Tumor Stroma-Derived TGF-β Limits Myc-Driven Lymphomagenesis via Suv39h1-Dependent Senescence

Maurice Reimann,1,6 Soyoung Lee,1,6 Christoph Loddenkemper,3,6,7 Jan R. Dörr,1,6 Vedrana Tabor,2,6 Peter Aichele,4 Harald Stein,3 Bernd Dörken,1,2 Thomas Jenuwein,5,8 and Clemens A. Schmitt1,2,*

1Charité - Universitätsmedizin Berlin/Molekulares Krebsforschungszentrum der Charité - MKFZ, 13353 Berlin, Germany
2Max-Delbrück-Center for Molecular Medicine, 13125 Berlin, Germany
3Charité - Universitätsmedizin Berlin/Department of Pathology, Campus Benjamin Franklin, 12200 Berlin, Germany
4Department of Immunology, University Hospital Freiburg, 79104 Freiburg, Germany
5Research Institute of Molecular Pathology, 1030 Vienna, Austria
6These authors contributed equally to this work
7Present address: Technische Universität München, Institute of Pathology, 81675 Munich, Germany
8Present address: Max-Planck-Institute of Immunology, 79108 Freiburg, Germany
*Correspondence: clemens.schmitt@charite.de

SUMMARY

Activated RAS/BRAF oncogenes induce cellular senescence as a tumor-suppressive barrier in early cancer development, at least in part, via an oncogene-evoked DNA damage response (DDR). In contrast, Myc activation—although producing a DDR as well—is known to primarily elicit an apoptotic countermeasure. Using the Eμ-myc transgenic mouse lymphoma model, we show here in vivo that apoptotic lymphoma cells activate macrophages to secrete transforming growth factor β (TGF-β) as a critical non-cell-autonomous inducer of cellular senescence. Accordingly, neutralization of TGF-β action, like genetic inactivation of the senescence-related histone methyltransferase Suv39h1, significantly accelerates Myc-driven tumor development via cancellation of cellular senescence. These findings, recapitulated in human aggressive B cell lymphomas, demonstrate that tumor-prompted stroma-derived signals may limit tumorigenesis by feedback senescence induction.

INTRODUCTION

Mitogenic oncogenes provoke checkpoint-mediated cellular countermeasures such as apoptosis or premature senescence, a terminal G1 arrest involving the p53 and p16INK4a tumor suppressors that is characterized by typical transcriptional, biochemical and morphological alterations (Campisi and d’Adda di Fagagna, 2007; Hemann and Narita, 2007). RAS- or BRAF-initiated senescent lesions in vitro and in vivo exhibit chromatin changes that include the transcriptionally repressive trimethylation mark at H3K9 (H3K9me3) and focal enrichment of HP1 proteins for which H3K9me3 provides a docking site (Bartkova et al., 2006; Braig et al., 2005; Collado et al., 2005; Lachner et al., 2001; Michaloglou et al., 2005; Narita et al., 2003). Mechanistically, hypophosphorylated retinoblastoma (Rb) protein, bound to growth-promoting E2F transcription factors, may recruit H3K9 methyltransferase activities such as Suv39h1 to direct heterochromatinization to the vicinity of E2F-responsive promoters, thus silencing S-phase genes (Narita et al., 2003). Increasing evidence points towards an oncogene-induced DDR as critical upstream trigger of the senescence program (Bartkova et al., 2006; Di Micco et al., 2006; Mallette

Significance

Cancer entities with constitutive Myc expression, among them aggressive B cell lymphomas, typically display high levels of apoptosis. So far, cellular senescence as another oncogene-inducible safeguard program has been recognized in RAS/BRAF-driven scenarios, but not as a bona fide Myc-evoked anticancer mechanism. Utilizing the genetically tractable Eμ-myc transgenic mouse lymphoma model and presenting supportive evidence from human aggressive B cell lymphoma samples, this study establishes a network of tumor/host immune cell interactions in which apoptotic tumor cells launch a paracrine response in non-malignant bystanders that limits lymphomagenesis by cellular senescence. Our data expand the relevance of oncogene-induced senescence to Myc-driven cancers, and highlight the tumor stroma as a critical contributor and potential therapeutic target in this process.
et al., 2007). Indeed, Myc and RAS oncogenes cause DNA damage by inducing reactive oxygen species (ROS) and generating stalled DNA replication intermediates (Di Micco et al., 2006; Lee et al., 1999; Reimann et al., 2007; Vafa et al., 2002). However, both prototypic oncogenes produce very different outcomes—i.e., predominantly cellular senescence following RAS/BRAF and apoptosis in response to Myc activation—when activated in primary cells in vitro (Evan et al., 1992; Serrano et al., 1997).

So far, there has been no clear evidence that Myc induction in primary cells may cause senescence under physiological conditions in vitro or in vivo (Feldser and Greider, 2007; Grandori et al., 2003; Guney et al., 2006). One cell-autonomous explanation for Myc’s primarily proapoptotic action might be that Myc favors apoptosis over arrest by influencing p53-dependent transactivation processes in response to DNA damage (Seoane et al., 2002).

The purpose of this study was to determine the contribution of cellular senescence as a tumor-suppressive mechanism in a transgenic mouse model of Myc-driven lymphomagenesis reminiscent of aggressive B cell lymphomas in humans. Given the well-established predominantly apoptotic response to Myc activation in primary cells in vitro, we specifically aimed to dissect cell-autonomous and non-cell-autonomous components of Myc-related senescence in vivo.

RESULTS

Suv39h1-Dependent Cellular Senescence Limits Myc-Induced Lymphomagenesis

To determine the role of cellular senescence in Myc-driven tumorigenesis, we studied the impact of senescence-compromising Suv39h1 loss in Eu-myc transgenic mice (Adams et al., 1985; Braig et al., 2005), where genetic disruption of apoptosis strongly promotes B cell lymphomagenesis (Egle et al., 2004; Schmitt et al., 2002b; Strasser et al., 1990). Mice that lacked one or both Suv39h1 alleles developed lymphomas significantly faster than mice without a targeted defect at the Suv39h1 locus (p < 0.0001 for either comparison, Figure 1A). Moreover, lymphomas that formed in Suv39h1+/− female mice invariably lost expression of the X-chromosomally encoded Suv39h1 transcript, thereby explaining the indistinguishable tumor onset in Suv39h1+/− and Suv39h1−/− (i.e., Suv39h1+/− male and Suv39h1−/− female) mice (Figure 1A, insert). Importantly, the frequency of apoptosis measured as TUNEL reactivity, a hallmark of Myc-driven lymphomas, was virtually identical in Suv39h1-deficient lymphomas when compared to control lymphomas (i.e., those that arose in Eu-myc mice without a targeted Suv39h1 lesion; Figure 1B). Furthermore, control and Suv39h1-deficient lymphomas presented with indistinguishable gross pathology, formed at comparable stages of B cell development, both expressed Suv39h2 transcripts, and displayed similar near-normal chromosome counts, unlike the previously reported chromosome-misegregated B cell lymphomas that form in the absence of both Suv39h1 and Suv39h2 alleles in nontransgenic mice (Peters et al., 2001) (data not shown). Thus, neither compromised apoptosis nor overt aneuploidy accounts for the accelerated lymphoma onset in Suv39h1-deficient Eu-myc mice.

To directly assess oncogene-induced senescence as a potential component of delayed lymphoma manifestation, senescence-associated β-galactosidase (SA-β-gal) activity (Dimri et al., 1995) was analyzed in Suv39h1-deficient and control lymphomas. Virtually none of the cells in the Suv39h1−/− lymphoma sections, but an average of about 14% of the control lymphoma cells, stained (often in a focal pattern) positive for SA-β-gal (P < 0.001; Figures 1B and 1C; see Figures S1A and S1B) available online for further evidence that senescent cells are indeed B lymphoma cells). Moreover, coanalysis of the proliferation marker Ki67 or bromodeoxyuridine (BrdU) incorporation, indicating DNA synthesis, with SA-β-gal or H3K9me3 staining confirmed the growth-arrested nature of SA-β-gal- or H3K9me3-positive cells (Figure 1D). Immunoblot analyses of bulk lymph node lysates indicated no differences in the expression levels of Myc and the cell-cycle inhibitor p21CIP1 between control and Suv39h1−/− lymphomas, while significant amounts of hypophosphorylated/G1-phase Rb and of H3K9me3 were only found in control lymphomas, which also displayed slightly reduced levels of the CDK4/6 inhibitor p16INK4a and the E2F target cyclin A (Figure 1E). Other histone modifications such as H3K4me3, acetylated H3K9, H3K27me3, or H4K20me3 appeared globally unaffected by Suv39h1 status (data not shown), underscoring the specific role of the Suv39h1-mediated H3K9me3 mark in the senescence process. Moreover, spleen samples derived from young, lymphoma-free Eu−/−myc mice (termed “preneoplastic,” albeit consisting of Myc-overexpressing normal B cells) as compared with spleen sections from nontransgenic mice exhibited signs of cellular senescence in a strictly Myc- and Suv39h1-dependent fashion, indicating that oncogene-related senescence may delay tumorigenesis already at a premalignant state (Figure S1C). Thus, aggressive Myc-driven lymphomas develop and manifest with a significant fraction of cells that lack any proliferative activity and display marks of cellular senescence.

All Myc-lymphomas developing in Suv39h1+/−;p53+/− or Suv39h1−/−;p53+/− backgrounds selected against the remaining p53 wild-type allele (12/12 cases tested “p53-null”; Figure 1F), as known from lymphomas forming in Eu−/−myc;p53+/− mice (Schmitt et al., 1999), and, thus, against p53-dependent apoptosis. Suv39h1 RNA expression was mostly retained in Suv39h1+/−;p53−/−-derived lymphomas (7/9 cases tested; Figure 1F), indicating that p53 loss coablates an apoptosis-independent tumor-suppressive function otherwise governed by Suv39h1. Accordingly, additional inactivation of Suv39h1 produced no further acceleration of Eu−/−myc lymphomagenesis in a p53−/− background (data not shown). Notably, and different from p53-null lymphomas, DDR-defective ATM−/− lymphomas displayed only a partial reduction of the senescent fraction at manifestation (Figure 1G and Figure S1D, showing, in addition, control lymphoma-comparable senescence in p16INK4a-deficient INK4a−/− and p21CIP1-deficient CIP1−/− lymphomas, but compromised senescence in ARC−/− lymphomas). Taken together, Myc-induced senescence presents in vivo as a p53-, Suv39h1−, and partly ATM-dependent program that complements apoptosis as an antioncogenic safeguard mechanism in Eu−/−myc lymphomagenesis.

Activated Myc Promotes ATM/p53-Dependent Senescence

Myc activation is known to produce marks of DNA damage in vivo (Reimann et al., 2007), at least in part via ROS, which
may link Myc via a DDR to Suv39h1-dependent senescence. Notably, Suv39h1 had no impact on g-H2AX-marked DNA lesions and the DDR signature in preneoplastic E\textsubscript{\textit{M}}-\textit{myc} transgenic B cells, or in lymphoma cells exposed to g-irradiation (Figure S2A-C). However, in contrast to wild-type B cells, primary B cells lacking the DDR mediators ATM or p53 largely failed—like Suv39h1-deficient B cells—to senesce in response to acute Myc overexpression in vitro (Figure 2A). If senescence detected in control lymphomas in situ is initiated via a Myc-evoked DDR, then genetic or pharmacological interference with the DDR should impact on the senescence response. Comparable to the ATM\textsuperscript{--} scenario (Figure 1G), exposure of E\textsubscript{\textit{M}}-\textit{myc} transgenic mice to the ROS scavenger N-acetyl-cysteine (NAC) or to the ATM/ATR inhibitor caffeine, both of which blunt an oncogene-evoked DDR in vivo (Bartkova et al., 2006; Reimann et al., 2007), resulted in a profound, albeit only partial reduction of senescent lymphoma cells in situ (Figure S2D-F, also showing that ROS levels are Myc, but not Suv39h1 dependent).
To directly address the cell-autonomous potential of Myc to drive senescence, we tested whether a conditional p53 moiety would suffice to convert constitutive Myc signaling into a robust senescence response in apoptosis-incapable cells. To this end, we employed Em-myc mice carrying a 4-OH-tamoxifen (4-OHT)-inducible p53ERTAM knockin allele, encoding a p53-estrogen receptor fusion protein that is inactive in the absence of 4-OHT (Martins et al., 2006). Expectedly, Em-myc;p53ERTAM/+ lymphomas that arose in the absence of 4-OHT typically selected against the remaining p53 wild-type allele (termed p53ERTAM/−; 5/5 cases tested (data not shown and Martins et al., 2006), thereby generating p53-null lymphomas in which p53 activity is restorable upon provision of 4-OHT in vitro (Figure 2C). Importantly, pharmacological scavenging of ROS or ablation of the DDR attenuated and, when combined, almost completely blocked the senescence induction of Em-myc;p53ERTAM/−/bcl2 lymphoma cells in response to 4-OHT in vitro (Figure 2D). Thus, acute overexpression of Myc in primary cells or p53 reactivation in the presence of constitutive Myc signaling unmasks the cell-autonomous, DDR-mediated prosenescent capability of Myc.

**TGF-β Induces Senescence of Myc-Driven Lymphoma Cells**

Because neither ATM deficiency nor pharmacological DDR ablation was sufficient to fully abrogate senescence of Myc-driven lymphoma cells in vivo, we aimed to identify an additional stimulus that may complement oncogene-induced DDR signaling in vivo. Genome-wide transcriptional profiling of whole lymph node RNA preparations from Suv39h1-proficient versus Suv39h1-deficient Em-myc lymphomas identified TGF-β-induced gene (Tgfbr; also known as Big-h3, β-ig H3, or keratoepithelin) as...
Figure 3. TGF-β Induces Suv39h1-Dependent Cellular Senescence in Myc-Driven Lymphomas

(A) Focal TGF-β1 detected by immunostaining (left) and costaining (right) for TGF-β1 (red) and Ki67 (blue) in control versus Suv39h1−/− lymphomas in lymph node sections in situ (representative photomicrographs). Inserts show TGF-β1-rich areas at higher magnification, and percentages reflect the fraction Ki67-positive cells within those areas (n = 3 samples each). Scale bar represents 50 μm (identical magnification throughout the panel).

(B) Growth curve analyses of freshly isolated and stably bcl2-infected control and Suv39h1−/− lymphoma cells exposed to TGF-β1 (100 pM) versus untreated (as in [B]). Note the comparable proliferative capacities of Suv39h1-proficient and Suv39h1−/− lymphoma cells in the absence of TGF-β1 treatment. Scale bar represents 10 μm (identical magnification throughout the panel).

(C) Immunoblot analysis of cyclin A protein levels (α-tubulin as a loading control) in lymphomas as in (B) with or without preceding exposure to TGF-β1 (100 pM) for 5 days.

(D) Relative growth of Bcl2-expressing lymphoma cells of the indicated genotypes after 5 days of exposure to TGF-β1 (100 pM) versus untreated (as in [B]). At least three cases each. Error bars denote SD; *p < 0.05. See also Figure S3.

the most strongly differentially upregulated transcript. Tgfbi, a TGF-β target, was expressed 3.9-fold higher in Suv39h1−/− lymphomas, and encodes a secreted protein with cytostatic potential that was previously linked to cellular senescence (Dokmanovic et al., 2002) (see Experimental Procedures for details and the confirmatory quantitative reverse transcriptase polymerase chain reaction [RQ-PCR] analysis in Figure S3A). We found TGF-β1, known to induce cellular senescence in fibroblasts (Lin et al., 2004), to be detectable in a multi-focal pattern in lymphoma sections reminiscent of the distribution of SA-β-gal-positive cells in control lymphomas (Figure 3A, compare to Figure 1B). Importantly, costaining for the proliferation marker Ki67 unveiled that in areas with abundant TGF-β1 significantly less control cells were Ki67-positive when compared with Suv39h1−/− lymphomas (Figure 3A). Thus, TGF-β1 correlates with a cytostatic response selectively detectable in control lymphomas, and high Tgfbi levels in Suv39h1−/− cells are suggestive of a downstream defect in a TGF-β1-inducible senescence program.

We sought to directly test the potential of exogenous TGF-β1 to induce cellular senescence in a Suv39h1-dependent fashion in Myc-driven lymphoma cells that were stably bcl2-transduced to block apoptosis. TGF-β1 countered proliferation in a dose-dependent manner and led to a complete growth arrest with features of cellular senescence, i.e., SA-β-gal activity and H3K9me3 expression, in control, but not in Suv39h1−/− lymphoma cells, whose growth behavior remained largely unaffected by TGF-β1 treatment (Figure 3B). Lack of a cytostatic response in Suv39h1−/− cells was not due to a primary defect in TGF-β receptor signaling, because lymphoma cells of both genotypes exhibited phosphorylation of the intracellular TGF-β1 mediators Smad2 and Smad3 following TGF-β1 treatment in vitro (Figure S3B). In line with the transcriptionally repressive H3K9me3 mark selectively induced in control lymphomas (Figure 3B), TGF-β1-treated control lymphomas displayed reduced transcript levels of numerous E2F target genes, including MCM7 or Cyclin A by microarray analysis, as well as increased levels of transcripts that encode for components of the heterochromatinization machinery such as DNA methyltransferase 3B or HP1β (Figure S3C, and Figure 3C for cyclin A protein expression). The mechanism by which TGF-β utilizes Suv39h1, presumably in conjunction with Rb/E2F complexes (Laiho et al., 1990; Schwarz et al., 1995; Spender and Inman, 2009), to induce senescence appears to be indirect, because we were unable to detect a physical interaction between Suv39h1 and Smad proteins (Figure S3D). TGF-β1 was incapable of inducing p15INK4b or p21CIP1 mRNA and protein expression in lymphomas independent of their Suv39h1 status, probably because constitutive Myc expression firmly represses these promoters via Miz-1.
isolated lymphoma cells exhibited Smad3 phosphorylation in culture medium background levels (Figure S3I). However, freshly
importantly, lymphoma cells did not secrete TGF-β-inducing H2O2 or TGF-β in lymphomas of various genotypes exposed to senescence
and a senescence-reinforcing proinflammatory secretory phenotype, termed “SASP” (Acosta et al., 2008; see Figures S4H–S4J). Consistently, lymphomas harboring a robust Bcl2-mediated apoptotic block (control/bcl2; see also Schmitt et al., 2002b) presented with a much lower frequency of both infiltrating macrophages and senescent cells in vivo (Figure 4B and Figure S4C, see also Figure 1G and Figure S1D for a correlation between senescent cells and infiltrating macrophages in various lymphoma genotypes). The nearly complete absence of senescent control/bcl2 lymphoma cells in vivo despite their in vitro susceptibility to TGF-β-mediated senescence (Figure 3B) underscores the importance of non-cell-autonomous events such as attraction of macrophages (Lauber et al., 2003) and their subsequent activation by apoptotic lymphoma cells to secrete TGF-β1.

To further elucidate the prosenescent role of activated macrophages in vivo, we adoptively transferred PMA-stimulated Ana-1 macrophages into mice harboring Myc-driven lymphomas. GFP-tagged Ana-1 cells homed to lymphoma sites, and their presence correlated with enhanced TGF-β1 pathway activation (i.e., Smad3-P), induction of the TGF-β target and senescence indicator plasminogen activator inhibitor-1 (PAI-1), and, most notably, with a substantial increment of senescent lymphoma cells (Figure 4C, and Figures S4D and S4E). Conversely, systemic depletion of macrophages by repetitive provision of liposome-encapsulated clodronate (Aichele et al., 2003) significantly lowered the number of lymphoma-infiltrating macrophages, Smad3 activation (i.e., Smad3-P), and, most importantly, lymphoma cell senescence (Figure 4D, and Figures S4F and S4G; for effects of pharmacological inhibition of TGF-β production see Figures S4H–S4J).

To confirm the impact of TGF-β on senescence induction in vivo, we sought to locally block its action by expressing a soluable, secretable TGF-β1-neutralizing TGF-β type II receptor extracellular domain (TβR-II-ED), thereby restricting TGF-β1 inhibition to the vicinity of TβR-II-ED-expressing cells (Thomas and Massague, 2005). Importantly, transplantations of Eμ-myc transgenic hematopoietic stem cells stably transduced with TβR-II-ED into lethally irradiated recipient mice resulted in a profoundly accelerated onset of lymphomas (p < 0.0001); these lymphomas virtually lacked Smad3 phosphorylation and displayed, despite unaffected macrophage frequencies, much fewer senescent cells when compared with a mock-infected cohort (Figure 4E and 4F and Figures S4K–S4M). Tumor latency remained unchanged when the TβR-II-ED moiety was tested in Suv39h1-deficient hematopoietic stem cells, indicating that TGF-β1-mediated apoptosis has no significant tumor-delaying impact in this model (data not shown). Furthermore, when matched pairs of primary lymphomas were propagated in immunocompetent recipients, TβR-II-ED-expressing lymphomas always formed with lower senescence frequencies than the corresponding empty vector samples (Figure 4G). Thus, selective ablation of TGF-β1 action reduces lymphoma cell senescence in tumor development and in otherwise genetically identical lymphoma aliquots during tumor expansion in vivo. Importantly, these results, like the sharply reduced senescence frequency in Bcl2-protected lymphomas in vivo (Figure 4B), clarify that the non-cell-autonomous induction of senescence is quantitatively substantially more relevant than the cell-autonomous signaling cascade into senescence (as addressed in Figure 2).
Ultimately, we aimed to dissect the sequential process of lymphoma cell apoptosis-induced macrophage-derived TGF-β action on lymphoma cell senescence in a single in vitro experiment. To this end, we cocultivated Bcl2-protected lymphoma cells with macrophages, which were activated by exposure to apoptotic lymphoma cells beforehand, with or without a pharmacological TGF-β receptor type I inhibitor (TGFRI-I). Indeed, only apoptotic body-activated macrophages produced a more than 3-fold increase of SA-β-gal-positive lymphoma cells that was largely abolished in the presence of the TGFRI-I (Figure 4H and Figure S4N). Therefore, TGF-β secreted by macrophages upon their activation by apoptotic lymphoma cells indeed acts as a critical stroma-derived inducer of lymphoma cell senescence.

To test whether the proposed mouse model-deduced mechanism of non-cell-autonomous senescence induction may apply to human aggressive B cell lymphomas as well, we analyzed its central components in a panel of 30 diffuse large B cell lymphoma samples. The panel was subdivided based on Ki67 immunoreactivity into a very high proliferation (Ki67hi; > 80% Ki67-positive cells) group and a lower proliferation (Ki67lo; < 80% Ki67-positive cells) group. Indeed, Ki67lo samples exhibited a significantly higher frequency of H3K9me3-positive cells, indicative of cellular senescence in paraffin-embedded sections that cannot be examined for enzymatic SA-β-gal activity (Figure 5A and 5B). Importantly, the Ki67lo group also presented with a higher fraction of apoptotic cells, more lymphoma-infiltrating macrophages, and a stronger reactivity for the TGF-β signaling mediator.
Thus, these data strongly suggest that environmentally cocontrolled tumor cell senescence plays an important growth-restraining role in human aggressive B cell lymphomas as well.

**DISCUSSION**

Our data establish a model of senescence induction in an oncogenic context where the primary cellular response to the driving oncogene is overt apoptosis, not senescence. Elegant work elucidating signaling cascades involved in RAS-, BRAF-, or MEK-type oncogene-induced senescence demonstrated that an oncogene-evoked DDR (Bartkova et al., 2006; Di Micco et al., 2006; Mallette et al., 2007), a global negative feedback response attenuating RAS effector signaling (Courtois-Cox et al., 2006), and, most recently, proinflammatory cytokines acting as reinforcing networks (Acosta et al., 2008; Coppe et al., 2008; Kuilman et al., 2008; Wajapeyee et al., 2008) contribute to the senescence phenotype. However, all of these studies view senescence as a cell-autonomous phenomenon in which, if at all, cellular interactions or secreted factors promote the senescent arrest in a homotypic self-amplifying way. We report here an oncogene-initiated but non-cell-autonomous route into senescence. This process depends on the activation of TGF-β1-secreting nonneoplastic cells as a critical intermediate step, linking Myc-provoked cell-autonomous apoptosis to the subsequent senescence induction of a significant proportion of the remaining tumor cells by the stromal cytokine (Figure 5C). Hence, our data demonstrate that apoptosis and senescence are not simply two context-dependent choices of cellular stress responsiveness, but that they can be enforced in an interdependent fashion on the organismic level. In this regard, disrupted DNA damage signaling might not only compromise cell-autonomous induction of cellular senescence (Figures 1G and 2D and Figures S2 E and S2F), but might also anticipate impaired macrophage-related senescence due to reduced primary apoptosis. Importantly, DDR-defective tumor cells remain susceptible to non-DNA-damaging prosenescent stimuli that might be therapeutically exploited in the future. Moreover, our data underscore why p53 inactivation—blocking apoptosis,
preventing macrophage attraction, and rendering the cell insensitive to TGF-β-induced senescence— is a particularly efficient way to escape Myc-related senescence.

We would like to emphasize that 12%-20% senescent cells, which were detectable in control lymphomas at diagnosis, are indeed likely to account for a substantial delay in tumor formation. These frequencies reflect “snapshots” of a dynamic process that involves rapid clearance of senescent cells by the host immune system (J.R.D. and C.A.S., unpublished data), as recently reported for a mouse model presenting with senescent liver cancer cells (Xue et al., 2007). The profound impact on overall tumor growth of relatively small steady-state proportions of cells that exited the cycle is well established in the apoptosis field and seems to apply to senescent cells in a comparable way.

Of note, cellular and secreted components that delay tumor manifestation via senescence as shown here do not necessarily keep operating as tumor constraints during later steps of cancer progression, because there is ample evidence that both tumor-associated macrophages and TGF-β can produce deleterious effects by promoting tumor growth or by exerting tolerogenic immune effects (Dave et al., 2004; Thomas and Massague, 2005). However, TGF-β1 signaling has just been reported as a component of the prognostically favorable “stromal-1” signature in human diffuse large B cell lymphoma (Lenz et al., 2008), a frequently Myc-activated entity in which we identified here a subgroup with features highly reminiscent of the presented mechanism of macrophage-mediated senescence induction that we genetically dissected in the murine Eμ-myc model of aggressive B cell lymphoma.

Furthermore, our findings characterize the Rb-related Suv39h1-mediated H3K9me3/HP1 heterochromatin mark as a rather universal and essential downstream effector module of the senescence program that is still operational in the presence of constitutive Myc signaling. This chromatin mark is produced not only by activated oncogenes or DNA damaging chemotherapy (Braig et al., 2005; Collado et al., 2005; Michaloglou et al., 2005), but also by the cytostatic action of secretory TGF-β. Given the anticancer relevance of cellular senescence, the now demonstrated inducibility of senescence by a non-DNA-damaging cytokine opens the exciting perspective to utilize Suv39h1/H3K9me3-enforcing approaches for future cancer therapies.

**EXPERIMENTAL PROCEDURES**

**Lymphoma Analysis and In Vivo Treatments**

The use of human tumor biopsies primarily obtained for the initial diagnosis of diffuse large B cell lymphoma as anonymous samples was based on informed patient consent, and was specifically approved by the local ethics commission of Charité - Universitätsmedizin Berlin (reference EA4/085/07).

All animal protocols used in this study were approved by the governmental review board (Landesamt Berlin), and conform to the respective regulatory standards. Lymphomas with defined genetic defects were generated by intercrossing Eμ-myc transgenic mice with mice carrying loss-of-function alleles at INK4a/ARF, p53, the Ink4a/Arf, the Cip1, or the ATM locus, all in a C57BL/6 background (Adams et al., 1985; Barlow et al., 1996; Christophorou et al., 2005; Deng et al., 1995; Jacks et al., 1994; Kamijo et al., 1997; Krimpenfort et al., 2001; Peters et al., 2001). Genotyping of the offspring by allele-specific genomic PCR, monitoring of lymphoma onset, preservation of snap-frozen or formalin-fixed lymph node tissue and isolation of viable lymphoma cells, splenic B lymphocytes (via magnetic bead selection) provided an internal control, Malignant lymphomas (FLC, primary peritoneal macrophages (pMP), or mouse embryo fibroblasts (MEF) were carried out as described (Davies and Gordon, 2005; Reimann et al., 2007; Schmitt et al., 2002a, 2002b). Where indicated, B cells were prestimulated for 48 hours with 5 μg lipopolysaccharide (LPS)/ml (from Salmonella enterica; Sigma-Aldrich). Preneoplastic cells were obtained from approx 30-day-old Eμ-myc transgenic animals devoid of lymph node or spleen enlargement and with no evidence of leukemia by blood smear analysis. In some experiments, mice were exposed to specific drug treatments as described in the Supplemental Experimental Procedures.

Eμ-myc transgenic FLC as a source of hematopoietic stem cells were obtained to reconstitute (sublethally, i.e., a single 6 or 10 Gy dose of total body γ-irradiation) irradiated nontransgenic recipient mice. FLC, splenic B lymphocytes, isolated lymphoma cells (typically on irradiated NIH3T3 fibroblasts serving as feeders), macrophages, and MEFs were cultured in liquid medium or semisolid methylcellulose as described (Schmitt et al., 1999, 2000), and stably transduced with MSCV-c-Myc-IRES-GBP, MSCV-HA-Suv39h1-puro (kindly provided as pcDNA3.1-HA-Suv39h1-puro by A. Leutz), MSCV-bcl2-blasticidin, MSCV-bcl2-puro, pBabe-c-MycER(γ2)-puro (a generous gift from M. Elsas), or the GBP coencoding retroviruses MSCV-IRES-GBP and MSCV-TfR-II-ED-IRES-GBP (kindly provided as MSCV-TfR-II-ED-puro by J. Massagué) (Reimann et al., 2007; Schmitt et al., 1999, 2002b); the C57BL/6-derived Ana-1 macrophages (kindly provided by L. Varela) were GBP-transduced via nucleofection (nucleofector kit V, Lonza). In some settings, macrophages were treated in vitro with phorbol 12-myristate 13-acetate (PMA; Sigma), liposcin or adriamycin (Sigma) for the indicated times and at the indicated concentrations.

**Analysis of Growth Parameters, Chromosomal Abnormalities, and DNA Damage**

In some experiments, lymphoma cells were exposed in vitro to purified human TGF-β1 (R&D Systems) at 100 or 1000 pM, or were treated with 1 μM 4-hydroxy-tamoxifen (4-OHT; Sigma-Aldrich) or the equivalent volume of the ethanol-based solvent, or were incubated with H2O2 (100 μM; Sigma-Aldrich), or were exposed to the TGF-β1 R inhibitor VI (SD-208; 500 nM; Calbiochem/Merck) for the indicated times, or were treated with NAC or caffeine (Sigma-Aldrich) as stated. Viability and cell numbers were analyzed by trypan blue dye exclusion, cell-cycle parameters by BrdU and propidium iodide (PI) staining (Schmitt et al., 1999; Schmitt et al., 2002b). For numeric karyotypic analysis, at least twelve DAPI stained metaphases were counted per lymphoma sample (Schmitt et al., 2002b). Cytosine preparations of suspension cultures for subsequent SA-β-gal analyses or immunostainings, quantification of ROS by 2′,7′-dichlorodihydrofluorescein-based flow cytometric analyses, and quantification of DNA strand breaks in Annexin V-negative cells (MiltiCyto) by the Comet assay were carried out as previously described (Braig et al., 2005; Reimann et al., 2007). Detection of apoptotic DNA strand breaks by TUNEL (Roche) staining in paraffin-embedded tissue sections and assessment of SA-β-gal activity at pH 5.5 in cryosections or cytospin preparations of cell suspensions were carried out as described (Schmitt et al., 2002a; Schmitt et al., 1999).
control mRNA, and relative transcript levels (e.g., treated versus untreated) were then produced based on $2^{ΔDCt}$ with $ΔDCt = ΔCt_{treated} - ΔCt_{untreated}$.

Immunophenotyping by flow cytometry and antigen detection by immunofluorescence, immunohistochemistry, immunoblotting, and immunoprecipitation were carried out as described (Reimann et al., 2007; Schmitt et al., 2002a). A summary of the methods and the complete list of antibodies used can be found in the Supplemental Experimental Procedures. Staining intensities of Smad3-P or PAI-1 in situ were semiquantitatively assessed (− versus +, ++, or ++++), converted into numeric values 0, 1, 2, or 3 to calculate a mean in some experiments (where a value of around 0.5 would translate into ++++). TGF-β1 protein concentrations were also measured by enzyme-linked immunosorbent assay (Quantikine, R&D Systems) in HCl-activated cell-free culture supernatant in accordance with the manufacturer’s protocol.

**Statistical Evaluation**

Tumor onset data reflecting the latency between birth and first-time palpability of enlarged lymph nodes were compared using the log-rank (Mantel-Cox) test. Curve fitting analysis was done by linear regression with $R^2$ as the coefficient of determination. The unpaired t-test was used to compare means and standard deviations (SD). All quantifications from staining reactions (e.g., immunostainings, TUNEL, or 5-A-bromo-2′-deoxyuridine assays) were carried out by an independent and blinded second examiner, and reflect at least three samples with at least 200 events counted (typically in more than three different tissue areas) each.

**ACCESSION NUMBERS**

Details about the cDNA microarray protocols, the specific array design, and the respective data can be found at http://www.ebi.ac.uk/arrayexpress/ under accession number E-MEXP-1423 for the first set and E-MEXP-1424 for the second set of experiments.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.ccr.2009.12.043.

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