SPECIAL ISSUE REVIEWS–A PEER REVIEWED FORUM

Biochemistry of the Tale Transcription Factors PREP, MEIS, and PBX in Vertebrates

E. Longobardi,[1] D. Penkov,[2] D. Mateos,[3] G. De Florian,[1] M. Torres,[3] and Francesco Blasi[1]*

TALE (three amino acids loop extension) homeodomain transcription factors are required in various steps of embryo development, in many adult physiological functions, and are involved in important pathologies. This review focuses on the PREP, MEIS, and PBX sub-families of TALE factors and aims at giving information on their biochemical properties, i.e., structure, interactors, and interaction surfaces. Members of the three sets of protein form dimers in which the common partner is PBX but they can also directly interact with other proteins forming higher-order complexes, in particular HOX. Finally, recent advances in determining the genome-wide DNA-binding sites of PREP1, MEIS1, and PBX1, and their partial correspondence with the binding sites of some HOX proteins, are reviewed. These studies have generated a few general rules that can be applied to all members of the three gene families. PREP and MEIS recognize slightly different consensus sequences: PREP prefers to bind to promoters and to have PBX as a DNA-binding partner; MEIS prefers HOX as partner, and both PREP and MEIS drive PBX to their own binding sites. This outlines the clear individuality of the PREP and MEIS proteins, the former mostly devoted to basic cellular functions, the latter more to developmental functions. *Developmental Dynamics 243:59–75, 2014.* © 2013 Wiley Periodicals, Inc.

Key words: PREP, MEIS, and PBX; vertebrates; TALE proteins

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INTRODUCTION

TALE proteins share the same basic structure (see below) with a conserved atypical 60-residue-long helix-loop-helix homeodomain (HD), which has a three-amino-acids extension between the first and the second helix with respect to the classical homeobox, a feature that gives this class the name TALE (Three Amino acids Loop Extension) (Bürglin, 1997). The primary goal of this review is to provide readers with practical and tabulary information rather than a comprehensive view of the available information.

In the present review, we will focus on the structure and molecular interactions of the TALE class of transcription factors PREP, MEIS, and PBX, in particular from *Homo sapiens, Mus musculus*, and *Danio rerio*. A phylogenetic tree is summarized in Figure 1A showing the evolutionary diversification of the corresponding genes. The extent of conservation in certain regions is very high, but Figure 1 also indicates the diversification of some genes, like the zebrafish *Prep1.1*, which reflects the duplication of the single mammalian *Prep1* gene into *Prep1.1* and *Prep1.2* in zebrafish. It is interesting to note that the *Prep1.2* gene of zebrafish is inducible by retinoic acid (Vaccari et al., 2010). This suggests, therefore, that also in

[1]Fondazione Istituto FIRC di Oncologia Molecolare (IFOM), Milano, Italy
[2]Moscow State University, Moscow, Russia
[3]Cardiovascular Development and Repair Department, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain
Grant sponsor: COST Action; Grant number: BM0805; Grant sponsor: Associazione Italiana Ricerche sul Cancro (AIRC); Grant number: 8929; Grant sponsor: EU FP7 Prepobedia; Grant sponsor: Ministero dell'Università e Ricerca, MERIT; Grant number: MIUR-FIRB RBNE08NKH7; Grant sponsor: Cariplo Foundation; Grant sponsor: Italian Ministry of Health; Grant sponsor: Spanish Ministerio de Economia y Competitividad; Grant numbers: RD06/0010/0008; BFU2009-08331/BMC; Grant sponsor: Russian Ministry of Education and Science; Grant number: 02.740.11.0872; Grant sponsor: Russian Foundation for Basic Research; Grant number: 12-04-01659-a; Grant sponsor: Italian Foundation for Cancer Research (FIRC); Grant sponsor: Ministerio de Economia y Competitividad; Grant sponsor: Pro-CNIC Foundation.
***Correspondence to: Francesco Blasi, IFOM (Istituto Fondazione FIRC di Oncologia Molecolare), Via Adamello 16, 20139 Milano, Italy. E-mail: francesco.blasi@ifom.eu**

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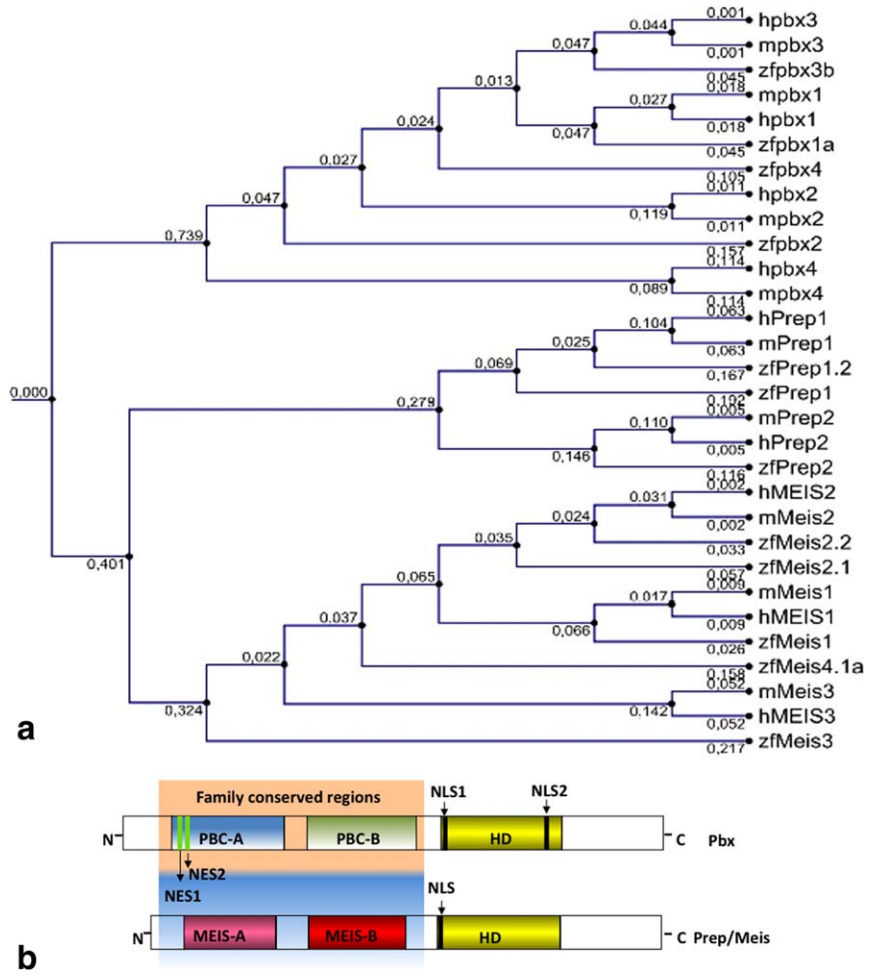

Fig. 1. Phylogenesis and basic structure of the TALE proteins. **A:** Cladogram representing the phylogenetic relationship between the TALE-superfamily members of zebrafish, mouse, and human. The alignment of peptide sequences was performed using "ClustalW" software, the amino acid substitutions per site are indicated in every branch. Individual proteins are identified by the species (*Hs*, *Homo sapiens*; *Mm, Mus musculus*; *Dr, Danio rerio)*. **B:** Basic features of the TALE proteins. The homeodomain (HD) is conserved throughout the whole group. The amino-terminal regions are family specific, since PBC-A and -B domains are found only in the PBX family while MEIS-A and -B in both Prep and Meis subfamilies. The location of the NES and NLS is also shown.

mammals some of the functions of Prep1 may be induced by retinoic acid; this possibility has, however, not yet been explored.

TALE proteins class includes two families, PBC and MEINOX (a contraction of MEIS and KNOX), the latter further sub-divided in PREP and MEIS sub-families. When we use the term MEINOX, we include both MEIS and PREP. A single MEINOX gene (*hth*) is present in *Drosophila* and is frequently thought to be the ancestral MEINOX ortholog. However, other insects like the malaria mosquito, the honey-bee, and the red flour beetle express both *Meis* and *Prep* orthologs. Possibly, therefore, a common *Prep* and *Meis* precursor was present in a common bilaterian ancestor and the *Prep* ortholog has been lost in *Drosophila* (Mukherjee and Bürglin, 2007).

Table 1 summarizes the symbols of the various proteins, their relationship to the single *Drosophila melanogaster* ortholog, and their correspondence in the various species. Notice that in zebrafish, most genes are duplicated: the single mammalian *Prep1, Meis1*, and *Meis2* genes are split into *Prep1.1* and *Prep1.2*, *Meis1* and *Meis4.1*, and *Meis2a* and *Meis2b*. Table 1 also highlights the correspondence of the nomenclature in zebrafish and mammals. For example, the zebrafish equivalent of mammalian *Pbx1* is called Lazarus (*Lzr*) or *Pbx4* (zfin.org).

In mammals, the PBC family includes four PBX proteins, whereas the PREP and MEIS subfamilies include two PREP and three MEIS proteins. PBX is the acronym for Pre-B-cell leukemia homeobox since it was isolated as a fusion protein present in a large fraction of human pre-B Acute Lymphocytic Leukemia (Kamps et al., 1990; Nourse et al., 1990); MEIS is the acronym for Myeloid Ecotropic Integration Site since it was discovered by viral insertion mutagenesis in the mouse (Moskow et al., 1995; Steelman et al., 1997). PREP stands for PBX Regulatory Protein (Berthelsen et al., 1998a) a name that is widely used instead of the initially proposed PKNOX (Pbx/Knotted 1 homeobox), which, however, is still the official acronym of this protein in the data banks. The originally proposed *PKNOX1* acronym for the gene (Chen et al., 1997) stems from the homology with the plants Knotted homeobox family of transcription factors; however, the symbol *PREP1* has gained consensus since *PKNOX* can be confused with a different KNOX family of plants transcription factors (Mukherjee and Bürglin, 2007) while PREP has a distinct and functionally correct connotation. Despite the clear differences underlined in the reports describing their isolation, many authors have considered *PREP* and *MEIS* as members of the same gene family. However, the actual analysis shows that they belong to different sub-families of the MEINOX family (Fognani et al., 2002; Mukherjee and Bürglin, 2007).

Although outside the scope of this review, a brief summary of the function of these proteins, as it appears from genetic studies, is necessary. The first TALE gene to be knocked out in mouse was *Pbx1*, whose deletion gave an embryonic lethal phenotype at E15.5 with hypoplasia or aplasia of several organs and homeotic transformation of elements of the second into elements of the first branchial arch (Selleri et al., 2001). Mice missing the *Pbx3* gene died within one day after birth due to central

TABLE 1. Correspondence of TALE Gene Symbols Between *Danio Rerio, Mus musculus* and *Homo Sapiens*

Drosophila melanogaster ancestor	Vertebrate family	Vertebrate subfamily	*Danio Rerio* proteins	*Mus musculus* proteins	*Homo sapiens* proteins
Hth	MEINOX	Prep (pKnox)	Prep1.1Prep1.2	Prep1	PREP1
Hth	MEINOX	Prep (pKnox)	Prep2	Prep2	PREP2
Hth	MEINOX	Meis	Meis1Meis4.1a	Meis1	MEIS1
Hth	MEINOX	Meis	Meis2.1Meis2.2	Meis2	MEIS2
Hth	MEINOX	Meis	Meis3	Meis3	MEIS3
Exd	PBC	Pbx	Pbx1a	Pbx1	PBX1
Exd	PBC	Pbx	Pbx2	Pbx2	PBX2
Exd	PBC	Pbx	Pbx4 (lazarus)	Pbx3	PBX3
Exd	PBC	Pbx	Pbx3	Pbx3	PBX3
Exd	PBC	Pbx	[a]	Pbx4	PBX4

[a]No *Danio rerio* ortholog of mammalian Pbx4 (Wagner et al., 2001) has been identified. In fact *lazarus,* although called Pbx4 (Pöpperl et al., 2000), is an ortholog of mammalian Pbx3 and its function during embryonic development is correlated to the function of mammalian Pbx1.

hypoventilation (Rhee et al., 2004). The *Pbx2* gene, on the other hand, appears to be dispensable as the *Pbx2* KO mice have no phenotype (Selleri et al., 2004). This suggested that *Pbx* genes can at least in part compensate each other, a hypothesis supported by studies on *Pbx1-Pbx2* double KO mice, which showed a much more severe phenotype than *Pbx1* KO alone (Capellini et al., 2006).

Deletion of the *Meis1* gene also causes an embryonic lethal phenotype, with death at E14.5, deficiency in definitive hematopoietic stem cells generation, vascular and ocular abnormalities (Hisa et al., 2004; Azcoitia et al., 2005; Carramolino et al., 2010). Importantly, *MEIS1* overexpression has an important inducing role in the generation of human and murine leukemias and tumors in general (Moskow et al., 1995; Nakamura et al., 1996; Wang et al., 2006; Wong et al., 2007; Crijins et al., 2007; Grubach et al., 2008; Somervaille et al., 2009).

On the other hand, *Prep1* null mouse embryos show a very severe phenotype with uterine death before E7.5, lack of gastrulation, and apoptosis of the epiblast (Fernandez-Diaz et al., 2010). An hypomorphic *Prep1* mutant ($Prep1^{i/i}$) producing about 2% of the normal mRNA level, has a less severe phenotype, with a leaky E17.5 embryonic lethality, hematopoietic stem cells defects, and vascular anomalies (Ferretti et al., 2006; Di Rosa et al., 2007). Even though the *Meis1* null and $Prep1^{i/i}$phenotypes affect the same organs, *Meis1* and *Prep1* do not interact genetically as double heterozygous mice are normal (Penkov et al., 2013).

Interestingly, comparison of the phenotypes of the Prep-null ($Prep1^{-/-}$, embryonic lethal before E7.5), hypomorphic ($Prep1^{i/i}$, E17.5) and trans-heterozygous ($Prep1^{-/i}$, E12.5) mutants (Rowan et al., 2010), indicates that *Prep1* has multiple critical and essential functions during embryogenesis. In addition, the few adult $Prep1^{i/i}$ mice (25%) that escape embryonic lethality, live a normal-length life but display a variety of phenotypes. Importantly, they develop tumors at high frequency that, together with other properties, defines *Prep1* as a haplo-insufficient tumor suppressor (Longobardi et al., 2010). Further studies have shown that *Prep1* in fact protects cells from DNA damage (Iotti et al., 2011).

ANATOMY OF THE TALE PROTEINS

The comprehensive study on TALE genes by Mukherjee and Bürglin (2007) can be used as a reference for genomic structural features, which therefore will not be discussed in this report. However, some data for human, mouse, and zebrafish PREP, MEIS, and PBX genes/proteins are summarized in Table 2. Chromosome position, gene length, number of exons, length of the major transcript, protein length, and length of the 5′ and 3′ untranslated regions (5′-UTR and 3′-UTR), are indicated.

The overall structural organization of the three TALE protein subfamilies PREP, MEIS, and PBX is similar, containing a DNA-binding homeodomain (HD) towards the carboxy-terminus and two protein–protein interaction domains towards the amino terminus (MEIS-A, B in PREP and MEIS, and PBC-A, -B domains in PBX) (Fig. 1B). The HD is conserved in all these proteins whereas the MEIS-A, B domains are conserved only within the MEINOX and the PBC-A, -B domains only within the PBC family (see below). PREP and MEIS proteins belong to different subfamilies. Indeed, sequence similarities are only found within the homeodomain and in two shared domains, MEIS-A and MEIS-B, whereas within each sub-family there is extensive similarity throughout the entire gene/protein (Fognani et al., 2002; Mukherjee and Bürglin, 2007). In fact, both biochemical and genetic studies have highlighted their differential functions (Berthelsen et al., 1998a; Fognani et al., 2002; Penkov et al., 2013, see below). The PBC-A and -B domains of PBX also display a MEIS-A-like box (Bürglin, 1997), but this feature has so far not received attention although it further indicates the common origin of these protein families.

In mammals, the MEINOX family includes three *MEIS* (*Meis1–3*) genes and two *PREP* (*Prep1—2*) genes. The two highly conserved MEIS-A and

TABLE 2. Gene Structure, Chromosomal Location, Transcript, and Protein Length[a]

Gene	Chromosome	Gene length (kbp)	Transcript length (bp)	Protein length (aa)	5′UTR (bp)	N of exons	3′UTR (bp)	N Isoforms
Hs-PREP1[b]	21	59	5,003	436	188	11	3,498	1
Mm-PREP1	17	41	2,583	436	145	11	1,083	1
Dr-PREP1.1	9	18	2,350	435	22	11	116	3
Dr-PREP1.2	1	17	1,293	431	NK	10	NK	2
Hs-PREP2	11	269	3,730	472	314	13	2,057	1
Mm-PREP2	9	256	3,488	474	223	13	1,840	1
Dr-PREP2	10	19	1,206	401	NK	8	NK	1
Hs-MEIS1	2	137	3,180	415	382	13	1,550	1
Mm-MEIS1	11	138	3178	390	729	13	1,276	2
Dr-MEIS1	13	79	2,258	391	383	14	1,700	2
Hs-MEIS2	15	209	3,687	381	1076	13	1,465	7
Mm-MEIS2	2	203	3,718	401	1026	13	1,486	7
Dr-MEIS2.1	20	21.3	3,104	393	694	14	1,184	1
Dr-MEIS2.2	17	99	3,169	390	544	13	1,511	1
Hs-MEIS3	19	16	2,149	421	447	13	436	1
Mm-MEIS3	7	11	1,781	378	177	13	467	2
Dr-MEIS3	15	22	1,110	369	NK	10	NK	1
Hs-PBX1	1	292	6,635	430	188	9	5,159	2
Mm-PBX1	1	272	1,293	430	NK	9	NK	2
Dr-PBX1A	2	32	1,029	342	NK	7	NK	3
Hs-PBX2	6	5.4	3,213	430	271	9	1,649	1
Mm-PBX2	17	5.1	3,068	430	292	9	1,477	1
Dr-PBX2	16	24	1,212	403	NK	8	NK	1
Hs-PBX3	9	220	2,823	434	67	9	1,450	6
Mm-PBX3	2	200	2,393	434	117	9	972	4
Dr-PBX3B	8	137	1,053	350	NK	7	NK	3
Hs-PBX4	19	57	1,459	374	24	8	290	1
Mm-PBX4	8	40	2,251	378	38	8	1,076	2
Dr-PBX4	3	50	834	278	NK	5	NK	1

[a]The length of the transcript refers to the longest transcript present in the data bank, which is also the most abundant form. Details: the data derived from ENSEMBL database (23.11.2007) except where specified. The length of the genes is counted from the starting point of the transcription. The length of the 5′ UTR may not be not accurate because in most genes the exact starting point of transcription has not been experimentally determined. In many genes also the 3′ UTR has not been established. Hs, *Homo sapiens*; Mm, *Mus musculus*; Dr, *Danio rerio*. NK, not known.
[b]Based on direct determination of the transcription start site (Bernardi et al., 2010).

MEIS-B domains are shown in Figure 1B. The sequences of the regions conserved in MEIS and PREP are shown in Figure 2, i.e., MEIS-A, MEIS-B, and the HD domain. At the C-terminus of the HDs, the amino acids sequence diverges in all proteins.

Mammalian cells contain four *PBX* genes (1–4) that code for six proteins, because of two alternatively spliced isoforms of PBX1 and PBX3. The gene products are extremely well conserved throughout the entire amino acids sequence (Fig. 3). PBX1-3 are about 430 residues long, while PBX4 is shorter, missing the first 78 residues including part of the PBC-A domain and a 30-residue stretch in the C-terminal domain. Starting from the N-terminus, all PBX proteins show high conservation including the homeodomain (HD), identifying the 75-residue-long PBC-A domain, PBC-B of 88, and the HD (Fig. 3). The three domains are very conserved (Table 3 and Fig. 3): PBC-A shows 3–9/75 differences in PBX1-4; PBC-B 2–3/88 differences and the HD 0–-5/60 (Table 3). The shorter PBC-A of PBX4 allows for restricting the MEIS/PREP binding region to 51 residues since this domain is functional in PBX4 (Wagner et al., 2001). Moreover, in PBC-B also the five serine residues involved in the regulation of PBX1 nuclear export (Kilstrup-Nielsen et al., 2003) are conserved, together with the four not yet characterized threonines (see below).

An important feature of TALE proteins is the formation of dimers between MEINOX and PBC proteins, even in the absence of DNA. The domains participating in this interaction are MEIS-A and -B in MEINOX, and PBC-A and -B in PBC. In addition, PBC proteins can also form dimers with the anterior HOX proteins but for this interaction the region containing the HD is indispensable (Fig. 1B). Since PBC proteins can interact with MEINOX and HOX proteins through separate interaction surfaces, ternary MEINOX-PBC-HOX complexes are formed that are able to bind DNA (see below). Finally, the MEIS sub-family of the MEINOX proteins can also directly interact with the posterior HOX proteins, again using a different interaction surface. These properties and the specific domains involved will be discussed below.

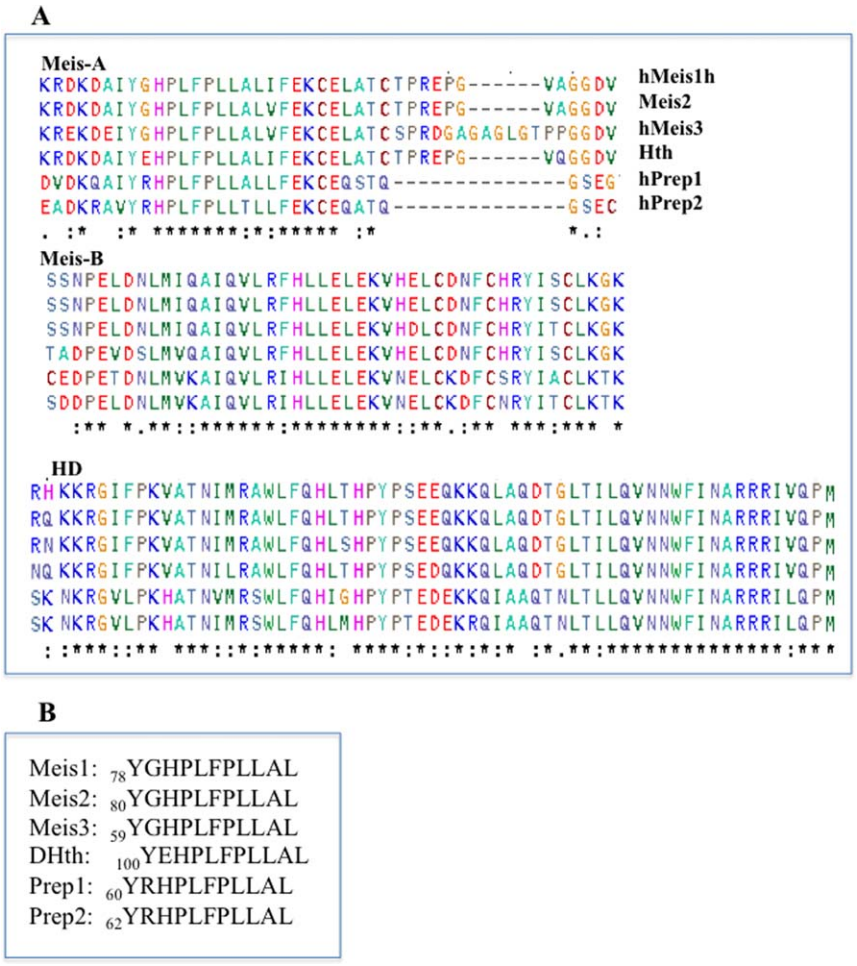

Fig. 2. Conserved domains in human Meis and Prep proteins. **A:** Comparison of the main domains in human Meis1, Meis2, Meis3, Prep1, Prep2, and Drosophila hth proteins. On top the aminoacid sequence of Meis-A domain. The list on the right indicates the corresponding protein. In the middle, the Meis-B domain; at the bottom, the HD domain. In Meis-A domain, the binding sequences for Pbx1, P160, and 4EHP are highlighted in green. Accession numbers are: hMeis1: O00470; hMeis2: O14770; hMeis3: Q99687; Hth: NP_476578; hPrep1: NP_004562; hPrep2: CAD01142.

TALE Proteins Alternative Splicing Isoforms

MEIS genes can form multiple protein isoforms, due to alternative splicing. MEIS1 comes in two major variants of the C-terminus, MEIS1a and MEIS1b, whose differential biochemical function has not been determined (Oulad-Abdelghani et al., 1997). However, MEIS1b has been specifically found to regulate postnatal cardiomyocyte cell cycle exit (Mahmoud et al., 2013). *MEIS1* can also produce HD-less isoforms, which have not been characterized (Crist et al., 2011).

Human and murine *MEIS2* are expressed in many different isoforms (8 in *Homo sapiens*) of various lengths containing essentially all the conserved regions except the isoform "8" that is missing part of the MEIS-A and the isoform "5" lacking part of the HD domain. Meis2b isoform "3" is required for the activity of a PDX1:PBX1b:MEIS2b complex in pancreatic acinar cells involved in the transcriptional activation of the ELA1 enhancer; the complex binds to the enhancer B element and cooperates with the transcription factor 1 complex (PTF1) bound to the enhancer A element (Liu et al., 2001). Probably in complex with PBX1, MEIS2 isoform d is involved in transcriptional activity of KLF4 (Bjerke et al., 2011). In cooperation with a PBX protein (such as PBX2), MEIS isoform "2b" has been proposed to act in the transcriptional activation of EPHA8 in the developing midbrain (Shim et al., 2007). Moreover, MEIS isoform "2b" may regulate myeloid differentiation (Fujino et al., 2001).

Mouse *Prep1* also produces an alternatively spliced isoform (Fernandez-Diaz and Blasi, unpublished results) but again its function has not been investigated. Both mouse and human *PREP1* come with 3′-UTR of two different lengths (unpublished data). Mouse *Prep2* was cloned from an E8.5 embryo cDNA library and shown to have three different isoforms by Northern analysis (Haller et al., 2002). Specific antibodies against the C-terminal and the N-terminal part of Prep2 showed five different bands by immunoblotting. Interestingly, the shorter mRNA form of mouse *Prep2* produces an HD-less protein (Prep2ΔHD). Although the different Prep2 isoforms have a different subcellular localization, there is no clue about their biological roles (Haller et al., 2004). The production of alternatively spliced isoforms by human *PREP2*, cloned from HeLa cells RNA, has not been investigated (Fognani et al., 2002).

Also some *PBX* genes present alternatively spliced protein isoforms, in particular *PBX1* and *PBX3*. PBX1a and 1b, and PBX3a and 3b derive from the same genes but express a protein different in the C-terminus, i.e., after the homeodomain (Monica et al., 1991) (Fig. 4). In *H. sapiens*, the sequence of PBX1b is 100% identical to PBX1a up to residue 333, lacks the subsequent 92 residues, and then contains 18 additional residues in the C-terminal tail, bringing the total length of PBX1b to 348 residues. The same form is also found in the mouse, where PBX1b includes residues 1–333 of PBX1 with 23 additional residues in the C-terminal region, bringing the overall size again to 347 residues. PBX3, tumor-specific, isoforms have been described (Milech et al., 2001).

Functional differences between alternatively spliced forms has been very little addressed in vertebrates. However, a deeper study is available for the Hth gene of *Drosophila melanogaster* (Noro et al., 2006). However, since further, still unpublished,

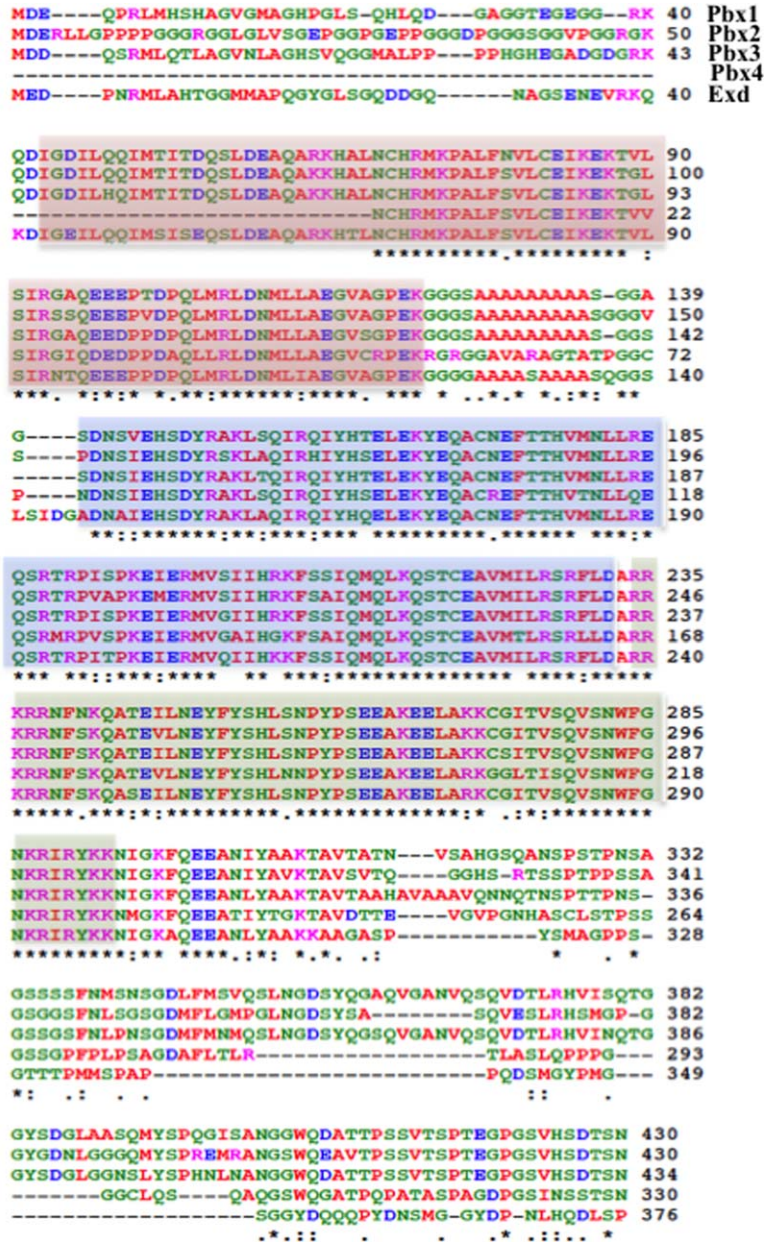

Fig. 3. Sequence comparison of human Pbx proteins and Drosophila Exd. Alignment of full-length proteins was performed using ClustalW2 software. Stars indicate conserved amino acids. Magenta, the PBC-A domain; blue, the PBC-B domain; green, the HD domain Accession numbers are: Pbx1: AAG30941; Pbx2: P40425; Pbx3: P40426; Pbx4: CAC28212; Exd: AAC37182.1

studies are now in progress, we have chosen not to review this issue at this time.

FUNCTIONAL DOMAINS

Both PREP and MEIS proteins (like Hth in *D. melanogaster*) (Johnson et al., 1995) have been shown to interact with PBX, and the interaction is essential for many of their functions. In fact, PREP1 from HeLa cells is purified as a complex with PBX1 and PBX2 (Berthelsen et al., 1998a). This type of interaction requires the N-terminal moiety of PREP1 or MEIS1. Likewise, MEIS can also form similar complexes with PBX (Jacobs et al., 1999).

The sequences required for the interaction with PBX are the MEIS-A and MEIS-B domains that are extremely conserved in all MEIS and PREP sub-family members. The sequence and length of the MEIS-A domain is similar in PREP and MEIS proteins but MEIS3 contains an insert of six residues in the carboxy-terminal half of the MEIS-A domain whose functional relevance is unknown (Fig. 2).

Table 4 summarizes the extent of identity of the three conserved domains of the MEINOX proteins in *H. sapiens, M. musculus*, and *D. rerio*. The extent of identity is very high, with the unique exception of the zebrafish Prep1.1, which is slightly less conserved.

MEIS-A and MEIS-B domains are required for the interaction of PREP/MEIS (and Hth) with PBX (Exd) proteins (Knoepfler et al., 1997; Ryoo et al., 1999; Jacobs et al., 1997; Berthelsen et al., 1998b). MEIS-A and MEIS-B domains from PREP or MEIS are thought to interact with the PBC-A and PBC-B domains of PBX; however, structural studies are still not available. While the requirement for MEIS-A seems absolute for the formation of a PREP1-PBX1 complex, MEIS-B, though structurally similar to MEIS-A, has not yet a totally clear function and may be (in part) dispensable for Pbx binding. In fact, deletion of the MEIS-A domain of PREP1 essentially abolishes the interaction (Berthelsen et al., 1998b).

Formation of a MEIS/PREP-PBX complex does not interfere with DNA binding, in fact it stimulates it and increases the selectivity (Knoepfler et al., 1997; Ryoo et al., 1999; Jacobs et al., 1999; Berthelsen et al., 1998b). When a MEIS/PREP-PBX complex binds DNA, both HD are required as the mutation in a single HD is sufficient to prevent binding. Thus, both PREP and MEIS HDs may have to contact DNA.

In PREP1, a binding site for PBX (and MYBBP1A, see below) has been localized to the sequence ^{64}LFPLLALL^{71} of the MEIS-A domain (Fig. 2) (Diaz et al., 2007) while the overlapping sequence ^{60}YRHPLFPL^{67} is required for binding 4EHP (see below) (Villaescusa et al., 2009). Mutations in residues Y60, L64, L67, and L68 strongly impair not only PBX1 but also MYBBP1a and 4EHP binding (Diaz et al., 2007; Villaescusa et al., 2009). Binding to MYBBP1a is also a feature of MEIS1a (Dardaei and Blasi, unpublished data). Whether MEIS2, MEIS3, and PREP2

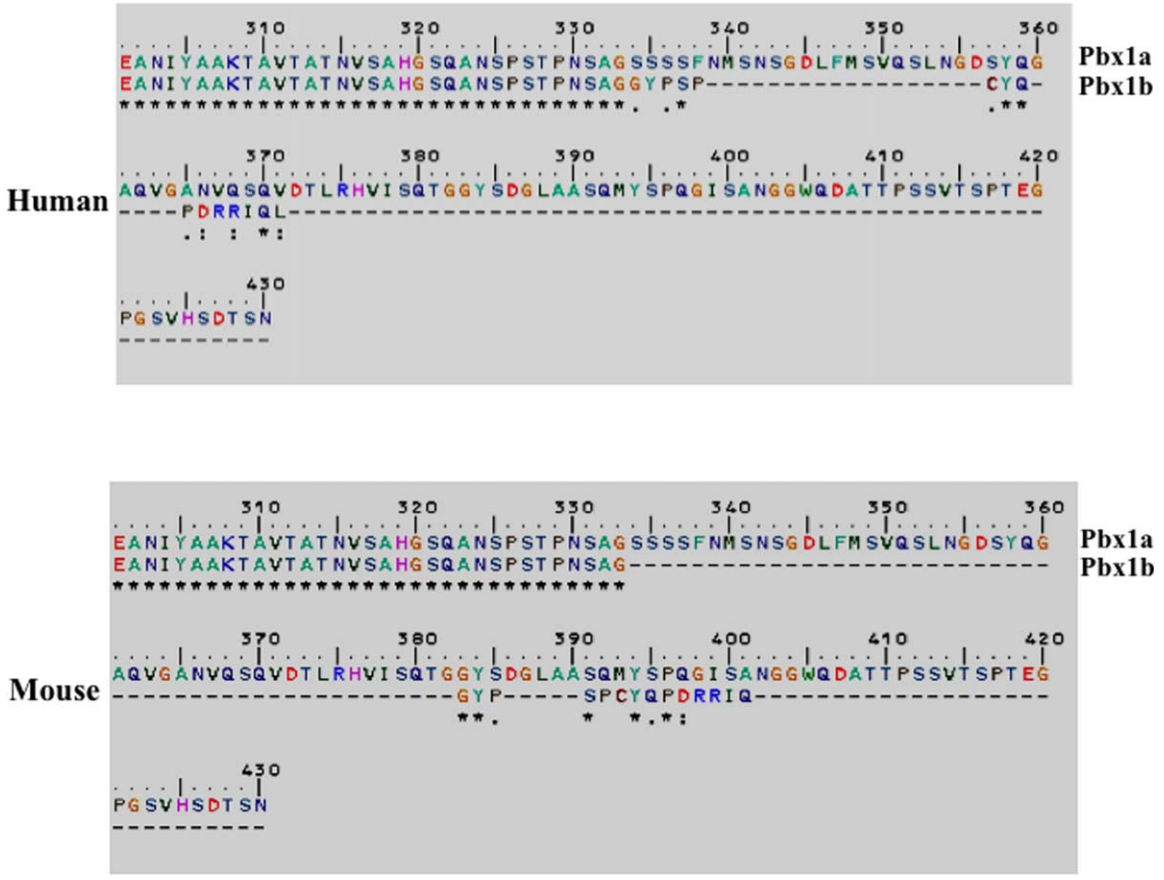

Fig. 4. The a and b isoforms of human and mouse Pbx1. The "a" forms are 430, the "b" forms 358 (*H. sapiens*) and 357 (*M. musculus*) residues long.

also bind MYBBP1a and 4EHP remains to be investigated.

In addition to MEIS-A and MEIS-B, the MEIS subfamily has three additional internally conserved regions of unknown function: MEIS-C (about 30 aa-long) and MEIS-D (20 residues) immediately upstream and downstream of the HD, respectively, and MEIS-N located at the N-terminus (Mukherjee and Bürglin, 2007).

NUCLEAR LOCALIZATION

As transcription factors, TALE proteins must act in the nucleus; however, in some cells/tissues they have a cytoplasmic localization. The nuclear localization of PBX proteins has been mainly studied in Drosophila. Both Exd and Hth have a nuclear localization signal (NLS), but apparently the one on Hth is dispensable (Rieckhoff et al., 1997; Ryoo et al., 1999). Exd and its homologs have two putative NLSs (NLS1 and NLS2) within the homeodomain (Abu-Shaar et al., 1999; Saleh et al., 2000a) (Fig. 1B). The weaker NLS1 is located in the N-terminal arm (amino acids 234–239: RRKRR) while the stronger NLS2 is in helix 3 (residues 285–294: KRIRYKKNI), and is less conserved. Notice that Figure 1B also indicates the NLS for MEIS. This, however, has been marked because of its correspondence with a canonical NLS sequence (KK/R-X-K/R), but has not been experimentally proven. No clear NLS has been found in PREP1 (Berthelsen et al., 1999).

The nuclear localization of Exd/PBX depends on the balance (Stevens and Mann, 2007) between nuclear

TABLE 3. Sequence Characterization of Human Pbx Proteins[a]

Length of protein or protein domain	PBX1a	PBX2	PBX3a	PBX4
Protein	430	430	434	330
PBC-A	75	75	75	51
PBC-B	88	88	88	88
HD	60	60	60	60
% Identity with PBX1	**PBCA**	**PBCB**	**HD**	
Pbx2	69/75	80/88	59/60	
Pbx3a	72/75	86/88	60/60	
Pbx4	66/75	75/88	55/60	

[a]The size of the full-length proteins indicates the longest isoform (like PBX1a and PBX3a) or the most frequent of the known isoforms (in the case of PBX2 and PBX4).

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TABLE 4. Amino Acids Sequence Identity in Human, Mouse, and Dr Meinox Protein Domains

Protein domain	Human /mouse	*D. rerio*/mouse
Prep1 MEIS-A	31/32	26/32[a]
Prep1 MEIS-B	45/45	38/45[a]
Prep1 HD	60/60	57/60[a]
Prep2 MEIS-A	32/32	30/32
Prep2 MEIS-B	45/45	44/45
Prep2 HD	60/60	60/60
Meis1 MEIS-A	40/40	39/40
Meis1 MEIS-B	45/45	45/45
Meis1 HD	60/60	59/60
Meis2 MEIS-A	40/40	39/40[b]
Meis2 MEIS-B	45/45	44/45[b]
Meis2 HD	60/60	60/60[a]
Meis3 MEIS-A	45/46	32/51
Meis3 MEIS-B	44/45	44/45
Meis3 HD	60/60	59/60

[a]The data refer to *D. rerio* R Prep1.1.
[b]For this comparison, the sequence of *D. rerio* Meis2.1 has been used.

import and export, which is mediated by the NLSs and the NES and the nuclear import and export pathway (Stevens and Mann, 2007; Abu-Shaar et al., 1999; Berthelsen et al., 1999; Saleh et al., 2000a). The PBC-A domain of PBX contains a NES-like sequence that, however, is not directly interacting with the exportin but inhibits nuclear localization indirectly, by binding intramolecularly to its own homeodomain, masking the NLSs (Saleh et al., 2000a). Moreover, phosphorylation of serine residues in the PBC-B domain of PBX1 regulates Pbx nuclear localization (Kilstrup-Nielsen et al., 2003). The NES sequence of Exd/PBX (IHKKFSSIQM) is highly conserved among *Drosophila, M. musculus*, and *D. rerio*.

The nuclear localization of PBX is also dependent on its dimerization status. Indeed, in the absence of Hth, Exd remains in the cytoplasm (Abu-Shaar et al., 1999; Jaw et al., 2000). Moreover, even though PREP1 has no functional NLS, mammalian PBX1 requires PREP1 to be stably localized in the nucleus as PREP1 appears to mask the NES from the exportin (Berthelsen et al., 1999).

Also PREP1 nuclear localization requires dimerization with a PBX protein. Interestingly, in the mouse thymus (which expresses almost uniquely PREP1 and PBX2 among the TALE proteins), expression of a mutant PBX1 missing the NLS but still capable of binding PREP1 is capable to completely deplete PREP1 from the nucleus and localize it in the cytoplasm, with functional consequences similar to the absence of PREP1 (Penkov et al., 2005).

PREP AND MEIS INTERACTORS

TALE transcription factors interact with each other and with proteins of other families. The most studied interactions are between MEINOX and PBC, PBC and HOX, and MEIS and HOX proteins. However, other interactors have been discovered. For some of them, the interaction has been validated in vivo and specific phenotypes have been identified.

MEINOX proteins are major interactors of PBX. This interaction involves a well-defined sequence, the MEIS-A domain, whose tertiary structure is however not known yet. Mutations in the sequence outlined in Figure 2B abolish or strongly decrease the binding, as well as prevent the nuclear localization of both MEINOX and PBC proteins (Diaz et al., 2007; Villaescusa et al., 2009). In the case of PREP1, this same sequence has been shown to bind also to MYBBP1A (Myb-binding protein 1A) and 4EHP (eukaryotic translation initiation factor 4E homolog protein) (see below). These proteins in fact compete for binding to PREP1 and affect its transcriptional activity (Diaz et al., 2007).

Dimerization of PBX with PREP or MEIS affects its stability in vivo. While the detailed mechanism is not known, it has been observed that in the absence of PREP1 the stability of PBX1, PBX2 and MYBBP1a is decreased (Longobardi and Blasi, 2003; Diaz et al., 2007; Oriente et al., 2008).

MYBBP1A is a non-DNA-binding transcriptional regulator of PGC1A (Fan et al., 2004). The binding of PREP1 to MYBBP1A increases protein stability, and indeed its concentration in mouse muscle is *Prep1*-dose-dependent, whereas its mRNA level is not. Hence, in the absence of PREP1, MYBBP1A fails to inhibit the transcriptional activity of PGC1A in muscle cells, increasing insulin sensitivity (Oriente et al., 2008). MYBBP1A acts as a tumor suppressor and its down-regulation in HeLa cells leads to drastic mitotic abnormalities, a block in G2/M, and increased sensitivity to oncogenic transformation (Mori et al., 2012).

4EHP is a cytoplasmic protein that in mouse oocytes interacts with the MEIS-A domain of cytoplasmic PREP1 (Villaescusa et al., 2009). The interaction of 4EHP with homeobox-containing proteins is not unique to PREP1 and might be conserved from Drosophila to man. Indeed, in *Drosophila* the homeodomain factor Bicoid (Bcd) interacts with 4EHP to repress translation of Caudal (*cdx*) mRNA and to drive *Drosophila* embryo development (Cho et al., 2005). In agreement with the presence of a Bcd-like 4EHP-binding sequence in PREP1, cytoplasmic PREP1 binds both 4EHP and the 3′-UTR of at least two *Hox* mRNAs (HoxB4 and HoxB8), inhibiting their translation, a molecular function that may be important in the maturation of oocytes (Villaescusa et al., 2009).

Other PREP1 interactors include (Table 5) SMAD2-4 (Bailey et al., 2004) and PAX6 (Mikkola et al., 2001), the ribosomal protein S3a/Fte, the splicing cofactors PSF and p54/NRB/NonO, and the cytoskeletal proteins beta-actin and myosin NMMHCIIa (Diaz et al., 2007; Ferrai

TABLE 5. PREP1 and MEIS1 Interactors

PREP1 interactor	Nuclear/ cytoplasmic	Interacting site		Reference
		PREP1 motif[a]	Interactor motif	
PBX	N	63LFP**L**LAL**L**70	PBCA	Berthelsen et al., 1998[b] Diaz et al., 2007
Smads 2,3,4	N			Bailey et al., 2004
Pax6	N			Mikkola et al., 2001
p160MBP	N	63LFP**L**LAL**L**70	LXXLL domains, N terminus	Diaz et al., 2007
4EHP	N	58**Y**RHPLFP**LL**66		Villaescusa et al., 2009
RFX3	(2HS)			Ravasi et al., 2010
ZBTB3	(2HS)			Ravasi et al., 2010
TLX1	(2HS)			Ravasi et al., 2010
β ACTIN[b]	N			Diaz et al., 2007; Ferrai et al., 2009
NMMHCIIA[b]	N			Diaz et al., 2007
Oct1	N	HD		Rave-Harel et al., 2004
p54/Nrb/NonO[b]	N			Ferrai et al., 2009
PSF[b]	N			Diaz et al., 2007
MEIS1 interactor	**Nuclear /cytoplasmic**	**Interacting site**		**Reference**
		MEIS1 motif	**Interactor motif**	
PBX	N	aa 64-202	PBCA	Chang et al., 1996
HOX9-13	N	C terminal		Williams et al., 2005
PKA	C	C terminal		Huang et al., 2005
TLX1	(2HS)[c]			Ravasi et al., 2010
TLX2	(2HS)[c]			Ravasi et al., 2010
EMX2	(2HS)[c]			Ravasi et al., 2010

[a]In bold are the residues whose mutation into Ala drastically decrease binding (Diaz et al., 2007; Villaescusa et al., 2009).
[b]In these proteins it is not known whether they interact with Prep/Meis or Pbx.
[c]Mammalian 2-Hybrid system.

et al., 2009). Although no structural information is available, some of these factors may interact with PREP1 through the PBX moiety of a PREP1-PBX1 complex (Ferrai et al., 2009; Naum-Ongania et al., unpublished data). The interaction of PREP1 with beta-actin is essential for the retinoic-acid induction of *HOX* genes expression in human N-TERA2 terato-carcinoma cells. In this context, beta-actin is part of a larger complex containing RNA polymerase II, PREP1, PBX1, the actin polymerizing agent N-WASP, and the splicing cofactors PSF and p54/NRB/NonO. This complex, in fact, allows the polymerization of nuclear actin and its binding to the enhancer of at least the *HOXB2* gene, and is required for the initiation of colinear transcription of the *HOXB* genes (Ferrai et al., 2009). The molecular details for the requirement of the polymerized nuclear actin in *HOX* gene expression are, in fact, not known.

Also Oct1 can interact with Prep1 in pull-down experiments in vitro. In this case, it appears that the HD domain is essential for the interaction (Table 5). This finding is rather curious as Oct1 appears to be an interactor of Pbx1 in vivo (Rave-Harel et al., 2004) (see Table 6).

The carboxy-termini of MEIS and PREP are totally divergent. In the case of MEIS, the carboxy terminus is the site for further direct interactions and was previously called transactivation region, i.e., a site for interaction with proteins that will induce activation or repression of target gene expression. For example, MEIS1 C-terminus is required to directly interact with posterior HOX11-13 proteins, a property unique to the MEIS members of the MEINOX family. For this interaction, MEIS uses carboxy-terminal sequences (18 residues in MEIS1 and 93 residues in MEIS2) (Williams et al., 2005). Moreover, MEIS can also bind PBX-HOX complexes (in particular those involving HOXD4 and HOXD5) without directly contacting DNA, again a feature requiring MEIS carboxy-terminal sequences (Shanmugan et al., 1999).

MEIS1 C-terminus harbors transcriptional activation domains that respond to chromatin structure and signaling pathways and that regulate transactivation. Indeed, four regions of the carboxyterminal 56 residues, required for transcriptional activation, are responsive to the HDAC inhibitor TSA and to CBP-dependent Protein Kinase-A. Mutations in all four sites eliminated the response to TSA and to protein kinase-A. C-terminal deletions impair transactivation but do not disturb DNA binding or MEIS-A-mediated formation of HOX or PBX complexes (Huang et al., 2005). Likewise, it has been shown that the 49 C-terminal residues of MEIS1 contain two domains responsible for leukemia induction and *HOXA* gene activation (Mamo et al., 2006).

Not much is known of the carboxy-terminal domains of PREP1 and PREP2 that are not highly conserved. Indeed, addition of the carboxyterminal domain of MEIS1 to the full-length PREP1 protein transforms it from a tumor suppressor into an oncogene; intriguingly, the same result is

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TABLE 6. Pbx Interactors

Interactor	Notes	Reacting site in PBX	Reacting site in the interactor	Reference
Hox 1–10	Clustered homeodomain transcription factors	HD, C terminal	Hexapeptide (usually YPWMX),	Piper et al., 1999
Prep1	Pbx regulating protein-1 (pKnox1)	PBC-A, N terminal	63LFPLLALL70	Berthelsen et al., 1998[b] Diaz et al., 2007?
Prep2	Pbx regulating protein-2 (pKnox2)	PBC-A, N terminal	HR1, N terminal	Fognani et al., 2002
Meis1	Myeloid Ecotropic Insertion site-1	N terminal (aa 1–88)	N terminal (aa 30–60)	Chang et al., 1996
MyoD and bHLH proteins (Myf5, E2a, Myogenin, Mrf-4)	Necessary for induction of muscle differentiation.	HD, C terminal	CL-X-W motif	Knoepfler et al., 1997; Berkes et al., 2004; Maves et al., 2007
Pdx1	Forms ternary complex with Pbx and Prep1/Meis1. *Pdx1*$^{+/-}$ *Pbx1*$^{+/-}$ have insulin resistance and a pre-diabetic phenotype .	HD, C terminal	Hexapeptide (FPWMK), N terminal	Peers et al., 1995; Kim et al., 2002
NMHCA (non-muscle heavy chain Myosin IIa)	Involved in Pbx1 retention in the cytoplasm	PBC-B	Coiled-coil tail (aa 161–177)	Huang et al., 2003 Diaz et al., 2007
Hox11	Same interaction as for clustered Hox	HD, C terminal	Hexapeptide (FPWME), N terminal	Allen et al., 2000; Brendolan et al., 2004
PbxIP1 (PBX1-interacting Protein1)	Inhibits the formation of the Pbx-Hox complexes on DNA	HD (aa 160–230)		Abramovich et al., 2000
ZF-Pip (Zinc Finger PBX1 Interacting Protein)	Prevents HOXA9-Pbx binding to DNA	aa 215–241		Laurent et al., 2007
Rnx	Forms a DNA binding complex with Pbx3	HD, C terminal		Rhee et al., 2004
Protein Kinase A	Phosphorylate-specific serines in the PBC-B domain	PBC-B, N terminal		Kilstrup-Nielsen et al., 2003
HDAC1 (histone deacetylase 1)	HDACs recruits to Pbx a corepressor complex containing N-CoR/SMRT and mSIN3B	N terminal (aa 89–172)		Saleh et al., 2000[b]
Oct-1	Coactivator of Pbx1b	C terminal		Rave-Harel et al., 2004
FoxC1	Transcription factor important for neural crest–derived tissue and mesenchymal mesoderm cells. Inhibits Pbx1a transactivation.			Berry et al., 2005
P54/Nrb (p54/NonO)	Accessory splicing factor playing a role in actin-dependent transcription-regulating role of Hox	N terminal		Ferrai et al., 2009; Ongania et al. (unpublished data)
NR3C1 (glucocorticoid receptor)	2HS[b]			Ravasi et al., 2010

TABLE 6. *Continued*

Interactor	Notes	Reacting site in PBX	Reacting site in the interactor	Reference
Pax6	2HS[b]			Mikkola et al., 2000
ZNHit3 (Zinc Finger, HIT-type containing 3)	2HS. HNF-4a coactivator; myosin head domain containing 1; thyroid hormone receptor interactor 3[b]			Ravasi et al., 2010
ZDHHC7 (zinc finger, DHHC domain containing 7)	2HS. GABA-A receptor-associated membrane protein 2; palmitoyltransferase[b]			Ravasi et al., 2010
Smads 2, 3, 4	Intracellular mediators of TGFbeta signaling.			Bailey et al., 2004

[a]Hox A9 and Hox 10 do not have a classical YPWMX hexapeptide.
[b]2HS: Mammalian 2 hybrid system.

not obtained by substitution of the PREP1 C-terminus with that of Meis1 (Bisaillon et al., 2011).

Other uncharacterized interactors of MEIS are LOBE (EPOLM), KLF4, and TLX1 (HOX11). In *Drosophila*, Hth is involved in defining the boundary between the eye and the head cuticle on the ventral margin. In this function, Hth interaction with Lobe, homolog of human EPOLM (epilepsy, occipitotemporal lobe, and migraine with aura) through the MEIS-A domain is required, while the HD is not (Singh et al., 2011). Kruppel-like factor KLF4 is implicated in tumorigenesis and maintaining stem cell pluripotency. PBX1 and MEIS2 homeodomain proteins interact with KLF4 and are recruited to DNA elements comprising a KLF4 site or GC box, with adjacent MEIS and PBX sites (Bjerke et al., 2011). The interaction details are not known. Aberrant expression of the TLX1 (aka HOX11) proto-oncogene is associated with a significant subset of T-cell acute lymphoblastic leukemias (T-ALL). TLX1 and MEIS proteins both interact and are co-expressed in T-ALL (Milech et al., 2010). The details of the interaction are not known but it has also been confirmed by mammalian 2-Hybrid analysis, in which both MEIS1 and PREP1 were identified as TLX1 interactors (Ravasi et al., 2010). This interaction is particularly interesting since PBX1 is required in mouse spleen formation where it is involved, among others, in the expression of TLX1, together with a MEINOX protein (Brendolan et al., 2005). The MEINOX protein was suggested to be Prep1, but maybe the newly discovered interaction with Meis1 will bring to light subtle regulatory features, i.e., the possibility that both PREP1-PBX1-TLX1 and MEIS1-PBX1-TLX1 complexes regulate *TLX* gene expression, possibly in different directions or in different cells.

A recent analysis using mammalian 2Hybrid System has identified, in addition to the classical ones, further novel interactions (Table 5) of PREP and MEIS proteins (Ravasi et al., 2010).

PBX INTERACTORS

In the presence of a HOX-PBX-responsive sequences, PBX and most

anterior HOX1-10 proteins form complexes that strongly increase the DNA-binding activity and selectivity of HOX proteins (Chan et al., 1994; Johnson et al., 1995; Merabet et al., 2009). The X-ray structure of DNA-bound PBX1-HOXb1 and PBX1-HOXA9 dimeric homeodomains (Gehring et al., 1994; Passner et al., 1999; Piper et al., 1999; LaRonde-LeBlanc and Wolberger, 2003) has revealed that each HD binds one half of an octameric DNA sequence, and that the third helix of each HD enters perpendicularly into the main groove of the DNA double helix. HOX proteins contribute to stabilize this interaction in most cases through a short amino acids sequence (the tryptophan-containing hexapeptide) located N-terminally of the HD; in some *Drosophila* Hox proteins and C-terminally of the HD, a UbdA motif is also involved in the interaction with Exd. PBX proteins, on the other hand, provide HOX-interacting motifs located likewise N-terminally (Chang et al., 1996) and C-terminally (Piper et al., 1999; LaRonde-LeBlanc and Wolberger, 2003). The DNA-bound complex hence forms a structure that embraces the DNA double helix. The DNA-sequence specificity of the PBX-HOX complex is given by the binding of the PBX and HOX moieties to the 5′ and 3′ tetranucleotide of an octanucleotidic sequence of the TGATTXXT type (Gehring et al., 1994; Piper et al., 1999). The sequence divergence of the HOX proteins HDs and of their target sequence explains how they have evolved to perform more recent unique fuctions while keeping common ancestral functions (Sambrani et al., 2013; Hudry et al., 2012; Saadaoui et al., 2011; Slattery et al., 2011a; Lelli et al., 2011).

Full-length PBX1, or any fragment of it, is unable on its own to modulate transcription. However, the 39–232 residues region specifically represses transcription induced by the transactivation domain of the SP1 transcription factor, but not of VP16 or p53. C-terminal sequences present in the PBX1a (but not PBX1b) isoform (see above) block this repressor function (repression domain), the core of which may be a sequence of nine contiguous alanine residues. Interestingly, repression does not require the HD, implying that it is exerted via other proteins and not by competing for target DNA (Knoepfler et al. 1996; Lu and Kamps, 1997).

In complex with HOXB1, PBX1 undergoes a switch from repressor to activator of transcription, upon inhibition of histone deacetylases. Indeed, a region in the amino-terminus of PBX1 (residues 89–172) recruits the repressing HDAC-1 or -3-mSIN3B-N-CoR/SMRT complex, while the HOX moiety recruits the CREB co-activator protein (CBP) (Saleh et al., 2000b). Interestingly, this region overlaps also parts of the PBC-A and PBC-B domains. Whether this entails an effect on the interaction with Prep or Meis proteins is not known.

PBX can form ternary complexes with both HOX and PREP/MEIS (Jacobs et al., 1999; Ryoo and Mann, 1999; Ferretti et al., 2000) because different interaction surfaces of PBX are required (the PBC-A domain for PREP/MEIS and the HD for Hox). Not much is known of the structure of the ternary complexes. In order to bind as a trimer, PREP/MEIS, PBX, and HOX select a DNA sequence that can accommodate all three HDs. In vitro experiments have shown the requirement for both an octanucleotide of the HOX-PBX-responsive element-type, and of a hexanucleotide like TGACAG. These sequences are required and functional in responding to the correct cues in vivo (Jacobs et al., 1999; Ryoo et al., 1999; Ferretti et al., 2000, 2005). However, in the case of MEIS, the TGACAG sequence might not be always necessary since MEIS binding to PBX may stabilize the complex without directly contacting the DNA (Shanmugan et al., 1999).

Besides the PBX-MEINOX interactions, many other proteins have been shown to interact with PBX, but their interaction surface is much less defined (Table 6).

DNA SEQUENCE SELECTIVITY AND TARGET GENES SELECTION IN VIVO

While a fair amount of knowledge has been accumulated on the genetics and biochemistry of the TALE proteins, the complete picture of their in vivo interactions is still far from complete. Due also to the numerous and redundant members of the families, the definition in vivo of the protein–DNA interactions, the proteins distribution on the genome, its variation in different cell lineages, and the functional outcome of these interactions, are still unanswered questions. Other questions are: is there a preference between PREP and MEIS in forming dimers with PBX and do these recognize the same genes and DNA sequences? While in some cells only (or mostly) one member of a family/subfamily is expressed (for example Prep1 and Pbx2 in mouse thymocytes), in other cells many and even all genes of the families are expressed. Since the sequence of the HD is totally conserved within a subfamily, which of the PREP, MEIS, or PBX proteins is bound to a specific gene? The answer to this question is important since, for example, in the analysis of a specific KO mouse we do not know whether the binding sites on DNA remain free or are occupied by other orthologs. Recent data have allowed a step forward in understanding the complex in vivo biochemistry of this network of transcription factors. Finally, an in-depth analysis of the differences in binding sites between *Drosophila* in which a single MEINOX gene is present (*hth*) and mammals, would likely contribute to put the mammalian data into a simpler but functionally significant frame.

The Target Genes and Recognition Sites

ChIPseq analysis (Chromatin Immunoprecipitation followed by parallel sequencing of the precipitated DNA) for Prep, Meis, and Pbx1 proteins has recently been performed on the whole trunk tissues of E10.5 mouse embryos (Penkov et al., 2013) identifying several thousand genomic sites (peaks) for each transcription factor. Only a fraction of the sites were bound by more than one factor, and in these cases they represented DNA sequences bound by one specific dimer. This analysis has allowed us to draw some general rules that are summarized in Table 7. Since the antibodies used can recognize both Meis1 and Meis2, these rules apply to the binding of

TABLE 7. General Rules Drawn From the Prep1, Meis1+2 and Pbx1 ChIPseq in E10.5 Embryo Trunks[a]

	Prep1	Meis	Pbx1
Number of binding sites[b]			
Functional Preference	Promoter	Intra and intergenic promoters and enhancers	Depends on Prep or Meis
Major partner(s)	Pbx1	Pbx1 and Hox	Prep1, Meis1+2 and Hox
% overlaps with Meis	5.58	NA	12.6
% overlaps with Prep1	NA	7.17	19
Consensus DNA sequence	Decanucleotide	Octanucleotide and hexanucleotide	Undefined when alone. Decanucleotide (Prep1–Pbx1) octanucleotide and hexanucleotide (Meis1+2-Pbx1).
% overlap with HoxC9[c]	2.9	11	4.4
% overlap with HoxA2[c]	3.9	15.3	6.0
Overlap of thymus and embryos binding sites[d]	98	NA	43

[a]Based on Penkov et al. (2013).
[b]Based on a P value of $10<10^{-6}$.
[c]The data on HoxC9 and HoxA2have been obtained from specific tissues whereas those of Prep1, Meis 1 and Pbx1 have been obtained in the whole embryo. Therefore, the overlaps must not be considered absolute as they may exist only in certain tissues and not in others. However, the basic rule derived, i.e., that many Meis-binding sites are in fact Hox-binding sites, remains very likely.
[d]Numbers represent the degree of overlap of thymocytes Prep1 and Pbx2 binding sites with those of Prep1 and Pbx2 in total E10.5 embryo trunk. The data are based on a ChIP-on-ChIP analysis with anti-Prep1 and anti-Pbx2 antibodies of mouse thymocytes in which Prep1 and Pbx2 represent the largely predominant form of TALE protein expressed.

both proteins. Likewise, the anti-Prep1 antibody recognizes in part also Prep2 and hence the same rules may also apply to Prep2.

Prep1-Pbx1 and Meis1/2-Pbx1 DNA-binding peaks frequently overlap whereas binding sites uniquely bound by Meis1/2 or Prep1 very rarely overlap. Alignment of the peaks with the mouse genome sequence showed that Prep1 largely prefers promoters while Meis1 binds preferentially to intra- and intergenic sites, which indicates a different mechanism of action. Moreover, Prep1 appears to be the main partner of Pbx1 as the number of Prep1-Pbx1 peaks is about three-fold more than those for Meis1/2-Pbx1.

ChIPseq analysis allows the identification of the DNA sequences bound by a specific transcription factor. This has revealed that in the mouse embryo the peaks bound exclusively by Pbx1 do not have a very strict DNA-binding consensus, whereas those bound by dimers with Prep1 or Meis1 do (see below) (Penkov et al., 2013). Since Pbx1 may have additional DNA-binding interactors, the absence of a binding site consensus may also depend on dimerization with others partners that, as Prep1 and Meis1, may direct Pbx1 to their specific binding site, a possibility that has not yet been explored.

Prep1 and Meis1-2 select the same specific DNA sequences both when binding alone and when in combination with Pbx1 (Penkov et al., 2013). Prep1 and Prep1-Pbx1 bind preferentially a decameric consensus with a general structure TGAXTGACAG, as expected (Knoepfler et al., 1997), whereas Meis1-Pbx1 binds mostly both an octameric TGATTGXX and an hexameric TGACAG sequence. Hence, the DNA sequence selectivity resides in Prep1 or Meis1 and not in Pbx1. This is new, since all three such sequences were previously considered equally likely to be bound by either Prep1 and Meis1. In fact, the list of genes targeted by Prep1 and Meis1 is quite different and can be grouped in different Gene Ontology categories: Meis1–2 targets preferentially embryonic development genes whereas Prep1 targets mainly basic cellular functions. Despite the low level of overlap and the clearly different and separate functional categories, Prep and Meis still show a low level coordination, for example, in the regulation of expression of *Hox* genes (Penkov et al., 2013).

The binding sites identified by the above study indeed confirm previously recognized regulatory regions. Meis1/2, Pbx1, and Prep1 bind several times in the *Hox* clusters often together, suggesting an important degree of cross-talk. Meis peaks are the most abundant, being most frequent in the HoxA and least frequent in the HoxC cluster. Pbx1 shows less binding sites and Prep1 the lowest number of binding sites among the three factors. In all cases Pbx1, Meis and Prep1 peaks coincide. Interestingly, all peaks are concentrated in the 1 to 9 paralog region, subdividing the Hox clusters in Meis/Pbx/Prep-interactive and Meis/Pbx/Prep-non-interactive regions. Previous biochemical and genetic studies had identified 6 TALE protein-bound regions as important regulatory sites in the Hox clusters, often in cooperation with HoxB1 and functional in auto- and cross-regulatory interactions (Gould et al., 1997; Jacobs et al., 1999; Lampe et al., 2008; Manzanares et al., 2001; Popperl et al., 1995; Tumpel et al., 2007). Despite that those studies were carried out at a different developmental stage and in specific tissues, 4 out of the 6 regions were found to coincide with the described regions (Penkov et al., 2013).

One other important novel finding obtained in such a study relates to the

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octameric TGATTGXX sequence that is present in a large fraction of the peaks bound by Meis1 (exclusively or in combination with Pbx1). Interestingly, these peaks identify a consensus sequence previously thought to bind Pbx-Hox (Piper et al., 1999). This suggests that Hox are major partners of Meis1 in addition to Pbx1. Indeed, analysis of the recently published ChIP seq analysis for Hoxa2 and Hoxc9 (Jung et al., 2010; Donaldson et al., 2012) reveals that a rather large percent of these binding sites overlaps with Meis1 peaks, and that this is more frequent than with Prep1 or Pbx1 (Penkov et al., 2013). Therefore, a large fraction of the Meis peaks corresponds in fact to Hox target genes. The fact that Hox peaks' identification was carried out in different tissues does not affect this conclusion.

In agreement with the above finding, cytological analysis of genome-wide chromosomal binding sites of *Drosophila* Hth (Cohen and Salzberg, 2008), and ChIP-chip analysis of Ubx and Hth-bound regions in the haltere and T3 leg imaginal discs (Slattery et al., 2011b) has revealed a remarkable amount of tissue-specificity of binding and of tissue-specific overlap. Unfortunately, data on the co-binding of Exd are still outstanding.

The amino acids sequence of the HDs within the various sub-families is nearly identical; hence, one might expect that in the absence of a specific TALE protein another one of the same sub-family would take its place. For example, in the absence of Pbx1 a different Pbx isoform might be bound to the Pbx1 sites. Indeed, a ChIP-on-chip analysis of mouse thymocytes (which express almost exclusively Pbx2 and Prep1 among all TALE proteins) shows that 90% of the Prep1 sites are co-occupied by Pbx2 and that these overlap with the sites bound by Pbx1 and Prep1 in the E10.5 embryo trunk ChIPseq. Thus, Pbx2 can substitute for Pbx1 (Penkov et al., 2013), a property that might be shared by Pbx3 and Pbx4 since their amino acids sequence in the HD is identical (Fig. 3).

The above rules were drawn from data obtained in the entire E10.5 embryo trunk (Penkov et al., 2013), which represents a mixture of a large variety of differentiated cells, and hence of the binding sites occupied in the various cells that compose the embryo at that specific stage. The results may be different in different types of cells, for example stem cells, or at different embryonic times or in the adult. However, the rules themselves are not likely to be susceptible to changes.

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