Max deletion destabilizes MYC protein and abrogates Eµ-Myc lymphomagenesis

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Although MAX is regarded as an obligate dimerization partner for MYC, its function in normal development and neoplasia is poorly defined. We show that B-cell-specific deletion of Max has a modest effect on B-cell development but completely abrogates Eµ-Myc-driven lymphomagenesis. While Max loss affects only a few hundred genes in normal B cells, it leads to the global down-regulation of Myc-activated genes in premalignant Eµ-Myc cells. We show that the balance between MYC–MAX and MNT–MAX interactions in B cells shifts in premalignant B cells toward a MYC-driven transcriptional program. Moreover, we found that MAX loss leads to a significant reduction in MYC protein levels and down-regulation of direct transcriptional targets, including regulators of MYC stability. This phenomenon is also observed in multiple cell lines treated with MYC–MAX dimerization inhibitors. Our work uncovers a layer of Myc autoregulation critical for lymphomagenesis yet partly dispensable for normal development.

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The MAX protein was first identified as a specific dimerization partner with members of the MYC oncprotein family [MYC, MYCN, and MYCL]. Like MYC, MAX is a member of the basic region helix–loop–helix zipper (bHLH) class of transcriptional regulators, and the association of MYC with MAX is mediated by heterodimerization between their two HLH domains. MYC–MAX heterodimers bind DNA through direct contact of each bHLH basic region with the major groove of E-box DNA sequences (CANNTG) [for reviews, see Conacci-Sorrell et al. 2014; Carroll et al. 2018]. MYC does not homodimerize or bind DNA under physiological conditions and, aside from MAX, no other bHLH proteins have been compellingly demonstrated to dimerize with MYC. Because mutations in the MYC bHLH that prevent association with MAX also block MYC’s major biological activities, it has been generally assumed that MAX is required for MYC function. Indeed, studies in the 1990s demonstrated that heterodimerization with MAX is required for MYC’s DNA-binding and transcriptional activities as well as for cell transformation [Blackwood and Eisenman 1991; Blackwood et al. 1992; Kretzner et al. 1992; Amati et al. 1993]. Moreover, targeted deletion of Max in mice results in early postimplantation lethality, consistent with essential functions for Myc and MycN during embryonic development [Shen-li et al. 2000]. In addition to dimerizing with MYC family proteins, MAX also forms E-box DNA-binding heterodimers with the MXD family and MNT and MGA proteins, all of which act as transcriptional repressors.

Despite the apparent centrality of MAX for the functions of multiple bHLH transcription factors, there is evidence that MAX loss of function can be tolerated and even oncogenic in several biological contexts. For example, pheochromocytoma cell lines can proliferate in the absence of MAX, and a subset of familial pheochromocytomas is strongly associated with inactivation of MAX [Hopewell and Ziff 1995; Comino-Méndez et al. 2011]. In addition, ~6% of human small cell lung carcinomas [SCLC] exhibit loss of MAX, and introduction of MAX into human SCLC lines lacking MAX arrests growth [Romero et al. 2014]. Last, in Drosophila melanogaster, larval development is less compromised by loss of MAX than by loss of MYC, and several critical activities of MYC appear unaffected by MAX inactivation [Steiger et al. 2008]. These findings suggest that there are functions of MYC independent of MAX and that loss of MAX in some settings can promote oncogenic conversion.

To investigate a MAX-independent role in MYC-induced oncogenesis, we turned to Eµ-Myc transgenic mice, which model the 8;14 translocation found in Burkitt’s B-cell lymphomas and have provided many insights...
into MYC-driven lymphomagenesis. The overexpression of MYC produces a polyclonal increase in pre-B cells in young mice, accompanied by reduced differentiation to mature B cells [Harris et al. 1988]. Earlier work using an Env-Max transgene established that overexpression of MAX alone in murine lymphoid cells is nononcogenic and results in reduced B-cell proliferation and numbers. Importantly, in the context of an Env-Myc transgene, augmented expression of MAX also attenuated B-cell lymphomagenesis and reduced lymphoproliferation [Lindeman et al. 1995], indicating that the ratio of MYC:MAX expression levels can influence MYC function. However, the requirement for endogenous MAX in MYC-induced tumorigenesis has not been determined. To address these questions, we generated a conditional Max allele to elucidate MAX function in lymphomagenesis and in B-cell homeostasis.

Results

Max deletion partially impairs B-cell development

We constructed a Max targeting vector by inserting loxP sites flanking exon 4 within a full-length Max genomic clone. This region encodes nearly the entire helix 2 leucine zipper region of Max necessary for dimerization with MYC and other bHLHZ proteins [Fig. 1A], and its Cre-mediated deletion results in a frameshift and truncation within exon 5, leading to a 127-amino-acid protein lacking the HLHZ domain. Expression of Cre in Maxfl/fl embryos results in a frameshift and truncated protein [MaxΔ/+], and these ES cells were used to produce chimeric mice. Extensive intercrossing of MaxΔ/+ F1 mice failed to produce any homozygous Max-null offspring, consistent with a previous report [Supplemental Fig. S1A; Shen-li et al. 2000].

We next crossed MaxΔ/Δ mice with hemizygous MaxΔ/Δ mbl-Cre transgenic mice. Expression of the mbl gene is restricted to B cells beginning at the early pro-B-cell stage, and mbl-Cre and has been used extensively to study B-cell development and function (Hobeika et al. 2006). MAX protein was detected in B220+ splenocytes in MaxΔ/Δ mice (referred to here as Max WT [wild type]) using an antibody against the C terminus, while mbl-Cre; MaxΔ/Δ cells (referred to as Max knockout) did not express any protein reactive with the antibody (Fig. 1B).

We examined the consequences of Max deletion on normal B-cell development by comparing Max WT with Max knockout mice. Using flow cytometry to assess cell subpopulations in the B-cell lineage [Supplemental Fig. S1B], we noted a significant decrease in the numbers of B220-positive, IgM+, and IgM+ B cells from Max knockout relative to WT [Fig. 1C]. Notably, B220+ IgM+ B cells [pre-B cells] were nearly 10-fold lower in Max knockout samples than in Max WT [Fig. 1D; Supplemental Table S1]. More detailed analysis of different stages of B-cell development showed that while the proportions of prepro-B and pro-B cells were approximately the same in mice of the two genotypes, the percentage of pre-B, immature B, and mature B cells was strikingly diminished in Max knockout mice, indicating that loss of Max results in a significant block in pro-B-to-pre-B-cell differentiation [Fig. 1D; Supplemental Table S1]. This block in development is similar to that seen upon Myc loss in B cells (Habib et al. 2007). In

Figure 1. Conditional deletion of Max in the B-cell lineage. (A) Schematic depicting the location of loxP sites at the Max locus. (B) Representative immunoblots for MAX in B220+ splenocytes from MaxΔ/Δ [wild-type [WT]] and MaxΔ/Δ mbl-Cre [knockout [KO]] animals. (C) Representative flow plots showing B220+ and IgM+ populations in CD45 gated bone marrow (BM) cells. (D) Quantification of B lymphocyte precursor populations in Max WT (n = 8) and knockout (n = 6) BM. (E) Dual immunofluorescence (IF) for MAX and B220 in spleens. Quantification of MAX+ B220+ cells from Max knockout spleens. n = 3. Yellow arrowheads indicate MAX+ B220+ cells in Max knockout. Total number of splenocytes [F] and CD19+ B220+ cells [G] in Max WT and knockout mice. WT n = 8; knockout n = 9. (H) IF staining for B220 and proliferation marker Ki67 in Max WT and knockout spleens. [I] IF staining of germinal centers [PNA] for Max WT and knockout spleens. Representative image. n = 3 animals per genotype. Scale bars, 100 µM. All error bars represent SEM.
addition, bone marrow (BM) precursors from Max knockout mice failed to efficiently differentiate into B220+ cells upon treatment with IL-7 in vitro [Supplemental Fig. S1C, D]. We also noted a compensatory increase in the percentages of CD3+ T cells and CD11b+ myeloid cells [Supplemental Fig. S1E]. To study mature B-cell populations, we examined spleens of Max knockout mice. A majority (~86%) of B220+ cells lacked detectable MAX staining in their nuclei [Fig. 1E], accompanied by reduced numbers of total [Fig. 1F] and CD19+ B220+ splenocytes in Max knockout spleens [Fig. 1G, Supplemental Fig. S1F]. Indeed, B220+ areas in the spleen displayed reduced Ki67 staining, especially in regions corresponding to germinal centers (GCs) [Fig. 1H, Supplemental Fig. S1G]. Since MYC is known to play a critical role in GC formation and maintenance [Calado et al. 2012], we stained spleens for PNA, a GC B-cell marker. Although nonimmunized mice have relatively few GCs, we still observed positive staining for PNA in Max WT mice, which was absent in Max knockout spleens [Fig. 1I], suggesting that MAX plays a critical role in GC formation. Our results suggest that Max is not essential for B lymphocyte development and differentiation. Of note, our data on BM development, splenic B-cell numbers, and GC phenotypes are largely consistent with a recent study from the de Alborn laboratory [Pérez-Olivares et al. 2018] using CD19-cre to delete Max.

We also found that depletion of Max in the T-cell lineage using lck-cre led to marginally impaired differentiation of double-negative (DN) to double-positive (DP) thymocytes [Supplemental Fig. S1H]. Taken together, our data indicate that Max loss attenuates overall lymphocyte development rather than completely abolishing it.

Requirement for Max in activated lymphocytes and Eμ-Myc-induced lymphomagenesis

To study the requirement for Max in situations where Myc expression is elevated, we activated B220+ B cells in vitro using bacterial lipopolysaccharides (LPSs). We found activation to be severely compromised in Max knockout mice, and B cells exhibited little increase in cell size [Fig. 2A]. This was accompanied by reduced cell numbers, viability, and apoptosis compared with LPS-treated controls [Fig. 2B–D, Supplemental Fig. S2E]. Max knockout B cells also failed to proliferate when activated with IgM-µ or a combination of anti-CD40/ IL-4 ex vivo [Supplemental Fig. S2A–E]. Similar effects on proliferation and cell size were observed in Maxfl/fl lck-Cre CD3+ T lymphocytes stimulated with anti-CD3 and anti-CD28 [Supplemental Fig. S2F, G].

To ascertain whether Max loss affects MYC-driven lymphomagenesis, we crossed our Max conditional allele with Eμ-Myc mice in which the Myc transgene is predominantly restricted to the B lymphoid lineage [Adams et al. 1985; Harris et al. 1988]. While all of the Eμ-Myc Max WT animals developed B-cell lymphomas with a median survival of 97 d, none of the Eμ-Myc Max knockout mice developed lymphoma even out to 300 d [Fig. 2E, data not shown]. Premalignant Eμ-Myc BM B-cell precursors exhibited developmental defects, including a block at the pre-B-cell stage [Langdon et al. 1986]; however, our analysis of BM populations failed to show an expansion of a pre-B-cell population in Eμ-Myc Max knockout mice [Fig. 2F, Supplemental Table S1]. In addition, B220+ cells from Eμ-Myc Max knockout mice were smaller than Eμ-Myc controls and exhibited decreased total RNA content [Supplemental Fig. S2H, I]. Augmented spleen size and splenocyte numbers are typical of Eμ-Myc-induced B-cell lymphomagenesis [Harris et al. 1988]. Compared with Eμ-Myc Max knockout mice, Eμ-Myc WT mice exhibited increased spleen size and significantly increased numbers and cell size of total and B220+ CD19+ splenocytes [Fig. 2G–J]. Eμ-Myc Max knockout B220+ splenocytes also had an increased proportion of mature IgM- and IgD-positive B cells when compared with WT controls [Fig. 2K], indicating that the Max knockout cells do not exhibit the defects in differentiation characteristic of premalignant Eμ-Myc cells. These data demonstrate that Eμ-Myc lymphomagenesis is severely compromised in the absence of Max.

Max loss affects E2F targets and proinflammatory pathways in B cells

To determine the effects of Max loss on the transcriptional program of normal and Eμ-Myc-expressing B cells, we performed RNA sequencing [RNA-seq] using B220+ cells of the four genotypes described above (normalized counts in Supplemental Table S2). Strikingly, Max deletion in normal B cells doesn’t completely phenocopy Myc loss in B cells. First, Max deletion does not perturb the expression of B-cell lineage transcription factors [Supplemental Fig. S3A], in contrast to Myc loss, which was shown previously to down-regulate expression of factors such as EBF and PAX5 [Vallespinós et al. 2011]. Second, Max knockout B cells exhibit a significant up-regulation of genes involved in proinflammatory pathways [Fig. 3A; Supplemental Fig. S3B]. Max knockout B220+ cells were also consistently larger than controls [Supplemental Fig. S3C]. Taken together, our data suggest that these cells are in a quiescent state, possibly related to the loss of repressive MAX dimers [e.g., MNT–MAX see below], compensating for the absence of MYC–MAX function. Interestingly, a majority of genes that exhibit decreased expression upon Max knockout are cell cycle-related [Fig. 3B] and include E2F targets. Consistent with the loss of GC cells [Fig. 1H, I], a group of genes crucial for GC maintenance is also down-regulated in Max knockout B cells [Supplemental Fig. S3D]. Not surprisingly, several gene sets that were significantly enriched for in our analysis overlapped with those reported recently in Max knockout B cells [Supplemental Fig. S3E, F; Pérez-Olivares et al. 2018]. We think it is likely that any differences in the differentially expressed genes in our study compared with that of Pérez-Olivares et al. [2018] are due to the use of distinct Cre drivers [CD19 vs. mb1].

To identify direct MYC–MAX and MNT–MAX targets, we carried out genomic occupancy analysis using CUT&RUN (cleavage under targets and release using nuclease) on Max WT and knockout B cells. CUT&RUN sequencing uses a combination of antibody targeted
controlled cleavage and nuclease-based release of DNA fragments to analyze protein occupancy on DNA (Skene and Henikoff 2017; Janssens et al. 2018). Peaks that were called in two independent experiments were used for analysis. MAX was found to bind to ∼11,000 gene loci [within ±5 kb of the transcription start site (TSS) or within the gene body] in WT B cells. There was an overall reduction in MAX and MNT binding in Max knockout cells when compared with WT (Fig. 3C). MYC occupancy appeared to be lower than MAX and MNT occupancy in WT B cells but was also markedly decreased in MAX-null B cells (Fig. 3C). The decrease in MAX binding at a representative gene (Cbx5) is shown in Figure 3D. The residual MAX binding observed in Max knockout B cells was most likely derived from a fraction of B cells that escaped Cre-mediated deletion of Max [-14%] (see Fig. 1E). In addition to the E-box motif, MEME and HOMER analysis revealed a significant enrichment for E2F motifs in MAX-bound regions in WT B cells compared with the IgG control (Fig. 3E; Supplemental Fig. S3B). Gene set enrichment analysis revealed that cell cycle, E2F target, and MYC target gene sets were enriched for in the MNT–MAX–MYC-bound gene populations (Fig. 3F). Around 40% of inflammatory response-related genes appeared to be directly bound by MNT–MAX. Therefore, the effects of Max inactivation on up-regulation of inflammatory genes [Fig. 3A; Supplemental Fig. S3B] are likely direct.

When we correlated MAX occupancy with gene expression changes in MAX-null cells, we found that ∼76% of the genes down-regulated in MAX-null cells were occupied by MAX in WT cells, while ∼65% of up-regulated genes were directly bound by MAX (Fig. 3G). Remarkably, 84% of MAX-bound genes were not differentially expressed in Max knockout B cells, including the majority of MAX-bound E2F target genes. Expression of E2F1-3 and phosphorylation of Rb were also unaffected by Max loss [Supplemental Fig. S4C,D]. The lack of change in expression of a majority of MAX-bound genes may be due to loss of binding by both the transcriptionally activating [MYC] and repressive [MXD, MNT, and MGA] heterodimerization partners of MAX and is consistent with the weak effects of MAX deletion on B-cell differentiation. Consistent with this idea, we observed that the expression of MYC and E2F target genes that are cobound by MYC, MNT, and MAX remained unchanged upon the deletion of Max [Supplemental Fig. S4E]. Another possibility is that E2Fs themselves can compensate for loss of MYC–MAX at key promoters. Indeed, we see E2F1 occupancy at target genes Cbx5 and Ncl in Max knockout cells, although at reduced levels. This is in contrast to the near elimination of MYC, MAX, and MNT occupancy [Fig. 3D; Supplemental Fig. S4F].

Max loss destabilizes MYC protein

To determine whether the gene expression changes and decreased MYC occupancy in Max knockout cells are solely due to the inability of MYC to bind DNA without MAX, we measured MYC levels in Max knockout B cells. While Myc mRNA levels were not affected by Max deletion [Fig. 4A], we observed a striking reduction in MYC protein levels in MAX knockout cells [Fig. 4B; Supplemental Fig. S4G]. This result was surprising in light of previous studies indicating that MYC is subject to negative autoregulation in normal B cells (Grignani et al. 1990).
Importantly, we found that treatment of B cells with the proteasomal inhibitor MG132 resulted in near-complete restoration of MYC levels in Max knockout B cells (Fig. 4C,D). Taken together, these results strongly suggest that MAX influences MYC stability.

Phosphorylation of AKT (p-AKT) and subsequent phosphorylation of GSK3β (p-GSK3β) at Ser9 is known to increase MYC stability (Cross et al. 1995; Farrell and Sears 2014). Because levels of both p-AKT Ser473 and p-GSK3β [Ser9] appeared to be higher in MAX knockouts than in WT B cells [Supplemental Fig. S4D,H], we surmise that MAX loss regulates factors independent of GSK3β-mediated regulation of MYC stability. To obtain a snapshot of MYC stability at a single cell level, we stained splenic

Figure 3. Gene expression profiling and genomic occupancy of MAX in B cells. (A,B) Hallmark gene set enrichment for pathways up-regulated [A] and down-regulated [B] in Max knockout [KO] B cells relative to WT B cells. (C) Next-generation sequencing [NGS] plots depicting genomic occupancy of MYC, MAX, MNT, and E2F1 in Max WT and knockout B cells ranked on expression changes. (D) Representative peaks for MAX, MYC, and MNT at E2F target Cbx5. (E) Motifs significantly enriched at MAX-bound genes. (F) Gene set enrichment for pathways enriched in MNT–MAX–MYC-bound genes. (G) Overlap of MAX-bound genes with genes that are differentially expressed in Max knockout B cells (false discovery rate <0.05 cutoff for differential expression).

Figure 4. MYC stability upon MAX loss in normal and premalignant B cells. [A,B] mRNA [A] and protein [B] levels of Myc in WT and knockout [KO] B cells. n = 4 for WT and knockout. (C,D) Immunoblot (C) and quantification (D) of MYC levels following 2 h of MG132 treatment of Max WT and knockout B cells. n = 3 for WT and knockout. (E,F) Representative micrographs [E] and mean fluorescence intensity quantification [F] of MYC staining in sorted B220+ splenocytes from Max WT and knockout mice. n = 54 WT cells; n = 61 knockout cells. Scale bar, 100 µm. (G,H) Myc mRNA levels [G] and MYC protein levels [H] in Eµ–Myc Max WT and knockout cells. All error bars represent SEM.
B220+ cells from Max WT and knockout mice to assess MYC protein levels and observed a significant reduction in MYC levels in cells from Max knockout mice, similar to immunoblots on whole-cell extracts (Fig. 4E,F; Supplemental Fig. S4I).

We next examined whether Max deletion in premalignant Eµ-Myc cells impacts MYC levels in a similar fashion. Although mRNA levels were reduced by 50% (Fig. 4G; Supplemental Fig. S5A), we observed a striking decrease in MYC protein levels and half-life (Fig. 4H; Supplemental Fig. S5B–D). Overall, this indicated that Max deficiency has a profound effect on MYC stability in both normal and premalignant settings.

**Max loss leads to a global down-regulation of the MYC signature in Eµ-Myc premalignant cells**

Given the profound effect on MYC protein levels, we hypothesized that the loss of MYC stability would have a widespread effect on the transcriptional profile of premalignant Max-null cells. Indeed, in contrast to normal B cells, where the loss of Max affects the expression of ∼550 genes (twofold change cutoff, false discovery rate [FDR] < 0.05), MAX depletion in Eµ-Myc mice causes a dramatic shift in the expression of thousands of genes (Fig. 5A), with Eµ-Myc Max WT cells occupying a space distinct from the other genotypes in a principle component analysis plot (Fig. 5B). Whereas genes up-regulated in
Eu-Myc Max knockout cells showed a close alignment with signatures enriched in normal B cells lacking Max (Fig. 5C), genes down-regulated in Eu-Myc Max knockout cells revealed a significant enrichment for MYC signatures (Fig. 5D). This translated to robust differences in expression where Eu-Myc Max knockout and Max WT profiles closely resemble each other and are nearly the inverse of the Eu-Myc Max WT (Fig. 5E). Interrogation of a publicly available ChIP-seq (chromatin immunoprecipitation [ChIP] combined with high-throughput sequencing) data set in B cells (Sabò et al. 2014) revealed that 73% of the genes known to be bound by MYC in Eu-Myc premalignant cells are differentially expressed in Eu-Myc Max knockout B220+ cells (Fig. 5F,G; Supplemental Fig. S5E). We also observed that in contrast to normal B cells, a substantial proportion of MAX-regulated inflammation-related genes are directly bound by MYC (Fig. 5H). In addition, a large fraction of MYC-bound E2F targets (Fig. 5I; Kuleshov et al. 2016) are down-regulated in Eu-Myc Max knockout cells. This may be due partly to the decrease in E2F1-3 expression in knockout cells (Supplemental Fig. S5F).

MAX loss or inhibition of MYC–MAX dimerization results in repression of MYC stability factors

Since MAX loss appeared to have a significant impact on MYC stability, we wanted to identify effectors downstream from MYC–MAX that may form a positive feedback loop to maintain MYC protein levels. While MYC proteins generally have short half-lives (on the order of 20–30 min), MYC half-life increases in several Burkitt’s lymphoma lines and ES cells (Hann and Eisenman 1984; Gregory and Hann 2000; Cartwright et al. 2005). The rate of MYC protein degradation is mediated by several factors that interfere with signals triggering MYC ubiquitination and proteasomal degradation (Farrell and Sears 2014). We noted in our RNA-seq data that genes encoding MYC stability factors, including Btrc, Cip2a, and Set, are up-regulated in Eu-Myc cells relative to normal B220+ cells and down-regulated in Eu-Myc Max knockout B cells (Fig. 6A). Moreover, the promoters of these genes are directly bound by MYC in premalignant cells (Fig. 6B; Supplemental Fig. S6A). CIP2A and SET are inhibitors of protein phosphatase 2A (PP2A), which normally dephosphorylates the stabilizing phospho-Ser62 (S62) within the conserved Myc box 1 phospho-degron. CIP2A and SET are often overexpressed in tumors and block PP2A activity, resulting in persistence of phospho-S62 and MYC activity, contributing to the decreased stability of MYC.

To confirm whether these genes are regulated by MYC–MAX in tumor lines, we treated Daudi and P493 human B-cell lymphoma lines (Schuhmacher et al. 2001) with 10058-F4 [referred to here as Myci], a small molecule reported to inhibit MYC–MAX heterodimerization (Yin et al. 2003). We observed a decrease in MYC protein levels, accompanied by reduced proliferation and a down-regulation of the stability factors in Myci-treated cells (Fig. 6C).

MYC degradation in inhibitor-treated cells is dependent on FBW7 and phosphorylation of MYC T58

To confirm that the decrease in MYC protein levels is indeed through the loss of multiple stability factors that affect Fbw7 activity, we examined MYC levels in HCT116 colon cancer cells lacking the FBW7 ubiquitin ligase known to target MYC for degradation (Welker et al. 2004; Yada et al. 2004). We found that MYC turnover in FBW7−/− HCT116 is largely unaffected by treatment with the dimerization inhibitor compared with the rapid turnover in control HCT116 cells treated with inhibitor (Fig. 7A–C). Moreover, CIP2A levels are unchanged in FBW7-null cells upon Myci treatment, whereas they decrease in Myci-treated WT HCT116 cells (Fig. 7D; Supplemental Fig. S7I). This supports the notion that disruption of MYC–MAX dimerization does not affect MYC–MAX-mediated transcription alone; it also augments degradation of MYC in cells. Our data suggest that the activity of the FBW7–SCF ubiquitin complex significantly contributes to the decreased stability of MYC.

To complement these data, we asked whether overexpression of a T58A phospho-site mutant would have a similar effect. We ectopically expressed either MYC or MYC{T58A} in HCT116 cells and treated them with a higher concentration (75 µM) of Myci to compensate for higher levels of MYC in these cells. Cells were harvested after 24 h, and we found that MYC{T58A}-expressing cells maintained higher levels of MYC and CIP2A expression upon Myci treatment when compared with MYC-expressing controls (Fig. 7E,F; Supplemental Fig. S7K).
Yet more severe phenotype than the one that we observed. Several lines of evidence suggest that the loss of repression may also underlie the indirect activation of proinflammatory pathways in normal B cells, and its binding is partially attenuated in Eµ-Max-deficient retinal progenitor cells (Chen et al. 2009). Similarly, combined deletion of Myc and E2f1-3 disrupts crypt villus integrity in the intestine, whereas neither Myc ablation nor E2f1-3 ablation alone has an effect (Liu et al. 2015). Our own data suggest a similar compensatory mechanism might exist in B cells, as E2f1 still binds E2F targets such as Cbx5 and Ncl even upon Max deletion. However, we did observe nominal effects on normal B-cell development upon B-cell-specific deletion of Max. This is reasonable because a significant overlap between E2F- and Myc-regulated genes has been noted in several systems. For example, Myc drives proliferation in E2f1-3-deficient retinal progenitor cells (Chen et al. 2009). Simultaneously, combined deletion of Myc and E2f1-3 disrupts crypt villus integrity in the intestine, whereas neither Myc ablation nor E2f1-3 ablation alone has an effect (Liu et al. 2015). Our own data suggest a similar compensatory mechanism might exist in B cells, as E2f1 still binds E2F targets such as Cbx5 and Ncl even upon Max deletion.

### Discussion

Our in vivo studies on Max loss in normal B cells and Eµ-Myc-driven lymphomagenesis offer several insights into the context-dependent function of Max. Surprisingly, the major hallmarks of normal B-cell differentiation are largely unperturbed by Max loss. The fact that Myc protein is destabilized and nearly absent in the Max-null B cells makes it unlikely that Myc is functioning independently of Max. The likely scenario is that in Max knockout cells, loss of repressive Max–MXD heterodimers may serve to partially compensate for diminished Myc activity. For example, MNT binds thousands of promoters in normal B cells, and its binding is partially attenuated in Max knockout cells (Fig. 3C). This alleviation of repression may also underlie the indirect activation of proinflammatory pathways in Max-null B cells, although it is certainly possible that loss of Myc–Max activation might also contribute to this phenotype (Casey et al. 2016). Several lines of evidence suggest that the loss of repressive heterodimers might partly rescue the loss of Myc–Max. First, deletion of Myc in B cells has a similar yet more severe phenotype than the one that we observed upon depletion of Max (Habib et al. 2007; Vallespinós et al. 2011; Pérez-Olivares et al. 2018). Second, this result is similar to that seen upon inactivation of Max in Drosophila melanogaster, where larval development is less compromised by loss of Max than by loss of Myc (Steiger et al. 2008). Moreover, the arrest of larval growth as a result of Myc deletion is partially rescued by loss of Mnt. Another possibility is that the requirement for Myc activity is rather minimal or readily compensated for by factors such as E2Fs in normal B-cell progenitors. This is reasonable because a significant overlap between E2F- and Myc-regulated genes has been noted in several systems. For example, Myc drives proliferation in E2f1-3-deficient retinal progenitor cells (Chen et al. 2009). Simultaneously, combined deletion of Myc and E2f1-3 disrupts crypt villus integrity in the intestine, whereas neither Myc ablation nor E2f1-3 ablation alone has an effect (Liu et al. 2015). Our own data suggest a similar compensatory mechanism might exist in B cells, as E2f1 still binds E2F targets such as Cbx5 and Ncl even upon Max deletion. However, we did observe nominal effects on normal B-cell development upon B-cell-specific deletion of Max. This is evidenced by decreased B-cell population sizes in both the BM and spleen, with a nearly complete loss of GC cells in the latter. Overall, our findings suggest that Myc–Max genomic binding and transcriptional activity is not absolutely required for several key aspects of early B-cell differentiation. Nonetheless, deletion of Max attenuates certain processes where Myc is critically required, such as GC formation.

Consistent with the GC phenotype, we observed a heightened requirement for Max in situations where Myc levels are elevated. Max-null B cells fail to grow in size or proliferate upon ex vivo stimulation with agents such as LPS and CD40/IL-4. In the context of Eµ-Myc,
depletion of Max leads to an unstable pool of MYC and largely reverses the transcriptional effects of MYC, leading to complete abrogation Eµ–Myc-driven lymphomagenesis. In addition, we found evidence for MYC–MAX cooperation with E2F factors in driving the proliferation of premalignant B cells. Max loss leads to a down-regulation of E2F1-3 and subsequent expression changes of several E2F targets. The inability of Eµ-Myc Max knockout B220+ cells to undergo malignant transformation strongly suggests that Myc-driven tumorigenesis in vivo absolutely requires Max. This is in contrast to cancers such as GIST (gastrointestinal stromal tumors), where Max loss drives proliferation (Schaefer et al. 2017). A similar tumor-suppressive role has been ascribed to Max in SCLC (Romero et al. 2014). Based on our results, it is reasonable to assume that these tumors are not dependent on MYC for their growth. Indeed, inactivating mutations of MAX are mutually exclusive with MYC paralog amplifications (Romero et al. 2014). In such a situation, MXD–MAX genomic binding and repression might be more widespread than MYC–MAX activity. Hence, Max loss in these tumors could conceivably lead to derepression at subsets of E-box-containing promoters, resulting in activation of pro-oncogenic pathways.

Our experiments across a broad spectrum of tumor lines revealed that disruption of MYC–MAX dimerization has an effect on MYC stability similar to those seen in vivo. This is accompanied by a decrease in expression of genes that promote MYC stability. In addition, we observed that MYC degradation is reduced in inhibitor-treated FBW7−/− or MYC T58A cells. Taken together, these data indicate that MYC stability is at least partially regulated via an FBW7-dependent degradation pathway. Recent studies have shown that MYC forms phase-separated structures on chromatin (Boija et al. 2018). Failure to dimerize with MAX might hinder MYC transcription-independent functions such as Cip2a and Set. Disruption of this circuit via Max deletion leads to destabilization of MYC protein and loss of the MYC signature expression. Hence, no tumors arise in knockout mice.
the consequences of MYC inhibition in cancers where PP2A is lost and/or regulators such as CIP2A and SET are overexpressed. However, it is also possible that MYC activation of several factors simultaneously reinforces MYC stability so deregulation of any one factor might not be sufficient to reduce MYC levels.

In summary, our data suggest that loss of Max in vivo disables MYC activity through inhibition of direct MYC association with genomic DNA and through destabilization of the MYC protein itself. We surmise that increased MYC degradation is facilitated at least in part by decreased MYC–MAX activity at the promoters of genes such as Cip2a, whose protein products normally serve to stabilize MYC by attenuating FBW7-mediated proteosomal degradation [Fig. 7G; Junttila et al. 2007]. In this scenario, MYC–MAX heterodimers drive a feed-forward circuit that reinforces high MYC expression in tumors as diverse as B-cell lymphomas and colon adenocarcinomas. In fact, a recent study shows that MYC regulates MAX and the broader MYC network.

Ma and the integrated context-dependent functions of circuitry and the feedback regulation might not be as crucial except in contexts where B cells are activated (Fig. 7G). Taken together, our data underscore the complex circuitry of the MYC network.

Materials and methods

Mouse strains and tissue processing

All mice were housed and treated according to the guidelines provided by the Fred Hutchinson Institutional Animal Care and Use Committee. For Max conditional mice, clonal G418R targeted ES cell lines were produced, and integration of the targeting vector was verified by genomic PCR and Southern blotting. Eµ-Myc mice were purchased from Jackson Laboratories. Max<sup>fl/fl</sup>, m1-cre, Eµ-Myc mice were maintained on a mixed 129/C57BL6 background after crossing. For BM studies, cells from 5- to 10-wk-old mice were flushed from femurs and tibiae using a 27.5-gauge needle under sterile conditions for subsequent use in stimulation experiments and flow cytometric analyses. Splenocytes and thymic tissue were harvested under aseptic conditions from 5- to 10-wk-old mice, and single-cell suspensions were made for isolation of purified populations. For immunohistochemistry and immunofluorescence, spleens were fixed in formalin and embedded in paraffin blocks.

Cell culture and in vitro experiments

Daudi, P493-6, and SKNBE cells were grown in RPMI with 10% FBS. NCI-H23, P53N, and HCT116, PT67, and 293FT lines were maintained in DMEM with 10% FBS. NCI-H2141 cells were cultured in DMEM with 20% FBS supplemented with insulin and pyruvate. For MYC and MYC T58A expression, retrovirus was made using the PT67 packaging line with pBabe-puro-MYC and pBabe-puro-MYCT58A retroviral constructs. HCT116 cells were then transduced with pBabe-puro-MYC and pBabe-puro-MYCT58A retrovirus, and experiments were performed after puromycin selection for at least 3 d. For Omomyc studies, pSLIK-hygro-OMOMYC and pSLIK-hygroGFP lentiviral particles were made using 293FT cells and p-VSV-G and pPAX2 packaging vectors. HCT116 cells were transduced, and experiments were performed after 100-µg/mL hygromycin selection for 1 wk. Omomyc expression was induced by treatment with 2 µg/mL doxycycline for 2 d.

Flow cytometry and cell sorting

For B-cell developmental studies, cells harvested from the BM and spleen were stained with fluorochrome-conjugated antibodies against B-cell lineage-specific markers (B220, CD19, IgM, and IgD), pan-T cell marker CD3, and myeloid marker CD11b. Viability was assessed using DAPI, and leukocytes were gated upon using forward and side scatter parameters. For mature B cell population studies, splenocytes were stained with fluorochrome-conjugated antibodies against B220, CD19, IgM, and IgD. B cells were sorted from spleens using AutoMACS mouse B220<sup>+</sup> beads. Purified B220<sup>+</sup> cells were then used for imaging, ex vivo stimulation, RNA-seq, and CUT&RUN experiments. For T lymphocyte studies, thymocytes were stained with antibodies against CD4 and CD8. All populations were analyzed on a BD FACs Canto II. See Supplemental Table S4 for details on the antibodies used.

Cell growth assays

Trypan blue-negative cells were counted at fixed time points to assess cell growth. Cell Titer Glo and Caspase Glo luciferase assays (Promega) were used to assess cell growth and apoptosis for ex vivo stimulated B cells. For growth curves of adherent cell lines, cells were seeded in 96-well dishes and imaged at fixed time intervals on either an Incucyte S3 or Incucyte zoom. Percent confluence was used as a measure of cell growth.

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed on 5-µm-thick paraffin-embedded mouse spleen sections. Following heat-induced antigen retrieval, sections were incubated with primary antibodies followed by Alexa-conjugated secondary antibodies [Invitrogen]. For immunofluorescent staining, sorted cells were cytospun on charged slides and fixed in 4% formaldehyde. Following primary incubation, Alexa-conjugated secondary antibodies [Invitrogen] were used, and slides were mounted using Prolong Gold antifade reagent with DAPI. Images were acquired using a Nikon E800 microscope, and image analysis and intensity measurements were performed using ImageJ. See Supplemental Table S4 for a list of the antibodies used.

Western blots and inhibitor studies

For Western blots, cells were lysed in RIPA buffer with protease and phosphatase inhibitors and reduced in LDS buffer. For inhibitor studies, cells were treated with the MYC inhibitor 10058-F4 or the Max probe MS-008 for 2 d prior to harvest unless noted otherwise. For cycloheximide chase experiments, cells were treated with 5 µg/µL cycloheximide and immediately lysed in RIPA buffer at specified time points. For MG132 studies, cells were treated with 10 µM MG132 for 2 h prior to lysis. Densitometry for all
blots was performed using ImageJ. See Supplemental Table S4 for details on the antibodies used.

**qRT-PCR, RNA-seq, and analysis**

All RNA isolation was performed using the Direct-zol RNA mini-prep kit (Zymo Research). For qRT-PCR, 500 ng to 2 µg of input total RNA was used, cDNA was generated using the ReverTaid cDNA synthesis kit (Thermo Fisher), and a Bio-Rad iCycler was used. Either SYBR Green or FAM probe-based methods were used. For RNA-seq, B220+ cells were purified using B220+ microbeads (AutoMACS) from spleens of 5- to 6-wk-old mice from all genotypes. Following RNA isolation, total RNA integrity was checked using an Agilent 4200 TapeStation and quantified using a Trinean DropSense96 spectrophotometer (Caliper Life Sciences). Libraries were prepared using the TruSeq version 2 RNA sample preparation kit with 500 ng of input RNA. Paired-end sequencing was performed on an Illumina HiSeq 2500. Reads that didn’t pass Illumina’s base call quality threshold were removed. Reads were then aligned to mm10 mouse reference genome using TopHat version 2.1.0. Counts were generated for each gene using htseq-count version 0.6.1p1 (using the “intersection-strict” overlapping mode). Genes that didn’t have at least one count per million in at least three samples were removed. Data were normalized, and comparisons were conducted using the exact test method in edgeR version 3.18.1. Gene ontology analysis was performed using hallmark data sets on mSigDB (Subramanian et al. 2005; Liberzon et al. 2015). Heat maps were generated using Morpheus [https://software.broadinstitute.org/morpheus].

**CUT&RUN studies**

For chromatin occupancy studies, primary mouse B cells isolated from spleens were prepared fresh. Cells were bound to ConA beads, permeabilized, and incubated overnight with antibodies against MYC, MAX, MNT, and E2F1. One million cells were used per immunoprecipitation, and the CUT&RUN protocol was followed (Janssens et al. 2018). Using an Illumina HiSeq 2500 instrument, 25 x 25 paired-end sequencing was performed (5 million–10 million reads), and sequences were aligned to the mm10 reference genome assembly using Bowtie2.

Normalization was performed based on library size. Peak calling used a threshold peak calling script to differentiate signal to noise (Kasinathan et al. 2014). This processing was carried out with Bedtools, custom R scripts defining genome position, and the GenomicRanges R package. Peaks were identified as being associated with a gene if they were within ±5 kb from the TSS. For MYC, MNT, and MAX peak calling in WT B cells, peaks called in two independent experiments were intersected to mitigate background issues. Genomic plots were made using ngs.plot (Shen et al. 2014) or the R package ggplot2. Heat maps for genomic binding were made ranking genes according to log fold change in expression from the RNA-seq experiment. De novo enrichment for sequence specificity was determined using Homer (Heinz et al. 2010) and MEME-Chip [Machanick and Bailey 2011].

**Data availability**

MYC-binding data from premalignant cells were obtained from Sabó et al. [2014] (Gene Expression Omnibus [GEO] accession no. GSE51004; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51004). RNA-seq data from this study are available from GEO under accession number GSE132773. CUT&RUN data from this study are available from GEO under accession number GSE132967.

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**Author contributions** H.M. and R.N.E. designed the study and cowrote the manuscript. H.M., P.-F.C., E.B., and J.T.C. performed and analyzed experiments. B.F. provided bioinformatics support and analyzed CUT&RUN data. D.J. and S.H. provided essential reagents and executed the CUT&RUN experiments. All authors discussed the results and read the manuscript.

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