Krüppel-like Factors: Three Fingers in Many Pies

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Running title: Multiple Roles for KLF Proteins
Krüppel-like factors (KLFs\(^1\)) are DNA-binding transcriptional regulators that play diverse roles during differentiation and development. They form a subset of the broad class of proteins containing Cys2/His2 zinc fingers, a motif that is the second most abundant seen in the human genome, and the most abundant in transcription factors, estimated to be present in just under 600 to over 700 proteins (1-3). The nomenclature is based on the homology of its founding member, EKLF (erythroid Krüppel-like factor; KLF1), to the *Drosophila* Krüppel protein (4). There are numerous finger proteins with such homology; however, the fact that KLF proteins contain additional conserved residues between each finger, that their fingers are three in number, and that these are found at the extreme carboxyl end serves to further define KLF proteins as a separate family. Subsequently, phylogenetic analysis of the fifteen human KLF members demonstrates that they form a clade distinct even from the closely related Sp1 and Krox zinc finger families. Structural considerations also place these members together, as three amino acids at specific locations ("XYZ" positions (5)) adjacent to the coordinating histidines play a determining role in target site selection and high affinity binding. As a result, all members of the family bind very similar "GT-box" or "CACCC element" sites on DNA, although the configuration is such that the site tends to be C-rich on one strand and G-rich on the other.

The homologies and relationships between KLF family members, as well as their biological properties, have been the subject of excellent comprehensive reviews in the past two years (6-8). This discussion will focus on an update of the family and on very recent experiments that demonstrate the wide and diverse range of cellular and molecular effects that are exerted by selected members (Fig. 2).
Cellular regulation of KLF gene expression

KLF6/ZF9/CPBP is rapidly induced in liver stellate cells that are activated after injury, leading to fibrogenesis and extracellular matrix formation (9). Consistent with this idea, KLF6 activates the collagen α1(I) promoter and the TGFβ promoters, establishing a link between KLF6 activity and cytokine responsiveness to injury (10). This response is also seen in injured arterial endothelial cells, where induced KLF6 expression upregulates the urokinase plasminogen activator, leading to proteolytic activation of latent TGFβ and subsequent tissue remodeling (11).

Vascular injury also plays a role in the induction of KLF5/BTEB2/IKLF expression in smooth muscle cells, and phorbol ester-sensitive protein kinase pathways (particularly MAP kinase induction of Egr1 binding to the KLF5 promoter) have been implicated in directing this response (12). In a similar (but not identical) way, KLF5 is a downstream target of Wnt1 signalling as judged by its induction after Wnt1 infection of cultured epithelia, and after comparison of KLF5 levels in transgenic Wnt1 mouse mammary cells to wild type controls (13). Induction is transcriptional and occurs via a β-catenin/TCF-independent mechanism that may involve protein kinase C activation.

Cytokines have also been implicated in KLF induction. First, investigation of TGFβ-driven effects on pancreatic epithelia, prostate, and brain cell growth led to the identification of KLF10/TIEG1/EGRα and KLF11/TIEG2/FKLF (14). These two proteins are not only most homologous with each other (Fig. 1, subgroup 2), but they are also immediate-early TGFβ responsive genes that behave as potent repressors via three uniquely conserved repression motifs (15). Overexpression of KLF10 or 11 in a pancreatic cell line or in transgenic mice reveals that the functional effect of this repression is inhibition of cell growth (14) and induction of apoptosis via formation of...
reactive oxygen species (16). Second, KLF4/GKLF is directly induced by IFNγ in a human colon carcinoma cell line, as mRNA induction is rapid and occurs in the absence of protein synthesis (17). As more fully described below, this link may explain the antiproliferative effects of IFNγ.

**Proliferation or differentiation?**

KLF4/GKLF has been most thoroughly investigated with respect to its role in cellular differentiation, initially within the gastrointestinal tract (18), where its expression is indicative of a growth-arrested state, and in the epidermis, where KLF4 is critical for late stage differentiation of skin cells (19). Three ways in which KLF4 accomplishes this have been proposed. In the first set of experiments, it was noted that KLF4 and p21 levels increase upon induction of growth arrest by serum deprivation, and that the kinetics of KLF4 expression slightly preceded that of p21 (20). Following the observation that the p21 promoter contains CACCC elements, KLF4 was shown to bind and directly transactivate the p21 promoter via these sites. Interestingly, this induction was dependent on p53, and thus also occurred after DNA damage with MMS. In addition, KLF4 physically interacts with p53. The resultant synergistic induction by p53 and KLF4 of p21 then leads to its inhibition of cyclin-dependent kinases and subsequent growth arrest. The second set of data demonstrate that the minimal cyclin D1 promoter also contains multiple CACCC elements that bind KLF4 in vitro, and that KLF4 binding results in in vivo repression of the promoter, an effect not seen after transfection of Sp1 (21). Finally, KLF4 activates late differentiation genes such as keratin 4 (22). Together these data argue that KLF4 levels play a critical role in the decision between proliferation and cell cycle arrest/differentiation.
Although originally considered to be ubiquitously expressed (23), a similar role has been postulated for KLF7/ULKF, particularly within developing and adult nervous systems (24). Expression of KLF7 at specific phases of early development correlated with the time when neuronal precursors exit the cell cycle and differentiate. Two sets of data implicate KLF7 in this process. First, KLF7 can modulate cell cycle regulators, as its induced overexpression results in a decrease in DNA synthesis, induction of p21 protein, inhibition of cyclin D1, and G1 arrest (24). Second, KLF7 may directly regulate expression of TrkA, which, as the receptor for NGF, is required for normal maturation and differentiation of sensory and sympathetic neurons (25).

**Possible role in cellular malignancy?**

The ability of molecules such as KLF4/GKLF to play critical roles in the proliferative state of the cell raised the issue of whether they play any role in the development of cancer. Intestinal samples from patients with familial adenomatous polyposis (FAP) and from multiple intestinal neoplasm (min) mice (a model of FAP) were monitored for expression of KLF4 (26). RT/PCR analysis revealed an inverse correlation between KLF4 levels and intestinal adenoma tumor size in min mice, and decreased levels of KLF4 in colonic adenomas from FAP patients compared to neighboring normal mucosa. As the KLF4 promoter contains binding sites for the Cdx-2 protein (27), a model has been proposed whereby mutated APC (adenomatous polyposis coli) can no longer induce Cdx-2, leading to low levels of KLF4 and accelerated growth in FAP samples. Consistent with this idea, KLF4 levels remain very low in the RKO colon cancer cell line, which contains wild-type APC but a mutated Cdx-2, a variant that also exerts a dominant negative effect on wild type Cdx-2 activation of the KLF4 promoter (28). Although a similar negative correlation is seen
between KLF4 level and growth in prostatic carcinoma, the role of KLF4 is not equivalent in all cancers; for example, KLF4 levels are upregulated during progression of human oral/pharyngeal and breast carcinomas (29).

**Tissue specific vs. general effects**

The biological roles of three KLF family members have been tested by genetic ablation. Consistent with its restricted expression and molecular properties, disruption of KLF1/EKLF leads to a directed effect on β-globin expression (see below). However, other members, more generally expressed, exhibit very specific phenotypes upon their disruption. In addition to expression in various tissues within the gut (see above), KLF4/GKLF is also expressed at high levels in the epidermis. However, its ablation leads to a specific deficiency in the barrier function of the skin, resulting in post-natal death (19). Although these data are consistent with KLF4 function in growth arrest and differentiation, the extent of normal development seen in its absence is paradoxical given the expression pattern and postulated role of KLF4 in the intestine and other endodermal tissues.

Similarly, genetic disruption of KLF2/LKLF, a molecule originally named by virtue of its high level of expression in the lung (30), led to defects in blood vessel organization and early embryonic death (31). Even though angiogenesis and vasculogenesis were normal, recruitment of pericytes and smooth muscle cells was deficient, leading to a low integrity vessel cell wall and severe, lethal hemorrhage.

The embryonic lethality of KLF2-null embryos made it difficult to analyze effects on other tissues. However, two other experimental approaches addressed this. To test the importance of KLF2 in lymphoid differentiation and at the same time avert embryonic lethality, KLF2-deficient embryonic stem (ES) cells were injected to
recombinase-deficient blastocysts to generate chimeric mice (32). B cell development was normal, but mature, single-positive T lymphocytes were susceptible to apoptosis and did not survive, implicating a role for KLF2 in quiescent T cells. To address the role of KLF2 in the lung, ES cells were again used to generate chimeric mice, and the contribution of the KLF2-deficient cells to a large number of tissues was determined (33). In this case, KLF2-deficient cells contributed to all tissues except the lung, and histopathological analysis of lungs from highly chimeric animals that died at birth showed deficiency in late stages of development and exhibited an abnormal pathology. A striking consistency in the KLF1, 2, and 4 knockouts is that they affect late stages of differentiation within their respective cellular environments.

**Molecular roles: transcriptional activation**

KLF1/EKLF was originally isolated by a subtractive cloning approach to identify genes important for erythroid differentiation (4). At present, it remains the best characterized member of the group (34,35), as its target binding sequence at the ß-globin promoter and its transcriptional activation properties were quickly identified. Its expression pattern has not, however, been a paradigm for the family, as KLF1 is most restricted in its expression, strikingly localized only to blood forming tissues during mammalian development (36). Its genetic ablation not only leads to absence of adult ß-globin expression and embryonic death (37,38), but also the loss of the chromatin hypersensitive site at the ß-globin promoter and a diminution of another strong hypersensitive site (HS3) located more than 50 kb away (39).

A molecular explanation for these results has followed from determining that KLF1/EKLF interacts with P300 and CBP (40), transcriptional coactivators that not only act as bridges to the basal transcription machinery, but that also contain an endogenous
acetyltransferase activity that acetylates histones and KLF1 itself. Such modifications appear to alter KLF1’s ability to interact with other proteins, such as components of the SWI/SNF, ATP-dependent chromatin remodeling complex (41-43). As a result, KLF1 has provided the clearest example of the role in transcriptional regulation and chromatin assembly that a KLF molecule can integrate.

Interestingly, the basic region adjacent to the zinc finger domain contains one of the KLF1/EKLF acetylation sites, and this region is most highly conserved between KLF1, KLF2/LKLF, and KLF4/GKLF (43). Indeed, KLF4 has also been shown to interact with the P300 and CBP coactivators in vitro, and the residues required for this interaction are also required for KLF4 to exhibit its growth suppressive effects in vivo (44). As KLF1, 2, and 4 form a closely-related subgroup (by sequence comparison) within the KLF family (Fig. 1, subgroup 3), KLF2 and 4 may also be targets for acetylation that alters their ability to interact with other proteins.

Another characteristic common to this subgroup follows from deletion analysis of their transactivation regions. Not surprisingly, in each case it can be whittled down to a minimal activation module (44-47). However, a region adjacent to their zinc fingers behave functionally as inhibitory modules (45-47), implying that the roles of KLF1, 2, and 4 in transcriptional control is complex and may be sensitive to selective modification and protein interactions.

Recently, KLF13/FKLF2 has been shown to interact with coactivators to stimulate transcription of the human γ-globin gene (48). Similar to KLF1, KLF13 is a substrate for acetylation by CBP and P300. However, unlike KLF1, P/CAF also acetylates KLF13, and this enzymatic activity is required for enhancement of KLF13 transcription. Part of this activation likely follows from the strong stimulation of DNA
binding by KLF13 in the presence of CBP or P/CAF. The cumulative data suggest that KLF factors are selective in their utilization of coactivators to stimulate transcription.

Molecular roles: transcriptional repression

A considerably different outcome arises from investigating the role of KLF3/BKLF, which primarily behaves as a strong transcriptional repressor. KLF3 was originally isolated by low stringency cDNA library screening with the KLF1 zinc finger region to identify other CACCC element proteins from erythroid cells (49). It is highly expressed in hematopoietic cells and the developing central nervous system, and to a lesser extent in many (but not all) adult tissues. It achieves repression by recruitment of CtBP2, a general corepressor protein that interacts with KLF3 by means of a Pro-X-Asp/Asn-Leu-Ser/Thr motif located in the KLF3 repression domain near the amino terminus (50). As preliminary data show that genetic ablation of KLF3 gives rise to a myeloproliferative disorder that infiltrates numerous tissues and effectively interferes with their normal growth (51), it appears that its repression function may play a role in preventing unobstructed cell expansion.

KLF3 is not unique in its interaction with the CtBP2 corepressor, as KLF8/BKLF3 also contains a region of homology to KLF3 (in addition to the zinc finger domain) with the appropriate CtBP2 interaction motif that enables it to repress transcription (52). KLF8 is broadly expressed, and endogenous target genes have not yet been determined for either KLF3 or 8. However, KLF12/AP2-rep, another KLF family member whose target promoter (in this case) is known, contains the same interaction motif, and functions as a repressor of the AP2α promoter in cotransfection assays (53). Although KLF12 was originally isolated from a brain cDNA library, it is most highly expressed in the kidney, and induction of KLF12 expression correlates with down-regulation of
AP2α gene expression during kidney development. Interestingly, KLF3, 8, and 12 are most closely related to each other simply by comparative sequence analysis in the absence of functional tests (Fig. 1, subgroup 1). As a result, it was not unexpected to find that KLF12 interacts with CtBP1 (54). However, the adenovirus E1A protein also contains the CtBP1 interaction motif, and recent experiments suggest that the ability of E1A to derepress the AP2α promoter results from its interaction with CtBP1, which prevents CtBP1 from productively interacting with KLF12, thus functionally inactivating KLF12 repression of AP2α (54).

**Antagonistic regulatory control by KLF family members**

Given these examples of molecular repressors and activators within the KLF family, it may not be completely surprising to find examples of cross-regulation. Three examples are illustrative. The original screen that had isolated KLF12/AP2-rep (above) had also identified KLF9/BTEB1 binding to the AP2α promoter CACCC element (53). Although KLF12 behaved as a repressor in transfection assays, KLF9 was a strong activator of the AP2α promoter. Their mutually exclusive binding leads to different reporter activities that are dependent on the relative levels of each protein. Unlike KLF12, KLF9 is expressed in many tissues. This provides an example of KLF factors whose differing modes of action (activation vs. repression) may play a role in regulating target gene expression from the same site.

KLF4/GKLF/EZF and KLF5/IKLF/BTEB2 have been implicated in antagonistic regulation of two gene systems. As already described, KLF4 plays a role in reduced proliferation and increased differentiation of the small intestinal epithelium via its effects on cell cycle regulators. Conversely, KLF5 is primarily expressed in the
proliferating cells of the crypt epithelium (55). KLF5 is induced by mitogens, and its overexpression in fibroblasts leads to their increased growth and a transformed phenotype. This leads to a model in which KLF4 and KLF5 play opposing roles in differentiation and proliferation in the intestine (55,56).

The α-smooth muscle and SM22α promoters contain CACCC elements in their promoters which were used in a yeast one-hybrid screen to isolate KLF4 (57). However, KLF4 was found to repress TGFβ induction of the α-smooth muscle actin and SM22α promoters in transfection assays; indeed, TGFβ treatment of smooth muscle cells in culture leads to a decrease in KLF4 levels. However, KLF5/BTEB2/IKLF (but not KLF2) strongly increased the activity of the α-smooth muscle and SM22α promoters in transient assays. As KLF5 is abundant in smooth muscle tissues and is preferentially activated during their proliferation (58), the regulatory model again is based on opposing effects of KLF4 and KLF5, this time during muscle differentiation.

A more subtle effect may be apparent during blood cell development, particularly with respect to regulation of globin gene switching (embryonic to fetal to adult) within the β-like cluster. As KLF1/EKLF appears dedicated to consolidating the switch from human fetal to adult β-globin expression, and since the other genes in the cluster also contain CACCC elements within their promoters, a search for related members that may play a role in embryonic and fetal β-like globin expression led to the identification of KLF11/FKLF (59) and KLF13/FKLF2 (60). KLF11 primarily activates the embryonic and KLF13 the fetal globin promoter, although KLF13 also stimulates non-globin promoters to a lesser extent. However, another factor highly expressed in erythroid cells is KLF3/BKLF. As discussed above, KLF3 primarily behaves as a repressor, but in addition may be indirectly regulated by EKLF (61). These data raise the
possibility that at different stages of red blood cell development, KLF11 and/or KLF13 compete with KLF1 to optimally activate their cognate high-affinity targets in the β-globin cluster, and at the same time, expression of KLF3 may serve to repress the embryonic and fetal members. Clearly, since all of these CACCC binding factors are present together in the red cell in development when globin gene switching is occurring, the mechanism of how a particular target globin promoter is specifically induced at the correct time in the midst of so many effector molecules remains perplexing.

Final thoughts

This review has concentrated on specific properties of selected KLF family members; however, it is not meant to imply that their roles are thereby limited. For example, KLF1 has historically been characterized as a strong transcriptional activator with specific roles in β-globin transcription and β-locus chromatin integrity; however, recent data indicate that it may also play a role in erythroid cell proliferation (62), and may even function as a transcriptional repressor in specific contexts (63).

How do these proteins exert their particular effects, given that they contain such similar DNA binding regions that bind to virtually identical sequences? Studies with KLF1 are instructive. At one level, specificity can follow from tissue-restricted expression. However, not only are a number of these factors expressed in multiple tissues, but most cells express more than one factor at any time. As a result, the second level of specificity is via their respective activation/repression domains, which are unique to each member, and thus likely determine their resultant protein/protein interactions. This has been directly tested for KLF1, where in vivo tests of β-globin promoter activation by transient transfection assays (64) and in transgenic mice (65)
demonstrate that the Sp1 transactivation domain cannot substitute for the KLF1 transactivation domain. Third, one should not also exclude the possibility that the zinc finger region can also play a role in KLF/protein interactions, whether bound to DNA (e.g., KLF1 interaction with DNA and SWI/SNF proteins (66)) or not (e.g., KLF1 behavior as a repressor (63)). Finally, there is a fourth level of control of specificity. Although the KLF DNA binding domains interact with specific nucleotides within the CACCC element that directly affect their binding affinity, it is also clear that the overall architecture and context within which this element is located can have a dramatic effect on the ability of KLF factors (e.g., KLF1 (67-69)) to bind and exert their transcriptional effects.

Although this review has focused on the mammalian members of the KLF family, it is clear from accumulated sequence analyses that large numbers of Cys2/His2 zinc finger proteins are also encoded by the Drosophila, C. elegans, and Danio rerio genomes (1-3). Significant amino acid homologies among KLF family members beyond their DNA binding regions exists only between very closely-related family members, and even then it is quite low. These families have expanded independently in different species, and thus direct functional analogies may be unobtainable by comparison of mammalian and non-mammalian KLF proteins. However, the localization of evolutionarily conserved domains, and any proteins and pathways with which these domains may interact and link with in non-mammalian systems, will prove quite useful for directing tests of homologous regions in their mammalian counterparts.

REFERENCES


ABBREVIATIONS

The abbreviations used are: KLF, Krüppel-like Factor; E, erythroid; L, lung; B, basic; G, gut; I, intestinal; U, ubiquitous; K, kidney; F, fetal; BTEB, Basic transcription element-binding; ZF, zinc finger; CPBP, core promoter binding protein; TIEG, TGFβ-inducible early gene; TGF, transforming growth factor; AP2rep, AP2 repressor; RFLAT, RANTES factor of late activated T lymphocytes; NGF, nerve growth factor; ES, embryonic stem; IFN, interferon; LCR, locus control region.

FIGURE LEGENDS

Fig. 1. Phylogenetic relationship among the fifteen KLF family members designated by the Human Gene Nomenclature Committee. The ClustalX/TreeTop programs (70) were used to align human KLF proteins and determine their relatedness (murine KLF14 (alias Sp6) is a partial sequence and was used because the human orthologue is not available). Murine KLF1 was included for comparison relative to human KLF1. Complete amino acid sequences were used. Also indicated are other names seen in the literature for the same proteins. Members are divided into four most related subgroups as discussed in the text.

Accession numbers for protein sequences used for this analysis are as follows:

hKLF1, U37106; mKLF1, M97200; hKLF2, Q9Y5W3; hKLF3, P57682; hKLF4, O43474; hKLF5, XP_007199; hKLF6, AF001461; hKLF7, XP_002291; hKLF8, NP_009181; hKLF9, XP_005584; hKLF10, NP_005646; hKLF11, AAF75793; hKLF12, CAB46982; hKLF13, NP_057079; mKLF14, CAC06698; hKLF15, BAA88561.
Developmental expression patterns of the murine homologues of many of these have also been determined: KLF1 (36), KLF2 (30,31), KLF3 (49), KLF4 (71,72), KLF5 (73), KLF6 (74,75), KLF7 (24,25), KLF9 (76), KLF13 (76), KLF15 (77).

Fig. 2. KLF factors play multiple roles in a diverse range of cellular events. A schematic summary of functions of selected KLF factors discussed in the text is shown. These include their induction by injury or cytokines, their stimulatory or antagonistic effects on the cell cycle, and their repression/activation of downstream gene expression. The specific role of KLF1 in chromatin remodeling and transcriptional activation of the β-globin promoter in the context of the complete locus (encompassing the locus control region (LCR) and embryonic (ε), fetal (γ), and adult (β) globin genes) is shown at the bottom.
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