Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants


The c-Myc oncoprotein promotes proliferation and apoptosis, such that mutations that disable apoptotic programmes often cooperate with MYC during tumorigenesis. Here we report that two common mutant MYC alleles derived from human Burkitt’s lymphoma uncouple proliferation from apoptosis and, as a result, are more effective than wild-type MYC at promoting B cell lymphomagenesis in mice. Mutant MYC proteins retain their ability to stimulate proliferation and activate p53, but are defective at promoting apoptosis due to a failure to induce the BH3-only protein Bim (a member of the B cell lymphoma 2 (Bcl2) family) and effectively inhibit Bcl2. Disruption of apoptosis through enforced expression of Bcl2, or loss of either Bim or p53 function, enables wild-type MYC to produce lymphomas as efficiently as mutant MYC. These data show how parallel apoptotic pathways act together to suppress MYC-induced transformation, and how mutant MYC proteins, by selectively disabling a p53-independent pathway, enable tumour cells to evade p53 action during lymphomagenesis.

Human tumours frequently show deregulated expression of the c-Myc proto-oncogene. In Burkitt’s lymphoma, this deregulation occurs through reciprocal translocations that juxtapose c-Myc with an immunoglobulin (Ig) promoter, leading to gross overexpression of c-Myc messenger RNA in the B cell lineage. In addition, point mutations are often found in the translocated MYC alleles, clustering in a conserved region known as MYC box I (refs 6–8). Although some mutations can increase MYC stability and transforming activity in vitro, their impact on the pathogenesis of Burkitt’s lymphoma is unclear. In fact, translocated c-Myc genes are subject to hypermutation in vivo that can also alter non-coding sequences, raising the possibility that these mutations are a consequence and not a cause of tumour development.

To examine the effects of MYC mutation on lymphoma development in vivo, we used a system for rapidly generating tissue-specific transgenic mice (see Supplementary Fig. 1). Two mutant MYC alleles commonly observed in Burkitt’s lymphoma (P57S and T58A) were cloned into a murine stem cell virus (MSCV)-based vector that co-expresses green fluorescent protein (GFP). Haematopoietic stem cells (HSCs) derived from normal fetal livers were transduced with retrovirus-expressing either wild-type or mutant MYC, and the genetically modified stem cells were then used to reconstitute the haematopoietic system of lethally irradiated recipient animals. This adoptive transfer strategy has several advantages over traditional transgenic approaches, such as (1) transgene expression is limited to the haematopoietic system; (2) various combinations of transgenes or stem cell genotypes can be rapidly analysed; and (3) only a fraction of the injected stem cells are infected, allowing tumorigenesis to occur in the context of an essentially normal haematopoietic compartment. Also, the strong MSCV promoter produces high levels of MYC expression that recapitulate that observed in Burkitt’s lymphoma (data not shown), and the co-expressed GFP enables assessment of infection efficiency and visualization of tumorigenesis using fluorescence imaging.

MYC mutants show enhanced oncogenicity in vivo

Although the control MSCV vector was not oncogenic (data not shown), recipients of stem cells infected with wild-type MYC developed tumours at low penetrance after a long latency (Fig. 1a; 2 out of 13 recipient mice at >100 days). The tumours that arose were aggressive pre-B cell lymphomas (Fig. 1b; see also Supplementary Fig. 2a), similar to those produced by the tissue-specific overexpression of MYC in transgenic mice. Recipients of stem cells infected with the tumour-derived MYC point mutants also developed pre-B cell lymphomas, but at a significantly higher penetrance (Fig. 1a; 9 out of 12 for P57S and 8 out of 11 for T58A; P < 0.005 for each mutant versus wild-type MYC) and a significantly reduced latency (56 ± 9 days for P57S and 66 ± 9 days for T58A). Flow cytometry for GFP fluorescence showed that the increased tumorigenicity of these mutants was not due to differences in stem cell infection efficiency or transgene expression (see Supplementary Fig. 2b).

As deregulated MYC expression coordinately induces proliferation and apoptosis, the increased oncogenicity of the mutant MYC proteins might reflect the altered activation of one or both of these processes. To investigate the proliferation of cells expressing wild-type and mutant MYC, we assessed BrdU incorporation in the bone marrow of pre-malignant mice after stem cell reconstitution. Although all MYC constructs induced an increase in proliferation relative to uninfected controls (data not shown), wild-type and mutant MYC showed indistinguishable BrdU incorporation profiles (Fig. 2a; 25 ± 3% for wild-type MYC, 24 ± 6% for P57S and 27 ± 5% for T58A BrdU-positive cells). To examine the impact of MYC expression on apoptosis, we compared the ability of wild-type and mutant MYC to induce tumours in the presence of a strong apoptotic block. Recipient mice were reconstituted with stem cells co-infected with a retrovirus expressing MYC constructs and a retrovirus expressing the anti-apoptotic protein Bcl2. Notably, when co-expressed with Bcl2, wild-type and mutant MYC produced

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aggressive B cell lymphomas with indistinguishable latency (34 ± 2 days for wild-type MYC, 35 ± 2 days for T58A and 33 ± 2 days for P57S) and penetrance (Fig. 2b, c). In addition, propidium iodide staining for DNA content revealed no differences in cell cycle profiles between lymphomas expressing wild-type and mutant MYC (Fig. 2d). Thus, in the absence of apoptosis, wild-type and mutant MYC are equally oncogenic.

Mutant MYC induces p19ARF and p53, but not Bim
Oncogene-induced apoptosis is often mediated by the induction of p19ARF and subsequent stabilization of p53. It is thought to sense hyperproliferative signals and prevent aberrant proliferation. To investigate the basis for the apoptotic defect of mutant MYC, we examined p19ARF and p53 expression in murine embryonic fibroblasts. A

Figure 1 | Tumour-derived MYC mutants show enhanced oncogenicity in vivo. a, Kaplan–Meier curve showing survival at various times after adoptive transfer. b, In vivo GFP imaging shows disseminated lymphomas in a mouse 60 days after reconstitution with HSCs transduced with P57S, but not wild-type MYC. c, Hematoxylin/eosin staining and immunohistochemical staining for B220 (a B cell lineage marker) of lymph node and liver sections of animals harbouring wild-type MYC and P57S lymphomas, showing an aggressive B cell disease with perivascular infiltration of B220-positive tumour cells into the liver.

Figure 2 | Wild-type and mutant MYC show apoptotic, but not proliferative, differences in vivo. a, Flow cytometry showing BrdU incorporation in GFP-positive bone marrow cells 24 days after reconstitution. Data are representative of three independent experiments. b, Kaplan–Meier curve showing survival following adoptive transfer with HSCs co-infected with the indicated MYC-expressing retroviruses and a Bcl2-expressing retrovirus. c, In vivo GFP imaging showing disseminated lymphomas in mice 35 days after adoptive transfer with HSCs co-transduced with wild-type or mutant MYC and Bcl2. d, Representative histograms of DNA content of wild-type MYC/Bcl2 and mutant MYC/Bcl2 lymphomas.
blasts (MEFs) and HSC populations infected with wild-type and mutant MYC retroviruses. Interestingly, both p57S and T58A induced p19ArfB and p53 as well as (or better than) wild-type MYC (Fig. 3a, c). This p53 was transcriptionally active, as cells expressing either wild-type or mutant MYC showed a similar increase in p53 phosphorylation and expression of the p53 transcriptional targets Bax, Puma and Noxa (Fig. 3c and data not shown). Furthermore, the apoptotic defect was unrelated to the established ability of MYC to repress Bcl-2 and Bcl-X£, because no consistent effect of MYC on Bcl-2 expression was observed and both wild-type and mutant MYC were capable of repressing Bcl-XL. Although p21WAF1/CIP1 levels were reduced in HSCs expressing wild-type MYC, they were substantially higher in cells expressing mutant MYC. However, this effect was not consistently seen in MEFs (Fig. 3c and data not shown), suggesting that the impact of p21WAF1/CIP1 repression on MYC-induced apoptosisB might contribute to, but is not essential for, the mutant MYC phenotype.

- A Western blot analysis showing ARF and p53 levels (a) or Bim levels (b) in MEFs stably infected with control (labelled C), wild-type MYC or mutant MYC vectors. The long (BimEL) and extra long (BimELR) isoforms of Bim are shown. C, Western blot analysis of HSCs using antibodies against the indicated proteins. D, Kaplan–Meier curve showing the time to death of mice (n = 6) infected with the indicated MYC-expressing retrovirus. E, In vivo GFP imaging showing disseminated lymphomas in mice 34 days after adoptive transfer with wild-type MYC (BD2B£) and BD2B£ HSCs infected with wild-type MYC. F, Western blot analysis showing the indicated MYC-expressing retroviruses. G, In vivo GFP imaging showing disseminated lymphomas in mice 34 days after adoptive transfer with wild-type MYC (BD2B£) and BD2B£ HSCs infected with wild-type MYC. H, Western blot analysis showing the indicated MYC-expressing retroviruses. I, In vivo GFP imaging showing disseminated lymphomas in mice 34 days after adoptive transfer with wild-type MYC (BD2B£) and BD2B£ HSCs infected with wild-type MYC. J, Western blot analysis showing the indicated MYC-expressing retroviruses. K, In vivo GFP imaging showing disseminated lymphomas in mice 34 days after adoptive transfer with wild-type MYC (BD2B£) and BD2B£ HSCs infected with wild-type MYC. L, Western blot analysis showing the indicated MYC-expressing retroviruses. M, In vivo GFP imaging showing disseminated lymphomas in mice 34 days after adoptive transfer with wild-type MYC (BD2B£) and BD2B£ HSCs infected with wild-type MYC. N, Western blot analysis showing the indicated MYC-expressing retroviruses. O, In vivo GFP imaging showing disseminated lymphomas in mice 34 days after adoptive transfer with wild-type MYC (BD2B£) and BD2B£ HSCs infected with wild-type MYC. P, Western blot analysis showing the indicated MYC-expressing retroviruses. Q, In vivo GFP imaging showing disseminated lymphomas in mice 34 days after adoptive transfer with wild-type MYC (BD2B£) and BD2B£ HSCs infected with wild-type MYC. R, Western blot analysis showing the indicated MYC-expressing retroviruses. S, In vivo GFP imaging showing disseminated lymphomas in mice 34 days after adoptive transfer with wild-type MYC (BD2B£) and BD2B£ HSCs infected with wild-type MYC.
The requirement to lose the wild-type p53 allele in tumors initiated by wild-type MYC may account for their delayed onset relative to tumors in recipients of mutant MYC-infected stem cells. Indeed, mice reconstituted with p53<sup>-/-</sup> HSCs expressing either the wild-type or mutant MYC formed tumors rapidly, with an identical penetrance and latency (Fig. 4b; 40 ± 2 days for wild-type MYC, 40 ± 4 days for T58A, and 41 ± 5 days for P57S). All of the p53<sup>-/-</sup> lymphomas were oligoclonal (see Supplementary Fig. 3b), and showed no obvious differences between wild-type and mutant MYC expression (Fig. 4b, inset). Thus, MYC mutations can facilitate tumour development in the absence of p53 inactivation.

**MYC, p53 and Bim status in Burkitt’s lymphoma**

These data predict that Bim expression should be reduced in Burkitt’s lymphomas expressing MYC mutations, and that p53 mutations should be under-represented in Burkitt’s lymphomas harbouring the P57S and T58A mutant MYC alleles. Indeed, seven out of seven Burkitt’s lymphomas expressing wild-type MYC were positive for Bim expression by immunohistochemistry, displaying substantially higher Bim levels than several diffuse, large B cell lymphomas (DLBCL) without MYC translocations (three out of three; Fig. 4c). In contrast, six out of seven Burkitt’s lymphomas harbouring MYC alleles with MYC box I mutations (including four out of five tumours with P57 and T38 mutations) were scored as Bim negative (Fig. 4c). Furthermore, none of the p53 mutant Burkitt’s lymphomas (9 out of 17) that we analysed or identified from the literature<sup>15,26</sup> had coincident mutations at MYC codons 57 and 58, despite their common occurrence in our dataset (12 out of 71) (see Supplementary Table and data not shown). Of note, two out of two lymphomas harbouring a Pro to Ser mutation at codon 60 of MYC had a p53 mutation at codon 248, suggesting that not all MYC mutations are equivalent. Nevertheless, our data demonstrate that two mutant MYC alleles commonly observed in Burkitt’s lymphoma can bypass p53 action during lymphomagenesis in mice, and provide evidence to suggest that this may also occur in humans.

**Discussion**

Our results reveal new insights into the biology of the MYC oncoprotein. For example, they suggest that tumour-derived MYC mutations are relevant to the pathology of Burkitt’s lymphoma because they are unable to upregulate Bim and efficiently inhibit Bcl2. Furthermore, they show that the MYC mutants we examined retain their ability to activate the p19<sup>ARF</sup>-p53 pathway and efficiently promote proliferation. Thus, MYC mutations alter MYC activity in both quantitative and qualitative ways. These qualitative changes may be particularly important for the development of tumours such as Burkitt’s lymphoma, which acquire independent alterations that quantitatively upregulate MYC. Such qualitative changes to MYC may also be important for normal cell growth; in this regard, some tumour-derived MYC mutations lead to constitutive phosphorylation of MYC at Ser62, a target of mitogen-stimulated kinases<sup>21</sup>. Perhaps MYC mutations ‘lock in’ a qualitative state that normally uncouples proliferation from apoptosis in response to mitogens, allowing tissue expansion.

Mutations that are selected for in malignant tumours pinpoint genes and processes that must be altered during tumour evolution. Thus, the fact that certain mutant MYC alleles selected for in human lymphomas are specifically impaired in their ability to promote apoptosis in vivo provides compelling genetic evidence that the phenomenon of oncogene-induced apoptosis is relevant to human tumorigenesis. Furthermore, our results demonstrate that the MYC-induced apoptotic programme is not linear, but instead involves p53-dependent and -independent signals that act in parallel to promote cell death and suppress MYC-induced tumorigenesis. That a cell expressing mutant MYC can activate p53 in response to hyperproliferative signalling but evade p53 action implies that MYC-induced apoptosis involves a threshold phenomenon, such that inactivation of any one of several MYC effectors can cause apoptotic firing to drop below the threshold level and allow unregulated proliferation. Consequently, certain mutations in MYC abrogate the requirement for a cooperating gene mutation during tumorigenesis.

**Figure 4** | Impact of mutations in MYC on p53 tumour suppressor action and Bim induction in human Burkitt’s lymphomas. a, Kaplan–Meier curve showing mouse survival after adoptive transfer with p53<sup>-/-</sup> HSCs infected with the indicated MYC retroviral constructs. Inset: Representative allele-specific PCR assay showing retention of the wild-type p53 allele in lymphomas produced by mutant but not wild-type MYC. b, Kaplan–Meier curve showing mouse survival after adoptive transfer with p53<sup>-/-</sup> HSCs infected as in a. Inset: Western blot analysis of representative lymphomas harbouring the indicated MYC allele. c, Bim expression in DLBCL and wild-type MYC-expressing and mutant MYC-expressing Burkitt’s lymphomas as assessed by immunohistochemistry using an anti-Bim antibody. The frequency of Bim-positive tumours in each category is indicated.
METHODS

Retroviral infection and cell culture. HAM (a methionine-rich variant of the widely used haemagglutinin tag)-tagged wild-type MYC and mutants were subcloned into MSCV-IRES-GFP\(^{48}\) for in vitro studies and MSCV-puro (Clontech) for cell culture studies. Primary MEFs were generated from embryonic 13.5 (E13.5) day embryos from a wild-type C57BL/6J background or from a Bcl2\(^{+/-}\) to Bcl2\(^{-/-}\) cross. Retroviral-mediated gene transfer was performed using Phoenix packaging cells as previously described\(^7\) Cells for in vitro studies were infected with MSCV-GFP, MSCV-GFP-WT MYC, MSCV-GFP-P575 or MSCV-GFP-T58A. Infected cells populations of FLS 12 cells and MEFs used for in vitro studies were selected in puromycin (2 μg·ml\(^{-1}\) for 2 days). Infected HSCs used for in vitro studies were diluted to equal infection efficiency (assessed by GFP) then sorted using magnetic beads to 70–80% purity with a lineage cell depletion kit (Miltenyi Biotec). For cell viability assays, MEFs were infected with MYC-expressing retroviruses as described, and were incubated for 24h in either 10% fetal bovine serum (high serum) or 0.1% fetal bovine serum (low serum) and analysed for viability by trypan blue exclusion.

Stem cell isolation and adoptive transfer. Pregnant E14.5 C57BL/6 mice from wild-type, p53\(^{-/-}\), Bim\(^{-/-}\) or Bcl2\(^{-/-}\) crosses were killed to obtain fetal livers. Stem cell isolation and transplantation were performed as described\(^8\). For stem cell reconstitution and BrdU analysis, total bone marrow was isolated 21–28 days after tail vein injection. In vivo BrdU incorporation was assessed using an APC BrdU Flow Kit (BD Pharmingen). Bone marrow was harvested 2h after an intraperitoneal injection of 200μl 10 mg·ml\(^{-1}\) BrdU, and BrdU incorporation was assessed for a population of GFP-positive cells at a defined GFP intensity. Flow cytometry analysis was performed on a Becton Dickinson LSRII cell analyser, with FACSVantage DiVa software.

Lymphoma monitoring and analysis. Reconstituted animals were monitored for illness by lymph node palpation, monitoring overall morbidity, and, in some cases, whole-body fluorescence imaging\(^9\). Overall survival was defined as the time from stem cell reconstitution until the animal reached a terminal stage and was killed. Statistical analysis was performed using a one-way ANOVA (analysis of variance) test using Graph Pad Prism version 3.0 (Graph Pad Software). Immunohistochemistry was performed using CD45R/B220–clone RA3-6B2 (BD Biosciences, Pharmingen) and rabbit anti-Bim (Stressgen) antibodies. Tumour cell DNA content was determined by FACS analysis with propidium iodide staining of ethanol-fixed cells\(^10\). Nested PCR analysis of variable, diversity and joining (V(D)J) recombination was performed as described\(^11\).

Protein analysis. Immunoblots were performed from whole-cell lysates obtained by boiling cell pellets that were solubilised in Laemmli sample buffer. Twenty micrograms of protein samples (Bio-Rad protein assay) were separated by SDS polyacrylamide electrophoresis (SDS–PAGE) and transferred to Immobilon-P membranes (Millipore). The antibodies used were anti-MYC (Ab1, Oncogene; 1:250), anti-p19-IRF\(^{37}\) (ab800–100, Abcam; 1:1000), anti-p21 (C–19, Santa Cruz; 1:500), anti-p53 (NCL-p53-505, Novocasta; 1:500), anti-phospho-p53 (Ser 15) (6G8, Cell Signalling; 1:1000), anti-Bim/BOP (AAD-330, Stressgen; 1:1000), anti-Bcl2 (N-19, Santa Cruz; 1:1000) and anti-Bcl-XL (S-18, Santa Cruz; 1:1000). Proteins were visualised using ECL (Amersham) or Lumli-light (Roche).