Supplemental Data

THE TEL-AML1 LEUKAEMIA FUSION GENE DYSREGULATES THE TGFβ PATHWAY IN EARLY B LINEAGE PROGENITOR CELLS

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Supplemental Methods

Isolation of TEL-AML1 inducible BaF3 cells

The GeneSwitch system (Invitrogen) (Wang et al. 1994), a mifepristone-regulated expression system for mammalian cells, was chosen to produce a cell line with inducible expression of the *TEL-AML1* gene. The IL-3-dependent murine pro-B cell line BaF3 (Palacios and Steinmetz 1985) was transfected with the pSwitch plasmid by electoporation and stable transfectants selected in semi-solid medium with 0.2mg/ml hygromycin. Positive clones were identified by Western blotting using an antibody to NF-kB (Santa Cruz). 3 clones were then stably transfected with the original pGene plasmid or the same vector containing the *TEL-AML1* cDNA and selected with 0.05mg/ml zeocin. 30 doubly transfected clones for pSwitch and *TEL-AML1* vectors were isolated in semi-solid medium and checked for 100% inducible expression of the fusion protein in liquid culture after treatment with $1x10^{-8}$M mifepristone (Invitrogen, as per manufacturers instructions) and staining with anti-AML1 (Calbiochem) or anti-V5 antibodies (Invitrogen) to detect the V5 epitope tag.

Immunocytochemistry

BaF3 cells were adhered to a glass slide by cytospin and fixed in chilled 4% paraformaldehyde for 20min. The cells on the slides were permealized with 0.2% Triton X-100 solution for 5min and after washing with TBS, they were quenched in 50mM Ammonium Chloride in TBS for 5min. Blocking was performed in 1% BSA for 1h followed by incubation with 1:400 mouse anti V5 antibody (Invitrogen) overnight (o/n) at 4°C. 1:100 FITC-conjugated anti mouse (Jackson Immuno Research) was used as the secondary antibody for 2h at room temperature (RT). After washing, the fixation procedure was repeated and the slides were incubated in 1:10000 TOPRO3 iodide (Molecular Probes) 3 times for 5min. Cover glasses were mounted with Vectashield (Vector Laboratories) and the slides were analysed by confocal fluorescence microscopy.

The percentage of TEL-AML1+ BaF3 cells was also measured by flow cytometry analysis after staining for intracellular V5, the tag fused at the 3' end of full-length *TEL-AML1*. The intrastaining was performed using the BD-Cytofix-Cytoperm kit (Becton Dickinson) and following the manufacturer's protocol. In particular, the goat polyclonal FITC conjugated anti V5 antibody (Abcam) was employed for 25min at 4°C at the concentration of 1:1200.

Transient transfection reporter gene assays

Annealed oligonucleotides containing sequences from the mouse Ig alpha promoter (5'-CGCGTCCCATGTGGTCAGACACACCTGTCTCCACCACAGCCAGACCAC AGGCCAGACATGACGTGGAGGC-3') were subcloned into the MluI and XhoI sites of the pGL3-Basic Vector (Promega) and acts as a minimal promoter. 1 x 10^6 cells of uninduced and 3 days induced BaF3 clones were co-transfected by nucleofection

(following the Amaxa protocol) with the Ig alpha reporter gene plasmid and the control reporter vector pRL-CMV (Promega) to normalize the results for transfection efficiency. After nucleofection the cells were plated in 5ml of culture medium in the presence or absence of 10ng/ml rhTGFβ for 18h (in the medium of the induced BaF3 clones was already 0.01nM Mifepristone). The cells were lysed and luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's recommendation.

Pre-B cell colony assays

To methylcellulose already enriched with IL-7 at 10ng/ml (Methocult M3630, StemCell Technologies) was added 50ng/ml Flt-3 ligand (R&D Systems), 100ng/ml murine SCF (Pepro Tech) and $1x10^5$ bone marrow cells per ml methylcellulose. 1.1ml methylcellulose was then dispensed into 35mm x 10mm petri dishes (Falcon) and incubated at 37°C, 5% CO_2 for 11 days. The number of colonies produced was counted after 9 days.

Replating and cell sorting was performed after 11 days, essentially 60 'tight' colonies and 60 'spread' colonies were sterilely picked and 30 of each replated in three 35mm x 10mm petri dishes in M3630 Methocult with supplemented 50ng/ml Flt3 and 100ng/ml mSCF. A further 30 colonies were similarly replated in medium supplemented as above with the addition of TGFβ at 10ng/ml (R&D Systems).

For additional Q-PCR analysis, 'spread' colonies were also separated into two populations of $CD11b^+$ and $CD11b^-$ fractions by a MACS separation column system and MACS CD11b microbeads (Miltenyi Biotech). Both the positive and the negative

fractions were plated in three 35mm x 10mm petri dishes in M3630 Methocult with 50ng/ml Flt-3 ligand + 100ng/ml mSCF and either with or without TGFβ (10ng/ml).

Quantitative real-time PCR (Q-PCR)

The inducible control clones and clones inducible for TEL-AML1 were grown in the presence or absence of 0.01nM Mifepristone and 10ng/ml rhTGFβ for 3 days. At the indicated incubation times 2 x 10^6 cells were harvested and lysed in 500µl Trizol (Invitrogen). Total RNA was extracted following the manufacturer's recommendation. 1 µg of RNA was subjected to cDNA synthesis with Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen), using random hexamers in accordance with standard procedures. *p27^{KIP1}* and the internal control *GAPDH* were amplified at the same time by using commercially available gene assay kits (Applied Biosystems). The PCR mix contained 0.7µl cDNA template, 12.5µl 2x pPCR Mastermix PLUS (Eurogentec), 1.25µl 20X specific primers and probe (Applied Biosystems) in a total volume of 25 µl. Standard reactions were performed using an Applied Biosystems PRISM 7700 or 7900HT Sequence Detection System. Transcript levels of *p27^{KIP1}* in control clones and induced TEL-AML1 clones were assigned the value 1 at the time point 0h. All experiments were carried out in triplicate.

Immunoprecipitation and immunoblotting

BaF3 cells were lysed in highsalt RIPA buffer (1% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS, 350nM NaCl in PBS) with Protease inhibitor cocktail (Sigma) and 0.25nM PMSF.

To test for TEL-AML1 expression, proteins were resolved on 4–12% NuPAGE gels (Invitrogen) run in MOPS SDS buffer (Invitrogen) for 1h at 200volt and transferred to PVDF membranes (Imobilon-P, Millipore) for 1h at 30Volts. The blot was blocked in

5% non-fat dry milk in PBS for 1h at RT and incubated o/n at 4°C with the mouse anti V5 antibody (Invitrogen). After washing, the membrane was incubated with the secondary goat anti-mouse IgG-HRP antibody (Santa Cruz) for 1h at RT, and subjected to ECL Western Blotting Detection Reagents (GE Healthcare).

The immunoprecipitations to verify TEL-AML1/Smad3 association were performed using the Catch and Release Immunoprecipitation System (Upstate) with anti-Smad3 antibody (Abcam) and following the manufacturer's protocol. The immune complexes were subjected to Western Blot analysis as described above using the rabbit anti-RHD antibody (Calbiochem) o/n at 4°C. After detection, the blot was stripped in 100mM 2-mercaptoethanol, 2% SDS and 62.5M Tris pH 6.7 for 30min at 50°C. The membrane was then washed, re-blocked and incubated with rabbit anti-Smad3 antibody o/n at 4°C or with rabbit anti-GAPDH antibody (Research Diagnostics Inc) for 1h at RT.

Western Blot analysis to test Smad2/3 phosphorylation was performed using rabbit anti-Phospho-Smad2 antibody (Cell Signaling) o/n at 4°C and after stripping, anti rabbit Smad2/3 antibody (Cell Signaling) also o/n at 4°C.

Phenotyping and cell sorting

Mouse bone marrow was harvested from the femurs of TEL-AML1 positive and wild type mice and placed in 0.5ml IMDM. The RBCs in the marrow were then lysed using 0.8% ammonium chloride solution with 0.1mM EDTA (StemCell Technologies) and incubated for 7 minutes at 4°C. Cells were then blocked with PBS containing 2% BSA for 10 min, before being incubated with the antibodies listed below conjugated with either PE or FITC. Antibodies were as follows: anti-CD-45R (B220, RA3-6B2), anti-CD19 (1D3), anti-CD135 (FLT3, A2F10.1), anti-Ly-51 (BP1, 6C3), anti-Ly6a/E (Sca-

1,D7), anti-CD117 (c-Kit, 2B8), anti-CD127 (IL7Ra, SB/199), anti-CD11b (M1/70) (PharMingen, BD Bioscience). Cells were washed with PBS containing 2% BSA and acquired in a LSRII flow cytometer (Becton Dickinson Biosciences). Files were analysed using WinMDI 2.9 software.

For cell sorting, a magnetic activated cell sorting (MACS) separation column system (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to separate the cells into $CD11b^+$ and $CD11b^-$ fractions, using MACS CD11b microbeads (Miltenyi Biotech). The two fractions were then sorted again using MACS B220 and Sca-1 microbeads (Miltenyi Biotec). Pellets were snap frozen in liquid nitrogen.

EMSA

EMSA was performed on extracts isolated from REH cells t(12;21) and control Raji cells t(8;14) pre-treated with 10ng/ml TGFβ for 30 mins using the Cell Lytic Nuclear Extraction kit (Sigma), the Gelshift AML1 kit (Active Motif) and a radio-labelled probe (Pardali etal 2000 JBC 275:3552-3560).

Figure 1. Construction of TEL-AML1 inducible BaF3 cells.

Schematic of the GeneSwitch System and expression of TEL-AML1 (see text).

The expression of TEL-AML1 is controlled by the presence or absence of the agonist mifepristone which brings about a conformational change in the pSwitch regulatory protein and causes its subsequent activation and dimerisation.

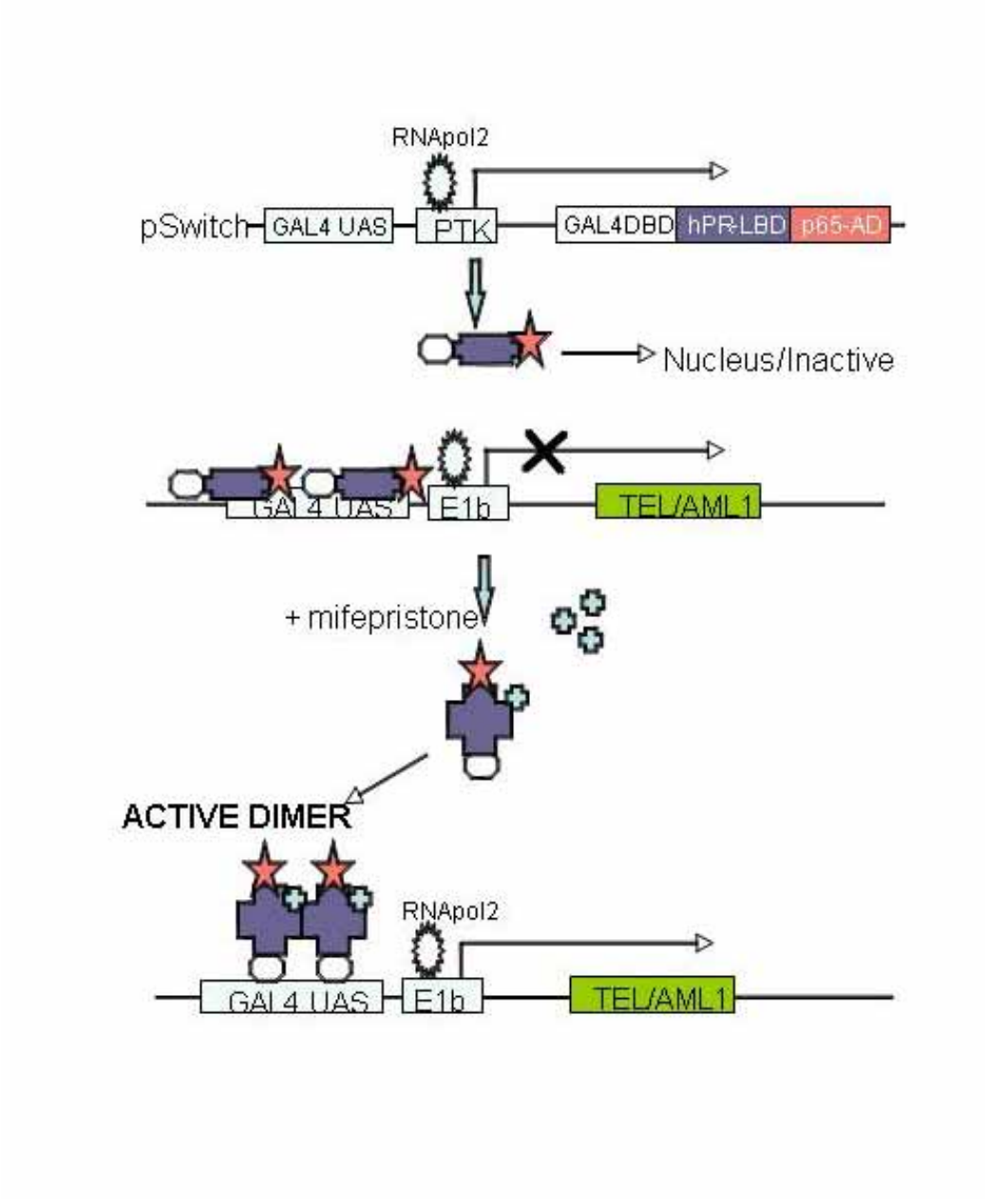


Figure 2. Inducible expression of TEL-AML1 in BaF3 cells.

Immunofluorescence staining after a time course of induced expression of TEL-AML1 for an alternative clone (1/28) after induction of TEL-AML1 by mifepristone and staining with anti AML1 RHD antibody (green) and DAPI (blue).

A) clone 1/28 uninduced **B**) clone 1/28 induced 6hrs

C) clone 1/27 induced 6hrs for comparison

D) clone 1/28 induced 72hrs showing nuclear staining

E) TEL-AML1 expressing cells co-stained with TOPRO3 and anti-V5 antibody (as a marker of TEL-AML1, LHP) or anti-V5 alone (RHP) to show mainly nuclear localisation.

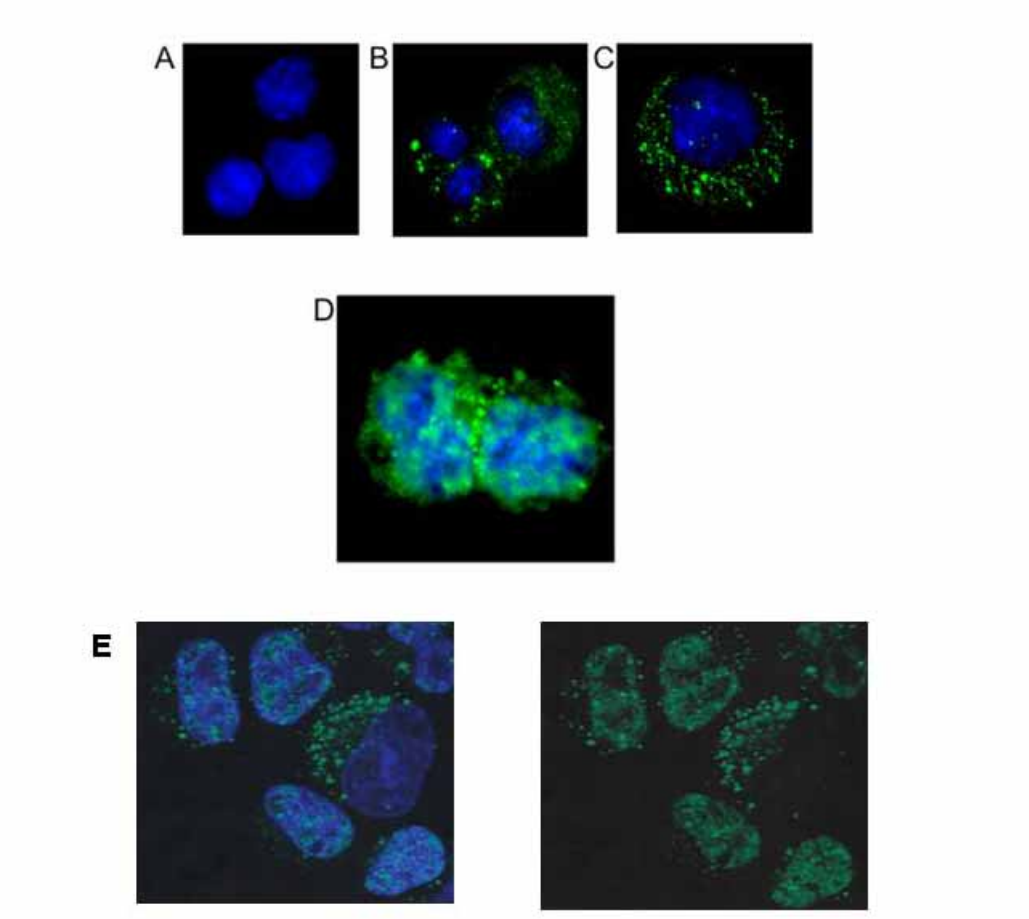


Figure 3. Growth characteristics of clone 1/28 inducible for TEL-AML1.

An alternative clone, 1/28, was also checked for growth response following expression of TEL-AML1 (M) and to TGFβ (T) under conditions described above. The growth response was identical to that of clone 1/27 (see manuscript figure 2).

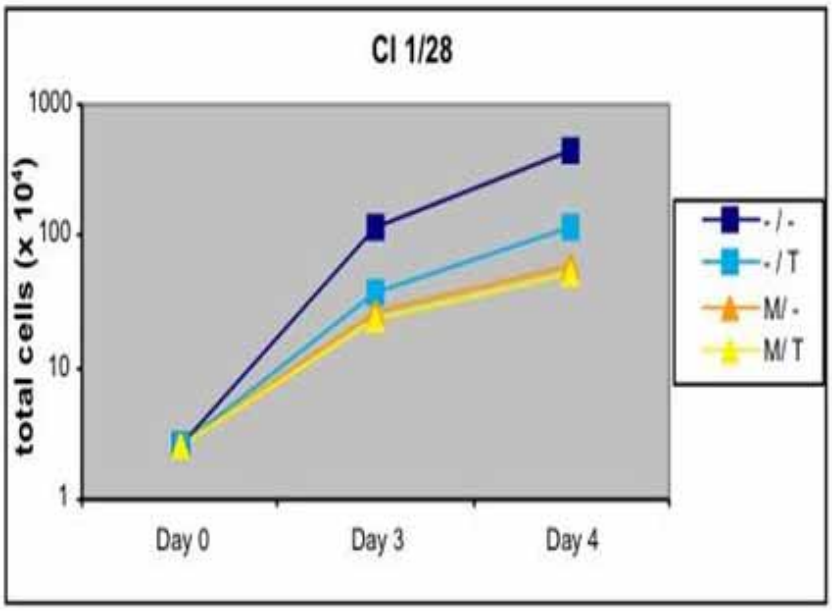
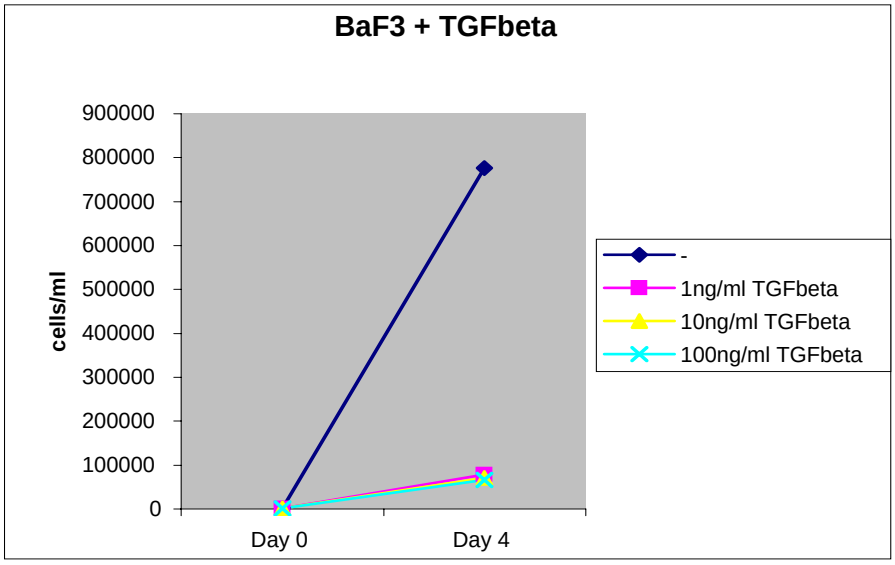


Figure 4. **Dosage Effect of TGFβ on parental BaF3 cells and effect of IL3-withdrawal on BaF3 cells un-induced or induced to express TEL-AML1.**

A) BAF3 cells were incubated with increasing amounts of TGFβ and analysed for cell growth. A concentration of 10ng/ml TGFβ was used in all subsequent experiments.



B) Uninduced and TEL-AML1 induced BAF3 cells do not show factor independence after withdrawal of IL3. M is mifepristone.

(This experiment was only performed once simply to look for IL3 independent growth induced by TEL-AML1 and no other assumptions should be made from the data).

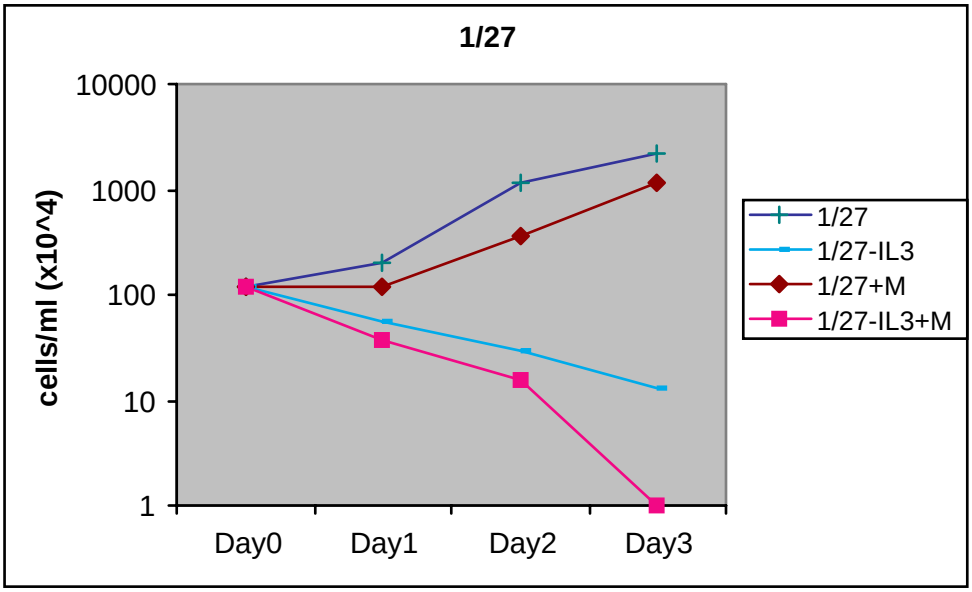


Figure 5. Relative changes in expression of cell-cycle control gene $p27^{kip1}$ in the presence of TEL-AML1 may explain the change in cell cycle.

The control clone (Cl1) and the clone inducible for TEL-AML1 (1/27) were grown for 3 days in the presence or absence of mifepristone (+M) and in the absence of TGFβ. Relative expression of $p27^{kip1}$ was assessed by Q-PCR. Expression of TEL-AML1 increases the expression of $p27^{kip1}$ when compared to the GAPDH control.

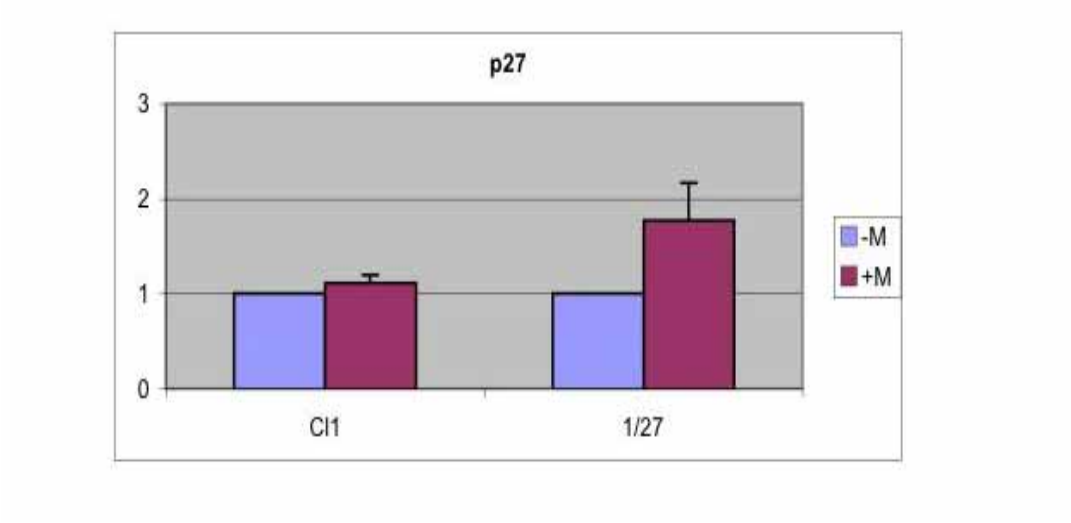


Figure 6. Analysis of endogenous SMAD2 phosphorylation in response to TGFβ.

Control and inducible cells were incubated with TGFβ for the indicated times in the presence or absence of inducer (Mif). Cells were then lysed and analysed for Smad2/3 phosphorylation by Western blot. The blot was later stripped and re-probed to check the total amount of Smad2/3 in the samples.

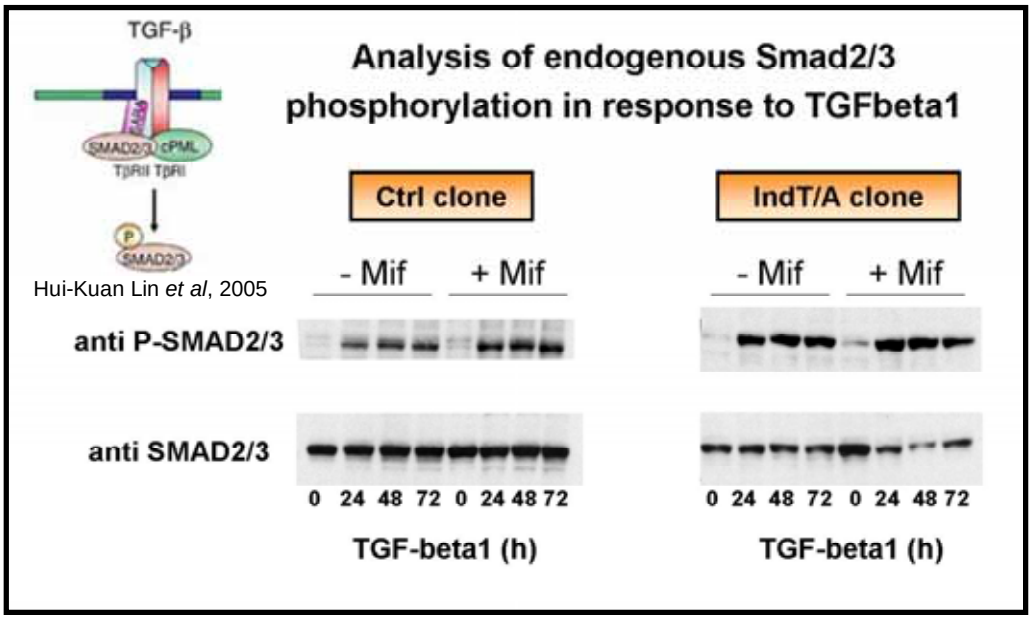
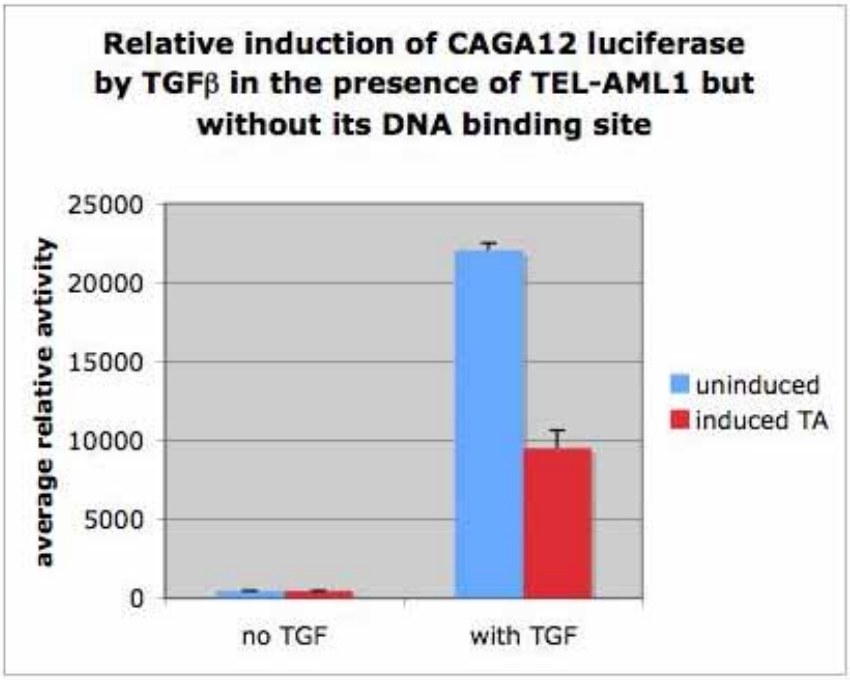


Figure 7. Repression of TGFβ signalling by TEL-AML1 occurs through SMAD3.

A). The $CAGA_{12}$ promoter reporter gene (a kind gift of Caroline Hill) contains 12 tandem copies of the DNA binding site for SMAD3 alone linked to luciferase and was used to assess the response to TGFβ in the absence of a binding site for (TEL-)AML1. In the presence of TEL-AML1 but the absence of its binding site, the normal activation of the $CAGA_{12}$ promoter by TGFβ is still repressed.



B). EMSA of Raji and REH protein extracts to the Ig-α promoter shows binding of TEL-AML1 and SMAD3 only in REH t(12;21) cells. In REH cells antibody supershift is seen to anti- AML1 and TEL and reduction of binding with a slight shift to anti-SMAD3 antibody. The competitor DNA 'self' is the AML1/SMAD3 oligo from Pardali et al., (2000, JBC 275:3552-3560), competitor 'AML1' contains only the AML1 binding site,

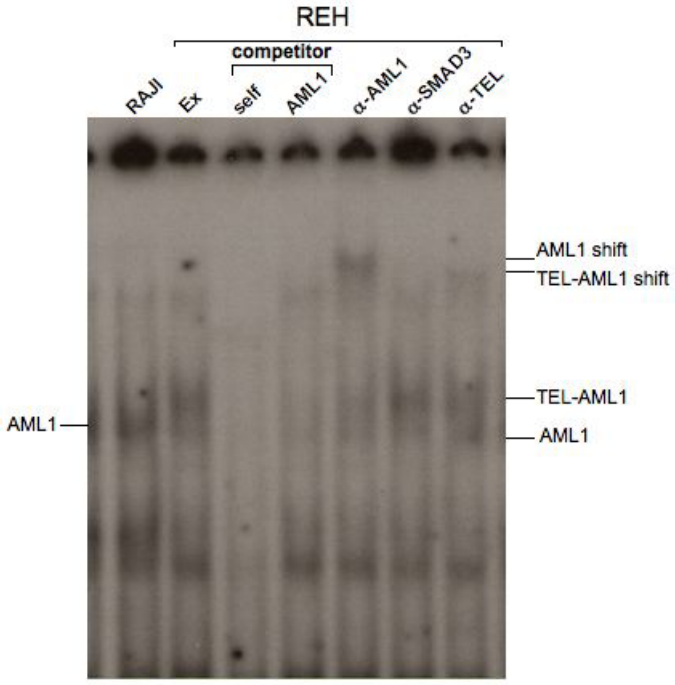


Figure 8. Impact of TGFβ on B cell progenitor numbers

A) Analysis of second round tight colonies produced from first round tight colonies. Error bars represent SD from 5 independent experiments.

B) Analysis of second round spread and tight colonies produced from first round spread colonies. Error bars represent SD from 5 independent experiments.

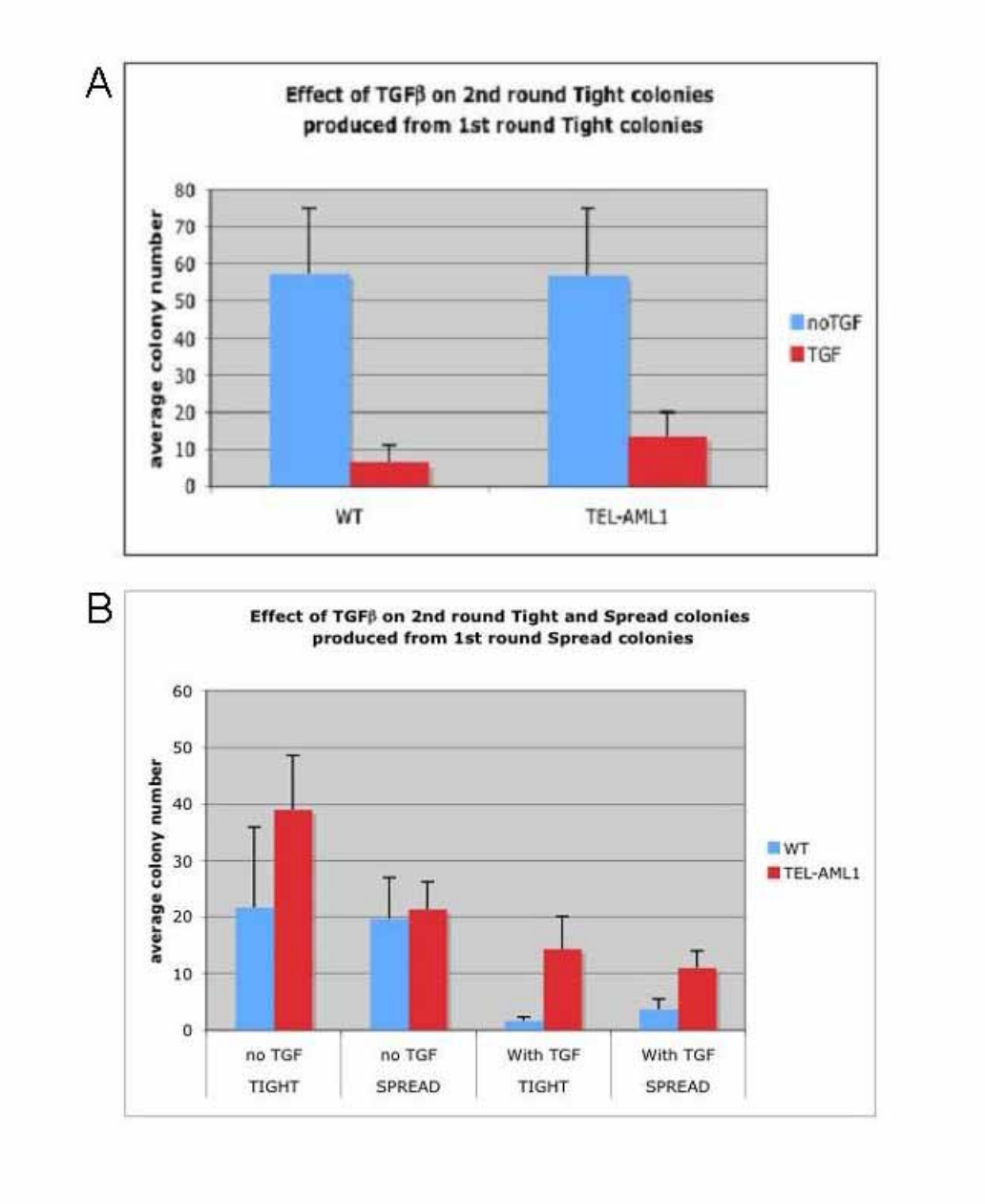
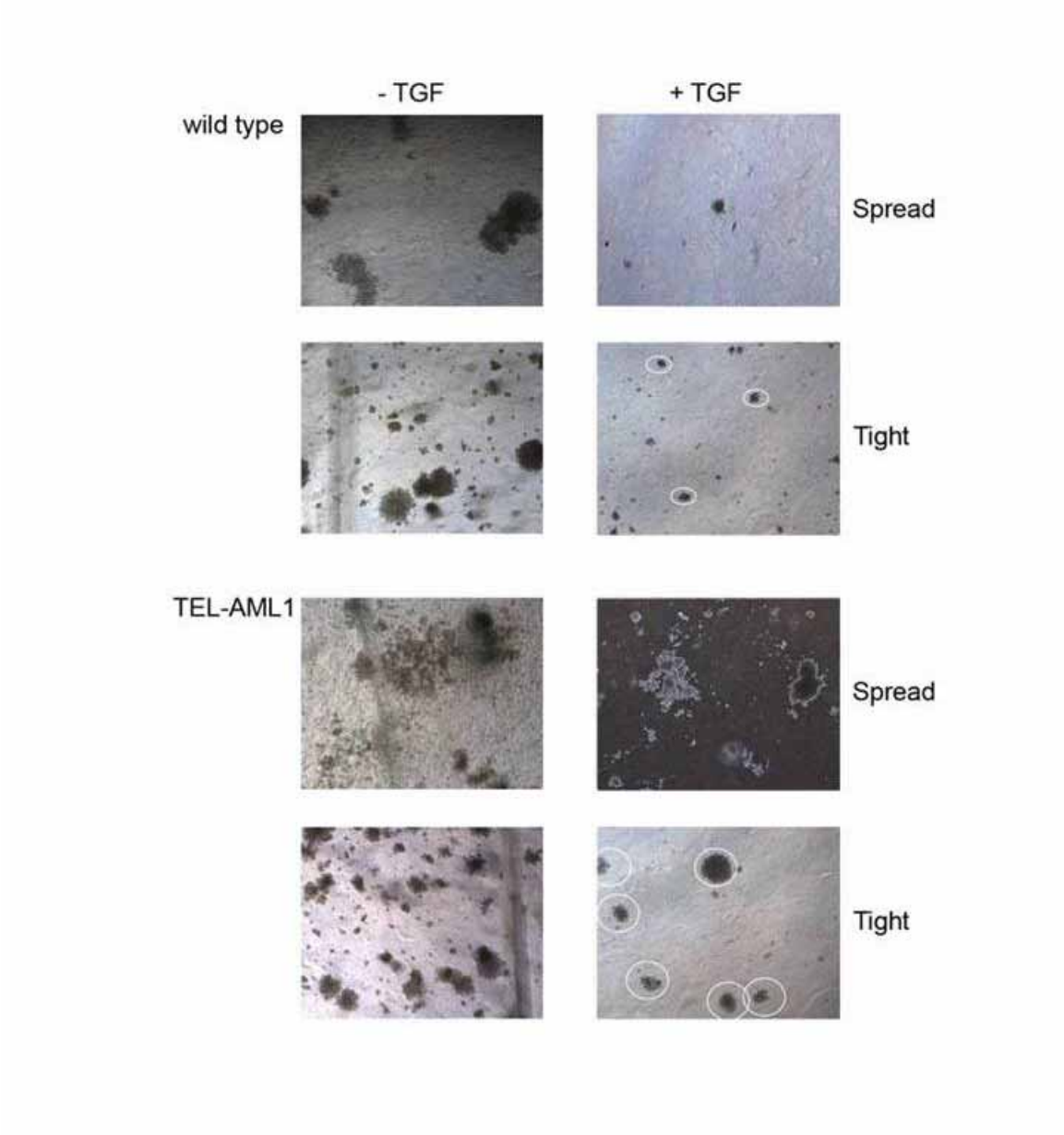


Figure 9. Impact of TGFβ on B cell progenitor colony morphology



Colonies were grown in the presence of IL7 with additional SCF and Flt-3 ligand and with or without TGFβ (TGF). Tight colonies scored in the presence of TGFβ are ringed.

Figure 10. Q-PCR analysis of tight and spread TEL-AML1 colonies for expression of lineage-specific markers and TEL-AML1.

cDNA was prepared directly from pooled tight colonies (tight) and from spread colonies further sorted into separate populations by CD11b (Spread CD11b- and Spread CD11b+ respectively). Respective cDNAs were normalized to GAPDH. M is mb1 (CD79a), R is Rag1 and TA is TEL-AML1. Vertical lines show corresponding Ct values.