Methods:

Mice

Eμ–Myc/+ transgenic mice, $L24^{+/-}$, $L38^{+/-}$ and $p53^{-/-}$ mice were all maintained on a C57/BL6 background and their offspring were bred in accordance with protocols approved by the committee for animal research at the University of California, San Francisco (UCSF), to obtain the genotypic combinations described in this paper. In all cases, Eμ–Myc/+ transgenic mice were maintained and studied on a heterozygote background. Mice were monitored twice a week for signs of morbidity and tumor development. Myc tumor initiation was scored by peripheral lymph node palpation as previously described[31]. Moribund mice (Eμ–Myc/+, Eμ–Myc/+; $L24^{+/-}$, Eμ–Myc/+; $L38^{+/-}$, $p53^{-/-}$, $p53^{-/-}$; $L24^{+/-}$) or mice with obvious tumors were killed, and tumors and different organs were analyzed by histology or processed for further analysis.

Cell culture and analysis of IRES dependent translation in mitosis

Primary B-lymphocytes were isolated from spleen or bone marrow from 4-5 week old mice utilizing an autoMACS separator (Miltenyi Biotec), according to the manufacturer's instructions. To synchronize primary B-lymphocytes in mitosis, 2 x 10^6 cells/ml were plated in culture medium (RPMI-1640 supplemented with 10 % foetal bovine serum (FBS), 1 % penicyllin/streptomycin, 0.5% β-mercaptoethanol, 5 μg/ml CD40 and 15 μg/ml IL-4). After 24 hrs, cells were resuspended in fresh culture medium supplemented with 2.5 mM of thymidine and incubated for 18 hrs to arrest cells in S phase. Cells were released from the thymidine block by washing in phosphate buffered saline supplemented with 2 % FBS and incubated in fresh culture medium for 6 hrs. Then, thymidine (2.5 mM) was added to the medium for 24 hrs. The cells were released from the second thymidine block as described above and incubated in fresh culture medium containing 1.5 μM nocodazole for 16 hrs to arrest cells in mitosis. Mouse embryonic fibroblasts (MEFs) were isolated from WT and L24+/- 13-14 d.p.c. embryos and infected with MycER[32]. Cells were transfected at steady state and in mitosis. For the steady state condition, MEFs were plated in 6-well culture plates at about $5x10^5$ cells per well and allowed to grow overnight to about 50% confluency. Transfection and subsequent steps were performed concurrently in both steady state and mitosis. Synchronization in mitosis was performed as previously described[33]. Briefly, upon release from the aphidicolin block, MEFs were transfected with the HCV-IRES or Cdk11/p58-IRES RNA bicistronic vector as previously described[30]. At this time MycER was activated by the addition of hydroxytamoxifen (OHT). Cells were harvested 12hrs hours in mitosis and Firefly and Renilla activities were quantified using the Glomax luminometer. Bicistronic mRNA levels were normalized by Q-PCR utilizing Rluc (5'-AACGCGGCCTCTTCTTATTT-3'; 5'-ATTTGCCTGATTTGCCCATA-3') and Fluc (5'-GAGGTTCCATCTGCAGGTA-3';5'-CCGGTATCCAGATCCACAAC-3') primers. IRES-dependent expression of the endogenous CDK11/p58 was performed by western blot utilizing rabbit polyclonal anti-CDK11/p58 from Abcam. Expression of β-actin was detected to confirm equal loading.

Analysis of global protein synthesis

Equal numbers of freshly isolated or cultured primary B-lymphocytes (synchronized in S phase or mitosis) from 4-5 week old mice were incubated in methionine-free DMEM for

45 min and then 50 μCi/well (25 uCi/mL) of [^{35}S] methionine (Perkin Elmer, Wellesley, MA) was added to the cultures for 35 minutes. Whole cell lysates were prepared with protein extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1mM DTT, 1mM EDTA, 1% Triton X-100, 1X protease inhibitor cocktails) by freezing in dry ice for 3 minutes and thawing at 37^{o}C for 3 minutes. 30 μg of protein was loaded on a 4-20% Tris-HCl gradient gel (Bio-Rad) and transferred onto a nitrocellulose membrane. In certain experiments, cells were pretreated with 200nM Rapamycin (Sigma) and incubated for 45 minutes at 37°C[34]. 30μCi of ^{35}S was added to each well and incubated for 90 minutes at 37°C. Radiolabeled proteins were visualized by exposure to X-ray film at -80^{o}C for 16 h. The radioactivity of each lane was quantified by densitometry analysis.

Cellular and molecular analysis of lymphocytes

Freshly isolated and cultured B-lymphocytes from 4-5 week old mice were fixed in 95 % ethanol, labeled with propidium iodide and cell cycle analyzed using a BD FACSCalibur^{TM} system. For in-vivo analysis of cell cycle rates, mice (4-6 weeks old) were adminstered 1 mg of BrdU (BD Biosciences BrdU Flow Kit) via i.p. injection 6 hrs before sacrifice. Spleenocytes were washed with PBS containing 3 % fetal bovine serum and 0.09% NaN_3 and B-lymphocytes were labeled with pacific blue-conjugated rat-anti mouse B220 (BD Biosciences). BrdU staining was performed using the BD Biosciences BrdU Flow Kit, following manufacturer's instructions. Samples were analyzed using a BD LSRII flow cytometer and the BD FACSDiva software. The percentage of BrdU positive B-lymphocytes was determined using the FlowJo 8.7.1 software. For the immunophenotypic analysis of blood and spleen from L24$^{+/-}$ and L38$^{+/-}$ mice, 100μl of peripheral blood and splenic cell suspensions obtained by mechanical disruption of the spleen, were processed and stained with the following combination of mAbs conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD19-PE/CD3-FITC, CD4-PE/CD8-FITC, CD43-PE/CD45R-B220-FITC. Fluorochrome-conjugated isotypic antibodies of irrelevant specificity were used as negative controls. Red blood cells were lysed immediately after labeling by incubation with 2 mL of FACS lysis solution (Becton Dickinson, San Jose, CA, USA) as recommended by the manufacturer. A minimum of 10,000 events/tube were acquired with a Becton-Dickinson LSRII flow cytometer. Mononuclear cells were gated and the percentage of each cell subset was determined using the CellQuest software (Becton Dickinson). Tunel assay was performed on freshly isolated lymphocytes by following manufacturer's instructions (Roche). Cell volume measurements were performed using a Coulter Model Z2 (Coulter). Cells were diluted in Isoton II (Beckman Coulter) at 100,000 cells/ml in 10 ml. A 1-ml sample was analyzed according to manufacturer's instructions. Fifty-thousand sorted cells were resuspended in 6 ml of Isoton II, and 1 ml was analyzed. Centrosome analysis was performed on cell cytospins fixed in cold methanol for 10 min and then briefly incubated in iced acetone. The antibodies used for immunofluorescence staining were anti-mouse γ-tubulin (T-6557 Sigma; 1:1,000) and Dapi. For western blot analysis total proteins were extracted in buffer A [150 mM NaCl, 20 mM NaH_2PO_4, 2 mM EGTA, 2 mM EDTA, 0.5 % Triton X-100, 1 mM DTT and a cocktail of protease inhibitors (Complete from Roche)]. 30 μg of proteins were employed. The membrane was probed with the appropriate antibody; Anti-mouse p27 (BD biosciences), Anti-rabbit cyclin D2 (Santa Cruz), Anti-mouse p21 (BD biosciences) Expression of β-actin was detected to confirm equal loading.

CGH analysis

Genomic DNA extracted from lymphomas of six Eμ–Myc/+ transgenic mice, and six Eμ–Myc/+;L24$^{+/-}$ mice was subjected to comparative genomic hybridization array analysis. High-molecular weight mouse DNA was extracted from mutant mice and normal tissue by standard methods and subjected to CGH according to the previously published method with some modifications[35]. Briefly, the test DNA and reference DNA were labeled by nick-translation with fluorescein-12-dUTP and Texas Red-5-dUTP (NEN-DuPont, Boston, MA), respectively. Equal amounts of test and reference DNA were coprecipitated along with 10 mg of mouse Cot-1 DNA (GIBCO/BRL, Gaithersburg, MD) and resuspended in the hybridization mix before in situ hybridization to mouse metaphase chromosome spreads Upon hybridization, the chromosomes were counterstained with DAPI to allow their identification. Then, 10 to 15 separate metaphases were captured for each case using a cooled charge-coupled devices (CCD) camera attached to a Nikon Eclipse 800 microscope. Copy number changes were detected based on the variance of the red: green ratio profile from the standard of one. Ratio values of 1.20 and 0.80 were used as upper and lower thresholds to define gains and losses, respectively.

Cytokinesis Analysis

A full-length Cdk11/p58 PITSLRE cDNA was generated from mitotic synchronized B cells using RT-PCR with the following primers: 5'-GAATTCTGAGGAAA TGAGTGAAGATGAAGAC-3'; 5'-GTCGACGACCTCAGAACTTGAGGCTGAA-3'. p58 PITSLRE cDNA was cloned in retroviral pBABE construct harboring hygromycin resistance. Primary MEFs were stably transfected with MycER harboring puromycin resistance or P58 cDNA plus MycER via a Phoenix viral vector and cultured in DMEM containing 10%FBS. Wt, MycER and P58;MycER cells were each plated in chamber slides at $2x10^{-4}$ cells per well. Synchronization in mitosis was performed as previously described[33]. Upon release from aphidicolin, Myc was activated by the addition of hydroxytamoxifen. At the 20 hour time point, cells were fixed in 4% paraformaldehyde and stained with an antibody against against α-Tubulin (Sigma) and Dapi. Binucleated cells were scored from more than four independent experiments with a minimal of 500 cell counts each. The slides were scanned utilizing a motorized X-Y stage and acquired images processed utilizing the taxonomy features in Nikon Elements software V3 to quantify the number of binucleated cells for each well.

Semi-quantitative and quantitative RT-PCR

Total RNA from B-lymphocytes was extracted with Trizol (Invitrogen) and purified with RNeasy (Qiagen), following manufacturer's instructions. From each sample, 3 μg RNA was treated with DNase (Turbo DNA Free, Ambion). Then, 1 μg was used for cDNA synthesis with the SuperScript III First-Strand Synthesis System (Invitrogen), following manufacturer's instructions and using 2.5 μg random primers (Promega) and 0.5 mM dNTPs and 400 U SuperScript III in a reaction volume of 100 μL. From each sample 1 μg RNA was used as negative control by omission of reverse transcriptase. For PCR, 1/10 (vol) of cDNA was used and 300 nM of the specific primers in a reaction volume of 25 μL. Quantitative PCR was employed for the analysis of p21 and p27

expression.SYBR GREEN PCR Master Mix (Applied Biosystems) was used and thermal profiles were as follows: predenaturation for 10 min at 95°C, followed by 42 cycles of denaturation at 95°C for 20 s, annealing at 57°C for 30 s and extension at 72°C for 30 s. Reactions were performed in duplicate in a Applied Biosystems 7300 thermocycler and the 7300 software system v1.4.0 was used for analysis. At the end of each PCR reaction, dissociation curves were generated to verify the formation of a specific amplicon. Primer sequences were as follows: p21, 5'- GACAGTGAGCAGTTGCG-3', 5'-CTCAGACACCAGAGTGC-3'; p27, 5'-TCAAACGTGAGAGTGTCTAACGG-3'; β-actin, 5'-CCTAGCACCATGAAGATCAAG-3', 5'-ATCGTACTCCTGCTTGCTG-3'. Forward and reverse primers utilized for Gadd45a Q-PCR were purchased from SuperArray cat.# PPM02927B. As a positive control for cellular stress and induction of GADD45α mRNA expression, 3T3 cells were incubated with 100 μg/ml MMS in culture medium (DMEM, 10 % FBS and 1% streptomycin/penicillin) for 4 hrs and total RNA was extracted with Trizol. Semi-quantitative PCR was employed for the analysis of Cyclin D2 expression. GoTaq Master Mix (Promega) was used and thermal profiles were as follow: predenaturation for 3 min at 94°C, followed by 25 cycles (denaturation for 30 s at 94°C, annealing for 30 s at 53°C (cyclin D2), and 57°C (β-actin), elongation for 1 min at 72°C) and final elongation for 10 min at 72°C. Primer sequences were as follows: cyclin D2, 5'-GTTCTGCAGAACCTGTTGAC-3', 5'-ACAGCTTCTCCTTTTGCTGG-3'. β-actin 5'-,GTATGGAATCCTGTGGCATC-3', 5'-AAGCACTTGCGGT GCACGAT-3'. Amplicons were resolved in 1.5 % agarose gel containing 40 mM Tris-acetate, 1 mM EDTA (pH8.0) and 0.5 μg/ml ethidium bromide. Gels were photographed by UV transilumination using an AlphaInnotec camera and software. Relative expression ratios were obtained by densitometry using the ImageJ software.

Methods References:

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