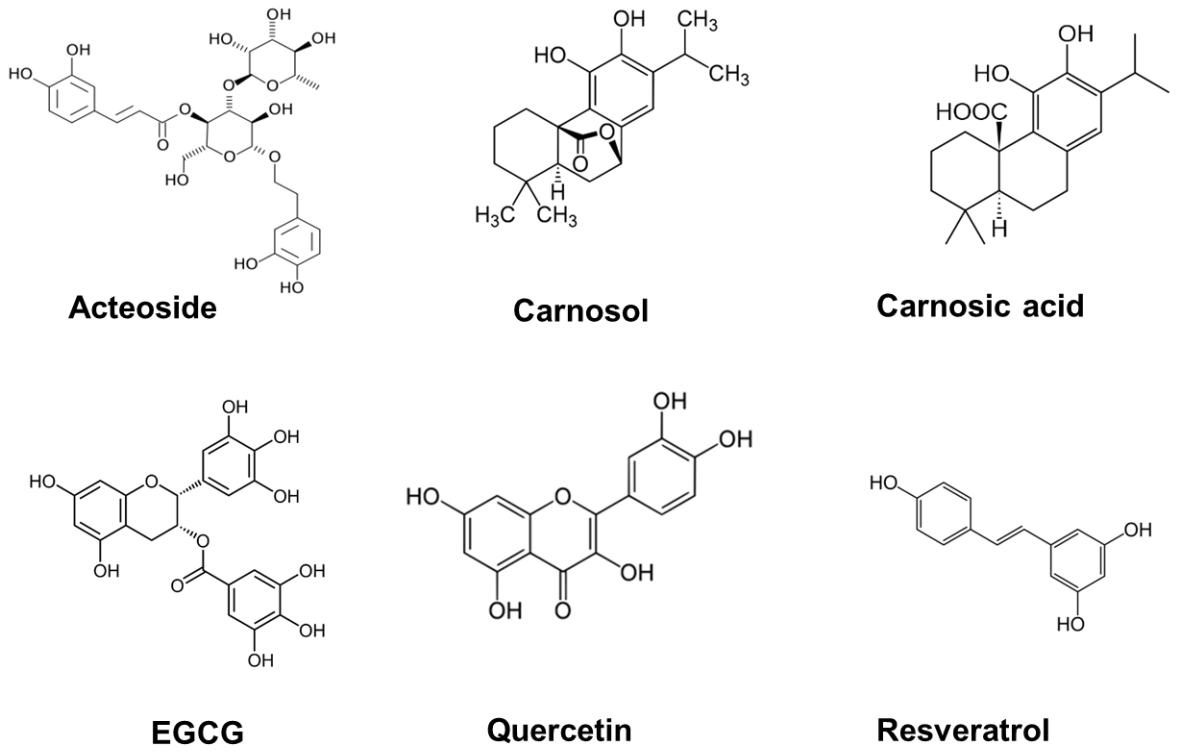

Figure 2. Chemical structures of selected polyphenols that interfere with p27/p57 expression and activity (see text for details).

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Table 1. Studies reporting the effects of selected polyphenols on Kip proteins.

Compound	Experimental model	Concentration	Effect on p27/p57	Reference
EGCG	A431 cell line	20-170 μM	p27↑	[144].
	LNCaP DU145 HeLa Caski SiHa	10-80 μM	p27↑ apoptosis↑	[145] [146]
Green tea extract	Hs578T MDA-MB-231	80-160 μg/ml (90-180 μM EGCG)	p27↑ apoptosis ↑	[111].
Resveratrol	LNCaP	5-10 μM	nuclear p27↓	[151]
	LNCaP	> 20 μM	p27↑	[152]
	Xenografts (PC-3 cells)	30 mg/kg	p27↑	[155]
	Leukemic cells	5-20 μM	p27↑	[157]
	MSTO-211H Xenografts (MSTO-211H)	60 μM 20 mg/kg	p27↓ apoptosis ↑	[159]
	Vascular smooth muscle cells	1-100 μM	p27↓ cell cycle arrest G1/S	[163]
Guggulsterone	Leukemia, Myeloma, Head and Neck Carcinoma, Lung, Melanoma, Breast, Ovarian, Embryonic Kidney cell lines	1-50 μM	p27↑ cell cycle arrest G1/S	[166]
Acteoside	HL-60	30 μM	p27↑ cell cycle arrest G1/S	[167]
Carnosic acid	HL-60 U937	2.5-10 μM	p27↑ cell cycle arrest G1/S	[168]
Carnosol	MDA-MB-231	25-100 μM	p27↓ cell cycle arrest G2/M	[169].
Theaflavin-3,3'-digallate	OVCAR-3	20-30 μM	p27↑ apoptosis ↑ cell cycle arrest G0/G1	[170]
Hydroxytyrosol + Cetuximab	HT-29 WiDr	10 μM 1 μg/ml	p27↓ apoptosis ↑ autophagy↑ cell cycle arrest G2/M	[171]
Green tea extract EGCG	NHEK human keratinocytes	200 μg/ml 50-200 μM	p57↑ differentiation↑	[173, 174] [177]
EGCG	SCC25 OSC2	15-200 μM	p27= apoptosis ↑	[173, 176]
Quercetin	MCF7	10-175 μM	p57↑ apoptosis↑	[179]

The involvement of p27 in mediating the anticancer effects of EGCG has been corroborated by other reports based on the administration of green tea extract, which is rich in polyphenols, including EGCG. As an example, in a rat model of mammary tumorigenesis induced by 7,12-dimethylbenz(a)anthracene (DMBA), the administration of a certified green tea extract (containing 11.79% of EGCG) given to rats as the unique fluid source (0.3%) for 17 weeks significantly reduced DMBA-induced tumor burden, invasiveness and increased latency of the first tumor. The effect of green tea beverage was confirmed on Hs578T and MDA-MB-231 triple negative breast cancer cell lines where 80-160 μg/ml of the extract or 90-180 μM EGCG similarly inhibited cell proliferation and induced apoptosis. At least in cancer cell lines, the antiproliferative effect of EGCG was associated to upregulation of p27. Particularly, lower doses of EGCG slowed cell growth by increasing p27 levels, which correlated with arrest of cells at the G1/S phase transition. Higher doses enhanced apoptosis [111]. Others suggested a different mode of action for some tea polyphenols, including EGCG [149]. In this case, they noted that tea polyphenols containing ester bonds (e.g., EGCG, EGC (2)2epigallocatechin-3-gallate, GCG, (2)2gallocatechin-3-gallate CG, (2)2catechin-3-gallate) could inhibit the proteasome activity, based on the evidence that the same

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ester bonds were present in conventional proteasome inhibitors (e.g., lactacystin -lactone). In fact, these polyphenols inhibited both in vitro and in vivo the chymotrypsin-like activity of the proteasome (IC50 values for ECG, GCG, and CG were 194, 187, and 124 nM, respectively). Because of this effect, EGCG in several malignant cell lines induced the "indirect" accumulation of two canonical proteasome substrates, p27 and IkappaB-alpha, which, in turns, mediated growth arrest in G1 phase of the cell division cycle [149].

3.1.2. Resveratrol

In 2002, for the first time, Reddy's group evidenced the double nature of resveratrol (RSV) in regulating cell cycle progression and its cell-type specificity [150] (Figure 2; Table 1). In fact, only in androgen-sensitive LNCaP cells, but not in androgen-independent DU145 prostate cancer cells or in NIH3T3 fibroblasts, RSV increased DNA synthesis pushing cells in S phase; however, this effect was measurable only at lower RSV concentration (5-10 μM) and was mediated by a decrease in the nuclear levels of p21 and p27. On the opposite, at concentration higher than 20 μM, RSV inhibited DNA synthesis, creating a "collision course" between two important cell cycle phases, e.g., entry of cells into S phase and progression through S phase. The authors speculated that this behavior of RSV could be exploited in the chemotherapy of prostate cancer by accelerating proliferation of cancer cells and making them more sensitive to chemotherapeutic agents and ionizing radiation [151]. Later, by comparing the apoptotic and cell cycle effects of RSV on LNCaP versus other prostate derived cells, namely PZ-HPV-7 (non-tumorigenic line) and PC-3 (androgen-insensitive cancer cell line), Benitez et al. [152] reported that RSV, at 1-150 μM concentration, differently affected cell cycle progression in these cell lines. In fact, the molecule blocked LNCaP cells almost exclusively in G1/S while a significant percentage of PC-3 cells were also arrested in G2/M with p21 and p27 expression increased by RSV only in LNCaP cells. Since, LNCaP and PC-3 cells mimic two different types of prostate cancer, it can be speculated that RSV triggered different regulators depending on their androgen-sensitivity and androgen-insensitivity. In supporting of this view, it has been reported that methyl ether analogs of RSV, present in edible plants, exhibited differential effects on LNCaP. As an example, RSV, pinostilbene, and pterostilbene induced cell cycle arrest at G1/S, while resveratrol trimethylether led to G2/M block [153].

A new wave of novelty on the effects of RSV on cell cycle regulation emerged in the biennium 2010-11, when several papers were published on the regulatory roles of RSV on the PI3K/AKT/FOXO pathway. As reported above, FOXO proteins are transcription factors whose inactivation is frequently observed in cancers [147]. In LNCaP cells, RSV (10-20 μM) inhibited PI3K/AKT pathway, stabilized the levels and enhanced the transcriptional activity of FOXO, finally resulting in the up-regulation of its gene products (TRAIL, TRAIL-R1/DR4, TRAIL-R2/DR5, Bim, p27 and cyclin D1). Consequently, the overexpression of FOXO genes (FKHR, FKHRL1 and AFX) enhanced these effects, while their inactivation abolished RSV-induced expression of TRAIL, TRAIL-R1/DR4, TRAIL-R2/DR5, Bim and p27. RSV had no direct effect on the expression of FOXO [154]. This finding was confirmed in mice xenografted with PC-3 cells where RSV (30 mg/kg) administered through gavage alone or in combination with TRAIL, inhibited tumor growth and angiogenesis, upregulated the expressions of TRAIL-R1/DR4, TRAIL-R2/DR5, Bax, p27, and inhibited the expression of Bcl-2 and cyclin D1 [155]. Comparable data were obtained for different cancer types, e.g. pancreatic cancer and leukemia. In the former, RSV reduced cell proliferation and induced caspase-3-dependent apoptosis in several pancreatic cancer cell lines (PANC-1, MIA PaCa-2, Hs766T, and AsPC-1). Cell cycle arrest was mediated by up-regulation of p21 and p27 expression and inhibition of cyclin D1 expression. When PANC-1 cells were orthotopically implanted in Balb C nude mice, RSV treatment (0-60 mg/kg body weight, through gavage) induced upregulation of Bim, p27, p21, cleaved caspase-3, and inhibited the expression of PCNA. In addition, RSV reduced the phosphorylation of PI3KTyr458, AKTSer473, FOXOSer256 and FOXO3aSer253 without significant changes in their total protein levels [156]. In a panel of leukemic cells (K562, U937, NB4, Daudi and Raji), not toxic concentration of RSV (5-20 μM) reduced the cytotoxicity of proteasome inhibitors. RSV in combination with MG132 (a potent cell-permeable

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proteasome inhibitor which reduces the degradation of ubiquitin-conjugated proteins in mammalian cells) induced cell cycle arrest at G1/S phase via p27 due to increased FOXO1-dependent expression at the transcriptional level. Knocking-down p27 by siRNA, almost abolished the protective effects of RSV. The authors concluded that MG132 and RSV synergistically induced p27 through enhanced recruitment of FOXO1 on the CDKN1B promoter [157]. As mentioned above, p27 is strongly upregulated by proteasome inhibitors' treatment, since the major mechanism of p27 level control is through ubiquitin/proteasome-dependent degradation acting in the nucleus and allowing the completion of S/G2 phases [44].

In human colon cancer cell, RSV at high concentration (100-150 μM) suppressed IGF-1 induced cell proliferation and increased apoptosis following G1/S phase cell cycle arrest through p27 stimulation and cyclin D1 suppression [158]. In human malignant pleural mesothelioma cells (MSTO-211H), RSV (0-60 μM) decreased cell viability and increased apoptotic with an IC50 of about 16 μM [159]. This study is of particular interest since a direct interaction between RSV and Sp1 transcription factor was reported. The binding of Sp1 to G-C rich promoters was inhibited by RSV, resulting in the reduced expression of cancer-related genes (p27, p21, cyclin D1, Mcl-1) under the control of Sp1. The data were also confirmed in BALB/c athymic (nu+/nu+) mice injected with MSTO-211H cells and treated with RSV (20 mg/kg daily for 4 weeks). In this model, Sp1 expression was inhibited with a parallel induction of apoptosis [159]. The unsolved paradox emerging from this work was the strong and unexpected reduction of p27, not clearly commented and explained by the authors.

Several examples have been published on the antiproliferative effects of RSV when combined with other natural or synthetic agents. In Caco-2 cells, derived from a human colon adenocarcinoma, RSV (50 μM) associated with butyrate (2 mM) enhanced the induction of p21 and attenuated the expression of p27 [160]. These data suggest that RSV magnified the differentiating effects of butyrate on Caco-2 cells and this capacity was mediated by p21 rather than p27. In several lung cancer cell lines, namely A549, EBC-1, and Lu65, RSV inhibited cell growth with an ED50 in the range 5-10 μM. Although RSV did not synergize with paclitaxel (Taxol®) on the same cell lines, the pre-treatment of RSV at a relatively low concentration (10 μM) for three days, significantly enhanced the subsequent apoptotic and antiproliferative effect of paclitaxel. Among the biochemical markers triggered by the combined effect of RSV and paclitaxel (p21, p27, E-cadherin, EGFR and Bcl-2), only p21 expression was increased of approximately 4-fold [161]. More recently, RSV (35-47 μM) in combination with docetaxel (10-31 nM) modulated apoptosis and cell cycle progression in the C4-2B and DU-145 prostate cancer cells, differently sensitive to docetaxel. The combined treatment up-regulated and down-regulated the pro-apoptotic and anti-apoptotic genes, respectively, in both cell lines. In C4-2B cells, more sensitive to docetaxel, p21 and p27 levels were increased leading to cell cycle arrest at G1/S transition. An additional block was evidenced in G2/M phase, matching with the suppression of CDK1 activity and cyclin B1 expression, suggesting that the cells that passed the G1/S checkpoint were blocked in G2/M. These effects were less pronounced in DU145 cells due to their reduced sensitivity to docetaxel [162].

Unexpectedly, in non-malignant cells, e.g. vascular smooth muscle cells, RSV arrested cells in S phase, but decreasing the expression of p21 and p27 and increasing the phosphorylation of Rb protein, which is normally under the control of these two factors [163]. Other authors confirmed that, in the same cellular model, RSV blocked the cell cycle in G1 phase, downregulated the levels of cyclin D1 and CDKs, upregulated the expression of p21, but did not increase p27 [164].

3.1.3. Other polyphenols

Hydrolysable tannins, generally called tannic acid, represent a class of plant-derived polyphenols with molecular weights ranging between 500-3000 daltons containing from 6 to 9 ester bonds. Tannic acid, similarly to a few other ester bond-containing tea polyphenols (e.g., EGCG) was able to inhibit the chymotrypsin-like activity of both purified 20S proteasome and Jurkat cells 26S proteasome. Since, as previously described, p27 is among the targets of the

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ubiquitin/proteasome-mediated degradation pathway, it is plausible that the direct inhibition of 26S proteasome by tannic acid generates the accumulation of p27 and the consequent induction of G1/S arrest and cell death [165] (Figure 2; Table 1).

Guggulsterone [4,17(20)-pregnadiene-3,16-dione] is a phenolic compound derived from the gum resin of the Commiphora mukul tree and used in traditional medicine as a remedy against inflammatory diseases. This molecule inhibited cell growth of a wide variety of tumor cells (but not normal human fibroblasts) blocking them in G1/S with down-regulation of cyclin D1 and upregulation of p21 and p27. It is of interest that the effects of Guggulsterone increased sensitivity to chemotherapy in drug-resistant cancer cells favoring apoptotic cell death [166].

Acteoside, the alpha-L-rhamnosyl-(1->3)- -D-glucoside of hydroxytyrosol, at approximately 30 μM concentration, arrested HL-60 human promyelocytic leukemia cells in G1 phase inducing differentiation via a mechanism that not only increased the mRNA and protein levels of p27 and p21, but also favored their binding to CDK4 and CDK6 contributing to the reduction of their kinase activities [167] (Figure 2; Table 1).

Carnosic acid is a polyphenolic compound present in rosemary, oregano and other culinary herbs. In human myeloid leukemia cells (HL-60 and U937) at 2.5-10 μM (EC50 about 6-7 μM), cell proliferation was inhibited without induction of apoptosis (Figure 2; Table 1). The G1/S arrest coincided with an increase of p21 and p27 proteins [168]. Carnosic acid is easily converted to carnosol by oxidation. Carnosol inhibited cell viability (25-100 μM) in MDA-MB-231 cell line thought multiple mechanisms. The block in the G2/M phase of the cell cycle was mediated by the increased expression of p21 and downregulation of p27. Beclin1-independent autophagy and apoptosis were also induced with autophagy that preceded apoptosis . At lower and not cytotoxic concentration (25 μM), carnosol stimulated a limited production of ROS, enough to activate γH2AX (Ser139-phosphorylated histone H2AX), a variant of histone H2A which plays an important role in the cellular response to DNA double-strand break, and induce autophagy. At higher concentration (100 μM), carnosol induced a massive production of ROS responsible for triggering autophagy followed by the activation of both intrinsic and extrinsic apoptotic pathways [169]. It remains to be clarified the role of cell cycle arrest in this scenario.

Theaflavin-3,3′-digallate is a unique polyphenol present in black tea produced by fermentation of EGCG and epicatechin gallate. In OVCAR-3 human ovarian carcinoma cells, this compound inhibited cell growth acting on parallel mechanisms: 1) at 20-30 μM concentration, it enhanced the phosphorylation of the checkpoint kinase 2 (Chk2) which, in turns, activated the intrinsic apoptosis independently of p53, increasing the Bax/Bcl-2 ratio; 2) theaflavin-3,3′-digallate (20 μM) caused G0/G1 arrest with an expected increase of p27 levels and a consequent dramatic down-regulation of CDK4, Cyclin D1, p-Rb and Rb [170]. It remains to be explained if and how these two events are independent or functionally related.

Hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol), one of the most studied polyphenol from olive oil, is known for its anti-inflammatory, antioxidant and anticancer activities. Among these, hydroxytyrosol was able to downregulate EGFR (epidermal growth factor receptor) expression and inhibit cell growth in both colon carcinoma cell lines and xenograft models with a mechanism that mimics the effect of the EGFR inhibitor cetuximab. In a recent work, Terzuoli et al. [171] demonstrated that the association between hydroxytyrosol (10 μM) and cetuximab (1 μg/ml) was 10-fold more efficient in reducing cell growth in HT-29 and WiDr colon cancer cells than the single compounds. The efficacy of the combined effect was mediated by cell cycle arrest at G2/M phase, associated with the induction of both p21 and p27. This event, with a mechanism still not fully clarified, induced caspase-independent apoptosis and autophagy activation, evidenced by a strong increase of Beclin-1 levels [171]. Previous studies carried out in the promyelocytic cell line HL60 demonstrate the cell growth inhibition and the apoptosis induction activities require the presence of two ortho-hydroxyl groups on the phenyl ring (Della Ragione et al., 2000).

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Finally, a mention is deserved to a recent study focused on the anticancer capacity of polyphenol metabolites generated through colonic microflora-dependent degradation of their parental aglycones, which are generally more unstable and present at nanomolar concentrations in the blood stream. 2,4,6-Trihydroxybenzoic acid (2,4,6-THBA) can be generated by degradation and subsequent oxidation of most flavonoids (anthocyanins, flavonols, flavones, flavanols). In cells expressing a functional SLC5A8 (a monocarboxylic acid transporter), this metabolite was able to accumulate in the cells at pharmacological concentrations (250-1000 μM), slowing down the rate of cell proliferation and massively increasing both p21 and p27. In addition, molecular docking studies indicated that 2,4,6-THBA was able to directly bind to Cdks 1, 2 and 4 and this prediction was confirmed by the inhibition of their kinase activities in in vitro Cdk assays employing the purified enzymes [172].

3.2. p57 a promising (putative) actor in polyphenol mechanism of action

The literature regarding the regulation of p57 by polyphenols is very limited and largely derives from the work done by Hsu and Schuster's group. These authors firstly demonstrated that polyphenols from green tea, either in form of a mixture of the four major catechins (epicatechin, epigallocatechin, epicatechin-3-gallate, and EGCG) or as purified EGCG, was able to induce the transient expression of p57 in normal human epidermal keratinocytes [173]. The p57 induction resulted in the stimulation of multiple survival pathways including cell differentiation [173, 174]. They subsequently reported that green tea catechins promoted the "re-energization" of aged keratinocytes, while the induction of p57 stimulated the differentiation of the keratinocytes in the basal layer of the epidermis. These effects were interpreted as combined, positive effects of green tea catechins in accelerating wound healing and regeneration of new skin tissues via p57 [175]. On the opposite, in cancer cells, the same green tea constituents generated a significantly different response. In the oral carcinoma cell lines SCC25 and OSC2, EGCG induced apoptosis, inhibited cell growth and invasion without altering the levels of p57 expression [173, 176]. In addition, retroviral-transfection of p57 in oral carcinoma cells increased resistance to catechins-induced apoptosis, suggesting that p57 was a key pro-survival factor to counteract apoptosis induced by green tea polyphenol [174]. In the following years, more details were added to the hypothesis that catechins activates differentiation in normal epidermal keratinocytes and apoptosis in tumor cells. Using NHEK (normal human epidermal keratinocytes), it was reported that EGCG induced differentiation by stimulating the expression of both p57 and caspase 14, a factor not directly involved in the typical apoptotic process, but associated with terminal differentiation of NHEK and barrier formation. The over-expression of p57 significantly preceded and was required to increase the expression level of caspase 14, suggesting that, in NHEK cells, p57 acted as a regulator for caspase 14 expression [177]. The precise mechanisms responsible for the upregulation of p57 by EGCG are not completely understood. Being MAPK proteins (p38, ERK and JNK) associated with EGCG signaling [178], the authors also demonstrated that EGCG induction of p57 in keratinocytes required p38 MAPK activity, since the use of SB203580, a p38 inhibitor, prevented p57 accumulation. but was independent of JNK or MEK activity [21].

On the opposite, in oral carcinoma cell lines (OSC2), EGCG induced a rapid activation of JNK followed by caspase-dependent apoptosis. Chemical inhibition of JNK activity abolished the pro-apoptotic effect of EGCG and an OSC2 subclone expressing high level of p57 failed to develop tumors in xenograft mice [21]. These results suggest that loss or reduced expression of endogenous p57 is associated with malignant transformation in oral carcinomas. The effect can be bypassed by JNK activation in response to EGCG treatment or over-expressing p57. The latter event promotes differentiation and abolishes EGCG-dependent activation of JNK and caspase 3. In other words, green tea catechins can act as a switch on different MAPK proteins inducing differentiation or apoptosis depending on the presence and level of expression of p57. In normal cells, catechins induce cell differentiation via p38 leading to the over-expression of p57 which, in turns, blocks the JNK-dependent apoptotic pathway stimulated by catechins. In cancer cells, the low/null expression of p57 frees catechins from the possibility to activate JNK and induce apoptosis.

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Only few data are available on the ability of other polyphenols in promoting CDKN1C transcription in cancer cell lines favoring cell cycle arrest and apoptosis. MCF7 breast cancer cells treated with quercetin showed an increase of p57 in parallel with p53 and the activation of the apoptotic pathway [179] (Figure 2; Table 1). In a previous work, we reported that resveratrol exerts strong antiproliferative activity in the micromolar range, upregulating Egr1 level [180]. Later, in a second report, we evaluated the effect of resveratrol on the expression of CDKN1C in K562 cells. Although it was confirmed that resveratrol determined an increase of Egr1, the compound did not influence the protein amount, suggesting that, at least in the used cell model, resveratrol-Egr1 induction is not involved in the modulation of CDKN1C transcription [130].

4. Conclusions and future directions

We learned from this review that both its "main characters", i.e. Kip proteins and polyphenols, share key qualities: a strong promiscuity and pleiotropy. In facts, although p27/p57 became originally popular in the field of cell cycle regulation for their pivotal role as CDKIs, now, their CDK-unrelated functions are increasingly maturing towards the control of multiple complex processes that include apoptosis, autophagy, DNA-damage repair, gene transcriptional control, cytoskeleton remodeling and many others, as we largely described in the above sections. Analogously, polyphenols are "by definition" pleiotropic compounds being able to hit and modulate multiple independent or complementary pathways that control the same key processes regulating cellular homeostasis and implicated in the physiopathology of complex diseases, such as cancer, cardiovascular and neurodegenerative disorders. In the present work, we attempted to harvest evidence on the existence of possible interplays between polyphenols and p27/p57 functions. We can conclude that, although this topic remains in its infancy and requires further studies, some interesting and promising outcomes are already emerging.

As a key issue, we need to understand if the described changes in the cellular levels/activity of p27/p57, observed in many cellular models, are the "cause" or the "consequence" of the phenotypical effects of polyphenols treatments. The latter appears the easiest answer. It is well known that polyphenols can arrest the cell cycle in cancer cells [181] and p27/p57 are part of the down-stream mechanisms controlling this process. Therefore, it is conceivable to expect an increase in their levels, together with those of other possible inhibitors of cell proliferation, because of the up-stream events triggered by polyphenols and ending up with cell cycle arrest. This simplistic explanation attributes a marginal and not-specific role to the interplay between polyphenols and p27/p57. This possibility cannot be excluded in the absence of strong evidence indicating: i. a direct binding between specific phenolic compounds and p27 or p57; ii. biochemical and/or genetic demonstrations that the effects of polyphenols on cell cycle arrest are abolished/reduced by inhibiting the expression of p27/p57. However, circumstantial evidence presented in this review are accumulating on the existence of more specific mechanisms activated by polyphenols to regulate p27/p57 functions. Among these: i. the observation that in some experimental settings p27 expression is down-regulated by polyphenols differently from what it is expected in terms of cell cycle arrest (summarized in Table 1); ii. the consolidated demonstration that EGCG and other polyphenols can specifically stimulate p27 accumulation by inhibiting the ubiquitin/proteasome-mediated degradation pathway, with the consequent induction of G1/S arrest and cell death; iii. data obtained in our laboratories suggest that a natural extract enriched in polyphenols arrests cell growth and activates autophagy via changes in p27 phosphorylation and its cellular localization with mechanisms probably independent from the cell cycle regulatory role of p27 (data not reported). This observation, although preliminary, goes in the direction of the novel role of p27 in autophagy commented above that will certainly represent the target of new studies in the field of Kip proteins.

A limitation of the present study, common to many reviews exploring the biological activities of polyphenols regards the observation that most of the cited articles are based on studies on cell lines and the concentration applied are in the tens or hundreds micromolar range. If, from one side,

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these features raise easy and routine criticisms to the biological significance and clinical applicability of bioactive polyphenols, from the other leave enormous space for further investigations in animal models and for clinical trials. We clearly expressed our point of view elsewhere on the strengths and weaknesses of using cell lines to study the bioactivities of polyphenols and their mechanism(s) of action and proposed guidelines in this direction [182]. Certainly, some observation commented above, such as the different effect of RSV in decreasing or increasing p27 expression in LNCaP cells depending on the applied concentration, below or above 20 μM, respectively, deserves an in deep study in animal model of prostate cancer [151, 152].

The different effects of polyphenols in regulating Kip proteins in normal cells vs malignant ones deserve a final consideration. We observed that, unexpectedly, in vascular smooth muscle cells, RSV decreased the expression of p27 and augments the phosphorylation of Rb protein, leaving space for future investigation on its role on the so-called "collision course" between two important cell cycle phases, e.g., entry of cells into S phase and progression through S phase. More intriguing the effect of p57 in normal human epidermal keratinocytes where EGCG and, generally, green tea catechins induce a transient expression of p57 hypothesizing a role of these polyphenols in the "re-energization" of aged keratinocytes. Although still speculative, this observation can open the doors to investigate more in deep the possibility that p57 participates in mediating the antiaging effects of polyphenols. The field appears extremely promising since we do believe and commented elsewhere [183] that the molecular mechanisms of the beneficial effects of polyphenols in aging and non-communicable diseases must be approached working on their applications at nutritional (not pharmacological) doses, like those present in polyphenols-enriched diets and/or nutraceuticals/functional foods.

In conclusions, as mentioned above, to shed light on the truth biological mechanisms is much more complex than what it appears and the future studies on the regulation of Kip proteins by polyphenols will be opened to new and unexpected perspectives.

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