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REVIEW

Forkhead Transcription Factors: Key Players in Development and Metabolism

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INTRODUCTION

Transcription factors are modular proteins where distinct functions, such as DNA binding, *trans*-activation or *trans*-repression, are often contained within separable domains. DNA-binding domains tend to be particularly well conserved and can be used as the basis for a classification that reflects phylogenetic relationships. Transcription factors with the same basic design in their interaction with DNA also tend to be related in function and to share properties such as the ability to heterodimerize or to convey certain intracellular signals.

Forkhead proteins are not among the largest transcription factor families, but display a remarkable functional diversity and are involved in a wide variety of biological processes. The name derives from two spiked-head structures in embryos of the *Drosophila fork head* mutant, which are defective in formation of the anterior and posterior gut (Weigel *et al.*, 1989). With the discovery in 1990 of a 110-amino-acid DNA binding domain that was almost perfectly conserved between FORK HEAD and the mammalian HNF-3 transcription factors, it became clear that this motif defined a novel transcription factor family (Weigel and Jackle, 1990). A comprehensive review on forkhead genes has been published by Kaufmann and Knochel (1996).

EVOLUTION OF THE FORKHEAD GENE FAMILY

The decade that has passed since the discovery of the first members has seen the identification of many forkhead genes in a variety of eukaryotic organisms, and with the recent completion of several genome sequencing projects, it is now possible to make a preliminary assessment of the size and distribution of this gene family. Forkhead genes have so far only been found in opisthokont organisms (animals + fungi), including several species of ascomycetic fungi and a wide variety of metazoans. Their absence in the *Arabidopsis* genome and failure to identify forkhead genes in any protist, support the view that this gene family is found exclusively in animals and fungi. Thus, the distribution of forkhead genes lends further support to opisthokonts as a well-defined evolutionary supergroup (Baldauf, 1999). *Mycetozoa (Myxozoa)* have been classified as fungi or protists and more recently as metazoans (Siddall *et al.*, 1995; Smothers *et al.*, 1994). However, the apparent lack of forkhead genes in *Dictyostelium* supports the recent reclassification of *Mycetozoa* as a separate clade close to, but distinct from, opisthokonts (Baldauf *et al.*, 2000). The involvement of forkhead proteins in many morphogenetic processes suggests that increasing complexity in body plan may have been a driving force behind the expansion of the forkhead gene family. Among the organisms for which the genome sequences are completed, or nearly so, there is indeed a correlation between anatomical complexity and forkhead gene number: 4 in *Saccharomyces* and *Schizosaccharomyces*, 15 in *Caenorhabditis*, 20 in *Drosophila*, and 39 in *Homo*.

NOMENCLATURE

In 2000, the nomenclature of chordate forkhead transcription factors was revised (Kaestner *et al.*, 2000). The new nomenclature, which uses Fox (for "Forkhead box") as the root symbol, ensures that the same name is used for orthologous genes in different species and reflects phylogenetic relationships by including a letter that indicates subfamily. Within a subfamily, each gene is identified by a number (e.g., *FoxF2*), the typography follows the conventions used in each species (*FOXF2* in *Homo*, *Foxf2* in *Mus*, and *FoxF2* in all others), and proteins are distinguished from genes by the use of roman type (e.g., FoxF2). New and old names, GenBank Accession Numbers, a phylogenetic tree, and other useful information can be found at the Web site http://www.biology.pomona.edu/fox.html. The most commonly used synonymes for human, mouse, and rat forkhead genes are listed in Table 1.

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TABLE 1
The More Commonly Used Synonyms of Human, Mouse, and Rat *Fox* Names

FoxA1	HNF3α
FoxA2	HNF3β
FoxA3	HNF3γ
FoxB1	Fkh5
FoxB2	Fkh4
FoxC1	FREAC-3, FKHL7, Mf1, Fkh1
FoxC2	Mfh1
FoxD1	FREAC-4, BF2
FoxD2	FREAC-9, Mf2
FoxD3	HFH2, Genesis
FoxD4	FREAC-5, Fkh2, HFH-6
FoxE1	FKHL15, TTF2
FoxE2	HFKH4
FoxE3	FREAC-8
FoxF1	FREAC-1, HFH-8
FoxF2	FREAC-2, Lun
FoxG1	BF-1
FoxH1	FAST1
FoxI1	FREAC-6, HFH-3, Fkh10
FoxJ1	HFH4
FoxK1	ILF, MNF
FoxL1	FREAC-7, Fkh6
FoxM1	Trident, HFH-11, INS1
FoxN1	Whn
FoxN2	HTLF
FoxO1	FKHR
FoxO3	FKHRL1
FoxO4	AFX1
FoxP1	QRF1
FoxQ1	HFH-1, HFH1L

Note. For a comprehensive list, see http://www.biology.pomona.edu/fox.html.

THE FORKHEAD DOMAIN

3D Structure

Burley and co-workers (Clark *et al.*, 1993) used X-ray crystallography to work out the first 3D structure of a forkhead domain (FoxA3) bound to a DNA target. They compared the fold with the shape of a butterfly and coined the term "winged helix" to describe the structure, which has a helix–turn–helix core of three α-helices, flanked by two loops, or "wings" (Fig. 1). "Winged helix" proteins are often used synonymously with forkhead proteins (Lai *et al.*, 1993). However, several phylogenetically unrelated proteins have similar 3D structure and are referred to as winged helix proteins in the literature as well; e.g., MotA from bacteriophage T4, LexA from *Echerichia coli*, E2F, DP, and RFX transcription factors as well as double-stranded RNA adenosine deaminase, ADAR1 (reviewed by Gajiwala and Burley, 2000). Care should be taken to distinguish between these topologically similar proteins of diverse evolutionary origin and forkhead proteins, which form a clearly defined, monophyletic group.

A large proportion of the amino acids in the forkhead domain are invariant or highly conserved (Fig. 1), which implies that there is only limited variation in 3D structure and mode of DNA recognition within the forkhead family. This has been confirmed by NMR structural analysis of the DNA binding domains of three additional forkhead proteins: FOXC2, Foxd3, and FOXO4 (Jin *et al.*, 1999; Marsden *et al.*, 1998; van Dongen *et al.*, 2000; Weigelt *et al.*, 2001). While Clark *et al.* (1993) identified three α-helices in FoxA3, the NMR structures all show a short fourth helix in the loop between helix 2 and 3. However, Weigelt *et al.* (2001), point out that the backbone fold in this region is nearly identical in all four structures (including FoxA3) and inclusion or omission of a fourth helix mostly reflects differences in interpretation. A 5-amino-acid insertion between helix 2 and 3 found in the FoxO subfamily adds a small extra loop, but has surprisingly little effect on the overall structure (Weigelt *et al.*, 2001). Binding to a DNA target site appears to cause only minor structural changes in the forkhead domain (Jin *et al.*, 1999), whereas circular permutation data indicate that a substantial bend is induced in the DNA (Pierrou *et al.*, 1994).

The structural basis for differences in sequence specificity between forkhead proteins remains elusive. Analysis of chimerical proteins identified regions close to the amino-terminal end of helix 3 (Overdier *et al.*, 1994; Pierrou *et al.*, 1994) and in the second wing (Pierrou *et al.*, 1994) as important for specificity. The recognition helix of Foxd3 is tilted compared with the other three proteins for which the structures have been solved, and Liao and co-workers propose that this alters the sequence specificity (Jin *et al.*, 1999; Marsden *et al.*, 1998). Wikström and collaborators argue that since FOXC2, FoxA3, and FOXO4 have close to identical 3D folds, variation in topology cannot generally explain distinct DNA binding specificities (van Dongen *et al.*, 2000; Weigelt *et al.*, 2001), and instead propose differences in charge distribution in the protein–DNA interface as a possible cause (Weigelt *et al.*, 2001).

DNA Binding

In contrast to most helix–turn–helix proteins, forkhead proteins bind DNA as monomers. Hence, the binding sites, which typically span 15–17 bp, are asymmetrical. The sequence specificity has been determined for several representatives of this protein family through selection of binding sites from pools of short, random-sequence duplexes (Pierrou *et al.*, 1995). A seven-nucleotide core corresponds to the major groove base contacts made by the recognition helix (helix 3). For the majority of forkhead proteins, the core conforms to the RYMAAYA (R = A or G; Y = C or T; M = A or C) consensus (Kaufmann *et al.*, 1995; Overdier *et al.*, 1994; Pierrou *et al.*, 1994), but the more distant outgroups also bind sequences with only partial match to this motif, e.g., the insulin response elements recognized by members of the FoxO subfamily (Brunet *et al.*, 1999; Kops *et al.*, 1999). An optimal core sequence is essential, but not

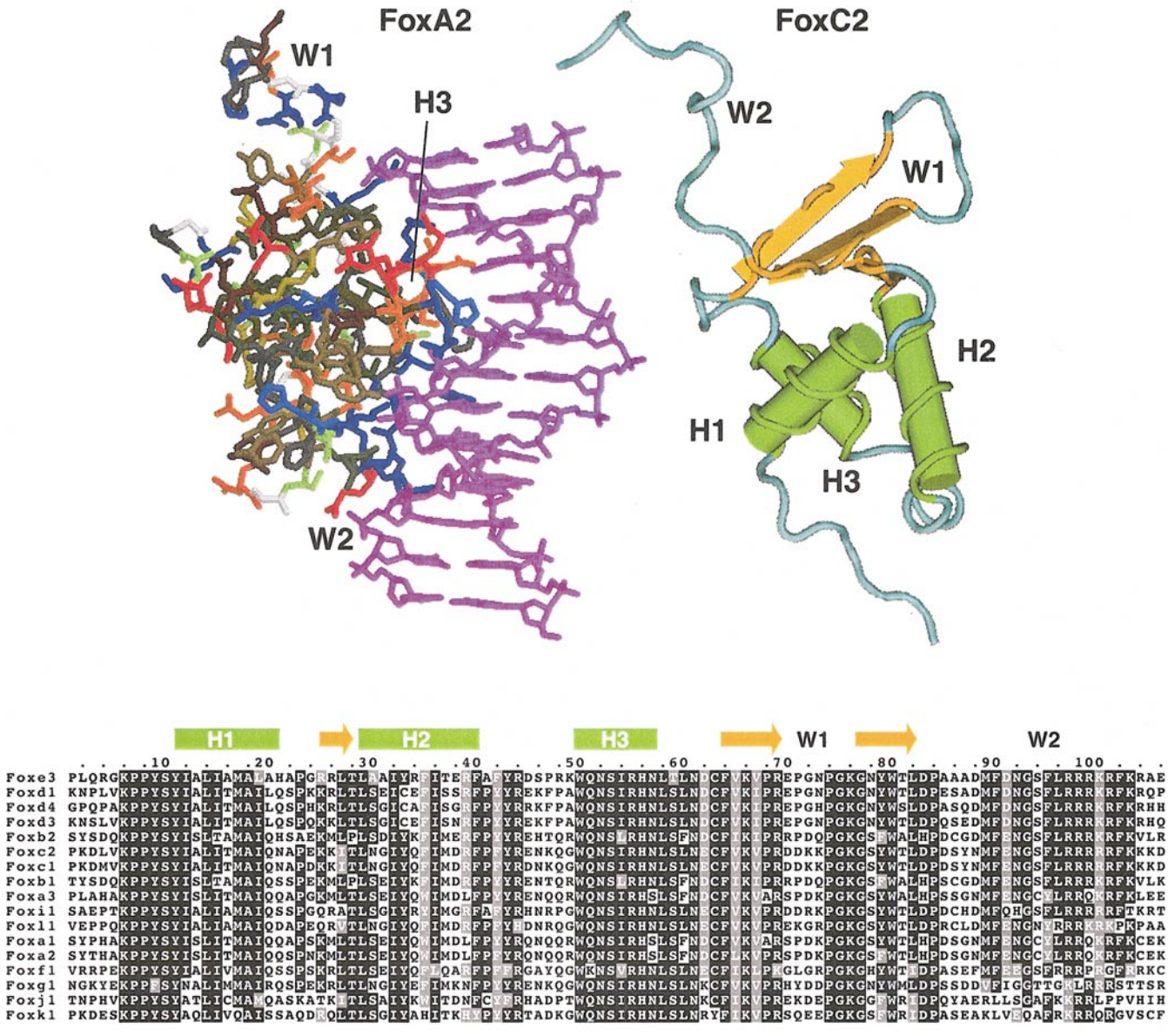


FIG. 1. Three-dimensional structure of the DNA-binding domains of FoxA3 bound to DNA (X-ray crystallography; Clark *et al.*, 1993) and FoxC2 (NMR; van Dongen *et al.*, 2000). The FoxA3 structure shows the recognition helix (H3) filling out the major groove of DNA (viewing angle parallel with the H3 helical axis). The first wing (W1) reaches upward, approximately parallel with the DNA helical axis and beyond the (3′) end of the oligonucleotide, whereas the second wing (W2) makes minor groove contacts in the 5′ end of the binding site. The FoxC2 NMR structure shows the helix–turn–helix motif viewed from the side facing away from the DNA with two helices, H1 and H2, stacked on top of the recognition helix (H3). The first wing (W1) consists of two antiparallel β-strands (yellow) separated by a short loop. In the bottom panel, alignment of 17 mouse forkhead domains illustrates the conserved regions in relation to helices (H1–H3) and β-strands (yellow).

sufficient for high affinity binding, which also depends on flanking sequences on both sides of the core (Kaufmann *et al.*, 1995; Overdier *et al.*, 1994; Pierrou *et al.*, 1994; Roux *et al.*, 1995).

Distantly related forkhead proteins can have completely nonoverlapping sequence specificity due to preference differences in both core and flanking positions (Overdier *et al.*, 1994). Others have overlapping, but nonidentical target specificities, as shown for FOXC1 and FOXD1 (Pierrou *et al.*, 1994); certain sequences are bound with equal affinity

by these two proteins, whereas others are preferentially bound by one or the other (Pierrou *et al.*, 1995). Pairs or small groups of forkhead proteins have DNA binding domains so similar that the specificities have to be assumed to be identical; for example FOXD1 and FOXD2 have forkhead domains with 100% amino acid identity (Ernstsson *et al.*, 1997), and FOXC1 and FOXC2 are 97% identical (Kume *et al.*, 1998). Mouse Foxf1, Foxf2, and human FOXF2 are 100% identical in the forkhead domain, whereas human FOXF1 differs by three conservative amino acid substitutions (Clevidence *et al.*, 1994; Hellqvist *et al.*, 1996; Miura *et al.*, 1998; Pierrou *et al.*, 1994). This exceptional degree of sequence conservation is confined to the forkhead domain—in FOXD1 and -2, for example, there are no discernable homologies between the rest of the proteins. Since experimental data suggest that a considerable sequence variation within the forkhead domain can be tolerated with no or little effect on sequence specificity, additional interactions and functions are likely to contribute to the selective forces that preserve the forkhead domains.

Chromatin Remodeling

A winged helix fold remarkably similar to that of forkhead proteins, except for the lack of a second wing, is found in the linker histones H1 and H5 (Brennan, 1993; Cerf *et al.*, 1994; Clark *et al.*, 1993; Ramakrishnan *et al.*, 1993). A series of elegant papers from Zaret and co-workers suggest that this structural similarity is functionally significant. FoxA proteins can determine the positioning of nucleosomes in the albumin enhancer (McPherson *et al.*, 1993, 1996; Shim *et al.*, 1998); they bind to DNA on one side of the nucleosome—in a manner similar to linker histones (Cirillo *et al.*, 1998)—and more efficiently to DNA wrapped up in nucleosomes than to naked DNA (Cirillo and Zaret, 1999). However, binding by FoxA1 does not compact chromatin, as linker histones do, but is instead correlated with an active chromatin structure in the albumin enhancer (Cirillo *et al.*, 1998). Furthermore, binding by FoxA proteins to nucleosomes is independent of histone acetylation and converts chromatin to a conformation where it can bind additional transcription factors (Cirillo and Zaret, 1999). These data suggest that the forkhead domain can promote gene activation directly, by opening up chromatin, and not just by bringing in a separate transcriptional activation domain. Additional support for a role of forkhead proteins in regulation of chromatin structure comes from the observation that DOMINA (a *Drosophila* protein most closely related to the FoxN subfamily) can suppress position–effect variegation, i.e., the position-dependent silencing of genes through spreading of heterochromatin (Strodicke *et al.*, 2000).

Nuclear Localization

The sequences responsible for nuclear localization have been mapped in FoxA2 and FOXF2 (Hellqvist *et al.*, 1998; Qian and Costa, 1995). In both proteins, the NLS is contained within the forkhead domain; sequences from its amino- and carboxy-terminal end have NLS activity, and both of these regions are needed for efficient nuclear localization. The carboxy-terminal part of this bipartite NLS consists of a cluster of basic amino acids, characteristic of many NLS motifs, but the amino-terminal part does not. The high degree of sequence conservation among forkhead domains suggests that this NLS structure is valid for all family members. However, in the FoxO subfamily, the NLS is regulated; subcellular localization is controlled by phosphorylation in response to extracellular signals (see below; Biggs *et al.*, 1999; Brunet *et al.*, 1999; Cahill *et al.*, 2000).

TRANSCRIPTIONAL EFFECTOR DOMAINS

Forkhead proteins have been shown to act mostly as transcriptional activators but not exclusively so. For example, *trans*-repression has been reported for FoxC2, -D2, -D3, and -G1 (Bourguignon *et al.*, 1998; Freyaldenhoven *et al.*, 1997; Sutton *et al.*, 1996). In *C. elegans*, the forkhead protein LIN-31 is thought to act as either repressor or activator, depending on its phosphorylation in response to MAP kinase signaling (Tan *et al.*, 1998). Mammalian FoxG1 represses transcription by forming a complex with transcriptional co-repressors of the Groucho family and histone deacetylases (Yao *et al.*, 2001). Specific binding to Groucho proteins has also been reported for FoxA2 (Wang *et al.*, 2000).

Using deletions and substitutions, the regions that contribute to transcriptional activation have been mapped in detail for several forkhead proteins, such as FoxA2, -F1, -F2, -N1, and others (Hellqvist *et al.*, 1998; Mahlapuu *et al.*, 1998; Pani *et al.*, 1992; Qian and Costa, 1995; Schuddekopf *et al.*, 1996). Like many other transcription factors, forkhead proteins often contain several activating regions, and these can be found in any location relative to the DNA binding domain.

The high degree of sequence homology within the DNA binding domain contrasts with the almost total lack of similarity between activation or repression domains in different forkhead proteins. Only within certain subfamilies can conservation of short activating motifs be recognized, e.g., "region II" in the FoxA subfamily (including FORK HEAD from *Drosophila*) (Clevidence *et al.*, 1994) and the C-terminal *trans*-activation domains in the FoxF subfamily (Hellqvist *et al.*, 1998; Mahlapuu *et al.*, 1998). In general, the described *trans*-activation and -repression domains lack distinctive features, such as enrichment for a particular amino acid. An exception is the C-terminal activation domain of Foxn1, which appears to be a typical "acidic blob" (Schuddekopf *et al.*, 1996).

Little is known about the mechanisms through which forkhead proteins interact with the transcriptional machinery. *In vitro*, FOXF2 binds the general transcription factors

TBP and TFIIB, and in cotransfection experiments, FOXF2 acts synergistically with TFIIB (Hellqvist *et al.*, 1998).

CHROMOSOMAL LOCALIZATION AND GENOMIC ORGANIZATION

In general, forkhead genes are distributed throughout the genomes and do not form physically linked clusters. Proximity, suggesting recent duplication, is seen in some pairs of closely related genes; for example, the *Drosophila* genes *sloppy paired 1* and *2 (slp 1, 2)* map within 10 kb (Grossniklaus *et al.*, 1992). Human *FOXC1* and *FOXF2* are located in the same region of chromosome 6 (6p25), whereas *FOXC2* and *FOXF1* are close on chromosome 16 (16q24) (Blixt *et al.*, 1998; Kaestner *et al.*, 1996; Larsson *et al.*, 1995). The mouse orthologs are organized in a similar way (Avraham *et al.*, 1995; Chang and Ho, 2001; Hong *et al.*, 1999; Kaestner *et al.*, 1996; Labosky *et al.*, 1996). A possible interpretation of this arrangement is that duplication of a primeval gene—followed by divergence of the two copies—gave rise to ancestral *FoxC* and *FoxF* genes (Fig. 2). A more recent duplication of the entire locus, transfer of one copy to a different chromosome, and additional sequence divergence then gave rise to the present four genes. The last duplication apparently took place after the separation of protostomes and deuterostomes; in *Drosophila*, there is just one homolog each of *FoxC* (*corocodile*) and *FoxF* (*biniou*). In *C. elegans*, the situation is less clear, but an obvious *FoxC* homolog is missing and the *FoxF* homolog, *F26B1.7*, is among those most similar to *FoxC/crocodile*. Thus, the first (hypothetical) duplication may have coincided with the appearance of primitive coelomate animals. Evidently, evolution has found independent uses for all four mammalian genes, since knockout in mouse of any of these results in embryonic lethality (Iida *et al.*, 1997; Kume *et al.*, 1998; Mahlapuu *et al.*, 2001b; N. Miura, personal communication). Nevertheless, overlaps in function have also been retained, as shown by the severe defects in combined *Foxc1/c2* (Kume *et al.*, 2000b, 2001) and *Foxf1/f2* (M. Ormestad, N. Miura, and P. C., unpublished observations) mutants.

The genomic organization of forkhead genes varies, but locations of introns are usually conserved in orthologs from different species and between closely related genes. Most of the vertebrate genes are comparatively small with few introns. Quite a few are intronless (e.g., *FoxC1, -C2, -D1, -D2, -D4, -E3,* and *-G1*) (Blixt *et al.*, 2000; Hatini *et al.*, 1996; Kaestner *et al.*, 1995; Miura *et al.*, 1997; Xuan *et al.*, 1995), whereas in others the forkhead box is interrupted by an intron, e.g., *FoxII, -J1,* and *-N1* (Brody *et al.*, 1997; Clevidence *et al.*, 1993; Murphy *et al.*, 1997; Pierrou *et al.*, 1994; Schorpp *et al.*, 1997). In *FOXO1* and *-O3*, the intron that splits the forkhead box is particularly large, 90 and 130 kb, respectively (Anderson *et al.*, 1998), which is probably an important reason why translocations with breakpoints in this intron are such a common cause of alveolar rhabdomyosarcoma (see below). *FoxF* genes are interrupted by an intron 3′ of the forkhead box (Blixt *et al.*, 1998; Chang and Ho, 2001; Mahlapuu *et al.*, 1998; Miura *et al.*, 1998), whereas *FoxA* genes have one or two introns on the 5′ side (Kaestner *et al.*, 1994).

FoxA3, -C1, -D1, and *-D4* are transcribed from conventional TATA promoters (Ernstsson *et al.*, 1996; Kaestner *et al.*, 1994, 1995; Mears *et al.*, 1998), but no TATA-box is located near the transcription start in *FoxA1, -F1, -F2,* or *J1*. Instead, CpG islands surround the transcription start regions of these genes (Blixt *et al.*, 1998; Brody *et al.*, 1997; Kaestner *et al.*, 1994; Mahlapuu *et al.*, 1998).

Several forkhead genes, such as *FoxC1, -K1, -M1, -N1,* and *P3*, give rise to multiple mRNAs, due to alternative start or polyadenylation sites, or to differential splicing of primary transcripts (Jeffery *et al.*, 2001; Nishimura *et al.*, 1998; Schorpp *et al.*, 1997; Yang *et al.*, 1997; Ye *et al.*, 1997).

The human genome also contains a few intronless sequences related to *FOXO* genes, *FOXO1b* and *FOXO3b*, that appear to be processed pseudogenes (Anderson *et al.*, 1998).

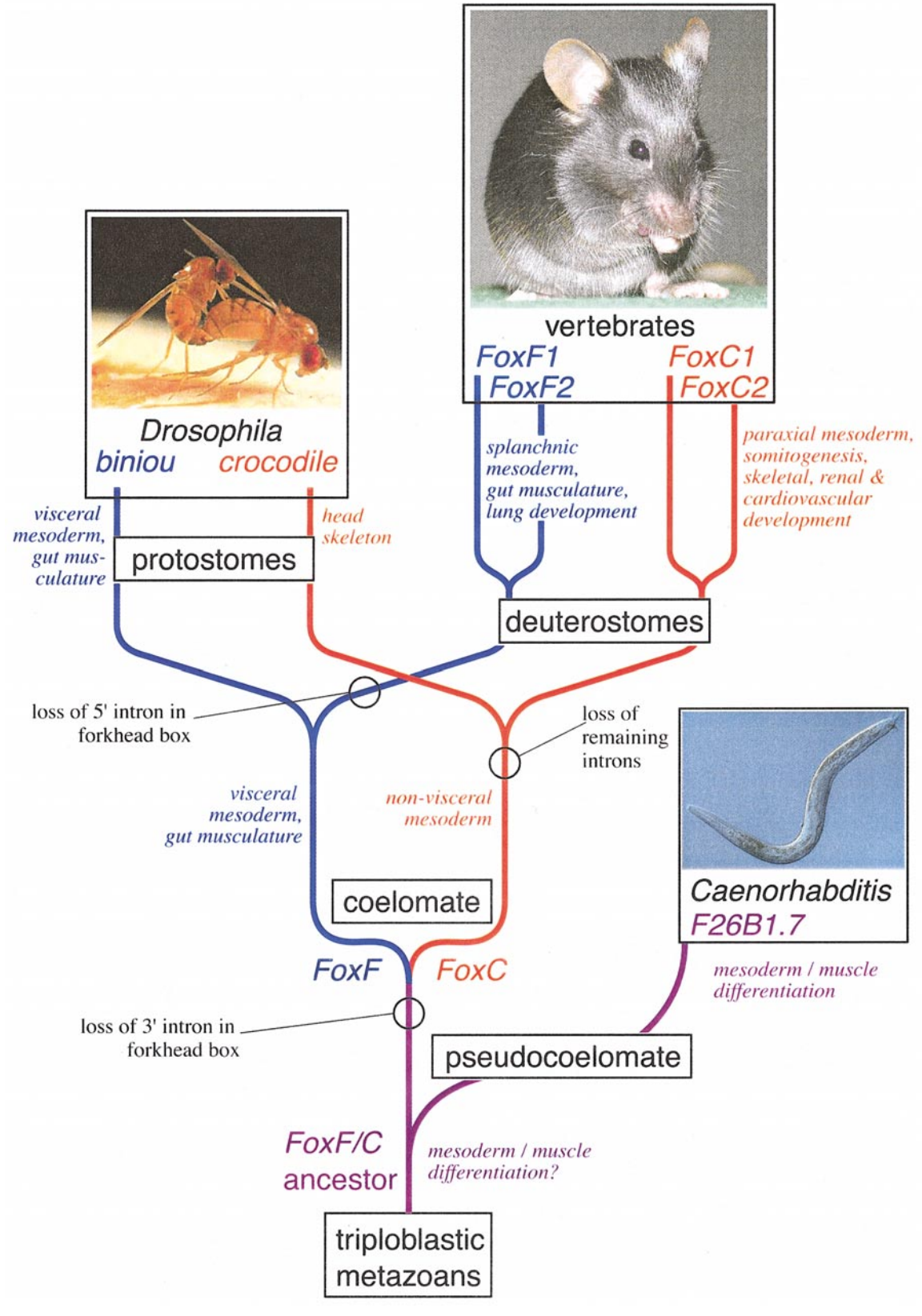
FORKHEAD PROTEINS AND SIGNAL TRANSDUCTION

TGFβ-Smad

FoxH1 is an important inducer of mesoderm specification (Watanabe and Whitman, 1999). It was first identified as a protein that binds to an activin response element in the promoter region of the mesoendodermal homeobox gene *Mix.2* (Chen *et al.*, 1996). In the absence of activin signaling, FoxH1 binds constitutively, but does not activate transcription (Fig. 3A). In the presence of activin—a member of the TGFβ superfamily—a complex containing FoxH1, Smad2, and Smad4 assembles on the DNA and transcription is activated (Chen *et al.*, 1996, 1997; Liu *et al.*, 1999; Yeo *et al.*, 1999). Mammalian FoxH1 homologs have been identified and also mediate TGFβ-type signaling through interaction with activated Smads (Labbe *et al.*, 1998; Liu *et al.*, 1999; Weisberg *et al.*, 1998; Zhou *et al.*, 1998). Mouse embryos without Foxh1 do not respond to nodal signaling and have defects in the node and anterior primitive streak (Hoodless *et al.*, 2001; Yamamoto *et al.*, 2001). In contrast to FoxH1, FoxG1 inhibits TGFβ-type signaling (Dou *et al.*, 2000; Rodriguez *et al.*, 2001). The inhibition is independent of the FoxG1 DNA binding domain, and according to Dou *et al.* (2000), the mechanism consists of an inhibitory interaction between FoxG1 and FoxH1. Rodriguez *et al.* (2001), on the other hand, found that the C-terminal part of FoxG1 binds to the MH2 domain of Smads and inhibits their association with DNA.

MAP Kinase

The *C. elegans* forkhead protein LIN-31 (Miller *et al.*, 1993, 2000) is a nuclear target for a receptor tyrosine



kinase/RAS/MAPK signaling cascade in vulval precursor cells (Tan *et al.*, 1998). The inductive signal is encoded by the *lin-3* gene, which is related to epidermal growth factor (EGF). The receptor for this ligand is the *let-23* product, which is a tyrosine kinase of the EGF receptor family. Interaction between LIN-3 and LET-23 leads to activation of RAS/MAP kinase signaling (reviewed by Kornfeld, 1997). LIN-31 and ETS transcription factor LIN-1 physically interact when unphosphorylated and inhibit vulval fates. Upon MAP kinase phosphorylation, the LIN-31/LIN-1 complex is disrupted, and phosphorylated LIN-31 then acts as a transcriptional activator, promoting the expression of vulval genes (Tan *et al.*, 1998).

Akt/PKB

Forkhead proteins of the FoxO subfamily are targets for PI3K/PDK1/PKB signaling initiated by insulin or insulin-like growth factor I receptors (for reviews, see Brunet *et al.*, 2001; Kops and Burgering, 1999). FoxO proteins regulate transcription of target genes involved in metabolism, but also mediate the survival factor function of growth factors by controlling expression of apoptosis genes, such as *FasL*. The initial clues about a connection between insulin-like signaling and forkhead transcription factors came from the nematode *C. elegans*. Inactivation of the *C. elegans daf-2* gene, which encodes a homolog of the insulin receptor, causes animals to arrest as dauers, shifts metabolism to fat storage, and prolongs the life span of the worm (Kenyon *et al.*, 1993; Kimura *et al.*, 1997). Mutations in the FoxO homolog *daf-16* suppress the dauer arrest, the metabolic shift, and the longevity phenotypes of *daf-2* mutants, indicating that DAF-16 is a negatively regulated target of *C. elegans* insulin receptor-like signaling (Lin *et al.*, 1997; Ogg *et al.*, 1997). DAF-16 is negatively regulated by DAF-2 through phosphorylation via AGE-1 (PI3K-like protein), PDK-1 (homolog of the mammalian PDK1), and AKT1/AKT2 (PKB-like kinases) (Morris *et al.*, 1996; Paradis *et al.*, 1999; Paradis and Ruvkun, 1998). This signaling pathway has been conserved between worm and mammals, where the homologs of DAF-16—FoxO1, FoxO3, and FoxO4—are targets for P13K/PKB phosphorylation (Brunet *et al.*, 1999; Kops *et al.*, 1999; Rena *et al.*, 1999; Takaishi *et al.*, 1999; Tang *et al.*, 1999). PKB/AKT inhibits transcriptional activation by FoxO/DAF-16 proteins through control of subcellular localization; when phosphorylated, FoxO/DAF-16 relocate from the nucleus to the cytoplasm through interaction with 14-3-3 proteins (Biggs *et al.*, 1999; Brunet *et al.*, 1999; Cahill *et al.*, 2000; Henderson and Johnson, 2001; Lee *et al.*, 2001; Lin *et al.*, 2001). Mutation of the AKT phosphorylation sites in DAF-16 leads to its stable nuclear localization, independent of DAF-2 signaling; Lee *et al.* (2001) found this to correlate with a constitutive dauer phenotype in a *daf-2*+ background, whereas Lin *et al.* (2001) failed to see an effect on either life span or dauer formation. Hence, there is still controversy as to whether regulation of DAF-16 nuclear localization through phosphorylation by AKT is the only output of DAF-2 signaling, or whether other mechanisms act in parallel. Under dauer-inducing conditions, *daf-7*—encoding a TGF-β-like ligand—also affects DAF-16 localization, which suggests DAF-16 as an integration point for insulin- and TGF-β-like pathways (Lee *et al.*, 2001; Ogg *et al.*, 1997).

Inhibition of FoxO proteins by PI3K/PKB is necessary for cell cycle entry in G_1 (Jones *et al.*, 1999; Klippel *et al.*, 1998; Kops *et al.*, 1999; Medema *et al.*, 2000), but reactivation in G_2 is essential for proper G_2/M, M/G_1 transitions and cytokinesis (Alvarez *et al.*, 2001). FoxO4 can also be phosphorylated by a PKB-independent mechanism that requires Ras signaling (Kops *et al.*, 1999; Medema *et al.*, 2000), which further emphasizes the role of forkhead proteins as crossroads for different signaling pathways.

Hedgehog

Several mammalian forkhead genes depend on the Hedgehog–Patched (Ptch)–Smoothened (Smo)–Gli signaling pathway for their expression. Sonic hedgehog (Shh) secreted from the notochord induces expression of *Foxa2* in the floorplate of the neural tube and Foxa2 maintains *Shh* expression in a positive feedback loop (Chiang *et al.*, 1996; Echelard *et al.*, 1993; Hynes *et al.*, 1997; Sasaki *et al.*, 1997). *Foxc2* and *Foxd2* are induced in presomitic mesoderm by Shh from the notochord (Furumoto *et al.*, 1999; Wu *et al.*, 1998). Expression of *Foxf1* in lung and foregut mesenchyme and in sclerotomes depends on Shh signaling from endodermal epithelia and notochord, respectively (Mahlapuu *et al.*, 2001a). In embryos lacking Shh, residual *Foxf1* expression is

FIG. 2. A model for evolution of *FoxF* and *FoxC* genes from a common ancestor based on sequences, exon–intron distribution, chromosomal localization, expression patterns, and mutant phenotypes. *C. elegans* has a single gene that appears to be the ancestor of both *FoxC* and *FoxF* genes and which is involved in muscle differentiation. In animals with a true coelom, distinct *FoxC* and *FoxF* genes are present in both protostomes and deuterostomes. The duplication of a common ancestor gene, followed by divergent evolution to generate *FoxC* and *FoxF*, is therefore likely to have occured prior to, or soon after, the appearance of primitive coelomate animals. Since the *FoxF* genes in both mammals and *Drosophila* are involved in differentiation of the visceral/splanchnic mesoderm, whereas *FoxC* genes are restricted to nonvisceral mesoderm, it is possible that functional divergence of *FoxC* and *FoxF* was intimately connected to the evolution of the coelom. An additional duplication has taken place in the deuterostome lineage, and signs of this can still be seen in the human genome, where *FoxC1* and *FoxF2* are located close to each other on chromosome 6, while *FoxC2* and *FoxF1* are adjacent on chromosome 16. *C. elegans* picture courtesy of Catarina Mörck.

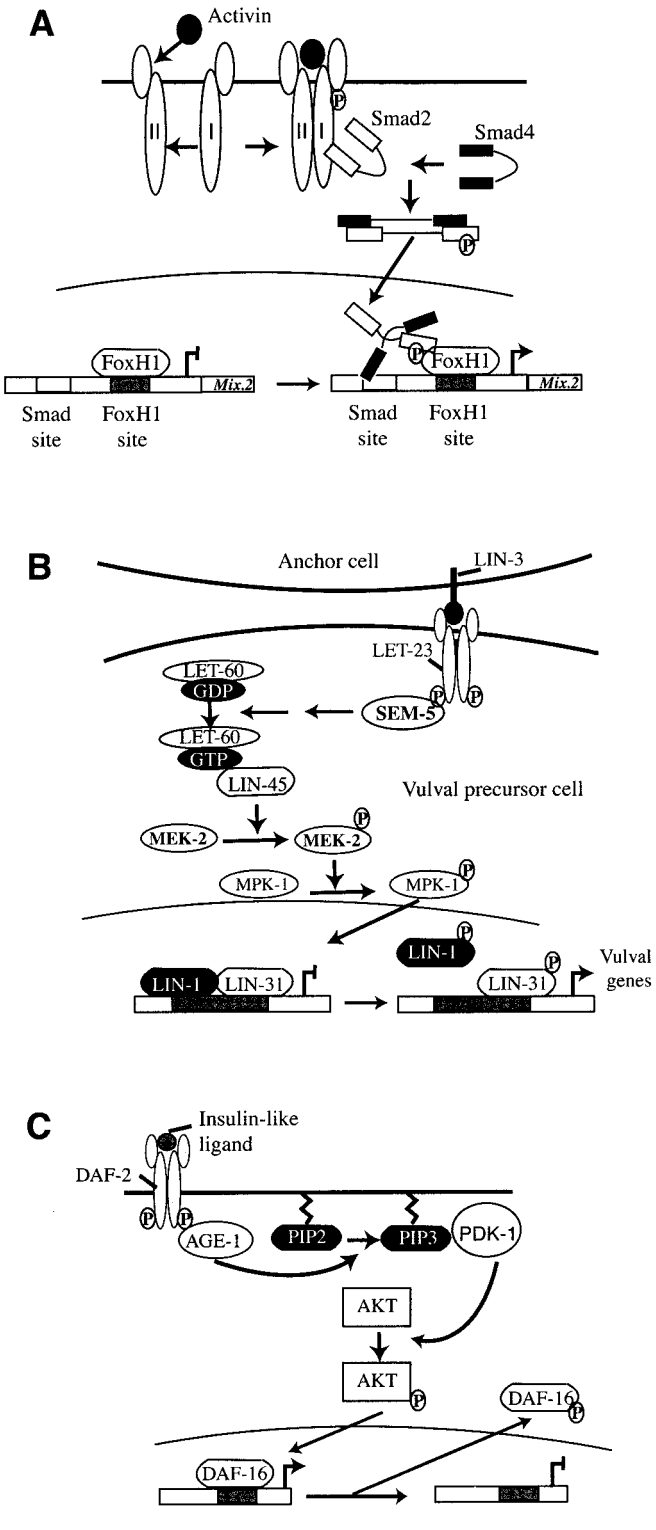


FIG. 3. (A) Model for activin activation of *Mix.2* transcription by the forkhead protein FoxH1 in the early *Xenopus* embryo. Activin induces heterodimerization followed by *trans*-phosphorylation of *trans*-membrane type I and type II serine/threonine kinase receptors (reviewed by Massague, 1998). The activated type I receptor then directly interacts with and phosphorylates the receptor-regulated Smad, Smad2. Phosphorylation of Smad2 stimulates its interaction with the common Smad, Smad4, and the transport of the resulting heteromeric complex to the nucleus (reviewed by Attisano and Wrana, 2000; Wrana and Attisano, 2000). In the absence of activin signaling, FoxH1 binds constitutively to an activin response element in the promoter region of the *Mix.2* gene, but does not activate transcription. In the presence of activin, the Smad2/Smad4 complex translocates to the nucleus and is recruited by FoxH1 to the promoter of *Mix.2*, which leads to activation of transcription (Chen *et al.*, 1996, 1997). Analysis of the FoxH1/Smad complex shows that Smad2 interacts directly with FoxH1 and brings Smad4 into the complex. Smad4 then binds DNA at a site adjacent to the FoxH1 binding site and stabilizes the Smad/FoxH1/DNA complex (Liu *et al.*, 1997). (B) Model for forkhead protein LIN-31 function in vulval development of *C. elegans*. LIN-3, a protein similar to EGF, is produced by gonadal anchor cell and initiates vulval development by activating the EGF receptor tyrosine kinase homolog LET-23 in the closest vulval precursor cell. Activation of the LET-23 triggers a conserved Grb2/Ras/Raf/MEK/MAP kinase cascade, the components of which are encoded by genes *sem-5*, *let-60*, *lin-45*, *mek-2*, and *mpk-1*, respectively (reviewed by Kornfeld, 1997). If the MAP kinase is inactive in cells, the LIN-1/LIN-31 complex binds to the promoter of target genes and represses transcription. Active MAP kinase enters the nucleus and directly phosphorylates both LIN-1 and LIN-31 proteins. Phosphorylation of LIN-31 disrupts the LIN-1/LIN-31 complex, and phosphorylated LIN-31 acts as a transcriptional activator, promoting vulval cell fates (Tan *et al.*, 1998). (C) Model for phosphorylation-dependent inhibition of forkhead protein DAF-16-activated transcription in *C. elegans*. Activation of DAF-2 receptors triggers a conserved signaling cascade in the worm involving AGE-1, PDK-1, and AKT1, -2 proteins (homologs of the mammalian PI3K, PDK1, and PKB, respectively) (reviewed by Kops and Burgering, 1999). Activated AKT kinases move to the nucleus and phosphorylate the DAF-16 protein, which results in redistribution of DAF-16 from the nucleus to the cytoplasm and in inhibition of target gene expression (Cahill *et al.*, 2000; Henderson and Johnson, 2001; Lee *et al.*, 2001; Lin *et al.*, 2001).

seen in mesoderm of the hindgut and yolk sac (Mahlapuu *et al.*, 2001a), which neighbor sites of Indian hedgehog secretion (Bitgood and McMahon, 1995; Farrington *et al.*, 1997; Maye *et al.*, 2000).

Wnt

Foxl1 knockout mouse embryos develop severe structural defects in the gastrointestinal tract due to overproliferation of the intestinal epithelium (Kaestner *et al.*, 1997). The hyperproliferation correlates with activation of the Wnt/β-catenin/TCF signaling pathway (Perreault *et al.*, 2001), which is important for the control of intestinal epithelial proliferation and the dysregulation of which is a major cause of human colorectal cancers. Rather than affecting the signal-transducing intracellular components, the normal function of Foxl1—which is expressed in the intestinal mesenchyme—appears to be to restrict deposition of the

extracellular proteoglycans which act as coreceptors for Wnt.

Cell Cycle Regulation

The yeast forkhead proteins Fkh1 and Fkh2 regulate the expression of the *CLB2* cluster genes, whose transcription peaks early in mitosis (Spellman *et al.*, 1998; Zhu *et al.*, 2000). Fkh1 and Fkh2 are constitutively bound to promoters of *CLB2* cluster genes, in complex with the MADS-box protein Mcm1, and function by providing a permanent platform for further regulatory inputs (Futcher, 2000; Lydall *et al.*, 1991; Pic *et al.*, 2000). One of these regulatory proteins recruited by the Mcm1–Fkh complex in the G_2/M phase is Ndd1, which is needed for activation of transcription (Koranda *et al.*, 2000). The Fkh proteins are phosphorylated in a cell cycle-dependent manner, which may control activation of the Mcm1–Fkh–Ndd1 complex (Pic *et al.*, 2000). *In vivo*, Fkh1 and Fkh2 occupy different promoters, and *in vitro* data suggest that they differ in their ability to bind synergistically with Mcm1 (Hollenhorst *et al.*, 2001).

In mammals, expression of the forkhead gene *FoxM1* is confined to cycling cells and is regulated in a cell cycle-dependent manner, with activation upon entry into S phase (Korver *et al.*, 1997). FoxM1 is phosphorylated in M phase, which implies regulation at both transcriptional and protein levels. Inactivation of *Foxm1* in mice leads to uncoupling between S phase and mitosis, with polyploidy as a result (Korver *et al.*, 1998). Its function therefore appears to be prevention of DNA re-replication during the G_2 and M phases.

FoxO4, which is negatively regulated by growth factors (see above), blocks cell-cycle progression at G_1, independent of the retinoblastoma protein, by transcriptional activation of the cdk inhibitor $p27^{kip1}$ (Medema *et al.*, 2000).

The Forkhead Associated (FHA) Domain

The FHA domain was discovered as a region of sequence homology between a set of proteins that includes, but is not restricted to, four forkhead proteins, Fhl1, Fkh1, and -2 from *Saccharomyces* and mammalian FoxK1 (Hofmann and Bucher, 1995). It contains three conserved blocks of amino acids separated by more divergent spacer regions, which make the total size variable. Most of the proteins that contain the FHA domain are nuclear and involved in cell cycle, checkpoint control, or signal transduction (e.g., Rad53, Fkh1, -2, Dun1, Spk1, Ki67, Mek1, Chk2). The FHA domain binds proteins phosphorylated on serine or threonine and mediates protein–protein interactions (Durocher *et al.*, 2000; Li *et al.*, 2000); i.e., it is for phosphoserine and phosphothreonine what the SH2 domain is for phosphotyrosine. In contrast to the forkhead domain, the FHA domain is present in several *Arabidopsis* proteins and thus appears to be a more ancient evolutionary invention.

FORKHEAD GENES IN DEVELOPMENT

This overview of embryonic development focuses on vertebrates. Although the pioneering work that indicated a role for forkhead genes in early patterning was performed in *Xenopus* (Dirksen and Jamrich, 1992; Knochel *et al.*, 1992; Ruiz i Altaba and Jessell, 1992), the emphasis in this account reflects the recent advances based on work with mutant mice (Table 2). Where functions appear to have been conserved, comparisons are made with invertebrates. The first documented roles of forkhead genes in vertebrate development are in defining different populations of mesoderm immediately following gastrulation.

As described in the signal transduction section, FoxH1 functions as a Smad DNA-binding partner to regulate transcription in response to activin and nodal signaling. Deletion of *Foxh1* in mice partly phenocopies loss of nodal and results in failure to pattern the anterior primitive streak, to form node, prechordal mesoderm, notochord, or definitive endoderm (Hoodless *et al.*, 2001; Yamamoto *et al.*, 2001). Expression of *Foxa2* in the node is dependent on Foxh1 (Hoodless *et al.*, 2001) and, like the Foxh1 mutant, *Foxa2* null embryos lack notochord (Ang and Rossant, 1994; Weinstein *et al.*, 1994). The notochord is an important midline signaling center that secretes sonic hedgehog, and its absence in *Foxa2* null embryos leads to lack of floorplate and defects in dorsoventral patterning of the neural tube and somites.

Indirect evidence suggests that a third forkhead gene, *Foxj1*, is required for proper function of the node. Left–right asymmetry in the vertebrate embryo is initiated in the node and requires hedgehog signaling (Levin *et al.*, 1995; Zhang *et al.*, 2001). The asymmetric distribution of lateralizing signals requires cilial movements within the node (Nonaka *et al.*, 1998), and several observations implicate *Foxj1* in this process; it is expressed in ciliated node cells and *Foxj1* null mice have randomized left–right asymmetry (Brody *et al.*, 2000; Chen *et al.*, 1998). Although cilia are not absent in $Foxj1^{-/-}$ node cells, the defective cilia in airway epithelial cells in the same mutant and ectopic ciliogenesis caused by forced *Foxj1* expression (Tichelaar *et al.*, 1999) show that this forkhead gene is important for cilial function.

Nascent mesodermal cells formed in different regions of the primitive streak differentiate into distinct populations along the mediolateral axis (corresponds to the dorsoventral axis of later stages) of the embryo, and each population expresses its characteristic subset of forkhead genes (Fig. 4). As described above, *Foxa2* is expressed in the anterior end of the primitive streak—the node—and specifies axial mesoderm (notochord). Four forkhead genes—*Foxb1, -c1, -c2,* and *-d2*—are expressed in the paraxial mesoderm, and in somites, their mRNAs occupy distinct, but overlapping, regions. *Foxb1* is expressed in the dorsal somite, but all somite-derived structures are negative for *Foxb1* expression at later stages (Labosky *et al.*, 1997). *Foxc1* and *-c2* are expressed throughout and *Foxd2* in the ventral region of epithelial somites; mRNA for these three genes becomes

TABLE 2
Mouse *Fox* Gene Null Mutant Phenotypes

Gene	Mutant phenotype	References
Foxa1 (*HNF-3α*)	Die postnatally with severe growth retardation and hypoglycemia. Reduced pancreatic glucagon production.	Kaestner *et al.*, 1999; Shih *et al.*, 1999
Foxa2 (*HNF-3β*)	Absence of node, notochord, and foregut. Embryos do not develop beyond E8.5. Conditional inactivation in pancreas causes hyperinsulinemic hypoglycemia.	Ang and Rossant, 1994; Sund *et al.*, 2000; Sund *et al.*, 2001; Weinstein *et al.*, 1994
Foxa3 (*HNF-3γ*)	Reduced expression of the hepatic glucose transporter GLUT2 leads to inefficient glucose efflux and fasting hypoglycemia.	Kaestner *et al.*, 1998; Shen *et al.*, 2001
Foxb1 (*Mf3*)	Variable phenotype including perinatal mortality, growth retardation, nursing defects, and defects in the central nervous system.	Alvarez-Bolado *et al.*, 2000; Labosky *et al.*, 1997; Wehr *et al.*, 1997
Foxc1 (*Mf1*)	Die at birth with multiple abnormalities including hydrocephalus, skeletal, ocular, renal, and cardiovascular defects. Heterozygotes have ocular defects. Together with Foxc2 required for somitogenesis.	Hong *et al.*, 1999; Kidson *et al.*, 1999; Kume *et al.*, 1998, 2000b, 2001; Smith *et al.*, 2000; Winnier *et al.*, 1999
Foxc2 (*Mfh1*)	Die pre- or perinatally with skeletal and cardiovascular defects. Heterozygotes have ocular defects. Together with Foxc1 required for somitogenesis.	Iida *et al.*, 1997; Kume *et al.*, 2000b, 2001; Smith *et al.*, 2000; Winnier *et al.*, 1997; Winnier *et al.*, 1999
Foxd1 (*Bf2*)	Die within 24 h after birth due to renal failure.	Hatini *et al.*, 1996
Foxd2 (*Mf2*)	Viable and fertile, but ca. 40% have renal abnormalities.	Kume *et al.*, 2000b
Foxe1 (*TTF-2*)	Die within 48 h of birth exhibiting cleft palate and either a complete or partial failure of thyroid gland development.	De Felice *et al.*, 1998
Foxe3	(*dysgenetic lens* mutant) Viable and fertile. Severe cataract and fusion of lens, cornea and iris caused by degeneration of lens epithelium.	Blixt *et al.*, 2000; Brownell *et al.*, 2000
Foxf1 (*FREAC1, HFH-8*)	Die around E9 due to absence of vasculogenesis in yolk sac and allantois as a result of defects in mesodermal differentiation. Heterozygotes have lung and foregut malformations.	Kalinichenko *et al.*, 2001; Mahlapuu *et al.*, 2001a,b
Foxg1 (*Bf1*)	Die around birth, with a severe reduction in the size of the cerebral hemispheres.	Xuan *et al.*, 1995
Foxh1 (*Fast*)	Embryonically lethal due to failure to form node, prechordal mesoderm, notochord, and definitive endoderm.	Hoodless *et al.*, 2001; Yamamoto *et al.*, 2001
Foxi1 (*Fkh-10*)	Malformations of the inner ear results in deafness and disturbed balance.	Hulander *et al.*, 1998
Foxj1 (*HFH-4*)	Majority die before weaning and show defective ciliogenesis as well as randomized left-right asymmetry.	Brody *et al.*, 2000; Chen *et al.*, 1998
Foxk1 (*MNF*)	Viable, but growth retarded. Incomplete muscle regeneration after injury due to defect proliferation and differentiation of myogenic stem cells.	Garry *et al.*, 2000
Foxl1 (*Fkh-6*)	The majority die before weaning with intestinal epithelial hyperplasia due to overactivation of the Wnt/β-catenin pathway.	Kaestner *et al.*, 1997; Perreault *et al.*, 2001
Foxm1 (*Trident, HFH-11*)	Die perinatally with cardiovascular defects and polyploidy in cardiomyocytes and hepatocytes.	Korver *et al.*, 1998
Foxn1 (*Whn*)	Exhibit all features of the original *nude* mutant, such as hairlessness and athymia.	Nehls *et al.*, 1994, 1996
Foxp3	(*scurfy* mutant) Overproliferation of $CD4^+$ $CD8^-$ T lymphocytes, extensive multiorgan infiltration and elevation of cytokines.	Jeffery *et al.*, 2001
Foxq1	(*satin* mutant) Have a silky fur coat due to defects in differentiation of the hair shaft.	Hong *et al.*, 2001

restricted to the sclerotome as the somites differentiate further (Kume *et al.*, 1998, 2000a, Winnier *et al.*, 1997). *Foxc1* and *-c2* are functionally redundant in the presomitic mesoderm. Although both null mutants are embryonically lethal with vascular and skeletal malformations (Iida *et al.*, 1997; Kume *et al.*, 1998; Winnier *et al.*, 1997), neither exhibits any overt defects in somitogenesis. The compound homozygotes, however, lack somites and segmentation of the paraxial mesoderm (Kume *et al.*, 2001). In the absence of *Foxc1* and *-c2*, transcription of *paraxis*, *Mesp1* and *-2*, *Hes5*, and *Notch1* is lost in the anterior presomitic mesoderm, as is the formation of sharp boundaries of *Dll1*, *Lfng*, and *ephrinB2* expression. *Foxc1* and *-2* thus appear to interact with the Notch–Delta signaling pathway in the prepatterning of anterior and posterior domains of the presumptive somites (Kume *et al.*, 2001). *Foxd2* homozygous null mice have no major developmental defects in somite derivatives (Kume *et al.*, 2000a), and only a minority of *Foxb1* mutants

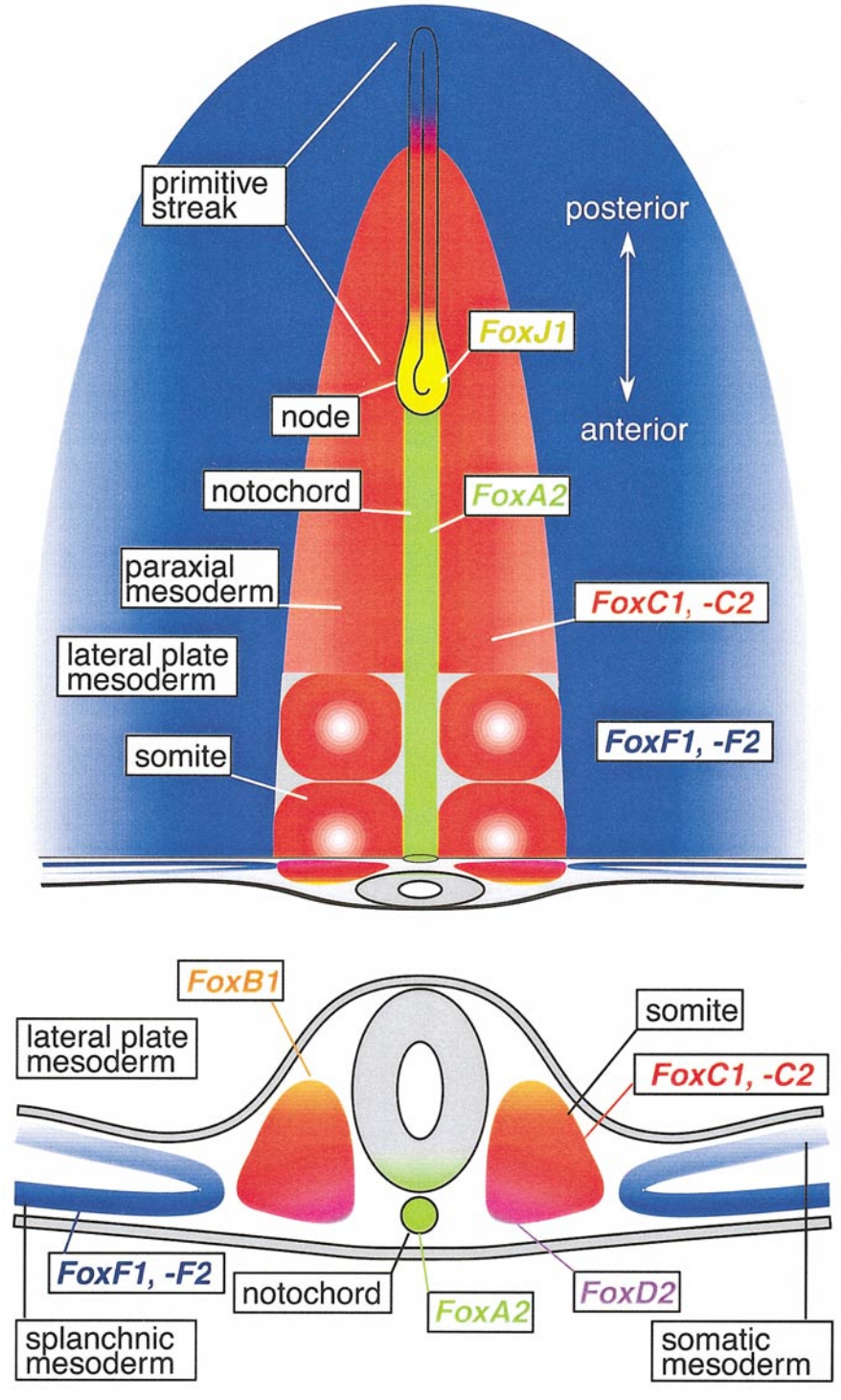


FIG. 4. Schematic view of how expression of *Fox* genes patterns mesoderm in the vertebrate embryo. The top panel shows a simplified version of a the posterior end of a vertebrate embryo, where distinct populations of mesoderm originate in different parts of the primitive streak. The lower panel shows patterning of somites and lateral mesoderm in a schematic cross section. The anterior end of the streak, the node, expresses *FoxJ1*. Additional forkhead genes expressed in the node, but not shown in this figure, include *FoxH1* and *FoxA2*. *FoxA2* expression in the notochord and the floor plate of the neural tube is maintained through a positive feedback loop with *Sonic Hedgehog*. Paraxial mesoderm expresses *FoxC1* and *-C2* and, as a result of the activity of these genes, becomes segmented into somites. The somites are patterned by expression of *FoxB1* dorsally, *FoxC1* and *-C2* throughout, and *FoxD2* medioventrally. The lateral plate mesoderm originates in the posterior primitive streak and expresses *FoxF1* and *-F2*. *FoxF* expression gradually disappears in the somatic mesoderm as the lateral plate differentiates anteriorly, but remains high in the splanchnic mesoderm.

show a reduction of the posterior part of the body that may be related to its expression in presomitic mesoderm (Labosky *et al.*, 1997).

The posterior primitive streak and lateral plate mesoderm express *Foxf1*, and laterally the expression continues into the extraembryonic mesoderm of amnion, allantois, and yolk sac (Mahlapuu *et al.*, 2001b; Peterson *et al.*, 1997). In extraembryonic mesoderm, inactivation of *Foxf1* leads to ubiquitous expression of the cell adhesion molecule VCAM1, with the result that VCAM1 is coexpressed with its ligand, α4-integrin (Mahlapuu *et al.*, 2001b). This is likely to contribute to the enhanced intramesodermal cohesion that prevents affected extraembryonic structures from expanding or reshaping, and to the abnormal adherence or fusion between amniotic and yolk sac mesoderm of *Foxf1*$^{-/-}$ embryos. Foxf1 promotes the division of the lateral plate into splanchnic and somatic mesoderm, and as differentiation proceeds, *Foxf1* expression becomes confined to the splanchnopleure (Mahlapuu *et al.*, 2001b). In *Foxf1* null embryos, the splanchnic mesoderm fails to separate completely from the somatic and expresses a marker for somatic mesoderm, the homeobox gene *Irx3*. The core function of *FoxF* genes in mesoderm differentiation has been conserved between vertebrates, *Drosophila* and *C. elegans* (Fig. 2). The *Drosophila FoxF* homolog *biniou (bin)* controls development of the visceral mesoderm (corresponds to the splanchnic mesoderm in vertebrates) and the derived gut musculature (Zaffran *et al.*, 2001). As in *Foxf1*$^{-/-}$ mouse embryos, there is a conversion of visceral to somatic mesoderm in the *bin* mutant. Conversely, ectopic expression of *bin* in the somatic mesoderm leads to activation of visceral mesoderm markers (Zaffran *et al.*, 2001). In *C. elegans*, the *FoxF* homologue, *F26B1.7*, is essential for normal development of several muscle types (M. Hellqvist, personal communication). Mouse *Foxf1* is required for normal expression of *Bmp4* in ventral (lateral) mesoderm (Mahlapuu *et al.*, 2001b), and in *Drosophila*, the same relation exists between *biniou* and *decapentaplegic (dpp)* (Zaffran *et al.*, 2001), although here it is expressed dorsally, consistent with the inversion of the dorsoventral axis between chordates and arthropods. Also in *C. elegans* is a TGFβ-like ligand, UNC-129, responsible for dorsoventral patterning, and a forkhead protein, UNC-130, establishes the gradient by inhibiting *unc-129* expression ventrally (Nash *et al.*, 2000).

The vertebrate endoderm expresses several forkhead genes, e.g., *Foxa1*, *-2*, and *-3*. Formation of the epithelial gut tube requires *Foxa2*—the definitive endoderm is formed in *Foxa2*$^{-/-}$ embryos, but foregut morphogenesis is severely affected (Ang and Rossant, 1994; Weinstein *et al.*, 1994). A role for this class of genes in differentiation of the anterior gut appears to be an ancient feature in metazoan development; the *FoxA* ortholog in *Drosophila*, *forkhead*, is essential for morphogenesis of the anterior digestive tract (Weigel *et al.*, 1989), and in *C. elegans*, the closest relative of *FoxA* genes is *pha-4*, which specifies the cells of the pharynx (Gaudet and Mango, 2002; Horner *et al.*, 1998; Kalb *et al.*, 1998). During organogenesis in the mouse embryo, additional forkhead genes are involved in differentiation of specialized local epithelia that evaginate or migrate from the primitive gut and give rise to different organs. *Foxe1* is expressed in the foregut epithelium and the migrating thyroid precursor cells; null mutations in this gene cause thyroid agenesis in both mouse and man (Clifton-Bligh *et al.*, 1998; De Felice *et al.*, 1998; Macchia *et al.*, 1999). The product of the nude mouse gene, *Foxn1* (Nehls *et al.*, 1994), is expressed in the precursor cells of the thymic epithelium and is essential for their differentiation into the subcapsular, cortical, and medullary epithelial cells of the thymus (Nehls *et al.*, 1996). Foxa1 and -2 regulate gene expression in the lung epithelium (reviewed by Costa *et al.*, 2001); *Foxj1* specifies the ciliated cells in the proximal airway epithelium (Brody *et al.*, 2000; Chen *et al.*, 1998; Tichelaar *et al.*, 1999) and *Foxp2* is preferentially expressed in the distal cells (Shu *et al.*, 2001).

Nonendodermal epithelia require other forkhead proteins for proper function. Foxe3 promotes proliferation and blocks premature differentiation of the ectodermally derived lens epithelial cells (Blixt *et al.*, 2000). It is also required for closure of the lens vesicle and survival of the anterior lens epithelium (Blixt *et al.*, 2000; Brownell *et al.*, 2000). *Foxi1* is expressed in the mesodermal epithelium of the distal renal tubuli (Overdier *et al.*, 1997) and in the ectodermal epithelium of the otic vesicle (Hulander *et al.*, 1998). Homozygous *Foxi1* null mice are deaf and circle due to a severe malformation of the vestibulum and cochlea in the inner ear (Hulander *et al.*, 1998).

Mesodermally expressed forkhead genes participate in the epitheliomesenchymal cross talk and frequently influence the development and differentiation of associated epithelia. *Foxl1* is expressed in the gut mesenchyme and controls the proliferation of the intestinal epithelium by interfering with growth factor signaling (Kaestner *et al.*, 1997; Perreault *et al.*, 2001). *Foxf1* is expressed in the lung mesenchyme in response to sonic hedgehog signaling from the epithelium (Mahlapuu *et al.*, 2001a), and *Foxf1* heterozygotes exhibit lung hypoplasia, defects in branching morphogenesis, and disruption of the tight association between endothelial and epithelial cells (Kalinichenko *et al.*, 2001; Mahlapuu *et al.*, 2001a). *Foxd1*, which is expressed in the stromal cells of the kidney, controls the production of signals that are required for the normal transition of induced mesenchyme into tubular epithelium and growth and branching of the collecting system (Hatini *et al.*, 1996). *Foxd2* is also expressed in the developing kidney (Wu *et al.*, 1998) and some *Foxd2* null mutants have kidney hypoplasia and hydroureter (Kume *et al.*, 2000a). *Foxk1* is expressed selectively in myogenic stem cells (satellite cells) in adult mice. Skeletal muscles of *Foxk1*$^{-/-}$ animals are atrophic, and timing of expression of cell cycle regulators and myogenic determination genes is dysregulated.

In the neuroectoderm, *Foxg1* is essential for development of the cerebral hemispheres (Xuan *et al.*, 1995). Telencephalic neuroepithelial cells are specified in the *Foxg1* mu-

TABLE 3
Human Developmental Disorders Caused by Mutations in *FOX* Genes

Gene	Phenotype	Disease transmission	References
FOXC1	Various developmental defects in the anterior segment of the eye; congenital glaucoma, Axenfeld–Rieger anomaly	Autosomal dominant	Lehmann *et al.*, 2000; Mears *et al.*, 1998; Mirzayans *et al.*, 2000; Nishimura *et al.*, 1998, 2001
FOXC2	Lymphedema combined with distichiasis, ptosis and/or cleft palate	Autosomal dominant	Fang *et al.*, 2000; Finegold *et al.*, 2001
FOXE1	Thyroid agenesis, cleft palate, and choanal atresia	Autosomal recessive	Clifton-Bligh *et al.*, 1998
FOXE3	Malformations in the anterior segment of the eye including Peters' anomaly	Autosomal dominant	Ormestad *et al.*, 2002; Semina *et al.*, 2001
FOXL2	Blepharophimosis/ptosis/epicanthus inversus syndrome (BPES); can be associated with ovarian failure (BPES type I)	Autosomal dominant	Crisponi *et al.*, 2001; De Baere *et al.*, 2001; Prueitt and Zinn, 2001
FOXN1	T cell immunodeficiency combined with alopecia and dystrophic nails	Autosomal recessive	Frank *et al.*, 1999
FOXP2	Severe speech and language disorder	Autosomal dominant	Lai *et al*, 2001
FOXP3	Immune dysregulation, polyendocrinopathy, enteropathy syndrome (IPEX)	X-linked recessive	Bennett *et al.*, 2001; Ramsdell *et al.*, 2001

tant, but their proliferation is reduced and premature differentiation leads to early depletion of the progenitor population. FoxG1 is also, together with FoxD1, involved in topographical mapping of retinal neurons on the tectum (Yuasa *et al.*, 1996). The temporal part of the retina expresses *FoxD1* and the nasal *FoxG1*. Misexpression of either gene causes misprojection of retinal neurons on the tectum along the rostrocaudal axis (Yuasa *et al.*, 1996). The closest homolog of *FoxD1* in *C. elegans*, *unc-130*, also controls neuronal fates (Sarafi-Reinach and Sengupta, 2000), and in *Drosophila*, JUMEAUX determines the distinct cellular identities of two sibling neurons in the central nervous system (Cheah *et al.*, 2000). *FoxD3* promotes differentiation of neural crest from neural tube progenitors and appears to act downstream of *Pax3* and independent of *Slug* (Dottori *et al.*, 2001; Kos *et al.*, 2001; Sasai *et al.*, 2001). In neural crest, FoxD3 inhibits melanoblast development and thereby facilitates differentiation of other neural crest-derived cell types (Kos *et al.*, 2001). *Foxc1* is expressed in mesenchyme derived from the cephalic neural crest; the null mutant has hydrocephalus and defects in chondrogenesis, skeletal, and eye development (Hong *et al.*, 1999; Kume *et al.*, 1998). The FoxC homolog in *Drosophila*, CROCODILE, controls patterning of the anterior-most head segment primordium and development of head skeletal structures (Hacker *et al.*, 1995).

Two forkhead genes, *Foxn1* and *-q1*, are important for mammalian hair follicle development. Satin mice have a defect in hair shaft formation, which has been linked to mutations in *Foxq1* (Hong *et al.*, 2001). *Foxn1* promotes proliferation and inhibits differentiation of hair follicle epithelial cells (Brissette *et al.*, 1996; Prowse *et al.*, 1999). This mode of action, which leads to a hypoplastic mutant phenotype due to depletion of precursor cells, is very similar to that of *Foxe3* in the lens and *Foxg1* in the telencephalon.

FORKHEAD MUTATIONS IN HUMAN DISEASE

Developmental Genetic Disorders

Analysis of mutant phenotypes in mice has facilitated identification of mutations in human forkhead genes that cause congenital malformations. So far, mutations in eight different forkhead genes have been associated with human developmental disorders, including immune, skeletal, circulatory, and craniofacial defects (Table 3). Notably, four of the disorders include eye abnormalities. Mutations in *FOXC1* and *-E3* have been identified in patients with defects in development of the anterior chamber of the eye (Lehmann *et al.*, 2000; Mears *et al.*, 1998; Mirzayans *et al.*, 2000; Nishimura *et al.*, 1998, 2001; Ormestad *et al.*, 2002; Semina *et al.*, 2001). Mutations in *FOXL2* cause variable eyelid defects, sometimes associated with ovarian failure (Crisponi *et al.*, 2001; De Baere *et al.*, 2001), and mutations in *FOXC2* lead to distichiasis, or double rows of eyelashes, together with lymphedema (Bell *et al.*, 2001; Erickson, 2001; Fang *et al.*, 2000; Finegold *et al.*, 2001).

An intriguing spectrum of symptoms is exhibited by persons with mutations in *FOXP2* (Lai *et al.*, 2001). Affected individuals have a severe impairment of the selection and sequencing of fine orofacial movements, which are necessary for articulation. They have also deficits in language processing—such as the ability to break up words into phonemes—and grammatical skills, including comprehension of syntactical structure. Some have a nonverbal IQ close to the population average, which suggests that *FOXP2*

is only essential for neural mechanisms specifically involved in language and speech development.

The majority of mutations in forkhead genes that have been linked to developmental disorders in humans are substitutions or frameshifts that disable or remove the DNA binding domain. They are therefore, most likely, loss-of-function alleles. Defects due to mutations in *FOXE1* (thyroid agenesis, cleft palate, and choanal atresia) or *FOXN1* (alopecia and T cell immunodeficiency) have an autosomal recessive inheritance (Clifton-Bligh *et al.*, 1998; Frank *et al.*, 1999), and *FOXP3* mutations [immune dysregulation, polyendocrinopathy, enteropathy (IPEX) syndrome] are X-linked (Bennett *et al.*, 2001; Ramsdell *et al.*, 2001). However, *FOXC1*, *-C2*, *-E3*, *-L2*, and *-P2* exhibit an autosomal dominant mode of inheritance, presumably due to haploinsufficiency, which indicates that gene dosage is critical for normal development. Interestingly, *FOXC1* causes eye malformations, not only when the gene dosage is decreased, but also when it is increased due to chromosomal duplications (Lehmann *et al.*, 2000; Nishimura *et al.*, 2001). This is one of only three known examples where duplication and deletion of a single gene both cause disease, which further emphasizes the importance of precise control of forkhead gene expression levels in eye development (Caplen, 2001). The reason for the widespread sensitivity to alterations in gene dosage in the forkhead family can only be speculated on, but recent data on the *C. elegans Pha-4* gene in pharynx development suggest a possible mechanism (Gaudet and Mango, 2002). PHA-4 controls transcription of many pharyngeal genes directly, and the concentration of PHA-4 increases gradually during pharynx morphogenesis and differentiation. The relative affinity for PHA-4 of binding sites in promoters of pharyngeal genes appears to correlate primarily with the time point for onset of transcription and not with expression level. In complex morphogenetic processes where several cell types are involved, such as eye development, precise timing of gene activation is crucial. Changes in expression level of a transcription factor, caused by an altered gene dosage, may therefore result in premature or delayed activation of target genes and force morphogenetic processes out of step.

Tumor Diseases

The oncogene *Qin* of avian sarcoma virus 31 (ASV-31) is responsible for the transforming activity of the virus (Li and Vogt, 1993; reviewed by Vogt *et al.*, 1997). Its cellular counterpart, FoxG1, as well as the viral protein inhibit transcription of target genes, but the viral protein is a more potent repressor (Freyaldenhoven *et al.*, 1997; Li *et al.*, 1995). The colocalization of transforming and repressing domains in Qin suggests that this protein induces oncogenic transformation by down-regulating the expression of anti-mitotic genes (Li *et al.*, 1995).

A majority of chromosomal translocations that cause acute lymphoid leukemia (ALL) disrupt the gene encoding the transcription factor MLL (McCabe *et al.*, 1992). The oncogenic proteins that result from such chromosomal breaks are often fusion proteins consisting of the DNA-binding domain of MLL fused to the *trans*-activation domain of another transcription factor. Two of those factors are the forkhead proteins FOXO3 and FOXO4 (Borkhardt *et al.*, 1997; Hillion *et al.*, 1997; Parry *et al.*, 1994).

In alveolar rhabdomyosarcomas, chromosomal translocations generate chimeric transcripts that fuse the PAX3 or PAX7 DNA-binding domain with the *trans*-activation domain of FOXO1 (reviewed by Barr, 2001; Galili *et al.*, 1993). The chimeric proteins retain PAX3/PAX7 DNA binding specificity, but are more potent transcriptional activators than the wild-type proteins (Bennicelli *et al.*, 1999; Fredericks *et al.*, 1995; Sublett *et al.*, 1995). The fusion proteins are therefore believed to function as oncogenic transcription factors mainly through enhanced activation of normal PAX3/PAX7 targets. There are, however, indications that the specificity may also be altered; the PAX3–FOXO1 fusion protein upregulates the gene encoding the PDGFα receptor, although this gene is not normally a target for FOXO1 or PAX3 (Epstein *et al.*, 1998). The PDGFα receptor is a potent activator of PI3K (Porter and Vaillancourt, 1998), which in turn will inactivate FOXO proteins by inducing a shift in their subcellular localization. FOXO4 blocks cell cycle progression by activating the Cdk-inhibitor $p27^{kip1}$, and inactivation of FOXO4 stimulates proliferation (Medema *et al.*, 2000). FOXO1 and FOXO3 have been shown to regulate apoptosis (Brunet *et al.*, 1999; Dijkers *et al.*, 2000; Tang *et al.*, 1999). The transforming potential of the PAX3–FOXO1 fusion protein could therefore, at least in part, be due to its ability to indirectly inactivate native FOXO proteins.

CONTROL OF METABOLISM AND GENE EXPRESSION IN DIFFERENTIATED TISSUES

Many metazoan forkhead genes that control morphogenesis or differentiation in the embryo have distinct functions in the adult. In particular, metabolic processes, including glucose, lipid, and energy homeostasis, appear to be controlled by members of this gene family. As a majority of forkhead null mutants in mice are embryonically lethal, much of our understanding of target gene regulation in differentiated tissues is based on transient transfections in cell lines, *in vitro* DNA binding assays, etc., but recently the use of transgenic mice and conditional knockouts have provided new insights.

The most extensively studied are the FoxA proteins and their roles in liver, lung, and pancreas metabolism (reviewed by Costa *et al.*, 2001; Kaestner, 2000). FoxA1–A3 were discovered as proteins binding to the α1-antitrypsin and transthyretin promoters (Costa *et al.*, 1989). Subsequently, FoxA binding sites have been discovered in regulatory regions of more than 100 genes expressed in liver, pancreas, lung, and intestine. These putative target genes

include hepatic and pancreatic enzymes, surfactant proteins, serum proteins, and hormones (Cereghini, 1996; Costa *et al.*, 2001; Kaestner, 2000).

Foxa2-deficient embryos do not develop beyond E8.5 (Ang and Rossant, 1994; Weinstein *et al.*, 1994), and even chimeric embryos obtained from tetraploid embryo/ *Foxa2*$^{-/-}$ ES cell aggregates lacked foregut and midgut endoderm (Dufort *et al.*, 1998). Hence, the null mutant cannot be used to investigate the role of Foxa2 in organogenesis or metabolism. Instead, the function of Foxa2 was studied in visceral endoderm of embryoid bodies; an *in vitro* system that mimics fetal liver and pancreas (Duncan *et al.*, 1998). In this model, lack of *Foxa2* resulted in reduction of the mRNA levels for the POU-homeodomain transcription factor HNF-1α and the orphan nuclear receptor HNF-4α, and in loss of mRNA for Foxa1 as well as for serum lipoproteins. This result suggests that Foxa2 regulates a transcription factor network required for differentiation and metabolism in early liver and pancreas. However, a conditional knockout inactivating *Foxa2* specifically in hepatocytes toward the end of fetal development did not interfere with normal liver function or the overall hepatic transcriptional program (Sund *et al.*, 2000). Thus, Foxa2 seems to play a critical role in early liver development, but not to be required for maintenance of adult hepatocyte function. Deletion of *Foxa2* in pancreatic β-cells, on the other hand, results in hyperinsulinemic hypoglycemia, which demonstrates that Foxa2 is involved in control of insulin secretion in the differentiated pancreas (Sund *et al.*, 2001). The genes encoding both subunits of the β-cell ATP-sensitive K(+) channel [K(ATP)]—the most frequently mutated genes in familial hyperinsulinism in man—were identified as Foxa2 targets in islets (Sund *et al.*, 2001).

Mice homozygous for a *Foxa3* null mutation develop normally and are fertile (Kaestner *et al.*, 1998). The expression of several putative FoxA target genes in liver (phosphoenolpyruvate carboxykinase, transferrin, tyrosine amino-transferase) was reduced by 50–70%, indicating that Foxa3 is an activator of these genes *in vivo*. The *status quo* in mRNA levels of other hepatic genes—implicated as Foxa3 targets by *in vitro* assays—could be explained by compensatory binding of Foxa1 and Foxa2, the levels of which are increased in *Foxa3*$^{-/-}$ mice (Kaestner *et al.*, 1998). When fasted, *Foxa3*$^{-/-}$ mice exhibit a substantial drop in blood glucose, in spite of normal secretion of pancreatic hormones and upregulation of gluconeogenic enzymes (Shen *et al.*, 2001). Hepatic expression of the plasma membrane glucose transporter GLUT2 is significantly decreased in the mutant, which suggests that the hypoglycemia is caused by inefficient efflux of newly synthesized glucose from hepatocytes.

Hypoglycemia is seen also in *Foxa1*$^{-/-}$ mice, which die soon after birth with severe growth retardation. In this case, the hypoglycemia derives from a marked reduction in circulating levels of the gluconeogenetic hormone glucagon, which correlates with a 50–70% decrease in pancreatic islet mRNA levels for proglucagon (Kaestner *et al.*, 1999; Shih *et al.*, 1999).

Another example, which illustrates the functional switch from embryonic morphogenerator to adult, metabolic regulator, is FoxC2. As described above, *FoxC2* controls multiple aspects of mesoderm differentiation; null mice die *in utero* from vascular, skeletal, and kidney defects, and haploinsufficiency in man causes eye and lymphatic defects. In adults, however, high level expression of *FoxC2* is restricted to adipocytes (Cederberg *et al.*, 2001). Transcriptional regulation of *Foxc2* by insulin and TNFα and a selective hypoplasia of brown adipose tissue (BAT) in *Foxc2*$^{+/-}$ mice suggested that this gene is involved in controlling the balance between energy storage and dissipation. Transgenic overexpression of *FOXC2* in brown and white adipose tissue (WAT) has a remarkably pleiotropic effect on the gene expression profile (Cederberg *et al.*, 2001). Expression of the BAT-specific uncoupling protein is induced in WAT, which exhibits increases in lipolysis, mitochondrial content, and oxygen consumption. Circulating levels of free fatty acids, triglycerides, glucose and insulin are reduced, insulin sensitivity is enhanced and total body fat content is decreased. FoxC2 appears to regulate metabolic efficiency in response to the energy content of the diet; a high-fat diet upregulates *Foxc2* expression and induces less weight gain in the transgenic animals than in normal controls. An important mechanism behind many of these effects appears to be increased sensitivity to β-adrenergic stimuli, caused by an isoenzyme shift in adipocyte PKA holoenzyme, which lowers the threshold concentration required for PKA activation. Elevated expression of FoxC2 thus counteracts most symptoms associated with obesity which predisposes to insulin resistance and type 2 diabetes.

The role of proteins in the FoxO subfamily in insulin/IGF signaling, discussed above, is another example of control of glucose and energy metabolism by forkhead proteins.

CONCLUSIONS AND FUTURE PERSPECTIVES

As should be evident from this overview, there is no simple answer to the question of what forkhead proteins do. If a unifying theme is to be found, it is likely to be in the mechanisms of interaction with chromatin and the transcription machinery, although studies of many more proteins will be needed to confirm this. A reasonable assumption is that the first forkhead genes arose in unicellular, or very simple multicellular, organisms and that their function was in fundamental cell metabolism. This pattern is seen today in *Fkh1* and -2 in yeast, and in mammalian *FoxO* and *-M1* genes, which are ubiquitously expressed and involved in cell cycle and growth regulation. In sequence alignments, these genes represent outgroups in the forkhead family, which supports their anciennity. The metazoan forkhead main group, on the other hand, appears to have undergone a more recent expansion, presumably

linked to the evolving anatomical complexity of animal body plans. Members of this group have tissue-specific expression patterns and are in general involved in cell-type determination and differentiation. A typical example is the subdivision of mesoderm in distinct populations, each expressing their characteristic subset of forkhead genes. In the differentiation process, forkhead proteins are often involved in sustaining proliferation of determined precursor cells, as well as in expression of differentiated traits. In many cases, genes responsible for differentiation processes during embryonic development are later recycled and control metabolism in the adult. At the sequence level, a strong conservation of the DNA binding domain often contrasts with an extensive divergence in other regions, indicating that the forkhead domain is compatible with multiple arrangements of transcriptional effector or signal transduction domains.

The number of forkhead genes for which we have loss-of-function data has increased dramatically in the last 5 years, and within the next 5 we can expect to have some kind of description of the mutant phenotype of all forkhead genes in the major model organisms. The next challenges will be to resolve issues of functional redundancy by creating combined mutants and overcome embryonic lethality with conditional knockouts to analyze functions in later stages or adults. In these exciting areas, the first papers have just recently been published. Another important subject will be to analyze in greater depth the mechanisms of transcriptional control, interactions with chromatin modifying enzymes, signal transducing molecules, etc. A better understanding of the molecular mechanisms of target gene interactions and transcriptional regulation may explain why the expression level (gene dosage) is so critical for many developmental processes in which forkhead proteins are involved.

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