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Role of DMP1 and its future in lung cancer diagnostics

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Abstract

Lung cancer is the most lethal carcinoma worldwide. Mutations of p53, inactivation of $p16^{INK4a}$, and overexpression of cyclins E, A and B are independently associated with poor prognoses of patients, while the prognostic value of cyclin D1 or RB expression is inconclusive. Cyclin D binding myb-like protein 1 (Dmp1) encodes a DNA binding protein that receives signals from oncogenic Ras and functions as a tumor suppressor by activating the Arf-p53 pathway. Dmp1 has been shown to be haplo-insufficient for tumor suppression in mouse models including K-ras-mediated lung carcinogenesis. The human DMP1 gene is located on chromosome 7q21, and our recent results revealed that the *hDMP1* gene is deleted, but not mutated or silenced, in approximately 40 % of human non-small-cell lung carcinomas. These cases typically retained

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wild-type *ARF* and *p53* and expressed very low levels of the *h*DMP1 protein. Thus, *hDMP1* loss could be a novel diagnostic marker for non-small-cell lung carcinomas.

Keywords

ARF; DMP1l; haploid insufficiency; immunohistochemistryl; LOHl; loss of heterozygosity; lung cancer; $p16^{INK4a}$; p53; Ras; tumor-suppressor gene

Lung cancer is the second most common human malignancy regardless of ethnic origin or sex [1]. In the USA, there are approximately 215,000 new patients and 162,000 deaths per year due to lung cancer, accounting for approximately 30% of total cancer deaths [1]. Novel anticancer therapies including novel cytotoxic agents and molecular-targeted reagents are developed each year, but the prognosis for lung cancer patients is still extremely poor, with overall 5-year survival of approximately 15% [1–4]. Lung cancer is categorized into two major histopathological groups: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). Approximately 80% of human lung cancers are NSCLC and they are further classified into adenocarcinomas, squamous cell carcinomas and large-cell carcinomas [3]. NSCLC and SCLC show striking differences in histopathologic characteristics that can be explained by the differential patterns of genetic alterations found in both tumor types [3,5–8]. The diagnostic and prognostic values of molecules that are involved in normal and malignant cell cycles have been extensively studied for human lung cancer in the past 20 years. In this review, we will briefly discuss the diagnostic values of known markers for cell cycle regulators in human NSCLC and then we will focus on the roles of Dmp1 in lung carcinogenesis and its possible diagnostic value.

Physiological cell cycle regulators

In nontransformed cells, cell cycle division is regulated in an ordered, securely regulated process involving multiple checkpoints that respond to extracellular growth signals, cell size and DNA integrity [9–12]. The replication of DNA occurs in the S phase and segregation of the chromosomes into daughter progeny occurs in the M phase (mitosis). There are two 'gap' phases in the mammalian cell cycle, named G_1 and G_2. During the G_1 phase, cells prepare for DNA synthesis and, during G_2, cells prepare for mitosis [9–12]. Cyclin/CDK complexes are formed during distinct phases of the cell cycle and are specifically involved in the phosphorylation of target proteins, including pocket proteins (RB, p107 and p130) (Figure 1). Mammalian G_1 cyclins D and E mediate progression through the G_1/S phases. Three D-type cyclins exist (cyclin D1, D2 and D3), which are expressed differently in various cell lineages, with most cells expressing cyclin D3 and either D1 or D2 (Figure 1). E-type cyclins (cyclins E1 and E2) are expressed during late G_1 to the end of S phase of the cell cycle. The activity of cyclin E plays critical roles in the passage of cells through the restriction point, which marks an irreversible point for cells to complete the rest of the cell division cycle. Expression of cyclin E is regulated at the level of gene transcription mainly by E2F proteins and by its degradation via the proteasome pathway. Cyclin E binds and activates the kinase CDK2 to phosphorylate pocket proteins and initiate a cascade of events that leads to the expression of S phase-specific genes (Figure 1) [9–13]. Aside from this specific function as a regulator of S phase entry, cyclin E plays distinct roles in the initiation of DNA replication, the control of genomic stability and the duplication of the centrosome. Mitotic cyclins A and B mediate progression through the S/G_2 to M phases. Cyclin A2 is expressed in proliferating somatic cells, while cyclin A1 is specifically detected in the testis and early embryogenesis. Cyclin B1 plays general roles in M phase progression, while cyclin B2 has a special function in Golgi remodeling during mitosis. *Cyclin B2*-null mice develop normally and are fertile whereas *cyclin B1*-null mice die *in utero*.

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The product of the retinoblastoma susceptibility gene (*RB*), plays a central role in the G_1–S transition (Figure 1) [11,12]. In its unphosphorylated state, RB prevents progression from G_1 to S phase by binding the key transcription factor, E2Fs1–3/DP-1 [11–13]. Once the RB protein is phosphorylated by the cyclin D/Cdk complex, E2F is released, thus allowing transcription of a battery of genes that regulate DNA synthesis. The p107/p130 proteins are required for the repression of distinct sets of genes, potentially due to their selective interactions with E2F4 and E2F5 that are engaged at specific promoter elements [13]. In addition to the regulation of E2F–responsive genes, pocket proteins contribute to silencing of genes in cells that are undergoing senescence or terminal differentiation. Pocket proteins also affect the G_1–S transition through E2F–independent mechanisms, such as by inhibiting CDK2 or stabilizing $p27^{KIP1}$ and these mechanisms have been implicated in the control of G0 exit, DNA replication and genomic re-replication [11–13].

The CIP/KIP ($p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) and INK4 families ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) represent two distinct families of CDK inhibitors that share no primary sequence similarity in spite of their binding to common targets, CDK4 and CDK6 (Figure 1) [14,15]. The binding mode and CDK specificity are different between these two families of inhibitors. While $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ bind to and form ternary complexes with cyclin D/CDK4 or CDK6, cyclin E/CDK2, cyclin A/CDK2, cyclinA/CDC2 and cyclin B/CDC2, the INK4 proteins bind exclusively to, and form tight binary complexes with, CDK4 and CDK6. Moreover, the expression pattern of each CDK inhibitor gene is differentially regulated by distinct antiproliferative signals and does not appear to be coordinated in most cases. For instance, p53 directly binds and activates the $p21^{CIP1}$ promoter while pRB represses $p16^{INK4a}$ transcription [15]. TGF-β treatment stimulates the transcription of $p15^{INK4b}$, but not $p16^{INK4a}$ or $p14^{ARF}$ although these three genes are located on the same genomic locus 9p21 in humans [15]. The transcription of $p18^{INK4c}$ or $p19^{INK4d}$ is not affected by these antiproliferative stimuli. These distinct transcriptional regulations in response to different antiproliferative signals together with their tissue-and developmental stage-specific expression patterns, established the concept that different CDK inhibitors are regulated by different growth inhibitory pathways, as in the case of sequential cyclin expression and CDK activation. Therefore, alterations in any one of these cell cycle regulatory proteins could lead to failure of cell cycle arrest, which will eventually contribute to neoplastic transformation of cells.

Prognostic values of the retinoblastoma susceptibility gene in human NSCLC

Inactivation of *RB* (by truncation, gene deletion, nonsense mutation or splicing alterations), together with loss of the wild-type *RB* allele, have been demonstrated in lung cancers, with protein abnormalities detected in approximately 90% of SCLC and 15–30% of NSCLC [16,17]. Whether the absence of RB expression is associated with poor prognosis in NSCLC is controversial. A study conducted by immunohistochemical detection of pRB in more than 100 patients with stage I and II NSCLC showed that the median survival was 32 months for patients with RB-positive tumors and 18 months for individuals in whom expression of RB protein was absent or altered [18]. However, later studies failed to show an independent prognostic value of RB status in NSCLC [19,20]. Nonetheless, it was reported that pRB; p53 combined status was a predictive factor of overall survival [18,21]. Patients with pRB(−); p53(+) tumors had a median survival of only 12 months, whereas those with pRB(+); p53(−) tumors had a median survival of over 40 months [18,21]. Zagorvski *et al.* studied the roles of RB loss in tumorigenic proliferation and sensitivity to chemotherapeutics in NSCLC cells [22]. Downregulation of RB by shRNA led to a proliferative advantage *in vitro* and aggressive tumorigenic growth in xenograft models with increased chemosensitivity. However, this response was transient and a durable response was dependent on prolonged

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chemotherapeutic administration [22]. They concluded that although RB loss enhances sensitivity of NSCLC cells to chemotherapeutic agents, efficient and sustainable response was highly dependent on the specific therapeutic regimen in addition to the molecular environment [22]. So far, no correlation between the RB status and patients' survival has been reported in SCLC, possibly because there are very few patients with SCLC with intact RB [17,23].

Impact of cyclins & CDK inhibitors in NSCLC

Upregulation of the cyclin D1 proto-oncogene is known to play key roles in G_1–S progression of the cell cycle as described earlier. An increase in this gene's expression permits loss of G_1 restriction point integrity. The impact of cyclin D1 overexpression in NSCLC is again a topic of debate [24,25]. Of the four main prognostic studies of cyclin D1 in NSCLC, two of them showed improved survival, whereas the other two showed shorter survival. In a study with 106 patients with stages I and II of NSCLC, cyclin D1 expression was associated with shorter survival and the cumulative survival rate of cyclin D1(+), $p16^{INK4a}$(−) patients was significantly lower than that of cyclin D1(−), $p16^{INK4a}$(+) patients (logrank test, $p = 0.0004$; Wilcoxon test, $p = 0.0002$) [24]. In contrast to cyclin D1, over-expression of cyclin E, cyclin A or cyclin B has been reproducibly associated with shorter survival among stage I–IIIA NSCLC patients undergoing curative surgical resection [25].

The prognostic value of expression of CDK inhibitor has also been examined. In two studies that adequately controlled for disease stage, $p21^{CIP1}$ expression was associated with improved survival [25]. Studies evaluating the effect of $p27^{KIP1}$ expression have also demonstrated a favorable effect on lung cancer survival in NSCLC with $p27^{KIP1}$ expression [25]. Among the four INK4 family proteins, the impact of lung cancer patients' survival has been studied exclusively on $p16^{INK4a}$. The absence of $p16^{INK4a}$ protein expression as detected by immunohistochemistry or Western blotting has reproducibly shown shorter survival, although two of seven studies did not reach statistically significant differences [25]. Additionally, Kratzke *et al.* reported an inverse correlation between pRB and $p16^{INK4a}$ expression in 65% of NSCLC cases ($p = 0.00019$) [26]. The observation that lack of $p16^{INK4a}$ expression was associated with a worse prognosis was consistent with the increased incidence of $p16^{INK4a}$ mutations observed in metastatic NSCLC. Other studies have also reported $p16^{INK4a}$ mutations with advanced stage (stage III and IV) in NSCLC [27]. The frequency of deletions of the $p15^{INK4b}$ gene was 12% (four of 34 cases) and no point mutations in the $p15^{INK4b}$ gene were detected in the NSCLC [28]. For the $p18^{INK4c}$ gene, no abnormality was detected in human NSCLC [28]. Alterations of $p19^{INK4d}$ or $p57^{KIP2}$ have not been reported in human lung cancer. In summary, loss of expression of the inhibitors $p16^{INK4a}$, $p27^{KIP1}$ and $p21^{CIP1}$ and/or overexpression of the cyclins A, E and B1 predict a poor prognosis of NSCLC patients after surgery [25]. Conversely, the impact of the expression of pRB and cyclin D1 on patients' survival has not been determined in human NSCLC.

Involvement of the ARF–p53 pathway in NSCLC

The *p53* tumor-suppressor gene has been reported to be mutated in approximately 50% of all human cancers. p53 responds to a variety of stress signaling including DNA damage, overexpression of oncoproteins and metabolic limitations to regulate a battery of target genes that induce cell cycle arrest, apoptosis, DNA repair and metabolism [29]. The importance of *p53* mutations in the pathogenesis of human lung carcinoma is very well established. Since wild-type p53 has a very short half-life (10–20 min), it is usually undetectable by standard immunostaining of normal tissues. By contrast, most mutant p53 proteins have prolonged half-lives, thus allowing visualization of the protein by

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immunohistochemistry. The significance of the p53 protein expression on the prognosis of NSCLC patients has been extensively studied by many different groups [27,30–39]. Approximately half of the studies found an increased risk for shorter survival with p53 expression, while high p53 expression had no effect, or was associated with favorable disease outcome, in the other half of studies. This controversy is, at least in part, due to the methodological differences in the detection of p53 proteins in lung cancer (i.e., differences in the antibodies or protocols for immunohistochemistry and/or in different criteria for the grading of p53-positive signals in tissues).

In good contrast to the controversial studies with p53 protein expression, genetic analyses of *p53* have consistently demonstrated that NSCLC with mutated *p53* had an adverse effect on the survival of patients with NSCLC [27,38–50]. Most genetic analyses have been conducted by single-strand conformation polymorphism for screening followed by nucleotide sequencing or p53 GeneChip® assay [42]. One report demonstrated that *p53* mutations at exons 7 and 8 were the most predictive for poor clinical outcome [40], while another group reported that *p53* mutations in exon 5 were associated with poor prognosis of NSCLC patients [49]. Although the results were different depending on the patient population and the methods they used, *p53* mutations detected by molecular genetic analyses are generally a more reliable predictor of poor outcome than p53 protein overexpression in patients with stage I–IIIA NSCLC.

The activity of p53 is positively regulated by $p14^{ARF}$ ($p19^{Arf}$ in mice) in response to oncogenic stress (Figures 2 & 3) [51–53]. $p14^{ARF}$ is an alternative reading frame gene product generated from the *INK4a/ARF* locus which also encodes the cyclin-dependent kinase inhibitor $p16^{INK4a}$ [54]. $p14^{ARF}$ directly binds to Hdm2, thereby stabilizing and activating p53, whereas $p16^{INK4a}$ binds to cyclin-dependent kinase 4 to inhibit Rb phosphorylation (Figure 2) [51–55]. Since this single genetic locus encodes two independent tumor-suppressor proteins that regulate the p53 and the RB pathways, it is very frequently (~40%) disrupted in human cancer [56]. The ARF induction by potentially harmful growth-promoting signals forces early-stage cancer cells to undergo p53-dependent and p53-independent cell cycle arrest or apoptosis, providing a powerful mode of tumor suppression [51–53]. The *Arf* promoter is activated by latent oncogenic signals *in vivo* [57] and thus *Arf*-null mice are highly prone to spontaneous tumor development [58]. $p19^{Arf}$ (or $p14^{ARF}$) interacts with nucleophosmin, E2F1, DP1 and numerous other proteins, showing the p53-independent functions of Arf [53]. In human lung cancers, $p14^{ARF}$ is more frequently inactivated in SCLC (~65%) than in NSCLC (~20%) [6]. Promoter hypermethylation of *ARF* has been reported in approximately 10% of NSCLC, but is much less frequent than that of $p16^{INK4a}$ (~40%) on the same locus [6]. Point mutations for *ARF* are very rare in human NSCLC.

The prognostic value of $p14^{ARF}$ has rarely been studied in human NSCLC. Wang *et al.* made a striking discovery that overexpression of p53 is associated with low expression of Hdm2 ($p < 0.001$) and high expression of $p14^{ARF}$ ($p = 0.001$) [62]. The overexpressed p53 proteins detected in their study were considered to be mutant p53 since wild-type p53 increases the Hdm2 levels by transactivation of the *Hdm2* (and also *Mdm2*) promoter and repression of the $p14^{ARF}$ promoter (Figure 3) [59–61]. Both overexpression of p53 and absence of Hdm2 expression were associated with squamous cell carcinoma, advanced stages and shorter survival of NSCLC patients (all $p < 0.05$), suggesting that disruption of the ARF–Hdm2–p53 pathway is important in the pathogenesis and outcome of NSCLC [62].

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Novel transcription factor Dmp1 is a regulator of the ARF–p53 pathway

Among known *Arf* activators, cyclin D-binding myb-like protein-1 (Dmp1), also called cyclin D-binding myb-like transcription factor 1 (Dmtf1), is a unique tumor suppressor [63–71]. Dmp1 was originally isolated in a yeast two-hybrid screen of a murine T-lymphocyte library with cyclin D2 as bait (Figure 1) [63]. Importantly, Dmp1 directly binds to the *Arf* promoter to activate its expression, thereby inducing p53-dependent cell cycle arrest (Figures 2 & 3) [64,65]. Dmp1 also binds to and activates the *CD13/aminopeptidase N* promoter through interaction with the c-Myb protein, suggesting its role in hematopoietic cell differentiation [66]. *Dmp1*-null mice are prone to spontaneous tumor development, which was accelerated when the animals were neonatally treated with ionizing radiation or dimethylbenzanthracene [67,68]. Although *Dmp1*-knockout mice develop a broad spectrum of epithelial and non-epithelial tumors, lung adenomas/adenocarcinomas were the most frequently found tumors in *Dmp1*-null and *Dmp1*-heterozygous mice (Figures 4A & 4B). The wild-type *Dmp1* allele is very often retained and expressed in tumors arising from $Dmp1^{+/-}$ mice, demonstrating a typical haplo-insufficiency for tumor suppression, although the molecular mechanisms are not clear [68,69]. Tumors from Eμ-*Myc*; $Dmp1^{-/-}$ or $Dmp1^{+/-}$ mice rarely show mutations, deletions, or silencing of $p19^{Arf}$ or p53, suggesting that Dmp1 is a critical regulator of the *ARF*–p53 tumor-suppressor pathway in living animals [68,69]. We have recently characterized the *Dmp1* promoter [70–74]. The *Dmp1* promoter is activated by the oncogenic Ras–Raf–MEK–ERK–Jun pathway. It is well known that continuous oncogenic Ras activation upregulates $p19^{Arf}$ and induces p53-dependent cell cycle arrest. Our results demonstrated that the induction of *Arf* by mutant Ras was Dmp1-dependent (Figures 2 & 3) [72]. On the other hand, the *Dmp1* promoter is repressed by overexpression of E2Fs and also by physiological mitogenic signaling [73]. Thus, Dmp1 is a marker of cells that have exited from the cell cycle [73]. Our most recent study shows that the *Dmp1* promoter is repressed by genotoxic stimuli (daunomycin, doxorubicin or UVC) that activate NF-κB through phosphorylation of the p65 subunit, and that the repression of the *Arf* promoter by genotoxic stress was Dmp1-dependent [74]. Thus, Dmp1 is a sensor to convey some forms of oncogenic and nononcogenic stress to the ARF–p53 pathway (Figure 3).

Roles of Dmp1 in *K-ras^{LA}* medicated lung cancer development

Dmp1-null mice were crossed with *K-ras^{LA}* mice to demonstrate the interactions between *Dmp1*-loss and oncogenic *K-ras* activation *in vivo* [75]. *K-ras$^{LA1/+}$* and *K-ras$^{LA2/+}$* are unique mouse models of lung cancer where the *K-ras* gene is controlled by its own promoter and is activated during spontaneous recombination events in the whole animal [76]. We found that the survival of *K-ras^{LA}* mice was significantly shortened in both $Dmp1^{+/-}$ and $Dmp1^{-/-}$ mice, with little difference between the two cohorts [75]. The lung tumor cells from $Dmp1^{+/-}$, *K-ras^{LA}* mice expressed *Dmp1* mRNA and protein in most cases, clearly demonstrating the haploid-insufficiency of Dmp1 in lung cancer suppression in these mice models. However, *K-ras^{LA}* lung tumors are different from Eμ-*Myc* lymphomas because bi-allelic *Arf* deletion or Mdm2 overexpression was not found in any tumors regardless of the genotype of *Dmp1* [75]. Moreover, none of the known *Ink4a/Arf* repressors, such as Bmi1, Twist, Tbx2, Tbx3 and Pokemon, were overexpressed in *K-ras^{LA}* lung tumors, ruling out the possibility of the contribution of these *Ink4a/Arf* modulators for *K-ras*-induced lung tumor development [75,77–81]. Approximately 40% of lung tumors from wild-type *K-ras^{LA}* mice showed mutations of the *p53* gene, recapitulating the molecular genetic alterations of p53 in human NSCLC [75]. Interestingly, *p53* mutations were rarely found in lung tumors from $Dmp1^{+/-}$, $Dmp1^{-/-}$, *K-ras^{LA}* mice; thus, it was assumed that *Dmp1*-deletions might have similar effects to *p53* mutations. In fact, we have found that tumors present in $Dmp1^{+/-}$, $Dmp1^{-/-}$, *K-ras^{LA}* mice tended to show malignant features of carcinomas, such as

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intravascular and/or intrabronchial invasion (Figures 4C & 4D) [75]. Moreover, the *Dmp1*$^{+/-}$, *Dmp1*$^{-/-}$, *K-ras*^{LA} group frequently developed types of tumors other than lung carcinomas [75]. Of note, the *Ink4a/Arf* locus is rarely inactivated by homozygous gene deletion or silencing in *K-ras*^{LA} lung tumors [75,76]. Thus, *Dmp1*-deletion and *p53* mutations play major roles in the development of *K-ras*^{LA} lung carcinomas.

Human DMP1 is a critical tumor suppressor in human lung cancer

The human *DMP1* (h*DMP1*) gene is located on human chromosome 7q21. The 7q21–31 region has been reported to be a hot locus of genomic DNA deletion in human carcinomas and hematopoietic malignancies [82–84]. Bodner *et al*. studied the copy numbers of the *hDMP1* locus by FISH analysis in leukemic samples with chromosome 7q abnormalities. The results demonstrated that one allele of the *hDMP1* locus was invariably deleted in tumor cells with 7q alterations, suggesting that the *hDMP1* locus was critically involved in 7q– leukemias [84]. Later, Tschan *et al*. characterized the *hDMP1* splicing variants, *hDMP1*α, β and γ [85]. The β- and γ splicing isoforms do not bind to DNA since they lack most of the DNA-binding domain of DMP1 [85]. The full-length *h*DMP1α is equivalent to full-length murine Dmp1, which directly binds to the *Arf* promoter and positively regulates the p19^{Arf}–p53 pathway. Interestingly, Tschan *et al*. showed that these variant isoforms are specifically expressed in immature hematopoietic cells and that *h*DMP1β inhibited *CD13/aminopeptidase N* promoter transactivation by *h*DMP1α [85]. Notably, stable and ectopic overexpression of *h*DMP1β efficiently blocked phorbol-12-myristate-13-acetate-induced terminal differentiation of U937 cells to macrophages, which resulted in maintenance of proliferation [85]. Therefore, in humans, the *hDMP1* α isoform has tumor-suppressor activity and the β and γ proteins are regarded as dominant negative isoforms for *h*DMP1α [85].

Previous studies have shown differential involvement of the *INK4a/ARF, RB* and the *p53* locus in human lung cancers. For instance, *RB* is inactivated in approximately 90% of human NSCLC, while *p16*^{INK4a} is deleted and/or promoter silenced in more than 50% of NSCLC. Promoter hypermethylation or deletion of *ARF is* relatively rare in NSCLC; however, *ARF is* inactivated in approximately 65% of human SCLC [6]. The *p53* gene is mutated in 90% of SCLC and in 50% of NSCLC [6]. In order to demonstrate the involvement of *hDMP1* in human lung cancer, we have recently conducted genomic DNA deletion analyses of *hDMP1, INK4a/ARF* and *p53* by loss of heterozygosity (LOH) assays in more than 50 cases of human NSCLC (total 51 patients: 33 cases of lung adenocarcinoma, 16 cases of squamous cell carcinoma and two cases of adenosquamous carcinoma) [75]. This is the first report of human cancer analysis for *hDMP1*. LOH of *hDMP1* was found in approximately 35% (average of two different sets of primers; 41% if we use relaxed criteria) of NSCLC (Figure 5) [75]. LOH for the *INK4a/ARF* or *p53* locus was also found in 30–50% of the same samples (Figure 5) [75]. Interestingly, LOH of the h*DMP1* locus and that of the *INK4a/ARF* or *p53* locus occurred in a mutually exclusive fashion ($p = 0.0035$ for h*DMP1* vs *INK4a/ARF*; $p = 0.027$ for h*DMP1* vs *p53*), consistent with our hypothesis that hemizygous deletion of h*DMP1* may be inactivating the ARF–p53 pathway in human NSCLC [75]. Of note, the LOH for *INK4a/ARF* and that of *p53* were overlapping at a higher frequency than random ($p = 0.0045$), possibly because the *INK4a/ARF* locus regulates both RB and p53 pathways and because p14^{ARF} has p53-independent function for tumor suppression.

Importantly, the region that was deleted in human lung cancer was confined to the h*DMP1/MGC4175* locus in approximately 80% of the cases that showed LOH for h*DMP1*. Although it was very difficult to dissect the contribution of h*DMP1* deletion and *MGC4175* deletion in NSCLC, h*DMP1* was considered to be a key player, since *MGC4175* encodes a

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mitochondrial protein that is involved in taxol- and doxorubicin-resistant malignant phenotypes in human cancer cell lines and, therefore, deletion of this gene would result in tumor regression rather than progression. Point mutations, and promoter methylations that inactivate *h*DMP1 functions, were very rare (<10%) in our NSCLC samples. Importantly, ectopic expression and activation of Dmp1:ER in an ARF^{+}, *p53* wild-type lung cancer cell line strongly inhibited the growth of the cells, while other lung cancer cells with deletion for *ARF* or *p53* were relatively resistant to the effects of Dmp1:ER [75]. In summary, our recent study demonstrated that the h*DMP1* gene is inactivated in a significant percentage of human NSCLC, especially those which hold the status of wild-type *ARF*, and *p53* and h*DMP1* deletion plays a key role in human lung cancer development.

Detection of the *h*DMP1 protein in human lung cancer

Although Dmp1 (or *h*DMP1) lacks nuclear localization signals, the endogenous product is localized in the nucleus in normal tissues, NIH 3T3 cells, H460 cells and approximately 30% of lung cancer samples (Figure 6). We found a significant correlation between the intensity of *h*DMP1 staining in the nucleus and the absence of h*DMP1* deletion [75]. However, when we extended our study to approximately 40 NSCLC samples, we noticed that there are many cases where *h*DMP1 is cytoplasmically localized or localized in both the nucleus and cytoplasm in NSCLC (Figures 6E, F & G). Cytoplasmic localization of the *h*DMP1 protein was confirmed by immunohistochemistry with two different antibodies to DMP1 in case #2541 (Figure 6). Although the mechanisms of cytoplasmic mislocalization of *h*DMP1 in cancer cells remain to be determined, there are a few possibilities. One possibility is that lung cancer cells lack binding partner(s) for *h*DMP1 that normally interact and transport *h*DMP1 from the cytoplasm to the nucleus. The other possibility is that *h*DMP1 proteins expressed in tumor cells lack physiological post-translational modification(s) that are essential for their nuclear localization. Physiological cytoplasmic localizations of transcription factors have been reported in repressive E2Fs and NF-κB [86,87]. In repressive E2Fs, the proteins use nuclear localization signals of their binding partners (DPs and pocket proteins) for nuclear transport [86]. The NF-κB dimers are bound by inhibitory IκB molecules and stay in the cytoplasm in unstimulated cells. Since transcriptional activation by NF-κB requires its nuclear translocation, signal-induced degradation of IκB molecules by phosphorylation at serine residues 32 and 36 is considered to be critical in NF-κB activation [87]. Thus, it will be essential to identify physiological *h*DMP1-binding partners by mass spectrometry analyses and/or binding assays with known molecules to clarify the mechanisms of nuclear localization of DMP1.

Our recent study demonstrated that stimulation of Dmp1:ER with 4-hydroxytamoxifen showed a major shift of the band of Dmp1:ER when the protein translocated from the cytoplasm to the nucleus in H460 cells [75]. These results suggested that post-translational modification(s) may also mediate Dmp1's nuclear translocation or prevent its nuclear export, the mechanisms of which may be altered in human lung cancer cells.

h*DMP1* as a biomarker of NSCLC?

The diagnostic or prognostic value of *h*DMP1 has never been tested in the literature. However, our recent study shows that LOH of h*DMP1* was typically found mutually exclusively with that of the *INK4a/ARF* locus or that of *p53*. Our study has also demonstrated that LOH of *INK4a/ARF* is often associated with silencing of the $p16^{INK4a}$ or $p14^{ARF}$ promoter, suggesting biallelic inactivation [75]. Our results are consistent with previous reports that showed good correlation between LOH of 9p21 and methylation of $p16^{INK4a}$ promoter in NSCLC [88,89]. We also conducted sequencing analyses of the *p53* cDNA in NSCLC when RNA was available. All of the four *p53* LOH(+) cases showed

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mutations for *p53* while none of the two *p53* LOH(−) cases showed *p53* mutations [75], consistent with the results from other groups [90,91]. Therefore, it is possible that NSCLC with h*DMP1* deletion existed in the historical group of NSCLC patients without *p53* mutation and also in that without $p16^{INK4a}$ alterations. Since previous studies have consistently demonstrated that mutations of *p53* or absence of the $p16^{INK4a}$ protein is associated with shorter survival and worse prognosis of patients with NSCLC, LOH of *hDMP1* or low expression of the *h*DMP1 protein in immunohistochemistry might be associated with relatively better prognoses of patients. Nevertheless, we still believe that *hDMP1* will a useful bio-marker for human NSCLC and LOH assays should be carried out for genotyping for the following reasons:

- There are small numbers of cases of NSCLC where LOH for *hDMP1* and that of *INK4a/ARF* or *p53* occurred simultaneously (10–20%) [75]. There are also cases of NSCLC where none of the *hDMP1, INK4a/ARF* or *p53* loci are involved (13% of total) [75]. Thus, some *hDMP1* LOH(+) cases might have existed in NSCLC with p53 or $p16^{INK4a}$ alterations.
- The primers used for LOH assays of the *INK4a/ARF* and the *p53* loci have been carefully designed by us to accurately evaluate gene deletions for these genomic loci and, thus, our LOH assays are unique. Therefore, although our results show mutually exclusive inactivation of *hDMP1* and *INK4a/ARF or p53 in* the vast majority of NSCLC cases, our results cannot be directly compared with those from historical studies conducted by other groups who used published microsatellite markers located more than 1 Mbp away from the *INK4a/ARF* or *p53* locus.
- The *INK4a/ARF* locus encodes another important tumor suppressor, $p14^{ARF}$, which has been considered the direct target of *h*DMP1. We speculate that this is the major reason why LOH for *hDMP1* of *INK4a/ARF are* mutually exclusive in approximately 90% of the cases [75]. Since the prognostic value of p14 ^{ARF} inactivation in NSCLC has not been reported in the literature, it is not possible to predict the prognostic value of *hDMP1* LOH just from the mutual exclusiveness with the LOH of the *INK4a/ARF* locus. It is not known whether $p16^{INK4a}$ is a direct target for hDMP1.
- We have found increased metastasis of $K\text{-}ras^{LA}$ lung tumors in *Dmp1*-hetrozygous mice [75]. Thus, it is possible that DMP1 regulates other genes that are involved in angiogenesis and/or metastasis of lung cancer cells. These targets will be regulated independently of the ARF-p53 pathway.

Hence, the diagnostic and prognostic values of *hDMP1* deletion and its correlation with other biomarkers have to be extensively studied in the near future using lung cancer patients' samples with known prognostic data.

Expert commentary

Lung cancer has been the leading cause of cancer mortality in the world and, thus, it is the most challenging topic for cancer research. The impact of cell cycle regulators, such as cyclins E,A and B and CDK inhibitors $p16^{INK4a}$, $p21^{CIP1}$ and $p27^{KIP1}$, in NSCLC have been well established. The prognostic values of *p53* mutations as detected by molecular genetic approaches have also been established in human NSCLC, although the impact of p53 detection by immunohistochemistry on patients' survival has been very controversial. The prognostic significance of $p14^{ARF}$ on clinical stage and/or patients' survival has not been reported in the literature for NSCLC.

Crossbreeding of $K\text{-}ras^{LA1}$ and $K\text{-}ras^{LA2}$ mice with *Dmp1*-null mice showed significant acceleration of lung carcinogenesis and shortened survival of $K\text{-}ras^{LA}$ mice [75]. Thus our

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study has established that Dmp1 plays significant roles in the prevention of *K-ras*-induced lung adenocarcinomas. The survival of $Dmp1^{+/-}$, $K\text{-}ras^{LA}$ and $Dmp1^{-/-}$, $K\text{-}ras^{LA}$ mice were not significantly different and lung tumors from $Dmp1^{+/-}$ mice retained and expressed the wild-type *Dmp1* allele as studied by competitive and real-time PCR [75]. Moreover, our immunohistochemical data showed expression of the Dmp1 protein in lung tumors from $Dmp1^{+/-}$ mice [75]. Importantly, lung tumors from $Dmp1^{+/-}$ or $Dmp1^{-/-}$, $K\text{-}ras^{LA}$ mice rarely showed mutations of the *p53* gene, which was found in 40% of wild-type $K\text{-}ras^{LA}$ lung tumors [75]. Thus, Dmp1 is considered to be a nonclassical, haplo-insufficient tumor suppressor gene which plays a critical role in the Ras–Arf–p53 signaling cascade.

The *Dmp1* (or h*DMP1*) gene is often inactivated by deletion in NSCLC cells with wild-type Arf or p53. Our immunohistochemistry results demonstrate that the *h*DMP1 protein is significantly downregulated in NSCLC cells that show LOH for the h*DMP1* locus. Since NSCLC with *p53* mutations have been shown to be associated with shorter survival of patients, it is reasonable to predict that NSCLC samples with LOH for h*DMP1* and/or low expression of the *h*DMP1 protein in immunohistochemistry will have a more favorable outcome in comparison to those with *p53* mutations.

Five-year view

Recent studies show improving prediction of drug efficacy and patient survival using molecular biological techniques. Lung cancers, *p53* mutations, *K-Ras* mutations and EGF receptor mutations may become indicators for the success of anticancer therapy and prognosis (reviewed in [92–94]). p53, anti-p53 antibodies, EGF receptor and Ras have been detected in the serum of lung cancer patients. However, routine use for these serum biomarkers for early detection of occupationally derived lung carcinomas is currently controversial [95]. HER2 overexpression has been shown to be a poor prognostic factor [96] and low expression of the excision repair cross-complementation group 1 gene was associated with improved survival within cis-platinum-based chemotherapy for NSCLC [97].

Our study has demonstrated the inactivation of h*DMP1* in approximately 40% of human NSCLC. Future studies should focus on the determination of prognostic values of h*DMP1* deletion and/or *h*DMP1 protein expression in NSCLC samples with patients' data for response to therapy and survival. In addition, the significance and prognostic values for cytoplasmic mislocalization of the *h*DMP1 protein should be analyzed/evaluated. Cancer-specific splicing alterations of h*DMP1* and their relationship with LOH of h*DMP1*, *INK4a/ARF* and *p53* loci should be studied with a large number of patients' samples. Since NSCLC cells invariably (>90%) retain one intact h*DMP1* allele, h*DMP1* gene activation within cancer cells with some naturally occurring or synthetic chemicals will be a possible approach for novel cancer therapy. Indeed, we have recently reported the activation of the *Dmp1* promoter by trichostatin A, which is a potent inhibitor of histone deacetylases. We hope that analysis of the h*DMP1* gene or proteins will help to plan an individualization of the patient treatment protocols for lung cancer.

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Key issues

- Dmp1 is a novel transcription factor that directly binds and activates the *Arf* promoter and induces Arf-p53-dependent cell cycle arrest in primary cells.
- Dmp1 is haplo-insufficient for tumor suppression.
- *Dmp1*-deficient mice are prone to develop lung adenomas/adenocarcinomas.
- Oncogenic Ras activates the *Dmp1* promoter through the Raf-MEK-ERK-Jun pathway which, in turn, stimulates the *Arf* promoter to stop cell proliferation.
- E2Fs and genotoxic stimuli mediated by NF-κB repress the *Dmp1* promoter.
- The human *DMP1* gene (h*DMP1*) is located on chromosome 7q21 and is hemizygously deleted in approximately 40% of human non-small-cell lung cancer (NSCLC). This *hDMP1* deletion is generally mutually exclusive with deletion, LOH or silencing of *INK4a/ARF* or *p53*.
- The nuclear *h*DMP1 protein levels correlate well with the genetic status of *hDMP1* in NSCLC.
- There are cases where the *h*DMP1 protein is mislocalized in the cytoplasm of NSCLC cells.
- Further study is expected if *h*DMP1 becomes a novel prognostic factor for the lung cancer and a novel target for gene-induction therapy.

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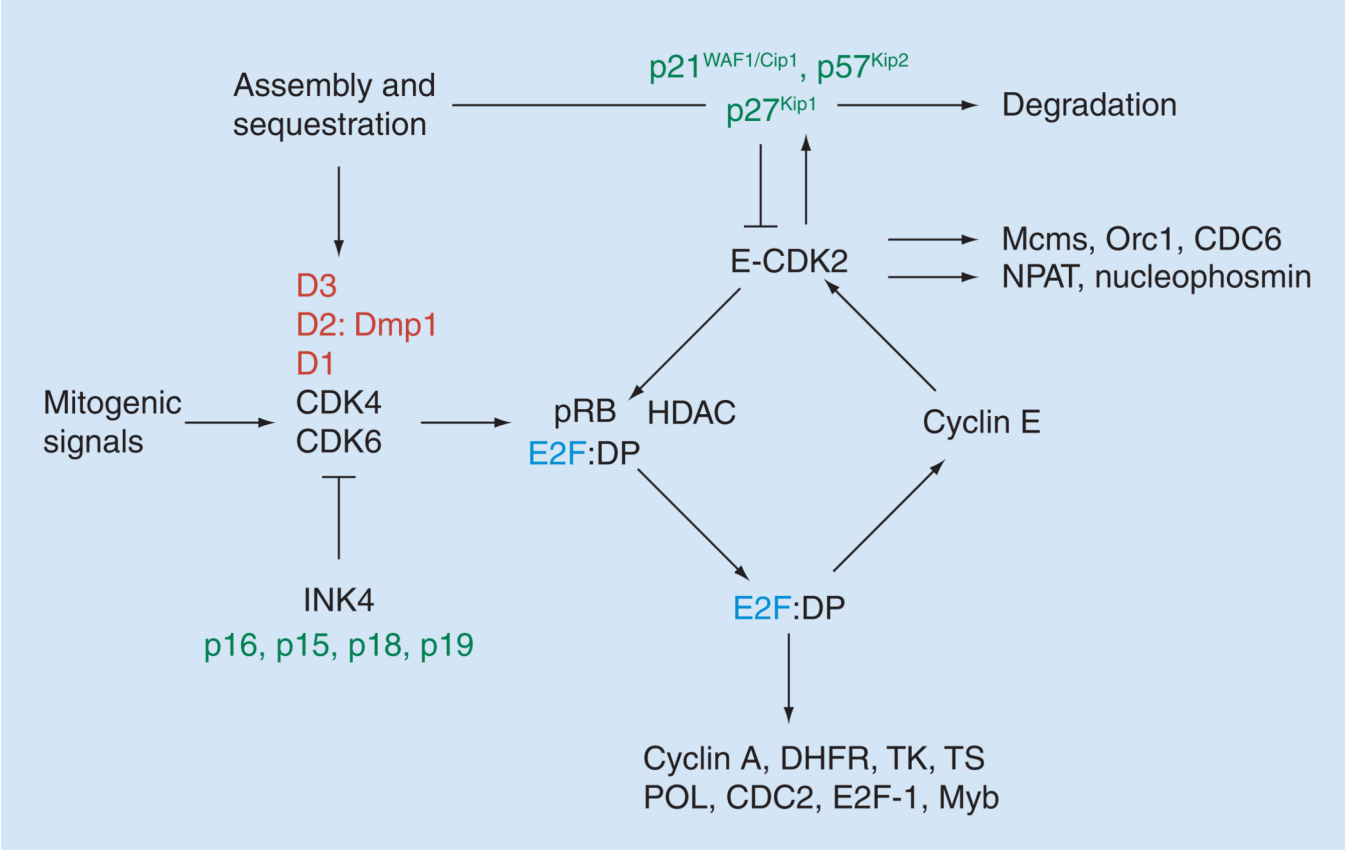

Figure 1. Restriction point control and the G_1–S transition

D-type cyclins are induced as delayed early responses to mitogenic signals. Among the three D-cyclins, only cyclin D1 is Ras-responsive. Dmp1 is a novel transcription factor that was isolated in a yeast two-hybrid screen with cyclin D2 as bait. The cyclin D/CDK 4/6; $p21^{CIP1}/p27^{KIP1}$ complexes assemble, sequestering CIP/KIP proteins from cyclin E–CDK2. Cyclin D- and E- dependent kinases phosphorylate the HDACs and the RB, resulting in release and activation of E2F transcription factors, which are necessary for the transcription of genes required for S phase progression. The targets of E2Fs include DHFR, TK, TS, POL, CDC2, E2F1, cyclin E and cyclin A, creating a positive feedback loop at the G_1–S boundary. This will eventually cause irreversible restriction point transition to the S phase. Cyclin E–CDK2 opposes the inhibitory effect of $p27^{KIP1}$ by phosphorylating it. This allows cyclin A–CDK2 and cyclin E–CDK2 to start S phase. E2Fs also target c-Myb, B-Myb (activation) and Dmp1 (repression). Other cyclin E–CDK substrates include Mcms, Orc1 and CDC6, all of which assemble into pre-initiation complexes to start DNA replication. Cyclin E-CDK2 also phosphorylates $p220^{NPAT}$ and nucleophosmin. As cells age, $p16^{INK4a}$ is induced, which inhibits CDK 4/6, causing release and degradation of D-type cyclins and redistribution of $p21^{CIP1}/p27^{KIP}$ proteins to cyclin E–CDK2.

DHFR: Dihydrofolate reductase; Dmp1: Cyclin D-binding myb-like protein 1; DP: Dimerization partner, E2F dimerization partner; CDC: Cell division cycle; CDK: Cyclin-dependent kinase; HDAC: Histone deacetylase; Mcm: Minichromosome maintenance; NPAT: Nuclear protein, ataxia-telangiectasia locus; Orc1: Origin recognition complex 1; POL: DNA polymerase α; RB: Retinoblastoma protein; TK: Thymidylate kinase; TS: Thymidine synthase.

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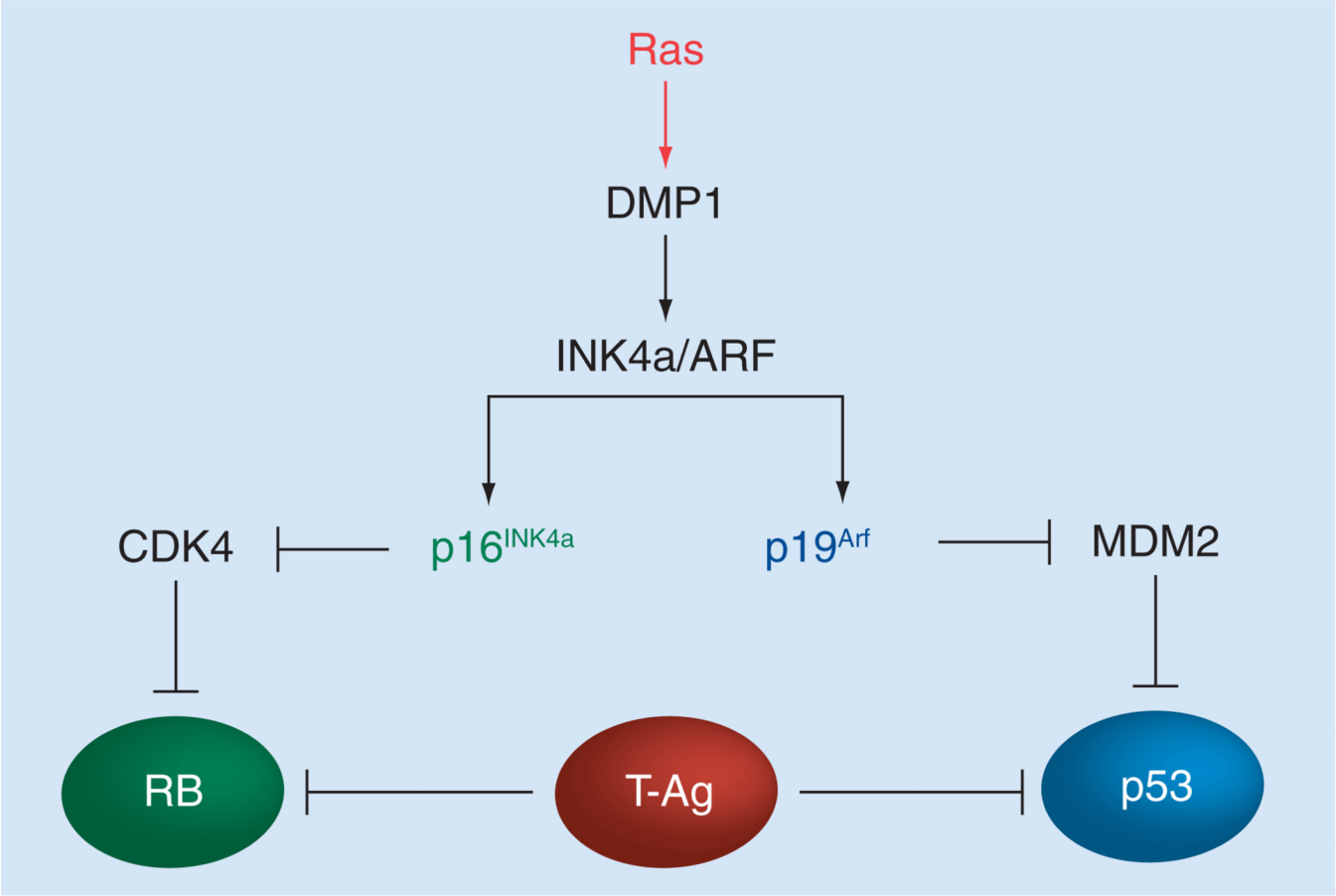

Figure 2. Regulation of the RB and p53 pathways by proteins encoded from the INK4a/ARF locus and DMP1

The *INK4a/ARF* locus located on human chromosome 9p21 encodes two tumor-suppressor genes, namely $p16^{INK4a}$ and $p14^{ARF}$. $p16^{INK4a}$ binds to cyclin-dependent kinase 4 to inhibit RB phosphorylation, while $p14^{ARF}$ ($p19^{Arf}$ in mice) directly binds to Hdm2 (Mdm2 in mice), thereby stabilizing and activating p53 [51–55]. Since the single genetic locus encodes two independent tumor-suppressor proteins that regulate the RB and the p53 pathways, the locus is very frequently disrupted in human cancer [56]. Dmp1 directly binds to the *Arf* promoter and activates its gene expression. Since high-affinity Dmp1-binding sites are also found in the genomic locus between exon 1β and exon 1α, Dmp1 may stimulate $p16^{INK4a}$ transcription. SV40 T antigen binds to both RB and p53 to neutralize their tumor-suppressor activity.

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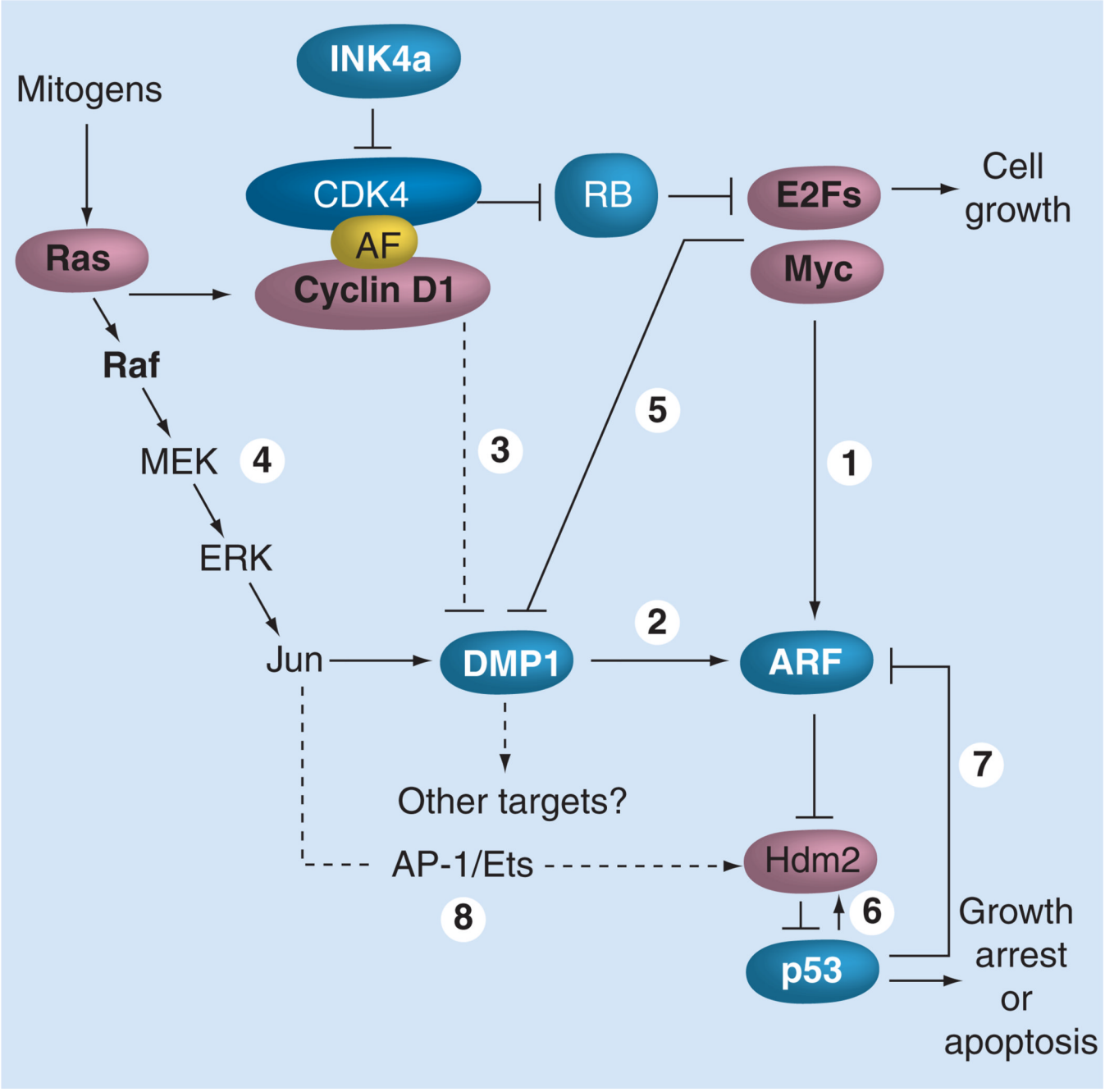

Figure 3. Signaling pathways involving the Dmp1 transcription factor

$p19^{Arf}$ is induced by potentially oncogenic signals stemming from overexpression of oncogenes such as c-Myc, E2F1 and activated Ras **(1)**. This Arf induction quenches inappropriate mitogenic signaling by diverting incipient cancer cells to undergo p53-dependent and -independent growth arrest or cell death. Dmp1 is unique in that it directly binds and activates the *Arf* promoter and induces cell cycle arrest in an *Arf*-dependent fashion **(2)**. Both *Dmp1*-null and heterozygous mice show hypersensitivity to develop tumors in response to carcinogen DMBA and γ-irradiation. This phenotype could be explained by the inactivation of the Arf–Mdm2–p53 pathway in the absence of the functional Dmp1 protein, although it is possible that Dmp1 has other targets than *Arf*. D-

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type cyclins inhibit Dmp1's transcriptional activity in a CDK-independent fashion when E2Fs do not bind to the same promoter; however, D-cyclins cooperate with Dmp1 to activate the *Arf* promoter **(3)**. The *Dmp1* promoter is efficiently activated by the oncogenic Ras–Raf–MEK–ERK–Jun pathway **(4)**, but is repressed by overexpressed c-Myc, E2Fs and by physiological mitogenic signaling **(5)** [72,73]. Our study shows that the induction of Arf by oncogenic Ras is dependent on Dmp1 [72]. The Dmp1–Arf pathway is inhibited by NF-κB proteins in response to genotoxic stress signaling. Both *Mdm2* and *Hdm2* are direct targets of p53 **(6)**, and both human and murine *Arf* promoters are repressed by p53 **(7)** [59–61]. It was reported that the *Hdm2* promoter is also responsive to oncogenic Ras signaling **(8)**, which can antagonize the Arf induction by the Dmp1 pathway. However, the Arf induction by Ras eventually overrides the Mdm2 activity in normal cells, which undergo p53-dependent cell cycle arrest. AF: Assembly factor.

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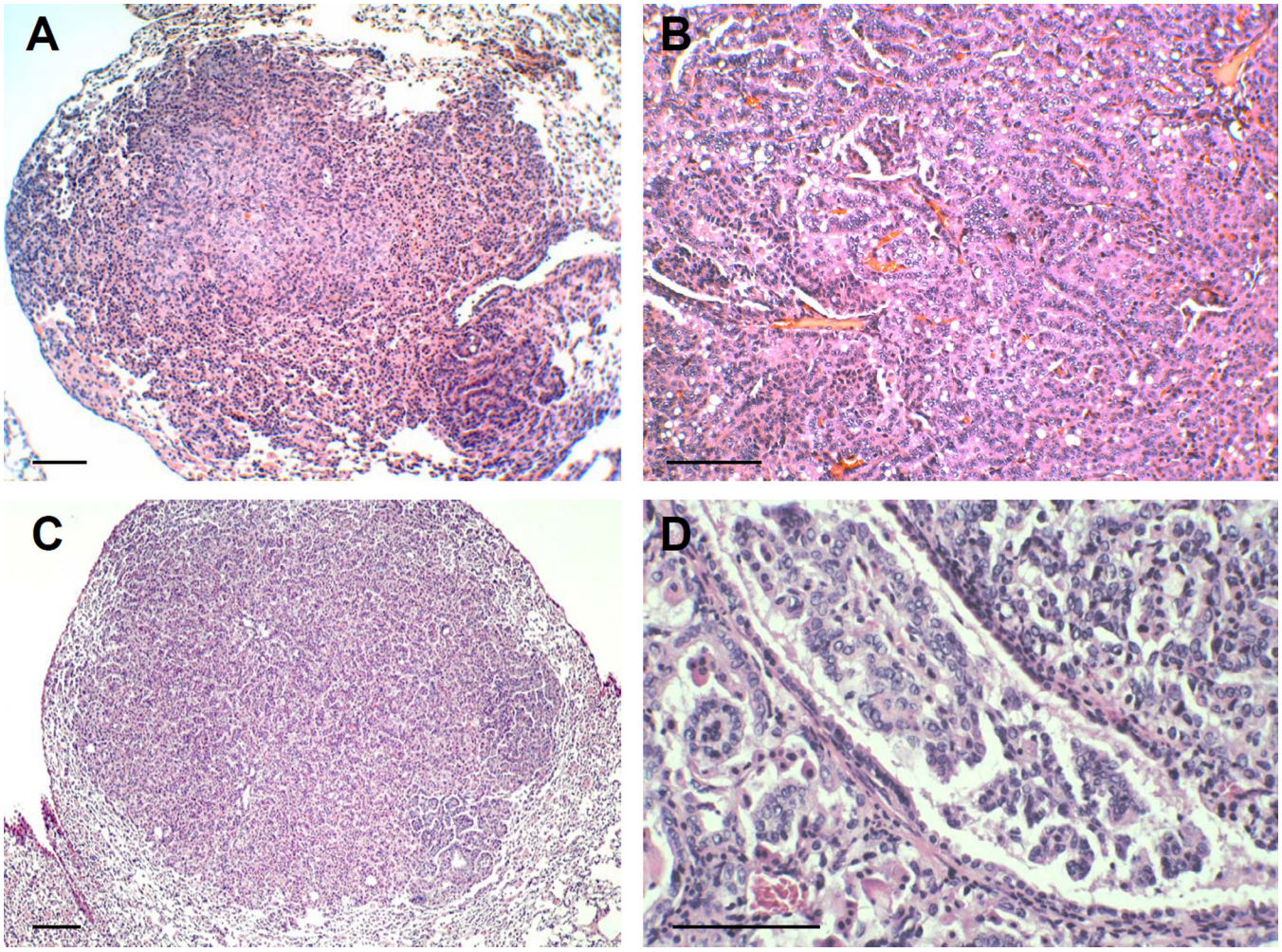

Figure 4. Lung tumors found in Dmp1 deficient mice

(A) Spontaneous lung adenoma found in an untreated *Dmp1*-null mouse (60-weeks old). **(B)** Lung adenocarcinoma found in a DMBA-treated *Dmp1*-null mouse (40-weeks old). **(C)** Lung adenoma observed in a wild-type *K-ras*$^{LA1/+}$ mouse (50-weeks old). **(D)** Invasive lung adenocarcinoma found in a *Dmp1*$^{+/-}$; *K-ras*^{LA1} mouse (35-weeks old). The scale bar in A–D is 100 μM.

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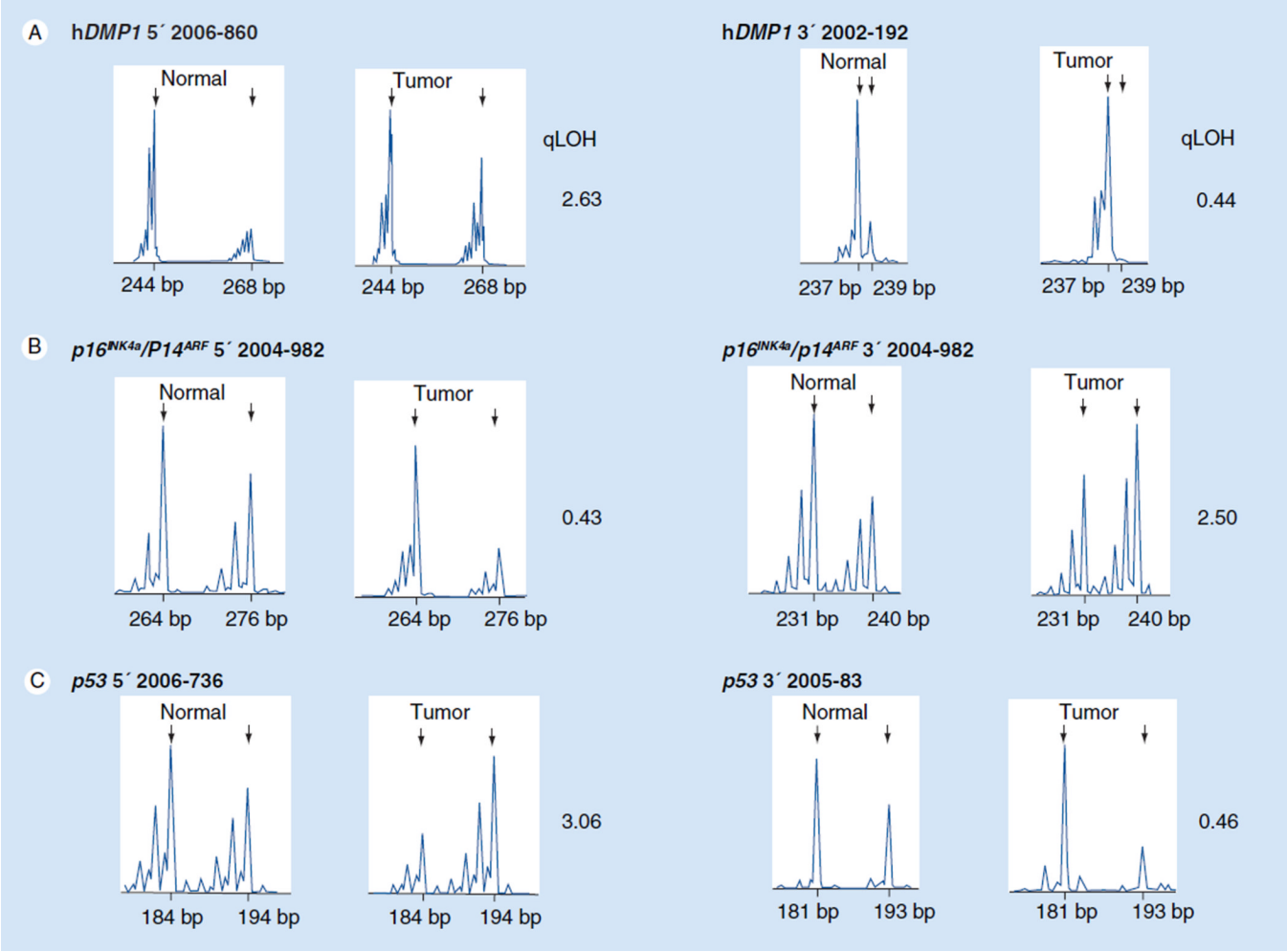

Figure 5. Representative patterns of LOH for h*DMP1 INK4a/ARF* and *p53* in human non-small-cell lung carcinoma

Genomic DNA was extracted from non-small-cell lung carcinomas and their normal counterparts and PCR was conducted with 6-FAM-labeled primers that amplify the dinucleotide repeats within (or close to) each locus [75]. The area peaks of the PCR products were quantitated by ABI 3730xl DNA analyzer. The qLOH values were determined through the following equation: qLOH = area peak 1/area peak 2 (normal tissue) divided by area peak 1'/area peak 2' (tumor tissue). The arrows indicate the peak that was lost in tumor cells. The sample was considered to have LOH when the value was >2.0 or <0.5 [75,98]. Two different sets of primers were used (set 1 sense: 5'-CCCAAAGAAGCCAACCAGAG-3' and antisense: 5'-GGCAAATGTGGGAGGTAAGG-3'; set 2 sense: 5'-GAGTGAAAGAGAGTGAGACAG-3' and antisense: 5'-TCTCACTGTCTCGCTCTGTG-3') to evaluate the LOH for the 3' region of h*DMP1* to increase the chance of finding a polymorphism. **(A)** LOH analysis of non-small-cell lung cancer with h*DMP1* primer sets. **(B)** LOH analysis of non-small-cell lung cancer with *INK4a/ARF* primer sets. **(C)** LOH analysis of NSCLC with *p53* primer sets. LOH: Loss of heterozygosity; qLOH: Quantitative LOH.

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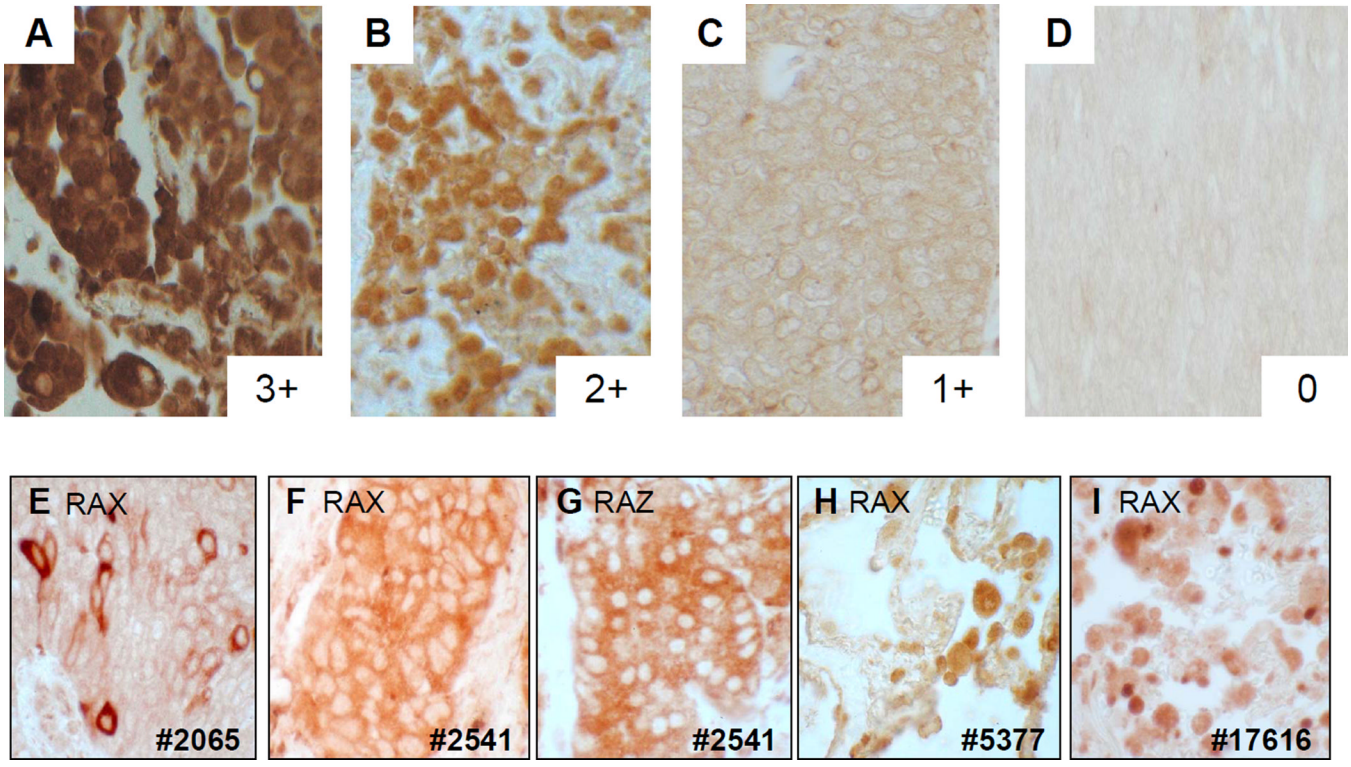

Figure 6. Immunohistochemical detection of the hDMP1 protein in human lung cancer

Pictures **(A–D)** show the grading of nuclear staining of *h*DMP1 in different lung cancer cells. It was graded as 3(+), strongly positive; 2(+), positive; 1(+), weakly positive; and 0, negative. Non-small-cell lung cancer samples without LOH for h*DMP1* showed significantly stronger signals than LOH(+) samples. **(E–I)** show abnormal subcellular localization of the *h*DMP1 protein in lung cancer. **(E–G)** Immunohistochemical detection of the *h*DMP1 protein in human non-small-cell lung cancer samples. **(H & I)** Detection of *h*DMP1 in normal human lung tissue. The patients' numbers are listed at the bottom. Paraffin sections were stained with the Dmp1-specific antibody, RAX [73] except for panel **(G)**. Dmp1 antibody to the carboxyl-terminus (RAZ) was used for **(G)**, confirming the cytoplasmic localization of *h*DMP1 in tumor cells. LOH: Loss of heterozygosity.