SHORT COMMUNICATION

The NF-κB subunit c-Rel regulates Bach2 tumour suppressor expression in B-cell lymphoma

JE Hunter1, JA Butterworth1, B Zhao2, H Sellier1, KJ Campbell3, HD Thomas4, CM Bacon4, SJ Cockell5, BE Gewurz2 and ND Perkins1

The REL gene, encoding the NF-κB subunit c-Rel, is frequently amplified in B-cell lymphoma and functions as a tumour-promoting transcription factor. Here we report the surprising result that c-rel−/− mice display significantly earlier lymphomagenesis in the c-Myc driven, Eμ-Myc model of B-cell lymphoma. c-Rel loss also led to earlier onset of disease in a separate TCL1-Tg-driven lymphoma model. Tumour reimplantation experiments indicated that this is an effect intrinsic to the Eμ-Myc lymphoma cells but, counterintuitively, c-rel−/− Eμ-Myc lymphoma cells were more sensitive to apoptotic stimuli. To learn more about why loss of c-Rel led to earlier onset of disease, microarray gene expression analysis was performed on B cells from 4-week-old, wild-type and c-rel−/− Eμ-Myc mice. Extensive changes in gene expression were not seen at this age, but among those transcripts significantly downregulated by the loss of c-Rel was the B-cell tumour suppressor BTB and CNC homology 2 (Bach2). Quantitative PCR and western blot analysis confirmed loss of Bach2 in c-Rel mutant Eμ-Myc tumours at both 4 weeks and the terminal stages of disease. Moreover, Bach2 expression was also downregulated in c-rel−/− TCL1-Tg mice and RelA Thr505Ala mutant Eμ-Myc mice. Analysis of wild-type Eμ-Myc mice demonstrated that the population expressing low levels of Bach2 exhibited the earlier onset of lymphoma seen in c-rel−/− mice. Confirming the relevance of these findings to human disease, analysis of chromatin immunoprecipitation sequencing data revealed that Bach2 is a c-Rel and NF-κB target gene in transformed human B cells, whereas treatment of Burkitt’s lymphoma cells with inhibitors of the NF-κB/κB kinase pathway or deletion of c-Rel or RelA resulted in loss of Bach2 expression. These data reveal a surprising tumour suppressor role for c-Rel in lymphoma development explained by regulation of Bach2 expression, underlining the context-dependent complexity of NF-κB signalling in cancer.

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INTRODUCTION

The tumour-promoting role of the NF-κB pathway is well established and results from its ability to regulate the expression of genes involved in multiple aspects of cancer cell biology.1 This is also true in haematological malignancies2 and in several B-cell lymphoma types, such as activated B-cell-like-diffuse large B-cell lymphomas,3 primary mediastinal large B-cell lymphoma,4,5 and classical Hodgkin lymphoma.6 NF-κB activity is required for survival and proliferation. However, the contribution of individual NF-κB subunits is generally not known. In particular, whereas NF-κB subunits have been reported to exhibit characteristic of tumour suppressors in vitro,1 it has not been investigated whether these properties have relevance to lymphoma development in vivo.

There are five NF-κB subunits in mammalian cells, RelA/p65, RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). RelA and c-Rel function as effector subunits for the IkB kinase B-dependent, canonical NF-κB pathway.7 Of these NF-κB subunits, c-Rel is most closely associated with lymphoma and was first identified as the cellular homologue of the avian Rev-T retroviral oncogene v-Rel.8,9 c-Rel is ubiquitously expressed in B cells regardless of developmental stage, although the highest levels are observed in mature B cells.10–12 c-Rel knockout mice developed normally with no effects on B-cell maturation but do exhibit some immunological defects, including reduced B-cell proliferation and activation, abnormal germinal centres and reduced number of marginal zone B cells.13–17 c-Rel is distinct from other NF-κB family members in its ability to transform chicken lymphoid cells in vitro.18–20 Moreover, genomic and cytogenetic studies of human lymphomas have shown gains of chromosome 2p13, which encodes the REL gene. Amplifications and gains of REL have been detected in ~50% of HL21–23 and 10–25% or 50% in two studies of primary mediastinal large B-cell lymphoma.24,25 REL has also been identified as a susceptibility locus for HL,24 whereas c-Rel nuclear localisation has been identified as a poor prognostic factor in both activated B-cell-like- and germinal centre B-cell-like-diffuse large B-cell lymphomas.26

Despite this, relatively little is known about the role of c-Rel or other NF-κB subunits in c-Myc-driven lymphomas. However, a recent study of Myc-driven B-cell lymphoma in mice revealed a tumour suppressor role for RelA.27 Here, short hairpin RNA silencing of RelA did not affect progression of established lymphomas, but after cyclophosphamide treatment its loss resulted in chemoresistance as a consequence of impaired induction of senescence.27 Similarly, NF-κB was required for both therapy-induced senescence and resistance to cell death in the Eμ-Myc mouse model of B-cell lymphoma upon expression of a degradation-resistant form of IkBa.28 c-Rel can also inhibit expression of NF-κB2, and loss of this NF-κB subunit in the Eμ-Myc mouse model resulted in moderately earlier onset of disease as a consequence of impaired apoptosis.29 By contrast, deletion of NF-κB1 displayed no effects on Eμ-Myc lymphoma.
These results imply a more complicated role for NF-κB in Myc-driven lymphoma, with both tumour-promoting and -suppressing functions being reported, although any role for c-Rel has not been established.

Here, we have investigated the role of c-Rel in mouse models of B-cell lymphomagenesis. We demonstrate that, opposite to the expected result, c-rel−/− Eμ-Myc and TCL1-Tg mice exhibit earlier onset of lymphoma and that this result can be explained by c-Rel-
dependent regulation of the B-cell tumour suppressor BTB and CNC homology 2 (Bach2).

RESULTS

NF-κB is active in Eμ-Myc-derived lymphoma

To determine if there are significant levels of NF-κB activity in Myc-driven B-cell lymphoma, with the potential to affect disease driven by this oncogene, we crossed 3 × b2-luc (NF-κB-luc) reporter mice onto Eμ-Myc transgenic mice, allowing in vivo visualisation of NF-κB activity. The median onset of aggressive lymphoma in Eμ-Myc mice is between the ages of 3 and 6 months but they exhibit the hallmarks of Myc overexpression by 4 weeks. This analysis revealed significantly higher levels of NF-κB activity in Eμ-Myc mice at 8 weeks of age, in lymphoid organ sites, including mesenteric/inguinal lymph nodes and thymus (Figures 1a and b).

Loss of c-Rel results in earlier onset of Eμ-Myc-driven lymphoma

To investigate the role of c-Rel in MYC-induced lymphomagenesis, Eμ-Myc/c- rel–/– mice were generated. Western blot analysis confirmed no significant effects on the other NF-κB subunits or c-Myc in splenic tumour B cells, although slightly lower levels of the non-canonical NF-κB subunits p52 and RELB were found in c- rel–/– cells (Figure 1c). Eμ-Myc/c-rel+/– mice, despite having intermediate levels of c-Rel mRNA (Figure 1d), had almost no detectable c-Rel protein in Eμ-Myc lymphoma cells (Figure 1e).

Given the known tumour-promoting role of c-Rel in B-cell lymphoma, we were surprised to find that Eμ-Myc/c- rel–/– mice had a significantly shorter overall survival (median survival 79 days) than Eμ-Myc mice (median survival 115 days; Figure 1f). Earlier onset of disease was also seen in heterozygote Eμ-Myc/c-rel+/– male mice (median onset 75.5 days; Figure 1g). Although survival times of male and female Eμ-Myc/c-rel–/– mice were similar (77 vs 83 days, respectively; Figures 1h and i), this effect appeared more pronounced in male c-Rel mutant mice due to gender differences.

**P = 0.0017, **P < 0.001, unmannered Student’s t-tests. For all tests, where appropriate, analyses were undertaken to test for normal distribution.

Reimplanted Eμ-Myc tumours grow equally well in wild-type and c- rel–/– mice

These results revealed an apparent tumour suppressor role for c-Rel, but it was unclear if this resulted from an effect intrinsic to the tumour cells or from other effects of the c- rel–/– mice. Therefore, to investigate whether non-tumour cells in the wild-type and c- rel–/– mice might contribute to earlier onset of disease in c-Rel null mice, we performed a series of reciprocal tumour reimplantation studies. Tumours derived from either wild-type or c- rel–/– male Eμ-Myc mice were transplanted into either C57BL/6 or c- rel–/– male host mice. Importantly, whether the host mice were wild type or c- rel–/– did not affect the rate of c- rel–/– lymphoma growth (Figures 2a and c). A more mixed effect was seen with reimplanted wild-type Eμ-Myc cells, where increased lymphoma growth was seen at some sites but not others in the c- rel–/– host mice (Figure 2c). Reimplanted c- rel–/– lymphomas were also slower to develop than wild type (–4 weeks vs 2 weeks) but this may reflect the reduced viability of Eμ-Myc/c- rel–/– tumour cells after thawing frozen samples (Figure 2d). This analysis does not rule out a contribution from the non-tumour background in the development of Eμ-Myc lymphoma in these mice. However, given that we saw no effects of the host animal on the growth of reimplanted c- rel–/– cells, we investigated if there were intrinsic differences between wild-type and c- rel–/– lymphoma cells.

**Figure 1.** c-Rel functions as a tumour suppressor in Eμ-Myc-driven B-cell lymphoma in mice. (a) Representative image of in vivo NF-κB bioluminescence (radiance) of age-matched littermates of NF-κB-x/luc and Eμ-Myc/NF-κB-luc mice. Eight-week-old mice underwent in vivo imaging using the IVIS Spectrum system (Perkin Elmer, Beaconsfield, UK) after being intraperitoneally administered with 150 mg/kg VivoGlo c-luciferin (Promega, Southampton, UK) dissolved in sterile phosphate-buffered saline. Ten-min post-c-luciferin-administration, mice were imaged using a photon emission over 5 min, under isoflurane anaesthesia. Luminescence was seen in the thymic area and also in the tails and other exposed regions of the Eμ-Myc/NF-κB-luc mice, the latter likely due to a higher number of circulating lymphocytes with increased NF-κB activity. (b) Quantification of NF-κB bioluminescence (radiance) of thymic regions in NF-κB-luc (n = 12) and Eμ-Myc/NF-κB-luc (n = 13) mice. Bioluminescence was quantified using the Living Image software version 4.3.1 (Perkins Elmer) and region of interest tool. Data shown as mean ± s.e.m., **P < 0.01, unpaired Student’s t-tests. For all tests, where appropriate, analyses were undertaken to test for normal distribution. (c) Western blot analysis of the NF-κB subunits, c-Myc, RelA, RELB, p100/p52 and p50 together with c-MYC in extracts prepared from Eμ-Myc and Eμ-Myc/c- rel–/– mouse tumorigenic spleens. Whole-cell extracts were prepared from Eμ-Myc or Eμ-Myc/c- rel–/– tumour cell suspensions. Cell pellets were washed with ice-cold phosphate-buffered saline and lysed using PhosphoSafe Extraction Reagent (Mercck Millipore, Watford, UK). Antibodies used were c-Rel (sc-71 Santa Cruz), Insight Biotechnology, Wembley, UK), c-Myc (sc-42 Santa Cruz), RelA (sc-372 Santa Cruz), RelB (4954 Cell Signalling, Hitchin, UK), p50 (06-886 Mercck Millipore), p100/p52 (sc-848 Santa Cruz) and β-Actin (A5441 Sigma-Aldrich, Gillingham, UK). (d) Quantitative-PCR analysis showing relative Rel expression in end-stage tumorigenic spleens from Eμ-Myc (n = 20), Eμ-Myc/c- rel–/– (n = 12) and Eμ-Myc/c-rel+/– (n = 11) mice. Data shown as mean ± s.e.m., each point is an individual mouse. (e) Western blot analysis of c-REL levels in tumorigenic spleens from Eμ-Myc, Eμ-Myc/c-rel+/– and Eμ-Myc/c- rel–/– mice. (f–j) Reduced survival of Eμ-Myc/c-rel+/– and Eμ-Myc/c-rel–/– mice. Kaplan–Meier plots showing survival curves for Eμ-Myc and (f) Eμ-Myc/c- rel–/– mice, (g) Eμ-Myc/c- rel+/– male mice, (h) Eμ-Myc/c- rel+/– female mice and (i) Eμ-Myc/c- rel–/– female mice and relative survival of male versus female Eμ-Myc mice is shown in (j). P-values (Mantel–Cox test) and hazard ratios are shown. (k) Kaplan–Meier plot showing reduced survival of TCell1/c- rel–/– mice. Animal handling, husbandry and experimentation were undertaken in compliance with UK Home Office regulations under project licences and approved by the local ethical review committee. All mice used in these experiments were on C57BL/6 background and bred at the Comparative Biology Centre, Newcastle University. c- rel–/– mice were provided by Dr Fiona Oakley (Newcastle University). NF-κB-luc (NF-κB-luc+/+) reporter mice were a gift from Professor Matthew Wright (Newcastle) and originated in the laboratory of Professor Harald Carlsten (Norwegian University of Life Sciences). Eμ-Myc and TCL1-Tg mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Eμ-Myc/c- rel+/– offspring were generated by mating c- rel+/– female mice with Eμ-Myc male mice. Eμ-Myc/c-rel–/– mice were then generated by crossing Eμ-Myc/c-rel+/– males with c- rel+/– female mice. In TCL1-Tg mice, a human TCL1 coding sequence is expressed from a B29 minimal promoter, coupled with the IgH intronic enhancer resulting in B- and T-cell expression. TCL1/c- rel+/– offspring were generated as for Eμ-Myc by mating c- rel+/– female mice with TCL1-Tg male mice. All mice were designated to an experimental group-dependent on their strain and no blinding was undertaken during analysis. For survival analysis, mice were monitored daily and were killed at predetermined end points, defined as the animal becoming moribund, losing bodyweight/condition and/or having palpable tumour burden at any lymphoid organ site, at which point animals underwent necropsy. Kaplan–Meier survival curves were drawn using GraphPad Prism (Version 5.0, GraphPad Software, La Jolla, CA, USA).
Figure 2. Eμ-Myc/c-rel−/− tumours grow equally well in wild-type and c-rel−/− mice and are more sensitive to apoptotic stimuli. (a, b) Reimplanted Eμ-Myc/c-rel−/− tumours grow equally well in wild-type and c-rel−/− mice. Lymph node tumours derived from three different Eμ-Myc/c-rel−/− mice were reimplanted in parallel into either three wild-type (C57Bl/6) or three c-rel−/− host mice. Four weeks after implantation, the mice were killed and tumour sizes at different sites were assessed. Data shown here are from the spleen (a) and cervical lymph nodes (b). Data representing mean ± s.e.m. and P-values were calculated using Student’s unpaired t-tests. (c) Tumour burden in lymphoid organs (weight of organ/bodyweight of animal in gram) following reimplantation of either Eμ-Myc or Eμ-Myc c-rel−/− tumour cells into either C57Bl/6 or c-rel−/− mice. Data shown are the means of three independent tumours each implanted into three mice ± s.e.m. *P < 0.05 in an unpaired Student’s t-test, but otherwise there were no significant differences between tumour burden in wild-type and c-rel knock-out animals at any of the sites assessed. (d) Cell viability of Eμ-Myc and Eμ-Myc/c-rel−/− tumour cells grown ex vivo. Cell viability was measured using the trypan blue exclusion assay over a 4-h period after freeze thawing. (e) Eμ-Myc/c-rel−/− tumour cells are more sensitive to apoptotic stimuli. Freshly isolated Eμ-Myc or Eμ-Myc/c-rel−/− lymph node tumour cells (5 × 10⁵ per well) were seeded into 96-well plates. Increasing concentrations of the chemotherapeutic agents, doxorubicin (Sigma-Aldrich) or vincristine (Sigma-Aldrich) or solvent controls were added to three replicate wells. After 96 h, viability was quantified using the CellTiter96 AQueous One Solution Cell Proliferation Assay (MTS; Promega), according to the manufacturer’s instructions. Single-cell suspensions were prepared from tumour-bearing organs of Eμ-Myc and Eμ-Myc/c-rel−/− mice upon necropsy. These were then used for downstream analyses or frozen in 90% fetal bovine serum/10% dimethyl sulfoxide for long-term storage and transplantation. For reciprocal microenvironment experiments, 2 × 10⁶ Eμ-Myc/c-rel−/− lymph node tumour cells from male mice were transplanted intravenously via the lateral tail vein into 8-week-old male C57BL/6 or c-rel−/− recipients. Mice were necropsied when they became moribund and the tumour burden assessed. C57BL/6 mice used for reimplantation studies were purchased from Charles River (Margate, UK).
c-rel−/− B-cell lymphomas are more sensitive to apoptotic stimuli than c-Rel and the other NF-κB subunits can contribute towards tumorigenesis by inducing the expression of antiapoptotic genes and, consistent with this and the results in Figure 2d, we found that when cultured ex vivo, tumour cell isolates from Eμ-Myc/c-rel−/− mice showed increased sensitivity to the R-CHOP therapy components doxorubicin and vincristine (Figure 2e). Therefore, Eμ-Myc/c-rel−/− cells appear more prone to apoptosis.
when compared with their wild-type equivalents. These effects are consistent with the known antiapoptotic effects of c-Rel but did not explain the earlier onset of disease in c-Rel null mice.

The tumour suppressor Bach2 is a c-Rel target gene. The p53 and ARF pathways are frequently disrupted in Eμ-Myc lymphoma. However, we found that mRNA levels of p53 target genes, such as Mdm2 and Bax, as well as the CDKN2A gene that encodes the ARF protein were similar across end-stage Eμ-Myc and Eμ-Myc/c-rel+/- tumour cells (not shown), suggesting that c-Rel loss does not lead to further disruption of these pathways. Moreover, we did not observe significant differences in BCL2L1 mRNA, an NF-κB target gene that encodes the antiapoptotic protein Bcl-xL, from data in end-stage tumorigenic spleens from TCL1-Tg (Figure 3a). Of these, the loss of expression of Bach2 in c-Rel mutant mice was of particular interest. Bach2 is a lymphoid-specific transcription factor with a role in B-cell development and the response to oxidative stress. Bach2 has also been identified as a tumour suppressor in acute lymphoblastic leukaemia. Importantly, quantitative PCR analysis confirmed that Bach2 mRNA expression is lost in B cells from 4-week-old Eμ-Myc/c-rel+/- and Eμ-Myc/c-rel+/- mice (Figure 3a). Of these, the loss of expression of Bach2 in c-Rel mutant mice was of particular interest. Bach2 is a lymphoid-specific transcription factor with a role in B-cell development and the response to oxidative stress. Bach2 has also been identified as a tumour suppressor in acute lymphoblastic leukaemia. Importantly, quantitative PCR analysis confirmed that Bach2 mRNA expression is lost in B cells from 4-week-old Eμ-Myc/c-rel+/- and Eμ-Myc/c-rel+/- mice (Figure 3a). Of these, the loss of expression of Bach2 in c-Rel mutant mice was of particular interest. Bach2 is a lymphoid-specific transcription factor with a role in B-cell development and the response to oxidative stress. Bach2 has also been identified as a tumour suppressor in acute lymphoblastic leukaemia. Importantly, quantitative PCR analysis confirmed that Bach2 mRNA expression is lost in B cells from 4-week-old Eμ-Myc/c-rel+/- and Eμ-Myc/c-rel+/- mice (Figure 3a). Of these, the loss of expression of Bach2 in c-Rel mutant mice was of particular interest.

To determine the generality of these effects we also analysed Bach2 levels in the spleens of TCL1-Tg mice, where we observed a reduction in mRNA and protein levels (Figures 3f and g). Furthermore, in a separate NF-κB knock in mouse model, where the RelA subunit was engineered to contain a Thr505Ala mutation in its transcription domain, a site previously shown to affect NF-κB function, loss of Bach2 expression was also seen in end-stage lymphoma cells (Figure 3h) but not in 4-week B cells from Eμ-Myc mice (Figure 3i). The RelA T505A mouse will be described in more detail elsewhere.

Although these data indicated that Bach2 expression is regulated by c-Rel, Bach2 has not been previously described as a direct NF-κB target gene. To address this, we analysed chromatin immunoprecipitation sequencing (ChIP-Seq) data from the Epstein–Barr-virus-transformed human lymphoblastoid B-cell line GM12878. This revealed that the Bach2 promoter is bound by c-Rel together with the other NF-κB subunits, RelA, RelB and p52 (Figure 4a). Moreover, further analysis of ChIP-Seq data obtained for the RelA NF-κB subunit by the Encode consortium confirmed that Bach2 is an NF-κB target gene in multiple B-cell lines (not shown). Consistent with these data, analysis of the human Burkitt lymphoma cell line Daudi, where NF-κB subunits had been depleted by CRISPR/Cas9 mutagenesis, revealed that loss of either Bach2 mRNA levels are uniformly low in all Eμ-Myc/c-rel+/- and c-rel+/- lymphoma samples analysed, we observed a wide range of Bach2 mRNA expression in end-stage wild-type Eμ-Myc tumours (Figure 3c). We were therefore interested in whether this would correlate with survival of these wild-type Eμ-Myc mice. Significantly, we found that Eμ-Myc mice with below-the-median level of Bach2 mRNA displayed decreased survival, with a median survival of 85.5 versus 135 days for mice with high Bach2 levels (Figure 3e). Therefore, wild-type mice with reduced levels of Bach2 have a very similar pattern of lymphoma onset to that seen in the c-rel+/- mice, providing a potential mechanism that allows this NF-κB subunit to function as a tumour suppressor in this model of c-Rel-driven B-cell lymphoma (Figure 3e).

Figure 3. Expression of the B-cell tumour suppressor Bach2 is dependent on c-Rel in Eμ-Myc lymphoma. (a) Table showing genes whose expression is regulated by c-Rel from microarray analysis of bone marrow-derived B cells from 4-week-old Eμ-Myc, Eμ-Myc/c-rel+/- and Eμ-Myc/c-rel+/- mice. Fold changes shown are compared with equivalent wild-type cells and are in log2 (a positive number indicates higher expression in wild-type cells). Bone marrow-derived B cells were purified from 4-week-old Eμ-Myc or Eμ-Myc/c-rel+/-, Eμ-Myc/c-rel+/- mice using CD19 microbeads (MACS Miltenyi Biotec, Surrey, UK). Total B-cell RNA, purified using the PepGold total RNA extraction kit (Peqlab, WVR, Lutterworth, UK), was then used for microarray analysis at Cambridge Genomic Services (University of Cambridge, Cambridge, UK) using the Illumina mouse WG-6 Expression BeadChip system (San Diego, CA, USA). These data were background corrected in Illumina GenomeStudio and subsequent analysis proceeded using the lumi and limma packages in R (Bioconductor, Seattle, WA, USA). Variant stabilisation transform and robust spline normalisation were applied in lumi. Differential expression was detected using linear models and empirical Bayes statistics in limma. A list of genes for each comparison was generated using a Benjamini–Hochberg false discovery rate-corrected P-value of 0.05 as a cutoff. (b) Bone marrow-derived B cells from Eμ-Myc (n = 10), Eμ-Myc/c-rel+/- (n = 9) and Eμ-Myc/c-rel+/- (n = 9) mice and (c) end-stage tumorigenic spleens from Eμ-Myc (n = 30), Eμ-Myc/c-rel+/- (n = 12) and Eμ-Myc/c-rel+/- (n = 11) mice. q-PCR was performed in triplicate on 20 ng cDNA (Reverse Transcriptase kit, Qiagen, Crawley, UK), using predesigned Bach2 QuantiTec Primer assays (Qiagen). Samples were run and analysed on a Rotor-gene Q system (Qiagen), using murine Gapdh primers as an internal control. All cycle threshold values were normalised to Gapdh levels using the Pfaff method. Data represent mean ± s.e.m. *P < 0.01, **P < 0.001 (unpaired Student’s t-test). (d) Bach2 protein levels are reduced in Eμ-Myc/c-rel+/- mice. Whole-cell extracts were prepared from Eμ-Myc or Eμ-Myc/c-rel+/- tumorigenic spleens. Cell pellets were washed with ice-cold phosphate-buffered saline, and lysed using PhosphoSafe Extraction Reagent (Merck Millipore), according to the manufacturer’s protocols. Western blot analysis was performed using antibodies to Bach2 (ab83364 Abcam, Cambridge, UK) or the loading control β-ACTIN (AS441 Sigma-Aldrich). (e) Low levels of Bach2 mRNA correlate with poor survival in wild-type Eμ-Myc mice. Kaplan–Meier analysis of the survival of mice with below and above the median levels of Bach2 mRNA (from data in c). Also shown for comparison is the survival data from Eμ-Myc/c-rel+/- mice shown in Figure 1f. (f) Bach2 mRNA levels are c-Rel regulated in TCL1-Tg mice. q-PCR showing relative Bach2 expression in end-stage tumorigenic spleens from TCL1-Tg (n = 11) and TCL1-Tg/c-rel+/- (n = 7) mice. Data represent mean ± s.e.m. *P < 0.05, **P < 0.001 (unpaired Student’s t-test). (g) Bach2 protein levels are reduced in TCL1-Tg/c-rel+/- mice. Whole-cell extracts were prepared from TCL1-Tg or TCL1/c-rel+/- tumorigenic spleens and western blot analysis was performed as indicated. (h, i) Low Bach2 mRNA levels in RelA T505A mice. q-PCR showing relative Bach2 expression in end-stage tumorigenic spleens from Eμ-Myc (n = 30) and Eμ-Myc/relaT505A (n = 8) mice and (i) bone marrow-derived B cells from Eμ-Myc (n = 10) and Eμ-Myc/c-relaT505A (n = 8) mice. Note, data from wild-type Eμ-Myc mice are the same as shown in c. Data represent mean ± s.e.m. **P < 0.01 (unpaired Student’s t-test). RelA T505A knock-in mice were generated by Taconic Artemis (Cologne, Germany) using C57BL/6 J cells.
c-Rel or RelA reduced Bach2 mRNA levels (Figures 4b and c). However, no effect on Bach2 protein level was seen (not shown) suggesting functional compensation between c-Rel and RelA in these cells, as has been reported previously for these subunits.42 Treatment of Daudi cells with the IκB kinase-β inhibitors BMS 345541 or TPCA-1, which inhibit the classical NF-κB pathway and so target both RelA and c-Rel, did result in loss of both Bach2 mRNA and protein (Figures 4d and g), and similar results were seen in the Burkitt cell line BL41 treated with TPCA-1 (Figures 4h and i).

The role of c-Rel in B-cell lymphoma
Given the large number of studies indicating tumour-promoting roles for c-Rel in lymphoma,2–6,21–26,43 our results showing earlier onset of disease in c-Rel mutant mice were surprising. However, a number of in vitro studies have, in addition to their known tumour-promoting activities, revealed tumour suppressor functions for NF-kB subunits.1 Moreover, previous reports using mouse models of c-Myc-driven lymphoma have demonstrated that through induction of therapy-induced senescence, NF-kB can function as a tumour suppressor in this context.27,28 Importantly,
dependent senescence reported in Eκκ see if c-Rel can also contribute to the regulation of NF-κκ

Nonetheless, this demonstrates the complex interplay between NF-κκ in some contexts.45 Since the tumour suppressor stages of B-cell lymphoma development. However, some reports

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lymphoid blast crisis in chronic myeloid leukaemia.44 Although we cannot rule out contributions from the other c-Rel-regulated genes we identified, we propose that induction of Bach2 expression by c-Rel/NF-κκ binds one mechanism that allows these factors to function as tumour suppressors in the early stages of B-cell lymphoma development. However, some reports have suggested that Bach2 may also contribute towards malignancy in some contexts.45 Since the tumour suppressor functions of Bach2 are associated with p53, it is possible that p53 loss or mutation is also the trigger for a change in Bach2 function. Therefore, the consequences of NF-κκ regulation of Bach2 expression may vary depending on the stage of lymphoma development.

Accession numbers

NF-κκ ChIP-Seq data sets have been published41 (gene expression omnibus, accession code GSE55105).

Microarray data have been submitted to ArrayExpress, accession code E-MTAB-2774.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

JEH performed the majority of the experimental work and contributed to concept and design of experiments and manuscripts. JAB and HS assisted with protocols involving Eμ-Myc mice. BG provided advice on working with Eμ-Myc mice and assisted with data analysis. HDT provided training and assisted with lymphoma reimplantation studies. CB provided advice on B-cell lymphoma and contributed to experimental concepts and design. SJ performed bioinformatics analysis of microarray data. BEG performed analysis of ChIP-Seq data and contributed to experimental concepts and design. NDP contributed to concept and design of experiments and manuscripts.
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