EKLF/KLF1 CONTROLS CELL CYCLE ENTRY VIA DIRECT REGULATION OF E2F2

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Running head: EKLF directly regulates E2F2 and cell cycle entry

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Differentiation of erythroid cells requires precise control over the cell cycle to regulate the balance between cell proliferation and differentiation. The zinc finger transcription factor, erythroid Krüppel-like factor (EKLF/KLF1), is essential for proper erythroid cell differentiation and regulates many erythroid genes. Here we show that loss of EKLF leads to aberrant entry into S-phase of the cell cycle, during both primitive and definitive erythropoiesis. This cell cycle defect was associated with a significant reduction in the expression levels of E2f2 and E2f4, key factors necessary for the induction of S-phase gene expression and erythropoiesis. We found and validated novel intronic enhancers in both the E2f2 and E2f4 genes which contain conserved CACC, GATA and E-Box elements. The E2f2 enhancer was occupied by EKLF in vivo. Further, we were able to partially restore cell cycle dynamics in EKLF-/- fetal liver upon additional genetic depletion of Rb, establishing a genetic causal link between reduced E2f2 and the EKLF cell cycle defect. Finally, we propose direct regulation of the E2f2 enhancer is a generic mechanism by which many KLFs regulate proliferation and differentiation.

Erythroid Kruppel-like factor (EKLF/KLF1), the founding member of the Kruppel-like factor (KLF) family of C2H2 zinc finger transcription factors, is essential for erythropoiesis (1-3). EKLF activates a diverse set of erythroid genes that include components of the erythroid membrane and cytoskeleton such as dematin (band 4.9) (4-6), heme synthesis enzymes, the erythroid chaperone α-hemoglobin stabilizing protein (AHSP) (7,8) and other transcription factors such as basic Kruppel-like factor (BKLF/KLF3) and TIEG/KLF10 (9).

Erythropoiesis is a complex process that requires a co-ordinated balance between proliferation/self renewal and differentiation, a process tightly linked to cell cycle control (10). In particular, regulation of the G1/S checkpoint is essential for both terminal differentiation (G0 entry) and proliferation (S-phase entry) (10). Gene targeting of specific regulators of this checkpoint has shown some of these to be critical for erythropoiesis in mice. In particular, components of the Rb-E2F complex that controls S-phase entry are essential for proper erythropoiesis. Loss of a functional Retinoblastoma (Rb) gene in mice leads to defective erythropoiesis and death in utero prior to E16 (11-13). Furthermore, loss of both E2f2 and E2f4, which are expressed at high levels during erythroid differentiation leads to the development of anemia in mice and a failure of proper erythroid expansion and maturation (14-16).

Transcriptional regulation of the E2f2 gene is not well understood, particularly in erythroid cells. There are E-box and E2f binding sites in the proximal promoter which can bind c-myc and E2Fs in response to growth signals, but there are no conserved CACC box elements or SP1 binding sites (17). During the preparation of this manuscript, a study by Pilon et al. suggested that the E2f2 promoter is bound by EKLF resulting in gene activation (18). The erythroid specific transcription factor GATA-1 has been shown to regulate the cell cycle by activation and repression of critical cell cycle control genes, including G1 cyclins and c-myc (19,20). Indeed, extensive circumstantial evidence exists for cooperation between GATA-1 and EKLF in erythroid gene regulation, suggesting the two
factors could work together to control aspects of the cell cycle (21) in particular the expression of E2f2.

We previously identified E2fs as potential EKLF target genes in a global profiling study (5, data accessible via http://spring.imb.uq.edu.au, Username: Hodge_etal_2005, Password: blood). The focus of this study was to examine how loss of EKLF altered the cell cycle, and to interrogate possible direct links between EKLF and the E2fs. Like Pilon et al. we show the major cell cycle defect of EKLF-/- cells is defective S-phase entry, and identify abnormal expression of E2f2 and E2f4 as the likely cause. However, in contrast to Pilon et al., we suggest that EKLF binding to a previously undescribed intronic enhancer is critical for E2f2 gene regulation. We suggest that our newly discovered enhancer region is likely to act in co-operation with the promoter to drive appropriate E2f2 expression. We also show partial rescue of the cell cycle phenotype in EKLF-/- mice is achieved by depletion of the E2F-binding protein Rb, providing further evidence for a genetic link between EKLF and the E2F-Rb G1/S checkpoint.

**Experimental Procedures**

**Mouse Studies**- Rb +/- EKLF +/- mice were obtained by mating EKLF +/- mice (3) and Rb +/- mice (12). Rb +/- EKLF +/- mice were intercrossed at 8-12 weeks of age. Genotyping of mouse lines and embryos was performed by genomic PCR using primers indicated in Supplementary Table 1.

**Draq5 Cell Cycle Profiling**- Peripheral blood collected at E10.5 (primitive red cells) or cells from homogenised E14.5 fetal livers (definitive red cells) were washed twice in FACS buffer (PBS containing 2% FCS) and filtered through a 70µm cell strainer. Cells were resuspended in FACS buffer at a concentration of 1 x 10⁶ cells/mL and stained by addition of the cell permeant DNA dye Draq5 (Biostatus) to a concentration of 2.5µM and incubated at room temperature in the dark for 10 minutes. Co-staining with 7-AAD provided an assessment of DNA content. FACS analysis was performed using a LSR II flow cytometer (BD Biosciences), and BD FACSDiva (BD Biosciences) or FlowJo (Treestar) software.

**In Vivo BrdU Incorporation Assays**- Intraperitoneal injection of 200µL of BrdU solution (10mg/mL in PBS) was performed on pregnant female mice 1 hour prior to sacrifice. Peripheral blood at E10.5, or fetal livers at E13.5 and E14.5 were collected from embryos and stained using a FITC BrdU Flow Kit (BD Pharmingen) according to the manufacturer’s recommendations. FACS analysis was performed using an LSR II flow cytometer (BD Biosciences), and BD FACSDiva (BD Biosciences), or FlowJo (Treestar) software.

**Gene Expression Analyses**- Total RNA was obtained using Trizol reagent (Invitrogen) according to the manufacturer’s instructions from E10.5 peripheral blood cells, homogenised E14.5 fetal liver, or K1-ER cells induced by addition of 4-hydroxytamoxifen (4-OHT, Sigma) as previously described (7). cDNA was prepared from total RNA by reverse transcription using Superscript III reverse transcriptase (Invitrogen) and oligo dT primers. Quantitative real-time RT-PCR reactions were performed as previously described (5) using primers designed to cross an intron-exon boundary. Primer sequences are described in Supplementary Table 1.

**Bioinformatics**- The UCSC Genome Browser (22) and the Evolutionary Conserved Region (ECR) Browser (23) were used to align the genic and intergenic sequences of E2f2 and E2f4 between the mouse, human and dog genomes and identify ECRs of greater than 100bp length and 70% similarity. Using the rVISTA (24) user-defined motif search tool we identified EKLF consensus binding sites (5'-CCNCNCCCN-3') within these regions. ECRs from the first intron of E2f2 and fifth intron of E2f4 were identified as potential erythroid specific enhancer regions and aligned using AlignX software (Invitrogen). The 7 species regulatory potential (RP) track available on the UCSC Genome Browser was also used to find putative erythroid specific enhancers (25).

**Luciferase Reporter Assays**- The XhoI restriction site of the pGL2-Promoter vector (Promega) was used to clone a 502bp fragment of murine E2f2 intron 1 (shown in Fig. 3) or a 222bp fragment including murine E2f4 intron 5 (shown in Supp. Fig. 1) in either the forward (5’-3’) or reverse (3’-5’) orientation upstream of the ubiquitous minimal SV40 promoter driving expression of luciferase. Constructs were transfected into murine
erythroleukemia (MEL) cells using Lipofectamine LTX (Invitrogen) according to the manufacturer’s recommendations, or Drosophila SL2 cells together with pPAC-EKLF or empty pPAC vector as previously reported (26). Lysates were prepared and assayed for luciferase activity using the Luciferase Assay System (Promega) and a VICTOR Light Luminescence Counter (PerkinElmer).

Chromatin Immunoprecipitation (ChIP)- The erythroid cell line K1zf-ER was created by immortalisation of EKLF -/- erythroid progenitors with J2 retrovirus as previously described (27), followed by infection with MSCV expressing the zinc finger DNA binding region (amino acids 273-376) of murine EKLF as an ER™ fusion. These cells or E14.5 fetal liver erythroid cells were used to determine in vivo chromatin occupancy of EKLF. K1zf-ER cells were induced for DNA binding activity by treating with 4-hydroxytamoxifen (4-OHT, Sigma). ChIP was performed as previously described (26) using a specific ERα antibody (Ab-10; Neomarkers) together with an irrelevant mouse IgG1 control antibody, or a specific EKLF antibody (3) together with pre-immune rabbit serum as a control. ChIP assays to address GATA-1 binding were performed in a similar manner using a specific monoclonal GATA-1 antibody (sc-265, Santa Cruz Biotechnology) or an irrelevant rat IgG control. Enrichment of transcription factor binding was determined by real-time PCR using specific primers designed to amplify putative enhancer regions. Primer sequences are provided in Table 1.

Electrophoretic Mobility Shift Assays (EMSA)- EMSA were performed as previously described (28). Nuclear extracts were derived from COS7 cells that had been transfected with an EKLF expression plasmid (pSG5-EKLF) (1) or from MEL cells. Supershifts were performed using a rabbit polyclonal antibody specific for EKLF (3) or a rat monoclonal antibody specific for GATA-1 (sc-265, Santa Cruz Biotechnology). Oligonucleotide sequences used to generate radiolabelled probes are provided in Supplementary Table 1 with only the forward strand shown. Reverse oligonucleotides were annealed in excess after labelling of the forward strand with T4 polynucleotide kinase (Promega) and [γ-32P] ATP (PerkinElmer).

RESULTS

Loss of EKLF impairs S-phase entry during primitive and definitive erythropoiesis. Forced over-expression studies in cell lines (27), expression profiling experiments (4,5) and the morphology of EKLF -/- erythroid cells (3-5) suggested a cell cycle defect caused by a loss of EKLF. To examine cell cycle phasing of erythroid cells in vivo, we used the cell permeable DNA dye Draq5. There was a significant reduction in cells with S-phase DNA content during primitive (E10.5 peripheral blood, Fig. 1A) and definitive (E14.5 fetal liver, Fig. 1B) erythropoiesis in EKLF -/- (Ek) embryos when compared to wild-type littermates (WT). In order to determine if this was due to a shortened S-phase or an inability of cells to enter S-phase we injected pregnant females with BrdU solution 1h prior to sacrifice and collected primitive blood or fetal livers to determine the amount of BrdU incorporation by FACS. There was a marked reduction in BrdU incorporation in EKLF -/- embryonic (Figs. 1C & E) and definitive red cells (Figs. 1D & F) compared with litter mates, which is consistent with the Draq5 DNA content data. In addition, there was a significant increase in cells in G2-M phase in EKLF -/- fetal liver cells, suggesting loss of EKLF in the erythroid compartment impairs progression through G2-M directly, or indirectly by eliciting a G2/M checkpoint (compare Ek and WT in Figs. 1E & F).

Expression of S-phase genes E2f2 and E2f4 is reduced in EKLF -/- mice. In order to explain the defect in S-phase entry for EKLF -/- erythroid cells we focused our studies on the E2F family of transcription factors as potential EKLF target genes, due to their critical role in S-phase progression. E2f5 and E2f6 showed no difference in expression at the mRNA level between EKLF -/- and wild-type E14.5 fetal livers (0.98 and 1.098 respectively, Ek vs WT, data not shown), while E2f1 and E2f3 showed only a modest decrease in mRNA levels in EKLF -/- (0.708 and 0.717 respectively, Ek vs WT, data not shown). In contrast, E2f2 and E2f4 both showed a significant reduction in mRNA expression in EKLF -/- fetal livers at E14.5 when compared to wild-type (0.28 and 0.38 respectively, Ek vs WT, Fig. 2B). In order to confirm that E2f2 and E2f4 were likely to be critical EKLF target genes during...
erythropoiesis, we investigated the mRNA expression levels during primitive erythropoiesis at E10.5 when the phenotype is subtle. E2f2 expression was also significantly reduced at E10.5 in the EKLF -/- (0.4, Ek vs WT, Fig. 2A). However, E2f4 expression was unaffected at this time (1.03, Ek vs WT, Fig. 2A). We also sought to confirm that the change in E2f2 expression levels was a direct effect of EKLF activity by utilising the K1-ER cell line system, which contains a 4-OHT (Tamoxifen) inducible form of EKLF (27). E2f2 is rapidly induced by 4-OHT, even faster than ß-globin and AHSP in these cells (Fig. 2C) (7).

Intronic regions of high conservation within E2f2 and E2f4 contain EKLF and other erythroid transcription factor binding sites. To investigate the possibility of direct regulation of E2f2 and E2f4 by EKLF, we looked for EKLF consensus binding sites (5'-CCNCNCCCN-3', (29)) that were conserved between the mouse, human and dog genomes. We identified a highly conserved region within the first intron of E2f2, with a high regulatory potential (RP) score (>0.3) (25) that contained EKLF consensus sites as well as consensus sites for the erythroid specific transcription factors GATA-1 and SCL/TAL1 (Fig. 3A). This region represents a putative erythroid specific enhancer of E2f2 expression (hereafter referred to as E2f2-i1en). A multiple sequence alignment of this region shows that it contains three independent conserved EKLF consensus sites (designated CACC-1, CACC-2 and CACC-3, Fig. 3B) as well as a conserved GATA-1 consensus site (GATA, Fig. 3B) and five separate E-Box binding sites for SCL/TAL1 and related proteins (E-BOX, Fig. 3B). Investigation of the E2f4 gene locus also showed a region containing conserved EKLF and GATA-1 consensus sites within intron 5 (hereafter referred to as E2f4-i5en) which has also been assigned a high RP score (>0.3, Supp. Fig. 1A). However, this region is not as highly conserved across the three genomes (Supp. Fig. 1B).

Intronic regions of E2f2 and E2f4 act as enhancers in erythroid cells and show EKLF dependence. We next sought to confirm the regions E2f2-i1en and E2f4-i5en were functional enhancers of gene expression using reporter assays. Enhancer constructs containing either E2f2-i1en or E2f4-i5en in forward and reverse orientations were tested for activity in the murine erythroleukemia cell line, MEL. Both E2f2-i1en and E2f4-i5en were found to enhance transcription of the luciferase gene in MEL cells 10-30 fold relative to a basal vector containing the SV40 promoter only (Fig. 4A). Interestingly, both E2f2-i1en and E2f4-i5en seem to display a degree of directional preference, forward for E2f2-i1en and reverse for E2f4-i5en.

To address whether these enhancers were directly responsive to EKLF, we performed further reporter assays in Drosophila melanogaster SL2 cells which lack any SP/KLF proteins. E2f2-i1en shows a significant response (2-3 fold induction) upon co-transfection with an EKLF expression plasmid in either the forward or reverse orientation when compared to empty vector (Fig. 4B). However, the E2f4-i5en enhancer does not display a significant response in either orientation when compared to empty vector containing the basal SV40 promoter alone (Fig. 4B), suggesting it may not be directly EKLF responsive.

EKLF binds to CACC sites within intron 1 of E2f2 in vivo and in vitro. To determine if the identified putative enhancers (E2f2-i1en and E2f4-i5en) were occupied by EKLF in vivo we performed a ChIP assay (Fig. 5). Using a cell line containing an inducible form of the EKLF DNA binding domain (K1zf-ER, inducible by addition of 4-OHT), we found there is specific in vivo occupancy of E2f2-i1en but not regions 1kb upstream or downstream, and that this occupancy is dependent on 4-OHT (EKLF induced, Tamox/black bars, Fig. 5A). In contrast, there is no occupancy of E2f4-i5en by K1zf-ER in these cells (Fig. 5B). This is consistent with our observations made by reporter assay in SL2 cells (Fig. 4B). As a positive control, a previously identified site of EKLF binding in the AHSP gene promoter (7) was also occupied by K1zf-ER in these cells (data not shown).

We were also able to show occupancy by endogenous EKLF at E2f2-i1en in primary fetal liver cells (Supp. Fig. 2). Pilon et al. recently showed EKLF occupancy broadly at the E2f2 proximal promoter (18). Even though the CACC sites within the E2f2 proximal promoter are not conserved, we also found EKLF was enriched close to the transcriptional start site (Supp. Fig. 2).

ChIP assays have limited resolution which is dependent on efficiencies of DNA shearing;
ChIP cannot reliably distinguish occupancy of sites within ~50-100bp. Thus, to determine EKLF binding at E2f2-i1en at higher resolution we used EMSA and specific probes designed for each of the bioinformatically identified EKLF binding sites (CACC-1, CACC-2, CACC-3, Fig. 3B). EKLF is only able to bind to the CACC-3 and CACC-2 probes in vitro (Fig. 5C) as confirmed by supershift with a specific EKLF antibody. EMSA using a probe for the CACC site of E2f4-i5en confirmed what was observed in vivo by ChIP, as EKLF also failed to bind to this site (data not shown). Taken together this data suggests direct regulation of E2f2 by EKLF is likely to occur via binding to the enhancer region, E2f2-i1en, which contains two EKLF binding sites (CACC-2 & CACC-3). Direct regulation via binding to the promoter could also occur as suggested by Pilon et al. (18). However, we suggest that transcriptional regulation of E2f2 probably occurs via co-operating contributions of both the promoter and the E2f2-i1en element (Supp. Fig. 2).

Additional genetic depletion of Rb rescues impaired cell cycle entry in EKLF-/- erythroid cells. We hypothesised that the defects in S-phase entry observed in EKLF-/- embryos at E10.5 in the peripheral blood and E14.5 in the fetal liver were primarily caused by a loss of EKLF dependent E2f2 gene expression. We postulated much of the abnormal erythroid differentiation phenotype in EKLF-/- embryos could be the result of aborted entry into S-phase. In order to test this, we generated Rb/EKLF double heterozygous mice (3,30) and crossed these mice for in vivo BrdU incorporation assays. Rb normally binds E2f2 and prevents its inappropriate activity, until Rb phosphorylation by CDK2, CDK4 or CDK6 allows the release of E2f2 and subsequent activation of S-phase. Thus, loss of Rb in EKLF-/- embryos should permit activity of residual E2f2 and hence S-phase entry. Previous studies have employed this genetic strategy to partially rescue the erythropoietic defects present in E2f2-/- mice (31).

At E10.5 (embryonic erythropoiesis) there was no significant improvement in the number of cells entering S-phase in the combined Rb/EKLF -/- compared to the EKLF -/- peripheral blood (Fig. 6A, compare Ek and Rb/Ek). This result was not unexpected as BrdU incorporation into Rb -/- embryos was no different to that of wild-type (WT) embryos in embryonic red cells (Fig. 6A, compare WT and Rb). EKLF -/- and Rb/EKLF -/- embryos were significantly different to wild-type in all three cell cycle phases (Fig. 6A). Thus, it is likely that Rb does not play a significant role during embryonic erythropoiesis as Rb -/- embryos were found to be phenotypically normal at this stage, as previously reported (13).

In contrast, additional loss of Rb in EKLF-/- definitive fetal liver cells resulted in partial rescue of the cell cycle at E13.5 (Fig. 6B). While EKLF -/- embryos showed a reduction of cells entering S-phase compared to wild-type at E13.5 as expected (55% and 61% for Ek and WT respectively, Fig. 6A), the combined loss of Rb and EKLF provided a rescue such that there was no significant difference in S-phase entry compared to WT (62% for Rb/Ek, Fig. 6B). These observations demonstrated that a lack of EKLF activation of E2f2 was the primary cause of the cell cycle defect during definitive erythropoiesis. This result prompted us to look for signs of a phenotypic rescue to erythropoiesis. However, we found no indications of a phenotypic rescue to erythropoiesis by FACS using antibodies to Ter119 and CD71, or by morphology in May-Grunwald Giemsa stained peripheral blood cytospins (data not shown). It was interesting to note that the proportion of cells in G1-phase between the Rb/Ek and WT embryos was significantly different (Fig. 6B). We attribute this difference is due to the increased proportion of cells trapped in G2/M-phase in the Rb/Ek embryos, independent of the restored G1/S transition. We conclude that a combined loss of EKLF and Rb does provide a partial rescue to cell cycle dynamics during definitive erythropoiesis, however many other aspects of erythroid development remain perturbed.

**DISCUSSION**

EKLF is a master regulator of erythropoiesis. EKLF’s essential role in erythropoiesis was initially thought to be primarily via direct regulation of the β-globin gene (2,3). However, it is becoming increasingly clear that EKLF is essential for other aspects of erythroid differentiation such as cytoskeletal integrity (4-6), transmembrane blood group and other non-globin
protein expression (4,5), and erythroid versus megakaryocyte lineage specification (32-34). In our study we have established a role for EKLF in erythroid differentiation via direct regulation of components of the cell cycle machinery.

We previously demonstrated a role for EKLF in cell cycle control by direct regulation of the cyclin-dependent kinase inhibitor, p18\textsuperscript{INK4c} (26), which is also a target of GATA-1 (19). In this study we have shown that one of the major defects of EKLF -/- red cells is an inability to enter into S-phase of the cell cycle during definitive erythropoiesis (confirmation of the results of Pilon et al.) and also during primitive erythropoiesis (Fig. 1). This was at odds with loss of p18\textsuperscript{INK4c} which would be predicted to result in increased S phase entry, and suggested additional EKLF target genes were responsible for the cell cycle phenotype.

Direct regulation of E2f2 is a critical function of EKLF in erythropoiesis. Herein, we have identified E2f2 as a direct target gene of EKLF via an intronic enhancer region we have called E2f2-i1en. Recently published work suggests that regions of the E2f2 gene promoter are also required for EKLF regulation (18). However, we hypothesise that the E2f2-i1en region may also be responsive to other erythroid transcription factors such as GATA-1 and SCL/TAL1 due to the presence of conserved motifs, and occupancy by GATA-1 \textit{in vivo} and \textit{in vitro} by ChIP assay and EMSA respectively (Supp. Fig. 3). As such, E2f2-i1en represents an erythroid specific enhancer similar to that shown for intron 8 of the Alas2 gene (35).

E2f2 and the closely related transcription factor E2f4 are the primary members of the E2F transcription factor family expressed during erythropoiesis (31). A loss of either transcription factor in mice leads to anemia which is associated with defects in erythroid maturation and an inadequate expansion of the erythroid compartment (14-16,36). However, our studies suggest E2f2 to be the only member of the E2F family to be a direct target gene of EKLF. The reduced levels of E2f4 mRNA observed in EKLF -/- mice may be in part, due to the failure of proper erythroid differentiation rather than as a consequence of impaired EKLF activity.

Based on the organisation of E2f2-i1en, it is likely that GATA-1 and perhaps SCL/TAL1 co-operate with EKLF to regulate E2f2 gene expression, although we are not aware of ChIP experiments which confirm \textit{in vivo} occupancy of this enhancer by SCL. To add to the complexity of erythroid cell cycle circuitry, GATA-1 and E2Fs themselves serve as transcriptional regulators of p18\textsuperscript{INK4c} gene expression (37). GATA-1 also regulates EKLF gene expression (38), suggesting a complex regulatory network impinges upon the G1/S checkpoint in erythroid cells. Nevertheless, the consequence of loss of EKLF is a dramatic reduction in S-phase entry.

While the regulation of E2f2 during erythropoiesis depends upon EKLF acting at the transcriptional level, regulation of E2F2 protein function occurs by association with the Rb protein to sequester its activity (39). It is interesting to note that E2F4 does not associate with Rb, but rather with the other two members of the pocket protein family, p107 and p130 (40). We attempted to restore the cell cycle balance of EKLF -/- red cells by genetically engineering additional loss of Rb. A similar experiment was recently used to show that the E2F2-Rb interaction during erythropoiesis is critical for control of S-phase entry and erythroid maturation (31). We hypothesized that a similar result might be obtained by restoring the ability of cells to enter S-phase in the EKLF -/- mice. Indeed, the combined loss of EKLF and Rb during erythropoiesis provided a rescue to S-phase entry during definitive erythropoiesis suggesting reduced levels of E2F2 are responsible (Fig. 6B). The failure to rescue erythroid differentiation during definitive erythropoiesis was not surprising since EKLF regulates many other critical target genes which are not dependent on the function of Rb. We also failed to improve survival of Rb -/- mice with additional loss of EKLF. This is also not surprising, since a critical role for Rb in the erythropoietic niche and the placenta has been demonstrated (41,42). Thus, reduction of E2F2 levels in erythroid cells (indirectly via loss of EKLF) would not be expected to rescue these aspects of the Rb -/- phenotype.

The KLF family as cell cycle regulators. Our study has identified a novel mechanism whereby EKLF, the founding member of the KLF family, regulates the cell cycle during erythropoiesis via direct binding to a novel E2f2 enhancer. There are 17 members of the KLF family and 8 members of the
Some act primarily as transcriptional activators and others act as repressors via differential recruitment of co-activators and co-repressors such as CBP/p300 and CtBP. Since all KLFs and SP1-like proteins bind very similar GC-rich or CACC-box motifs, we suggest they might perform a similar function in cell cycle regulation via E2f2 in other tissue types. Consequently, we interrogated recent ChIP-seq data for KLF4 occupancy in ES cells and found occupancy directly over the same E2f2-i1en (44), which coincides with specific trimethylation of H3 lysine 4 (45) (Supp. Fig. 4). In these studies no such occupancy of KLF4 was observed in the promoter of E2f2. We suggest that a critical role played by KLF2 and KLF4 in ES cell self-renewal, and induction of iPS cells from differentiated adult cells (46), might be primarily enacted via regulation of E2f2 and that this regulation is likely to occur via the E2f2-i1en region alone (44,47,48). As further KLF ChIP-seq and ChIP-chip data become available, we predict other members of the family will be found to directly bind E2f2-i1en in vivo. We hypothesize this will be a generic mechanism by which the KLF family of proteins control the balance between self renewal and differentiation.

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**FOOTNOTES**

"The authors wish to thank Natalie Eriksson for help with maintenance and genotyping of the EKLF and Rb mouse colonies and Tyler Jacks (MIT) for the kind gift of the Rb mutant mice. The authors are also grateful to Tom Whittington for assistance with bioinformatics and use of the UCSC Genome Browser, and to Simon Wilkins for critical review of the manuscript. This work was supported by a grant from the Cancer Council Queensland (519718) (A.C.P.), and an ARC Discovery Grant (DP0770471) (A.C.P.). M.R.T. is the recipient of an Australian Postgraduate Award. J.R.K. was supported by a research fellowship from the Cooley’s Anemia Foundation and P.O.H. is a RD Wright Fellow of the Australian NH&MRC.

The abbreviations used are: AHSP, α-hemoglobin stabilizing protein; BKLF, basic Kruppel-like factor; CBP/p300, CREB binding protein; CDK, Cyclin dependent kinase; ChIP, Chromatin immunoprecipitation; ChIP-seq, Chromatin immunoprecipitation sequencing; COS7, African green monkey kidney fibroblast cell line; CtBP, C-terminal binding protein; ECR, Evolutionary conserved region; EKLF, erythroid Kruppel-like factor; ER, estrogen receptor; HPRT, hypoxanthine phosphoribosyltransferase; MEL, murine erythroleukemia cell line; MSCV, murine stem cell virus; NRS, normal rabbit serum; Rb, retinoblastoma gene/protein; RP, regulatory potential; SCL/TAL1, stem cell leukemia hematopoietic transcription factor; SL2, Schneider’s *Drosophila* line 2.

**FIGURE LEGENDS**

Fig. 1. Disruptions to the cell cycle during primitive and definitive erythropoiesis in EKLF -/- mice. Cell cycle profiles were obtained by Draq5 DNA staining and FACS analysis of wild-type (WT) and EKLF -/- (Ek) embryos. (A) Representative DNA histogram of E10.5 peripheral blood. (B) Representative DNA histogram of E14.5 fetal liver. Representative FACS contour plots of E10.5 peripheral blood (C) and E14.5 fetal liver (D) from *in vivo* BrdU incorporation assay show a reduction in
cells containing BrdU. Quantification of BrdU incorporation assay data using gates shown in C and D for E10.5 peripheral blood (E) and E14.5 fetal liver (F). Mean+SEM, n≥3 for each genotype, * P<0.05 compared to WT by Student’s t test. Percentages of cells in G2-M-phase are indicated.

**Fig. 2.** E2f transcript levels are reduced in EKLF -/- erythrocytes. Levels of E2f2 and E2f4 mRNA were determined by quantitative real-time RT-PCR in E10.5 peripheral blood (A) and E14.5 fetal liver (B). Mean+SEM relative to housekeeping gene HPRT and WT expression levels, n≥3 for each genotype, * P<0.05 vs WT levels by Student’s t test. (C) Induction of E2f2 gene expression by 4-OHT. K1-ER erythroid cells were used to show a rapid induction of E2f2 gene expression by RT-PCR upon addition of 4-OHT (Tamox) but not upon addition of vehicle control (EtOH). E2f2 mRNA expression levels normalised to pre-treatment controls and the housekeeping gene HPRT. Each point is represented as Mean±SEM, n=4 at each time-point.

**Fig. 3.** Identification of a potential intronic enhancer in E2f2. The murine E2f2 gene from the UCSC Genome Browser. Gene structure is indicated by the Refseq track with exons shown by numbered black boxes and narrow portions indicating UTR. Conserved CACC (EKLF binding) sites and enhancer regions identified using the ECR browser are grossly represented on separate tracks. Tracks utilizing conservation data (RP 7 Species (25) & Cons M v H v D) are also shown to highlight the specific intronic enhancer region. Scale bar is shown. Three-way species alignment generated (mouse, human, dog) for the shaded region of E2f2 (B) provides a more specific picture of the binding motifs present for EKLF (CACC, 5’-CCNCNCCCN-3’), GATA-1 (GATA, 5’-A/TGATAA/G-3’) and SCL/TAL1 (E-BOX, 5’-CANNTG-3’). Nucleotide positions relative to the canonical start of transcription are indicated.

**Fig. 4.** Intronic regions of E2f2 and E2f4 enhance expression in erythroid cells. The regions of E2f2 and E2f4 identified to be potential intronic enhancers (E2f2-i1en & E2f4-i5en respectively) cloned into the pGL2-promoter vector (positions relative to transcriptional start are indicated) were transfected into MEL cells to show erythroid activity (A). Mean+SEM, n=3, * P<0.05 vs empty vector by Student’s t test. The same E2f2-i1en and E2f4-i5en constructs were transfected into Drosophila SL2 cells with or without an EKLF expression vector (pPAC-EKLF and Control respectively, (B)). Mean+SEM, n=3, * P<0.05 vs empty vector by Student’s t test.

**Fig. 5.** EKLF occupies the E2f2 intronic enhancer in vivo and in vitro. ChIP assays in an inducible EKLF cell line (K1zf-ER) show specific EKLF binding to the CACC enhancer region (E2f2-i1en) of E2f2 and not to regions upstream or downstream in the presence of 4-OHT only (Tamox/black bars, (A)). In this same system no binding was observed across the E2f4 enhancer region (E2f4-i5en, (B)). The mean relative occupancy for positive (ERα) vs negative (IgG1) antibodies for two independent experiments is shown. Error bars represent the standard deviation between experiments. (C) EMSA using probes specific for the three predicted EKLF binding sites (CACC-1, CACC-2, CACC-3) in the E2f2-i1en region as well as a control probe from the β-globin promoter (β-globin). Nuclear extracts from COS7 cells alone or COS7 cells transfected with an EKLF expression vector (pSG5-EKLF) were used. EKLF binding was confirmed by supershift with specific EKLF antisera (αEKLF).

**Fig. 6.** Combined loss of EKLF and Rb rescues S-phase entry during definitive but not primitive erythropoiesis. BrdU incorporation assays were performed on embryonic peripheral blood at E10.5 (A) and fetal liver at E13.5 (B). Genotypes are as indicated (wild-type (WT), Retinoblastoma -/- (Rb), EKLF -/- (Ek)). BrdU incorporation assay data was gated as shown in Fig. 1 and plotted as Mean+SEM, n≥3 for each genotype, * P<0.05 compared to WT by Student’s t test. Percentages of cells in G2-M-phase are indicated for clarity.

**Supp. Fig. 1.** Identification of a potential intronic enhancer in E2f4. The murine E2f4 (A) gene from the UCSC Genome Browser. Gene structure is indicated by the Refseq track with exons shown by
numbered black boxes and narrow portions indicating UTR. Conserved CACC (EKLF binding) sites and enhancer regions identified using the ECR browser are grossly represented on separate tracks. Tracks utilizing conservation data (RP 7 Species(25) & Cons M v H v D) are also shown to highlight a specific intronic enhancer region. Scale bar is shown. Three-way species alignments generated (mouse, human, dog) for the shaded region of E2f4 (B) provides a more specific picture of the binding motifs present for EKLF (CACC, 5’-CCNCNCCC-3’) and GATA-1 (GATA, 5’-A/TGATAA/G-3’). Nucleotide positions relative to the canonical start of transcription are indicated.

**Supp. Fig. 2.** EKLF occupies sites across the E2f2 gene locus *in vivo*. A ChIP assay was performed using a specific EKLF antibody (EKLF) or non-specific rabbit serum (NRS) as a control in E14.5 fetal liver cells. Primer sets covering both the E2f2 proximal promoter and the E2f2-i1en region are indicated as A-H on the E2f2 locus together with a track showing all sites that match the EKLF consensus (A). Results are represented as relative occupancy for EKLF antibody vs control (NRS) for a single ChIP experiment (B).

**Supp. Fig. 3.** GATA-1 occupies E2f2-i1en *in vivo* and *in vitro*. A ChIP assay was performed using a specific GATA-1 antibody or an irrelevant rat IgG as a control in E14.5 fetal liver cells (A). Primers to assay binding across E2f2-i1en were used as described for Fig. 5. Results are presented as relative occupancy for GATA-1 vs control (ratIgG) for a single ChIP experiment. Binding of GATA-1 to E2f2-i1en was confirmed by *in vitro* EMSA using the CACC-3 probe that contains a GATA-1 consensus binding site and nuclear protein extracts from MEL cells (B). The specific GATA-1 interaction with the CACC-3 probe was confirmed by supershift with a specific GATA-1 antibody.

**Supp. Fig. 4.** Potential regulation of E2f2 by KLF4 in murine ES cells via E2f2-i1en. The murine E2f2 gene was visualised using the UCSC Genome Browser together with two sets of previously published ES cell data. Tracks are shown for all regions matching the EKLF consensus binding site 5’-CCNCNCCC-3’ (All CACC Sites), KLF4 binding sites determined using ChIP-seq by Chen et al. (44) (KLF4 Binding), and trimethylation of H3 lysine 4 in both ES cells (ES Cell H3K4me3) and mouse embryonic fibroblasts (MEF H3K4me3) from data generated by Mikkelsen et al. (45). Other tracks are shown as for Fig. 3.
### Supplementary Table 1. Oligonucleotides

|                | Forward Primer | Reverse Primer       |
|----------------|----------------|----------------------|
| **Genotyping** |                |                      |
| Rb WT          | 5'GCATCTGCATCTTTATCGC | 5'CCCATGTTCGGTCCTCATA |
| Rb KO          | 5'GCATCTGCATCTTTATCGC | 5'GAAGAACGAGATCGACGAC |
| EKLF WT        | 5'AGGCTTGGTGAAGCCTGCTTTCGG | 5'TTGGAATAGCCTTTCGCCAGCTT |
| EKLF KO        | 5'AGGCTTGGTGAAGCCTGCTTTCGG | 5'CCGCTATCAGAATCGTGGC |
| **Gene expression** |                |                      |
| E2f2           | 5'CCCCAAAACCCCCACGTCCT | 5'ACTCGCTAGGAGGTATAGACT |
| E2f4           | 5'GCAAGATGGCTTTGCTGGGAAAT | 5'TGCTGGACTTCTCTGCCCCAG |
| HPRT           | 5'GCAGTACAGGCCCAAAATGG | 5'ACCAAGTCTGGCCTGTGGAAG |
| **EMSA probes** |                |                      |
| β-Globin CACC  | 5'TAGAGGCCACACCTGCCTGTAAG |                      |
| E2f2 CACC-1    | 5'GGGGGATCTGGGCTGGGAGGAGAAGAT | 5'TGCTGGATAGGAACTCCG |
| E2f2 CACC-2    | 5'TCCCTGGAGCTGGGAGGAGGAAGAAGAT | 5'ACCAAGTCTGGCCTGTGGAAG |
| E2f2 CACC-3    | 5'AGGGAACAGCTGCTGCTGCTGCTG |                      |
| **ChIP primers** |                |                      |
| E2f2 CACC (G)  | 5'TGGCCAGGAGGGAGAAGTG | 5'ACACCTGGGACTCCCTG |
| E2f2 1kb 5' (F) | 5'GTTGTTGAGGGAGGAGGAGAAGAT | 5'ACACCTGGGACTCCCTG |
| E2f2 1kb 3' (H) | 5'TGGCCAGGAGGGAGAAGTG | 5'ACACCTGGGACTCCCTG |
| E2f4 CACC      | 5'GGCAGAGCTGGGAGGAGGAGAAGAT | 5'ACACCTGGGACTCCCTG |
| E2f4 1kb 5'    | 5'AGGGGAGGAGGAGGAGAAGAT | 5'ACACCTGGGACTCCCTG |
| E2f4 1kb 3'    | 5'AGGGGAGGAGGAGGAGAAGAT | 5'ACACCTGGGACTCCCTG |
| AHSP CACC      | 5'AGGGAACAGCTGCTGCTGCTG | 5'ACACCTGGGACTCCCTG |
| E2f2 Promoter (A) | 5'TCTTGGAGGGAGGAGAAGAAGT | 5'ACACCTGGGACTCCCTG |
| E2f2 Promoter (B) | 5'TCTTGGAGGGAGGAGAAGAAGT | 5'ACACCTGGGACTCCCTG |
| E2f2 Promoter (C) | 5'TCTTGGAGGGAGGAGAAGAAGT | 5'ACACCTGGGACTCCCTG |
| E2f2 Promoter (D) | 5'TCTTGGAGGGAGGAGAAGAAGT | 5'ACACCTGGGACTCCCTG |
| E2f2 Promoter (E) | 5'TCTTGGAGGGAGGAGAAGAAGT | 5'ACACCTGGGACTCCCTG |

*EKLF consensus binding sites are shown underlined.
Figure 1

A

E10.5 Peripheral Blood
(Primitive)

WT

Ek

B

E14.5 Fetal Liver
(Definitive)

WT

Ek

C

WT

Ek

D

WT

Ek

E

% of Cells

WT

Ek

F

% of Cells

WT

Ek

G1

S

G2-M

0

20

40

60

80

100

0.8

1.2

G1

S

G2-M

1.6

0.6
Figure 2

A

E10.5 Peripheral Blood (Primitive)

Expression Relative to HPRT

|          | WT | Ek |
|----------|----|----|
| E2f2     |    |    |
| E2f4     |    |    |

B

E14.5 Fetal Liver (Definitive)

Expression Relative to HPRT

|          | WT | Ek |
|----------|----|----|
| E2f2     |    |    |
| E2f4     |    |    |

C

K1-ER Cell Line Induction

- Tamox
- EIOH

Fold Induction Relative to HPRT

| Induction Time (h) | 0   | 8   | 16  | 24  |
|--------------------|-----|-----|-----|-----|
| Tamox              |     |     |     |     |
| EIOH               |     |     |     |     |
Figure 5

A

B

C

COS7
pG05-EKLF
eEKLF

Free probe

β-globin
CACC-3
CACC-2
CACC-1

Relative Occupancy (ERα//lg0)

Relative Occupancy (ERα//lg0)

Relative Occupancy (ERα//lg0)

1kb 5’
E2f2-1en
1kb 3’

1kb 5’
E2f4-1en
1kb 3’

1kb 5’
E2f4-1en
1kb 3’

EKLFL
Figure 6

A. E10.5 Peripheral Blood (Primitive)

B. E13.5 Fetal Liver (Definitive)
Supplementary Figure 1
Supplementary Figure 2

A

B

Relative Occupancy (EKL/NRS)

0 5 10 15

A B C D E F G H
Supplementary Figure 3
Supplementary Figure 4
EKLF/KLF1 controls cell cycle entry via direct regulation of E2f2
Michael R. Tallack, Janelle R. Keys, Patrick O. Humbert and Andrew C. Perkins

*J. Biol. Chem.* published online May 20, 2009

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