

## TUMOR SUPPRESSING FUNCTIONS OF p16<sup>INK4a</sup> – A REVIEW

Auerkari EI\*, Suwelo IS\*, Tjarta A\*\*, Cornain S\*\*, Rahardjo TW\*,  
Eto K\*\*\*, Ikeda-MA\*\*\*

\*Faculty of Dentistry-University of Indonesia

\*\*Faculty of Medicine University of Indonesia

\*\*\*Faculty of Dentistry-Tokyo Medical & Dental University

Auerkari EI, Suwelo IS, Tjarta A, Cornain S, Rahardjo TW, Eto K, Ikeda-MA: Tumor Suppressing Functions of p16<sup>INK4a</sup>-A review. Jurnal Kedokteran gigi universitas indonesia. 2003; 10 (Edisi Khusus):510-519

### Abstract

DNA damage can have particularly severe carcinogenic influence if it incapacitates the cellular machinery normally protecting the cell from the effects of genomic damage. The protective functions involve not only DNA repair and apoptosis (programmed cell death), but also regulation of the cell cycle and proliferation. Therefore, carcinogenesis can be promoted by inactivating or altering key regulatory proteins like p16<sup>INK4a</sup>, which has the capability to arrest the cell cycle in the G<sub>1</sub> phase and prevent inappropriate proliferation. Functional cyclin-dependent kinase (Cdk) inhibitor p16<sup>INK4a</sup> binds to Cdk-4 and Cdk-6, thereby preventing the Cdk-cyclin complexes from promoting phosphorylation of pRb and releasing the transcription factor E2F, needed for the cell cycle to proceed to the S phase. Arrest in G<sub>1</sub> accounts for a minority of arresting cells after DNA damage, the majority of arrests taking place in G<sub>2</sub> without recognized p16<sup>INK4a</sup> contribution. However, inactivating alterations of p16<sup>INK4a</sup> are common in cancers, possibly because of additional functions of p16<sup>INK4a</sup> in senescence and inhibition of the spreading and migration of cancer cells. Since oncogenic initiation is insufficient for growing significant tumors without spreading and angiogenesis, this could partly explain why inactivated p16<sup>INK4a</sup> is frequently exhibited in clinical tumors in spite of apparently less exclusive role in cell cycle arrest. On the other hand, multiple oncogenic events are usually necessary to develop cancer, and generally both pRb and p53 pathways are impaired in tumors. This suggests that growth regulation in G<sub>1</sub> and therefore also its key molecular components including p16<sup>INK4a</sup> are important in carcinogenesis.

Key words: Tumor; DNA

### Introduction

In general, carcinogenesis results from a failure of the cellular defences to DNA damage or oncogene activation. This

may happen due to some specific weakness in the defence mechanisms, or by chance with sufficient time to accumulate damage.

### Cell cycle arrest

DNA damage is particularly severe if it can inactivate the mechanisms that are normally protecting the cell from genomic damage. The protective functions include several mechanisms of DNA repair, apoptosis or programmed cell death in case of irreparable damage, and arresting of the cell cycle before undue proliferation. Arresting of the cell cycle is also important to allow for the necessary time for DNA repair. Therefore, carcinogenesis can be promoted by inactivating or altering key regulatory proteins like p16<sup>INK4a</sup>, which can mediate arrest of the cell cycle in the G<sub>1</sub> phase.

The progression of the cell cycle is controlled normally by partly interconnected and tightly regulated processes, mediated by a network of promoter and inhibitor proteins (Fig 1). An important part of this cellular machinery is the pRb/E2F pathway, which is involved in the cell cycle regulation at the G<sub>1</sub>/S boundary both in stressed and unstressed cells.<sup>1</sup> Active dephosphorylated pRb inhibits the G<sub>1</sub>/S transition and cell proliferation by binding the E2F transcription factors. Full phosphorylation of pRb will release E2F and promote progression to the S phase. The phosphorylation is promoted by complexes of cyclin-dependent kinases Cdk 4/6 and

Cyclin D, and inhibited by cyclin-dependent kinase inhibitors (Cdi) like p16<sup>INK4a</sup>, which prevent the complex formation by binding themselves to the Cdk, arresting the cell cycle at G<sub>1</sub>.<sup>2,3,4,5</sup> If an alteration in the Cdi prevents its inhibitory function, the cell cycle can proceed to proliferation, and in case of sufficient number of oncogenic events, possibly eventually initiating cancer.<sup>7,8,9,10</sup> In the pRb/E2F pathway, pRb binds to E2F1, E2F2, E2F3 and E2F4, and parallel to this, the pRb family proteins p130 and p107 bind to E2F4 or E2F5.<sup>1,2,6</sup> For p16<sup>INK4a</sup> dependent arrest in G<sub>1</sub>, pRb function alone is not sufficient without the action of either p130 or p107.<sup>1</sup> Non-functional p16<sup>INK4a</sup> can appear in cancer cells regardless of the status of p53, which controls much of the cellular defences after DNA damage.<sup>2,3,4</sup>

The p16 protein and other INK family proteins contain four or five tandemly repeated ankyrin motifs, each about 32 amino acids in length.<sup>10,11,12</sup> The functional binding locus of p16<sup>INK4a</sup> appears to reside by the third ankyrin repeat, extending somewhat beyond it, and binding adjacent to the ATP-binding site of the Cdk catalytic cleft. This prevents binding of ATP and induces structural changes that propagate to the cyclin-binding site of Cdk.<sup>19,20,21</sup>

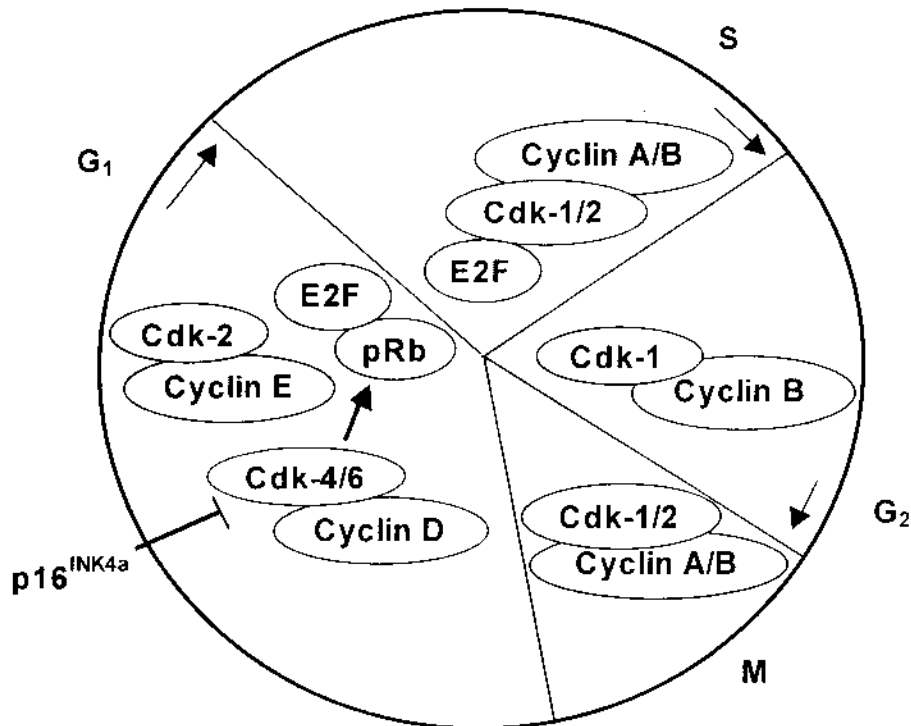


Fig 1. A simple model of cell cycle, including Cdk-cyclin complexes and the Cdk-4/6 inhibitor p16<sup>INK4a</sup> which can mediate cycle arrest at G<sub>1</sub>. With inhibitor action, the Cdk-cyclin complexes cannot promote phosphorylation of the pRb family of proteins to release the E2F transcription factors for G<sub>1</sub>/S transition. Note that there are other pathways to G<sub>1</sub> arrest (see Fig 2), and that parallel processes can also arrest G<sub>2</sub> before entering mitosis (M).

There are several other important Cdi proteins (Fig 2) inducing G<sub>1</sub> arrest, like p21<sup>Waf1/Cip1</sup>, which is activated in p53 dependent and independent manner after DNA damage or oncogene activation, and p27<sup>Kip1</sup>, which can bind to and inhibit Cdk-2.<sup>3,4,5,6,15</sup> In spite of the potential complementary action of at least three Cdi molecules, G<sub>1</sub> arrest accounts only for 15-40% of arrested cells after DNA damage.<sup>3,2</sup> A second set of defence mechanisms can arrest the cell cycle in the G<sub>2</sub> phase, preventing the cell from initiating mitosis. Blocking G<sub>2</sub> accounts for 60-85% of the arrested cells after DNA damage, and is largely mediated by p53 dependent regulation without known influence by p16<sup>INK4a</sup>.<sup>2,15</sup> Studies on tumors with inactive p16<sup>INK4a</sup> have attributed the inactivation to allelic loss (homozygous deletion) and to transcriptional silencing by subtle mutations or DNA methylation.<sup>11,12,13,16,17,27</sup> DNA

methylation occurs in about one third of all samples, particularly affecting the 5' CpG island of the gene. Silencing by methylation may prevent transactivation of p16<sup>INK4a</sup> by AP-2, a DNA-binding protein implicated in signaling terminal differentiation and in transactivating also p21<sup>Waf1</sup>.<sup>14,18</sup>

Apart from the assumed transactivation by AP-2, at least the *ras* family of genes, expressed as four 21 kD proteins H-, N-, KA and KB-*ras*, appear to induce the Cdi proteins p16<sup>INK4a</sup> and p21<sup>Waf1</sup>.<sup>14,22,23</sup> Oncogenic changes cooperating with *ras* tend to neutralise the functionality of these kinase inhibitors, and can also suppress the third important kinase inhibitor p27<sup>Kip1</sup> via the MAP kinase pathway.<sup>14,23</sup>

Some of the p16<sup>INK4a</sup> and pRb related regulatory proteins of the cell cycle and their chromosomal mapping are summarised in Table 1.

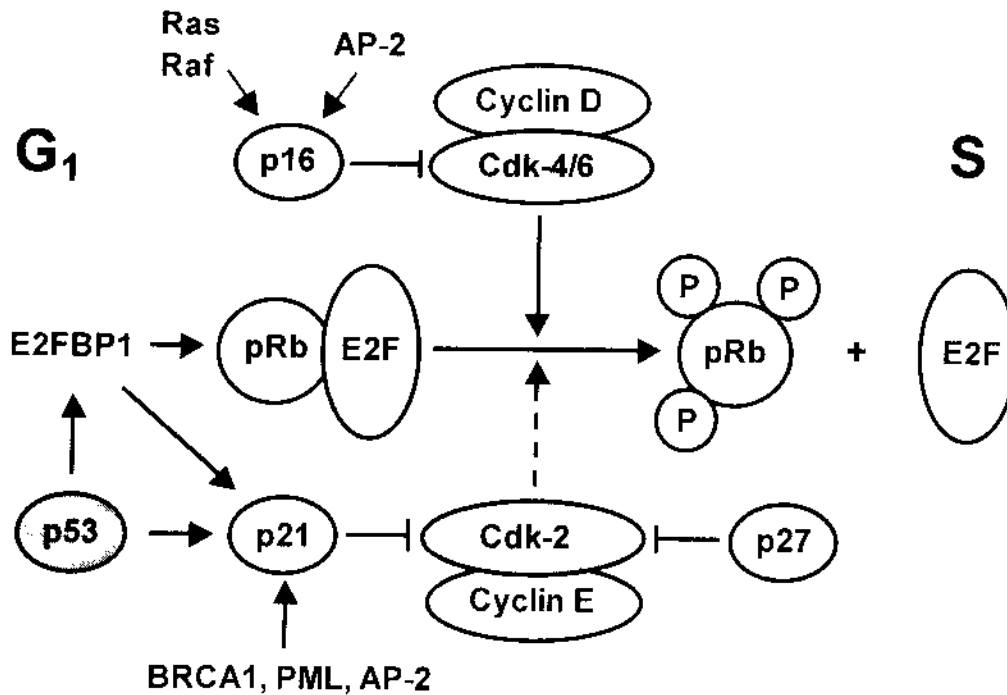


Fig 2. Simple model of the regulatory network for cell cycle arrest in  $G_1$  by cyclin-dependent kinase inhibitors (Cdi)  $p16^{INK4a}$ ,  $p21^{Waf1/Cip1}$  or  $p27^{Kip1}$ . An arrow signifies activation/promotion or process direction, a line with a terminal bar inhibition. Any of the three shown Cdi's can arrest the cycle, since the action of both Cdk-cyclin complexes are needed for passage from  $G_1$  to S. Several of the indicated regulatory molecules appear in reality as a family of proteins with partly parallel functions, e.g. the INK family of kinase inhibitors includes  $p16^{INK4a}$ ,  $p15^{INK4b}$ ,  $p18^{INK4c}$  and  $p19^{INK4d}$ . Also, to arrest  $G_1$  in a  $p16^{INK4a}$  dependent manner, parallel function by other members of the pRb family (p130 or p107, not shown) is also needed in addition to pRb.

The three kinase inhibitors  $p16^{INK4a}$ ,  $p21^{Waf1/Cip1}$  and  $p27^{Kip1}$  generally respond to different sets of stimuli and are not of equal importance under particular circumstances to arrest the cellular cycle. The Cdi's also show unequal rates of carcinogenic alterations. In observed cases,  $p16^{INK4a}$  has been found frequently mutated or otherwise altered in tumors, but  $p21^{Waf1/Cip1}$  and  $p27^{Kip1}$  much less often.<sup>14</sup> Tumorigenesis is thought to require on average about six oncogenic events<sup>7,8</sup>, but these events can be both

complementary and exclusive. For example, silencing of  $p16^{INK4a}$  in tumors is usually accompanied with alteration of some of the central molecules of the p53 pathway (such as p53 itself), demonstrating the powerful carcinogenic effect of combined inactivation of p53 and pRb pathways. Within the pRb pathway either  $p16^{INK4a}$  or pRb (or other pRb family proteins p130 or p107) can be altered, but generally not both, i.e. expression and alteration of these components are inversely correlated.<sup>25,26,28,29</sup>

Table 1. Some important regulatory components in the cell cycle and defence systems against DNA damage, with main functions and chromosomal locations.

Gene/expression	Main function	Chromosomal mapping
p53	Damage checkpoint, arrest/apoptosis	17p13.1
p14 <sup>ARF</sup>	Binds MDM2 to stabilise p53 and arrest G <sub>1</sub>	9p21-22 <sup>1)</sup>
p16 <sup>INK4a</sup>	Induces G <sub>1</sub> arrest mediated by pRb, p130, p107	9p21-22 <sup>2)</sup>
p21 <sup>Waf1</sup>	Inhibits Cdk-cyclin, arrest of G <sub>1</sub>	6p21.2
p27 <sup>Kip1</sup>	Inhibits Cdk-2, arrest of G <sub>1</sub>	12p12 - 12p13.1
pRb	Retards G <sub>1</sub> to S transition with E2F	13q14.2
Cyclin D	Control of G <sub>1</sub> to S transition	11q13

1) reading exons 1 $\beta$ , 2 and 3; 2) alternative reading exons 1 $\alpha$ , 2 and 3

### Inhibition of cell migration

The p16<sup>INK4a</sup> protein is a small molecule and its function as a G<sub>1</sub> kinase inhibitor can be mimicked by a 10 amino acid peptide, which corresponds to the third ankyrin-like repeat of the full-length protein.<sup>10</sup> This peptide can be synthesized and directly delivered to cells, with 100 % efficiency in arresting the cells reversibly at G<sub>1</sub>. However, the peptide also shows matrix-specific inhibition of cells spreading on vitronectin, without affecting the capacity of the cells to attach to this substratum. This effect is related to dissociation of the vitronectin integrin receptor  $\alpha$ v $\beta$ 3 from the focal adhesion contacts. Similar effect has been observed using peptides derived from the kinase inhibitor p21<sup>Waf1/Cip1</sup>, suggesting that the G<sub>1</sub> associated kinase inhibitors regulate  $\alpha$ v $\beta$ 3 function.<sup>19</sup> It has also been found that transfection of glioblastoma and melanoma cells with full length p16<sup>INK4a</sup> gene construct results in inhibition of spreading and cell migration. As  $\alpha$ v $\beta$ 3 is strongly associated with invasive cancer and

angiogenesis, it is possible that p16<sup>INK4a</sup> (and some other G<sub>1</sub> kinase inhibitors) can inhibit tumor growth as well as initiation.<sup>10</sup>

The G<sub>1</sub> arrest accounts only for a minority, about 15-40% of arresting cells, the majority of arrests taking place in the G<sub>2</sub> phase where p16<sup>INK4a</sup> has no appreciable influence.<sup>2,14</sup> However, mutations or other inactivating alterations of p16<sup>INK4a</sup> are relatively common in cancers. One possible reason for this could be the suggested additional role of p16<sup>INK4a</sup> in inhibiting the spreading and migration of cancer cells, and possibly even angiogenesis.<sup>10,24</sup> Particularly solid tumors cannot grow beyond a size of about 1-2 mm without additional new blood vessels.<sup>9</sup> Although not applicable to all cancer types, initiation of such a small stagnant tumor is usually insufficient to develop a clinically significant disease without spreading and angiogenesis. This could partly explain why inactivated p16<sup>INK4a</sup> is frequently exhibited in clinical tumors, as clinically significant large and growing tumors are much more likely to be

detected than small stagnant tumors. There appears to be little comparative evidence on alterations of p16<sup>INK4a</sup> in small stagnant and larger growing tumors. General comparisons of premalignant and malignant tumors<sup>29</sup> do not provide such evidence, since most studies on premalignant tissue have not included follow-up on stagnancy. However, absence of p16<sup>INK4a</sup> tends to correlate with reduced survival, while e.g. loss of pRb expression does not, in spite of observed inverse correlation between loss of pRb and p16<sup>INK4a</sup> expressions<sup>1,25</sup>. This suggests influence by other functions of p16<sup>INK4a</sup> than cell cycle arrest on the pRb pathway.

#### Control of cellular proliferative life span

Normal somatic cells have a limited life span in terms of the number of possible cell divisions. This limit is generally attributed to gradual shortening of telomeres until they are too short to sustain further cell divisions. However, loss of function of p16<sup>INK4a</sup> or p53 can considerably extend the limit, and in this extension the effect of these two proteins is additive.<sup>11,30</sup> Eventually such cells stop dividing and reach a state called crisis. Small number of cells may escape the crisis limit and become immortalised by activating telomere maintenance, usually with the telomerase enzyme. In this process loss of p16<sup>INK4a</sup> hence appears to occur before immortalisation<sup>14</sup>.

Immortalisation is a common though not exclusive feature of tumor cells. Frequent loss of p16<sup>INK4a</sup> has been observed in immortalised tumor cell lines, while also several immortalised but not tumorigenic *in vitro* cell lines have been found to lack functional p16<sup>INK4a</sup>.<sup>10,11,14</sup> Induced expression of p16<sup>INK4a</sup> (or p21<sup>Waf1/Cip1</sup>) in immortalised human fibroblasts leads to senescence-like growth arrest.<sup>31</sup> Spontaneous loss of p16<sup>INK4a</sup> expression has been seen in small colonies of normal human mammary epithelial cells grown on standard serum-free medium, resulting in additional 40-50 divisions beyond the normal limit, until final permanent growth arrest.<sup>32,33</sup> Such spontaneous loss of

p16<sup>INK4a</sup> expression has been attributed to methylation of the CpG island of the gene, and treatment of the cells with the methylation inhibitor 5-aza-2'-deoxycytidine has been shown to restore p16<sup>INK4a</sup> expression with the accompanying normal growth arrest.<sup>34</sup>

In certain cells like normal fibroblasts and retinal pigment epithelial cells, transduction of the telomerase catalytic subunit (hTERT) cDNA is sufficient for a large extension of the life span.<sup>35,36</sup> However, other cell types like keratinocytes or human mammary epithelial cells only show this extension if either pRb or p16<sup>INK4a</sup> is inactivated. The cell type dependent differences of the extension appear not to be well understood.<sup>14,37</sup>

In several human cell types the levels of p16<sup>INK4a</sup> tend to accumulate, so that a senescent cell may contain up to 40 times the levels observed in early passage cells.<sup>38</sup> The continuous accumulation with the number of cell divisions suggests that the increase in p16<sup>INK4a</sup> levels is not a consequence of reaching senescence, and it has been speculated that accumulation of p16<sup>INK4a</sup> could be involved in triggering senescence or possibly related to progressive telomere shortening or other mitotic clock mechanisms.<sup>14</sup>

Expression of *ras* or *raf* oncogenes can induce accumulation of p16<sup>INK4a</sup> (and p53), and premature senescence.<sup>22,23</sup> This function of induction of premature senescence has also been found from other cyclin-dependent kinase inhibitors.<sup>39</sup> The kinase inhibitors therefore show partially shared complementary functions to provide redundancy for the cellular defences. These functions link the mechanisms of senescence to those of cell cycle arrest.

#### Discussion

High reactivity of the human genome can result in more than 100 modifications a day.<sup>7,8</sup> This is significant attack in spite of the large size of the genome, also because the modifications and

their consequences are not random. They inflict particularly severe carcinogenic consequences if they can disable some of the key mechanisms in the cellular defences to damage or inappropriate oncogene activation. Therefore, the observed alterations in tumors concentrate on key regulatory proteins like p16<sup>INK4a</sup> and other tumor suppressors in the defence networks.<sup>1,2,12</sup> Alteration or loss of p16<sup>INK4a</sup> has been often implicated in a wide range of different types of cancers, including head and neck, esophageal, lung, brain, breast, colon, bladder and bone cancers as well as gliomas, T-cell acute lymphoblastic leukemias and melanomas.<sup>16,17,18</sup> In general, albeit insufficient to initiate cancer alone, defective p16<sup>INK4a</sup> is the most common type genomic damage in human cancer after defective p53, and frequently correlated with reduced survival.<sup>1,24,28</sup>

Understanding of the functions of p16<sup>INK4a</sup> has been complicated by the unusual character of the corresponding gene locus. Through alternative reading frames this locus encodes two distinctly different regulatory proteins, p16<sup>INK4a</sup> and p14<sup>ARF</sup>, of which the latter induces p53-dependent growth arrest by binding MDM2, stabilising p53 and thereby promoting p21<sup>Waf1/Cip1</sup> to inhibit kinase-cyclin complexes required for G<sub>1</sub>/S transition.<sup>2,14</sup> Genomic alteration affecting p16<sup>INK4a</sup> can also have an impact on p14<sup>ARF</sup>, and therefore without checking it is possible that phenomena due to alterations in both proteins are incorrectly assigned to only one of them. There is evidence suggesting that the expression of both of these proteins is regulated by a common transcriptional repressor bmi-1<sup>15</sup>, directly linking the growth arrest functions of the pRb and p53 pathways.

G<sub>1</sub> arrest accounts only for a minority, about 15-40% of arresting cells after DNA damage, the majority of arrests taking place in G<sub>2</sub> where p16<sup>INK4a</sup> has no known influence<sup>2,14</sup>, and yet mutations or other inactivating alterations of p16<sup>INK4a</sup> are relatively common in cancers. A possible explanation can be found in the suggested functions of p16<sup>INK4a</sup> in inhibiting the

spreading and migration of cancer cells, and possibly even angiogenesis.<sup>12</sup> Solid tumors are believed to have a maximum size of about 1-2 mm without angiogenesis or additional blood vessels to maintain growth. Initiation of a single tumor would therefore not lead to a clinically significant disease without spreading and angiogenesis. In addition, loss of p16<sup>INK4a</sup> function may facilitate avoiding cellular senescence, which is also an important defence mechanism against genomic damage and oncogenesis. The roles in tumor growth and senescence could partly explain why dysfunctional p16<sup>INK4a</sup> is frequently exhibited in clinical tumors.

On the other hand, a chain of about six oncogenic events are believed to be necessary to develop cancer, and within this chain generally both the pRb and p53 pathways are impaired in tumors.<sup>7,8</sup> This alone suggests that growth regulation in G<sub>1</sub> is significant in carcinogenesis, and therefore potentially also the functional role of p16<sup>INK4a</sup> as a cyclin-dependent kinase inhibitor. Within this explanation, the importance of p16<sup>INK4a</sup> is emphasised by the observation that alterations of p16<sup>INK4a</sup> and pRb are inversely correlated in tumors.<sup>25,26,29</sup> This suggests that the tumor suppressor function of p16<sup>INK4a</sup> has considerable uniqueness in spite of existing other members of the INK family of proteins, so that p16<sup>INK4a</sup> exhibits limited redundancy within the pRb pathway. Although there are other cyclin-dependent kinase inhibitors of both p53 dependent and independent pathways, such as p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup>, these do not share same routes of promotion or impact with p16<sup>INK4a</sup>. Also, little redundancy to p16<sup>INK4a</sup> or pRb functions is provided by p130 and p107, which have been also found to be altered in some tumors.<sup>9</sup> Not much redundancy can be expected from p14<sup>ARF</sup> either, since due to shared coding locus both protein expressions can be dysfunctional when p16<sup>INK4a</sup> is altered.

The performance and redundancies in the network of cellular defences are characterised by the number of oncogenic

events required for carcinogenesis, as well as the relative difficulty by the oncogenic agents to induce them. Individual molecular components of the defences are much less redundant than the system, and with unequal oncogenic weight. Therefore molecules like p16<sup>INK4a</sup> with multiple important functions are often dysfunctional in tumors. For reversing the processes that support tumor initiation and growth, it may hence suffice to interfere with the dysfunction of very few key molecules within the cellular defences. This is also an essential starting point for much of the current effort towards new therapeutic drugs.

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