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Non-random subcellular distribution of variant EKLF in erythroid cells

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Abstract

EKLF protein plays a prominent role during erythroid development as a nuclear transcription factor. Not surprisingly, exogenous EKLF quickly localizes to the nucleus. However, using two different assays we have unexpectedly found that a substantial proportion of endogenous EKLF resides in the cytoplasm at steady state in all erythroid cells examined. While EKLF localization does not appear to change during either erythroid development or terminal differentiation, we find that the protein displays subtle yet distinct biochemical and functional differences depending on which subcellular compartment it is isolated from, with PEST sequences possibly playing a role in these differences. Localization is unaffected by inhibition of CRM1 activity and the two populations are not differentiated by stability. Heterokaryon assays demonstrate that EKLF is able to shuttle out of the nucleus although its nuclear re-entry is rapid. These studies suggest there is an unexplored role for EKLF in the cytoplasm that is separate from its well-characterized nuclear function.

Keywords

nuclear-cytoplasmic shuttling; nuclear transcription factor; EKLF; erythropoiesis; heterokaryon assay

Introduction

Transcription factors, whose primary function is nuclear, are not necessarily localized solely to the nucleus. Many have a subcellular distribution that is tightly regulated. This regulation may take various forms. The accessibility of a protein's nuclear localization signal (NLS) to the import machinery can be modulated such as in the case of NF- κ B. Normally localized to the cytoplasm, it is bound to I κ B, which masks its NLS. Only when I κ B is phosphorylated and targeted for degradation (via the 26S proteasome pathway in the cytoplasm) is NF- κ B translocated into the nucleus [1]. Regulation of protein import can also occur by protein phosphorylation such as in the case of MAPK [2] or by direct binding of a lipophilic hormone ligand to a steroid receptor located in the cytoplasm such as in the case of glucocorticoid receptor [3]. Additionally, an intermolecular interaction may prevent a protein from nuclear localization by actively retaining that protein in the cytoplasm such as in the case of *Xenopus* nuclear factor 7 (xnf7) [4]. In yet another example p53, which functions as a transcriptional activator in the cell nucleus, is exported out to the cytoplasm to be degraded

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[5]. Quite often these diverse mechanisms exist to integrate signal transduction pathways and molecular cues with temporal and specific protein translocation both into and out of the nucleus.

Erythroid Krüppel-like Factor (*EKLF*; *KLF1*) plays an essential role during erythroid development as a nuclear transcription factor [6,7]. *EKLF* mRNA is highly restricted in its expression pattern, limited to hematopoietic organs such as the yolk sac, fetal liver, adult bone marrow, and the red pulp of the spleen [8,9]. Molecular and genetic ablation studies demonstrate that *EKLF* is absolutely required for β -globin transcription and plays a prominent role in the final developmental switch to adult β -globin in definitive erythroid cells [10–14]. *EKLF* protein can interact with coactivators such as p300/CBP histone acetyltransferases as well as chromatin remodelers such as SWI/SNF to maximally transactivate the β -globin gene [15,16]. *EKLF* also activates protein-stabilizing, heme biosynthetic pathway, and red cell membrane protein genes in both primitive and definitive cells [17–20]. Although *EKLF* predominantly serves as a transcriptional activator, protein-protein interactions between *EKLF* and corepressors, such as mSin3A and HDAC1 [21], can result in the stage-specific repression of *EKLF* target genes [22]. Recent studies demonstrate that *EKLF* plays a novel role in negatively regulating megakaryocyte lineage commitment [23]. *EKLF* is also post-translationally modified by phosphorylation [24], acetylation [16], ubiquitylation [25], and sumoylation [26]. Some of these alter protein/protein interactions and thus modify *EKLF* activity [22,26,27].

Based on its critical and varied functions during hematopoiesis, we addressed whether its intracellular localization is straightforward, or whether this might provide another point for cellular regulatory control of its activity. Unexpectedly we find that *EKLF* protein is localized to both the nucleus and cytoplasm of erythroid cells and that *EKLF* from these subcellular compartments have subtle yet distinct biochemical and functional differences, implicating a non-random distribution of variant *EKLF* forms. These observations raise intriguing questions about unexplored aspects of *EKLF* function in erythropoiesis.

Materials and methods

Cell preparations

Erythroid cells from E10.5 yolk sacs and E12.5 or E13.5 fetal livers were washed in PBS containing 50U/ml Heparin (Sigma) and resuspended in 1ml 1% BSA/PBS (indirect immunofluorescence) or 0.5ml NE-A buffer plus protease inhibitors (biochemical fractionation; see below). MEL 745A and 293T cells were maintained in Dulbecco's Modified Enriched Medium (Invitrogen) supplemented with 10% FBS. MEL differentiation was induced by the addition of 5 mM Hexamethylenebisacetamide (HMBA; Sigma) for up to 48 hours. Transfections with Δ PEST *EKLF* and anti-FLAG immunoprecipitation were as described [25]. Cell extracts were generated as described [9,25].

Indirect immunofluorescence

Indirect immunofluorescence was performed based on Elefanty et. al. [28] with minor modifications. Anti-*EKLF* 6B3 mouse monoclonal antibody [16] or anti-GATA-1 rat monoclonal antibody (N6; Santa Cruz) was used at 1:100 dilution in 1%BSA/PBS and incubated overnight at 4°C. The slides were washed twice with PBS/BSA followed by incubation with donkey anti-mouse conjugated to Texas Red or donkey anti-rat conjugated to FITC (Jackson Labs) for 2 hours in the dark. After washing with PBS, slides were mounted with Vectashield containing DAPI nuclear stain (Vector Laboratories). Images were visualized on a Zeiss Axiophot fluorescent microscope.

Sucrose cushion nuclear and cytoplasmic preparation

NE-A Buffer (10mM HEPES pH 7.9, 1.5mM MgCl₂, 10mM KCl, 2.8mM β-mercaptoethanol) including 0.25M sucrose and 0.2% NP-40 were mixed to a final volume of 2ml in 15ml falcon tubes. Actively growing cells were counted by hemacytometer and 10–15×10⁶ total cells were washed once with PBS and resuspended in 0.45ml NE-A Buffer plus protease inhibitors. The cells were then gently layered onto the sucrose cushion and centrifuged at 1000rpm for 5 minutes. 150μl cytoplasm was removed from the top layer and frozen at –80°C. The remaining solution was removed without disturbing the nuclear pellet which was then washed with 0.5ml NE-A plus protease inhibitors. The washed pellet was finally resuspended in 150μl 1x SDS loading buffer. The Bradford Protein Assay (Bio-Rad) using BSA as standard was performed to determine the protein concentration of all samples. Centricon filters were used where indicated to concentrate the cytoplasmic fraction 3-fold so that its cell equivalents equaled the nuclear fraction.

Hypotonic lysis and mechanical disruption of MEL cells

Actively growing cells were resuspended in 1ml Hypotonic Lysis Buffer (5mM HEPES pH 7.2, 10mM NaCl and 1.5mM MgCl₂) and incubated on ice for 1 minute. The cells were monitored under the light microscope to ensure that they were swollen but that the nuclei were not lysed. Ten strokes with a Dounce Homogenizer (pestle A) were followed by cell disruption with pestle B until nuclei were smooth yet not lysed. The material was transferred to a pre-chilled 15ml falcon tube and centrifuged at 700rpm for 5 minutes at 4°C. Cells were monitored after centrifugation under the microscope to ensure that the supernatant did not contain any nuclei and recentrifuged when necessary. Cytoplasm (800μl) was removed and frozen. The nuclear pellets were resuspended in 800μl hypotonic lysis buffer. The maximum cross-contamination between the nuclear and cytoplasmic fractions was 5%–10%.

Western blot analysis

Western blots used the following primary antibodies: anti-EKLF polyclonal antibody [9], anti-EKLF monoclonal antibody 6B3 [16], anti-GATA-1 rat monoclonal antibody (N6; Santa Cruz Biotechnology), anti-Hsp90 rabbit polyclonal antibody (H-114; Santa Cruz Biotechnology), anti-α-tubulin monoclonal antibody (T9026; Sigma), anti-β-actin monoclonal antibody (A5316; Sigma), anti-ERK1 monoclonal antibody (BD Biosciences, Transduction Laboratories), or anti-Flag M2 antibody coupled to HRP (A8592; Sigma). Secondary antibodies were: anti-rabbit/HRP (Jackson Labs), anti-mouse/HRP (Pierce) or anti-mouse, anti-rabbit or anti-rat conjugated to alkaline phosphatase (AP) (Promega). ECL Chemiluminescence (Pierce) was used for signal detection of HRP conjugated antibodies. AP activity was detected with NBT and BCIP (Promega).

Protein-DNA interactions

Annealing, labeling, and gel shift assays with double-stranded oligos were performed as previously described [8]. Some reactions included anti-EKLF antibody 4B9 [27].

For sodium deoxycholate (DOC) experiments, 0.125% to 1.2% DOC was added to protein extracts and incubated for 10 minutes on ice. For reconstitution experiments, equal parts nuclear and cytoplasmic extracts were mixed prior to incubation with DNA. For RNase experiments, 10ng to 100ng RNase was added to extracts for 20 minutes.

RNA analysis

RNA isolation, reverse transcription with oligo-dT, and semi-quantitative analyses were performed as described using number and cycle parameters previously established for linear

range assessment of β -globin, EKLF, and HPRT [9,29]. Primers were as previously described [9,30].

Interspecies heterokaryon shuttling assays—Heterokaryon assays were performed as described [31] with several modifications. A single 50–80% confluent 10cm dish of HeLa cells was transfected with GFP-fused Estrogen Receptor [32], full-length GFP-EKLF [33], or GFP-EKLF containing only the zinc finger domain [33] using FuGENE 6 (Roche Diagnostics) according to the manufacturer's protocol. Twenty-four hours post-transfection, 2×10^5 human (HeLa) cells were mixed at a 1:1 ratio with untransfected mouse (NIH 3T3) cells and seeded onto sterile 1.5mm glass coverslips (Corning) in a 6 well plate and co-cultured overnight at 37°C. The following day, the co-cultured cells were grown in DMEM containing 100 μ g/ml cycloheximide for 2.5 hrs. Cells were then washed twice with PBS and fused with 200 μ l prewarmed 50% polyethylene glycol 3500 (Sigma) in serum-free DMEM for 2 minutes at room temperature. Cells were washed twice with PBS and incubated in DMEM containing 100 μ g/ml cycloheximide for an additional 4hrs. Cells were fixed with 3.7% formaldehyde, rinsed three times for 5 minutes with PBS and counterstained with 10 μ g/ml Hoechst 33258 for 15 minutes at room temperature. Finally, cells were rinsed six times with PBS for 2 minutes each and coverslips were mounted onto Superfrost microscope slides (Fisher) with Vectashield Mounting Medium (Vector Laboratories). Shuttling was never observed in the absence of cell fusion.

To test for CRM-1-dependent shuttling, co-cultured cells were treated with 20nM or 200nM Leptomycin B (LMB) during the duration of cycloheximide treatment of cells. 100% ethanol was utilized as the control in untreated cells.

Cells were visualized on a Leica Scanning Confocal Microscope using differential interference contrast for detection of multinucleated cells and detection of intact nuclear membranes. Hoechst staining served to distinguish between mouse (NIH 3T3) and human (HeLa) cell nuclei.

Results

EKLF can shuttle between nuclei in heterokaryons

Our earlier studies had shown that transfected EKLF protein quickly enters the nucleus [33]. We now asked whether we could generate any evidence for EKLF movement out of the nucleus. To address this question we utilized the heterokaryon shuttling assay that takes advantage of the difference in nuclear Hoechst staining between human HeLa cells and murine NIH3T3 cells, enabling them to be easily distinguished by visual inspection [31]. For the present studies, HeLa cells were transfected with GFP-EKLF (which readily enters the nucleus due to its two NLSs) and mixed with untransfected 3T3 cells. After inhibition of protein synthesis and cell fusion, the fixed cells were stained with Hoechst 33258, and GFP fluorescence along with that of the Hoechst stain was monitored by confocal microscopy. Successful fusion was monitored by inspection of DIC images. We used transfected GFP-estrogen receptor (GFP-ER), a known shuttling protein [32], as a control for these studies.

Our quantitative analysis enabled us to differentiate between three types of results (Fig. 1a). Two were straightforward: fused HeLa and 3T3 nuclei that were both GFP-positive (indicating shuttling), and fused nuclei in which only HeLa was GFP-positive (indicating non-shuttling). One did not fit into either of these categories: 3T3 GFP-positive nuclei that were not near any HeLa GFP-positive nuclei (which was considered a false-positive).

Using this assay we were surprised to find that EKLF can readily shuttle from the HeLa to the 3T3 nuclei at a comparable level (~78%) to ER protein (~81%) (Fig. 1b, d, and f). We did not observe any cytoplasmic signal in any case.

We have previously shown that EKLF interacts with importin proteins that recognize classical nuclear localization signals (NLSs) [33]. Since protein traffic through the nuclear pore is bidirectional, EKLF could be undergoing active export from the nucleus into the cytoplasm, generating a cytoplasmic EKLF population. The most extensively studied and most commonly utilized protein export pathway is mediated by the chromosome region maintenance 1 (CRM1) protein [34,35]. This export receptor protein recognizes short, leucine-rich amino acid stretches termed nuclear export sequences (NESs) similar to that present in the HIV-1 Rev protein [36]. The ability of CRM1 to function as an export receptor can be inhibited by the metabolite leptomycin B (LMB) [34]. While EKLF contains two potent NLSs [33], it does not possess an obvious, putative NES based on primary sequence analysis. In any case, we tested whether the CRM1 pathway could export EKLF. Using two concentrations of leptomycin B, we find a dose-dependent inhibition of ER shuttling (non-shuttling levels increase from 13% to 68% at 200 nM), as expected (Fig. 1e and f). However, we see no effect on EKLF shuttling at 20 nM (in which non-shuttling levels remain at 14%), and a minimal inhibition at 200 nM (non-shuttling increased only to ~24%) (Fig. 1f). As the CRM1 export process is highly sensitive to leptomycin B, this assay suggests that EKLF shuttling does not rely primarily on this export mechanism.

We then addressed whether the two modular domains of EKLF, its transactivation and DNA binding (zinc finger) regions, contribute to shuttling. Because this assay must begin with a GFP signal that is maximally localized to the nucleus, we could not test the effect of removing the zinc finger region, which is the major EKLF NLS [33]. As a result, we tested removal of the EKLF transactivation domain (GFP-ZnF) and monitored for any effect on shuttling. We find it plays a significant role, as non-shuttling levels rise from ~8% to 37% in its absence; however, the zinc fingers retain a significant level of shuttling capability (~60%) (Fig. 1c and g). We conclude that EKLF shuttling is complex, as there is not a single region that accounts for this capability, and that the zinc fingers are the major contributor.

Significant levels of EKLF reside in the cytoplasm of primary erythroid cells

Given these results in non-erythroid cells, we wished to ascertain the normal subcellular localization of EKLF within the red cell environment. EKLF cellular localization was first tested by indirect immunofluorescence (IF) of primary cells from circulating d10.5 (primitive) erythroblasts or d12.5 dispersed fetal liver cells (definitive erythroid population). The results were unexpected: EKLF was primarily localized to the cytoplasm in both primitive and definitive erythroid cells (Fig. 2a). Intactness of the cellular preparations (which followed published procedures [28]) was demonstrated by monitoring GATA1, which remained exclusively nuclear.

To verify this observation with an independent assay, we developed a biochemical fractionation protocol that requires minimal perturbation of cells to generate cleanly separated nuclear and cytoplasmic fractions (Fig. 2b). Cells were gently resuspended in a low salt, magnesium-containing buffer and layered onto a sucrose cushion in the same buffer plus triton X-100. After low speed centrifugation in a swinging bucket rotor, the cytoplasm, visually bright red, cleanly separates from the nuclear pellet, which is completely pale. These fractions were directly monitored for the presence of EKLF via western blot analysis.

Using this procedure we separated primary erythroid cells into nuclear and cytoplasmic compartments, analyzed the extracted proteins, and obtained similar results to that seen by IF: significant levels of EKLF partitioned to both the cytoplasm and the nucleus (Fig. 2c). GATA-1

remained in the nuclear fraction and served as a control for its integrity. Hsp90 and α -tubulin clearly partitioned as expected exclusively into the cytoplasmic fraction [37]. After normalization to the number of cell-equivalents loaded into each lane, it is apparent that, consistent with the immunofluorescent data, a large majority (in the range of 50–80%) of the EKLF protein resides in the cytoplasm in both primitive and definitive cells. We can also conclude that EKLF does not appear to undergo any obvious developmental regulation of its localization during erythroid development.

The EKLF nuclear/cytoplasmic ratio is not altered by a variety of treatments in vivo

To further characterize parameters that may influence EKLF localization, we monitored EKLF partitioning in a tissue culture system that could be more readily manipulated. Murine erythroleukemia (MEL) cells are definitive pro-erythroblast erythroid cells that can be induced to terminally differentiate in culture by the addition of chemical inducers such as HMBA or DMSO [38]. EKLF mRNA and protein are expressed in actively growing MEL cells prior to terminal differentiation [8]. We fractionated MEL cells into nuclear and cytoplasmic compartments, monitored EKLF protein by western blot, and obtained results similar to that seen with primary cells: GATA1 remained nuclear, whereas EKLF partitioned to both the cytoplasm and the nucleus (Fig. 3a). To exclude any influence of the Triton X-100, we performed detergent-free fractionation of MEL cells using a hypotonic lysis buffer and mechanical disruption to generate nuclei and cytoplasm. Although not as cleanly separated as the sucrose cushion fractions, these manually fractionated cells had a maximal nuclear/cytoplasmic cross-contamination rate of about 5–10% as detected by western blot. However, as was seen with the sucrose cushion method, EKLF also partitioned to both the nucleus and cytoplasm under these conditions (Fig. 3b), thus ruling out any effect of detergent on our observations.

We next used MEL cells to attempt to alter the EKLF distribution by a variety of treatments. First we checked whether cytoplasmic EKLF could be redistributed to the nucleus of MEL cells during terminal differentiation when adult β -globin, a well-characterized activation target of EKLF, is induced [38]. Differentiation of MEL cells was induced with HMBA over 0–48 hours. At 20–24 hours post-HMBA induction, an increase in β major mRNA can be detected and expression of the gene increases 10–30 fold by 36–48 hours (Fig. 4a). Biochemical fractionation was performed on the induced MEL cells to determine if changes in the nuclear and cytoplasmic EKLF distribution had occurred. Although levels of both EKLF mRNA (Fig. 4a) and total protein (Fig. 4b) decreased by 5 hours post-induction prior to their increase by 24 hours, there was no significant change in the cytoplasmic to nuclear ratio during this time. Therefore, the erythroid terminal differentiation process does not appear to affect EKLF nuclear/cytoplasmic distribution.

We next asked again whether this process requires CRM1, as we could now address this question in the erythroid cell. Addition of increasing concentrations of LMB leads to no significant change in nuclear and cytoplasmic EKLF levels (Fig. 5), as EKLF still partitioned to the cytoplasm. Therefore, if nuclear EKLF can shuttle between the nucleus and the cytoplasm, it is not via the CRM1 pathway.

Finally, we examined whether the stability of EKLF may be differentially affected in the nucleus or the cytoplasm. While degradation of proteins can occur by various systems in both fractions [39], it is possible that one may be preferentially stabilized leading to a differential level of degradation in that compartment. To determine if this is the case with EKLF, MEL cells were treated with MG132, a potent pharmacological inhibitor of the 26S proteasome, prior to fractionation [40]. As we have previously observed, inhibition of the proteasome with MG132 leads to an increase in the accumulated steady state levels of EKLF in unfractionated MEL cells, where its normal half-life is <3 hours [25]. However, when nuclear and cytoplasmic

derived EKLF levels are monitored, both populations of EKLF are stabilized by MG132 addition (Fig. 6). These studies suggest that degradative mechanisms are not unique to cytoplasmic EKLF and do not serve to distinguish it from nuclear EKLF.

Cytoplasmic and nuclear EKLF display different biochemical properties

Although EKLF protein typically migrates as a doublet when monitored in whole-cell erythroid extracts [9]; unpublished observations), inspection of nuclear and cytoplasmic EKLF readily reveal an apparent molecular weight difference when well-resolved on SDS-PAGE gels (Fig. 3a). Even though the doublet is still evident in both, their relative intensity is inverted: i.e., the slower migrating form is more intense in the nuclear fraction, while the faster migrating form is more intense in the cytoplasmic fraction. Our initial impulse was to attribute this to phosphorylation differences, as we had previously shown EKLF is phosphorylated [24]. However, phosphatase treatment of these fractions does not result in a collapse of the doublet into a singlet in either fraction (data not shown). Acetylation will not give rise to such a difference in migration [16], and as the extracts were not prepared to favor retention of isopeptidase-sensitive modifications [25,26], we don't believe that this difference is due to modifications such as ubiquitinylation or sumoylation.

However, our earlier studies in non-erythroid cells had pinpointed two conserved PEST sequences that may play a role in EKLF stability in the absence of affecting its ability to be ubiquitylated [25]. Of relevance to the present studies, deletion of either of these domains leads to loss of the doublet that is apparent with full-length EKLF (Fig. 7), a result not seen by simply mutating the critical first amino acid (k74r) of the PEST 2 consensus site (Fig. 7) or by mutation of other individual lysines outside this sequence, including those that are acetylated [25,27]. This suggests that sequences within the PEST domains may be playing a role in generating the two forms of EKLF that are seen upon SDS-PAGE analysis.

To ascertain whether the biochemical differences in subcellular erythroid EKLF proteins as distinguished by SDS-PAGE may also be indicative of functional differences, fractionated EKLF protein derived from either the MEL nucleus or the cytoplasm was tested for DNA binding ability. Since the fractionation technique gave rise to different amounts of EKLF in the cytoplasm as compared to more concentrated nuclear fractions, protein levels were normalized by concentrating the cytoplasmic fraction with a microcon filter and equivalent amounts (as judged by western blot analysis) of cytoplasmic and nuclear EKLF were tested in gel shift assays using the mouse β -globin CACCC element as an oligonucleotide probe [8]. EKLF binding was localized on the gel in the midst of various CAC-site binding proteins by supershift analysis with anti-EKLF antibody. This assay revealed that the cytoplasmic EKLF/DNA complex migrates more slowly than the nuclear EKLF/DNA complex (Fig. 8, lane 3 compared to lane 1). The two distinct EKLF/DNA complexes remain even after the two fractions are mixed (Fig. 8, lane 5).

As a result of these analyses we conclude that there are subtle but significant differences between the DNA binding and biochemical properties of cytoplasmic versus nuclear EKLF, possibly involving sequences within the PEST domains.

Discussion

EKLF encodes a transcription factor that plays a critical role in erythroid gene regulation, particularly in consolidating the final developmental switch to adult β -globin expression in the definitive cell [6,7]. We have unexpectedly found that a majority of endogenous EKLF protein resides in the cytoplasm of the erythroid cell. This property remains true independent of developmental stage, differentiative status, inhibitor treatment, or source (primary cell or cell line) and is further supported by noting that high levels of EKLF reside in the cytoplasm of

lineage-depleted primary fetal liver cells that have not yet undergone terminal differentiation (Y Yien and JJB, unpublished observations). We have additionally observed that cytoplasmic and nuclear EKLF populations differ in biochemical properties as evidenced by their different migration upon denaturing electrophoresis and by the different complexes they form upon DNA binding. Together, these data demonstrate that the two EKLF forms are non-randomly segregated in the erythroid cell and suggest either that regulation of nuclear EKLF activity is kept under another layer of cellular control beyond post-translational modifications, or that there is an unexplored additional facet to EKLF function in the cell that is centered in the cytoplasm.

Other KLF factors also exhibit partitioning to the cytoplasm. For example, KLF6 is expressed in multiple spliced isoforms that are not equivalently segregated, leading to significant downstream consequences [41]. In the present studies, different forms of EKLF are enriched in the cytoplasm versus the nucleus as visualized during SDS-PAGE, which may arise from alternative splicing. However, this is not likely, as transfected EKLF derived from a cDNA clone also gives rise to two forms on SDS-PAGE analysis.

Our ability to investigate the molecular nature of EKLF nuclear/cytoplasmic partitioning is severely limited by the fact that virtually all exogenous EKLF migrates to the nucleus after transient or stable transfection into erythroid or non-erythroid cells [33,42]; unpublished observations). This is also evident in the present studies, where we never observed EKLF in the cytoplasm of any positive heterokaryon. Although we and others used this property to identify the EKLF NLS [33,43], this has curtailed our ability to identify an NES, particularly as there is not any obvious leucine-rich export sequence motif within the EKLF primary sequence. Coupled to this is our inability to efficiently inhibit endogenous or exogenous EKLF export by LMB. CRM-independent export mechanisms, although not common, have been observed before [44–46].

Our inability to maintain exogenous EKLF in the cytoplasm may imply the existence of a cytoplasmic anchor that functions in a stoichiometric manner. For example, ectopic p53 is rarely found in the cytoplasm unless PARC is also transfected into the cell [47]. Alternatively, microtubules play a role in tethering Smad proteins to the cytoplasm [48]. It is possible that there may exist such an analogous anchor(s) for EKLF in the erythroid cell. Of more potential relevance to red cell biology, heme has been shown to affect the nuclear-cytoplasmic shuttling of Bach1 [49], a heterodimer partner of MafK that is known to be replaced by the critical p45/NFE2 activator upon erythroid differentiation [50]. However, inhibition of heme synthesis by succinylacetone had no effect upon EKLF localization (unpublished observations).

As a result, a fundamental question that remains difficult to address given our inability to properly partition exogenous EKLF is whether its presence in the cytoplasm implies a “latent” form analogous to the many other examples of transcription factors that exhibit this property [51,52]. An alternative idea that must be considered is that EKLF maintains a different function in the cytoplasm, separate from its function in the nucleus. In this regard, its genetic ablation is not informative, as the resultant embryonic lethality derives from its function in the nucleus.

Even though the most striking effect of EKLF ablation is the lack of adult β -globin expression in the definitive erythroid cell [11,13], later studies suggested that EKLF is also transcriptionally active within the primitive erythroid nucleus [53,54]. Recent studies more directly show that there are other targets, including some in the primitive cell, that are also negatively affected by the absence of EKLF [17–20]. In the case of Band 4.9 and AHSP expression, this effect is as dramatic in the primitive cell as for β -globin in the definitive cell [17]. These data demonstrate that EKLF retains an important nuclear function in both cell types, and that subcellular partitioning is not apparently part of the developmental control program

for EKLF. Our studies are consistent with this, as we find EKLF in the nucleus of both the primitive and definitive erythroid cell.

During the course of these studies, Shyu et al. also demonstrated that EKLF can be found in the cytoplasm [55]. Although this conclusion is similar to ours, there are large inconsistencies in the details of the two studies that are hard to resolve but may be due to differences in experimental conditions and antibodies used. In addition, their inability to detect EKLF in the nucleus of undifferentiated MEL cells or particularly of primitive erythroid cells is difficult to reconcile with known EKLF nuclear function in these cells.

We have noted some inter- (but not intra-) experimental variability in the absolute levels of EKLF in the cytoplasm during the course of these studies, particularly as we have been very careful about monitoring cell-equivalents during our analyses. Although we always found EKLF in the cytoplasm, and in most cases as high as 80%, in some cases it was less than 50%. We have not been able to account for this variability. For example, use of actively growing versus saturated MEL cultures made little difference when tested within the same experiment, and we did not see any effect as a result of changes in media or serum.

The EKLF NLSs must be quite strong, as the heterokaryon assay never revealed any EKLF in the cytoplasm of the over 400 cells that were individually monitored. Given that the HeLa cell is fused for only four hours with the NIH3T3 cell, this implies a very quick shuttle back into the nucleus after a minimal amount of time in the cytoplasm, suggesting that the rate of EKLF nuclear import must be much greater than its rate of nuclear export. Whether the positive evidence for shuttling seen with this assay is simply reflective of a dynamic constitutive shuttling [52] or indicates a potential control point remains to be established.

Of tantalizing interest is the observation that nuclear and cytoplasmic EKLF are not equivalent as judged by electrophoretic methods. This is further complicated by the observation that nuclear EKLF, which mostly contains the slower migrating form of the doublet when compared to cytoplasmic EKLF, gives rise to the faster species when complexed with DNA. Although a number of post-translational modifications have already been ruled out by our studies, it is of interest that deletion of either of the PEST sequences obviates formation of the doublet, suggesting that these domains contain the putative modification site. However, even this interpretation is not straightforward, as deletion of *either* is sufficient to remove the doublet, indicating that the putative modification site may be located elsewhere. Both of these domains are located towards the EKLF amino terminus, distal from the zinc fingers located at the carboxyl end of the protein. They are present and similarly located in human EKLF, and the sequences within are highly conserved [25]. However, they do not code for any obvious motifs that might illuminate this issue. We are attempting to identify and locate the modification(s) that can account for the doublet by mass spectrometric analysis of purified nuclear and cytoplasmic EKLF protein.

These experiments imply that the story on EKLF function is not yet complete and still contains some unanticipated surprises. Determining whether and how EKLF nuclear function is kept in check by cytoplasmic sequestration, whether there is an alternate role for EKLF in the cytoplasm that is separate from its transcriptional responsibilities, and how the variant forms of EKLF in these two compartments relate to each other will likely provide novel insights into erythroid biology.

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Fig. 1. EKLF shuttles out of the nucleus in mouse-human heterokaryons
Heterokaryon assays were performed using human HeLa cells after transfection with GFP-EKLF or GFP-ZnF as indicated. Nuclear (Hoechst 33258) and EKLF signals were monitored by confocal microscopy after fusion with murine NIH3T3 cells. Cell localization and nuclear fusion were monitored by DIC visualization. The same field is shown within each panel. (A) Three categories of results are shown for GFP-EKLF: shuttling (top: fused HeLa and 3T3 nuclei that were both GFP-positive); non-shuttling (middle: fused nuclei in which only HeLa was GFP-positive); and other (bottom: 3T3 GFP-positive nuclei that were not near any HeLa GFP-positive nuclei). (B) Typical example of GFP-EKLF shuttling.

(C) Typical example of GFP-ZnF shuttling.

(D) Typical example of GFP-estrogen receptor (ER) shuttling observed in the absence (positive control) or presence (negative control) (E) of leptomycin B (LMB).

(F) Quantitation of results for GFP-EKLF or GFP-ER shuttling in the absence or presence of LMB; range of cell number that was inspected for each condition is indicated.

(G) Quantitation of results for GFP-EKLF or GFP-ZnF shuttling in the absence of LMB; range of cell number that was inspected for each condition is indicated.

A

B

C

Fig. 2. EKLFL is localized to both the nucleus and cytoplasm of primary erythroid cells

(A) Indirect immunofluorescence was performed on yolk sac primitive erythroid cells (d10.5 erythroblasts) or definitive erythroid cells (d12.5 fetal liver) using either a mouse monoclonal EKLFL antibody (Texas Red) or rat monoclonal GATA-1 antibody (FITC). DAPI (blue) signals indicate cell nuclei.

(B) Schematic of the sucrose cushion method used to separate cytoplasm from nuclei as described in the text.

(C) E10.5 yolk sac (primitive erythroid) and E13.5 fetal liver (definitive erythroid) cells were fractionated using the sucrose cushion method. Nuclear (N) and cytoplasmic (C) fractions were immunoblotted with EKLFL, α-tubulin or Hsp90 (cytoplasmic controls), or GATA-1 (nuclear control). Results are representative of three independent experiments. Yolk sac cell equivalents are $N=1.53 \times 10^6$ and $C=4.6 \times 10^5$ cells; fetal liver cell equivalents are $N=2 \times 10^6$ and $C=6 \times 10^5$ cells.

Fig. 3. EKLf is localized to both the nucleus and cytoplasm of MEL cells and do not migrate equivalently on a denaturing gel

Actively growing MEL cells were fractionated using either a sucrose cushion (A) or mechanical disruption (B). Hsp90 and GATA-1 served as cytoplasmic and nuclear fraction controls, respectively. Cell equivalents of nuclear (N) and cytoplasmic (C) fractions were 1:1 or approximately 4.7×10^5 cells per lane (A) or 5×10^5 cells per lane (B). Two independent MEL cell fractionations obtained using the sucrose cushion method are shown in (A).

Fig. 4. HMBA induced MEL cell differentiation does not alter EKLF nuclear and cytoplasmic distribution

Actively growing MEL cells were induced to terminally differentiate for 0–48 hours with 5 mM HMBA followed by fractionation with the sucrose cushion method.

(A) RNA was isolated from 5×10^5 MEL cells or from E13.5 fetal liver cells as a positive control, converted to cDNA, and amplified by semiquantitative PCR using gene-specific primer pairs as indicated in the presence of a radiolabeled tracer for a limited number of cycles [9,29]. Water served as a negative control. The analysis is representative of two independent experiments.

(B) Nuclear extracts were adjusted so that cell equivalents were loaded at a 1:1 ratio with the cytoplasm (4.7×10^5 per lane). EKLF, Hsp90, and GATA-1 signals are shown. Results are representative of three independent experiments.

Fig. 5. Leptomycin B treatment of MEL cells does not affect EKLf nuclear and cytoplasmic distribution

Actively growing MEL cells were treated with increasing concentrations of leptomycin B (LMB) for 3 hours prior to fractionation with the sucrose cushion method and western blot analysis. Nuclear extracts were adjusted so that cell equivalents were loaded at a 1:1 ratio with the cytoplasm (4.7×10^5 per lane). EKLf, Hsp90, and GATA-1 signals are shown. Results are representative of three independent experiments. As a positive control for LMB activity, addition of LMB to NIH3T3 cells resulted in the accumulation of p53 in the nucleus (data not shown).

Fig. 6. Both nuclear and cytoplasmic EKLf levels increase when the proteasome is inhibited
 MEL cells were treated 3 hours with MG132 (+) or DMSO vehicle control (-; 0.4% v/v). Total cell extracts were prepared and nuclear (N) and cytoplasmic (C) fractions were obtained using the sucrose cushion fractionation method. Nuclear extracts were adjusted so that cell equivalents were loaded at a 1:1 ratio with the cytoplasm (4.7×10^5 per lane). ERK served as a total cell (and cytoplasmic) protein loading control. Hsp90 served a cytoplasmic protein loading control. EKLf, Hsp90, and GATA-1 signals are shown.

Fig. 7. Removal of PEST domain sequences alter EKLF migration properties on a denaturing gel 293T cells were transiently transfected with Flag-EKLF (wt), Flag-EKLF mutated at lysine 74 (k74r), with a deletion of PEST 1 (amino acids 50–70; RSEETQDLGPGPPNPTGPSLH), or with a deletion of PEST 2 (amino acids 74–85; KSEDPSGEDDER) in two different experiments as shown. Equal protein amounts were immunoprecipitated with anti-Flag antibody and subsequent western blots were probed with anti-Flag antibody. A non-specific band is denoted with an asterisk (*).

Fig. 8. Nuclear and cytoplasmic EKLF DNA complexes are not equivalent

Actively growing MEL cells were biochemically fractionated with a sucrose cushion, cytoplasm was concentrated with microcon filters and fractions were subjected to western analysis to determine equivalent levels of nuclear and cytoplasmic EKLF. Nuclear (N), cytoplasmic (C), or mixed (N + C) fractions were incubated in the presence (+) or absence (–) of EKLF antibody prior to incubation with radiolabeled β -globin CACCC oligonucleotide. For all reactions, 1.2×10^6 cell equivalents were used. The EKLF/DNA bands are denoted with an asterisk (*).