Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation

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Summary

Checkpoints that control Myc-mediated proliferation and apoptosis are bypassed during tumorigenesis. Genes encoding polyamine biosynthetic enzymes are overexpressed in B cells from Eμ-*Myc* transgenic mice. Here, we report that disabling one of these Myc targets, *Ornithine decarboxylase* (*Odc*), abolishes Myc-induced suppression of the Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, thereby impairing Myc's proliferative, but not apoptotic, response. Moreover, lymphoma development was markedly delayed in Eμ-*Myc*;$Odc^{+/-}$ transgenic mice and in Eμ-*Myc* mice treated with the Odc inhibitor difluoromethylornithine (DFMO). Strikingly, tumors ultimately arising in Eμ-*Myc*;$Odc^{+/-}$ transgenics lacked deletions of *Arf*, suggesting that targeting Odc forces other routes of transformation. Therefore, *Odc* is a critical Myc transcription target that regulates checkpoints that guard against tumorigenesis and is an effective target for cancer chemoprevention.

Introduction

The *Myc* family of oncogenes (c-*myc*, N-*myc*, and L-*myc*) is activated in ~70% of human cancer by direct means such as translocations or amplifications or indirectly in response to alterations in upstream signaling pathways or tumor suppressors. To determine their roles in human cancer, transgenic mice have been engineered to express *Myc* genes in a variety of tissues, including the skin (Pelengaris et al., 1999), pancreas (Pelengaris et al., 2002), and prostate (Ellwood-Yen et al., 2003), and in all of these scenarios, enforced Myc expression provokes lethal malignancies. The Eμ-*Myc* transgenic mouse was one of the first mouse models of cancer (Adams et al., 1985), and overexpression of c-*myc* in the B cells of these mice by the immunoglobulin heavy chain enhancer (Eμ) mimics the overexpression of MYC in human Burkitt's lymphoma (BL) that harbors *MYC:Ig* translocations (Dalla-Favera et al., 1982). Importantly, the genetic alterations that accompany the development of B cell lymphomas in Eμ-*Myc* transgenics mimic those that occur in human BL (Eischen et al., 1999; Lindstrom et al., 2001; Wilda et al., 2004), underscoring the use of this transgenic as an important in vivo tool for understanding the molecular pathogenesis of Myc-driven cancers and as a platform to test agents that prevent or treat established disease.

Overexpression of Myc at levels found in cancer is sufficient to drive normal quiescent cells into cycle and to accelerate their rates of cell cycle traverse (Bouchard et al., 1998). These responses are, at least in part, dependent upon Myc's ability to downregulate the expression of the cyclin-dependent kinase (Cdk) inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ (Baudino et al., 2003; Herold et al., 2002; Vlach et al., 1996). However, cells respond to this hyperproliferative response by activating apoptosis (Askew et al., 1991; Evan et al., 1992), through the agency of the Arf-p53 tumor suppressor pathway (Eischen et al., 1999; Zindy et al., 1998), by suppressing the expression of the antiapoptotic proteins Bcl-2 and/or Bcl-X_L (Eischen et al., 2001a) and/or by various other pathways (Nilsson and Cleveland, 2003). Notably, bypass of these cell cycle checkpoints and apoptotic pathways is a hallmark of Myc-driven cancers, and their disruption in transgenic and knockout mouse models markedly accelerates Myc-induced tumorigenesis (Baudino et al., 2003; Egle et al., 2004; Eischen et al., 1999; Eischen et al., 2001b; Martins

SIGNIFICANCE

The ability of oncogenes to provoke cancer is harnessed by regulators that control cell proliferation or induce apoptosis, and bypass of these checkpoints is a hallmark of malignancies. Myc oncoproteins are overexpressed in ~70% of all cancers and induce numerous transcription targets that regulate cell growth, metabolism, and the ribosome machinery. In this report, we show that one select Myc target, *Ornithine decarboxylase* (*Odc*), the rate-limiting enzyme of polyamine biosynthesis, is a critical downstream regulator of Myc's ability to provoke accelerated growth and cancer. *Odc* heterozygosity or inhibition of Odc enzyme activity is shown to affect checkpoints bypassed during Myc-induced tumorigenesis, establishing a mechanism by which chemoprevention strategies targeting Odc can prevent the development of cancer.

and Berns, 2002; Pelengaris et al., 2002; Schmitt et al., 1999; Schmitt et al., 2002; Strasser et al., 1990).

Myc proteins are basic helix-loop-helix leucine-zipper (bHLH-Zip) transcription factors whose function relies on their dimerization with a small bHLH-Zip transcription factor coined Max to bind DNA. Myc-Max complexes transactivate genes carrying their recognition sequence CAYGTG, yet when in a ternary complex with other transcriptional factors such as Miz-1, Myc can repress some target genes (Eisenman, 2001), in particular the Cdk inhibitors $p21^{Cip1}$ and $p15^{Ink4b}$ (Herold et al., 2002; Staller et al., 2001). Which specific targets contribute to Myc's diverse biological effects is a real challenge, as recent genome-wide scanning approaches have shown as many as one-tenth of all genes carry CAYGTG (E box) sequences and thus may be bound by Myc-Max complexes (Patel et al., 2004).

Odc was one of the first identified transactivation targets of Myc (Bello-Fernandez et al., 1993; Wagner et al., 1993), and it encodes the rate-limiting enzyme in polyamine biosynthesis (Cohen, 1997). Polyamines are positively charged small molecules present in all living organisms that bind to and stabilize negatively charged cellular macromolecules, including nucleic acids, phospholipids, and proteins. However, polyamines also perform essential specific functions, for example in the joining of Okazaki fragments during DNA replication, as modifiers of ion transport channels and as regulators of protein translation (Gerner and Meyskens, 2004). Given their broad roles, intracellular polyamine levels are kept under tight control in cells through transport, export, synthesis, and catabolism (Cohen, 1997). Odc converts L-ornithine into putrescine, which is then converted into spermidine and then spermine by dedicated synthases. *Odc* has been used for a number of years as a surrogate marker for Myc function and fulfills all criteria for a bona fide Myc target, as it contains conserved CAYGTG E boxes (Bello-Fernandez et al., 1993), is bound by Myc-Max in growth-stimulated cells (Bello-Fernandez et al., 1993; Nilsson et al., 2004a), and can be conditionally activated by Myc independent of de novo protein synthesis (Wagner et al., 1993). However, the importance of Odc to Myc-induced apoptosis, cell proliferation, and tumorigenesis has not been evaluated in vivo.

Odc is essential for mouse development, as $Odc^{-/-}$ blastocysts die shortly after implantation (Pendeville et al., 2001), and treatment of pregnant mice with α-DFMO, an irreversible inhibitor of ODC, leads to resorption of the embryos by embryonic day 5–8 (Fozard et al., 1980). Nonetheless, $Odc^{+/-}$ mice are overtly normal (Pendeville et al., 2001), and chronic treatment of adult wild-type (wt) mice with DFMO is well tolerated (Lan et al., 2000). Thus, by using $Odc^{+/-}$ mice and DFMO as tools, we evaluated the role of Odc in Myc-driven tumorigenesis in the Eμ-*Myc* transgenic mouse model. Here, we report that activation of Odc is a hallmark of B cell lymphomas arising in these mice and in human BL. Importantly, impairing Odc disables Myc's ability to downregulate Cdk inhibitors, alters the route of Myc-induced transformation, and markedly delays Myc-induced lymphomagenesis. Collectively, these findings support the notion of targeting Odc in cancer chemoprevention.

Results

Myc induces the polyamine biosynthetic pathway in vivo

Biased and unbiased analyses of cell lines engineered to overexpress c-Myc have indicated that *Odc* (Bello-Fernandez et al., 1993) and the genes encoding S-adenosylmethionine decarboxylase (*Amd1*) and Spermidine synthase (*Srm*) (Myc target gene database, http://www.myccancergene.org) are Myc target genes. To test the scope of the response of polyamine metabolic enzymes to Myc in vivo, we submitted total RNA of $B220^+$ splenic B cells from 6-week-old precancerous Eμ-*Myc* transgenic mice and their wt littermates to gene-chip analysis (using Affymetrix 430A gene arrays) and clustered all of the genes of the pathway (Supplemental Figure S1 available in the Supplemental Data with this article online) that were present on the chip (Figure 1A). These findings were then confirmed by real-time PCR with total RNA from both spleen and bone marrow-derived B cells (Figure 1B). Notably, the expression of *odc*, *amd1*, *srm*, and spermine synthase (*sms*) were all elevated in B cells of Eμ-*Myc* transgenic mice, whereas mRNAs encoding enzymes in the catabolic arm of the pathway, spermine/spermidine N-acetyltransferase (*sat1*) and spermine oxidase (*smox*) were generally repressed in Eμ-*Myc* B cells (Figures 1A and 1B). The sole exception was the induction of polyamine oxidase (*paox*, Figure 1B), yet in the presence of reduced levels of *sat1*, which generates substrates for *paox* (Figure S1), this is a futile response. Therefore, Myc coordinately regulates enzymes of the polyamine metabolic pathway in a manner that should elevate polyamine levels in B cells. Indeed, direct measurements of polyamine levels demonstrated that elevated levels of putrescine, spermidine, and spermine were hallmarks of Eμ-*Myc* transgenic B cells (see below, Figure 3).

Odc overexpression is a hallmark of Myc-induced lymphoma

Overexpression of *Odc* is sufficient to transform immortal fibroblast cells (Auvinen et al., 1992) and to provoke benign papillomas in the skin of *Odc* transgenic mice (Megosh et al., 1995). Because Odc is the rate-limiting enzyme of polyamine biosynthesis, we predicted that Odc would be overexpressed in B cell lymphomas that arise in Eμ-*Myc* transgenic mice and in BL. We therefore determined the levels of Odc protein in precancerous B cells from 4- to 6-week-old Eμ-*Myc* mice and their wt littermates as well as in lymphomas that arise in Eμ-*Myc* transgenics. As expected from the RNA analyses, $B220^+$ precancerous Eμ-*Myc* B cells overexpressed Odc protein, and even higher levels of Odc were present in lymphomas (Figure 1C). Furthermore, 8/14 primary tumors from patients suffering from BL also overexpressed *Odc* transcripts (Figure 1D). Therefore, Odc overexpression is a trademark of Myc-driven lymphomas in mice and humans.

Disrupting Odc function impairs Myc-induced lymphomagenesis

Targeted deletion of *Odc* in mice leads to early embryonic lethality (Pendeville et al., 2001). However, $Odc^{+/-}$ mice appear completely normal, despite the fact that they express half the levels of Odc protein and enzyme activity (Guo et al., 2005). We therefore addressed whether loss of one *Odc* allele would affect Myc-induced lymphoma development. $Odc^{+/-}$ mice were bred onto a C57Bl/6 background (12 generations) and were then crossed to C57Bl/6 Eμ-*Myc* transgenics. Eμ-*Myc*;$Odc^{+/+}$ and Eμ-*Myc*;$Odc^{+/-}$ littermates were then followed for their course of disease. As expected (Eischen et al., 1999), most Eμ-*Myc*;$Odc^{+/+}$ mice succumbed to lethal lymphoma between 3 and 6 months of age (mean latency of 110 days, Figure 2A).

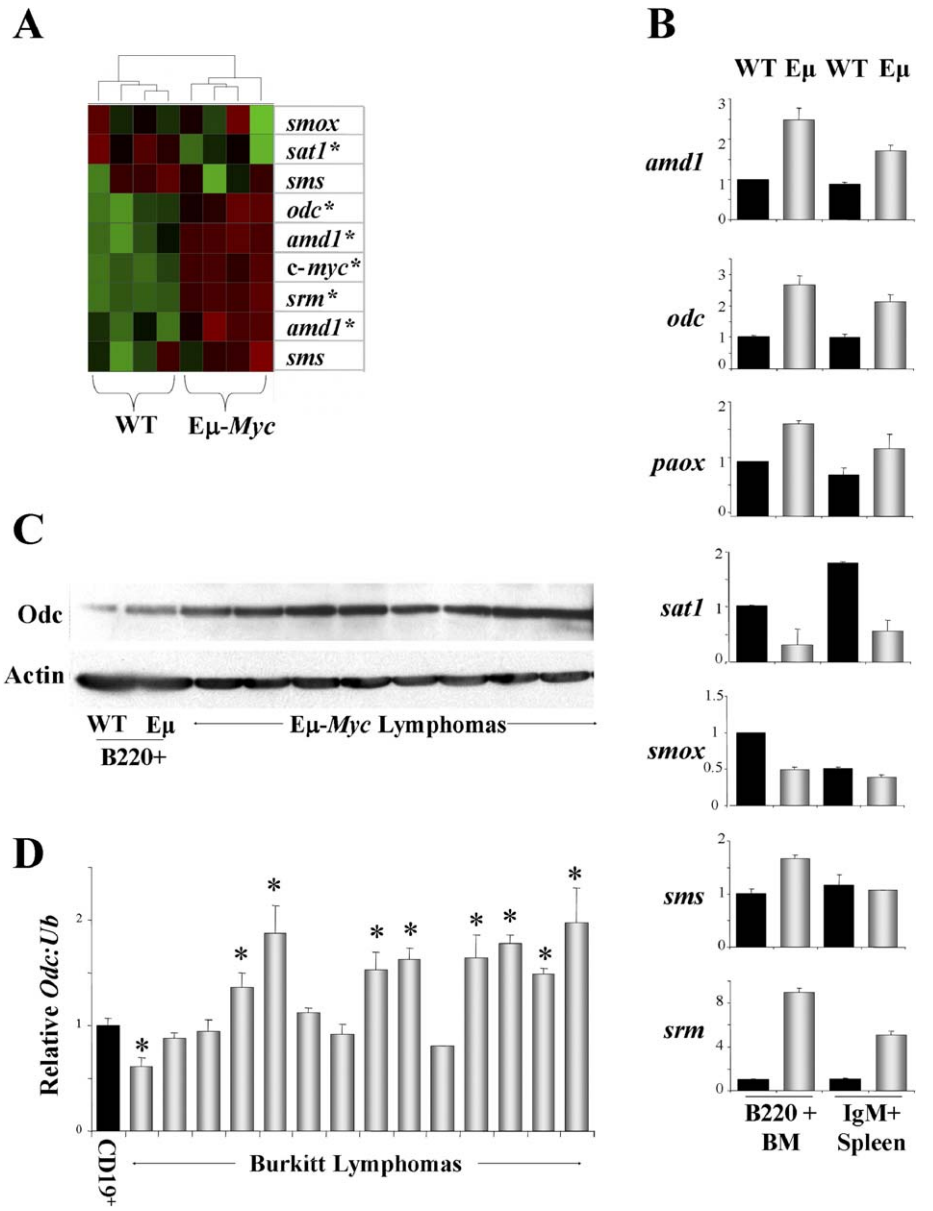

Figure 1. *Odc* and other genes encoding enzymes in the polyamine biosynthetic pathway are targeted by Myc in vivo

A: Affymetrix gene array showing expression of the indicated genes in B220+ splenic B cells from 4- to 6-week-old wild-type (wt) and Eμ-*Myc* littermates. Probe sets with an asterisk are genes that are significantly altered according to Anova analysis ($p < 0.05$).

B: SYBRgreen real-time PCR analysis on cDNA from bone marrow-derived (BM) B cells and from splenic IgM+ B cells using primers for the indicated genes. For the role of each individual gene product in polyamine synthesis or catabolism, see Supplemental Figure S1. The expression of each gene was correlated to that of *ubiquitin* (*ub*), which is not regulated by c-Myc. Mean expression is shown, and error bars represent analyses of three individual mice of each genotype.

C: Western blot analysis for Odc and Actin protein levels in B220+ MACS-sorted B cells from 6-week old wt and Eμ-*Myc* (Eμ) mice and in lymphomas arising in Eμ-*Myc* mice.

D: SYBRgreen real-time PCR analysis on cDNA from peripheral CD19+ B cells of a healthy donor and from tumors of Burkitt's lymphoma (BL) patients using primers directed against *Odc*. The expression of *Odc* was correlated to that of *Ubiquitin*, which is not regulated by c-Myc. An asterisk indicates tumors with statistically significant changes in the expression of Odc (Student's t test, $p < 0.05$).

Strikingly, their Eμ-*Myc*;*Odc*$^{+/-}$ littermates had a markedly delayed course of disease (mean latency of 320 days), and ~25% of these animals survived beyond 14 months of age (Figure 2A). Hallmarks of imminent disease in Eμ-*Myc* transgenics are elevated peripheral B cell counts and splenomegaly. 8-week-old Eμ-*Myc*;*Odc*$^{+/-}$ mice had reduced numbers of peripheral B cells and smaller spleens compared to their Eμ-*Myc*;*Odc*$^{+/+}$ littermates (data not shown). The delay in lymphoma development was not due to overt effects on lymphoid cells in *Odc*$^{+/-}$ mice, as their B cell subsets and numbers were normal (Table S1). Therefore, although halving the gene dosage of *Odc* has no overt effect on development, modest reductions in Odc activity markedly compromise Myc-mediated tumorigenesis.

The effects of *Odc* heterozygosity suggested that lymphoma development in Eμ-*Myc* mice would also be impaired by treating mice with DFMO, an enzyme-activated suicide inhibitor of Odc (Cohen, 1997). Originally developed in the 1970s as a potential therapeutic, DFMO irreversibly inactivates Odc in a highly specific manner. DFMO is stable in water and is therefore easily administered to mice in their drinking water (Fozard et al., 1980). Importantly, treatment of Eμ-*Myc* mice with 1% DFMO, from weaning age on, dramatically delayed lymphomagenesis (mean latency of ~350 days) compared to littermates provided with normal drinking water (mean latency of ~100 days, Figure 2B). DFMO had no effect on the peripheral blood counts or spleen size in wt mice, yet its effects on Eμ-*Myc* mice were dramatic, as 6–8 week old, DFMO-treated Eμ-*Myc* mice had normal white blood cell counts and lacked the splenomegaly that was evident in untreated Eμ-*Myc* mice (Figure 2C and data not shown). Finally, DFMO was continuously required to prevent lymphoma development, as when animals

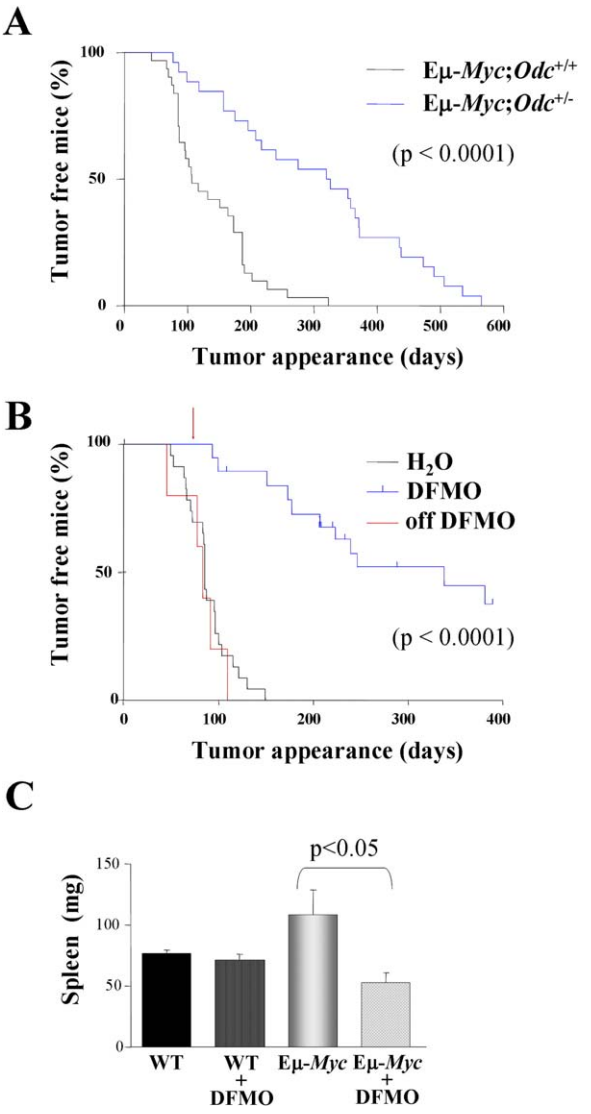

Figure 2. Targeting Odc impairs Myc-induced lymphomagenesis

A: Survival curve of Eμ-*Myc* mice of different *Odc* genotypes. Median survival time was 110 days for Eμ-*Myc*;*Odc*$^{+/+}$ and 320 days for Eμ-*Myc*;*Odc*$^{+/-}$ mice ($p < 0.0001$).

B: Survival curve of untreated (H_2O) or DFMO-treated Eμ-*Myc* mice. Mean survival time for control mice was 100 days and those on DFMO was 350 days ($p < 0.0001$). The red arrow indicates the time at which a group of DFMO-treated Eμ-*Myc* mice were taken off the drug, and the red line indicates their survival time after removal of DFMO (mean survival time 100 days).

C: Average spleen weights (n = 3) of 7-week-old wt and Eμ-*Myc* mice treated or untreated with DFMO. The treatment lasted for three weeks. Student's t test was employed for statistical analyses.

treated with the drug for 90 days were then taken off from DFMO they all succumbed to lymphomas. Furthermore, the onset of the tumors in animals taken off the drug occurred at the expected interval (3–4 months, red line Figure 2B). Collectively, these findings support a crucial role for Odc in Myc-induced lymphomagenesis.

Polyamine levels are in large part regulated by Odc activity but are also controlled by polyamine catabolism and active transport. The latter is carried out by a largely unknown mechanism, but in most cells, polyamine transport is enhanced in response to DFMO (Seiler et al., 1996). Endogenous stores of polyamines are available from standard diets, and thus it was somewhat surprising that the effects of DFMO on disease in Eμ-*Myc* mice were so profound. We therefore assessed whether Eμ-*Myc* transgenic B cells might be defective in polyamine uptake. Primary B cells were grown from precancerous Eμ-*Myc* bone marrow cells on S17 stroma in the presence of IL-7 (Eischen et al., 1999), and after ~2 weeks in culture, these cells were >95% pre-B cells, as determined by FACS with B cell-specific markers. These cells, and control 32D.3 myeloid cells (Askew et al., 1991), were then tested for effects of DFMO on polyamine uptake. As expected, DFMO treatment of 32D.3 cells enhanced polyamine uptake (~2-fold). Surprisingly, DFMO treatment reduced, rather than increased, polyamine uptake in B cells from Eμ-*Myc* mice (Figure 3A). Therefore, the B cells of Eμ-*Myc* transgenic mice are unable to compensate for reductions in Odc by increasing polyamine uptake.

Because Myc augments the expression of every enzyme involved in polyamine biosynthesis (Figure 1), an expectation was that polyamine levels would be elevated in B cells from Eμ-*Myc* mice. Indeed, elevated levels of putrescine, spermidine, and spermine were evident in the precancerous B cells of Eμ-*Myc* transgenic mice versus those of their wt littermates (Figure 3B). Furthermore, a 2-week exposure of mice to DFMO effectively reduced levels of putrescine in bone marrow (data not shown) and splenic transgenic B cells to those present in wt littermates. DFMO treatment also resulted in significant reductions in spermidine content but to compensatory increases in spermine levels in both wt and Eμ-*Myc* B cells (Figure 3B). Nonetheless, the collective effect of DFMO on putrescine and spermidine levels clearly counterbalances the biological effects of increased polyamine levels seen in response to Myc.

Odc is a critical regulator of Myc's proliferative response

In most cell lines, polyamine depletion induces G1 phase cell cycle arrest (Cohen, 1997). Myc accelerates cell cycle progression and increases proliferative rates in vivo (Baudino et al., 2003) and overrides cell cycle arrest in response to growth factor withdrawal (Askew et al., 1991). We therefore evaluated whether impairing Odc function would compromise Myc's ability to drive B cell proliferation in vivo. Four different cohorts of mice (wt untreated, wt + DFMO, Eμ-*Myc* untreated, and Eμ-*Myc* + DFMO) were injected with BrdU, and after 12 hr, B cells were isolated from bone marrow and spleen. The numbers of BrdU$^+$ and Annexin V$^+$ IgM$^+$ and IgM$^-$ negative splenic and bone marrow-derived B cells were then determined. There were much greater numbers of B cells in S phase in Eμ-*Myc* than in wt mice (Figure 4A). Although DFMO had essentially no effect on the proliferative rates of B cells from wt mice, DFMO treatment significantly reduced numbers of BrdU$^+$ cells in all B cell subpopulations of Eμ-*Myc* transgenic mice, especially those of proliferating IgM$^-$ splenic B cells (Figure 4A). A similar reduction in BrdU$^+$/IgM$^-$ splenic B cells was also evident in B cells derived from Eμ-*Myc*;*Odc*$^{+/-}$ mice compared to their wt littermates (Figure 4B). Therefore, DFMO treatment, or loss of one allele of *Odc*, impairs Myc's proliferative response.

In contrast to the obvious effects of DFMO and *Odc* heterozygosity on Myc's proliferative response, the effects of impairing Odc on Myc-induced apoptosis were not as evident. In Eμ-*Myc* mice, Myc's apoptotic response is mostly manifest in IgM$^+$

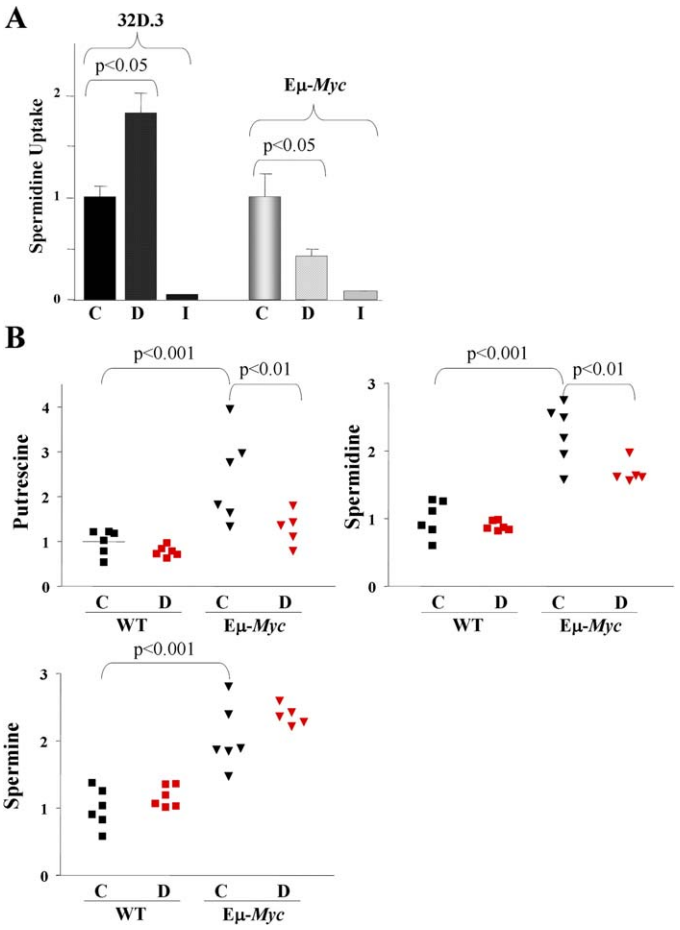

Figure 3. DFMO-treatment reduces polyamine uptake in Eμ-*Myc* B cells and restores proper levels of putrescine in Eμ-*Myc* transgenic B cells in vivo

A: Bone marrow cells from an Eμ-*Myc* mouse were cultured ex vivo on S17 stromal cells in medium containing IL-7. After establishment of a pure B cell culture, 10^6 cells were cultured in the presence (D) or absence (C) of DFMO for two days. ^{3}H-spermidine was added, and the cells were incubated for 1 hr at 37°C or on ice (I). Uptake of spermidine was determined with a scintillation counter. The experiment was carried out in triplicates. As a control, IL-3-dependent 32D.3 myeloid cells (Askew et al., 1991) were also analyzed. Student's t test was employed for statistical analyses.

B: Effects of DFMO on polyamine levels in splenic B cells in vivo. Levels of intracellular putrescine, spermidine, and spermine were determined in 10^6 splenic B cells from untreated (C) and DFMO-treated (D) 4- to 6-week-old wt (squares) and Eμ-*Myc* (filled arrowheads) mice. Five to six mice of each group were analyzed. Black symbols, untreated mice; red symbols, DFMO-treated mice.

B cells (Maclean et al., 2003). DFMO had no effect on the survival of wt B cells, and it did not impair Myc-induced apoptosis; rather, the higher rates of apoptosis of Eμ-*Myc* IgM-positive B cells were enhanced in DFMO-treated transgenics (Figure 4B). Furthermore, analyses of the apoptotic index of Eμ-*Myc*;*Odc*$^{+/-}$ transgenic B cells, and of wt *Odc*$^{+/-}$ B cells, indicated that *Odc* heterozygosity had no effect on B cell survival. Collectively, these findings support the concept that *Odc* is selectively haploinsufficient for Myc's proliferative response.

Odc is necessary for Myc-mediated suppression of p21^{Cip1} and p27^{Kip1}

Myc-driven tumors are dependent on sustained Myc expression (Jain et al., 2002; Pelengaris et al., 1999). Given the profound effect of *Odc* heterozygosity or DFMO treatment on lymphomagenesis, we initially assessed whether compromising Odc activity would affect the expression of the Myc transgene in Eμ-*Myc* B cells. However, expression profiling and real-time PCR of splenic B220$^+$ B cells (Figures 5A and 5B), and Western blot analyses of B220$^+$ B cells from bone marrow (data not shown) and spleen (Figures 6A and 6B), established that Myc activity was sustained in Eμ-*Myc* mice treated with DFMO (Figure 6A) or when crossed onto a *Odc* heterozygous background (Figure 6B). To analyze the expression of Myc target genes (http://www.myccancergene.org), we clustered those genes that differed significantly between splenic B cells from 7-week-old wt and Eμ-*Myc* mice (three mice per group). Their expression profiles were then compared to those of wt and Eμ-*Myc* littermates treated with DFMO for 3 weeks. These analyses established that the *Myc* transgene, rather than DFMO, was the major determinant of changes in the expression of these targets (Figure 5A). In addition, real-time PCR analyses established that the increased levels of c-*myc*, *odc*, and *rcl* transcripts in Eμ-*Myc* B cells were not appreciably affected by DFMO treatment, and loss of one allele of *Odc* only affected *odc* levels (Figure 5B). Therefore, impairing Odc function does not alter the expression of the Myc transgene nor its ability to regulate the majority of its transcription targets.

Myc's proliferative response has been associated with its ability to regulate cyclins and their kinases. Indeed, levels of cyclin D1 (*ccnd1*), *cdk4*, and *cdk2* were upregulated in Eμ-*Myc* B cells, but they were unaffected by DFMO treatment (Figure 6D), and expression of cyclin D2, a direct target of Myc in some cell contexts (Bouchard et al., 1999), was reduced in Eμ-*Myc* B cells and unaffected by DFMO (data not shown). Furthermore, the expression of cyclin E1 and E2 and cyclin A were not significantly altered by Myc or by treatment with DFMO (data not shown). However, Myc also accelerates cell cycle traverse by suppressing the expression of the cdk inhibitors p21^{Cip1} and p27^{Kip1}, and indeed, levels of p21^{Cip1} and p27^{Kip1} were repressed in B220$^+$ precancerous splenic B cells of Eμ-*Myc* mice (Figures 6A and 6B). DFMO, or loss of one *Odc* allele, had only very subtle effects on the expression of these Cdk inhibitors in B cells derived from wt mice (Figures 6A and 6B). Strikingly, levels of p21^{Cip1} and p27^{Kip1} protein were effectively restored back to those present in wt splenic B cells in Eμ-*Myc* mice treated with DFMO and in Eμ-*Myc*; *Odc*$^{+/-}$ mice (Figures 6A and 6B). Expression profiling indicated that the changes in at least p27^{Kip1} expression were manifest at the level of the protein and not its RNA, as levels of *cdkn1b* transcripts (encoding p27^{Kip1}) remained low in B cells from DFMO-treated Eμ-*Myc* mice (Figure 6D). Furthermore, DFMO treatment of primary pre-B cells engineered to express a conditionally activatable form of Myc, Myc-ER, induced p27^{Kip1} protein (Supplemental Figure S2A) without affecting the levels of *ckn1b* transcripts, which were suppressed after Myc activation with the estrogen receptor (ER) agonist 4-hydroxytamoxifen (Supplemental Figure S2B). The level at which disabling Odc leads to changes in p21^{Cip1} expression was less evident, as expression profiling called *cdkn1a* (encoding p21^{Cip1}) as absent and acute treatment of Myc-ER-expressing pre-B cells with DFMO had little effect on p21^{Cip1} protein levels (Supplemental Figure S2A). Therefore, impairing Odc abolishes Myc's ability to downregulate p27^{Kip1} protein and, perhaps through indirect means, p21^{Cip1} protein. Alternatively, disabling Odc

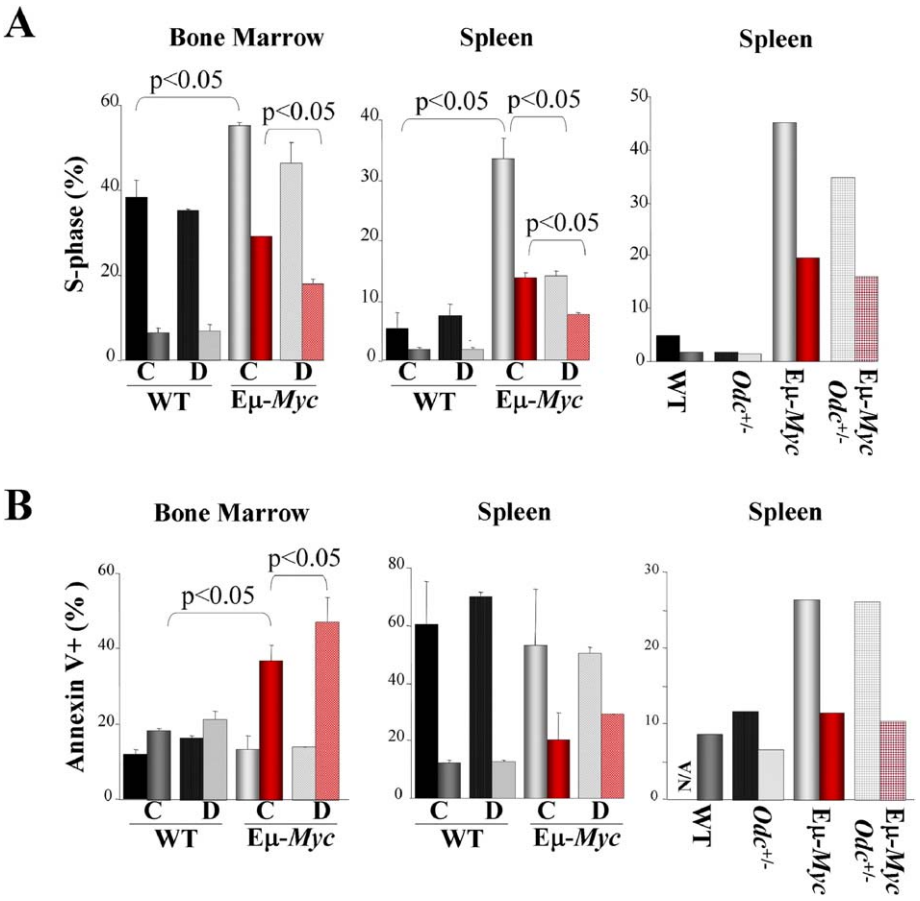

Figure 4. DFMO and *Odc* heterozygosity impair Myc's proliferative response

A: Effects of DFMO and *Odc* heterozygosity on Myc-driven B cell proliferation in vivo. Bone marrows and spleens from untreated (C) and DFMO-treated (for 3 weeks) (D) wt and Eμ-*Myc* mice were harvested 12 hr after BrdU injection (at 7 weeks of age). Three mice of each group were analyzed for BrdU incorporation in $B220^+$/surface IgM^- and $B220^+/IgM^+$ cells; i.e., pro/pre B cells and more mature B cells (black bars, wt IgM^-; dark gray, wt IgM^+; black stippled, wt IgM^+ DFMO-treated; gray stippled, wt IgM^- DFMO-treated; light gray, Eμ-*Myc* IgM^-; red, Eμ-*Myc* IgM^+; light gray hatched, Eμ-*Myc* IgM^- DFMO-treated; and red hatched, Eμ-*Myc* IgM^+ DFMO-treated). B cell proliferation of 8-week-old wt and $Odc^{+/-}$ Eμ-*Myc* transgenics were analyzed in a similar fashion (gray checkered bars, Eμ-*Myc*;$Odc^{+/-}$ IgM^-; red checkered, Eμ-*Myc*;$Odc^{+/-}$ IgM^+). Student's t test was employed for statistical analyses.

B: To determine apoptotic indices, aliquots of the same bone marrow and spleen samples as in (A) were stained for B220, IgM, and Annexin V. Student's t test was employed for statistical analyses.

may induce $p27^{Kip1}$ and $p21^{Cip1}$ protein through a pathway independent of Myc.

The very modest effects of DFMO and *Odc* heterozygosity on Myc's apoptotic response in vivo suggested that compromising Odc function would not alter Myc's ability to activate the Arf-p53 pathway. As expected (Eischen et al., 1999), there was an obvious upregulation of $p19^{Arf}$ and p53 protein in B cells derived from both bone marrow and spleen of precancerous Eμ-*Myc* mice compared to their wt littermates (Figure 6C and data not shown). Further, DFMO treatment (or *Odc* heterozygosity, data not shown) did not augment this response but slightly reduced levels of $p19^{Arf}$ and p53 in Eμ-*Myc* B cells (Figure 6C). Finally, Myc-induced apoptosis has also been associated with its ability to induce the expression of the pro-apoptotic Bcl-2 family proteins Bax (Mitchell et al., 2000) and Bim (Egle et al., 2004). Bax expression was comparable in wt and Eμ-*Myc* transgenic B cells +/– DFMO, and Bim expression also did not correlate with effects of DFMO treatment (Figure 6C and data not shown). Therefore, impairing Odc functions does very little to Myc's apoptotic response.

Impairing Odc functions alters the route of Myc-induced transformation

Hallmarks of tumors that arise in Eμ-*Myc* transgenics are alterations in the Arf-p53 tumor suppressor pathway, which occur through missense hot-spot mutations of *p53* that create dominant negative forms of the protein, and biallelic deletion of *Arf* (Eischen et al., 1999). Unlike wt p53, dominant negative p53 mutants have a long half-life, and tumors bearing p53 mutations express high levels of endogenous $p19^{Arf}$, due to loss of p53-mediated transcriptional repression of *Arf* (Robertson and Jones, 1998). Collectively, Western and Southern blot analyses indicated that the frequency of alterations in the Arf-p53 pathway was only slightly reduced in lymphomas arising in Eμ-*Myc*;$Odc^{+/-}$ mice versus their wt Eμ-*Myc* littermates (Figures 7A and 7B). However, tumors arising in Eμ-*Myc*;$Odc^{+/-}$ mice lacked deletions of *Arf* (Figure 7B, bottom), whereas 4/9 lymphomas arising in their Eμ-*Myc* littermates showed biallelic deletions in *Arf* (lanes with an asterisk, Figure 7B, top). Lymphomas that ultimately arose in DFMO-treated Eμ-*Myc* transgenics also lacked *Arf* deletions (data not shown). Thus, impairing Odc specifically biases against biallelic deletions in *Arf* during Myc-induced transformation.

Impairing Odc activity abolished Myc's ability to downregulate the expression of $p21^{Cip1}$ and $p27^{Kip1}$ (Figures 6A and 6B). We therefore assessed the expression of these cdk inhibitors in the lymphomas that arose in Eμ-*Myc*;$Odc^{+/-}$ versus Eμ-*Myc*;$Odc^{+/+}$ littermates. Very low to undetectable levels of $p21^{Cip1}$ and $p27^{Kip1}$ protein were present in lymphomas arising in wt Eμ-*Myc* transgenics, yet the levels of these inhibitors were markedly elevated in many of the tumors arising in Eμ-*Myc*;$Odc^{+/-}$ transgenics (Figure 7A). Furthermore, the levels of

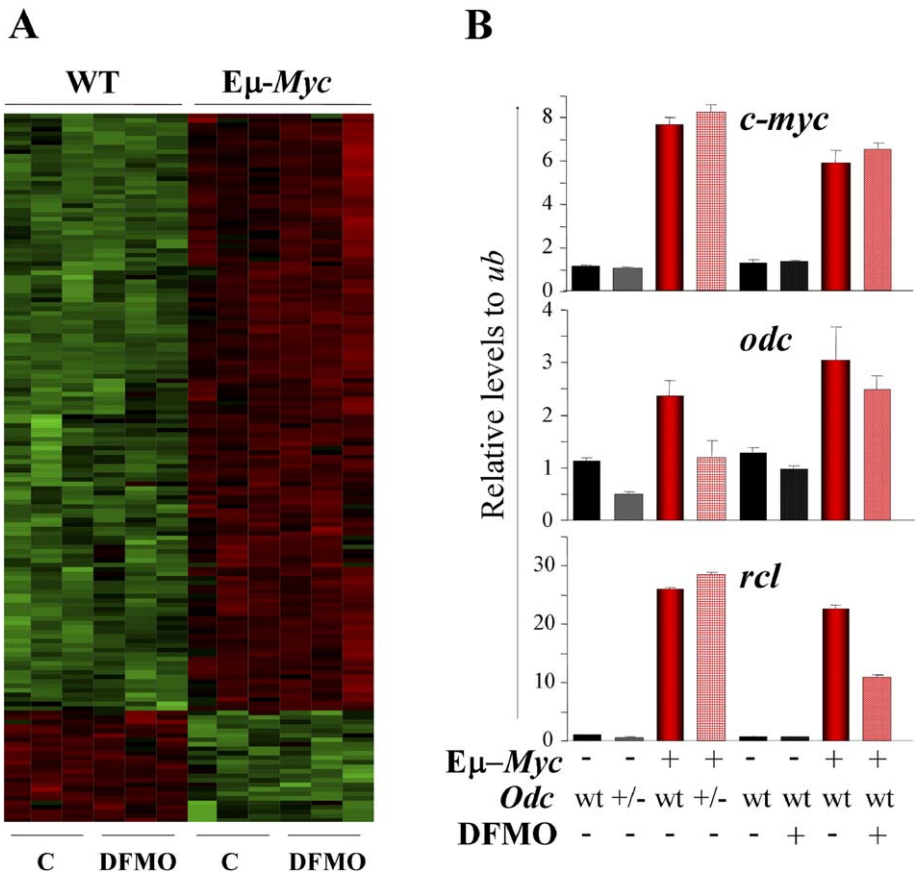

Figure 5. DFMO treatment and *Odc* heterozygosity do not affect Myc's transcriptional response

A: DFMO does not grossly affect the "Myc transcriptome." Hierchical clustering of significantly altered genes of the Myc target gene database is presented. RNA was prepared from $B220^+$ splenic cells of 7-week-old untreated and DFMO-treated (3 weeks) wt and Eμ-*Myc* mice and was subjected to analysis on a 430A Affymetrix chip (three mice were analyzed per group). Analysis was performed with the "treatment comparison" function of the Spotfire program. The gene names of the probe sets shown are given in Supplemental Table S3.

B: SYBRgreen real-time PCR analysis on cDNA from splenic B cells of wt, $Odc^{+/-}$, Eμ-*Myc* and Eμ-*Myc*;$Odc^{+/-}$ mice, and wt and Eμ-*Myc* mice treated with DFMO (for 2 weeks) was performed with primers (Supplemental Table S2) for *c-myc* and the direct Myc transcription targets *odc* and *rcl*. The expression of each gene was standardized to that of *ub*. Mean expression is shown, and error bars represent analyses of three individual mice of each genotype.

the antiapoptotic protein Bcl-X_L, which is only rarely increased in lymphomas arising in Eμ-*Myc* transgenics (Eischen et al., 2001a), were markedly elevated in several tumors arising in Eμ-*Myc*;$Odc^{+/-}$ transgenics (Figure 7A). Finally, we evaluated whether there might be a selection for alterations in the *Odc* gene (for example *Odc* amplification) in tumors arising in Eμ-*Myc*;$Odc^{+/-}$ transgenics. However, neither of these events was evident (Supplemental Figure S3). Therefore, the delay in tumor development in Eμ-*Myc*;$Odc^{+/-}$ transgenics is associated with specific differences in the expression of $p21^{Cip1}$, $p27^{Kip1}$, and Bcl-X_L and with a lack of deletions in *Arf*.

The failure of lymphomas arising in Eμ-*Myc*;$Odc^{+/-}$ and DFMO-treated Eμ-*Myc* mice to undergo alterations in *Arf* suggested that in cells where Arf function is compromised, the preventative effects of DFMO might be abolished. To test this notion, we bred Eμ-*Myc* mice to $Arf^{GFP/GFP}$ mice (Zindy et al., 2003), an *Arf* knockout strain that bears the gene for green fluorescent protein (GFP) in place of exon 1β of *Arf* in the *Ink4a/Arf* locus. Eμ-*Myc*;$Arf^{+/-}$ transgenic mice develop lymphomas at a greatly accelerated pace, and this is nearly always accompanied by loss of the remaining wt *Arf* allele, as expected for a tumor suppressor gene (Eischen et al., 1999). Indeed, all Eμ-*Myc*;$Arf^{+/GFP}$ died within 2.5 months of age (Figure 7C) and 4/5 of the lymphomas arising in these mice lost the wt *Arf* allele (Figure 7D). Strikingly, DFMO treatment (from 1 week of age) failed to significantly delay lymphoma development in Eμ-*Myc*;$Arf^{+/GFP}$ littermates (Figure 7C), and tumors that arose in these mice all displayed loss of the wt *Arf* allele (Figure 7D). Therefore, disabling Odc delays the onset of de novo deletions of *Arf*, but not gene conversions, in $Arf^{Gfp/+}$ mice. Importantly, these data indicate that targeting Odc is effective as a chemopreventative intervention but would be ineffective in therapy for cancers bearing mutations in the Arf-p53 tumor suppressor pathway.

Discussion

Chemoprevention strategies targeting the polyamine biosynthetic pathway in cancer have recently shown efficacy in a number of preclinical animal studies and also appear to show promise in clinical trials in human cancer (Gerner and Meyskens, 2004). However, the mechanisms by which targeting Odc prevents cancer have been a mystery. The findings reported herein provide clues to this puzzle and reveal that targeting Odc prevents Myc-induced tumorigenesis in two ways. First, Odc is necessary for Myc to accelerate cell cycle traverse. This response is specifically linked to Myc's effect on the Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, as when Odc is disabled, Myc's ability to suppress their expression is effectively counteracted. Second, although Odc does not overtly affect Myc's apoptotic response, full activity of the enzyme appears critical for events that lead to the biallelic deletions in *Arf* that often accompany Myc-induced lymphomagenesis, and compromising the Arf checkpoint inactivates Odc inhibitors as chemopreventative

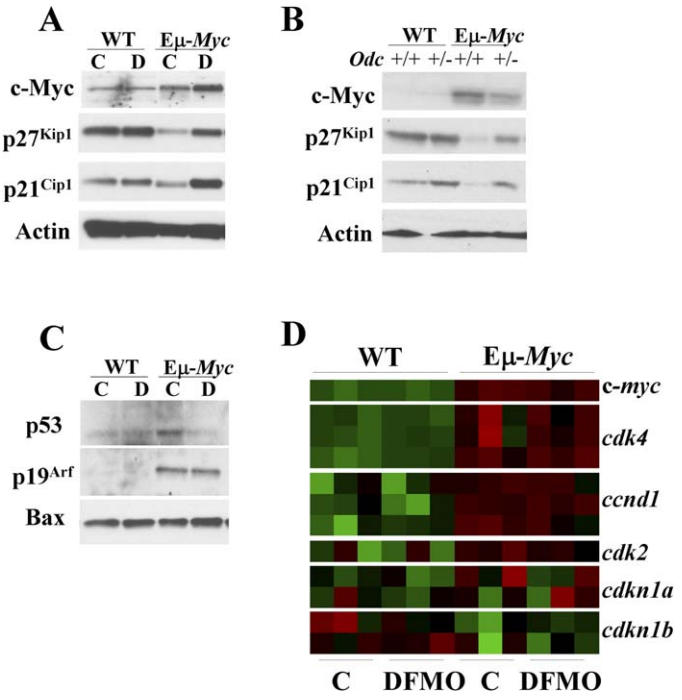

Figure 6. DFMO treatment and *Odc* heterozygosity abolishes Myc's ability to suppress the expression of the Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$

A: Western blot analysis of $p21^{Cip1}$ and $p27^{Kip1}$ expression in spleen-derived $B220^+$ B cells. The protein samples were from untreated (C) and DFMO-treated (3 weeks) (D) wt and Eμ-*Myc* mice.

B: Western blot analysis of $p21^{Cip1}$ and $p27^{Kip1}$ expression in spleen-derived $B220^+$ B cells from wt, $Odc^{+/-}$, Eμ-*Myc*, and Eμ-*Myc*;$Odc^{+/-}$ (8-week-old) littermates.

C: Western blot analysis of p53, $p19^{Arf}$, and Bax expression in spleen-derived $B220^+$ B cells. The protein samples were again from untreated (C) and DFMO-treated (3 weeks) (D) wt and Eμ-*Myc* mice.

D: Affymetrix gene array showing expression of the indicated genes in $B220^+$ splenic B cells from 4- to 6-week-old wt and Eμ-*Myc* littermates untreated (C) or treated with DFMO for 2 weeks.

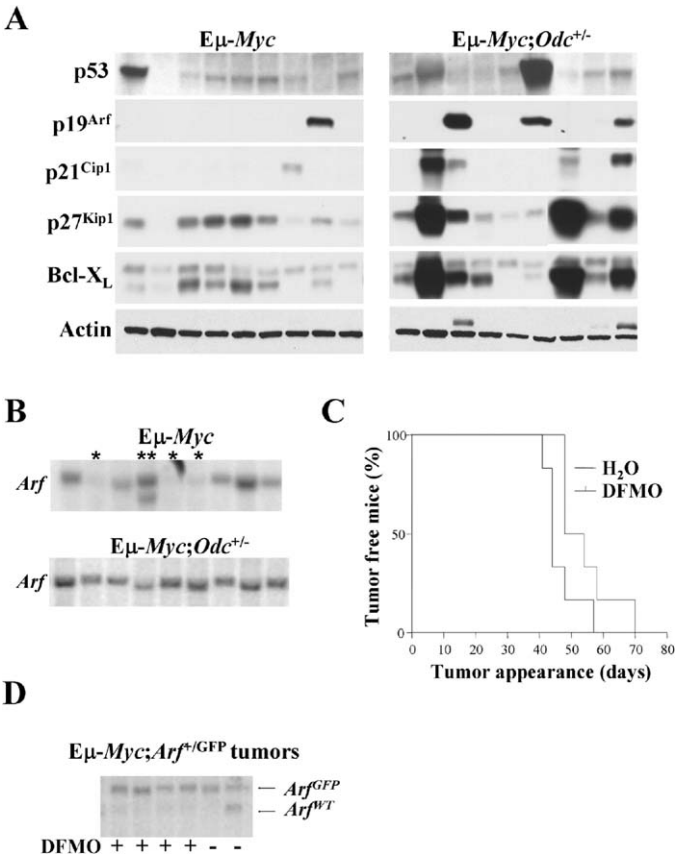

Figure 7. Targeting *Odc* alters the route of Myc-induced transformation

A: Western blot analysis of p53, $p19^{Arf}$, $p21^{Cip1}$, $p27^{Kip1}$, and Bcl-X_L expression in lymphomas arising in Eu-*Myc* and Eu-*Myc*;$Odc^{+/-}$ littermates.

B: Southern blot hybridization of genomic DNA from the same tumors as in (A) using a probe against the *Arf* locus. An asterisk indicates tumors showing biallelic deletion of *Arf* in Eu-*Myc* transgenics; two asterisks indicate a tumor undergoing alterations of one allele of *Arf*.

C: Survival curve of five untreated (H_2O) or five DFMO-treated Eu-*Myc*; $Arf^{+/GFP}$ mice.

D: Southern blot hybridization of genomic DNA from tumors arising in Eu-*Myc*;$Arf^{+/GFP}$ mice. The top band represents the targeted, GFP-containing allele and the lower band the wt allele that is lost during tumorigenesis.

agents. These findings underscore the limitations of targeting Odc in cancer, where DFMO is effective as a chemopreventative agent but has largely failed as a cancer therapeutic (Gerner and Meyskens, 2004). Nonetheless, the rather remarkable effects of DFMO or loss of one *Odc* allele in preventing Myc-induced lymphomagenesis establish *Odc* as one of the critical downstream targets of Myc that is necessary to drive cell proliferation and transformation.

Not all Myc targets are created equal

Mouse models such as Eμ-*Myc* transgenics provide valuable platforms to test the relevance of known downstream targets of oncogenes and/or tumor suppressors that may contribute to cancer development and/or maintenance. Based upon their ability to drive quiescent cells into cycle and to accelerate rates of cell cycle progression, critical targets of Myc were initially thought to be those that regulate the cell cycle. More recent in vivo assessments have shown that proposed Myc targets like *Id2* (Murphy et al., 2004; Nilsson et al., 2004b), *Cyclin D2* (P. Sicinski and M. Eilers, personal communication), and *Cdk4* (J.A.N. and J.L.C., unpublished data) are dispensable for Myc-induced tumorigenesis. However, there are obvious cell context-specific effects of these regulators as in the K5-*Myc* transgenic mouse model loss of *Cdk4* impairs skin tumorigenesis (Miliani de Marval et al., 2004), and loss of *E2f1*, which is also induced by Myc (Baudino et al., 2003), accelerates Myc-induced skin tumors (Rounbehler et al., 2002), yet impairs Myc-induced lymphomagenesis.

Characterization of the Myc transcriptome by SAGE, expression profiling, and genome-wide scans for sites of Myc bound chromatin have indicated that the majority of Myc targets are those thought to direct cell metabolism, cell growth (mass), and the ribosome machinery (Patel et al., 2004). The numbers of Myc targets that have come from these assays range up into the thousands (Patel et al., 2004), suggesting that focusing efforts on any single Myc target would be ineffective. Thus, our finding that intervention of a single metabolic target of Myc impairs lymphomagenesis is remarkable and suggests that the

downstream products of Odc, the polyamines, influence many of the metabolic and growth processes that are also necessary for cell division and transformation.

This study suggests that metabolic targets of Myc certainly deserve attention as targets in cancer chemoprevention and therapy. The case for targeting Odc is particularly compelling. Firstly, Odc expression is induced by various carcinogens (Cohen, 1997), and Odc overexpression is sufficient to induce transformation in vitro (Auvinen et al., 1992) and for tumor promotion in the skin (O'Brien et al., 1997). Secondly, Odc-induced tumorigenesis is reversed by DFMO treatment (Lan et al., 2000). Further, in addition to its impressive effect on Eμ-*Myc* transgenics, DFMO is also effective in blocking tumorigenesis in other mouse cancer models but heretofore has never been formally linked to a pathway leading to changes in Odc expression. Thirdly, overexpression of antizyme, which directs Odc to the proteasome for destruction (Murakami et al., 1992), blocks tumorigenesis (Feith et al., 2001). Fourthly, a single-nucleotide polymorphism in human *ODC* that resides between the two Myc-Max sites in *ODC* intron 1 regulates its expression (Guo et al., 2000) and correlates with improved clinical response in colon adenomas (Martinez et al., 2003). Finally, as shown here, and also in skin tumor models (Guo et al., 2005), *Odc* heterozygosity dramatically impairs tumorigenesis. Therefore, critical thresholds of Odc, and thus of polyamines, are important arbiters of tumor development. Indeed, simply reducing levels of putrescine to those present in wt B cells is sufficient to markedly impair lymphoma development.

Odc mediates Myc's proliferative response and directs the route of transformation

Perturbing Odc functions affects the Myc response at two levels. DFMO treatment or *Odc* heterozygosity abolishes the ability of Myc to accelerate B cell cycle traverse (Figure 4B), and this occurs without gross effects on Myc's transcriptional programs (Figure 5A). Rather, impairing Odc abolishes the ability of Myc to suppress the expression of $p21^{Cip1}$ and $p27^{Kip1}$ (Figures 6A and 6B and Supplemental Figure S2). Although Myc can repress the transcription of both *Cdkn1a* ($p21^{Cip1}$) and *Cdkn1b* ($p27^{Kip1}$) (Gartel et al., 2001; Herold et al., 2002; Yang et al., 2001), in the Eμ-*Myc* B cell, the regulation of at least $p27^{Kip1}$ appears to occur largely at a posttranscriptional level, as expression profiling and real-time PCR failed to document changes in *cdkn1b* transcripts in response to DFMO treatment (Figure 6D and Supplemental Figure S2). Degradative control is an important route of regulating $p21^{Cip1}$ and $p27^{Kip1}$ protein levels (Bartek and Lukas, 2001; Sherr and Roberts, 1999), and this level of regulation for $p27^{Kip1}$ is certainly important in the response to Myc (O'Hagan et al., 2000; Vlach et al., 1996). The ability of DFMO or *Odc* heterozygosity to effectively abolish this response thus suggests that changes in the thresholds of polyamines may also influence the turnover of these proteins, which is consistent with studies showing that polyamine depletion can lead to the induction of $p21^{Cip1}$ (Kramer et al., 2001). Importantly, a limiting role for $p27^{Kip1}$ in B cell transformation in Eμ-*Myc* transgenic mice has already been established, as loss of *p27Kip1*, but not of *p21Cip1*, accelerates lymphomagenesis (Martins and Berns, 2002), and loss of *E2f1* leads to the ectopic expression of $p27^{Kip1}$ and delays lymphomagenesis (Baudino et al., 2003). Thus, DFMO's chemopreventative properties likely include its ability to specifically restore $p27^{Kip1}$, which would suppress tumor development.

When faced with the inability to downregulate $p21^{Cip1}$ and $p27^{Kip1}$, such as occurs in Eμ-*Myc*;*Odc*$^{+/-}$ mice, alternative routes of transformation appear to be at play. Quite dramatically, very low to undetectable levels of $p21^{Cip1}$ and $p27^{Kip1}$ were revealed as a hallmark of lymphomas arising in Eμ-*Myc* transgenics, and Myc's ability to suppress their expression was abolished in many tumors arising in Eμ-*Myc*;*Odc*$^{+/-}$ transgenics. In addition, many of the lymphomas arising in these mice were also characterized by a marked upregulation of Bcl-X_L, a rare event in these tumors (Eischen et al., 2001a). Furthermore, although impairing Odc has no overt effects on Myc's apoptotic response in vivo, a striking finding was that none of the tumors arising in Eμ-*Myc*;*Odc*$^{+/-}$ or DFMO-treated Eμ-*Myc* transgenics bore biallelic deletions in *Arf*, which were readily evident in lymphomas arising in wt Eμ-*Myc* transgenics (Figure 7B). Finally, loss of even one *Arf* allele was sufficient to render Eμ-*Myc* B cells refractory to the protective effects of DFMO (Figure 7C). Thus, the ability of DFMO or *Odc* heterozygosity to impair transformation appears to occur at a very early step and likely involves blocking of lesions that provoke the loss of one allele of *Arf*, which can sometimes be captured in Eμ-*Myc* lymphomas (e.g., see the lane with two asterisks, Figure 7B), that then lead to loss of the entire *Arf* locus.

Relevance to human cancer

Myc oncoproteins are deregulated in the vast majority of human tumors, suggesting they are perfect targets for chemotherapeutic intervention. Unfortunately, their functions as transcription factors that regulate a large fraction of the genome clearly complicate this approach. Further, Myc is a member of the "Max network," where Max also dimerizes with at least six other partners to repress transcription (Eisenman, 2001), and Myc-induced target gene activation may rely on its ability to relieve Mnt-Mad-mediated transrepression (Nilsson et al., 2004a). Therefore, targeted therapies that block Myc binding to E boxes may also block Mnt-Mad functions and lead to activation of gene transcription through relief of transrepression, the opposite of the desired effect. We therefore propose that targeting the products of genes induced by Myc is a safer approach to treating Myc-driven tumors. Focusing on those gene products that are enzymes, like Odc, should facilitate drug design.

Myc-driven tumors require sustained expression of the oncogene and quickly regress when Myc activity is compromised (Jain et al., 2002; Pelengaris et al., 1999). If Odc were also a key mediator of Myc in tumor maintenance, then treatment of preexisting lymphomas with DFMO would be predicted to be of therapeutic benefit. However, we and others (Gerner and Meyskens, 2004) have seen little, if any, effect of DFMO on the progression of established malignancies. Still, DFMO as a chemopreventative agent is important in many scenarios of human cancer where tumors arise in a predictable manner. For example, in colon cancer, which undergoes an ordered progression from polyps to adenomas to frank carcinoma, combinations of COX-2 inhibitors with DFMO have proven efficacious in preventing adenoma recurrence and progression to carcinoma (Gerner and Meyskens, 2004). This represents a success story, but there are many as yet unexplored arenas that represent avenues for prevention. Finally, there is a growing cadre

of heritable malignancies where agents such as DFMO, which has limited toxicity, should be considered. We have shown that DFMO is effective at blocking tumor formation in a scenario where there is a single genetic lesion (Myc activation). Therefore, it is possible that this agent will also be effective in blocking secondary mutations occurring in heritable breast and ovarian cancer patients having mutations of *BRCA1*, *BRCA2*, or *CHK2* and in Li-Fraumeni patients having *TP53* mutations (Varley, 2003).

Experimental procedures

Mice and tumor analyses

Odc$^{+/-}$ mice were bred with Eμ-*Myc* transgenics (both on C57Bl/6 background) to generate F1 Eμ-*Myc*;*Odc*$^{+/-}$ and Eμ-*Myc*;*Odc*^{wt} offspring. Eμ-*Myc* transgenic male mice were also bred to *Arf*$^{GFP/GFP}$ knockin/knockout females (mixed background, kindly provided by Drs. Martine Roussel and Charles Sherr) to generate Eμ-*Myc*;*Arf*$^{+/GFP}$ mice (Zindy et al., 2003). Lastly, cohorts of Eμ-*Myc* transgenic mice were given either standard water or water containing 1% DFMO. All mice were observed daily for signs of morbidity and tumor development. Sick animals were sacrificed, and tumors and lymphoid organs were analyzed by histology and immunohistochemistry to confirm B cell lymphoma.

With institutional review board approval and after informed consent, RNA was extracted from tumors of 14 BL patients by using the RNA/DNA Kit from Qiagen.

Cell culture

Primary bone marrow-derived pre-B cell cultures were generated from 6-week-old C57Bl/6 mice as described (Eischen et al., 1999). After 2 weeks in culture on S17 stromal cells, the established B cell culture was maintained in conditioned medium from NIH-3T3 cells expressing IL-7. To test effects of DFMO on polyamine transport, cells were cultured in the presence of 5 mM DFMO for 2 days. IL-3-dependent 32D.3 myeloid cells were cultured as previously described (Askew et al., 1991). Generation of pre-B cell cultures expressing Myc-ER has been described (Nilsson et al., 2004b).

Polyamine transport

1×10^6 B cells or 32D.3 cells (Askew et al., 1991) were cultured ± 5 mM DFMO for 2 days. 1 μl ^{3}H-spermidine was added and the cells incubated for 1 hr at 37°C or on ice (negative control for nonspecific binding). After incubation, the cells were harvested, washed twice with PBS containing 100 μM cold spermidine, and lysed by the addition of 1 M NaOH. The lysate was mixed with scintillation fluid (UltimaGold, Packard Bioscience) and counted in a scintillation counter.

B cells from untreated and DFMO-treated wt and Eμ-*Myc* transgenic mice were analyzed for their polyamine content by high-performance liquid chromatography. Briefly, polyamines from frozen pellets of 1×10^6 cells were extracted with 0.6 N perchloric acid, dansylated, and analyzed as described (Chen et al., 2001).

FACS and magnetic-activated cell sorting of B cells

Rates of proliferation of B220$^+$/IgM$^+$ and B220$^+$/IgM$^-$ cells were determined by using a Flow Kit as described by the manufacturer (BD Biosciences Pharmingen). Animals were injected intraperitoneally with 100 μl of 10 mg/ml BrdU in sterile PBS. Animals were sacrificed 12 hr postinjection, and bone marrow and spleen were harvested. 1×10^6 cells were used for the BrdU proliferation assay, by incubation with antibodies against B220 (APC conjugated) and IgM (PE conjugated), followed by washes. Labeled cells were further processed and stained with FITC anti-BrdU antibody, washed, and analyzed by FACS.

The remainder of the bone marrow and spleen cells was incubated with beads conjugated to a B220 antibody (Miltenyi Biotech) and enriched by MACS for B cells according to the manufacturer's instruction. The same procedure was used to obtain splenic B cells as controls for Western blots.

RNA preparation and analyses

B cells were obtained from bone marrow or spleen by MACS using beads conjugated to antibodies against B220 or IgM, respectively. RNA was prepared by using the RNeasy Kit (Qiagen). For Affymetrix analyses, cRNA was synthesized by using the One-Cycle Target Labeling and Control Reagent package (Affymetrix, Inc.), and the reaction was probed to the 430A mouse Affymetrix chip. The scanned data output was imported into the Spotfire software. After normalization, selected probe sets for genes indicated in Figure 1 or in Figure 5 were clustered by using the Hierarchical Clustering function of Spotfire. Statistical analysis was performed in Spotfire with the Anova function.

For real-time PCR, cDNA was prepared from 1 μg RNA by using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed with an iCycler machine (Bio-Rad) and the iTaq SYBR Green Kit. Data analyses were performed with the ΔCt method, where *ubiquitin* served as the internal control. Sequences for primers are available in Supplemental Table S2.

Western blot analyses

Extracts from MACS-sorted B cells and lymphomas from Eμ-*Myc* mice were prepared as described (Eischen et al., 1999). Protein (30–50 μg per lane) was separated on a 15% SDS-PAGE gel, transferred to membranes (Protran, Schleicher & Schuell), and blotted with antibodies specific for c-Myc (N-262, Santa Cruz Inc.), ODC (from Drs. Anthony Pegg and Lisa Schantz), p21^{Cip1} (F-5, Santa Cruz Inc.), p27^{Kip1} (Transduction labs), p53 (Ab-7, Oncogene research), p19^{Arf} (Abcam), Bim (Stressgen Bioreagents), Bax (Santa Cruz Inc.), and β-actin (AC-15, Sigma Chemicals).

Supplemental data

Supplemental Data include three figures and three tables and are available with this article online at http://www.cancercell.org/cgi/content/full/7/5/433/DC1/.

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