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c-Myc, Genome Instability, and Tumorigenesis: The Devil Is in the Details

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Abstract The c-myc oncogene acts as a pluripotent modulator of transcription during normal cell growth and proliferation. Deregulated c-myc activity in cancer can lead to excessive activation of its downstream pathways, and may also stimulate changes in gene expression and cellular signaling that are not observed under non-pathological conditions. Under certain conditions, aberrant c-myc activity is associated with the appearance of DNA damage-associated markers and karyotypic abnormalities. In this chapter, we discuss mechanisms by which c-myc may be directly or indirectly associated with the induction of genomic instability. The degree to which c-myc-induced genomic instability influences the initiation or progression of cancer is likely to depend on other factors, which are discussed herein.

1 Introduction

1.1 Overview

Cells must overcome multiple barriers designed to limit growth and proliferation to become tumorigenic [1–3]. The aim of this chapter is to discuss accumulating evidence that expression of c-Myc and other oncoproteins can compromise genomic integrity, how this may contribute to tumorigenesis, and to consider some of the potential mechanisms involved. In addition to other chapters in this volume, we refer the reader to the following excellent reviews detailing the diverse biological effects of the c-Myc protein on cell growth, proliferation, apoptosis, and differentiation [4–10].

1.2 Genetic Instability and Cancer Progression

The genesis of a malignant cell is a multistage process requiring the progressive accumulation of genetic and epigenetic changes [3]. Debates have arisen over whether the large number of changes required for malignancy (typically 6–10) arise spontaneously or whether events occur during tumor progression that increase genomic instability [11–13]. Consistent with the latter idea, many human tumors exhibit structural chromosomal aberrations such as amplifications that harbor increased copies of the c-*myc* oncogene [14, 15], and this type of genetic instability is not detected at measurable frequencies in normal cells [16]. This suggests that the mechanisms that maintain structural chromosome integrity are compromised during tumor progression. Consistent with this, loss of p53 function occurs frequently during cancer progression and creates a permissive environment for gene amplification [17, 18].

Vogelstein and colleagues have suggested subdividing tumors with genomic instability into two broad categories; those displaying chromosomal instability (CIN) and those with microsatellite instability (MIN) [19]. CIN represents a numerical and/or structural change in the karyotype, while MIN describes the expansion or contraction of homopolymers or tandem short repeats throughout the genome [20, 21]. CIN may occur due to mutations in genes required for the partitioning of chromosomes during mitosis, in genes that control cell-cycle checkpoints, or in genes that participate in DNA metabolism and repair [22]. Structural aberrations leading to CIN-like chromosomal abnormalities can also occur following break-induced translocations. These translocations can be balanced, such as the Ig:myc translocation in Burkitt's lymphoma (BL) [23] or unbalanced, such as non-reciprocal translocations generated as a result of bridge-breakage-fusion

cycles [24]. MIN is typically caused by mutation or epigenetic inactivation of genes encoding proteins that participate in mismatch repair [25, 26]. As technology has improved the resolution at which karyotypic differences between normal and tumor can be determined, it has become clear that virtually all tumors exhibit abnormalities at the DNA level. In this review, other changes in the genome including point mutations, deletions, and base modifications will be included as manifestations of genomic instability.

Induction of cell-cycle arrest and activation of apoptosis are parts of the normal cellular defenses against oncogene-driven proliferation [27, 28]. It follows that inactivation of either of these two processes could enhance the likelihood of tumorigenesis. For example, variants with defective arrest or apoptotic machinery are more likely to survive oncogene activation than their "normal" counterparts. Chemical carcinogens and ionizing radiation, which accelerate tumorigenesis by increasing the frequency of somatic mutation [29], can increase the probability of generating such variants. Mutation rates are accelerated in mice following topical application of carcinogens [30]. Carcinomas arising in such mice frequently display mutations in the *H-ras* oncogene, a mutation also associated with human carcinomas [31, 32]. This strongly implicates induction of somatic mutations as an important factor in cancer progression. Viruses can also increase tumorigenicity, but for many years physical agents and oncogenic viruses were thought to work by different mechanisms [33]. Four decades ago, Nichols suggested that the mechanisms of radiation, chemical, and virus-driven oncogenesis may be shared, when he stated: "... it is possible that one of the earliest changes in tumor cells involves activation of a gene locus which increases the likelihood of non-disjunction or other mitotic error" [33]. Thus, Nichols proposed that, like chemical carcinogens and ionizing radiation, viruses might increase mutation frequency. This provided a conceptual framework expanded upon by Nowell [34] and Loeb [35] who suggested that genetic lability could accelerate tumor progression through mutation of genes that are essential for maintaining chromosomal integrity. Lesions in such genes would give rise to a "mutator phenotype" able to fuel further instability. The MIN phenotype (see above) is one specific example of the mutator phenotype. While the MIN phenotype was first identified in Lynch syndrome (hereditary non-polyposis colon cancer) [36], microsatellite instability has subsequently been observed in a variety of other cancers [37–39].

1.3 Viruses, Oncogenes, and Connections to Genome Destabilization

The link between tumor-associated viruses and perturbation of the genome is clear in birds and rodents, and accumulating data suggest viruses may

have a similar impact on genome stability in human cancer. Early work in this field by Nichols demonstrated that infection of cells with the oncogenic Rous sarcoma virus (RSV) induced strand breaks and chromosomal abnormalities [40]. RSV-induced tumorigenesis is attributed to expression of the oncogene *v-src* [41], and overexpression of cellular *c-src* can promote genomic instability [42]. Together these data indicate that oncogene activation by viruses and consequent genome destabilization may be important in tumorigenesis. Viruses can also induce neoplasia by deregulating the expression of endogenous proto-oncogenes [43]. Integration of retroviruses near the *c-myc* promoter leads to aberrant *c-myc* expression in avian and murine tumors [44, 45]. Similarly, retroviral integration increases transcription of *ras*, an oncogene implicated in the initiation or progression of human cancer [46].

Many human tumors associated with oncogenic viruses also display genomic instability. For example, chromosomal instability is observed in human papillomavirus (HPV)-associated cancers [47]. HPV-induced perturbation of the genome appears to precede the invasive stage of cancer [48]. Instability is almost certainly due to the virally encoded E6 and E7 proteins, which inactivate the tumor suppressors p53, pRB, and pocket proteins related to pRB [49]. Oncogenic HPV has been implicated in inducing strand breaks [51, 50], which are precursors of diverse types of structural chromosomal alterations (e.g., see Windle et al. [52]). Furthermore, activation of oncogenic ras in murine fibroblasts induces structural and numerical chromosomal aberrations within one cell cycle [53], as does Mos, an oncogene that activates the mitogen-activated protein kinase (MAPK) pathway [54].

Considerable data therefore indicate that oncogene activation may be a common mechanism by which genomic instability arises in tumors. In the following sections we will discuss the diverse mechanisms by which aberrant c-myc expression may also lead to genomic instability.

1.4 Activation of c-myc and Initiation of Instability

Many mechanisms can lead to the activation of c-myc during tumorigenesis, including enhanced transcription by other oncogenic signaling pathways [56, 55], chromosomal rearrangements [15, 57], and resistance of Myc protein to ubiquitin-mediated proteolysis [58, 59]. c-myc is deregulated in the majority of breast carcinomas and in the early and late stages of colorectal cancer [60–64]. Overexpression of *c-myc* is also associated with the etiology of hepatocellular carcinoma (HCC) [65].

Elevated *c-myc* expression and genomic instability appear to be correlated in the solid tumor types mentioned above [66–68]. This raises the intriguing

possibility that high-level *c-myc* expression in some situations might actually contribute to genome destabilization. In vitro and in vivo studies over the past decade strengthen this possibility. For example, Mai and colleagues showed that elevated c-myc increases the frequency of obtaining variants resistant to the antimetabolites *N*-(phosphonacetyl)-L-aspartate (PALA) and methotrexate via amplification of their respective target genes, *CAD* and *DHFR* [69–71]. This was recently confirmed by Felsher and Bishop [72]. *Cyclin D* and *ribonucleotide reductase R2* are also amplified following activation of c-myc in the absence of drug selection [73, 74], implying that c-myc function, and not the genome destabilizing effects of the selective agents [75], explains the observed increase in amplification frequency. While it has not been determined whether preferred regions are destabilized by *c-myc* overexpression, fluorescent in situ hybridization (FISH) and spectral karyotypic analyses indicate that *c-myc* overexpression may induce alterations at multiple genomic regions [74, 76]. This could have significant physiological impact since amplification of genes such as *mdm2*, *cyclin D*, and *c-erbB2* occur frequently in human cancers as the overproduced gene products provide cells with growth and survival advantages [77–79].

In vivo models of tumorigenesis support the notion that c-myc-induced instability contributes to the neoplastic phenotype. For example, Felsher and Bishop demonstrated that induction of instability in Rat1a fibroblasts by activation of c-myc rendered them tumorigenic in mice [72]. Importantly, c-myc was activated in cells under conditions where apoptosis would not be expected to occur (e.g., complete medium). Furthermore, cell lines derived from such tumors retained the ability to undergo c-myc-induced apoptosis. These data suggest that induction of genomic instability by c-myc does not always require a selection against apoptotic pathways. Transient activation of c-myc was sufficient to induce tumorigenesis and gene amplification. Therefore, initiation of genomic instability by c-myc likely contributes to neoplastic progression in this cell type. The genetic changes that occur following activation of c-myc also appear to be important during liver and breast carcinogenesis in vivo. For example, pre-neoplastic cells from both tissues contain non-random chromosomal rearrangements, including translocations and deletions that persist in late-stage HCC and mammary carcinomas [67, 80]. The early appearance of instability in these models correlates with deregulated c-myc activity. Persistence of chromosomal rearrangements into "mature" tumors suggests that a combination of c-myc-induced instability and subsequent selective pressure are important factors in the HCC and breast carcinoma models.

In other tumor types, it appears that inhibition of p53-induced apoptosis, rather than induction of instability, is the main block to c-myc-driven tumorigenesis. To illustrate, expression of *c-myc* under the control of the IgH [81] or

Igκ or γ [82] enhancers leads to B cell lymphoma with pre-B cell and B cell phenotypes, respectively. Both models show a protracted latency prior to onset of lymphoma, suggesting secondary events are required for c-myc-induced B cell tumors. Various genetic lesions that decrease p53 function, or that prevent induction of apoptosis, accelerate c-myc-induced lymphomagenesis [83–85]. It seems that large-scale genomic instability is not required in the Eμ-myc model of B cell lymphoma, since tumors in which c-myc-induced apoptosis was inhibited by dominant-negative caspase-9 were pseudodiploid [86]. Using an integrated LacZ reporter, Rockwood and colleagues analyzed the mutation and rearrangement rates in c-myc-driven lymphomas [87]. Strikingly, they found that chromosomal rearrangement but not mutation rate was enhanced in lymphomas compared to normal tissue, and that the p16Ink4a/p19arf locus was deleted. These data indicate that deregulated c-myc activity likely selects for cells with defects in the retinoblastoma (Rb) and p53 tumor suppressor pathways. While BL biopsies are usually pseudodiploid, comparative genomic hybridization and spectral karyotypic analysis have found that, similar to mouse models, numerous chromosomal aberrations, including deletions are present [88]. In summary, it appears that selection for somatic mutations in tumor suppressor pathways is the primary determinant in c-myc-induced B cell lymphomagenesis. Once cells resistant to apoptosis emerge, the growth and proliferative functions of c-myc are able to drive tumorigenesis.

2
Possible Mechanisms of c-myc-Induced Instability

The complex karyotype that is observed in biopsies from human tumors is a footprint of multiple genetic changes that have occurred during tumorigenesis. Therefore, it is not possible to conclude when during tumor progression such changes arose, and whether the instability is a continuing process or a reflection of a historic event. Consequently, it is not possible to derive cause and effect relationships between genomic instability and *c-myc* overexpression by analyzing archival human tumor samples. However, an examination of gene amplification mechanisms suggests how excess myc activity and genomic instability might be causally linked. The two mechanisms for amplification in mammalian cells are re-replication of target loci and induction of strand breaks [24, 52, 89–91]. Re-replication involves the initiation of multiple rounds of DNA replication within a single S-phase. Recent data demonstrate that high-level overexpression of cdc6 and cdt1 proteins, which are required for replication origin licensing, can induce re-replication at some frequency in cancer cell lines [92]. Since c-myc can transactivate genes encoding replica-

tion origin licensing proteins ([93, 94] and Sect. 2.5 below), it remains possible that it could induce amplification by a re-replication mechanism.

The second mechanism for gene amplification involves chromosome breakage, which can be induced in a number of ways [52, 95, 96]. Importantly, recent data show that elevated *c-myc* expression can lead to metaphase chromosome abnormalities including those that harbor amplified genes and that usually reflect breakage during G1 or S-phase [72]. Breakage has also been observed in G0/G1 arrested cells expressing the c-Myc/estrogen receptor fusion protein (Myc-ER) under conditions where apoptosis was not induced [97]. The same study showed phosphorylation of p53 on Ser15, an indicator of DNA damage. Finally, c-myc activation can lead to a delay in G2, which usually occurs in cells that have experienced DNA damage during

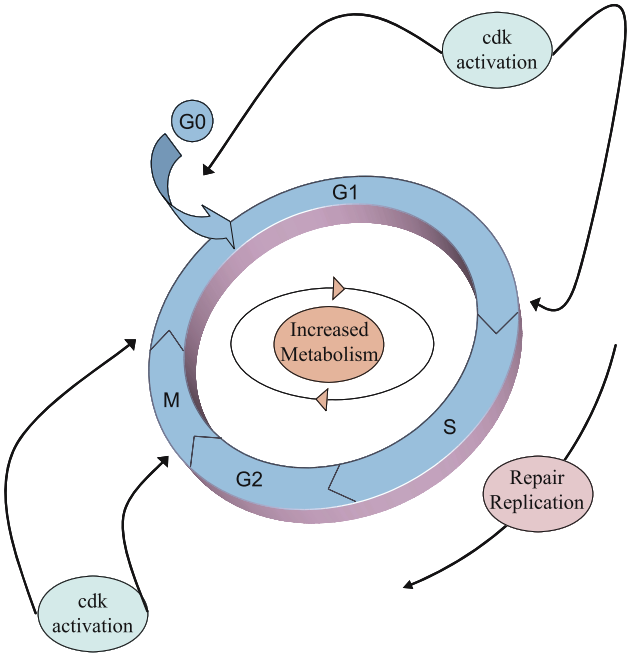


Fig. 1 Summary of potential sites of c-myc-induced DNA damage. Activation of cyclin/cdk complexes by c-myc can lead to premature entry into S-phase or exit from G2/M. Both these events may induce DNA damage as described in the text and in the following figures. Additionally, increased metabolic activity induced by c-myc can generate reactive oxygen species, which can contribute to DNA damage. High level c-myc expression also activates the transcription of DNA replication and repair components, which may impact the fidelity of these processes

S-phase and have arrested for repair [98]. Together, these data support the conclusion that elevated levels of c-myc can induce the types of DNA damage that precede gene amplification and other structural chromosome alterations. The available literature suggests that c-myc may destabilize the genome by multiple mechanisms. This section focuses on five we consider most likely: (1) cell growth and metabolism, (2) unscheduled entry into S-phase, (3 and 4) abrogation of stress-induced cell-cycle checkpoints at G1/S and G2/M, and (5) modulation of DNA damage response and repair pathways (Fig. 1).

2.1 Increased Metabolism and Induction of ROS

The mechanisms by which c-myc couples mitogenic stimulation to growth and proliferation are gradually being elucidated. Physiological activation of c-myc can be achieved in several ways. In quiescent B cells, *c-myc* expression can be activated by nuclear factor (NF)-κB and protein kinase C (PKC) signaling [99], whereas *c-myc* transcription is controlled by src and signal transducer and activator of transcription (STAT) signaling in platelet-derived growth factor (PDGF)-stimulated fibroblasts [100, 101]. Activation of c-myc induces growth of B cells in the absence of proliferation, and c-myc overexpression can increase cell size throughout the cell cycle [102, 103]. Concordant with these results, c-myc gene targets include rate-limiting enzymes in the glycolytic and respiratory pathways and in biosynthetic pathways [104–106].

The metabolic burst associated with emergence from quiescence and entry into S-phase is a potential source of reactive oxygen species (ROS). ROS are essential mediators of proliferative signals, but at high levels can cause oxidative base modifications and single- or double-stranded DNA breaks. If such lesions are not repaired, they may become fixed in the genome during DNA replication. ROS are estimated to induce up to 10,000 lesions per cell per day [107]. However, the mutagenic potential of these lesions is limited by a combination of antioxidants and DNA repair enzymes. It follows that since oncogenes such as *ras* and *c-myc* are key players in mitogenic pathways, aberrant signaling from either might create an oxidative burden. In support of this, activation of oncogenic ras can induce ROS in various cell lines in vitro [108, 109]. Adding to these data, other groups have found that activation of c-myc can increase intracellular ROS [110, 97]. While activation of c-myc is associated with induction of DNA damage in serum-deprived and cycling normal human fibroblasts, preincubation with antioxidant only appears to reduce damage in the former case [97, 111]. These data indicate that although ROS can contribute to c-myc-induced DNA damage under certain circumstances,

other mechanisms are also likely to be involved. Data from other studies also highlight the complex role of ROS as mediators of c-myc-induced effects. For example, ROS induced by c-myc in NIH3T3 cells do not appear to be cytotoxic unless the cells are cultured in low serum [110]. Additionally, ROS are mediators of c-myc-induced apoptosis in some human cell lines but are associated with induction of an arrested state resembling senescence in normal human fibroblasts [97, 112]. A similar senescent-like state has been described in normal human fibroblasts exposed to ionizing radiation [113], oxidative stress [114], and following telomere shortening [115]. Taken together, the data support the idea that in some normal cell types, inappropriate c-myc activation can induce sufficient DNA damage to elicit a stress response resulting in some cells undergoing permanent cell-cycle exit.

Elevated ROS are found in some human tumors and tumor-derived cell lines [116, 117]. In addition to their role in mitogenic signaling mentioned above, there is evidence that ROS can also contribute to mutations associated with tumor initiation or progression. For example, many of the point mutations found in tumor suppressor genes in human cancer can be induced by oxidative stress [118–121]. Furthermore, elevated frequency of such lesions can be found in the p53 gene in normal hepatocytes of individuals with Wilson's disease, a disorder associated with elevated ROS and increased risk of hepatocellular carcinoma [122]. There is also an elevated frequency of oxidative stress-related p53 mutations in ulcerative colitis, another disease that is linked to an increased risk of cancer [123].

Induction of MIN occurs predominantly through mutation of mismatch repair genes, but excessive ROS can also lead to MIN in vitro [124, 125]. MIN can generate frameshift mutations in tumor suppressor genes [126], such as those that inactivate the type II transforming growth factor-β receptor (TGF-βRII) [127]. This may allow colon epithelial cells to escape growth restriction mediated by ligation of TGF-β to TGF-βRII. Furthermore, oxidative stress can increase the frequency of frameshift mutations in lung and colorectal carcinoma cell lines [128, 129]. Together these data suggest that ROS may contribute to destabilization of the genome in certain malignancies.

Although many human cancers are associated with environmental agents such as those inhaled by smoking, the age-specific incidence of sporadic cancers of the ovary, pancreas, and colon does not vary significantly between populations [130]. This suggests that endogenous cellular processes may be involved in the initiation of some tumors. The ability of *c-myc* and other oncogenes to activate metabolic pathways leading to oxidative stress suggests they could be considered candidate pro-mutagens. However, whether ROS induced by c-myc in vivo is sufficient to induce somatic mutation remains untested. This is likely to be determined by the contributions of multiple

signaling pathways in the cell, which in turn will be influenced by cell type and the surrounding environment. As one example, in a mouse model of HCC, *c-myc* overexpression in hepatocytes results in liver tumors, with a latency of more than 1 year, suggesting that multiple changes are required for c-myc-induced HCC [131]. By contrast, when *TGF*-α is co-expressed with *c-myc*, the latency for tumor onset is decreased dramatically. Concomitantly, ROS levels and chromosomal and mitochondrial genome instability increased [133, 132]. Supplementing the diet of these mice with the antioxidant vitamin E reduced ROS levels and also reduced proliferation. Coincident with the block to proliferation, the amount of genomic instability was also significantly decreased. Additional data showed that mitochondrial DNA deletions were also reduced by vitamin E in this study, providing compelling evidence that ROS produced as a result of a combination of deregulated *c-myc* and *TGF*-α expression can induce DNA damage in vivo. These data suggest that inhibition of proliferation and DNA damage by antioxidants can prevent c-myc-induced instability and tumor progression.

2.2 Unscheduled Entry into S-Phase

In mammalian cells, c-myc activation can increase cell number as well as cell size, which may depend on the cell type [102, 134]. Studies in rodent cells demonstrate that the G1 interval is longer in *c-myc*-null cells when compared to wildtype [135]. These data suggest that c-myc facilitates progression through G1 into S-phase. In part, these observations may be explained by the ability of c-myc to downregulate inhibitors of cyclin/cdk complexes or to stimulate transcription of genes encoding cyclins. The activation of cyclin/cdk complexes removes the block to the transition from G1 to S-phase, which is mediated, at least in part, by the Rb protein [136]. Briefly, hypophosphorylated Rb prevents transcription of genes required for S-phase in two ways. First, Rb can sequester the transcription factor E2F1, which has been implicated in the control of S-phase entry [137]. Second, Rb can form a complex with E2F1 (and other E2F family members) that actively represses S-phase gene transcription [138]. This section will focus only on bypass of the cell-cycle checkpoints associated with the transition from G0/G1 to S-phase in the absence of exogenous stresses. The bypass of DNA damage-induced checkpoints will be addressed in Sects. 2.3 and 2.4).

Numerous mechanisms may promote the transition into S-phase [139–143]. For simplicity, the following illustrates a linear pathway in which c-myc activates cyclin E/cdk2 leading to S-phase entry independently of Rb status. Activation of cyclin E/cdk2 is important for entry into S-phase, although the

critical downstream targets are unknown [144–146]. c-myc can activate the cyclin E/cdk2 complex, primarily by altering the levels or distribution of the cyclin E/cdk2 inhibitor, p27. p27 loss is a poor prognostic indicator in tumors of the breast and in gastric and colon carcinoma; a feature of all these cancers is overexpression of *c-myc* [147, 148]. Furthermore, deletion of p27 reduces the latency to tumor onset in *c-myc* transgenic mice [149]. Cdk-2 dependent phosphorylation at threonine 187 is required for degradation of p27 [150]. The phosphorylation allows binding of the Skp1/Cul1/F-box (SCF) ligase complex, which ubiquitinates p27 and targets it for proteasome-mediated degradation [151–153]. Cul1, a component of the SCF ligase complex, is also required for efficient ubiquitination and degradation of p27 [154, 153]. In some systems, c-myc can induce Cul1, leading to p27 degradation and S-phase entry [155]. Together these data provide one explanation for the ability of c-myc to overcome a p27-induced cell-cycle block. Additionally, c-myc can directly target cyclin D2, leading to the sequestration of p27 into heat-labile complexes and permitting cyclin E/cdk2 activation [156]. The activation of cyclin E/cdk2 by c-myc is also sufficient to bypass the G1/S block imposed by hypophosphorylated Rb and p16 [157]. These data indicate one mechanism by which c-myc can bypass Rb-mediated checkpoints without Rb hyperphosphorylation.

Inappropriate cyclin E expression can induce genomic perturbations. For example, the bypass of an Rb-imposed cell-cycle block by c-myc and cyclin E is associated with endoreduplication [141], and cyclin E/cdk2 activity can induce chromosomal instability [158]. Although the mechanism for this is unknown, it is possible that excessive cdk activity might perturb replication origin licensing, which has been linked to instability [159–161]. Interestingly, inappropriate cyclin E/cdk activity appears to accelerate S-phase entry but actually slows replication [158, 162], raising the possibility that DNA damage and activation of the S-phase checkpoint may occur under such conditions. Studies in yeast indicate that precocious cyclin/cdk activity can delay firing of replication origins, leading to strand breakage and chromosomal abnormalities [163]. Whether this can occur in mammalian cells has yet to be shown. However, a reasonable speculation is that inappropriate entry to S-phase induced by c-myc in the absence of correct origin licensing might lead to DNA damage (Fig. 2).

2.3
Abrogation of G1/S Arrest Induced by DNA Damage

DNA damage activates checkpoints throughout the cell cycle that prevent the replication and transmission of mutated DNA [164]. Activation of a p53-dependent checkpoint at or prior to the restriction point can prevent entry

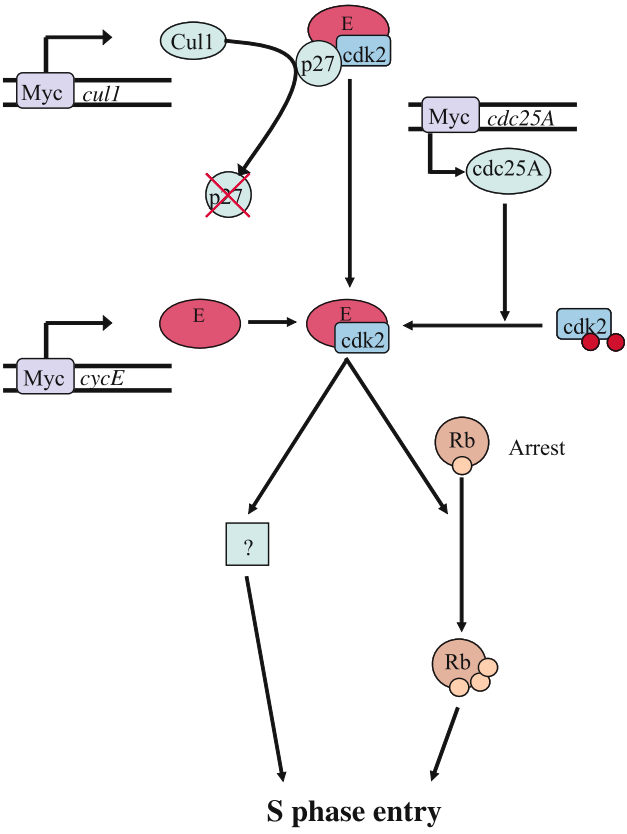


Fig. 2 c-myc can induce restriction point bypass by mulitple mechanisms. c-myc can activate cul1 transcription in some cell types, leading to degradation of the cyclin/cdk2 inhibitor, p27. Additionally, c-myc can transactivate cyclin E and cdc25A, a phosphatase which activates cdk2. Together, these activities activate cyclin E/cdk2 kinase, which in turn should inactivate Rb, release E2Fs and enable S-phase progression. c-myc can also activate a parallel pathway for S-phase progression, which requires cyclin E/cdk2 activation, but does not require inactivation of Rb. The downstream targets of cyclin E/cdk2 in this pathway are unknown

of cells with as few as one unrepaired double-strand break into S-phase [165, 166]. DNA lesions are recognized by specific protein complexes, which transduce the DNA damage signal to downstream effectors to elicit arrest. Below we briefly describe the activation of p53 in response to DNA strand breaks and present experimental data demonstrating that c-myc can attenuate this pathway in some cell strains.

Mre11/Rad50/Nbs1 (MRN) complexes are recruited rapidly to sites of breakage [167]. This termolecular complex is involved in the processing of DNA lesions that arise during replication and following DNA damage [168, 169]. Activation of the ATM kinase also occurs rapidly after strand breakage as a result of an intramolecular phosphorylation event [170]. However, the mechanism by which the break is detected and subsequently activates ATM remains to be determined. Although MRN is phosphorylated by ATM, it can be recruited to sites of damage in the absence of ATM activity, indicating that these two events are not linked [171]. ATM induces direct phosphorylation of p53 at Ser15, and indirectly induces phosphorylation of p53 at Ser20 by activating the damage checkpoint kinase chk2 [172, 173]. These modifications can activate p53 either by decreasing p53 binding to its negative regulator, mdm2, or by increasing association with the transcriptional co-activator p300/CBP [175, 174]. Activated p53 then regulates the transcription of numerous target genes leading to cell-cycle arrest, apoptosis, or increased repair, depending on the cell type and type of damage induced [166]. The inhibition of Rb phosphorylation by p21 is partially responsible for p53-dependent G1 arrest [176].

Constitutive overexpression of *c-myc* in epithelial cells can compromise ionizing radiation-induced arrest, forcing cells into S-phase prematurely [177]. The escape from radiation-induced G1 arrest is a direct result of c-myc action, and not the result of selection for checkpoint-deficient variants, as it occurs in a significant fraction of normal fibroblasts and epithelial cells expressing an inducible *c-myc-ER* construct [97, 177]. The replication of DNA strand breaks during S-phase is a potential source of continuing genomic instability, since break repair could generate dicentric chromosomes, which can then enter into bridge-breakage-fusion cycles (see Sect. 1.2 and [24]). Therefore, c-myc's ability to attenuate damage-induced checkpoints is likely to contribute to genomic instability.

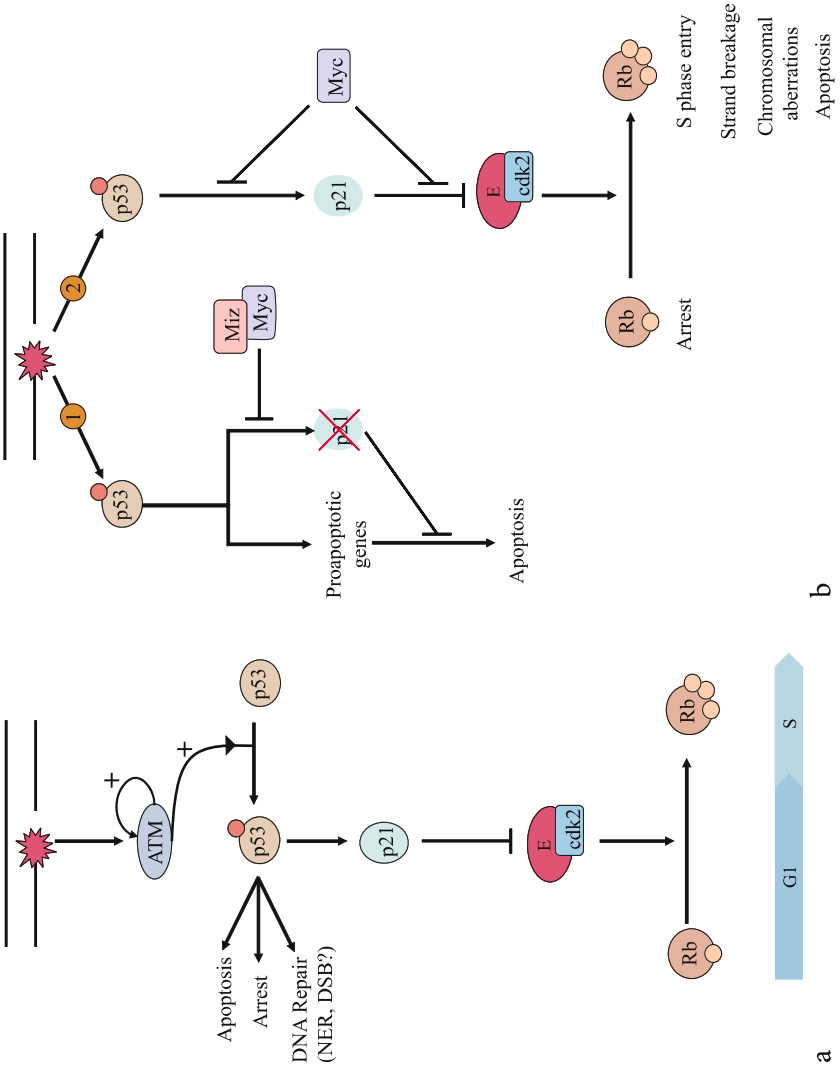
The abrogation of p53-dependent arrest by c-myc can lead to apoptosis in some cell types [178], which could provide a backup mechanism for limiting the emergence of genetically unstable variants. Recent data indicate that regulation of *p21* expression by c-myc is a determinant of the apoptotic response. For example, c-myc can specifically block the DNA damage-induced accumulation of p21 normally observed in colon carcinoma cells [179]. Concomitant with the decrease in p21 levels, the response of the cells to DNA damage was switched from arrest to apoptosis. These data suggest that in the context of a DNA damage signal, *p21* induction should be able to prevent apoptosis. A corollary is that the ability of c-myc to override a damage-induced arrest should require *p21* downregulation, and S-phase entry should induce apoptosis. However, cells overexpressing c-myc can escape damage-induced arrest

and enter S-phase with elevated p21 levels [97, 180]. Other studies show that the anti-apoptotic function of p21 does not necessarily require its ability to inhibit the cell cycle [181, 182]. This raises the possibility that cells with damaged DNA that enter the cell cycle due to deregulated *c-myc* expression may evade apoptosis if p21 levels are sustained. In turn, this may increase the possibility that DNA lesions become fixed in the genome during replication or repair.

Felsher and Bishop showed that aneuploidy could be induced by c-myc in exponentially growing Rat1a fibroblasts and normal human fibroblasts, but that damage associated with strand breakage (i.e., double minutes, polycentric chromosomes) was only observed in the Rat1a cells [72]. This is presumably because normal cells respond to strand breaks induced by c-myc by undergoing a p53-dependent arrest resembling senescence [183]. The Rat1a cells are immortal and have no p21 function due to methylation of the promoter [184]. A lack of p53-mediated arrest in rodent cells may create a permissive environment for a wide range of c-myc-induced chromosomal aberrations. Conversely, in human cells, activation of p53 may restrict the emergence of certain types of chromosomal defects, as noted. However, c-myc activity is still able to induce aneuploidy in normal human cells, indicating that it can compromise the fidelity of events associated with mitosis (see Sect. 2.4).

Fig. 3a, b Activation of c-myc can override damage-induced checkpoints. **a** The signaling pathway downstream of DNA damage is simplified for clarity. Following strand breakage, the ATM kinase is activated, although the mechanism by which break detection occurs is unknown. p53 is stabilized and activated by ATM-induced phosphorylation. Activated p53 induces the transcription of numerous target genes, among which are several that induce apoptosis, stimulate DNA repair, or promote cell-cycle arrest. For example, induction of the cyclin/cdk inhibitor, p21 inhibits cyclin-cdks such as cyclin E/cdk2, which prevents Rb hyperphosphorylation and inactivation, thereby blocking S-phase entry. Excess myc activity can attenuate the DNA damage response and induce cell-cycle progression downstream of p53 activation by inhibiting p21 function in some cell types, although in other situations c-myc-induced bypass occurs without apparent alterations of p21 levels (see **b**). For discussion of other components up- and downstream of p53 activation, see Wahl and Carr [166]. **b** Override of the p53-dependent DNA damage response by c-myc. DNA damage can lead to simultaneous, p53-dependent transcription of cell-cycle arrest and pro-apoptotic genes. In some cell types, the induction of p21 can inhibit p53-dependent apoptosis. c-myc can selectively inhibit p21 induction when bound to Miz protein at the p21 promoter, resulting in apoptosis (*1* and [179]). (*2*) In other cell types, c-myc-mediated inhibition of p21 appears to lead to cell-cycle entry, which is dependent on cyclin E/cdk2 activity, but does not involve Miz, and may rather be related to sequestration of p21 into other cyclin-cdk complexes. Under these conditions, replication of damaged DNA may lead to chromosomal abnormalities, which could trigger apoptosis or give rise to genetic variants

Activation of cyclin/cdk complexes by c-myc may also be involved in the abrogation of damage-induced checkpoints. The indirect activation of cyclin E by Myc could potentially participate in this process. Cyclin E and c-myc appear to activate some common elements of the DNA damage response. For example, activation of c-myc or overexpression of cyclin E in the absence of exogenous stress leads to an increase in p53 Ser15 phosphorylation in pri-



mary cells [97, 185]. This demonstrates that inappropriate proliferative signals induce DNA damage and elicit a classical p53-dependent damage response. However, the mechanisms by which c-myc and cyclin E override DNA damage-induced checkpoints are likely to be distinct. To illustrate, expression of cyclin E induces genomic instability in normal human fibroblasts and immortalized epithelial cells [158, 185]. However, induction of chromosomal instability by cyclin E requires abrogation of p53 or p21 function [185]. In contrast, c-myc can induce chromosomal instability in primary human cells with an intact p53 pathway [72]. Furthermore, c-myc can abrogate ionizing radiation-induced arrest, but cyclin E overexpression is unable to do so [97, 177, 185]. Taken together, these data suggest that activation of cyclin E may contribute to induction of genomic instability by c-myc, but that other activities of c-myc are likely required to bypass damage-induced checkpoints (Fig. 3).

2.4
Abrogation of Arrest at G2/M

The tight coupling of mitosis and DNA replication ensures the replication and faithful segregation to each daughter of only one complete genome per cell cycle [186]. Cell-cycle checkpoints in G2 and M function to maintain the structural integrity of the duplicated chromosomes and ensure their equal partitioning at cell division. Defective processes during mitosis can lead to an abnormal karyotype. For example, aneuploidy occurs following defects in chromosomal segregation. Additionally, abrogation of arrest induced at G2/M can also lead to endoreduplication (re-replication of the genome without cell division) [187–189].

Overexpression of c-myc has been correlated with endoreduplication and aneuploidy in several models. Prolonged arrest at mitosis following exposure to agents that perturb the mitotic spindle results in "mitotic slippage," leaving cells arrested with 4N DNA content in a G1-like biochemical state [190–193]. Overexpression of *c-myc* compromises this arrest, leading to endoreduplication [180]. In addition to drug-induced perturbation of microtubules, sequestration of E2F transcription factors can also lead to mitotic slippage, and c-myc is able to induce endoreduplication under these conditions [141, 177]. In primary cells, endoreduplication is countered by apoptosis [180]. However, in cells that are resistant to apoptosis, such genomic instability can be tolerated [194]. In summary, for cells that have reduced apoptotic responses, c-myc activation could induce cell-cycle progression and lead to endoreduplication, which could perpetuate instability and accelerate tumor progression.

The ability of c-myc activation alone to induce accumulation of cells with 4N DNA content [98] is consistent with its ability to induce sufficient DNA

damage to provoke a G2/M checkpoint arrest response. However, G2/M arrest in *c-myc*-expressing cells also seems to lead to increased ploidy. One potential explanation is that under these conditions elevated *c-myc* expression in cells arrested at G2/M may enable DNA synthesis to reinitiate in the absence of cell division to induce polyploidy. Although the mechanism for this is unclear, the data summarized above raise the possibility that it could involve premature activation of cyclin/cdk complexes and other factors involved in replication origin licensing and initiation of S-phase (Fig. 4; see also Sects. 2 and 2.3 above).

2.5
Modulation of DNA Damage Response and Repair Pathways

DNA damage response and repair pathways are present to ensure the faithful replication and segregation of genetic material. Conversely, attenuation of damage response or repair pathways contributes to genomic instability. A link between c-myc activation and DNA metabolism is particularly attractive when the effects of c-myc on replication and genomic instability are considered. This section summarizes recent analyses indicating that c-myc regulates the expression of genes involved in DNA replication and the DNA damage response and repair pathways.

Microarray analyses indicate that c-myc can upregulate genes involved in DNA replication including Topoisomerase I (*TOP1*), *mcm4*, *mcm6*, *mcm7* and *cdt1* [93, 94, 103, 155]. TOP1 is required during DNA replication to relax supercoils that are generated by passing replication forks [195]. Therefore, the induction of this enzyme by c-myc might facilitate S-phase progression. However, overexpression of *TOP1* can induce illegitimate recombination, and trigger instability [196]. Mcm6, mcm7, and cdt1 are required for firing of replication origins and can also induce genomic instability when expressed at high levels ([197] and see Sect. 1).

Although these data show a correlation between myc activation and gene expression, at present their biological significance is unclear. However, two recent reports suggest that components of the DNA repair machinery may be involved in the response to activation of c-myc. The first report focused on the Nbs1 protein, a component of the MRN complex involved in repair of replication and damage-associated breaks ([169] and Sect. 2.3 above). Chiang et al. [198] showed that small interfering (si)RNA-mediated knockdown of *c-myc* decreases Nbs1 levels, and they postulate that induction of *Nbs1* by c-myc is required during DNA replication. However, the length of S-phase is unaffected in *c-myc*-null rat fibroblasts compared to the parental line [135, 199]. Additionally, Nbs1 deficiency in transformed fibroblasts does not affect the rate of DNA synthesis [200]. Further work is therefore required to determine

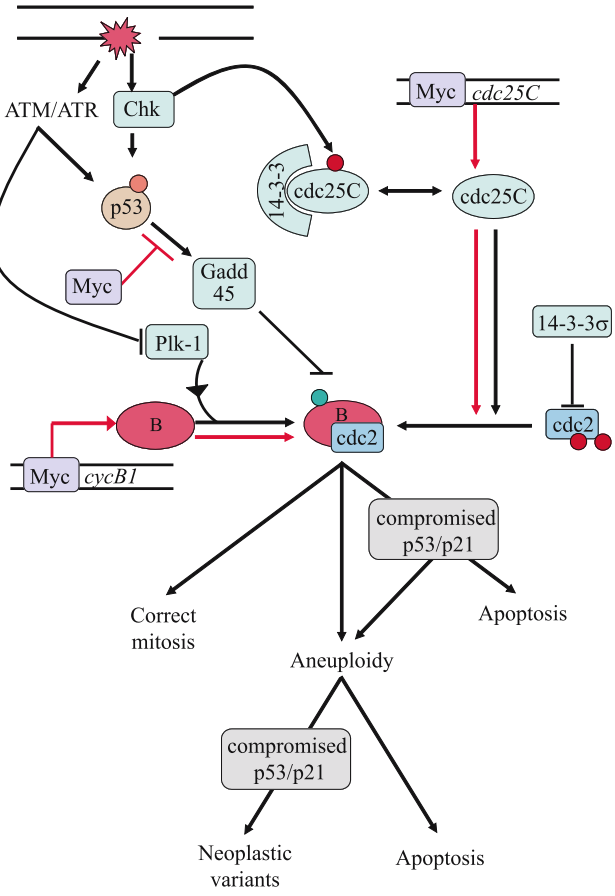


Fig. 4 c-myc Activation of cyclin B/cdc2 may contribute to chromosomal instability. Multiple regulatory pathways converge at cyclin B/cdc2 to control its mitosis-promoting activity. Inhibitory phosphorylations are removed from the cdc2 subunit by cdc25C phosphatase, and Plk-1 kinase phosphorylates cyclin B, leading to activation of the holoenzyme. Following induction of DNA damage by exogenous stresses or oncogene activation, several pathways lead to arrest at G2/M, presumably by inhibiting cyclin B/cdc2. Arrest pathways involve sequestration of cdc25C and cdc2 in the cytosol by 14-3-3 and 14-3-3σ proteins, respectively, and upregulation of the cyclin B/cdc2 inhibitor, Gadd45. c-myc can upregulate cyclin B and cdc25C, leading to activation of cyclin B/cdc2, which should lead to mitotic entry. Additionally, c-myc can attenuate p53 function, which has been implicated by several studies in the G2/M checkpoint. Since c-myc has been reported to induce aneuploidy and can activate cyclin B/cdc2, it is possible that c-myc overexpression perturbs events in G2-M to reduce the fidelity of chromosome segregation. See Sect. 2 for further details

whether c-myc and Nbs1 interact in pathways that affect DNA metabolism. A second study indicated that loss of the WRN protein (a DNA helicase involved in repair) leads to senescence in cells overexpressing *c-myc* [201]. The authors speculate that WRN activity may be required in certain cellular contexts to facilitate c-myc-driven proliferation during tumorigenesis.

It is unclear how c-myc-induced upregulation of DNA repair genes such as *Nbs1* or *WRN* might affect genomic integrity. During normal proliferation, induction of repair enzymes by c-myc might facilitate the resolution of breaks arising during replication and thus contribute to replication fork progression. However, it has also been suggested that inappropriate induction of repair enzymes during S-phase could promote unscheduled repair of replication intermediates and increase the probability of generating chromosomal aberrations [202]. Conversely, inhibition of scheduled DNA repair during the cell cycle can also lead to chromosomal defects. Interestingly, a recent report indicates that c-myc activation may suppress the repair of double-strand breaks in normal human cells [111]. The authors suggest that this may explain the increased frequency of chromosomal rearrangements following activation of c-myc. Whether c-myc inhibits repair directly via transactivation or repression of DNA damage response or repair genes or via a more indirect mechanism remains to be determined. Finally, conditions that accelerate or retard replication fork progression can induce chromosome breakage, suggesting that perturbation of S-phase progression could also increase the probability of chromosomal rearrangement. It is conceivable that *c-myc* overexpression could affect S-phase progression given the number of target genes it regulates with functions in DNA replication [93, 94].

3
Reversible Activation of Oncogenes and Genomic Instability

Loeb postulated that induction of a mutator phenotype initiates a genetically irreversible tumor progression [203]. This is because once genes critical for maintenance of genomic stability are mutated, re-establishment of a normal genome becomes impossible. Therefore, if c-myc is acting as an endogenous activator of the mutator phenotype, turning off *c-myc* expression should not lead to the re-emergence of cells with a normal karyotype. Furthermore, if the gene expression changes resulting from the rearrangements induced by *c-myc* overexpression were sufficient to sustain growth, turning *c-myc* off should not lead to tumor regression. Felsher and Bishop [72] showed that c-myc-induced gene amplification and tumorigenicity persisted in Rat1a cells following c-myc inactivation. These data suggest that, at least in the Rat1a cells, c-myc-driven

instability correlated with a durable tumorigenic phenotype that persists in the absence of the initiating event (i.e., c-myc activation).

By contrast with these data, other studies show a requirement for persistent c-myc activity to maintain tumor cells in vivo. T cell lymphomas initiated by *c-myc* activation undergo apoptosis and regression when *c-myc* is turned off [204]. Similarly, inactivation of c-myc in the skin and pancreas leads to regression of papillomatosis and β-cell hyperplasia, respectively, which are accompanied by apoptosis [205, 206]. Osteosarcomas and mammary carcinomas initiated by c-myc also revert after the *myc* transgene is turned off [207]. Mutations in the Wnt pathway leading to excessive Wnt signaling are associated with a number of human cancers [208, 209]. c-myc is positively regulated by the Wnt signaling pathway and may be required for Wnt-induced tumorigenesis [210, 211]. In support of this, activation of Wnt in the breast leads to carcinoma concomitant with elevated c-myc [212]. Similar to the reversible activation of c-myc, inactivation of Wnt is sufficient to induce tumor regression [213].

The regression mechanisms have not been elucidated. Loss of c-myc functions such as proliferation, angiogenesis, and inhibition of differentiation are likely to be important. Another possibility is that genomic instability could be a trigger for apoptosis once c-myc is inactivated. Perhaps c-myc can attenuate signaling from the damaged genome to the apoptotic machinery. Alternatively, c-myc may activate some enzymes involved in DNA metabolism (see Sect. 2), which would prevent apoptosis at the expense of initiating irregular repair. DNA damage could induce apoptosis and regression, but the downstream effectors of apoptosis remain unknown. To illustrate, inactivation of Wnt in the breast leads to regression regardless of p53 status, implying the involvement of p53-independent apoptotic mechanisms [213].

The studies outlined above suggest that *c-myc* expression is required for sustained tumorigenesis. Furthermore these data seem to indicate that genomic destabilization may not be sufficient to maintain tumorigenic potential in these models. Therefore, one might conclude that c-myc is not able to engender the classical mutator phenotype as described by Loeb (see above), since the tumorigenicity is reversible. However, following a period of remission, some tumors resumed growth in the absence of oncogene activity [204, 207]. Murine mammary carcinomas that relapsed in the absence of c-myc activity frequently exhibited ras mutations [207]. Complex chromosomal rearrangements were also observed in relapsed lymphoid tumors that had escaped dependence on c-myc [76]. Interestingly, all relapsed tumors displayed novel karyotypic aberrations compared to primary tumors. It is possible that in the breast model, pre-existing *ras* mutations are present in some of the c-myc-induced tumors and that these cells provide a selective

advantage for regrowth in the absence of c-myc activity. In contrast, there was no genetic lesion common to all relapsed lymphoid tumors. This raises the intriguing possibility that acquisition of specific genetic lesions induced by c-myc enhance the propensity for relapse in some tumors. Studies of Wnt-driven tumorigenesis indicate that p53 status is an important determinant of relapse. For example, loss of one p53 allele leads to a sevenfold increase in relapse frequency of breast tumors [213]. This suggests that attenuation of p53 function may be one mechanism by which genomically unstable tumors initiated by oncogenes could relapse. Does this mean that relapsed tumors are those that have sustained somatic mutations in p53 and now provide a selective advantage in the context of c-myc-induced chromosomal changes? Preliminary data from Karlsson et al. [76] suggests that *p53* and *arf* loci are intact in relapsed tumors, indicating that genetic inactivation of these tumor suppressors is not required for escape from oncogene dependence. However, it is possible that epigenetic inactivation of the p53 pathway may contribute to tumor progression in this model.

It is important to note that some tumors do not relapse once c-myc is turned off. For example, full regression of c-myc-driven hyperplasia is observed in the pancreas and skin [205, 206]. Furthermore, osteosarcomas driven by c-myc regress when the transgene is inhibited [214]. Therefore, in some cell types, genomic instability may be insufficient to phenocopy the required functions of c-myc. The basis of these differences is not understood. However, it is possible that hyperplasia in some tissues remains dependent on other functions of c-myc such as its role in stimulating angiogenesis. In addition, c-myc activation in the skin can inhibit or promote differentiation, depending on the cell type, further underscoring the complex response to c-myc in vivo [205, 215].

4 Summary

Oncogenic activation of c-myc affects multiple intracellular pathways, culminating in neoplastic transformation in many cell types. Frequently associated with deregulated c-myc activity are numerical and structural alterations of the karyotype. In certain tumors, comparison of normal and pre-neoplastic tissues reveals chromosomal aberrations specifically associated with c-myc activation. The persistence of these lesions during tumor progression indicates that they are selected for during tumorigenesis. Due to its ability to impact numerous biological functions, c-myc is carefully controlled in the non-pathological state. By extension, deregulated c-myc activity is potentially catastrophic for the cell. Activation of apoptosis in response to c-myc plays

a critical role in limiting its deleterious effects. However, should this pathway become disabled or desensitized, c-myc has the potential to wreak havoc on the genome. Mechanistic links between c-myc activation and genome destabilization are beginning to emerge from in vitro and in vivo studies. For example, disruption of cell-cycle checkpoints by c-myc can lead to aberrant DNA replication, a source of genomic instability. Other data indicate that metabolic effects of c-myc, which may be independent of its cell-cycle promoting ability, might also lead to DNA damage. Specifically, oxygen radicals produced following c-myc activation could precipitate genomic changes including break-induced rearrangements and oxidative base modifications. The ability of c-myc to compromise p53-dependent cell-cycle checkpoints indicates that, under certain conditions, genomic perturbations may occur even in the presence of tumor suppressor genes.

In vivo models have provided great insight into the complexities involved in c-myc-induced tumorigenesis. The reversible activation models have demonstrated that many tumors remain dependent on c-myc expression and undergo apoptosis once c-myc is turned off. These data indicate that there is a functional inactivation of the apoptotic pathway in the presence of c-myc activity, rather than a selection for cells that have lost the ability to induce cell death. The mechanism of apoptosis induction following c-myc inactivation is incompletely understood. Many explanations have been put forward, based on some of the known biological effects of c-myc. These include regression of vasculature, which would reduce tumor nutrient supply and re-establishment of differentiation, which may sensitize cells to programmed cell death. However, the link between genome destabilization and apoptosis might offer an alternative explanation. Perhaps DNA damage signaling pathways, which normally initiate apoptosis in response to karyotypic abnormalities, are attenuated while c-myc is expressed. Re-activation of these pathways once c-myc is switched off might lead to the rapid elimination of cells with abnormal genomes. Further studies that address the interaction of c-myc with components of the DNA damage response pathway are likely to provide valuable data in this emerging area of c-myc research. Determining the effect of c-myc expression in the context of DNA damage response/repair pathway deficiencies in vivo may provide further insight into the role of c-myc-induced instability in tumorigenesis.

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