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Myc, Cdk2 and cellular senescence

Old players, new game

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The aberrant activation of oncogenic pathways promotes tumor progression, but concomitantly elicits compensatory tumor-suppressive responses, such as apoptosis or senescence. For example, Ras induces senescence, while Myc generally triggers apoptosis. Myc is in fact viewed as an anti-senescence oncogene, as it is a potent inducer of cell proliferation and immortalization, bypasses growth-inhibitory signals, and cooperates with Ras in cellular transformation. Recent reports prompt re-evaluation of Myc-induced senescence and of its role in tumor progression and therapy. We have shown that the cyclin-dependent kinase Cdk2, although redundant for cell cycle progression, has a unique role in suppressing a Myc-induced senescence program: Myc activation elicited expression of $p16^{INK4a}$ and $p21^{Cip1}$, and caused senescence in cells lacking Cdk2, but not in Cdk2-proficient cells. We show here that suppression of Myc-induced senescence by Cdk2 does not occur through phosphorylation of its purported substrate residue in Myc (Ser 62). Additional cellular activities have been identified that suppress Myc-induced senescence, including the Wrn helicase, telomerase and Miz1. These senescence-suppressing activities were critical for tumor progression, as deficiency in either Cdk2, telomerase or Miz1 reduced the onset of Myc-induced lymphoma in transgenic mice. Other gene products like p53, SUV39H1 or TGFβ promoted senescence, which together with apoptosis contributed to tumor suppression. Paradoxically, Myc directly counteracted the very same senescence program that it potentially elicited, since it positively regulated Wrn, telomerase and Cdk2 activity. Furthermore, Cdk2 inhibition re-activated the latent senescence program in Myc expressing cells. Hence, while these molecules are instrumental to the oncogenic action of Myc, they may simultaneously constitute its Achilles' heel for therapeutic development.

Key words: Myc, Cdk2, senescence, tumor suppression, p53, INK4a, ARF, TGFβ

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Background: Oncogenic Activation of Myc and Tumor Suppression

Oncogenic mutations have double-sided effects on somatic cells: on the one hand, they promote cancer development through enhanced cell proliferation or survival; on the other, the same mutations generally trigger compensatory cellular responses such as programmed cell death (apoptosis) or permanent proliferative arrest (cellular senescence): both of these responses are thought to constitute important barriers to cancer progression.[1,2] Both apoptosis and senescence are under control of the p53 and the pRb/p16 tumor suppressor pathways: a wealth of clinical and experimental evidence indicates that prospective cancer cells are under strong selective pressure to accumulate inactivating mutations in these pathways, underscoring the relevance of bypassing apoptosis and/or senescence in the course of tumor development.[1,2]

The nature of the tumor suppressive response activated in preneoplastic cells seems to be largely dictated by the type of oncogenic lesion involved. One example is provided by activation of the RAS/MAP kinase pathway, which,

while transiently promoting a proliferative response, subsequently drives cells into senescence. Overexpression of the c-*myc* oncogene—another frequent event in cancer—also induces proliferation but concomitantly sensitizes cells to undergo apoptosis, unless artificially provided with an ample supply of survival factors.[3]

The c-*myc* product, Myc, is a transcription factor that acts as a potent inducer of cell growth and proliferation. Myc acts at multiple levels to promote cell cycle progression.[4,5] First, it directly activates several genes encoding positive cell cycle regulators, such as cyclins and cyclin-dependent kinases (CDKs) and represses genes encoding the two main classes of CDK-inhibitory proteins (or CKIs), including the Cip/Kip family (in particular $p21^{Cip}$ and $p27^{Kip1}$) and the Ink4 family ($p15^{INK4b}$). Second, Myc influences the turnover of cell cycle regulatory proteins, for example by inducing destabilization of $p27^{Kip1}$. Third, Myc can bypass cell cycle arrest upon overexpression of either class of CKIs.

Owing to these multiple levels of action, conditional activation of a chimeric form of Myc fused to the Estrogen Receptor hormone-binding domain (MycER)[6,7] caused activation of the G_1-S phase-specific CDK species Cdk2 and Cdk4/6, associated with either reentry of quiescent cells into the cell cycle or acceleration of cell cycle progression in proliferating cells, with concomitant sensitization to cell death by apoptosis.[8-10] Likewise, expression of a c-*myc* transgene in Eμ-*myc* transgenic mice[11] caused hyperproliferation of B cells, compensated in young mice by a p53-dependent apoptotic response. This was followed by the occurrence of clonal B-cell lymphomas that had frequently mutated p53 or its upstream activator ARF, leading to the bypass of Myc-induced apoptosis.[12] Based on these and similar observations in other tissue,[13,14] apoptosis was widely viewed as the main or sole tumor suppressor mechanism elicited upon oncogenic activation of c-*myc*. This view was reinforced by the observation that Myc cooperated with Ras in cellular transformation either in vitro[15] or in vivo,[16] attributed to the concept that primary cells senesce upon activation of Ras[17] and that Myc bypasses Ras-induced senescence.[18,19]

As discussed below, recent reports have revealed that Myc also sensitizes cells to senescence: first, a cell-autonomous senescence program may be potentially activated in Myc-expressing cells, but is maintained in a latent inactive state by cellular activities such as the Wrn helicase[20,21] or Cdk2 kinase.[22] Second, Myc-induced tumors were shown to be sensitive to senescence induced by autocrine or paracrine secretion of the cytostatic chemokine TGFβ.[23,24] These findings add an unexpected twist to tumor suppression and open new therapeutic perspectives in Myc-driven tumors.

Cdk2 as a Suppressor of Myc-Induced Senescence

One of the biggest surprises in the cell cycle field came with the generation of Cdk2 knockout (KO) mice: these animals were viable, with no apparent defect in somatic tissues, implying a redundant role of Cdk2 in the mitotic cell cycle. However, Cdk2 loss impaired meiosis at the prophase stage of division I, providing a rationale for its evolutionary conservation in higher eukaryotes.[25,26] Follow-up work indicated that the mitotic kinase Cdk1 took over the function of Cdk2 in G_1-S control and, as Cdk2 in wild-type cells, associated with cyclin E and the inhibitors $p21^{Cip1}$ or $p27^{Kip1}$ in Cdk2-null cells.[27-30]

Because previous evidence indicated that Cdk2 is robustly activated following Myc overexpression (see Background), we set out to address whether this kinase was critical for Myc-induced proliferation.[22] In line with the reported redundancy of Cdk2, the ability of MycER to accelerate S-phase entry in the short term (1–2 days) was unaltered in *Cdk2*$^{-/-}$ mouse embryo fibroblasts (MEFs) relative to *Cdk2*$^{+/+}$ controls. Quite surprisingly, however, Cdk2 was required for proliferation when MycER was activated for longer periods of time (4–5 days and beyond): in these conditions *Cdk2*$^{-/-}$ cells ceased to proliferate and became positive for senescence-associated β-Galactosidase activity (SA-β-Gal). Fully consistent results were obtained in vivo, as MycER activation in the pancreatic β-cells of *pIns-MycER*^{TAM} *RIP-Bcl-X*$_L$ double-transgenic mice[14] initially caused hyper-proliferation in both the *Cdk2*$^{-/-}$ and *Cdk2*$^{+/+}$ backgrounds, but subsequently caused β-cell senescence selectively in the KO mice. Thus, Cdk2 was not essential for Myc to induce cell cycle progression in the short term, but was required to prevent the subsequent onset of a Myc-induced senescence-like arrest.

Closer examination in vitro showed that requirement for Cdk2 was selective, since MEFs lacking both Cdk4 and Cdk6 were refractory to Myc-induced senescence. The arrest of Cdk2$^{-/-}$ cells was irreversible and did not depend upon continuous activation of MycER. Concomitant with proliferative arrest, MycER induced accumulation of $p21^{Cip1}$ and $p16^{INK4a}$ (and as shown at the mRNA level also of $p15^{INK4b}$) in *Cdk2*$^{-/-}$ but not *Cdk2*$^{+/+}$ MEFs. On the other hand, induction of $p19^{ARF}$ and p53 by MycER[31] occurred in both the wild-type and KO backgrounds. As expected, induction of the *Cip1* gene—but not that of *INK4a*—depended upon the ARF-p53 axis. These data place Cdk2 as a negative regulator of the two major tumor suppressor pathways in response to Myc activation, as shown schematically in **Figure 1** (red arrows).

Several important observations must be underlined here. (1) The endogenous activity of Cdk2 was not sufficient to prevent all forms of oncogene-induced senescence: consistent with earlier work,[17] Ras-induced senescence was equally effective in *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ MEFs. (2) The expression of a Wnt ligand—known to signal via c-*myc* in several cell types[32]—induced senescence selectively in *Cdk2*$^{-/-}$ cells.[22] (3) In the absence of any exogenous oncogene, *Cdk2*$^{-/-}$ cells were also sensitized to plating under ambient O_2 tension, a known trigger of senescence.[33] Hence, Myc, Wnt and ambient O_2 may induce a distinct form of stress that can be counteracted by Cdk2, possibly linked to the generation of reactive oxygen species.[34] Alternatively, Wnt, Myc and Ras may induce similar forms of stress, but Ras may do so more effectively, overcoming endogenous Cdk2 activity. (4) Albeit suppressed by Cdk2, Myc-induced senescence can still occur in wild-type cells, as observed in transgenic mice (see below) or human diploid fibroblasts.[35] This is consistent with the view that Cdk2—as well as other cellular activities—controls the

threshold of oncogenic stress required for activation of the senescence program.[36]

Suppression of Myc-Induced Senescence by Cdk2 Does Not Occur through Direct Phosphorylation of Myc on Serine 62

As outlined above, Cdk2 is redundant for mouse development, implying that most of its substrates can be targeted by other Cdk species. Yet, we must surmise that some substrates (perhaps even a single one) must be unique to Cdk2 in suppressing Myc-induced senescence. To date, we have not yet identified these substrates.

One of the proposed substrates of Cdk2 is Serine 62 (S62) of Myc itself,[37,38] although the same residue is targeted by MAP kinases.[39,40] In recent work, Larsson and collaborators showed that S62 phosphorylation is required for Myc to bypass Ras-induced senescence and proposed that Cdk2 is the key kinase in this regard.[38] Either mutation of Myc S62 to alanine (S62A) or pharmacological inhibition Cdk2 prevented the bypass of Ras-induced senescence. On the other hand, a Myc protein bearing the phospho-mimicking mutation S62D was able to bypass Ras-induced senescence in the face pharmacological Cdk2 inhibitors. Thus, Cdk2 acts two-fold: (1) to suppress senescence induced by Myc[22] and (2) to allow Myc bypassing senescence induced by Ras.[38] We show here that these are two unrelated effects of Cdk2:

(1) Exactly as observed with control "wild-type" MycER,[22] cultures of *Cdk2*$^{-/-}$ MEFs underwent proliferative arrest upon activation of a MycER^{S62D} mutant protein (**Fig. 2A**). Both forms of MycER caused accumulation of the *INK4a* and *Cip1* mRNAs in Cdk2$^{-/-}$ MEFs (**Fig. 2B**). Thus, the phospho-mimicking mutation S62D did not bypass the requirement for Cdk2 in suppressing Myc-induced senescence.

(2) Activation of a MycER^{S62A} mutant protein did not induce senescence in *Cdk2*$^{+/+}$ MEFs (**Fig. 2C**). Thus, lack of S62 phosphorylation was not a sufficient cause of Myc-induced senescence.

(3) Phosphorylation of MycER on S62 was equivalent in Cdk2$^{+/+}$ and *Cdk2*$^{-/-}$ MEFs (**Fig. 2D**). Note that as expected, the phospho-S62 signal was lost with MycER^{S62A}, but not with the MycER^{T58A} mutant. We conclude that in MEFs Cdk2 is not required for phosphorylation of Myc on Serine 62.

(iv) Phosphorylation of MycER on T58 was also independent from Cdk2, and was abrogated by the S62A mutation (**Fig. 2D**), as expected based on the priming role of S62.[40] It is worth noting that MycER^{T58A}, which is phosphorylated on S62 (**Fig. 1D**), induced senescence in *Cdk2*$^{-/-}$ cells like wild-type MycER.[22]

Altogether, the above data demonstrate that the function of Cdk2 in suppressing Myc-induced senescence is unrelated to the state of Myc T58/S62 phosphorylation.

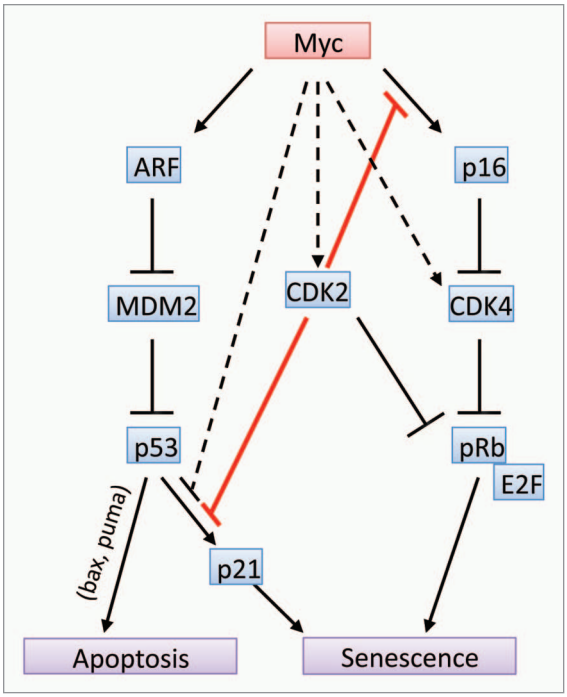

Figure 1. Schematic representation of the involvement of Cdk2 in Myc-induced senescence, as discussed in the text.

How Does Cdk2 Suppress Senescence?

The observation that p21^{Cip1} and p16^{INK4a} accumulate selectively in Cdk2$^{-/-}$ cells upon Myc activation implies that Cdk2 negatively regulates expression of these proteins upon Myc activation (**Fig. 1**). Diverse regulatory mechanisms may be proposed to explain this effect. First, one may envision a direct role of Cdk2 in regulating Myc-dependent transcriptional repression of *Cip1*. Myc induces ARF and p53, which in principle should induce *Cip1* transcription. However, Myc interacts with the transcription factor Miz1 to directly suppress *Cip1* transcription.[5] Hence, the accumulation of p21^{Cip1} in *Cdk2*$^{-/-}$, as opposed to Cdk2$^{+/+}$ cells, may results from an involvement Cdk2 in Myc-mediated repression of *Cip1*. The same principle may apply to p15^{INK4b}, which is regulated by the same Miz1/Myc-dependent mechanism.[5] In an analogous manner, we speculate that Cdk2 may also function to repress *INK4a*. For example, since repression of the *INK4-ARF* locus[41] is directly mediated by chromatin-associated Polycomb-group proteins and is lost in senescent cells,[42] it is tempting to speculate that Cdk2 may play a role in Polycomb-mediated repression, although we have so far obtained no conclusive evidence in this regard. An alternative scenario may be that Cdk2 modulates the response to the stress signals that activate *INK4a* and *Cip1*. Oncogenes other than Myc were shown to induce a DNA damage response (DDR) that is a prerequisite for senescence.[43,44] Myc also induced a DDR in cultured cells and transgenic

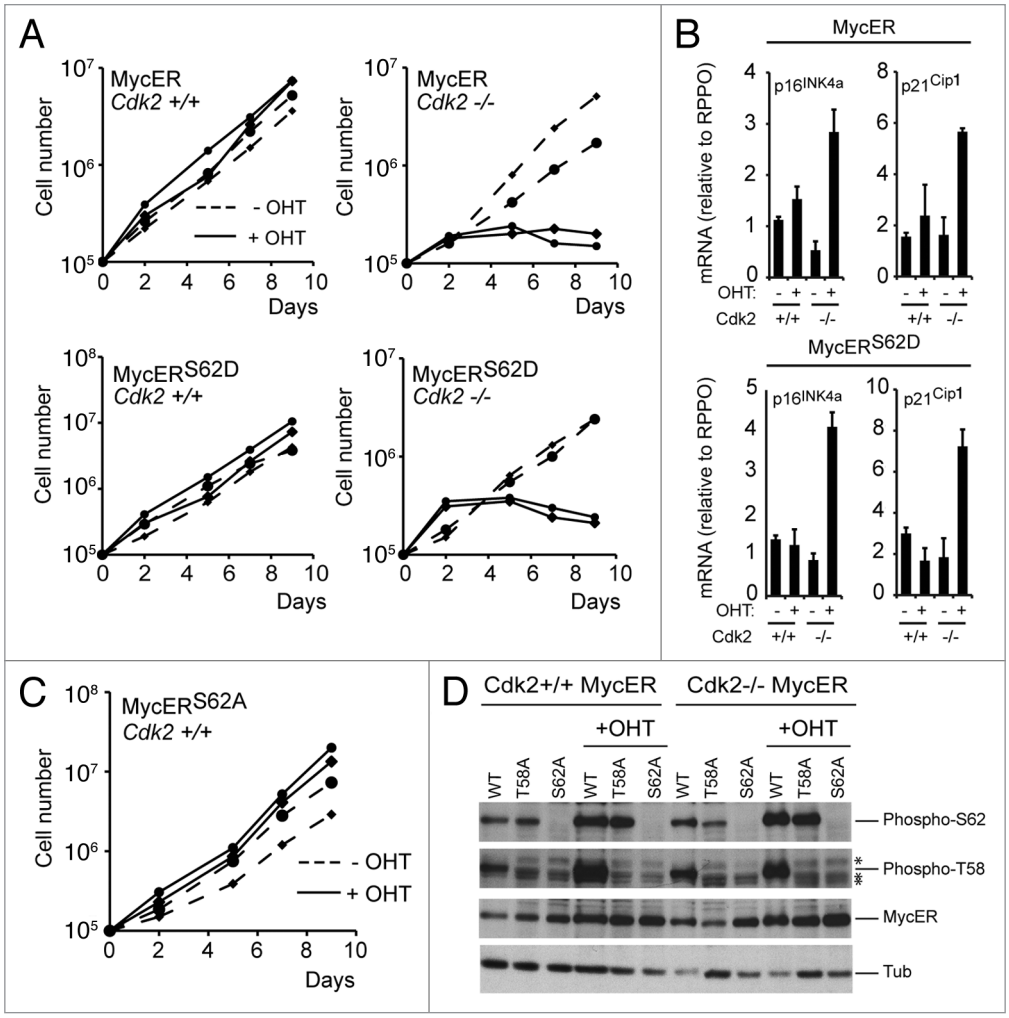

Figure 2. Cdk2 does not suppress Myc-induced senescence through phosphorylation of Myc Serine 62. (A) *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ MEFs infected with retroviruses expressing wild-type MycER or MycER^{S62D} were grown in the absence (dashed lines) or presence of OHT to activate MycER (solid lines) as previously described.[22] The growth curves show cumulative cell numbers for two independent MEFs preparations of each genotype. (B) RT-qPCR analysis of relative mRNA levels determined in cells treated as in (A) and collected at day 9. Bar graphs represent the mean values ± standard deviation (n = 3). Units on the y-axis are multiples of 10^{-3} for p16^{INK4a} and 10^{-2} for p21^{Cip1}. (C) MEFs expressing MycERS62A were grown as in (A). (D) Immunoblot analysis of primary *Cdk2*$^{+/+}$ and *Cdk2*$^{-/-}$ MEFs expressing MycER (WT), MycER^{T58A} (T58A) or MycER^{S62A} (S62A) treated with OHT for 24 hours, as indicated. Total MycER was detected with Myc-specific C-33 antibody (Santa Cruz Biotech). Phosphorylation on S62 and T58 were detected with custom-made phospho-specific antibodies. Asterisks in the phospho-T58 blot indicate non-specific background.

mice and genetic evidence connected this DDR to tumor suppression,[34,45-49] although it remains unknown whether senescence was involved. We obtained no evidence that Cdk2 modulates the Myc-induced DDR per se, since DDR markers were induced in a comparable manner in *Cdk2*$^{-/-}$ and *Cdk2*$^{+/+}$ MEFs. This notwithstanding, genetic or pharmacological bypass of the DDR indicated that it was required for Myc-induced senescence.[22]

The nature of the Myc-induced stress that triggers the DDR remains to be fully elucidated. This may include replicative,[50] oxidative,[34] as well as telomeric stress,[51] all of which have the potential to trigger a DDR and senescence.[52,53] Recent data suggest a role for Cdk2 in DNA repair,[28,54] and the Cdk inhibitor Roscovitine modulates DNA repair and induces senescence in some cancer cell lines.[55] Cdk2 inhibition was also shown to induce replicative stress in tumor cells.[56] In summary, Cdk2 may act at multiple levels by modulating either the senescence-inducing damage (or its repair), the resulting signals or expression of the key effectors p16^{INK4a} and p21^{Cip1}. The pervasive and tight nature of senescence seen in *Cdk2*$^{-/-}$ MEFs[22] appears consistent with a multi-faceted role of Cdk2 in regulating this cellular response.

Senescence Serves as a Tumor Suppressor Mechanism in Myc-I 1806 Rio Grande nduced Lymphoma

To explore the role of Cdk2 in Myc-induced tumors, we bred Eμ-*myc* transgenic mice into a *Cdk2* KO background. Consistent with the role of Cdk2 in suppressing Myc-induced senescence, Eμ-*myc* *Cdk2*$^{-/-}$ mice showed enhanced SA-β-Gal

activity in the spleen relative to Eμ-*myc Cdk2*$^{+/+}$ siblings and a significantly delayed onset of B-cell lymphoma.[22] On the other hand, the loss of Cdk2 did not affect Myc-induced apoptosis in pre-tumoral B-cells (the same was observed in pancreatic β-cells of *pIns-MycER*^{TAM} mice).

Earlier work by Feldser and Greider also showed that an enhanced senescence response suppressed progression of Myc-induced lymphoma.[57] This was achieved by crossing Eμ-*myc* mice with a KO strain lacking the RNA component of telomerase (*mTR*$^{-/-}$), resulting in telomere shortening, induction of senescence in pre-tumoral cells and a profound reduction in lymphoma onset.

Altogether, the above data indicated that increased senescence in pre-tumoral B-cells is an effective tumor suppressor mechanism. These data, however, did not address a possible role of senescence—even if secondary to apoptosis—in tumor suppression in wild-type Eμ-*myc* mice. This was a plausible scenario, as SA-β-Gal was induced by Myc in vivo, albeit at low levels, in either pre-tumoral B-cells or pancreatic β-cells.[22] This issue was directly addressed in independent work by Lozano and coworkers,[58] who exploited a knock-in mutant allele of mouse p53 (R172P, termed *p53*^{515C}),[59] corresponding to the cancer-associated mutation R175P in human p53, which disables the pro-apoptotic activity of p53 but leaves intact its ability to induce cell cycle arrest and cellular senescence. Given the prevalent view that apoptosis is the critical tumor suppressor mechanism elicited by Myc, the *p53*^{515C} allele would be expected to behave like a complete loss of function. Instead, *p53*^{515C} retained partial tumor-suppressive activity in Eμ-*myc* mice. Thus, besides apoptosis, other tumor suppressive responses, like senescence, must be triggered by Myc in a p53 dependent manner.

The relevance of a Myc-dependent senescence program in tumor suppression has been reinforced by recent work of Schmitt and coworkers,[23] who provided evidence for a tumor-stromal paracrine network involving the local recruitment and activation of macrophages by apoptotic tumor cells. Activated macrophages secreted TGFβ, which in turn unleashed a Myc-dependent senescence response in tumor cells. Senescence was p53-dependent and could be bypassed by deletion of the SUV39H1 gene, which also eliminated senescence-associated heterochromatin foci in tumor cells. As a result, Eμ-*myc* transgenic mice lacking SUV39H1 succumbed very rapidly to lymphomas.

As already mentioned, the notion that Myc is a strong suppressor of cellular senescence was partly based on the transcriptional repression of CKIs such as p21^{Cip1} or p15^{INK4b} via the interaction with Miz1. The role of this repressive complex in Myc-dependent tumorigenesis was recently addressed by Eilers and collaborators,[24] who devised an inducible allele of Myc^{V394D}, a mutant unable to bind Miz1. In this mouse model, which is prone to develop T-cells lymphomas, the V394D mutation considerably delayed tumor development compared to control mice expressing wild-type Myc. The lymphomas developing in Myc^{V394D} mice, showed increased SA-β-Gal staining, reduced BrdU incorporation, high levels of the heterochromatic marker H3K9me3 and elevated p15^{INK4b} expression. Of note, this enhanced senescence response was triggered by autocrine TGFβ signaling.

In summary, different types of senescence response can limit the progression of Myc-induced lymphomas. First, a cell-autonomous senescence program controlled by p53 co-exists with Myc-induced apoptosis in pre-tumoral B-cells: this program is maintained in a largely suppressed, latent state by cellular activities, such as Cdk2, telomerase or Wrn (as shown in different cell types) and, we surmise, others that remain to be identified: interesting candidates in this regard include antagonists of mTOR signaling, as mTOR can favor p53-dependent senescence over quiescence.[60-62] A key concept here is that Myc itself upregulates the very same activities that are required to suppress Myc-induced senescence, including Cdk2, Wrn and telomerase: in so doing, Myc contributes to bypass the very same senescence program that it potentially elicits.

Second, TGFβ-dependent senescence of tumor cells contributes to the constant pruning of cancer cells at more advanced stages. The source of TGFβ might be autocrine, paracrine or both and significantly contributes to limit tumor burden. Following the same concept as above, Myc itself antagonizes TGFβ-induced arrest,[63] in particular through its interaction with Miz1 and suppression of p21^{Cip} and p15^{INK4b}.[5] Thus, at either stage of tumor progression, the outcome constantly lies in the fine-balance between the anti- and pro-senescence activities set in place.

Senescence as a Therapeutic Opportunity in Myc-Driven Tumors

Myc-driven tumors mainly experience selective pressure for bypassing apoptosis: while some of the resulting lesions (e.g., loss of p53) may also affect senescence, others are predicted to leave it intact (e.g., loss of *CASP8* in neuroblastomas or Myc-T58A in Burkitt lymphomas).[22,64,65] Thus, tumor cells may retain a latent senescence program, which may be re-activated with therapeutic efficacy.[53,66-69] Important proof-of-principle for such strategy was provided with either chemotherapy,[68] or *myc* inactivation[69] in mice bearing Myc-induced lymphomas.

The fact that Cdk2 was dispensable for the proliferation of human tumor cell lines led to questioning its validity as a therapeutic target.[67] The role of Cdk2 in suppressing Myc-induced senescence, however, prompted us to reconsider this issue: our data in mouse or rodent fibroblasts, as well as in a human leukemic cell line (U937), showed that Myc overexpressing cells underwent senescence upon treatment with Cdk2-inhibitory compounds.[22] Thus, while Myc super-activates Cdk2, thereby suppressing its own senescence program (**Fig. 1**), this may simultaneously sensitize tumor cells to undergo senescence upon Cdk2 inhibition. We surmise that pharmacological targeting of Cdk2 may be effective in tumors driven either by direct activation of Myc or by oncogenic pathways that signal via Myc, such as Wnt or Notch.[32,71]

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