

Review

Escape from p53-mediated tumor surveillance in neuroblastoma: switching off the p14^{ARF}-MDM2-p53 axis

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A primary failsafe program against unrestrained proliferation and oncogenesis is provided by the p53 tumor suppressor protein, inactivation of which is considered as a hallmark of cancer. Intriguingly, mutations of the *TP53* gene are rarely encountered in neuroblastoma tumors, suggesting that alternative p53-inactivating lesions account for escape from p53 control in this childhood malignancy. Several recent studies have shed light on the mechanisms by which neuroblastoma cells circumvent the p53-driven antitumor barrier. We review here these mechanisms for evasion of p53-mediated growth control and conclude that deregulation of the p14^{ARF}-MDM2-p53 axis seems to be the principal mode of p53 inactivation in neuroblastoma, opening new perspectives for targeted therapeutic intervention.

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The p53 tumor surveillance network constitutes the core defense mechanism of the cell against loss of genomic integrity and malignant transformation. Evasion of p53 activity is, therefore, a prerequisite for tumor cells to survive and thrive, and this is attainable either through mutation of the *TP53* gene or through defects in the molecular components that govern or execute the various aspects of the p53 response. Elucidation of the mechanisms by which tumor cells override the p53-orchestrated failsafe program is not only important to gain insight into the ontogenesis of a tumor, but may also point to preferable modes of therapeutic intervention.

A striking feature of the childhood cancer neuroblastoma is the low frequency (<2%) of *TP53* mutations at diagnosis.¹ There is considerable evidence that *TP53* mutations may be acquired during chemotherapy and malignant progression of neuroblastoma.^{1–4} Accordingly, an increased frequency of *TP53* mutations is observed in multidrug-resistant neuroblastoma cell lines and in neuroblastoma cell lines established at relapse, but even in this context, the majority of cell lines remain characterized by a wild-type *TP53* gene.^{5,6} Furthermore, many studies indicate that the p53 signal transduction pathway is intrinsically intact in neuroblastoma,^{1,4,7–9} suggesting that circumvention of the p53 barrier in this tumor entity relies primarily on an inappropriately increased activity of inhibitors of p53 signaling or, alternatively, on a loss of positive regulators of p53 activity. This review summarizes our

current understanding of the mechanisms by which neuroblastoma cells escape from p53-mediated tumor surveillance and positions deregulation of the p14^{ARF}-MDM2-p53 axis as a central switch for p53 inactivation in neuroblastoma.

The p14^{ARF}-MDM2-p53 Axis and Lesions at the *MDM2* and *CDKN2A* (*p16^{INK4a}*/*p14^{ARF}*) Loci in Neuroblastoma

The MDM2 oncoprotein, a human homolog of the ‘mouse double minute 2’ gene product that was originally identified in a spontaneously transformed mouse cell line with double minute chromosomes,¹⁰ is a critical negative regulator of p53 stability and activity. It has been well established that p53 and MDM2 mutually control their cellular levels and form a tight autoregulatory feedback loop (Figure 1a). Under normal physiological conditions, p53 protein levels are very low because of MDM2-dependent proteasomal degradation.¹¹ Exposure of cells to harmful stimuli, such as DNA damage, hypoxia, telomere erosion, ribonucleotide depletion, or oncogene activation, results in a number of modifications on the p53 protein (e.g. phosphorylation and acetylation), which suppress the binding of p53 to MDM2 and which lead to accumulation and increased transcriptional activity of p53.¹² In addition to inducing expression of target genes involved in cell-cycle arrest, DNA damage repair, senescence, and apoptosis, p53 also transactivates the *MDM2* gene (Figure 1b). The resulting increase in *MDM2* expression limits

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Abbreviations: bHLH, basic helix-loop-helix; CHD5, chromodomain helicase DNA-binding protein 5; ChIP, chromatin immunoprecipitation; chromo, chromatin-organizing modulator; MDM2, mouse double minute 2 homolog; MEFs, mouse embryonic fibroblasts; p14^{ARF}, human alternate reading frame protein of 14 kDa; p19^{ARF}, murine alternate reading frame protein of 19 kDa; Parc, p53-associated parkin-like cytoplasmic protein; pRb, retinoblastoma protein; SNP309, single nucleotide polymorphism at position 309 (T>G) in *MDM2*; Wip1, wild-type p53-induced phosphatase 1

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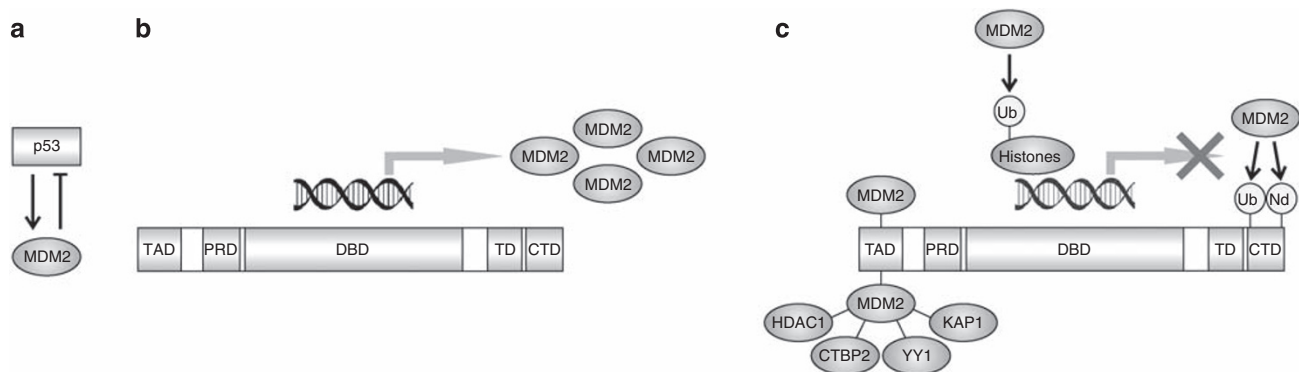


Figure 1 The p53-MDM2 autoregulatory feedback loop. **(a)** The p53 protein induces expression of MDM2, which negatively regulates the stability and activity of p53, providing a means to keep p53 levels and activity low in unstressed cells and to switch off p53 at the end of a stress response. **(b)** The p53-mediated expression of MDM2 results from binding of p53 to response elements in the *MDM2* gene and subsequent transactivation of *MDM2*. The domain structure of p53 is shown schematically: TAD, transactivation domain, amino acids 1–40; PRD, proline-rich domain, amino acids 61–94; DBD, DNA-binding domain, amino acids 100–300; TD, tetramerization domain, amino acids 324–355; CTD, C-terminal regulatory domain, amino acids 360–393. **(c)** The p53-inhibitory activity of MDM2 relies on multiple mechanisms. Binding of MDM2 to p53 conceals the TAD and consequently blocks the transcriptional activity of p53. MDM2 also recruits several corepressor proteins to p53, including HDAC1, CTBP2, YY1, and KAP1. The E3 ubiquitin ligase activity of MDM2 results in ubiquitination of lysine residues in the CTD of p53, preventing acetylation of p53, favoring nuclear export, and promoting proteasomal degradation (see text for details). Some of these lysine residues can also be neddylated by MDM2, resulting in inhibition of the transcriptional activity of p53. Finally, MDM2 may also serve as a p53-specific transcriptional silencer by binding and monoubiquitinating histone proteins in the proximity of p53-responsive promoters. Nd, NEDD8; Ub, ubiquitin. The color reproduction of this figure is available on the html full text version of the manuscript

the duration and intensity of a non-lethal stress response. There are several mechanisms by which MDM2 is capable of counteracting p53 activity and stability (Figure 1c). First, MDM2 binds to the transactivation domain of p53 and, therefore, directly interferes with recruitment of the basal transcriptional machinery and transcriptional coactivators.^{13–15} Second, MDM2 acts as an E3 ubiquitin ligase for p53 in a dosage-dependent manner. Low levels of MDM2 promote p53 monoubiquitination, which may both stimulate nucleocytoplasmic shuttling of p53 because of unmasking of a nuclear export signal and decrease p53 transactivation capacity owing to unavailability of the ubiquitinated lysine residues for acetylation. At higher levels, the activity of MDM2 results in polyubiquitination and subsequent proteasomal degradation of p53.^{11,16,17} Third, MDM2 also induces monoubiquitination of histone proteins in the vicinity of p53-responsive promoters, resulting in transcriptional repression.¹⁸ Fourth, MDM2 has been reported to inhibit p53 transcriptional activity by promoting conjugation of the ubiquitin-like protein NEDD8 to p53.¹⁹ Fifth, MDM2 may also contribute to p53 inactivation by recruiting several corepressor proteins, such as HDAC1,²⁰ CTBP2,²¹ YY1,²² and KAP1.²³

A central negative regulator of MDM2 is the tumor suppressor protein p14^{ARF}, which is an alternate reading frame product of the *CDKN2A* locus on chromosome 9p21. This locus encodes two structurally unrelated growth-inhibitory proteins, p16^{INK4a} and p14^{ARF}, that govern the activities of the pRb and p53 tumor suppressor pathways, respectively.²⁴ The p14^{ARF} protein serves as a key sensor of hyperproliferative signals generated by activated oncogenes and engages both p53-dependent and p53-independent pathways to protect cells from malignant transformation.²⁵ The importance of p14^{ARF}-mediated signaling of oncogene activity in the p53 tumor surveillance network is underscored by observations in mice models that the cancer-protective activity of p53 is abolished in the absence of the murine

homolog p19^{ARF}.^{26,27} The physical interaction between p14^{ARF} and MDM2 is in large part responsible for the ability of p14^{ARF} to stabilize and activate p53. p14^{ARF} prevents MDM2 from targeting p53 for degradation by inhibiting the E3 ubiquitin ligase activity of MDM2²⁸ and by blocking nuclear export of MDM2 and p53.^{29,30} It has also been firmly established that p14^{ARF}, which is predominantly a nucleolar protein, is capable of mobilizing MDM2 into the nucleolus, and it has, therefore, been proposed that p14^{ARF} releases nucleoplasmic p53 from the inhibitory grip of MDM2 by inducing nucleolar sequestration of MDM2.^{30,31} Although MDM2 redistribution to nucleoli may contribute to p14^{ARF}-induced p53 activity, several reports indicate that neither localization of p14^{ARF} in the nucleolus nor nucleolar sequestration of MDM2 is essential for stabilization and activation of p53 by p14^{ARF}.^{32–35} In this regard, it has been suggested that p14^{ARF} is stored within the nucleolus in complexes with nucleophosmin, regulating ribosome biogenesis, and displaced to the nucleoplasm by stress-induced nucleolar perturbation, in which it can efficiently counteract MDM2 and activate the p53 pathway.^{34–36} In addition, p14^{ARF} may also enhance p53 function by MDM2-independent mechanisms, for example by inhibiting the activity of another E3 ubiquitin ligase involved in p53 degradation, ARF-BP1/Mule,³⁷ and by neutralizing the p53-antagonizing NF- κ B pathway.³⁸ The mechanisms by which p14^{ARF} promotes p53 stability and activity are shown in Figure 2.

Not surprisingly, many forms of cancer develop defects in MDM2 or p14^{ARF} to escape from p53 control. Genetic aberrations of the *MDM2* locus as well as genetic or epigenetic disruption of the *CDKN2A* (p16^{INK4a}/p14^{ARF}) locus may account for inactivation of the p53 pathway in a subset of neuroblastoma tumors, mainly at relapse. Amplification of chromosome 12q-derived sequences encompassing the *MDM2* gene has been described almost exclusively in neuroblastoma tumors and cell lines that simultaneously have

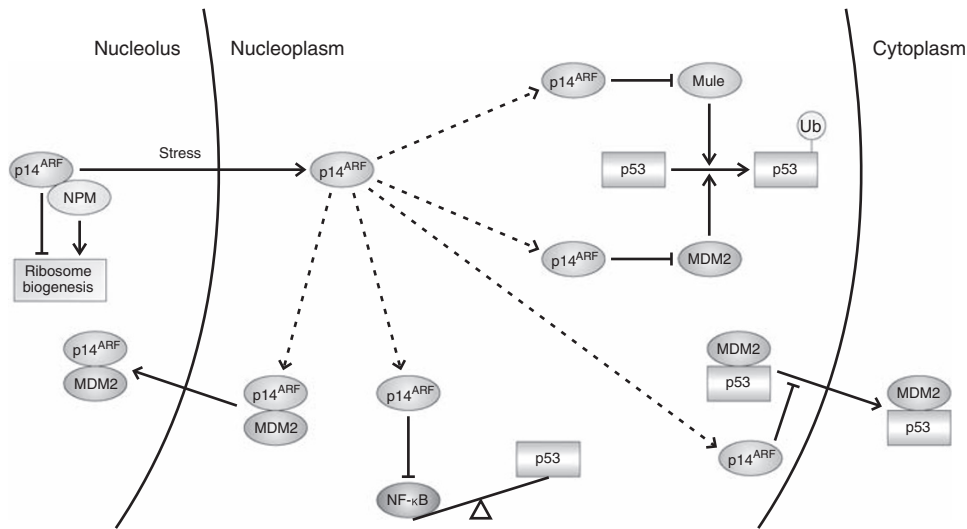


Figure 2 Schematic diagram of p53 stabilization and activation by p14^{ARF}. The p14^{ARF} protein is predominantly localized to the nucleolus, in which it is stabilized by binding to nucleophosmin within maturing pre-ribosomal particles, pointing to a function in the regulation of ribosome biogenesis. Nucleophosmin promotes the processing of ribosomal RNA precursors and the nuclear export of ribosomal subunits, whereas overexpression of p14^{ARF} or its murine homolog p19^{ARF} interferes with transcription and processing of ribosomal RNA, impedes nucleocytoplasmic shuttling of nucleophosmin, and inhibits ribosome nuclear export. However, the precise biological function of the nucleophosmin–p14^{ARF} complexes remains a subject of debate. Stress signals trigger the disruption of the interaction between p14^{ARF} and nucleophosmin, and induce translocation of p14^{ARF} to the nucleoplasm. This redistribution enables p14^{ARF} to interact with p53-bound MDM2 and to antagonize MDM2 function by inhibiting its E3 ubiquitin ligase activity and by blocking nucleocytoplasmic shuttling of MDM2 and p53, resulting in p53 stabilization. The p53-inhibitory activity of MDM2 may also be neutralized by p14^{ARF}-mediated mobilization of MDM2 into the nucleolus, although this mechanism is not strictly required for the p53-dependent functions of p14^{ARF}. Furthermore, the p14^{ARF} protein is capable of inhibiting the activity of another E3 ubiquitin ligase that targets p53 for degradation, ARF-BP1/Mule, and of counteracting the p53-antagonizing NF- κ B pathway. It should be noted that p14^{ARF} also exerts a potent tumor suppressor activity independently of p53, which is not illustrated in this figure. Mule, ARF-BP1/Mule; NPM, nucleophosmin; Ub, ubiquitin. The color reproduction of this figure is available on the html full text version of the manuscript

amplification of the *MYCN* oncogene on chromosome 2p24, and is associated with attenuated p53 transcriptional function and multidrug resistance.^{5,39–43} The *CDKN2A* (*p16^{INK4a}/p14^{ARF}*) locus at 9p21 is the most frequent target of homozygous deletion in both neuroblastoma cell lines⁴⁴ and primary tumors,⁴⁵ and has been found to be silenced by methylation in several neuroblastoma cell lines.^{46,47} It has been estimated that approximately half of all neuroblastoma cell lines established at relapse are subject to a genetic or epigenetic lesion of the *MDM2* or *CDKN2A* (*p16^{INK4a}/p14^{ARF}*) locus,⁶ but these findings await confirmation in a study that takes also neuroblastoma tumor samples into account.

A recent line of evidence supporting a role for MDM2 activity in the development and malignant behavior of neuroblastoma stems from epidemiological studies of a T > G single nucleotide polymorphism in the *MDM2* promoter (SNP309; rs2279744). The presence of this polymorphism increases the affinity of the *MDM2* promoter for a transcriptional activator, Sp1. This results in enhanced transcription of *MDM2*, overexpression of the MDM2 protein, attenuation of the p53 pathway, and may eventually lead to accelerated tumor formation.⁴⁸ Both individuals homozygous for SNP309 (G/G) and subjects heterozygous for SNP309 (T/G) have an increased risk for the development of neuroblastoma, and neuroblastoma patients carrying the SNP309 variant (G/G or T/G) present with a more advanced clinical stage and have a shorter 5-year overall survival than patients homozygous for the wild-type allele (T/T).⁴⁹ A survival study in children with stage 4 neuroblastoma yielded similar results, with patients homozygous for SNP309 (G/G) having a worse overall

survival and a worse survival after relapse than those homozygous for the wild-type allele (T/T), and with heterozygous SNP309 variant carriers (T/G) showing intermediate survival rates.⁵⁰ These findings suggest that an increased activity of MDM2 because of the presence of SNP309 has an adverse effect on the development, aggressiveness, and outcome of neuroblastoma, and provide a direct incentive for the development of novel therapeutic strategies aimed at MDM2 inhibition.

Transactivation of *MDM2* Expression by *MYCN*

Amplification of the *MYCN* oncogene plays a central role in the pathophysiology and clinical behavior of high-risk neuroblastoma. This genetic aberration is found in approximately 22% of all neuroblastoma tumors⁵¹ and is highly correlated with advanced stages of disease, rapid progression, treatment failure, and fatal outcome.^{52,53} *MYCN* amplification results in overexpression of the MYCN protein, which is a bHLH transcription factor that operates in a heterodimeric complex with Max family proteins to promote cell growth and proliferation.⁵⁴ The oncogenic effects of *MYCN* overexpression have been convincingly established in a variety of model systems. Enhanced expression of *MYCN* elicits neoplastic transformation of mammalian cells,^{55,56} induces autocrine growth factor activity and increases proliferative potential,⁵⁷ accelerates cell-cycle progression,⁵⁸ enhances tumor cell motility and invasiveness,⁵⁹ evokes genomic instability through disruption of the regulation of centrosome replication,^{60,61} diminishes expression of angiogenesis

inhibitors,^{62,63} and promotes immune escape in neuroblastoma by inhibiting the chemoattraction of natural killer T cells.⁶⁴ Direct evidence for a causative role of *MYCN* amplification in the pathogenesis of neuroblastoma is derived from the observation that transgenic mice with targeted expression of *MYCN* in normal neuroblasts develop tumors with a phenotype very similar to human neuroblastoma.⁶⁵

However, aberrant *MYCN* expression also potentially sensitizes neuroblastoma cells to drug- and stress-induced apoptosis,^{66–69} and, therefore, needs to be accompanied by a collateral impairment of the cell death program to provide a selective advantage for the tumor. This counterbalance to the intrinsic apoptosis-sensitizing effect of *MYCN* may be delivered by an increased activity of MDM2. A ChIP cloning approach combined with oligonucleotide pull-down and luciferase reporter assays has identified *MDM2* as a direct transcriptional target of *MYCN* in neuroblastoma cells.⁷⁰ In the same study, endogenous *MDM2* mRNA and MDM2 protein levels were rapidly upregulated on induction of *MYCN* in *MYCN*-conditional neuroblastoma cell lines, whereas targeted inhibition of *MYCN* in *MYCN*-amplified neuroblastoma cells resulted in reduced MDM2 levels with stabilization of p53 and induction of apoptosis. These data suggest that *MYCN*-driven expression of *MDM2* may constitutively debilitate the p53 pathway in *MYCN*-amplified neuroblastoma cells, providing both a possible mechanism for evasion of *MYCN*-primed apoptosis and an explanation for the low frequency of *TP53* mutations in these cells. This view is further strengthened by evidence that the closely related MYC (c-MYC) oncoprotein also relies on MDM2 to restrain p53-mediated apoptosis, as Myc-induced lymphomagenesis in mice is profoundly suppressed by haploinsufficiency of *Mdm2* because of a drastic increase in p53-dependent apoptosis.⁷¹

Suppression of p14^{ARF} and p53 by TWIST1

Another excellent candidate to explain escape from *MYCN*-dependent apoptotic sensitization is TWIST1. Just like *MYCN*, TWIST1 is a bHLH transcription factor with a fundamental role in embryonic and fetal development. This evolutionary conserved protein is involved in mesoderm formation and diversification, myogenesis, neurogenesis, and neural crest cell migration and differentiation.⁷² Loss-of-function mutations in the *TWIST1* gene have been identified as the main cause of the Saethre–Chotzen syndrome, an autosomal dominant disorder of craniosynostosis with craniofacial and limb abnormalities.^{73,74} In addition to its developmental function in mesodermal and neural crest cell populations, TWIST1 also acts as an oncoprotein in several cancer types. Neuroblastoma tumors with *MYCN* amplification consistently exhibit *MYCN*-driven overexpression of *TWIST1*, resulting in an oncogenic cooperation that protects neuroblastoma cells from the proapoptotic effect of *MYCN* and that increases tumorigenicity *in vivo*.⁷⁵ It could be shown that the protective effect conferred by TWIST1 was due to suppression of the p53 response and that the dampened p53 function was at least partially attributable to impaired p14^{ARF} activity.⁷⁵ These findings are in agreement with an earlier study that pointed to downregulation of p19^{ARF} expression by Twist1 as a mechanism for compensating the

apoptosis-priming properties of Myc.⁷⁶ Several other mechanisms may also contribute to the p53-inhibitory activity of TWIST1, including inhibition of acetyltransferases that serve as transcriptional coactivators for p53,⁷⁷ modulation of the activity of a transactivator of the *TP53* promoter,⁷⁸ prevention of p53 phosphorylation,⁷⁸ and direct suppression of the DNA-binding activity of p53.⁷⁹ Of note, the TWIST1 and TWIST2 proteins have also recently been shown to prevent oncogene-induced premature senescence with concomitant abrogation of p16^{INK4a} and p21^{WAF1/CIP1} activation, and to induce, in cooperation with activated mitogenic oncoproteins, epithelial-mesenchymal transition, suggesting a role as general inhibitors of multiple oncogene-induced safeguard programs.⁸⁰

Inactivation of the p14^{ARF}-p53 Pathway by BMI1

The Polycomb-group transcriptional repressor BMI1 has been proposed as another roadblock to *MYCN*-induced apoptosis by suppressing the p14^{ARF}-p53 signaling pathway.^{81,82} BMI1 is a component of the Polycomb repressive complex 1, which mediates transcriptional silencing through chromatin modifications and which is involved in embryonic and adult stem cell maintenance and in the development of several cancer types.⁸³ It has been convincingly shown that Bmi1 is indispensable for the self-renewal capacity and postnatal maintenance of hematopoietic and neural stem cells in mice by repressing the *Cdkn2a* (p16^{INK4a}/p19^{ARF}) locus.^{84–88} Notably, Bmi1 also collaborates strongly with Myc in murine lymphomagenesis,^{89–92} and the molecular basis of this oncogenic cooperation is the ability of Bmi1 to prohibit Myc-induced apoptosis by downregulating *Cdkn2a* (p16^{INK4a}/p19^{ARF}) expression.⁹³ Similar to TWIST1, the BMI1 oncoprotein inhibits oncogene-induced premature senescence and cooperates with activated H-Ras to induce neoplastic transformation and epithelial-mesenchymal transition.^{84,94} Thus, both the TWIST1 and BMI1 transcriptional regulators may overcome several oncogene-induced failsafe barriers and may serve as examples of corrupt exploitation of normal developmental programs by tumor cells.

BMI1 is strongly expressed in neuroblastoma cell lines and tumors,^{81,82} and has been shown to be essential for the tumorigenicity of neuroblastoma cells.⁸² BMI1 negatively regulates p53 expression in neuroblastoma cells, potentially inhibits the apoptotic activity of *MYCN*, and functions as an oncogenic partner of *MYCN* in the transformation of normal neural crest cells and in the malignant progression of neuroblastoma cells.⁸² These findings have been attributed to the ability of BMI1 to repress the *CDKN2A* (p16^{INK4a}/p14^{ARF}) locus, although it cannot be excluded that CDKN2A-independent pathways may also play a role. Interestingly, the collaborative activity between *MYCN* and BMI1 may be switched on by a single initiating event, as deregulated E2F1 activity, which is a characteristic lesion in highly proliferative neuroblastoma tumors,⁹⁵ is capable of directly driving the expression of both oncogenes.^{81,96} The role of BMI1 in neuroblastoma pathogenesis seems not to be limited to *MYCN*-amplified tumors, as *BMI1* is also expressed and required for tumorigenicity in neuroblastoma cells with a normal copy number of *MYCN*.⁸² In line with the requirement

of BMI1 activity in self-renewal of neural stem cells, it has been argued that BMI1 may be of critical importance for the maintenance of neuroblastoma stem cells by regulating clonogenic self-renewal and multilineage differentiation, offering an attractive target for therapeutic intervention.⁹⁷

Repression of p14^{ARF}-p53 Signaling by Loss of CHD5

Escape from p53 surveillance in neuroblastoma cells may also be accomplished by the loss of another chromatin-remodeling protein involved in transcriptional control of the *CDKN2A* (*p16^{INK4a}/p14^{ARF}*) locus. One of the most characteristic genomic lesions in neuroblastoma is deletion of the short arm of chromosome 1, which is found in 25 to 35% of primary neuroblastoma tumors and 80 to 90% of neuroblastoma cell lines.⁹⁸ The actual target of this deletion has remained elusive for a long time, but detailed analysis of the different genes located in the smallest region of deletion at 1p36.31 has recently identified *CHD5* as the strongest candidate tumor suppressor gene.^{99,100} *CHD5* encodes a protein with chromatin-organizing modulator (chromo), helicase, and DNA-binding motifs that is preferentially expressed in the nervous system and the adrenal gland.¹⁰¹ Expression of *CHD5* is very low or absent in neuroblastoma cell lines and is inversely correlated with 1p deletion, *MYCN* amplification, advanced clinical stage, unfavorable histology, and poor event-free and overall survival in neuroblastoma tumors.^{99–101} Homozygous deletion and mutational inactivation of *CHD5* are infrequent events,⁹⁹ but it has been shown that the remaining *CHD5* allele in neuroblastoma cells with heterozygous 1p deletion may be transcriptionally silenced by promoter methylation.¹⁰⁰ Reintroduction of *CHD5* in such neuroblastoma cells with 1p deletion and epigenetic *CHD5* silencing significantly reduced clonogenicity and *in vivo* tumorigenicity, validating *CHD5* as a *bona fide* tumor suppressor gene.¹⁰⁰

Of note, an independent study that used chromosome engineering to produce mouse strains with deletions or duplications of a region corresponding to human 1p36 identified *Chd5* as a potent tumor suppressor that controls proliferation, senescence, and apoptosis through the p19^{ARF}-p53 pathway.¹⁰² Silencing of *Chd5* by short hairpin RNA in MEFs severely compromised p53 function and promoted tumorigenesis, and these effects were associated with a substantial reduction in the basal and oncogene-induced expression levels of p16^{INK4a} and p19^{ARF}. Knockdown of p19^{ARF}, but not p16^{INK4a}, was capable of bypassing the proliferation defect of MEFs that harbored an engineered duplication of the 1p36-syntenic region, indicating that *Chd5*, which could be shown to be responsible for the proliferation-suppressive properties of the 1p36-syntenic region, exerts its antiproliferative activity by facilitating expression of p19^{ARF}. Altogether, the findings of this study support a model in which the chromatin-remodeling activity of *Chd5* is required for proper transcriptional activation of the *Cdkn2a* (*p16^{INK4a}/p19^{ARF}*) locus. Although a direct link between *CHD5* and the p14^{ARF}-p53 network remains to be established in the context of human neuroblastoma, it is tempting to speculate that loss of *CHD5* by 1p deletion and epigenetic silencing may

promote the pathogenesis of neuroblastoma by crippling the p14^{ARF}-p53 signaling pathway.

Deregulation of the p14^{ARF}-MDM2-p53 Axis by PPM1D (Wip1)

The most frequent and the prognostically most unfavorable genomic alteration in neuroblastoma is gain or amplification of genetic material from the long arm of chromosome 17.^{103,104} A gene at 17q23.2 encoding a key negative regulator of p53, *PPM1D*, has been put forward as the most likely target of 17q gain/amplification, based on its location in the minimal common region of gain/amplification, its consistent pattern of overexpression in neuroblastoma cell lines with 17q gain/amplification, its growth-promoting and antiapoptotic activity in neuroblastoma cells, and the adverse impact of its expression level on the prognosis of primary neuroblastoma.¹⁰⁵ Similarly, *PPM1D* has been blamed as the culprit oncogene behind gain or amplification of 17q23 in breast cancer,^{106–108} ovarian clear cell adenocarcinoma,¹⁰⁹ and medulloblastoma.^{110,111}

The protein encoded by *PPM1D* is a serine/threonine phosphatase that is transcriptionally induced by wild-type p53 in response to DNA-damaging stimuli such as ionizing radiation, and it has, therefore, been given the name Wip1 (wild-type p53–induced phosphatase 1).¹¹² The p53-dependent expression of Wip1 creates a negative feedback loop that helps to turn off p53 at the end of a stress response, as Wip1 suppresses p53 activity and stability through multiple mechanisms (Figures 3a–c). First, Wip1 dephosphorylates and inactivates several kinases that mediate p53 stabilization and activation after genotoxic stress, for example p38 MAPK,¹¹³ Chk1,¹¹⁴ Chk2,^{115–117} ATM,^{118,119} and probably ATR.¹²⁰ In addition, Wip1 dephosphorylates p53 itself at serine 15, thereby probably promoting both p53 degradation and inactivation.¹¹⁴ The most important block provided by Wip1 on p53 function is mediated through stabilization and enhanced p53 binding of MDM2, which result from Wip1-induced dephosphorylation of MDM2 at serine 395 and which argue for a role of Wip1 as a molecular gatekeeper in the p53-MDM2 autoregulatory feedback loop.¹²¹ Finally, studies using *Ppm1d*-null MEFs have shown that Wip1 is also capable of suppressing p19^{ARF} levels through a p38 MAPK–dependent mechanism, which seems to involve transcriptional repression of the *Cdkn2a* (*p16^{INK4a}/p19^{ARF}*) locus, thus offering an additional explanation of how Wip1 may keep p53 in check.¹²²

The potent inhibitory activity of Wip1 on p53 provides, in principle, an appealing opportunity for tumor cells to escape from p53 control. Indeed, as discussed above, copy number gain/amplification and overexpression of *PPM1D* are observed in a variety of human tumors including neuroblastoma, which then typically retain wild-type p53 and often carry a poor prognosis.^{105,107–110} Overexpression experiments have shown that Wip1 induces malignant transformation in collaboration with other oncogenes, protects against oncogene-induced premature senescence and apoptosis, and accelerates tumorigenesis *in vivo*.^{106,107,123,124} Conversely, *Ppm1d*-null MEFs and mice are resistant to oncogene-induced transformation and to spontaneous

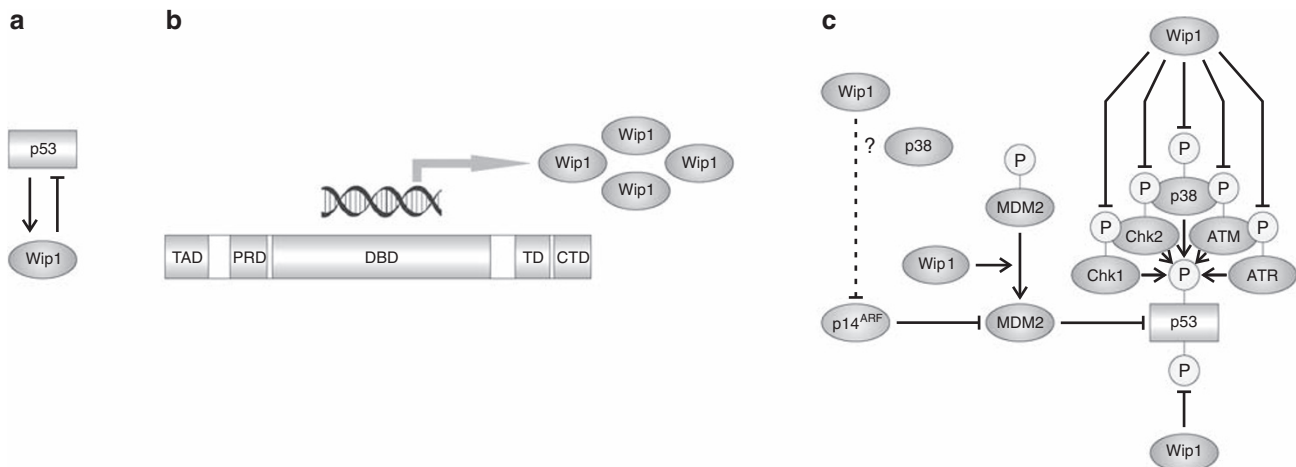


Figure 3 The p53-Wip1 autoregulatory feedback loop. (a) The p53-inducible expression of Wip1 sets up a negative feedback loop that helps to restrain p53 at the end of a stress response. (b) Binding of p53 to a response element in the 5' untranslated region of the *PPM1D* (*WIP1*) gene results in transcriptional activation and expression of Wip1 after exposure to DNA damage and other environmental stresses. The domain structure of p53 is shown schematically: TAD, transactivation domain, amino acids 1–40; PRD, proline-rich domain, amino acids 61–94; DBD, DNA-binding domain, amino acids 100–300; TD, tetramerization domain, amino acids 324–355; CTD, C-terminal regulatory domain, amino acids 360–393. (c) Wip1 is a phosphatase that negatively regulates p53 activity and stability by several mechanisms, including dephosphorylation and inactivation of p53-targeting kinases (p38 MAPK, Chk1, Chk2, ATM, and probably ATR), dephosphorylation of p53 itself, and dephosphorylation of MDM2. The latter activity of Wip1 stabilizes MDM2 and facilitates its access to p53, and has been proposed to be the most important contributor to the p53-inhibitory effect of Wip1. In addition, Wip1 may also decrease the expression of p14^{ARF} through a p38 MAPK-dependent, but incompletely understood mechanism. P, phosphate group; p38, p38 MAPK. The color reproduction of this figure is available on the html full text version of the manuscript

and oncogene-driven tumorigenesis, respectively.^{119,122,123} Although most of the oncogenic properties of Wip1 are ascribed to its ability to suppress the p53 pathway and the DNA damage response, concomitant inhibition of pRb tumor suppressor activity because of transcriptional repression of p16^{INK4a} expression may also play a role.¹²² Of note in the context of neuroblastoma is the observation that homozygous or heterozygous deficiency of *Ppm1d* in mice confers protection against Myc-induced lymphomagenesis in a p53- and ATM-dependent manner, indicative of a strict requirement for Wip1 activity in the suppression of Myc-triggered apoptosis.¹¹⁹ This is reminiscent of the critical role of Mdm2 in evasion of Myc-primed apoptosis in murine lymphomagenesis,⁷¹ which is a concept directly transferable to human neuroblastoma and MYCN.⁷⁰ It could, therefore, be speculated that increased dosage of *PPM1D* provides another mechanism for escape from MYCN-stimulated apoptosis and a molecular explanation for the strong association between 17q gain and *MYCN* amplification in neuroblastoma cells.¹⁰⁵

Cytoplasmic Sequestration of p53

Aberrant cytoplasmic localization of wild-type p53 has been proposed as another mechanism for p53 inactivation in neuroblastoma cells. Although controversy exists on the frequency and functional relevance of this phenomenon, it has been extensively documented that cytoplasmic p53 sequestration does occur in at least some cases of neuroblastoma. Interestingly, as will be discussed below, cumulating evidence indicates that an increased activity of MDM2 or a dysfunction of its functional counterpart HAUSP, a principal p53-deubiquitinating enzyme, lies at the molecular basis of cytoplasmic p53 retention in neuroblastoma, further under-

scoring the importance of MDM2 deregulation as a means to escape from p53 control.

An initial study found cytoplasmic p53 sequestration in 96% of undifferentiated neuroblastoma tumors, whereas this phenotype was absent in differentiated neuroblastoma tumors.¹²⁵ However, other studies have reported a predominant nuclear localization of p53 in undifferentiated neuroblastoma tumors, and both cytoplasmic and nuclear p53 in differentiating neuroblastoma.^{9,43,126} Conflicting results also exist for neuroblastoma cell lines, as the subcellular localization of p53 has been reported to be exclusively cytoplasmic (e.g. in IMR-32 and SK-N-SH cells),¹²⁷ primarily cytoplasmic and weakly nuclear (e.g. in IMR-32 and SK-N-SH cells),^{126,128} equally cytoplasmic and nuclear (e.g. in SK-N-SH cells),⁷ predominantly nuclear (e.g. in IMR-32 cells),⁴³ and completely nuclear (e.g. in IMR-32 and SK-N-SH cells).¹²⁹ Some of the discrepancies may be explained by cross-reactivity of the antibodies used to detect p53 and by different methods of tissue fixation and cell preparation.^{9,43,126} Nonetheless, it is generally accepted that some cytoplasmic p53 does exist in neuroblastoma, although the prevalence and importance of cytoplasmic p53 sequestration remain a subject of debate.^{9,130} It has been reported that abnormal cytoplasmic p53 localization may attenuate the DNA damage-induced G₁ checkpoint function¹²⁷ and the apoptotic activity^{131,132} of wild-type p53 in some neuroblastoma cells. On the contrary, many studies have shown that the DNA-binding and transactivation capacity of p53 and the p53 signal transduction pathway are intact in neuroblastoma cells with wild-type p53,^{1,4,7–9} indicating that cytoplasmic retention of wild-type p53 is either an infrequent anomaly or a relative block on p53 that can be overcome by appropriate p53-inducing stimuli.

Proposed mechanisms for abnormal p53 accumulation in the cytoplasm of neuroblastoma cells include hyperactive

nuclear export of p53, cytoplasmic tethering of p53, resistance of p53 to proteasomal degradation, and possibly impaired nuclear re-import of p53. A unifying theme common to these diverse mechanisms may be the involvement of a disrupted MDM2/HAUSP regulation of p53. It has been firmly established that cytoplasmic p53 sequestration in neuroblastoma cells is at least in part caused by enhanced nuclear export¹³³ and that MDM2 plays an important role in this nuclear exclusion of p53.^{131,134} Once transported to the cytoplasm, p53 may be held in this compartment by a cytoplasmic anchor protein, such as Parc.¹²⁸ Neuroblastoma cells express high levels of Parc, which have been shown to prevent nuclear localization of p53 and a normal apoptotic response to the genotoxic drug etoposide.¹²⁸ A comparable cytoplasmic anchoring function may be exerted by the large T antigen from human polyomavirus BK,^{135,136} by the glucocorticoid receptor,¹³⁷ and by the MDM2-related protein MDM4 (also known as MDMX).¹³⁸ It has also been shown that p53 in neuroblastoma cells is aberrantly ubiquitinated because of an impaired interaction between p53 and the deubiquitinating enzyme HAUSP, and that this hyperubiquitination contributes to cytoplasmic p53 sequestration.¹³⁰ As both Parc and HAUSP interact with the carboxy terminus of p53, competition between Parc and HAUSP for p53 binding has been postulated to underlie the impaired p53–HAUSP interaction in neuroblastoma cells, although this remains to be formally proven.¹³⁰ The defective deubiquitination of p53 results in the appearance of (multi)monoubiquitinated p53 species, which are relatively resistant to proteasomal degradation¹³⁹ and which are subject to increased nuclear export and possibly to diminished re-import, thus yielding a phenotype of cytoplasmic p53 sequestration.¹³⁰ In keeping with the deregulation of MDM2/HAUSP, interference with p53 hyperubiquitination by targeted inhibition of the p53–MDM2 interaction in neuroblastoma cells has been shown to relocate p53 from the cytoplasm to the nucleus and to restore the transcriptional and apoptotic activities of p53.¹³⁰

Conclusions

The rarity of *TP53* mutations in neuroblastoma has been a puzzling issue to many investigators given the potent antitumor capacity of wild-type p53 protein. A substantial number of alternative p53-inactivating lesions have been identified in neuroblastoma during the past few years, many of which interfere with proper functioning of the p14^{ARF}–MDM2–p53 axis (Figure 4). A recent mouse study underscores the importance of direct inhibition of p53 by MDM2 and suppression of p19^{ARF} in the pathogenesis of neuroblastoma.¹⁴⁰ However, it should be kept in mind that cellular decisions of growth, survival, and death result from the integration of a complex network of intertwined signaling cascades and, therefore, that also pathways that do not impinge directly on the core p53 machinery may still provide a means to oppose or neutralize p53 activity. Full characterization of the nature and relative importance of the different blocks on the p53 pathway in neuroblastoma cells awaits genome-wide experimental approaches in well-controlled model systems.

It may not be that surprising, after all, that turning off the p14^{ARF}–MDM2–p53 axis is a preferential mode of p53

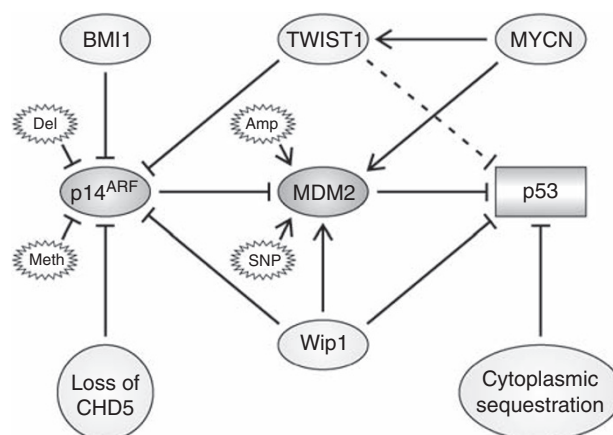


Figure 4 Mechanisms by which neuroblastoma cells escape from p53-mediated growth control. A variety of lesions that result in inactivation of p14^{ARF} or increased activity of MDM2 may provide a molecular explanation for the low frequency of *TP53* mutations in neuroblastoma cells (see text for details). Amp, amplification; Del, deletion; Meth, methylation; SNP, SNP309 in the *MDM2* gene. The color reproduction of this figure is available on the html full text version of the manuscript

inactivation in neuroblastoma cells. It has been convincingly shown that deregulated *MYC* expression is a potent trigger for induction of the p14^{ARF} protein, and it is very likely that the same holds true for *MYCN*.^{25,141} In addition, aggressive neuroblastoma tumors typically express high levels of the E2F1 transcription factor,⁹⁵ which is capable of inducing p14^{ARF} expression through binding to an E2F-responsive element in the p14^{ARF} promoter.^{142,143} Hence, acquisition of defects that inactivate p14^{ARF} or that uncouple p14^{ARF} from its p53-dependent effector pathway (i.e. through uncontrolled MDM2 activity) may provide the most effective route to non-mutational p53 inactivation in neuroblastoma cells by directly dismantling the molecular circuitry that signals the malicious identity of these cells to the p53 guardian.

In conclusion, it has become increasingly clear in recent years that inappropriately increased activity of MDM2 is the primary culprit for p53 inactivation in neuroblastoma cells. Preclinical work from our laboratory and others has shown that small-molecule MDM2 inhibitors are capable of eliciting potent antitumor effects against neuroblastoma by selectively and non-genotoxically reactivating p53.^{8,130,144,145} These findings may provide a new therapeutic avenue for the treatment of children with high-risk neuroblastoma.

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