TITLE

E2F4 plays a key role in Burkitt lymphoma tumorigenesis

RUNNING TITLE

E2F4 deregulation in Burkitt lymphoma

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ABSTRACT

Sporadic Burkitt lymphoma (sBL) is a rapidly growing B-cell non-Hodgkin's lymphoma whose treatment requires highly aggressive therapies that often result severely toxic. Identification of proteins whose expression or function is deregulated in sBL and play a role in its formation could facilitate development of less toxic therapies. We have previously shown that *E2F1* expression is deregulated in sBL. We have now investigated the mechanisms underlying *E2F1* deregulation and found that the E2F sites in its promoter fail to repress its transcriptional activity in BL cells and that the transcriptional repressor E2F4 barely interacts with these sites. We also have found that E2F4 protein levels, but not those of its mRNA, are reduced in sBL cell lines relative to immortal B-cell lines. E2F4 protein expression is also decreased in 24 of 26 sBL tumor samples from patients compared to control tissues. Our data demonstrate that enforced E2F4 expression in BL cells not only diminishes E2F1 levels, but also reduces selectively the tumorigenic properties and proliferation of BL cells, while increasing their accumulation in G2/M. Our results therefore point to E2F4 as a target for developing novel and less toxic treatments for sBL.

KEYWORDS

Burkitt; Lymphoma; E2F4; E2F1; Cancer; Cell cycle

INTRODUCTION

Burkitt lymphoma (BL) is a highly aggressive form of mature B-cell non-Hodgkin's lymphoma. The World Health Organization (WHO) recognizes the existence of three clinical BL variants: endemic, sporadic, and immunodefficiency-associated (1). BL is one of the most rapidly growing pediatric tumors, doubling its size every 24 hours (2), and it is rapidly fatal if untreated. Intensive combination chemotherapy is the primary treatment for BL together with intrathecal and systemic chemotherapy for Central Nervous System (CNS) prophylaxis, due to high risk of CNS involvement (3). Radiation therapy is limited to the treatment of overt CNS disease unresponsive to chemotherapy and in certain emergencies (3). Unfortunately, the toxicities reported from these intensive therapies are significant, including neurotoxicities from intrathecal therapy, hematologic toxicity, severe mucositis, cardiac disease, and infertility (4). The design of more efficient, more specific, and less toxic therapies relies on the identification of genes and proteins whose expression or function is affected in BL cells. Since the etiology of sporadic Burkitt lymphoma (sBL) remains largely unknown, a better understanding of the molecular mechanisms underlying sBL lymphomagenesis would undoubtedly help to identify such genes and proteins.

The major pathogenic alteration known to occur in sBL is the translocation of the *C-MYC* locus to one of the three different immunoglobulin loci that leads to C-MYC over-expression (5). However, several studies examining the role of C-MYC in the pathogenesis of BL have concluded that *C-MYC* translocation is not the only critical event (6, 7). In fact, C-MYC over-expression in the absence of additional alterations elicits either cell death (8) or cell growth arrest in the G2 phase of the cell cycle (9). Hence, additional factors likely cooperate with C-MYC in BL lymphomagenesis. We have recently shown that the transcription factor and cell cycle regulator E2F1 is highly

expressed in 100% of BL cell lines and sBL lymphoma specimens tested so far, relative to control cell lines and tissues, and that its elevated expression is required for the formation of this tumor (10).

E2F1 is the founding member of the E2F family of transcription factors. This family is essential for the regulation of cell growth and play an important role in almost every phase of the cell cycle (11, 12). Eight different *E2F* genes (*E2F1* to *E2F8*) and three different *DP* genes belong to this family in mammals (13). E2F factors are divided between transcriptional activators (E2F1, E2F2, and E2F3a) and transcriptional repressors (E2F3b and E2F4 to E2F8). Transcriptional repression by E2F3b, E2F4 and E2F5 seems to be dependent upon their association with members of the pocket protein family that includes the Retinoblastoma protein (pRB), p107 and p130 (14). The interaction of these proteins with E2F factors not only inhibits E2F-mediated transactivation (15), but also recruits histone deacetylases and other chromatin-modifying proteins to the promoters of E2F-regulated genes to enforce their active repression (16).

E2F4 accounts for the majority of E2F proteins throughout the cell cycle (17). In quiescent cells, E2F4 is primarily nuclear and represses transcription of E2F-regulated genes through its recruitment of the Retinoblastoma family members p107 and p130 (14). When Retinoblastoma family members are phosphorylated at the end of G_1 by Cyclin-Dependent Kinases (CDKs) in response to mitogens (18, 19), E2F4 unbinds DNA, dissociates from pocket proteins, and is exported to the cytoplasm (11, 20). Thus, although E2F4 has a transactivation domain, its transcriptional activity is restrained because of its interaction with pocket proteins in the nucleus or because of its translocation to the cytosol when E2F4 is not associated to them (21).

While E2F factors show bimodal actions in rodent models, functioning either as oncogenes or as tumor-suppressors (13), it remains largely unknown how E2F family members affect human tumors. We have recently shown that E2F1 is over-expressed in sBL and that its deregulated expression is central to the formation of this tumor (10). However, the precise mechanisms leading to E2F1 over-expression in sBL have not been identified yet. Here, we show evidence supporting that down-regulation of E2F4 in sBL prevents repression of the *E2F1* promoter, thus leading to increased expression of E2F1 and tumorigenesis in this lymphoma. Hence, these results point to E2F4 as a tumor suppressor in sporadic BL.

MATERIALS AND METHODS

Patients and cells.

All cases consisted of existing frozen de-identified anonymous biopsy specimens obtained from the Spanish Tumor Bank Network in Centro Nacional de Investigaciones Oncológicas (CNIO). Institutional review board approval was obtained for these studies. Biopsy specimens from sBL cases were reviewed by M.A. Piris and S. Montes-Moreno, and characterized according to morphology, C-MYC translocation identification by Fluorescent in situ hybridization and immunostaining of CD10, BCL2, BCL6, Ki67, TCL1, and CD44. Cases with atypical Burkitt Lymphoma morphological features were included only when the rest of phenotypical and cytogenetic criteria were consistent with a diagnosis of BL, according to the criteria of WHO (1, 22). Twenty-six specimens corresponding to sBL from patients, together with reactive tonsils were finally selected. Cases associated with HIV or HCV infections or previous immunosuppressive treatments were excluded. Representative areas of sixty-seven specimens corresponding to Diffuse Large B-Cell Lymphoma were selected to construct tissue microarrays.

BL cell lines DG75, Ramos, Mutu-I, Raji, Rael, Akata, and BL2, as well as lymphoblastoid B-cell lines (LCL) X50-7, JY, Dana, and IB4, were cultured in RPMI-1640, whereas human embryonic kidney 293-T, human breast cancer MCF-7 and mouse fibroblasts NIH-3T3 cell lines were cultured in DMEM. Both media were supplemented with 10% FCS (Life Technologies), 2mM glutamine, 100U/ml penicillin, and 100µg/ml streptomycin.

Quantitative PCR analysis.

Real-time quantitative RT-PCR (q-PCR) was performed as described (10) using TaqMan assays specific for human *E2F1, E2F4, E2F8* and *β-ACTIN* (Life

Technologies). *β-ACTIN* was chosen as a control gene on the basis of its homogeneous expression in used cell lines. Calculations were made from measurements of 3 replicates of each sample. For mRNA stability assays, cell cultures were incubated with the transcription inhibitor actinomycin D (Sigma-Aldrich).

Transfections and reporter gene assays.

BL and LCL cells were transfected by electroporation (23). Transfections included 10μg of pGL2-E2F1-WT or pGL2-E2F1-Null (24) plus either 0.1μg pSV-Renilla and 20μg carrier plasmid [Bluescript (Stratagene)], or 10μg pCEFL-βGal and 10μg carrier plasmid (Bluescript). Firefly and Renilla luciferase activities were assayed using Dual Luciferase Reporter Assay System (Promega) 48 hours after transfection. ß-galactosidase activity was determined as described (25). Firefly luciferase activity was normalized with that of Renilla luciferase or that of β-galactosidase.

Preparation of nuclear extracts and electrophoretic mobility shift analysis (EMSA) assays.

Nuclear extracts were prepared and gel shifts were performed as reported (26) with labeled oligonucleotides encompassing the distal E2F element from the E2F1 promoter (5′-CTGGAGCTCTTTCGCGGCAAAAAGGAG-3′ and 5′-CAGGCTCCTTTTTGCCGCGAAAGAGCT-3′). Antibodies against E2F4, E2F5, pRB, p107 and p130 were from Santa Cruz. Anti-DP1 and pre-immune serum were described (23).

Immunoblotting and immunohistochemistry.

Anti-E2F1, anti-E2F4-E2F6, anti-p130 (all from Santa Cruz), or anti-Tubulin (Sigma-Aldrich), followed by peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Sigma-Aldrich) were used. Chemiluminescent detection reagent (PerkinElmer) was used and the membrane exposed to X-Ray Medical film. For protein

stability assays, cell cultures were incubated with the transduction inhibitor cycloheximide (Sigma-Aldrich).

Paraffin-embedded tissue samples and tissue microarrays were stained with anti-E2F4 (GeneTex), anti-p130 (Santa Cruz), or anti-E2F1 (Santa Cruz) and counterstained with hematoxylin. The specimens were analyzed using an Olympus BX60 microscope (Olympus Optical). Images were photographed using an Olympus DP50 camera (Olympus Europe) and Axiovision version 4.6 software (Imaging Associates), and were adjusted using Adobe Photoshop version 9.0 software (Adobe Systems).

Retrovirus and lentivirus production and infections.

For retrovirus production, human embryonic kidney 293-T cells were co-transfected with pCL-Anfo and either pBabe-puro or pBabe-puro-HA-E2F4 expression vectors using the calcium-phosphate method (23). Lentiviruses bearing *E2F1*-specific shRNAs or scrambled shRNA sequences were produced as described (10) employing MISSION pLKO.1-puro-based vectors (Sigma-Aldrich) TRCN0000039658 and SHC002, respectively. Cell transduction was achieved as described (10). Transduced cells were washed and selected with 1μg/ml puromycin for at least 96 hours.

Cell proliferation and cell cycle analysis.

Cell cycle analysis was performed as described (26). For BL cells, 5 x 10^4 cells in 200μl of culture medium were added to each well of a 96-well flat-bottomed microtiter plate and cultured in triplicate. Cell proliferation was then estimated by [^{3}H]dThd incorporation (27). NIH-3T3 and MCF-7 cell proliferation was estimated by EdU incorporation during 16 hours of culture. Cells were harvested and the incorporated EdU was detected using the Click-iT EdU Alexa fluor 488 Flow Cytometry Assay Kit (Invitrogen).

Transformation assays *in vivo*.

DG75, Mutu-I, or Ramos cells (2 x 10^6 in 0.1ml of phosphate-buffered saline) were injected into 8- to 10-week-old female NOD.CB17-*Prkdc*^{scid}/J mice (Charles River Laboratories). Tumor masses were removed after 3 weeks and weighted. All animal procedures were approved by the institutional review board.

Statistical analysis

All values are expressed as means ± S.D. Differences were evaluated using the Student t test. Statistical significance was assigned at $p<0.05$.

RESULTS

Increased *E2F1* promoter activity in BL cell lines.

We have previously established that E2F1 levels are higher in sBL tumor samples and cell lines than in control tissues and cell lines and that its elevated expression is involved in BL lymphomagenesis (10). To investigate the mechanisms implicated in *E2F1* deregulation in BL, we compared *E2F1* mRNA stability in BL and immortalized, but non-transformed, Lymphoblastoid B-cell lines (LCL). Transcription was blocked in these cells with actinomycin D before assessing RNA expression by qPCR analyses. We found no major differences in reduction of *E2F1* mRNA levels between BL and LCL cell lines following transcription inhibition (Figure 1A). Since these results suggested that *E2F1* mRNA half-life was similar in BL and LCL cell lines, we investigated whether its promoter was more active in BL than in control cell lines. Firefly luciferase reporter plasmids driven by a wild-type (wt) human *E2F1* promoter were transfected in different BL and LCL cell lines. For normalization, Renilla luciferase or ß-galactosidase reporter plasmids under the control of the SV40 promoter or the CMV promoter, respectively, were cotransfected. The normalized activity of the wt *E2F1* promoter was 10-100 times higher in BL than in LCL cell lines when either Renilla luciferase (Figure 1B) or ß-galactosidase (Figure S1) were used as reference. The *E2F1* promoter contains two E2F binding elements (Figure 1C) that negatively regulate its activity in fibroblasts (24). We compared the activity of the wt *E2F1* promoter with that of a modified version (mut) with inactivated E2F elements in both BL and LCL cell lines. The activity of the mut version was higher than that of the wt version in LCL cell lines (Figure 1D), thus suggesting that these E2F elements play a repressor role in these cells. In contrast, the activities of wt and mut versions of the *E2F1* promoter were almost identical in BL cell lines (Figure 1D), indicating that these

E2F sites lacked repressor activity in these cells. Together, these data suggest that the higher expression of *E2F1* in BL relative to LCL cell lines might be due to impaired transcriptional repression through the E2F elements from the *E2F1* promoter in BL cells.

BL and LCL cells show different DNA-protein complex formation patterns.

To investigate whether differences in *E2F1* promoter repression between BL and LCL cell lines might be caused by differences in the set of proteins that interact with the E2F elements, we compared the pattern of protein-DNA complex formation between a radiolabeled oligonucleotide corresponding to the distal E2F element from the *E2F1* promoter (E2F1-d) and nuclear extracts from BL and LCL cell lines. Several groups, including ours, have described the formation of 4 major retarded complexes between E2F sites and nuclear extracts from several cell types, including LCL cell lines (26, 28, 29). These complexes contained E2F associated to p107 and p130 (complex I), E2F bound to pRB (complex II), and either E2F4 (complex III) or E2F5 (complex IV) 'free' from association with pRB family members (26, 28, 29). Accordingly, the interaction of nuclear extracts from various LCL cell lines and the labeled E2F1-d probe gave rise to 4 major retarded complexes (Figure 2A) whose formation was inhibited by an excess of the same unlabeled oligonucleotide (Figure 2B). Remarkably, complex III formation was barely detected when nuclear extracts from BL cell lines were employed (Figures 2A and S2). Formation of complexes I-IV was affected by addition of antibodies against various E2F and pRB family members (Figures 2C, 2D and S2). In particular, formation of complexes I and III was inhibited by addition of antibodies against DP1 and E2F4 subunits when nuclear extracts from LCL cell lines were employed (Figures 2D and S2). Of note, anti-E2F4 antibodies barely inhibited complex I formation when nuclear extracts from BL cell lines were used, but its formation was impaired by addition of

antibodies to DP1, p107 or p130 (Figures 2C and S2). These results suggested that either E2F4 from BL lost its capacity to interact with DNA or that it was almost absent in these cells.

E2F4 is downregulated in sBL.

We compared E2F4 levels in BL and LCL cell lines by immunoblotting and found that E2F4 expression was markedly lower in BL (Figure 3A). In contrast, *E2F4* mRNA levels were similar in both cell types (Figure 3B). Hence, E2F4 protein expression down-regulation in BL cells is not caused by decreased mRNA expression. To ascertain whether protein stabilization was involved in differences in E2F4 expression between these cell types, we analyzed the effect of translation inhibition with cycloheximide on its expression. We found that E2F4 half-life was markedly shorter in BL than in control cell lines (Figure 3C).

We next used immunohistochemistry to compare E2F4 protein levels in control tissues and a collection of 26 biopsy sBL specimens that met the combined morphological, immunohistochemical and cytogenetic criteria for BL according to the WHO classification. We used germinal centres of reactive tonsils as control tissues because they are formed almost exclusively by proliferating B cells. We employed qPCR (Figures 4A and S3A) or immunohistochemistry (Figure S3B) to confirm that E2F1 expression was increased in these samples relative to control tissues. As expected in asynchronously growing cells, E2F4 was readily detected in the nucleus and cytosol of most cells in the germinal center of reactive tonsils (Figure 4). E2F4 levels were much lower in 20 sBL specimens (Figure 4A), moderately lower in four additional samples (Cases 5, 13, 21, and 22; Figure 4A) and relatively normal only in Cases 2 and 14. Of note, E2F4 location was mostly cytosolic in numerous cells of Case 13 (Figure 4). Hence, E2F4 levels or location were altered in 24 of 26 sBL samples (92.3 %). We

determined *E2F4* mRNA expression in 23 of these sBL specimens and found that it was similar to that observed in the control tissues (Figure S4). Hence, in accordance with our results using cell lines, E2F4 downregulation in sBL samples was not due to reduced mRNA expression levels.

Since E2F4 negatively regulates E2F1 transcription in concert with p130 (30, 31), we hypothesized that p130 expression might be downregulated in BL tumor samples with relatively normal E2F4 levels. To address this issue, we used immunohistochemistry to analyze its expression in these samples. While p130 was readily detected in the nucleus of most cells in germinal centers of reactive tonsils, it was almost undetectable in the sBL cases herein studied (Figure 5A). However, its expression was similar in BL and LCL cell lines (Figure 5B), suggesting that these cell lines are not valid to assess p130 role in BL formation. We also assessed E2F4 and p130 levels in 67 DLBCL specimens and found that, as opposed to sBL, most Cases were positive for both proteins (Table SI). Together, our data suggest that the E2F4-p130 axis is downregulated in most sBL cases included in our study, but not in DLBCL.

E2F4 regulates E2F1 expression and BL tumor formation.

Our results suggest that down-regulation of the E2F4-p130 pathway in sBL cells might hamper repression of the *E2F1* promoter, thus leading to E2F1 overexpression in these cells. According to this hypothesis, forced expression of E2F4 in these cells should reduce E2F1 levels. To confirm it, three BL cell lines (DG75, Ramos, and Mutu-I) were transduced with a retrovirus encoding E2F4 employing conditions that rendered E2F4 levels similar to those present in control cells (Figure S5). Protein expression was determined in transduced cells following puromycin selection. We found that ectopic E2F4 caused a sharp reduction of E2F1 protein levels without affecting those of Tubulin

(Figure 6A). Accordingly, *E2F1* mRNA expression, but not that of *GAPDH*, decreased in DG75 cells transduced with E2F4 (Figure S6).

BL cell lines produce malignant tumors in immunodeficient mice (32). We have shown that tumors formed by inoculation of BL cell lines, such as DG75 or Ramos, into these mice are solid masses consisting of tumor lymphoid cells (10). To investigate whether deregulation of the E2F4-p130 pathway is involved in BL tumor formation *in vivo*, we enforced E2F4 expression in BL cell lines by retroviral transduction. The capacity of these cells to form tumors was subsequently determined through their inoculation into immunodeficient mice. DG75, Ramos, and Mutu-I cells transduced with a mock retrovirus elicited formation of tumors >0.3g in mass within three weeks (Figure 6B). In contrast, mice inoculated with BL cell lines transduced with retroviruses encoding E2F4 formed no tumors or very small tumors (Figure 6B). Therefore, E2F4 over-expression in BL cells severely inhibited their tumor formation capacity, thus pointing to E2F4 as a key player in BL lymphomagenesis.

E2F4 inhibits proliferation and elicits G_2/M accumulation of BL cells.

To investigate the mechanism of tumor formation inhibition by E2F4, we compared cell proliferation rates of BL cell lines (DG75, Ramos, and Mutu-I) with normal or augmented E2F4 levels. We found that BL cells that overexpress E2F4 incorporated 5-fold less [^{3}H]dThd than mock-transduced cells (Figure 7A). Remarkably, E2F4 overexpression in a breast carcinoma cell line (MCF-7) or in a fibroblast cell line (NIH-3T3) barely affected E2F1 levels and did not inhibit their proliferation (Figures 7B-7C), suggesting that E2F4 levels were not a limiting factor for E2F1 expression and cell growth regulation in these cells.

C-MYC overexpression in normal cells arrests them in the G_2 phase of the cell cycle (9) and E2F1 knock-down in sBL cells leads to their accumulation in G_2/M (10).

316 Since E2F1 levels are drastically reduced in BL cells that overexpress E2F4, we
317 hypothesized that these cells might also be arrested in G_2/M. Cell cycle distribution
318 analysis of mock- and E2F4-transduced cells revealed that 26% of mock-transduced BL
319 cells were found in G_2/M, whereas >38% of E2F4-transduced BL cells accumulated in
320 G_2/M (Figures 7D-7E). Accordingly, the amount of cells in G_0/G_1 and S was reduced in
321 BL cells that over-expressed E2F4 compared to control cells (Figures 7D-7E). These
322 results were similar to those obtained with BL cells expressing an shRNA specific for
323 E2F1 (Figures 7D). Together, our results indicate that enforced E2F4 expression in BL
324 cells reduces their proliferation capacity and leads to their accumulation in the G_2/M
325 phase of the cell cycle.
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DISCUSSION

The identification of molecular hits leading to sBL formation may facilitate the development of more specific, less toxic therapies than those currently in use. Unfortunately, the pathways underlying sBL formation are not completely understood. We have reported recently that E2F1 was overexpressed in 100% of the sBL cases studied and that its overexpression was required for tumor formation (10). We have now investigated the mechanisms deregulating E2F1 levels in BL and found that the transcriptional repression pathway mediated by E2F4 is down-regulated in BL cell lines and sBL tumor samples relative to control cell lines and tissues and that this defect is critical for E2F1 deregulation in this tumor and for lymphomagenesis.

These results are seemingly in conflict with a previous report showing that the absence of *E2f4* delays tumor onset in an *Eμ-myc* mouse model (33). There is no conflict, however, because while the *Eμ-myc* mouse is useful to study the role of c-myc in lymphomagenesis (34), this mouse is not a BL model. Indeed, Eμ-myc mice develop primarily pre-B cell tumors whose histopathologic features are consistent with the diagnosis of lymphoblastic lymphoma rather than BL (35).

Since we found that *E2F1* mRNA stability was similar in BL and control cells and that the *E2F1* promoter was more active in BL than in control cells, we propose that deregulation of this promoter accounts for the elevated expression of E2F1 in BL. This promoter contains two E2F-binding sites that repress its transcription in immortalized fibroblasts (24). Accordingly, we found that these sites also act as transcriptional repressors in immortalized B-cell lines. Remarkably, these sites failed to repress *E2F1* transcription in BL cells likely because E2F4 expression was down-regulated in these cells. Indeed, restoration of relatively normal E2F4 level in BL cells reduced E2F1 expression. However, we cannot rule out that additional mechanisms might also

facilitate E2F1 deregulation, including C-MYC-mediated activation of CDKs (36) or the existence of a positive feedback loop mediated by E2F1.

Since E2F4 is the most abundant E2F family member (17), its reduced levels in BL cells might readily increase E2F1 expression in them. Although other repressor E2F factors could potentially inhibit *E2F1* transcription, only E2F4 levels were markedly lower in BL than in control cells. Indeed, E2F2, E2F3, E2F5, and E2F6 expression was similar in both cell types [(10) and Figure S7] and E2F7 was undetectable in these cells (our unpublished observations). Of note, *E2F8* mRNA levels were higher in BL than in LCL cells (Figure S7). E2F1 binds and activates the *E2F8* promoter whereas E2F8 binds and repress the *E2F1* promoter (37). Therefore, E2F1 deregulation might account for a higher expression of E2F8 in BL cells. Whether E2F8 down-modulation in these cells would further increase E2F1 expression remains to be elucidated. Finally, enforced E2F4 expression in BL cells decreased E2F1 levels. Therefore, even if E2F4 is not the only repressor E2F family member that modulates *E2F1* transcription in other cell settings, its downregulation in BL is likely responsible of *E2F1* overexpression in this tumor. This regulatory mechanism might be common to other tumors, such as anaplastic thyroid cancers, in which E2F1 and E2F4 expression are also inversely regulated (38).

E2F4 has a transactivation domain and activates transcription and gene expression in certain scenarios (39, 40). Its transcriptional activity is restrained by interaction with pocket proteins. Moreover, E2F4 binding to pRB family members is required for its repressor role (41). In particular, recruitment of E2F4/p130 complexes to the E2F sites in the *E2F1* promoter is critical for *E2F1* transcriptional repression (30, 42). In addition, the presence of strong nuclear export signals in E2F4 can promote its cytosolic location and thus restrain its transcriptional activity (11, 20). Since E2F4 levels were lower in most sBL biopsy specimens herein studied than in control tissues,

we propose that E2F1 expression is deregulated in these cases through a defect in transcriptional repression. E2F4 levels were similar or only moderately lower to those of control tissues in only 2 and 3 samples, respectively, of 26 specimens and might, therefore, activate or repress E2F1 expression in these samples. Because p130 was almost undetectable in these biopsy specimens, it seems likely that E2F4 might not be able to repress *E2F1* transcription in these cases. In one additional sample (case 13), E2F4 levels were similar to those of control tissues, but its location was mostly cytosolic in most cells, thus arguing against a transcriptional repressor activity of E2F4 in this sample. Together, our findings strongly support that E2F4 might be unable to repress E2F1 transcription in sBL tumor samples.

A recent study documenting expression profiles of BL samples and germinal center cells revealed that they were intimately related, differing for molecules involved in cell proliferation, immune response, and signal transduction (43). *E2F4* was not among the genes deregulated in BL relative to normal cells. Accordingly, we detected no differences in *E2F4* mRNA expression between BL and control cell lines. These results raise the possibility that E2F4 level is regulated post-transcriptionally in sBL samples. Indeed, our findings revealed that differences in protein stabilization likely account for E2F4 deregulated expression in BL.

E2F1 and E2F4 are proteolyzed through the ubiquitin-proteasome pathway and their binding to pRB family members protects them from degradation (23, 44-46). Since p130 is one of the major partners of E2F4 and its expression is downregulated in some of our sBL cases, E2F4 might be destabilized in BL relative to control tissues because of the low abundance of p130. However, p130 expression was similar in BL and LCL cell lines. We have also sequenced E2F4 cDNA from 3 BL cell lines and found no single mutation in its coding sequence (our unpublished obervations), thus ruling out

that its decreased protein stability is due to mutations. Instead, unidentified yet posttranslational modifications might perhaps facilitate its ubiquitilation and proteolysis. Additional research is required to uncover these modifications.

E2F4 is generally considered as an inhibitor of cell proliferation. Accordingly, our results support a negative role for E2F4 in BL cell proliferation. However, accumulating evidence suggest that E2F4 can also activate cell proliferation in certain contexts, such as fetal erythropoiesis (47), heart development (48), or colon epithelium homeostasis (49). Hence, E2F4 plays a dual role in proliferation regulation that might depend on cell context or the expression level of other E2F family members.

The hallmark of BL cells is the reciprocal translocation between one of three immunoglobulin gene loci and the *C-MYC* gene that leads to deregulated C-MYC expression (50). This translocation is not the only critical event in BL pathogenesis (7). Indeed, C-MYC overexpression alone cannot sustain proliferation of normal cells but, instead, either arrests them in G_2 (9) or leads them to undergo apoptosis (8). Additional hits should therefore be involved in the biology of BL. In this regard, we have shown previously that elevated E2F1 expression in BL cells might facilitate their escape from C-MYC-induced G2 arrest (10). We show now that E2F4 down-regulation in these cells might also facilitate their escape from C-MYC-induced G2 arrest and their capacity to form tumors. Together, our results strongly support that insufficient E2F4 levels in BL cells might cause elevated E2F1 expression and, hence, enable them to overcome C-MYC-induced growth arrest and to form tumors. Therefore, E2F4 is a potential target for therapeutic intervention in BL. Drugs or compounds that inhibit its proteolysis, such as Bortezomib, might restore normal E2F4 levels in BL cells and inhibit their growth. A better characterization of the mechanisms involved in E2F4 proteolysis in BL might provide more specific therapeutic tools.

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CONFLICT OF INTEREST

The authors have no conflicting commercial interests.

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FIGURE LEGENDS

Figure 1. Different *E2F1* transcription rates account for different *E2F1* mRNA expression levels in sBL and LCL cell lines. (**A**) Representative qPCR analysis of *E2F1* levels in the indicated cell lines after actinomycin D (10 μg/ml) treatment during the indicated periods of time. Expression is shown relative to non-treated cells (0h) and normalized by *β-ACTIN*. (**B**) A firefly luciferase reporter plasmid containing the *E2F1* promoter was co-transfected with pRL-SV40 into the indicated cell lines. Firefly luciferase activity is shown relative to Renilla luciferase activity for each cell line. The average and SD of four independent experiments is shown. *P<0.05, **P<0.01 and ****P<0.0001 vs X50-7. (**C**) Schematic representation of the *E2F1* promoter indicating the proximal and distal E2F sites in close proximity to the transcription start site. (**D**) Luciferase reporter plasmids containing wt or mutated E2F sites from the *E2F1* promoter were co-transfected with pRL-SV40 into the indicated cell lines. Firefly luciferase values were normalized by Renilla activity. Normalized luciferase values for the mutant reporter (solid columns) are shown relative to those of the wt reporter (empty columns) in the indicated cell lines as average + SD of four independent experiments. ***P<0.005 vs wild-type.

Figure 2. Analysis of DNA-binding species that interact with the distal E2F element within the *E2F1* promoter reveals a different binding pattern in BL and LCL cells. (**A**) Complex formation employing nuclear extracts from the indicated cell lines and radiolabeled distal E2F element from the *E2F1* promoter (E2F1-d) was analyzed by EMSA. Reaction mixtures were pre-incubated in the absence (-) or in the presence (+) of a 50-fold excess of the unlabeled oligonucleotide (**B**) or in the presence of Abs against the indicated proteins (**C** - **D**) for 20 min prior to addition of the labeled probe. A DP1 pre-immune rabbit polyclonal Ab was used as control. The position of complexes I-IV is indicated. Free probes are not shown to do not unnecessarily enlarge figures size.

Figure 3. E2F4 shows lower expression levels and protein stability in BL than in LCL cells. (**A**) Representative immunoblotting of four analysis performed of E2F4 and tubulin protein expression in the indicated cell lines. (**B**) qPCR analysis of *E2F4* mRNA expression in the indicated cell lines. Expression is shown relative to that found in the LCL cell line IB4 and normalized by *β-actin*. *Columns*, average (n=3); *bars*, SD. (**C**) Representative immunoblotting of three analysis performed of E2F4 and tubulin protein expression in the indicated cell lines treated with 100 μg/ml cycloheximide during the indicated times.

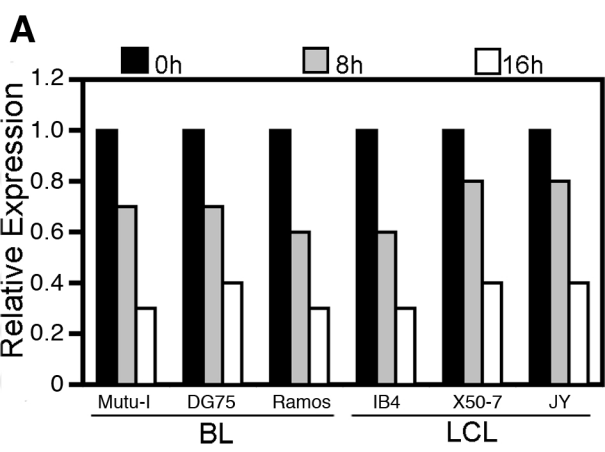
Figure 4. E2F4 expression is downregulated in sBL biopsy specimens. (**A**) E2F1 and E2F4 expression were assessed by qPCR and immunohistochemistry, respectively, in a reactive tonsil and the indicated sBL tumor samples. E2F1 levels are shown relative to the control tissue. +, <2 fold; ++, 5-10 fold; +++, 10-15 fold; ++++, >15 fold (see also Figure S3A). POS, Cases that could no be analyzed by qPCR, but were positive for E2F1 immunohistochemistry staining (see also Figure S5). E2F4 levels were determined by immunohistochemistry. 0, No positive cells; +, 0-30% positive cells and/or low level; ++, 30-70% positive cells; +++, >70% positive cells; C, Cytosolic staining in most cells. (**B**) Representative images of E2F4 staining of a reactive tonsil (section of a germinal centre) and the indicated sBL cases. Please, note that despite morphology of Case 1, the rest of its phenotypical and cytogenetic characteristics are typical of BL. Scale bar, 10 μm.

Figure 5. The expression of p130 is down-regulated in sBL biopsy specimens. (**A**) Representative images of p130 staining of a reactive tonsil (section of a germinal centre) and the indicated sBL cases. Scale bar, 10 μm. (**B**) Representative immunoblotting of three analysis performed of E2F4 and tubulin protein expression in the indicated cell lines.

Figure 6. E2F4 inhibits E2F1 expression and tumor formation. DG75, Ramos, and Mutu-I BL cell lines were transduced with retroviruses bearing an empty expression vector

(mock) or an E2F4 expression vector (HA-E2F4). (**A**) Representative immunoblotting of four experiments performed with the indicated antibodies. (**B**) Weight of tumors extracted from immunodeficient mice inoculated with the indicated cells (n=7, n=12 and n=5 for DG75, Ramos and Mutu-I, respectively).

Figure 7. E2F4 reduces cell proliferation and elicits G2/M accumulation in BL cells. (**A**) DG75, Ramos and Mutu-I cells or (**B**) MCF-7 and NIH-3T3 cells were transduced with retroviruses bearing an empty expression vector (mock) or an E2F4 expression vector (HA-E2F4). (**A**) [^{3}H]dThd incorporation in DG75 cells are shown relative to mock-transduced cells as average + SD of four independent experiments performed in triplicate. ****P<0.0001 vs mock. (**B**) Representative immunoblotting of three analyses performed with the indicated antibodies. (**C**) Edu incorporation in these cells is shown relative to mock-transduced cells as average + SD of three independent experiments performed in triplicate. (**D**) Representative cell cycle profiles of DG75 cells transduced with retroviruses bearing an empty expression vector (mock), an E2F4 expression vector (HA-E2F4), or DG75 cells transduced with lentiviruses encoding a shRNA specific for *E2F1* (E2F1-shRNA). G_2-M phase of the cell cycle is indicated (arrowhead). (**E**) *Columns*, average (n = 4) of the percentage of each of the indicated transduced cells in G_0-G_1, S, and G_2-M; *bars*, SD. ****P<0.0001 vs mock.



B

	Cell Line	Average	SD	
BL	Mutu-I	24.20	3.57	****
BL	DG75	15.63	1.85	****
BL	Ramos	187.00	10.27	****
LCL	IB4	0.57	0.09	**
LCL	X50-7	1.89	0.22	
LCL	JY	1.32	0.13	*

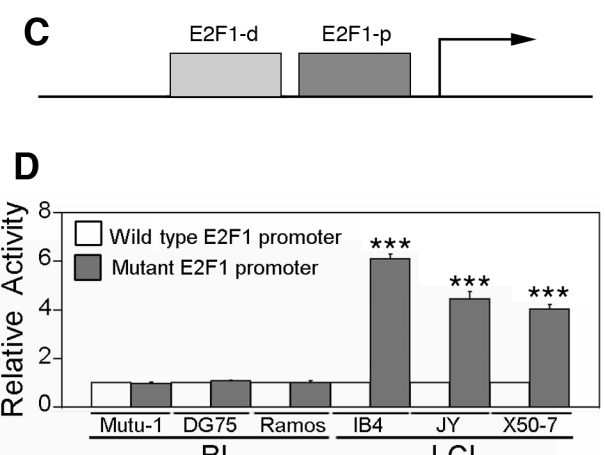


Figure 1

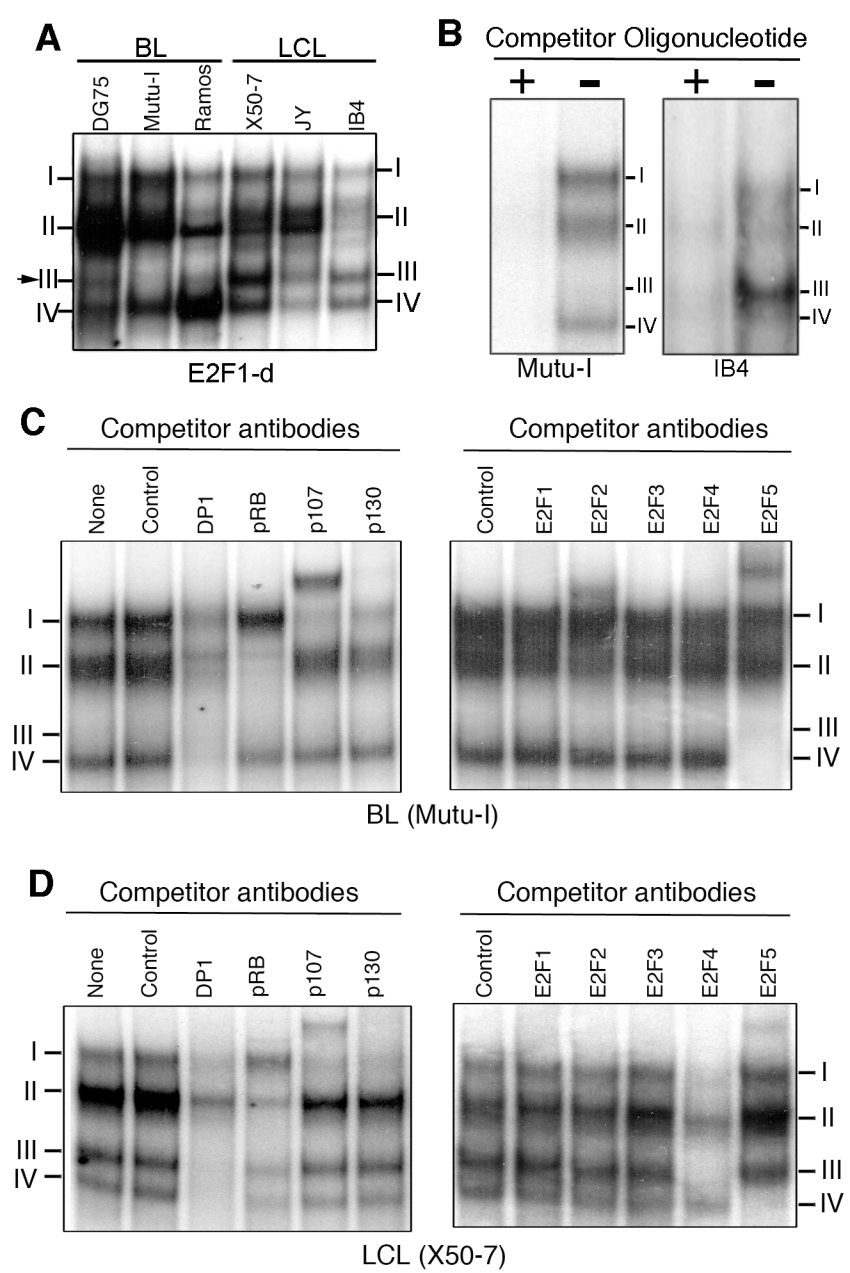

A
BL
LCL
DG75
Mutu-I
Ramos
X50-7
JY
IB4
I
II
III
IV
E2F1-d
B
Competitor Oligonucleotide
+
-
+
-
Mutu-I
IB4
C
Competitor antibodies
None
Control
DP1
pRB
p107
p130
Competitor antibodies
Control
E2F1
E2F2
E2F3
E2F4
E2F5
BL (Mutu-I)
D
Competitor antibodies
None
Control
DP1
pRB
p107
p130
Competitor antibodies
Control
E2F1
E2F2
E2F3
E2F4
E2F5
LCL (X50-7)

Figure 2

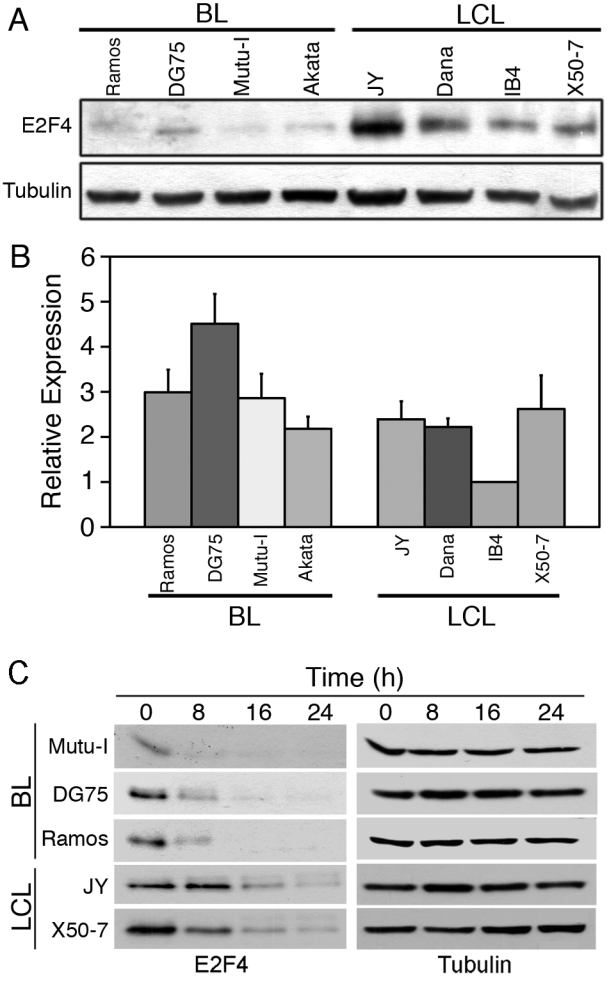

A
BL
LCL
Ramos
DG75
Mutu-I
Akata
JY
Dana
IB4
X50-7
E2F4
Tubulin
B
Relative Expression
0
1
2
3
4
5
6
Ramos
DG75
Mutu-I
Akata
JY
Dana
IB4
X50-7
BL
LCL
C
Time (h)
0
8
16
24
0
8
16
24
Mutu-I
DG75
Ramos
JY
X50-7
BL
LCL
E2F4
Tubulin

Figure 3

A

Case	E2F1	E2F4
Tonsil	±	+++
Case 1	++++	+
Case 2	++	+++
Case 3	+++	+
Case 4	POS	+
Case 5	++++	++
Case 6	++	+
Case 7	++	0
Case 8	++++	0
Case 9	+++	+
Case 10	POS	+ C
Case 11	POS	0
Case 12	++++	+
Case 13	POS	++ C
Case 14	++	+++
Case 15	++	+
Case 16	+++	+
Case 17	+++	+
Case 18	+++	+
Case 19	++++	+
Case 20	++++	0
Case 21	++	++
Case 22	+++	++
Case 23	++++	0
Case 24	++	0
Case 25	+++	+
Case 26	++	+

B

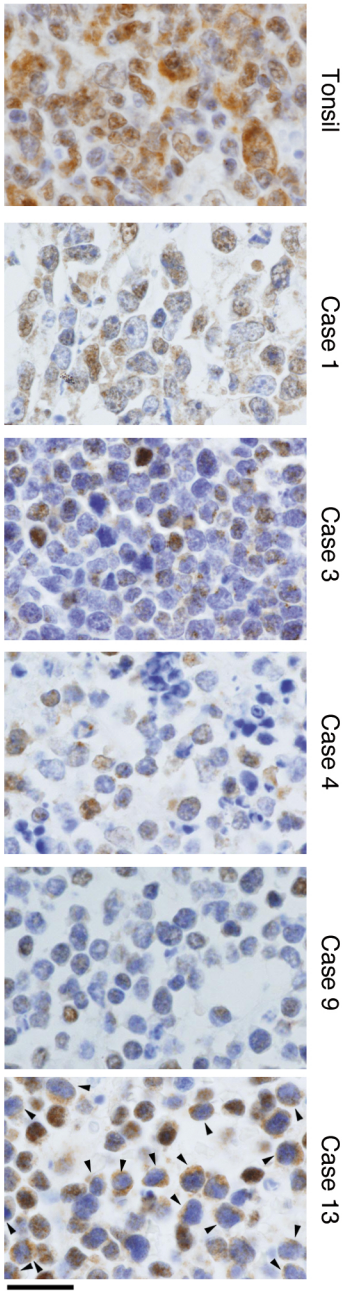


Figure 4

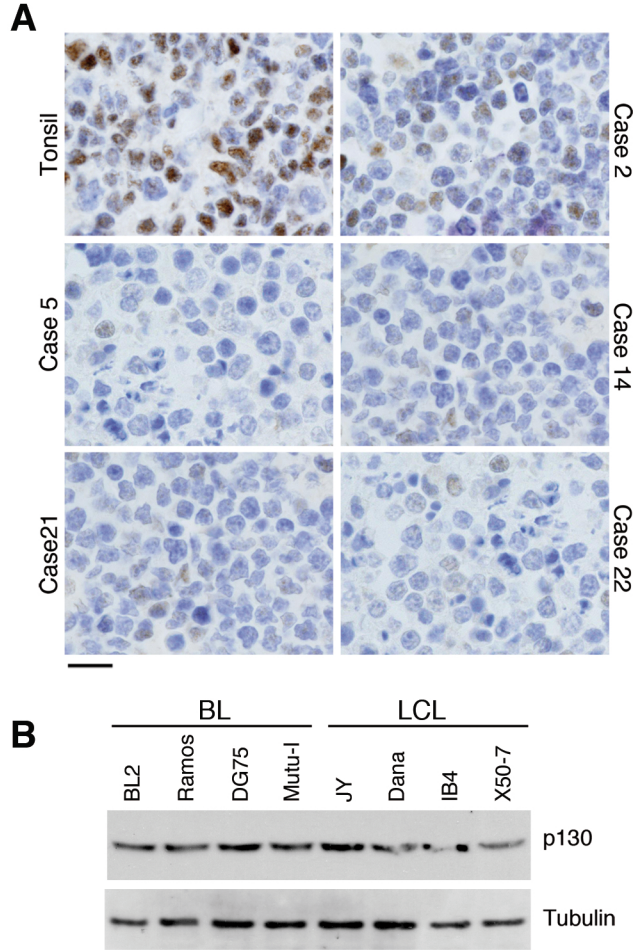

A
Tonsil
Case 2
Case 5
Case 14
Case21
Case 22
B
BL
LCL
BL2
Ramos
DG75
Mutu-I
JY
Dana
IB4
X50-7
p130
Tubulin

Figure 5

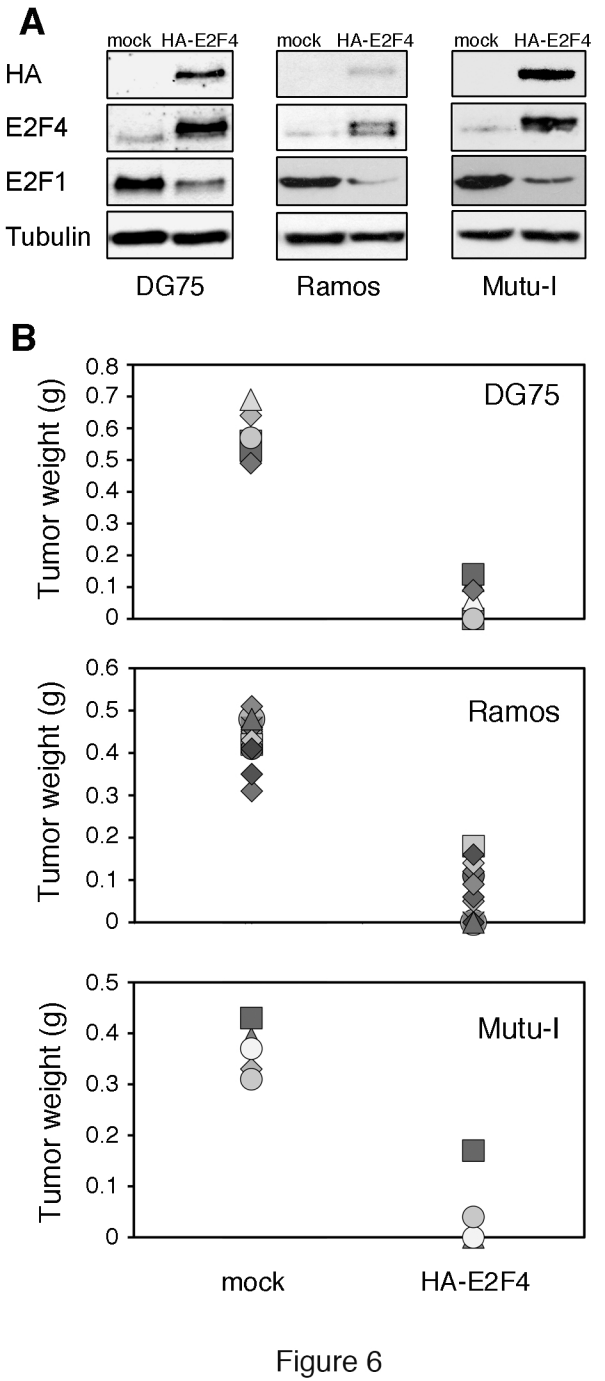

A
mock HA-E2F4
mock HA-E2F4
mock HA-E2F4
HA
E2F4
E2F1
Tubulin
DG75
Ramos
Mutu-I
B
Tumor weight (g)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DG75
Tumor weight (g)
0.6
0.5
0.4
0.3
0.2
0.1
0
Ramos
Tumor weight (g)
0.5
0.4
0.3
0.2
0.1
0
Mutu-I
mock
HA-E2F4

Figure 6

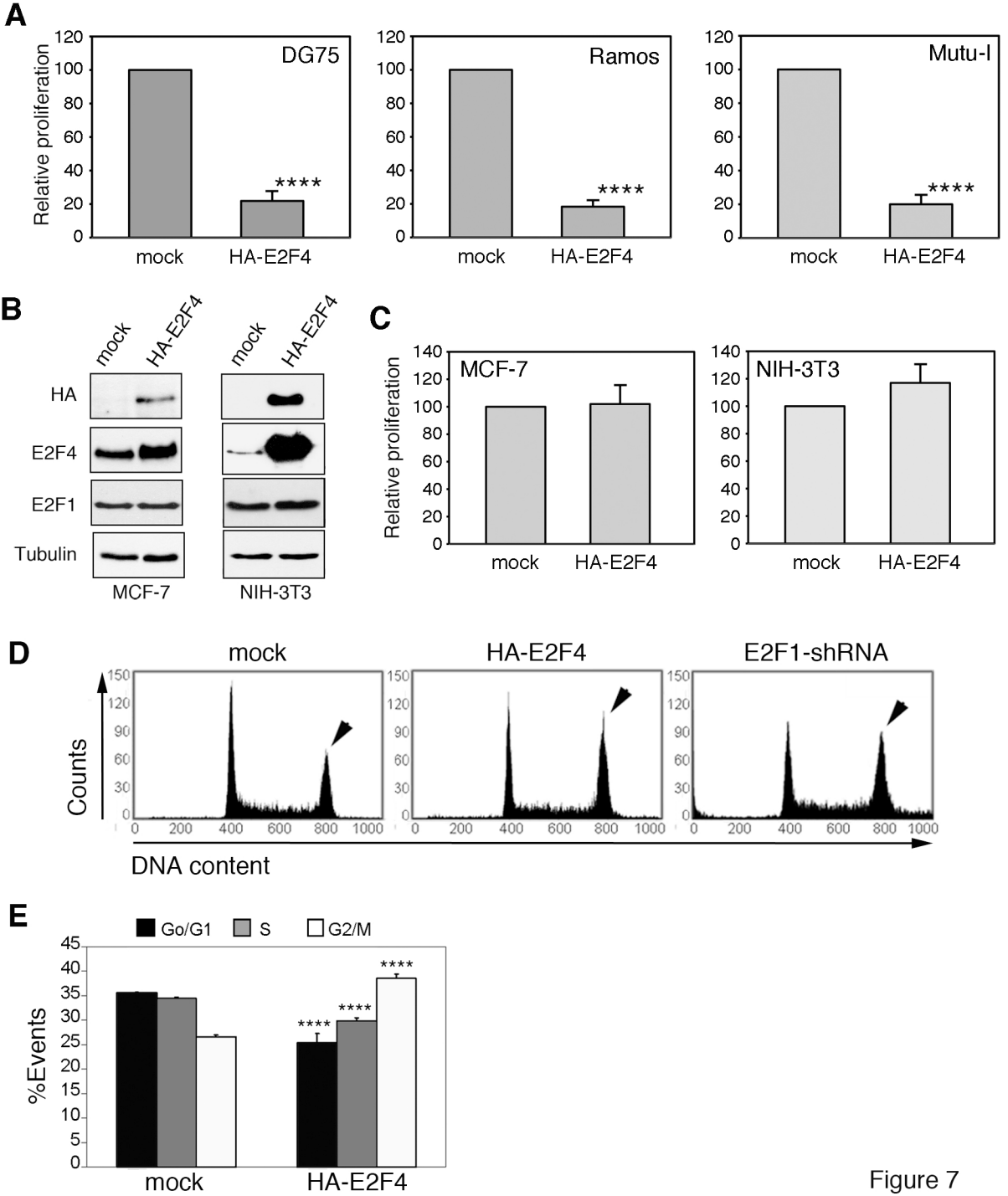

A
Relative proliferation
DG75
Ramos
Mutu-I
****
mock
HA-E2F4
B
mock
HA-E2F4
HA
E2F4
E2F1
Tubulin
MCF-7
NIH-3T3
C
Relative proliferation
MCF-7
NIH-3T3
D
mock
HA-E2F4
E2F1-shRNA
Counts
DNA content
E
Go/G1
S
G2/M
%Events

Figure 7

SUPPLEMENTAL INFORMATION

Supplemental Figure 1. Increased *E2F1* promoter activity in BL. A firefly luciferase reporter plasmid containing the *E2F1* promoter was co-transfected with pCMV-βgal into the indicated cell lines. Firefly luciferase activity is shown relative to ß-galactosidase activity. The average and SD of four independent experiments is shown. ****P<0.0001 vs X50-7.

Supplemental Figure 2. Analysis of DNA-binding species that interact with the distal E2F element within the *E2F1* promoter. Complex formation employing nuclear extracts from DG75 (BL) and JY (LCL) cell lines and radiolabeled distal E2F element from the *E2F1* promoter was analyzed by EMSA. Reaction mixtures were pre-incubated in the presence of Abs against the indicated proteins for 20 min prior to addition of the labeled probe. A DP1 pre-immune rabbit polyclonal Ab was used as control. The position of complexes I-IV is indicated. Free probes are not shown to do not unnecessarily enlarge figures size.

Supplemental Figure 3. Analysis of *E2F1* expression in sBL tumor samples. (**A**) qPCR analysis of *E2F1* mRNA levels in the indicated sBL cases and reactive tonsils is shown relative to that found in reactive tonsil 1 and normalized by *β-actin*. Columns, average (n = 3); bars, SD. (**B**) Representative images of E2F1 immunohistochemistry staining of a reactive tonsil (section of a germinal centre) and the indicated sBL cases are shown. Scale bar, 10 μm.

Supplemental Figure 4. Analysis of E2F4 expression in sBL tumor samples. qPCR analysis of *E2F4* mRNA levels in the indicated sBL cases and reactive tonsils is shown relative to that found in reactive tonsil 1 and normalized by *β-actin*. Columns, average (n = 3); bars, SD.

Supplemental Figure 5. E2F4 inhibits E2F1 expression in BL cells. Ramos and DG75 BL cell lines were transduced with retroviruses bearing an empty expression vector (mock) or an E2F4 expression vector (E2F4). Immunoblotting performed with the indicated antibodies is shown. X50-7 and JY (LCL cell lines) and BL2 (BL cell line) are shown as controls.

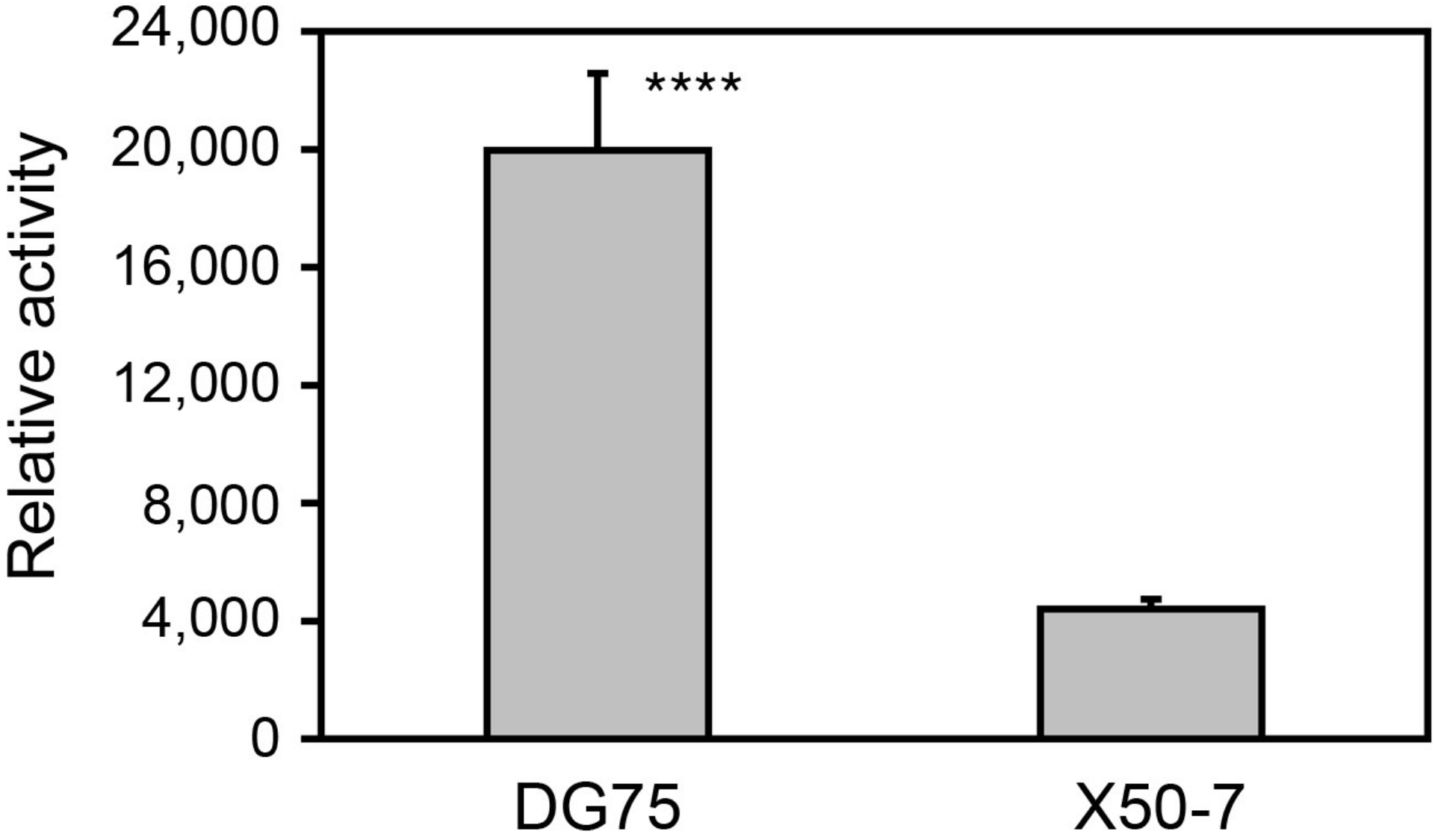
Supplemental Figure 6. E2F4 inhibits *E2F1* transcription in BL. DG75 cells were transduced with retroviruses bearing an empty expression vector (mock) or an E2F4 expression vector (HA-E2F4). qPCR analysis of *E2F1* and *GAPDH* mRNA levels in these cells is shown relative to that found in mock-transduced cells and normalized by *β-actin*. Columns, average (n = 3); bars, SD. ****P<0.0001 vs mock.

Supplemental Figure 7. Analysis of E2F5, E2F6 and E2F8 expression in BL and LCL cell lines. Representative immunoblotting of three analysis performed of E2F5 (**A**) and E2F6 (**B**) protein expression in the indicated cell lines. Tubulin is shown as loading control. (**C**) qPCR analysis of *E2F8* mRNA expression in the indicated cell lines. Expression is shown relative to that found in the LCL cell line IB4 and normalized by *β-actin*. *Columns*, average (n=3); *bars*, SD. *P<0.05 vs IB4.

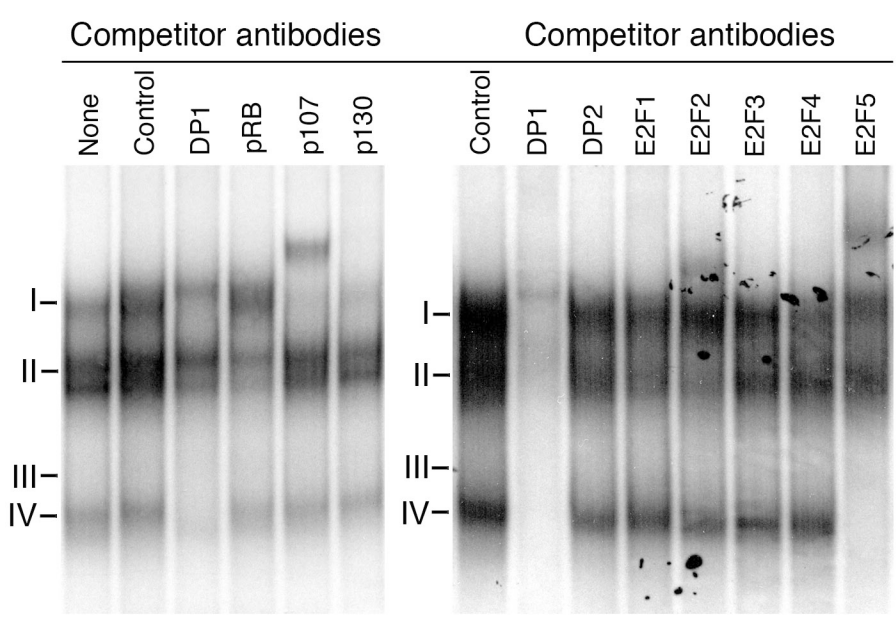
Case	E2F1	E2F4	P130
1	+	+++	+++
2	0	+++	+++
3	+	+++	+++
4	0	++	+++
5	+	+++	+++
6	0	+++	+++
7	0	0	0
8	0	++	+++
9	0	+++	+++
10	+++	++	+++
11	0	+++	+++
12	0	+++	+++
13	+	+++	+++
14	+++	+++	+
15	+	+++	+++
16	0	+++	0
17	++	+++	+++
18	+	+++	0
19	+	0	+++
20	0	+++	+++
21	0	+++	+++
22	0	+++	+++
23	+++	+++	+++
24	+	+++	+++
25	0	+++	+++
26	+	+++	+++
27	+++	+++	+++
28	0	+++	+++
29	+++	+++	+++
30	++	+++	+++
31	++	+++	+++
32	+	++	+++
33	++	+++	++
34	0	+++	+++
35	0	+++	+++
36	0	+++	++
37	0	+++	+++
38	0	+	+
39	0	+++	+++
40	+	++	0
41	n.d.	+++	0
42	+	+++	+++
43	0	++	+++
44	++	+++	+++
45	0	+	0
46	+	n.d.	+++
47	0	0	++
48	++	C	+++

49	0	+++	+++
50	0	0	+++
51	+	+++	+++
52	0	0	++
53	0	+++	0
54	0	+++	0
55	+	+++	+++
56	+	+++	++
57	+	++	+
58	0	+++	+++
59	+++	+++	+++
60	++	+++	+++
61	++	+++	+++
62	+	+++	+++
63	0	+	0
64	0	+++	+++
65	++	+++	+++
66	0	+	+
67	+	+++	+++

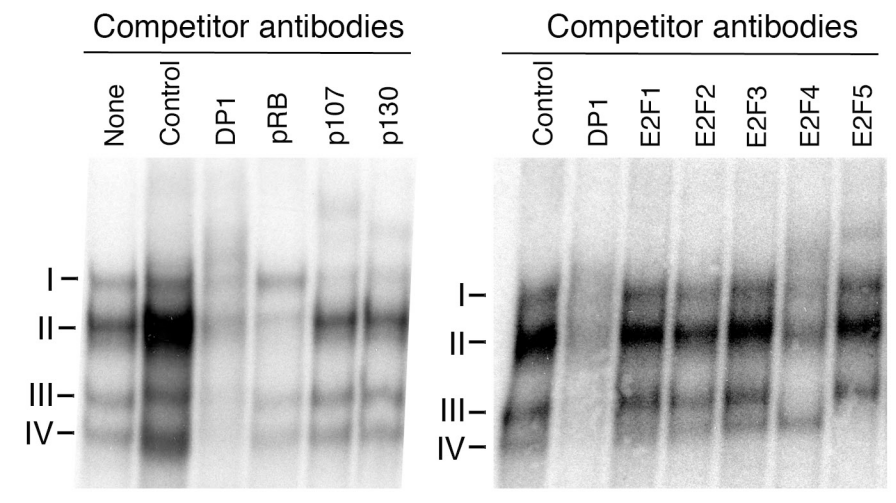
Supplemental Table 1. Assesment of E2F1, E2F4 and p130 expression in DLBCL cases. E2F1, E2F4 and p130 expression were assessed by immunohistochemistry in the indicated DLBCL tumor samples. 0, No positive cells; +, 0-30% positive cells and/or low level; ++, 30-70% positive cells; +++, >70% positive cells; C, Cytosolic staining in most cells; n.d., not determined.



Supplemental Figure 1

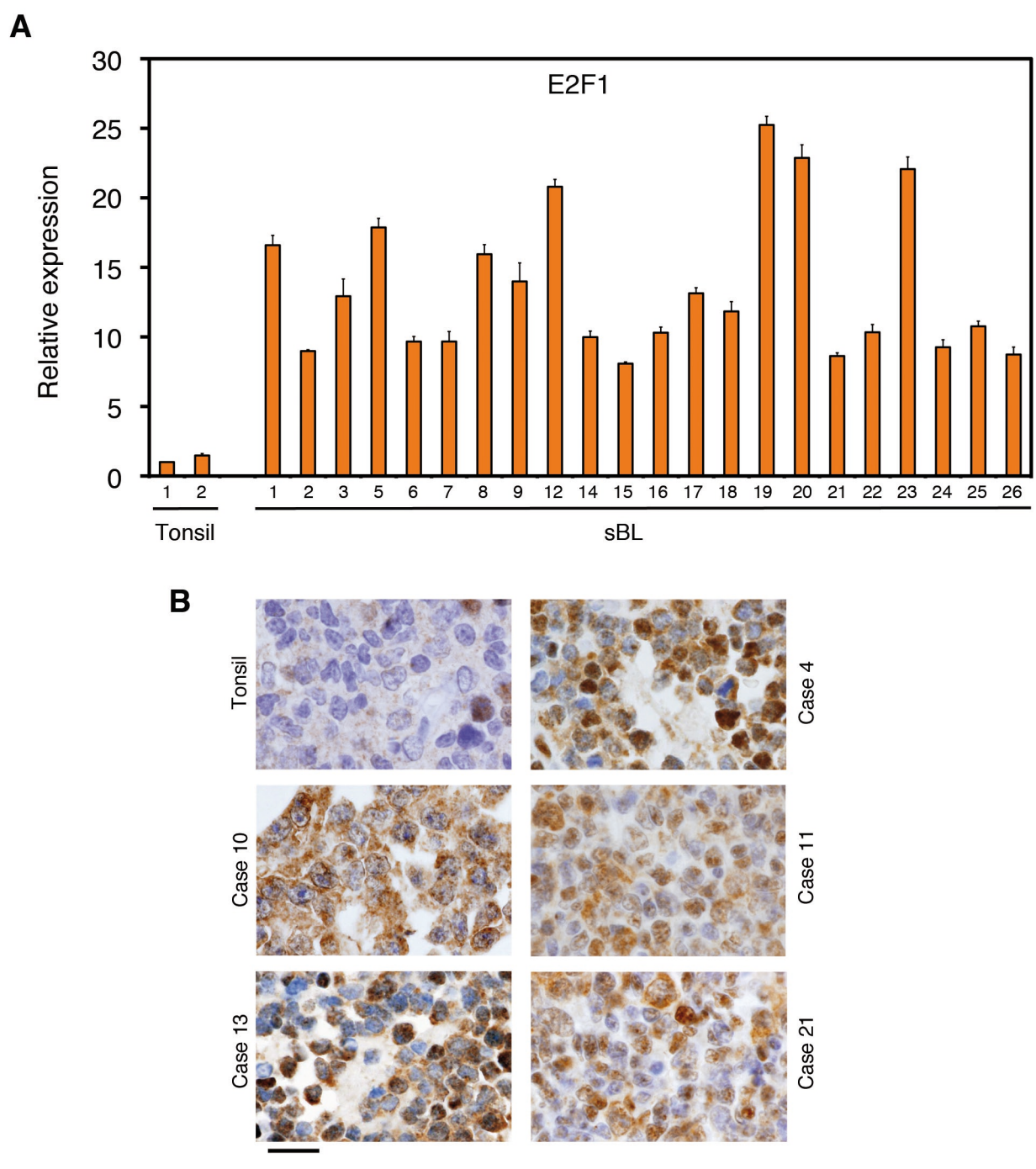


BL (DG75)

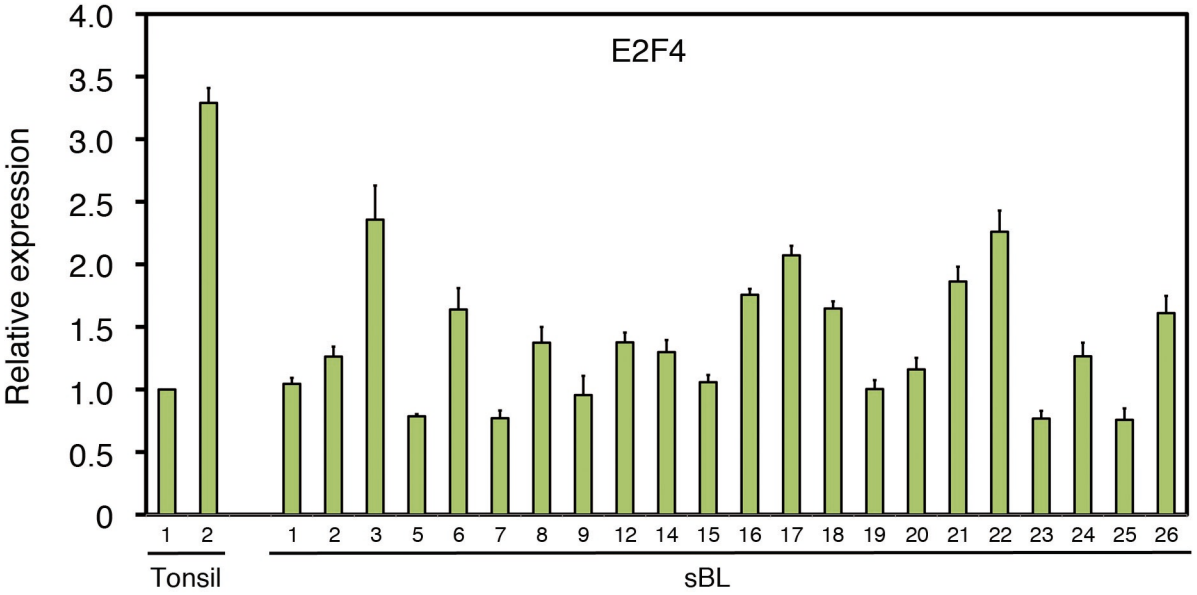


LCL (JY)

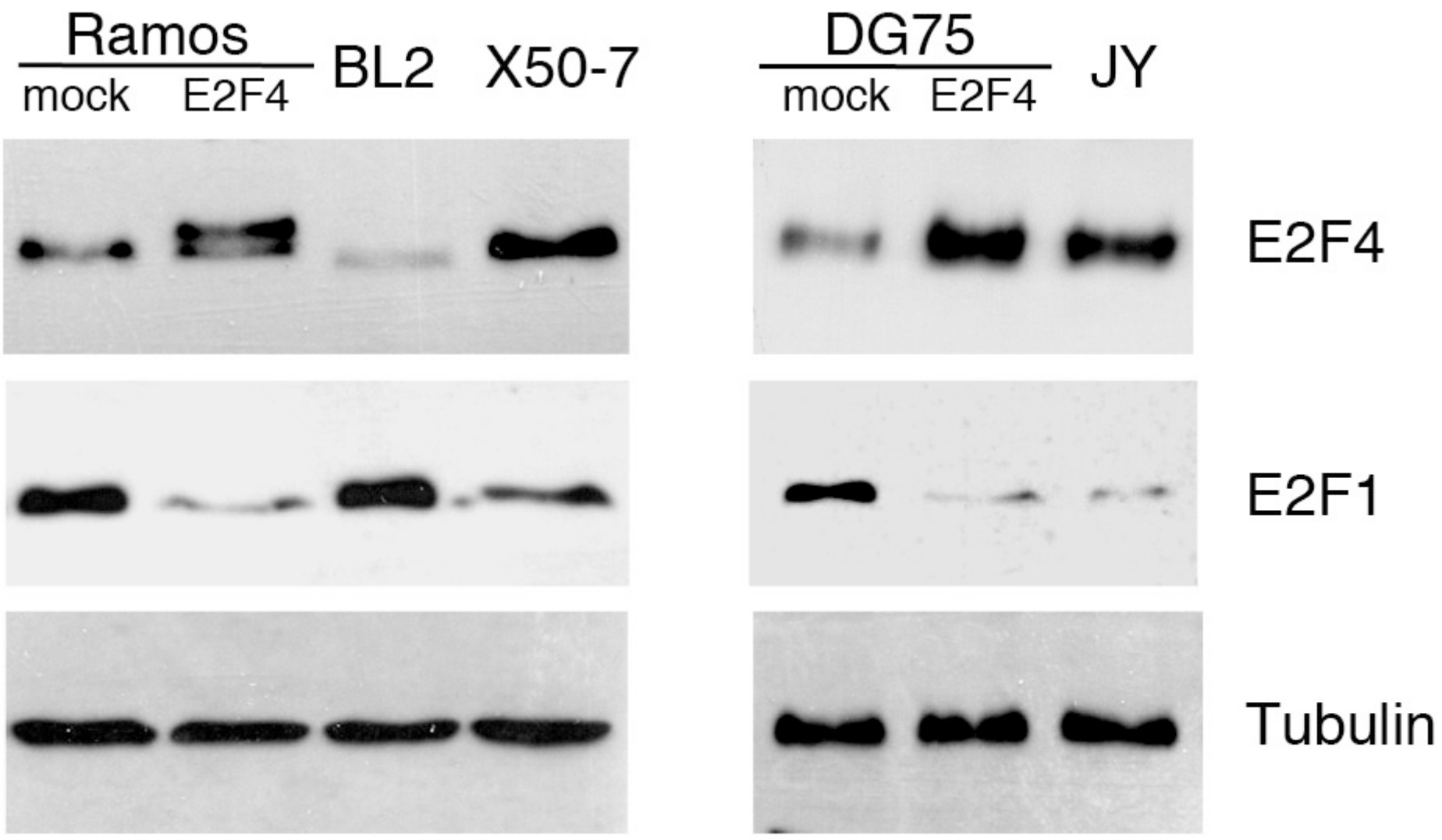
Supplemental Figure 2



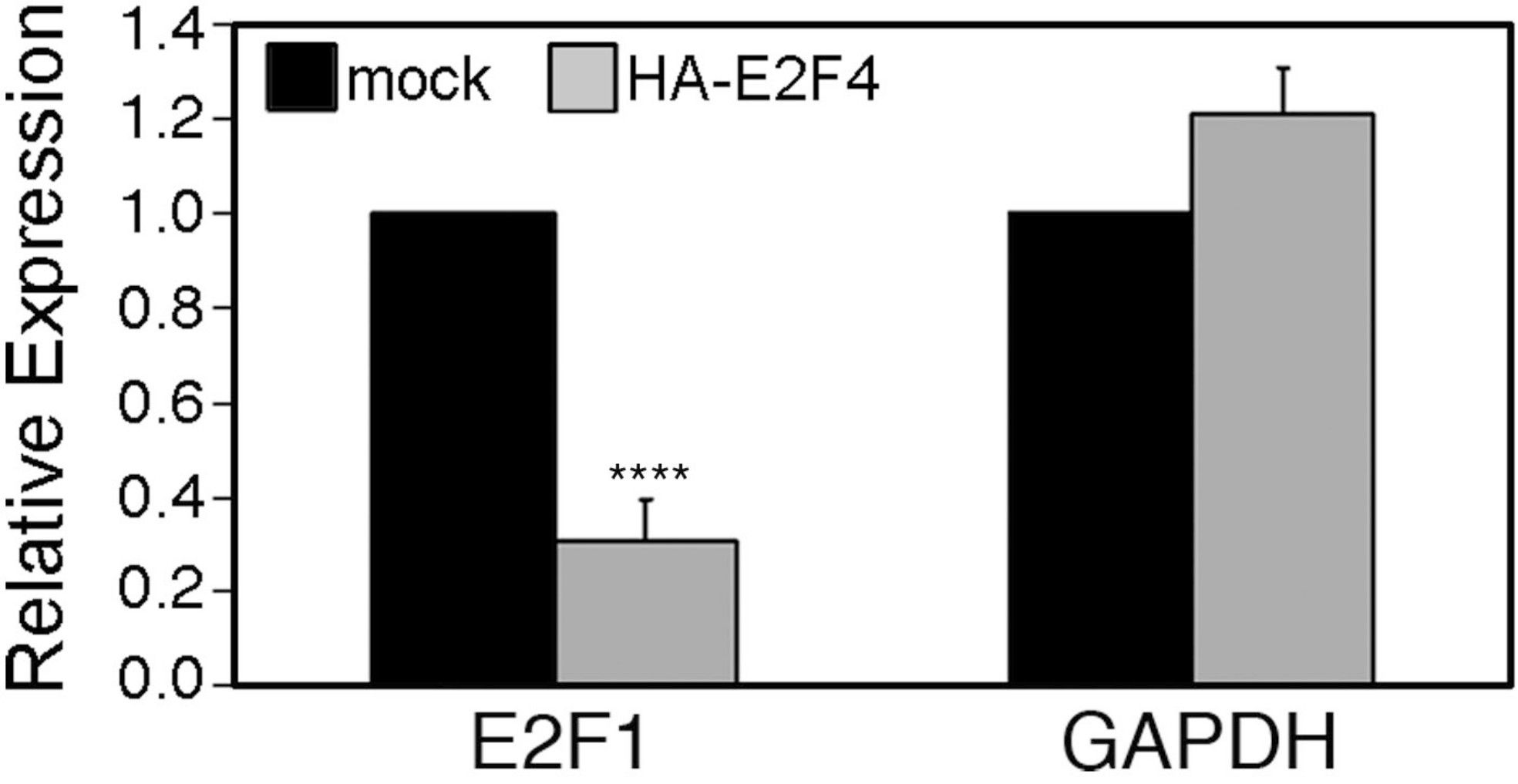
Supplemental Figure 3



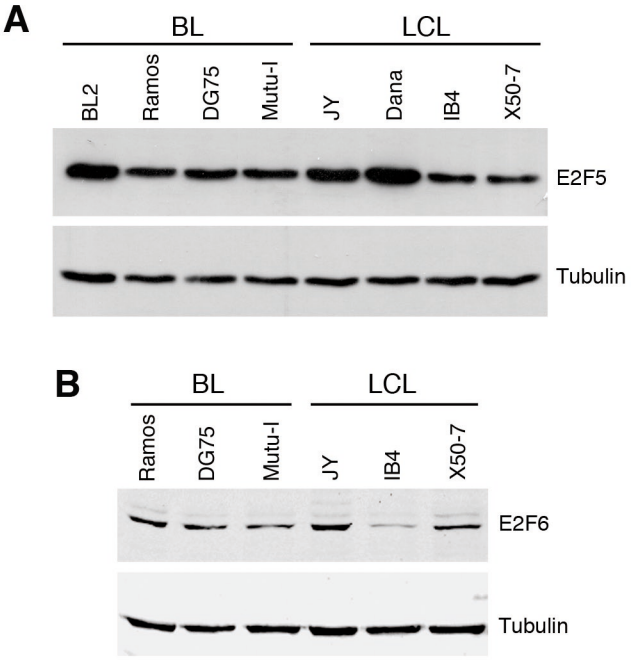
Supplemental Figure 4

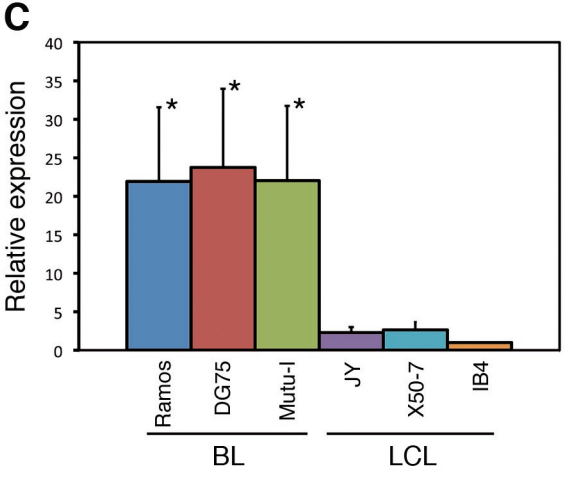


Supplemental Figure 5



Supplemental Figure 6





Supplemental Figure 7