REVIEW ARTICLE

The family feud: turning off Sp1 by Sp1-like KLF proteins

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Sp1 is one of the best characterized transcriptional activators. The biological importance of Sp1 is underscored by the fact that several hundreds of genes are thought to be regulated by this protein. However, during the last 5 years, a more extended family of Sp1-like transcription factors has been identified and characterized by the presence of a conserved DNA-binding domain comprising three Krüppel-like zinc fingers. Each distinct family member differs in its ability to regulate transcription, and, as a consequence, to influence cellular processes. Specific activation and repression domains located within the N-terminal regions of these proteins are responsible for these differences by facilitating interactions with various co-activators and co-repressors. The present review primarily focuses on discussing the structural, biochemical and biological functions of the repressor members of this family of transcription factors. The existence of these transcriptional repressors provides a tightly regulated mechanism for silencing a large number of genes that are already known to be activated by Sp1.

Key words: co-repressor, gene regulation, Krüppel-like factor (KLF), Sp1, transcription factor, zinc-finger domain.

INTRODUCTION

Sp1, the founding member of the family of proteins described in the present review, represents the most characterized transcriptional activator in mammalian cells as a result of intense investigations throughout the last two decades. One of the first mammalian transcription factors to be cloned, this protein binds to GC-rich promoter elements, which are essential for proper expression of a large variety of important cellular genes [1–5]. On the basis of these initial observations, along with the broad expression pattern of Sp1, a predominant model for understanding the function of this protein assumed that Sp1 performed the important role of participating in the regulation of most physiological processes. However, our current understanding of Sp1 and its related family members, described in this review, dismisses this initial assumption as quite an oversimplified view and replaces this model with a new paradigm.

Following the momentum of the cloning era, several Sp1-like proteins were identified by the presence of their conserved DNA-binding domain, comprising three Krüppel-like zinc fingers similar to Sp1 [6–12]. Subsequently, these proteins were classified into structural subfamilies derived from variant sequences within their N-terminal domains. As commonly occurs within the scientific community, many of these proteins were unsystematically named by their various discoverers, which promoted the development of a new nomenclature by the HGNC (Human Gene Nomenclature Committee) that sequentially designates each KLF (Krüppel-like factor) protein by number [13]. The recent discovery of several repressor KLF proteins has led us to propose a further classification into two functional subgroups, activators and repressors, based on their divergent transcriptional regulation of heterologous promoters; however, we fully recognize that some of these transcription factors may belong to both categories. Thus functional classification may offer the proper guidance for successful experiments intended to identify the co-activator/co-repressor systems utilized by these proteins.

The discovery of Sp1-like transcriptional repressors represents a significant step in the transcriptional field towards changing the early paradigm of ‘Sp1 activates all GC-rich sites’. The new, more accurate paradigm emphasizes that GC-rich sites are not necessarily the target of Sp1, but instead may be activated or repressed depending on the family member by which it is recognized. Evidence accumulating in this field has highlighted the complex nature of the biological effects generated by the existence of various KLF proteins, thereby justifying active investigation into the role of these transcription factors in biochemistry and cell biology. Several excellent reviews on Sp1-like proteins, in general, have been published previously [6–8,10–12,14]. Thus the reader is encouraged to consult these articles for more detailed information regarding the activator members of this family. The present review will provide an up-to-date, provocative view exclusively centred on KLF repressor proteins.

PAINTING A FAMILY PORTRAIT: A CLOSER LOOK AT THE BASIC STRUCTURAL FEATURES OF KLF PROTEINS

Extensive work, performed by many dedicated laboratories worldwide, has resulted in the identification of more than 20 proteins with DNA-binding domains highly similar to Sp1, and therefore belonging to the KLF/Sp1-like family of proteins. To first understand the function ascribed to these transcription factors, it is important to revisit, in more detail, a few basic structural properties of these proteins. A minimum of three domains are required to comprise any of these site-specific transcription factors: namely, the previously mentioned DNA-binding domain,

Abbreviations used: AP-2α, activator protein-2α; BKLF, basic KLF; BTE, basic transcription element; BTEB, BTE-binding protein; CBP, CREB (cAMP-response-element-binding protein)-binding protein; CtBP, C-terminal-binding protein; CYP1A1, cytochrome P4501A1; EGF, epidermal growth factor; FHL, four and half LIM domain family; HDAC, histone deacteylase; KLF, Krüppel-like factor; mSin3, mammalian Sin3; NLS, nuclear localization signal; PAH, paired amphipathic helix; SAP18 and SAP30, Sin3-associated polypeptides 18 and 30; SID, Sin3-interacting domain; SV40, simian virus 40; TGF-β, transforming growth factor-β; TIEG, TGF-β-inducible early gene.

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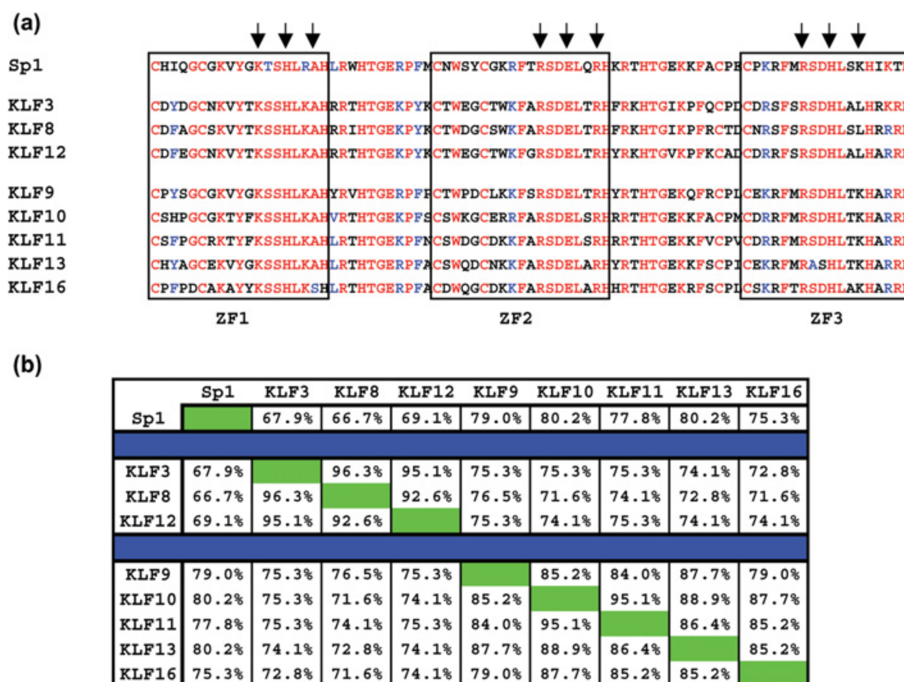

	Sp1	KLF3	KLF8	KLF12	KLF9	KLF10	KLF11	KLF13	KLF16
Sp1		67.9%	66.7%	69.1%	79.0%	80.2%	77.8%	80.2%	75.3%
KLF3	67.9%		96.3%	95.1%	75.3%	75.3%	75.3%	74.1%	72.8%
KLF8	66.7%	96.3%		92.6%	76.5%	71.6%	74.1%	72.8%	71.6%
KLF12	69.1%	95.1%	92.6%		75.3%	74.1%	75.3%	74.1%	74.1%
KLF9	79.0%	75.3%	76.5%	75.3%		85.2%	84.0%	87.7%	79.0%
KLF10	80.2%	75.3%	71.6%	74.1%	85.2%		95.1%	88.9%	87.7%
KLF11	77.8%	75.3%	74.1%	75.3%	84.0%	95.1%		86.4%	85.2%
KLF13	80.2%	74.1%	72.8%	74.1%	87.7%	88.9%	86.4%		85.2%
KLF16	75.3%	72.8%	71.6%	74.1%	79.0%	87.7%	85.2%	85.2%	

Figure 1 Sequence alignment of the zinc-finger domains of KLF proteins

(**a**) The sequence of the zinc-finger domains of Sp1 was compared with the corresponding regions of the three CtBP-mediated and the five Sin3-mediated KLF repressor proteins. The three consensus zinc fingers (ZF1, ZF2 and ZF3) are outlined by boxes, and the amino acid residues predicted to participate in interactions with DNA are indicated by arrows [16]. Identical residues are in red, similar residues in black and different residues in blue. (**b**) For each KLF zinc-finger domain, the percentage similarities were calculated in comparison with the equivalent domain of Sp1 and the other KLF proteins. All sequences are available in the NCBI human genome database (http://www.ncbi.nlm.nih.gov/).

an NLS (nuclear localization signal) and a transcriptional regulatory domain. In the DNA-binding domain, the sequence identity among the Sp1-like/KLF family members is higher than 65 %, suggesting a role in the regulation of similar promoters (Figure 1) [14]. This domain comprises three Cys_2His_2 zinc-finger motifs, each of 25–30 amino acid residues [15,16]. The N-terminal portion shapes into two β-pleated sheets and the C-terminal region forms an α-helix, joined at the base by the co-ordination of a zinc atom. In addition, this domain resembles the segmentation gene product of *Drosophila melanogaster*, Krüppel, and shares a highly conserved seven amino acid inter-finger spacer, TGE(R/K)(P/k/r)(F/y)X [17,18]. In all of the KLF family members, each motif is equal in length, with the first two zinc fingers comprising 23 amino acids and the third one encompassing 21 amino acids. The functional implications of this difference, however, remain to be characterized. Individual zinc-finger motifs make contact with the major groove of DNA, binding a nucleotide triplet [16,19,20]. Interestingly, several biochemical and structural studies have demonstrated that the identity of three residues at positions -1, $+3$ and $+6$ within each α-helix region determines the type of nucleotides recognized by a particular finger (Figure 1) [16,21–23]. However, some zinc-finger proteins recognize slightly different DNA sequences from the one that is predicted from its amino acid sequence. This phenomenon is likely to be due to a mechanism of co-operative binding, in which the interaction of one finger with DNA modifies the selectivity of another finger. Alternatively, DNA recognition by these zinc fingers may reflect a 'wobbler effect' similar to the one that operates during peptide synthesis. Nevertheless, to date, Sp1-like proteins have been divided into two groups based upon the selectivity for one of two highly similar GC-rich sites, containing either a CGCCC or CACCC core sequence [24–29]. Whether proteins within these groups compete against each other for recognition sites or acquire different binding selectivity by post-translational modifications or combinations of homo-/hetero-dimerization remains to be determined. However, the discovery of multiple Sp1-like proteins, with similar overall binding selectivity, should provide caution against adopting an Sp1-centric assumption, and encourage investigators to search meticulously for the exact proteins that may regulate such a promoter through GC-rich sites. Since there are several thousands of these sites throughout the genome, this information should be useful to further the research of a large number of studies that are focused on the mechanism of expression of distinct genes.

In contrast with the conservation of the 'Sp1-like domain' in all the members of this family, the structure and function of the N-terminal transcriptional regulatory domain, as well as the location of the NLS, are variable. The location of the NLS has also been used by some investigators to divide these proteins into two major groups, one containing the NLS within the zinc-finger domain and another with the signal immediately upstream of this region [30,31]. In agreement with the structural differences at the N-terminal domain, distinct family members have been shown to diverge in their functional ability to regulate transcription, and, as a consequence, to influence cellular processes. In contrast with the function of Sp1 as one of the best-characterized transcriptional activators currently known, KLF11, for example, behaves as a potent transcriptional repressor [32]. Furthermore, several KLF proteins have been observed to perform as both activators or repressors, as determined by the cellular environment. The functional distinctions between the members of this family are embedded within the high level of variability in the N-terminal portion of protein. Specific activation and repression domains

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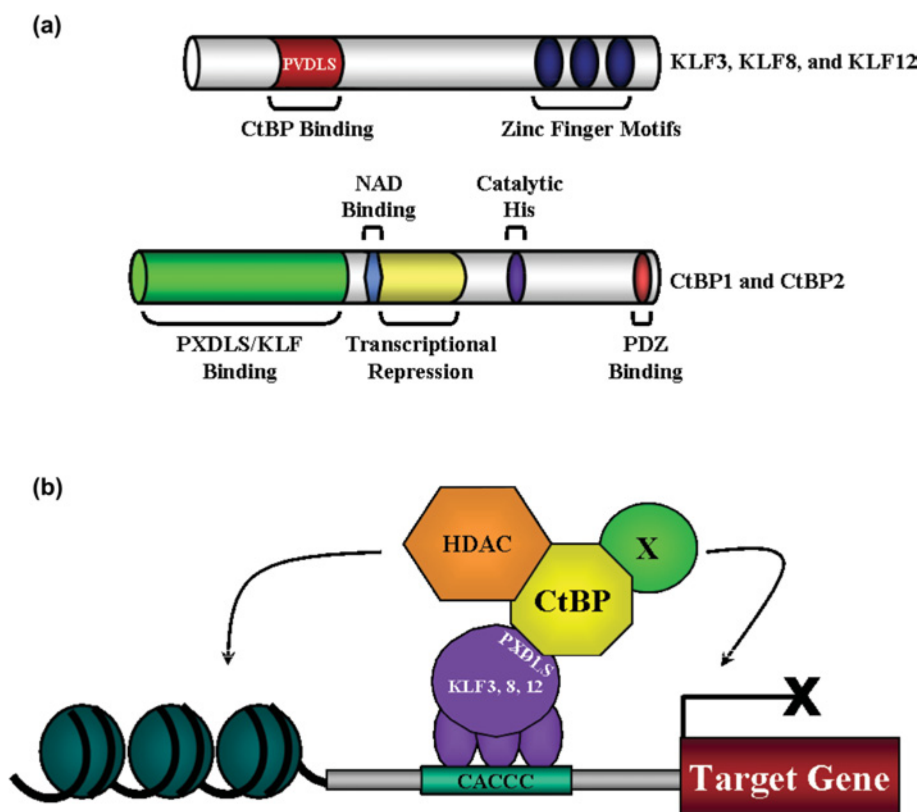

Figure 2 CtBP and CtBP-mediated KLF

(**a**) Structural properties of the CtBP-mediated KLF proteins and CtBP family members. The highly homologous DNA-binding domains of the CtBP-mediated KLF, which are characterized by three Cys_2His_2 zinc-finger motifs, are indicated (blue). The region required for CtBP binding, containing the PXDLS recognition motif, is indicated in red. For the CtBP family members, the putative catalytic His^{315} residue is shown in purple. The regions essential for transcriptional repression of CtBP1 and PXDLS motif/KLF binding are shaded in yellow and green respectively [48]. (**b**) Model of CtBP-mediated repression. The CtBP-mediated KLF binds to its respective recognition sequence in the promoter of a particular target gene. This protein is then able to recruit a CtBP protein via a conserved PXDLS motif. As a result, CtBP influences repression through association with proteins that include the chromatin-condensing HDACs and/or other inhibitory factors (X), such as polycomb and Ikaros.

located within this region have been determined to interact with various co-activators and co-repressors, thereby regulating transcription in a distinct manner. Thus, in summary, although the structure of the DNA-binding domain classifies these proteins within the KLF family of transcriptional regulators, the N-terminal region provides the functional identity to each member of this family.

DIVULGING A FAMILY SECRET: DISCOVERY OF THE KLF TRANSCRIPTIONAL REPRESSORS

Almost simultaneously in 1998, the primary functional sub-families of transcriptional repressors were characterized. Turner and Crossley [33] reported the existence of a KLF/BKLF (basic KLF) subfamily that utilizes CtBP (C-terminal-binding protein) co-repressors, whereas our laboratory described the KLF/TIEG [TGF-β (transforming growth factor-β)-inducible early gene] subfamily of transcriptional repressors that functions via the Sin3 HDAC (histone deacteylase) system [32,34,35]. Subsequently, we also discovered an extended subfamily of Sin3-mediated repressors, known as the KLF/BTEBs [BTE (basic transcription element)-binding proteins) [36,37]. Originally, these three repressor groups of KLF proteins were classified entirely based on structural features. More detailed analyses later supported the notion that KLF/TIEGs and KLF/BTEBs may represent the same functional subfamily, which underscores the fact that mechanistic predictions based on primary sequence data, although useful, are to some extent incomplete [35]. Therefore, a supplementary classification for these proteins by consideration of their actual mechanism of action is necessary. In this article, we will review the KLF proteins according to their mechanisms of action, namely CtBP-dependent and Sin3-dependent KLF repressors. The structural, biochemical and biological functions of both subfamilies of KLF transcriptional repressors will be discussed.

BEARING A FAMILY RESEMBLANCE: CtBP-MEDIATED KLF REPRESSORS

Members of the KLF/BKLF subfamily, which include KLF3, KLF8 and KLF12, have been shown to repress transcription through the recruitment of a CtBP family member (Figure 2a) [33,38,39]. Interaction of these proteins with CtBP occurs through a 5-amino-acid motif PXDLS (Pro-Xaa-Asp-Leu-Ser). Interestingly, except for the small CtBP-recognition motif, no significant similarity occurs between the N-terminal domains of these three proteins. Since the repressor function of these proteins depends, at least in part, on the action of CtBPs, it is necessary to briefly describe a few key properties of this family. The first family member, CtBP, was originally characterized as the binding protein of the C-terminal portion of the adenovirus E1A protein [40,41]. CtBPs are highly conserved through evolution and share significant amino acid similarity to NAD-dependent 2-hydroxyacid dehydrogenases, including the residues believed to

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participate in catalysis and the characteristic core NAD^+-binding motif (Figure 2a) [41]. Although for many years the mechanism of CtBP function was unclear due to lack of evidence to support dehydrogenase or NAD-binding activities, subsequent studies led to biochemical and crystallographic data which revealed that CtBP is indeed a functional dehydrogenase [42]. Furthermore, Kumar et al. [42] demonstrated that the dehydrogenase domain is necessary and sufficient to mediate repression, while also identifying the NAD^+ and putative substrate interactions based on the structure of this domain in the presence of NAD^+.

The function of CtBPs as transcriptional co-repressors has been well established; however, their mechanism of action remains obscure [33,43–47]. CtBP proteins recruit HDACs, suggesting this pathway as one of the mechanisms for gene silencing (Figure 2b) [48,49]. Remarkably, however, transcriptional repression via CtBP also occurs in a HDAC-independent manner, which is probably due to interactions between these proteins with different co-repressors [49–51]. Indeed, other families of transcriptional repressors, such as Ikaros and members of polycomb, have been reported to interact with CtBP (Figure 2b) [49,52]. As these interacting proteins are integral parts of chromatin-remodelling complexes, these data suggest an additional possible mechanism of action for CtBP, namely gene silencing through the physical rearrangement of nucleosomes. The mechanism that operates in KLF-mediated repression, whether through one of the aforementioned or another yet undiscovered mechanism, remains poorly understood. Next we will discuss the most important characteristics of the biochemical and cellular function of these CtBP-mediated KLF repressors individually.

KLF3 was discovered as the result of efforts to identify additional CACCC-binding factors following the characterization of KLF1, an activator of the β-globin gene locus [27]. Gel-shift assays with mouse erythroleukaemia cell extracts revealed a distinct CACCC-bound complex from KLF1, which subsequently prompted the isolation of KLF3 cDNA through a low-stringency library screen [27]. KLF3 binds to CACCC sequences preferably over GC-rich sites, and the specificity of its DNA-binding domain is distinct from KLF1 on various promoters, although there is a general resemblance in the recognition sequence. KLF3 is commonly called BKLF and is distinguished from other KLF proteins by its basic charge. Whereas KLF1 has an isoelectric point of 7.0, KLF3, however, is appreciably unique with an isoelectric point of 10.2 [27]. Similar to KLF1, KLF3 is present in haematopoietic tissues, however, at even higher intensities. In contrast with the erythroid confinement of KLF1 mRNA, the KLF3 transcript is abundant in the developing nervous system and moderately expressed in several adult tissues [27]. Preliminary data from the generation of *KLF3*-knockout mice shows signs of myeloproliferative disorder, perhaps indicative of a crucial role for KLF3 in haematopoiesis [53].

KLF3 binds to many *cis*-regulatory sites containing the CACCC element, including the promoters of the β-globin, GATA-1, pyruvate kinase, carbonic anhydrase, porphobilinogen deaminase, α-fetoprotein and mitochondrial glycerol phosphate dehydrogenase genes, as well as globin locus control regions [27,54,55]. Initially, KLF3 was assumed to solely possess abilities to activate transcription due to its effects on a minimal promoter that contained one KLF3-binding site. However, this activation was of considerably less magnitude than other KLF proteins and was only achieved with excessive amounts of KLF3 [27]. Subsequent studies revealed that KLF3 also modulates gene expression as a potent repressor [33]. The domain responsible for the silencing activity of KLF3 was mapped to a 74-amino-acid region within the N-terminus, which sustained repression upon fusion to a heterologous DNA-binding domain [33]. Subsequently, investigations into the mechanism of KLF3 repressor function using a yeast two-hybrid screen identified CtBP2 as the co-repressor for this protein [33]. Intriguingly, the disruption of CtBP recruitment fails to completely eliminate transcriptional repression via KLF3, implicating additional cofactors in its regulation of transcription. This interesting observation led to a repeat two-hybrid screen with KLF3, which isolated FHL3, a member of the FHL (four and half LIM domain) family, as another partner protein facilitating repression [56]. Although FHL proteins were originally proposed to function in cytoskeletal organization, family members have recently been observed within the nucleus, participating in co-regulation of transcription [57–60]. Therefore, interactions between KLF3, CtBP2 and FHL3 suggest that large multiprotein complexes function in transcriptional repression of GC-rich promoters.

KLF8, a protein identified by its limited similarity to KLF3 through a database search, also associates with CtBP through a PVDLS recognition motif [38]. The N-terminus of KLF8 possesses repression activity, which is dependent upon the integrity of the CtBP interaction site [38]. However, as with KLF3, mutation of the CtBP-recognition motif does not fully abolish the capacity of KLF8 for transcriptional repression [38]. Similar to the case of KLF3, this phenomenon represents a yet unexplored indication of the existence of additional co-repressors for KLF8. Interestingly, expression of *KLF8* was detected in a broad range of human tissues, and considerable overlap was observed between the expression profiles of KLF3 and KLF8 [38]. Currently, however, it is unclear as to whether these proteins have similar or redundant cellular functions. Thus extensive functional analyses are still necessary to resolve this important biological question.

KLF12 encodes the third member of this subfamily of CtBP-mediated repressors, which, distinct from *KLF3* and *KLF8*, shows a pattern of expression restricted to kidney and brain [61]. In addition, KLF12 differs from both KLF3 and KLF8 by the fact that an endogenous target promoter for this protein is known. KLF12 was identified as a repressor of the AP-2α (activator protein-2α) gene, which also encodes a mammalian transcription factor [61]. KLF12 represses AP-2α expression through an N-terminal PVDLS sequence that promotes physical interaction with the co-repressor CtBP1 [39]. Therefore, KLF12 repression is tightly coupled with an effective CtBP interaction motif. However, a truncated KLF12 of the C-terminal portion with three intact zinc fingers maintained partial repression of the AP-2α promoter, indicating that the zinc-finger motif may accommodate a compatible transcriptional repressor function [62]. The possibility also remains that the C-terminal region may sterically interfere with binding of activators which recognize adjacent sequences. Interestingly, upon induction of *KLF12* expression during kidney development, down-regulation of AP-2α expression is subsequently detected, suggesting that repression, not activation, is the major function of KLF12 [61]. As KLF12 functions to suppress endogenous AP-2α expression, AP-2α reciprocates, acting as a negative regulator of *KLF12* expression [62]. These data suggest a mechanism of *trans*-regulation between these transcription factors, but does not explain the biological relevance of this phenomenon.

In contrast with other subfamily members (KLF3 and KLF8) that recognize the CACCC sequence, KLF12 interacts with CAGTGGG, which is similar to the high-affinity binding sites of the tumour suppressor WT-1 [62]. This selectivity is due to the fact that the DNA-binding domain of KLF12 also shares significant similarity to the equivalent region of WT-1/egr proteins [61]. Moreover, the presence of N-terminal Ser-Thr and Pro-Gln motifs, which possess structural similarity to WT-1, is also a unique characteristic of KLF12. These features suggest that KLF12 bears

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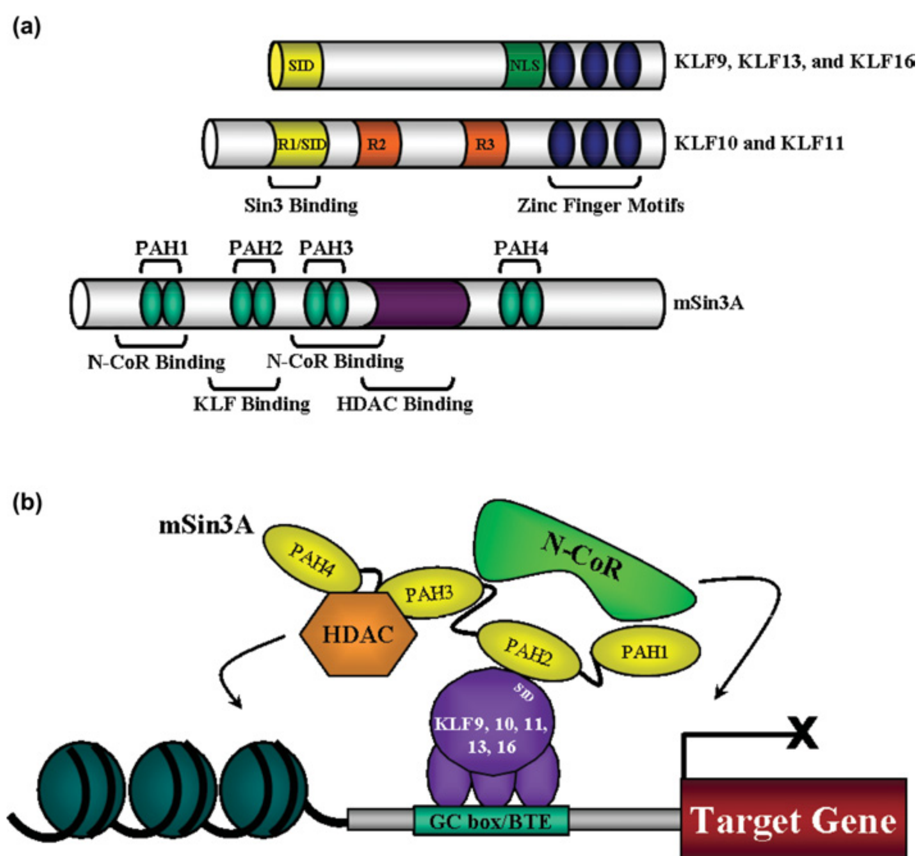

Figure 3 mSin3 and mSin3-mediated KLF

(**a**) Structural properties of the mSin3-mediated KLF proteins and mSin3 family members. As in Figure 2, the three conserved Cys_2His_2 zinc-finger motifs are shown in blue. The SID region involved in interactions with mSin3 is shaded yellow. KLF10 and KLF11 have three conserved N-terminal repression domains, including the previously mentioned SID (R1) along with the R2 and R3 domains (orange). For the mSin3 family members, the four PAH protein interaction motifs are represented by adjacent, green ovals. The locations of Sin3 regions required for interaction with KLF (PAH2), N-CoR (nuclear receptor co-repressor) and HDAC are indicated. (**b**) Model of mSin3-mediated repression. The Sin3-mediated KLF associates with its respective GC box/BTE site in the promoter of a target gene. The SID domain of these proteins facilitates the recruitment of mSin3A through interaction with PAH2. Consequently, mSin3A recruits N-CoR, which inhibits the basal transcriptional machinery, and HDACs, which modify chromatin structure to a more condensed state, thereby mediating transcriptional repression.

significant structural similarities at the DNA-binding domain to proteins that silence embryonic gene expression during terminal cell differentiation. Together, the interaction with AP-2α during kidney morphogenesis and the structural similarities with WT-1 support a role for KLF12 in regulating developmental processes. Thus, although KLF12 is a member of the CtBP-mediated transcriptional repressors, it shows distinct structural and functional differences from KLF3 and KLF8.

RECOGNIZING FAMILY TIES: Sin3-MEDIATED KLF REPRESSORS

A subgroup of KLF proteins, which includes KLF9, KLF10, KLF11, KLF13 and KLF16, represses transcription via the HDAC system through direct interaction with the scaffold co-repressor protein Sin3A (Figure 3a) [35]. Mammalian Sin3 (mSin3) proteins, orthologues of the Sin3p transcriptional repressor in *Saccharomyces cerevisiae*, are part of large, multi-protein complexes involved in local chromatin modification [63–66]. Most of the subunits in these mSin3–HDAC complexes have been identified, such as RBAP46 [Rb (retinoblastoma protein)-associated protein 46], RBAP48, HDAC1, HDAC2, SAP30 (Sin3-associated polypeptide 30) and SAP18, in addition to the mSin3A/mSinB components [67–69]. Sin3 itself is a substantial multi-domain protein proposed to function as a scaffold for assembly of the entire complex. Disruption of the HDAC core-binding site within Sin3, as well as treatment with HDAC inhibitors, were shown to significantly inhibit the repressor activity of the Sin3 complex, emphasizing the critical nature of the HDAC interaction for Sin3-medated repression (Figure 3b) [70,71]. In addition, there are four evolutionarily conserved imperfect repeats within Sin3, each of which is predicted to form a newly discovered four helix bundle fold, labelled PAH (paired amphipathic helix) regions (Figure 3) [64,72,73]. These PAH domains mediate binding with various transcription factors and accessory proteins, thereby facilitating recognition of a target sequence for the complex. Through direct demonstration with KLF10 (TIEG1) and structural comparisons to KLF9 (BTEB1), KLF11 (TIEG2), KLF13 (BTEB3) and KLF16 (BTEB4), a specific interaction with mSin3 was shown via its second PAH domain (Figure 3) [35].

Of the KLF proteins, KLF9 was the first identified and cloned, based upon its ability to bind the BTE, a single GC-box sequence in the *CYP1A1* (cytochrome P4501A1) gene promoter that is required for its constitutive expression [74,75]. Apart from a 72 % similarity to Sp1 within its zinc-finger domain, KLF9 displays minor to no further resemblance with Sp1 [5]. The activation domain of KLF9 is contained within two regions that are rich in hydrophobic residues, which differs from other KLF family members [76]. The zinc-finger motifs of KLF9 recognize

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the BTE with comparable affinity to Sp1 [77]. Although both KLF9 and Sp1 activate gene expression from promoters with multiple GC-box sequences, such as the SV40 (simian virus 40) early promoter and HIV-1 long terminal-repeat promoter, KLF9 represses the activity of the *CYP1A1* promoter, containing a single BTE [5,78]. This is in strong contrast with Sp1, which is a potent stimulator of *CYP1A1* gene expression [5]. As a ubiquitously expressed protein, KLF9 has been implicated in a wide variety of biological functions. The activation of collagen α1(I) gene expression in liver fibroblast-like stellate cells, which is induced by acetaldehyde upon injury, is reportedly mediated by KLF9 [74]. These studies emphasize the importance of KLF9 in a highly regulated process, such as stellate cell activation, demonstrating the existence of a cell-specific function for KLF proteins. In co-operation with the progesterone receptor, KLF9 has been shown to stimulate uteroferrin expression, which produces a pregnancy and epithelial-specific, endometrial secretory protein [79]. This transactivation of the uteroferrin gene promoter not only exhibits another example of cell-specific action, but also reveals regulation of pregnancy-associated transcription through a KLF protein. In addition, KLF9 has been demonstrated to have a role in neural development [80]. The thyroid hormone, T3, regulates *KLF9* expression specifically in neuronal cells of the developing nervous system, and this over-expression of KLF9 results in an increased length and number of neurites. These data suggest that KLF9 is required for the proper maturation of the central nervous system. This result has recently been confirmed by the analyses of *KLF9*-knockout mice [81]. Although, in addition to normal morphogenesis in the central nervous system, it is tempting to speculate that KLF9 may play a role in neurodegenerative diseases and regeneration, and these studies remain to be carried out.

Expanding the repertoire of BTEB-related KLF transcription factors, KLF13 (BTEB3) and KLF16 (BTEB4) were independently discovered through sequence comparisons with KLF9 and other KLF family members [34,36,37]. The expression patterns of *KLF13* and *KLF16* display variable levels in the majority of human tissues, with a notable difference in the liver where *KLF16* transcripts are enriched, but transcripts of *KLF13* are comparatively low [37,82,83]. KLF13 has been shown to activate the SV40, RANTES (regulated upon activation, normal T-cell expressed and secreted; a chemotactic cytokine) and β-globin promoters or repress promoters with a BTE site, such as *CYP1A1* [5,36,82,84,85]. Analogous to the highly related family member KLF9, KLF13 binds to BTE sites with an affinity similar to Sp1 [36,77]. In addition, KLF13 functions as a repressor of BTE-dependent transcription via mechanisms that include competition with Sp1 for DNA binding and recruitment of the co-repressor complex, mSin3A–HDAC [36]. With approx. 80 % similarity to both KLF9 and KLF13, KLF16 also behaves as a BTE-binding transcriptional repressor, exhibiting repression of the *CYP1A1* promoter and interaction with mSin3A [36]. Thus the evolution of this group of BTEB-related KLF proteins may have occurred, at least in part, to counteract the activating function of Sp1 on the *CYP1A1* promoter.

KLF10 (TIEG1) and KLF11 (TIEG2) are two significantly related Krüppel-like factors [32]. Ubiquitously expressed, KLF10 was originally identified by differential-display PCR as the product of a TGF-β-inducible gene in a human osteoblastic cell line [86]. Furthermore, *KLF10* expression was induced upon treatment with a limited number of other growth-stimulating factors, including bone morphogenetic factor 2 and EGF (epidermal growth factor) [86]. Independently, *KLF10* was also identified as a growth-factor-inducible gene from the prostate, brain and rat pancreas, thereby receiving the names of EGR-α (early growth response gene-α), GIF (glial cell-derived neurotrophic factor inducible transcription factor) and rat TIEG respectively [87,88]. In addition to TGF-β and other growth factors, various hormones have also been shown to influence *KLF10* expression, such as the oestrogen 17β-oestradiol, which stimulates a rapid increase of *KLF10* expression in oestrogen-receptor-positive human fetal osteoblastic cells [89]. Conversely, androgens, such as 5α-dihydroxytestosterone, result in the inhibition of *KLF10* expression in prostate cancer cell lines [87,90].

In many different cell types, TGF-β1 serves as a potent inhibitor of cell growth by two different mechanisms, namely cell-cycle arrest and apoptosis [91–95]. As a result, the stimulation of *KLF10* expression by TGF-β1 suggested that KLF10 may participate in the regulation of apoptosis. *In vitro* studies of KLF10 revealed that its over-expression in the epithelial TGF-β-sensitive pancreatic cell line PANC-1, indeed, inhibits cell proliferation and induces apoptosis [96]. Of note, even though the pro-apoptotic role of KLF10 is not specific to epithelial cells, mechanistic experiments performed in epithelial cell lines have resulted in a better understanding of the intracellular pathway underlying KLF10-induced apoptosis [97,98]. In these cells, *KLF10* expression subsequent to TGF-β1 treatment was observed to precede the typical morphological changes associated with apoptosis. However, cell death triggered by TGF-β1 treatment occurred without changes in the expression of genes encoding classical apoptotic regulatory proteins, such as Bax and Bcl-Xl [98]. In contrast, this apoptotic response was accompanied by an increase in the formation of reactive oxygen species and a loss of mitochondrial potential, which precede the morphological features of apoptosis [98]. These observations indicated that the increased intracellular levels of KLF10 mimic the anti-proliferative and apoptotic effects of TGF-β1 on epithelial cell growth, suggesting that KLF10 is an important factor for mediating TGF-β1 signalling, and that genes involved in the regulation of oxidative stress may be targets of this transcriptional repression.

KLF11/TIEG2, a structural relative to KLF10, was identified in our laboratory through the use of an *in silico* screening approach [32]. Sequence analysis of these two proteins revealed an approx. 90 % similarity within the C-terminal DNA-binding domain and 44 % overall similarity throughout the proline-rich N-terminal domain, with selected regions sharing as much as 70 % similarity [32]. Some mutual characteristics of KLF10 and KLF11 include the TGF-β inducible nature of their expression and their participation in growth regulation [32]. The tissue expression pattern of *KLF11* is also ubiquitous, with enrichment in the pancreas and muscle, comparable with *KLF10* [32]. Thus, on the basis of their sequence similarity and inducibility by TGF-β, KLF10 and KLF11 represent a distinct group of KLF transcription factors.

Initial biochemical characterization of these proteins using heterologous promoter systems revealed that the N-terminal domains of both KLF/TIEGs contain three distinct transcriptional repressor domains [34]. Within KLF11, the R1 (amino acids 24–41), R2 (151–162) and R3 (273–351) domains exhibited repression of reporter activity by a minimum of 75 % [34]. However, regions outside the R1, R2 and R3 domains failed to demonstrate any transcriptional regulatory capacity. The R1 domain had been predicted to adopt an α-helical conformation based on secondary-structure prediction algorithms. Subsequently, CD analysis confirmed that this domain has the propensity to form an α-helix [35]. Mutations within the central core of R1 (amino acids 30–39; AVEALVCMSS) disrupted the transcriptional regulatory activity, thereby defining a 10-amino-acid core sequence that is required for the R1 domain to function [34]. This core has been characterized as the α-helical repression

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motif SID (Sin3-interacting domain), which is discussed below in greater detail. Mutations in three consecutive residues, valine, isoleucine and arginine (aa 158–160), within R2 resulted in a complete loss of R2 repression activity [34]. However, these three residues alone were not sufficient to repress reporter activity, indicating that while the residues are essential for R2-mediated repression, the remainder of the R2 domain also contributes to its function. The R3 domain is proline-rich (20 %), a classic feature of activation and repression domains [34,99,100]. This domain also contains a core sequence of approx. 20 amino acids (311–328) that is highly conserved (67 % similar) between KLF10 and KLF11 [34]. Mutation analyses indicated that these proline residues are not essential for the ability of R3 to repress transcription; however, mutation of the conserved core abolished the ability of R3 to repress transcription. Investigation of the role of the four linker regions in between the DNA binding revealed the presence of numerous sites for post-translational modifications and demonstrated that KLF11 function is regulated by different signalling cascades [101].

The identification and functional characterization of a novel mSin3A-interacting domain, which is conserved in particular KLF proteins, initiated from the observation that R1 of KLF11 binds mSin3A with high affinity [35]. Detailed biochemical and functional analyses have demonstrated that the KLF11 SID interacts specifically with the PAH2 domain of mSin3A to repress transcription. The N-termini of KLF9, KLF13 and KLF16 also contain this conserved SID, analogous to the corresponding domain in KLF10 and KLF11 [35]. Therefore, the SID is a defining structural and functional feature of this subset of KLF repressors, linking the function of these proteins to HDAC-mediated transcription repression via mSin3A binding [67]. Besides the KLF repressors, interactions of this type have been only described for Mad1, Ume6 and Pf1 [102–104].

The SID of KLF proteins displays structural and functional resemblance to the characterized SID of Mad1, the basic helix–loop–helix protein that dimerizes with Max to antagonize the function of the c-Myc oncoprotein [35]. Members of the Mad family of repressor proteins, which include Mad1, Mad3, Mad4 and Mxi-SR, also associate with the PAH2 domain of mSin3A through their N-terminal SID [72]. CD and mutational analyses first demonstrated that the Mad1 SID adopts an amphipathic α-helical conformation, and this was confirmed through NMR structure analysis [102,105]. From these studies, it became evident that this α-helical structure interacts with PAH2 by docking into a hydrophobic pocket within the base of this four helix bundle structure. The KLF11 SID and Mad1 SID interaction with the same PAH2 domain of Sin3 prompted an investigation as to whether the interaction was based upon structural similarities [35,106]. Low-stringency sequence comparisons revealed a minimal level of homology between the KLF11 SID and the Mad1 SID with the presence of a core consensus sequence, A(A/V)XXL, and similar helical propensities [35]. Although structurally similar, the SID of the KLF repressor proteins displays an affinity for the Sin3A PAH2 domain that is lower than the affinity of the Mad1 SID for this domain. Molecular modelling experiments, combined with molecular dynamics simulation of the Mad1 SID–PAH2 complex, as compared with the KLF11 SID–PAH2 complex, suggest that this difference in affinities is a result of distinct binding mechanisms [106]. Thus this divergence may serve as the basis for the design of pharmacological agents that modulate the activity of different SID–PAH2 complexes. Overall, the KLF/TIEG and KLF/BTEB proteins have more similarity between their SIDs than to the Mad1 SID.

The SID that is a Sin3-interacting domain functions as a transcriptional repressor domain *in vivo* [35]. As a result, the KLF/TIEG and KLF/BTEB proteins are the first KLF transcription factors demonstrated to repress gene expression via the mSin3A–HDAC co-repressor complex. The SID of these KLF repressors represents a broader mechanism for transcriptional repression beyond the SID of the Mad subfamily of basic helix–loop–helix proteins. In addition, the functional similarity conferred by the SID present in a subset of KLF proteins encourages the formation of the new functional classification – the Sin3-mediated KLF repressors, to provide more information than the former classification of these proteins into different structural subfamilies.

THE RECESSIVE TRAITS: TYPICAL ACTIVATORS ACTING AS REPRESSORS

In addition to the aforementioned CtBP-mediated and Sin3-mediated repressors, there are emerging examples of prototypical KLF activators functioning as repressors via mechanisms not yet fully characterized. EKLF, or KLF1, is well-established as an erythroid-specific transcription factor that is essential for activation of β-globin gene expression [107–112]. Although much of the focus has been on its activator role on the β-globin locus, questions arose concerning the observations that KLF1 expression on day 7.5 is much earlier than the onset of adult β-globin expression [113]. This observation suggested that KLF1 had additional targets and thus different functions under different cellular contexts. Unexpectedly, full-length KLF1 and solely the zinc-finger domain displayed transcriptional repression *in vivo* when tethered to a GAL4 DNA-binding domain [114]. KLF1 requires recruitment to a promoter to exhibit repression; however, its zinc-finger domain is unable to directly bind DNA while simultaneously inhibiting transcription. As a result, several new mechanisms can be proposed, including the possibility that KLF1, as a repressor, associates with the promoter via other DNA-binding proteins and perhaps the target site does not contain the CACCC sequence commonly equated with KLF1 binding. Of further importance, KLF1 was found to interact with mSin3A and HDAC1, providing a potential model for repression [114]. At the same time, it exposed the deeper conundrum of the mechanism responsible for influencing KLF1 to selectively recognize these co-repressors or its known co-activators, CBP [CREB (cAMP-response-element-binding protein)-binding protein] and p300 [115].

KLF4 was initially identified as a potent activator of transcription under several conditions [29,116,117]. As KLF4 was associated with negative regulation of cellular growth, an interaction between CBP and KLF4 was established to couple transcriptional activation with growth suppression [117]. Interestingly, subsequent studies offered a connection between KLF4, growth suppression and transcriptional repression. Overexpression of KLF4 in colon adenocarcinomas was shown to reduce cyclin D1, a key regulator of cell-cycle progression, at the mRNA and protein levels, and direct binding of KLF4 suppressed the cyclin D1 promoter by 55 % [118,119]. The mechanism by which this repression occurs remains to be completely elucidated; however, data are indicative of a competitive interaction between KLF4 and Sp1 [118]. KLF4 can also inhibit the promoter activity of *CYP1A1* by this mechanism [120]. Therefore, KLF4 is another example of a pleiotropic Krüppel-like transcription factor with activation or repression functions in a context-dependent manner.

A FAMILY LEGACY: LOOKING TOWARD THE FUTURE

The existence of multiple KLF proteins with similar recognition sequences confers on the nucleus numerous possibilities of diverse

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biochemical mechanisms by which these proteins may function, allowing precise manipulation of gene expression under various physiological conditions. In fact, structural motifs within these proteins have evolved in a manner that provides a strategic opportunity for interaction with distinct cofactors. As a result, differential association of KLF proteins with a unique repertoire of cofactors not only allows for the regulation of a distinct subset of genes, but also divergent regulation of the same gene. Early studies have extensively characterized the activation function of several KLF proteins. The ensuing consensus has indicated that activation domains within these proteins interact with particular co-activator proteins to modulate either basal transcription machinery or chromatin structure. However, our understanding of the mechanisms underlying the repressive function of KLF proteins has just begun to emerge in recent years.

This review focused on two main subfamilies of KLF repressors, the CtBP- and Sin3-mediated repressors. However, whether two subfamilies are the full extent of repressor subfamilies in the KLF family and whether the members of these two families interchange their mechanisms of repression require further investigation. More extensive assessment of context-dependent actions would facilitate the comprehension of these issues, given that different cellular situations dictate the mechanism of KLF repression. This mechanism may be better understood once the number of endogenous promoters that are influenced by KLF repression increases. More frequent use of techniques, such as chromatin immunoprecipitation using antibodies to specific KLFs or selected cofactors, may accelerate the resolution of this complex network of activation and repression. Future studies should consider the possibility of pleiotropic function; perhaps most, if not all, KLF proteins have both activating and repressive activities. The characterization of many KLF transcription factors as possessing one predominant function may stem from its capture in a single scenario within the cell. For instance, a particular modification or signal may reveal an alternative function.

Much potential lies ahead with the examination of functional consequences related to post-translational modifications on KLF proteins, along with various signalling events that initiate their occurrence. Data has already accumulated on the acetylation of KLF1 on Lys^{288} and Lys^{302} [115,121]. Although neither modification affects site-specific DNA binding, mutagenesis of Lys^{288}, in particular, reduces activation of the β-globin promoter via KLF1 and eliminates the enhanced activation by p300 or CBP [115]. Moreover, the acetyltransferase capability of p300 or CBP is necessary for complete co-activation with KLF1. Interestingly, acetylated KLF1 has a higher affinity for the chromatin remodelling SWI–SNF complex, in addition to acting as a more potent transcriptional activator of reconstituted chromatin *in vitro*. In another study, the EGF signalling pathway, EGF–Ras–MEK1–ERK2, was shown to inhibit the SID domain of KLF11 through phosphorylation of four serine/threonine residues adjacent to this region [101]. The phosphorylation event was concluded to disrupt the KLF11–mSin3A interaction, thereby impeding the repressive function of KLF11. Consequently, these two studies have provided evidence for the controlled regulation of KLF activity by post-translational modifications and cellular signalling, rather than a constitutive function. Potentially, this paradigm may extend beyond a simple ‘on/off’ mechanism by determining which KLF binds to a multi-KLF recognition site or mediating some of the pleiotropic behaviour of the KLF proteins.

Taking these data into consideration, it is possible to speculate on how the regulation of KLF proteins can be utilized for designing both experimental and therapeutic strategies. For instance, the exploitation of the sequence specificity of KLF recognition sites may lend to the development of molecules with direct therapeutic benefit. Artificial manipulation of individual KLF-regulated promoters, such as those of cancer-related genes, may provide the accuracy required to target only detrimental gene expression and not the ‘healthy’ remainder. The significance of these potential applications underscores the importance to strive for complete comprehension of the functions and mechanisms of these proteins.

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