REVIEW ARTICLE
In control of biology: of mice, men and Foxes

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Forkhead proteins comprise a highly conserved family of transcription factors, named after the original forkhead gene in *Drosophila*. To date, over 100 forkhead genes have been identified in a large variety of species, all sharing the evolutionary conserved 'forkhead' DNA-binding domain, and the cloning and characterization of forkhead genes have continued in recent years. Forkhead transcription factors regulate the expression of countless genes downstream of important signalling pathways in most, if not all, tissues and cell types. Recent work has provided novel insights into the mechanisms that contribute to their functional diversity, including functional protein domains and interactions of forkheads with other transcription factors. Studies using loss- and gain-of-function models have elucidated the role of forkhead factors in developmental biology and cellular functions such as

metabolism, cell division and cell survival. The importance of forkhead transcription factors is underlined by the developmental defects observed in mutant model organisms, and multiple human disorders and cancers which can be attributed to mutations within members of the forkhead gene family. This review provides a comprehensive overview of current knowledge on forkhead transcription factors, from structural organization and regulatory mechanisms to cellular and developmental functions in mice and humans. Finally, we will discuss how novel insights gained from involvement of 'Foxes' in the mechanisms underlying human pathology may create new opportunities for treatment strategies.

Key words: cell cycle, development, forkhead, Fox, immunoregulation, transcription factor.

FORKHEAD TRANSCRIPTION FACTORS

Spatial and temporal gene expression patterns are tightly regulated by tissue-specific transcription factors that bind to regulatory DNA sequence elements located proximal or distal to the promoter region. Such transcription factors are often assigned to different families on the basis of the conservation of their DNA-binding domain. One such family comprises the forkhead family of transcription factors, named after the first gene identified containing this highly conserved DNA-binding domain in *Drosophila* [1–3]. Mutations in this gene cause homoeotic transformation of certain gut structures, resulting in replacement of both fore- and hind-gut by ectopic spike-formed head structures, hence the name forkhead [1]. Since the existence of a forkhead family was acknowledged, over 100 forkhead genes have been identified in species ranging from yeast to human, although not in plants. Interestingly, the number of forkhead genes in different species rises with increased anatomical complexity, from four known family members in *Saccharomyces cerevisiae* to over 35 in mouse and human. In 2000, a unified nomenclature was introduced which reflects the phylogenetic origin for all known chordate forkhead genes [4], and has replaced diverse names such as *fkh* (forkhead), *FREAC* (forkhead-related activator), *HFH* (hepatocyte nuclear factor 3/forkhead homologue) and *FKHR* (forkhead in rhabdomyosarcoma). In this nomenclature, each family member is designated, e.g. *Foxd2*, whereby Fox reflects the presence of the forkhead box, and the trailing letter refers to the subfamily, currently ranging from A to S, while the number

distinguishes between subfamily members. Species differences are indicated by the use of uppercase letters for human (e.g. *FOXD2*), a capital F for mouse (e.g. *Foxd2*) and the first and subclass letters uppercase for all other chordate species (e.g. *FoxD2*). The classification into subfamilies is based on the structural similarities and conservation levels in the forkhead DNA-binding domain (Figure 1).

The importance of forkhead transcription factors in embryonic development was quickly recognized (reviewed in [5,6]), and our knowledge of their function in developmental biology has expanded since then through the increased availability of mouse mutants. However, a wealth of data have accumulated in recent years that demonstrate an important role for forkhead proteins in the regulation of cellular processes such as metabolism and cell-cycle regulation. Also, the signalling pathways that govern forkhead function are starting to be elucidated, and provide insights into the mechanisms that contribute to their functional diversity. In this review, we provide an overview of current knowledge about forkhead transcription factors and their roles in the biology of mice and humans.

FORKHEAD ARCHITECTURE AND ORGANIZATION

Forkhead domain

All forkhead transcription factors share a highly conserved DNA-binding domain, named after the *Drosophila* forkhead gene. The three-dimensional structure of the forkhead domain has been

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                α1        β1   α2                    α3        β2   W1     β3    W2

Foxo3a  NAWGNLSYADLITRAIESSPDKRLTLSQIYEWMVRCVPYFKDKGDSNSSAGWKNSIRHNLSLHSRFMRVQNEG...TGKSSWWIINPDGGKSGK
Foxo6   NAWGNLSYADLITKAIESAPDKRLTLSQIYDWMVRYVPYFKDKGDSNSSAGWKNSIRHNLSLHTRFIRVQNEG...TGKSSWWMLNPEGGKTGK
Foxo1   NAWGNLSYADLITKAIESSAEKRLTLSQIYEWMVKSVPYFKDKGDSNSSAGWKNSIRHNLSLHSKFIRVQNEG...TGKSSWWMLNPEGGKSGK
Foxo4   NAWGNQSYAELISQAIESAPEKRLTLAQIYEWMVRTVPYFKDKGDSNSSAGWKNSIRHNLSLHSKFIKVHNEA...TGKSSWWMLNPDGGKGGK
Foxp1   EVRPPFTYASLIRQAILESPEKQLTLNEIYNWFTRMFAYFRR....NAAT.WKNAVRHNLSLHKCFVRVENV......KGAVWTVDEVEFQKRR
Foxp4   DVRPPFTYASLIRQAILETPDRQLTLNEIYNWFTRMFAYFRR....NTAT.WKNAVRHNLSLHKCFVRVENV......KGAVWTVDEREYKRR
Foxp2   DVRPPFTYATLIRQAIMESSDRQLTLNEIYSWFTRTFAYFRR....NAAT.WKNAVRHNLSLHKCFVRVENV......KGAVWTVDEVEYQKR
Foxp3   NMRPPFTYATLIRWAILEAPERQRTLNEIYHWFTRMFAYFRN....HPAT.WKNAIRHNLSLHKCFVRVESE.....KGAVWTVDEFEFRKKR
Foxn1   FPKPIYSYSILIFMALKNSKTGSLPVSEIYNFMTEHFPYFK.....TAPDGWKNSVRHNLSLNKCFEKVENKSGSSSRKGCLWALNPSKIDKMQEELQ
Foxn4   YPKPIYSYSCLIAMALKNSKTGSCPVSEIYSFM.KTLPYFK.....TAPDGWKNSVRHNLSLNKCFEKVETKSSGSSSRKGCLWALNLARIDKMEEEMH
Foxn2   TSKPPYSFSLLIYMAIEHSPNKCLPVKEIYSWILDRFPYFA.....TAPTGWKNSVRHNLSLNKCFQKVERSHGKVNGKGSLWCVDPEYKPNLMQALK
Foxn3   NCKPPYSFSCLIFMAIEDSPTKRLPVKDIYNWILEHFPYFA.....NAPTGWKNSVRHNLSLNKCFKKVDKERSQSIGKGSLWCIDPEYRQNLIQALK
Foxj2   DGKPRYSYATLITYAINSSPAKKMTLSEIYRWICDNFPYYKN.....AGIGWKNSIRHNLSLNKCFRKVPRPRDDP.GKGSYWTID....TCPDISRKRR
Foxj3   DGKPPYSASLITFAINSSPKKKMTLSEIYQWICDNFPYYRE.....AGSGWKNSIRHNLSLNKCFLKVPRSKDDP.GKGSYWAID....TNPKEDTLP
Foxj1   HVKPPYSYATLICMAMQASKATKITLSAIYKWITDNFCYFRH.....ADPTWQNSIRHNLSLNKCFIKVPREKDEP.GKGGFWRID....PQYAERLLSGA
Foxk1   ESKPPYSYAQLIVQAISSAQDRQLTLSGIYAHITKHYPYYRT.....ADKGWQNSIRHNLSLNRYFIKVPRSQEEP.GKGSFWRID....PASEAKLVEQAF
Foxa1   HAKPPYSYISLITMAIQQAPSKMLTLSEIYQWIMDLFPYYRQ.....NQQRWQNSIRHSLSFNDCFVKVARSPDKP.GKGSYWTLH....PDSGNMFENGC
Foxa3   HAKPPYSYISLITMAIQQAPGKMLTLSEIYQWIMDLFPYYRE.....NQQRWQNSIRHSLSFNDCFVKVARSPDKP.GKGSYWALH....PSSGNMFENGC
Foxa2   HAKPPYSYISLITMAIQQSPNKMLTLSEIYQWIMDLFPYRQ.....NQQRWQNSIRHSLSFNDCFLKVPRSPDKP.GKGSFWTLH....PDSGNMFENGC
Foxb1   DQKPPYSYISLTAMAIQSSPEKMLPLSEIYKFIMDRFPYYRE.....NTQRWQNSLRHNLSFNDCFIKIPRRPDQP.GKGSFWALH....PSCGDMFENGS
Foxb2   DQKPPYSYISLTAMAIQHSAEKMLPLSDIYKFIMERFPYYRE.....HTQRWQNSLRHNLSFNDCFIKIPRRPDQP.GKGSFWALH....PDCGDMFENGS
Foxf1   PEKPPYSYIALIVMAIQSSPSKRLTLSEIYQFLQARFPFFRG.....AYQGWKNSVRHNLSLNECFIKLPKGLGRP.GKGHYWTID....PASEFMFEEGS
Foxf2   PEKPPYSYIALIVMAIQSSPSKRLTLSEIYQFLQARFPFFRG.....AYQGWKNSVRHNLSLNECFIKLPKGLGRP.GKGHYWTID....PASEFMFEEGS
Foxd1   LVKPPYSYIALITMAILQSPKKRLTLSEICEFISSRFPYYRE.....KFPAWQNSIRHNLSLNDCFVKIPREPGNP.GKGNYWTLD....PESADMFDNGS
Foxd2   LVKPPYSYIALITMAILQSPKKRLTLSEICEFISGRFPYYRE.....KFPAWQNSIRHNLSLNDCFVKIPREPGNP.GKGNYWTLD....PESADMFDNGS
Foxd3   LVKPPYSYIALITMAILQSPQKKLTLSGICEFISNRFPYYRE.....KFPAWQNSIRHNLSLNDCFVKIPREPGNP.GKGNYWTLD....PQSEDMFDNGS
Foxd4   PAKPPYSYIALITMAILQSPHKRLTLSGICAFISGRFPYYRR.....KFPAWQNSIRHNLSLNDCFVKIPREPGHP.GKGNYWSLD....PASQDMFDNGS
Foxe1   RGKPPYSYIALIAMAIAHAPERRRLTLGGIYKFITERFPYYRD....NPKKWQNSIRHNLTLNDCFLKIPREAGRP.GKGNYWALD....PNAEDMFESGS
Foxe3   RGKPPYSYIALIAMALAHAPGRRLTLAAIYRFITERFAFYRD.....SPRKWQNSIRHNLTLNDCFVKVPREPGNP.GKGNYWTLD....PAAADMFDNGS
Foxi1   LVRPPYSYSALIAMAIHGAPDQRLTLSQIYQYVADNFPFYNK.....SKAGWQNSIRHNLSLNDCFKKVPRDEDDP.GKGNYWTLD....PNCEKMFDNGN
Foxi2   LVRPPYSYSALIAMAIQSAPLRRLTLSQIYQYVAGNFPFYKR.....SKAGWQNSIRHNLSLNDCFKKVPRDENDP.GKGNYWTLD....PNCEKMFDNGN
Foxc1   MVKPPYSYIALITMAIQNAPDKKITLNGIYQFIMDRFPFYRD.....NKQGWQNSIRHNLSLNECFVKVPRDDKKP.GKGSYWTLD....PDSYNMFENGS
Foxc2   LVKPPYSYIALITMAIQNAPEKKITLNGIYQFIMDRFPFYRE.....NKQGWQNSIRHNLSLNECFVKVPRDDKKP.GKGSYWTLD....PDSYNMFENGS
Foxl1   PQKPPYSYIALIAMAIQDAPEQRVTLNGIYQFIMDRFPFYHD.....NRQGWQNSIRHNLSLNECFVKVPREKGRP.GKGSYWTLD....PRCLDMFENGN
Foxs1   PTKPPYSYIALIAMAIQSSPGQRATLSGIYRYIMGRFAFYRH.....NRPGWQNSIRHNLSLNECFVKVPRDDRKP.GKGSYWTLD....PDCHDMFQHGS
Foxl2   AQKPPYSYVALIAMAIRESAEKRLTLSGIYQYIIAKFPFYEK.....NKKGWQNSIRHNLSLNECFIKVPREGGGE.RKGNYWTLD....PACEDMFEKGN
Foxg1   YEKPPYSYNALIMMAIRQSPEKRLTLNGIYEFIMKNFPYYRE.....NKQGWQNSIRHNLSLNKCFVKVPRHYDDP.GKGNYWMLD....PSSDDVFIGGT
Foxq1   RPKPPYSYIALIAMAIRDSAGGRLTLAEINEYLMGKFPFFRG.....SYTGWRNSVRHNLSLNDCFVKVLRDPSRPWGKDNYWMLN....PNSEYTFADGV
Foxh1   HDKPPYTYLAMIALVIQAAPFRRLKLAQIIRQVQAVFPFFRD.....DYEGWKDSIRHLSSNRCFHKVPKDPAKPQAKGNFWAVDVSLIPAEALR
Foxm1   SERPPYSYMAMIQFAINSTERKRMTLKDIYTWIEDHFPYFKH....IAKPGWKNSIRHNLSLHDMFVR....ETSANGKVSFWTIHPSANRYLTLD
        ●●●●●●  ●●  ●●  ●  ●●●  ●●      ●●●●      ●  ●●●●●●●●●● ●● ●●        ●●  ●  ●                  ●
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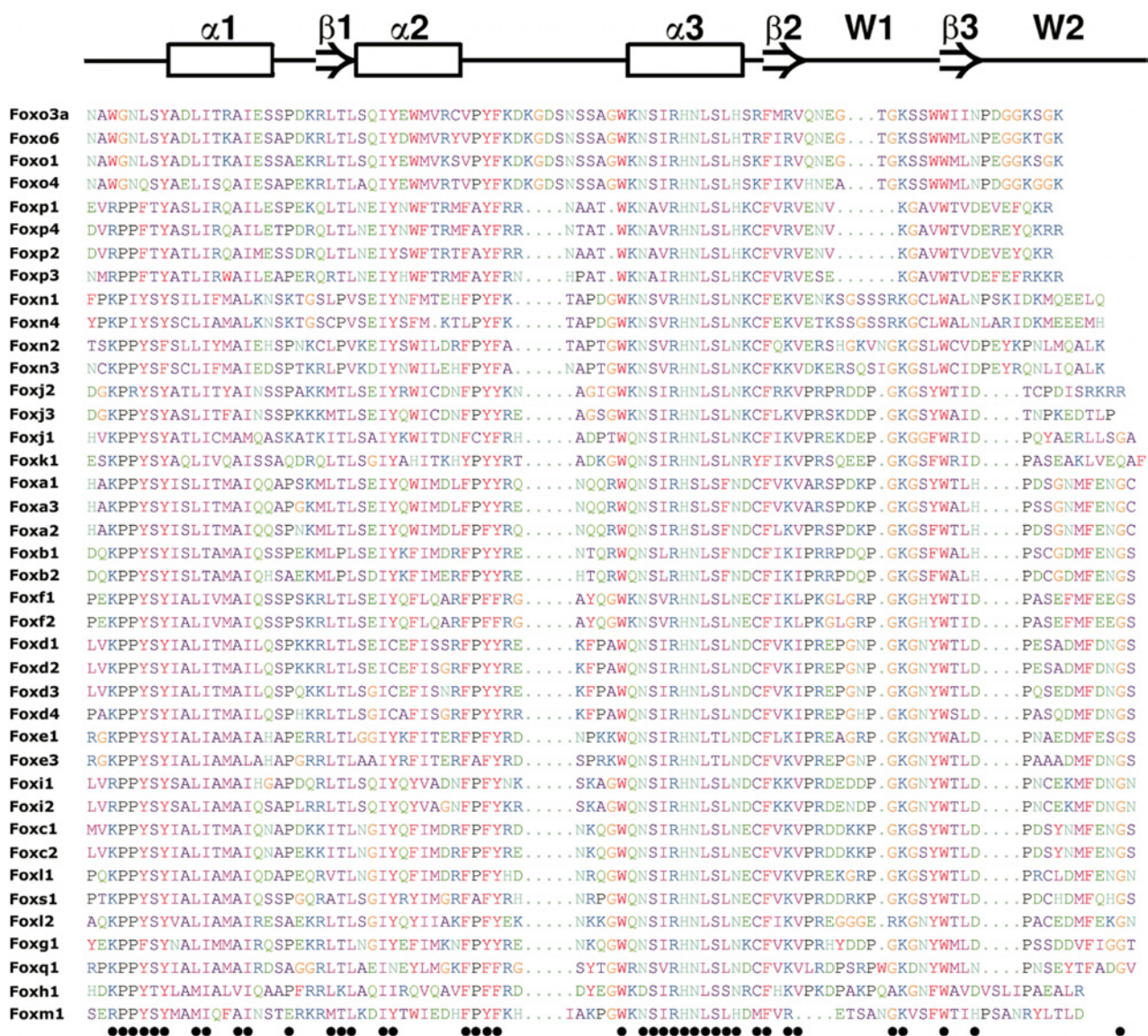
Figure 1   Alignment of murine forkhead domains showing the high degree of amino acid conservation

Above the alignment, the approximate position of the structural features are depicted, taken from the human FOXK2 factor [9]. Colour coding refers to the relative similarity between amino acid residues based on their physicochemical properties (e.g. the aromatic residues phenylalanine, tyrosine and tryptophan are depicted in red). Below the alignment, dots indicate residues that are highly conserved (similar in > 75 % of cases). Note the high degree of conservation in the third α-helix (α3), also referred to as the recognition helix. The short insertion in the Foxo subclass between helices 2 and 3 has little effect on domain structure [12]. α1–α3, α-helices 1–3; β1–β3, β-sheets 1–3; W1–W2, wings 1 and 2. Dots in the alignment represent gaps.

characterized by both X-ray crystallography and NMR spectroscopy. Its apparent resemblance to the shape of butterflies, in addition to the comparable composition with the helix–turn–helix motif, resulted in the label 'winged-helix domain' [7]. Not all winged-helix proteins are forkhead transcription factors, however, and other evolutionarily unrelated winged–helix proteins have been identified [8]. The canonical forkhead domain consists of three α-helices, three β-sheets and two loops or wings, typically arranged in an α1-β1-α2-α3-β2-W1-β3-W2 order (Figure 1). Variations on this order include additional α-helices [9,10] and missing β-sheets or wings [11,12] and, although DNA binding generally does not cause major structural changes, an additional helix is formed in Foxd3 upon DNA binding [13]. Conservation of the functional structure of the forkhead domain is mirrored at the amino acid level over approx. 100 residues (Figure 1). High sequence similarity exists in the α-helices and β-sheets, parti-

cularly in the third helix, but lower identity exists in the wings and in between the second and third helices, which are notably the locations of most structural variations.

Information on how binding specificity is conferred comes from work on the three-dimensional structure of forkhead domains bound to matching DNA sequences. Although multiple contacts with the sugar–phosphate backbone occur, the primary binding interaction is through the third α-helix, dubbed the recognition helix, which binds target DNA directly in the major groove [7,14]. Interactions with the minor groove through binding of the second wing can also influence binding stability and specificity. The first wing, although contacting DNA, does not contribute significantly to the protein–DNA interactions [8,13]. Considering the high degree of sequence and structural conservation, especially in the recognition helix (Figure 1), all forkhead transcription factors are thought to bind in a similar fashion. Since most residues

that are in contact with the target DNA are highly conserved, additional features must exist that govern sequence specificity, and variations in amino acid sequence N-terminal to the recognition helix and in the second wing are thought to be involved [9,15,16]. Differences in electrostatic distribution are also thought to contribute [12], but the mechanisms of DNA-binding specificity clearly require additional research. Unlike certain other transcription factor families, forkhead transcription factors usually bind target DNA sequences as monomers. The FoxP subfamily is an exception, however, as recent findings suggest that Foxp1, Foxp2 and Foxp4 require dimerization for DNA binding [17]. This dimerization, mediated by a leucine zipper domain, leads to swapping of part of the forkhead domain, including the recognition helix [10]. Interestingly, the three-dimensional structure of FOXP2 bound to DNA implies that FoxP dimers cannot bind adjacent DNA elements, suggesting that FoxP factors might require or regulate more intricate protein–DNA complexes and chromatin structure.

The conserved recognition helix binds to different DNA sequences, all complying with the core consensus sequence A/C-A-A-C/T-A, which is required but not sufficient for binding [12,15,16]. Additional flanking sequences are essential for ensuring highly specific binding, complicating formulation of longer consensus sequences useful for, e.g. *in silico* genomic screening. Members of the same forkhead subfamily, however, have the ability to bind to highly similar DNA sequences, e.g. FoxO factors, which can all recognize and bind to the consensus sequence T/C-G/A-A-A-A-C-A-A [18].

In contrast with the highly conserved forkhead domain, few similarities exist between regions outside the DNA-binding domain of different forkhead family members. Still, several conserved domains and motifs have been identified in multiple forkhead factors, usually within specific subclasses. Often, common features have first been identified in one forkhead subfamily, usually the extensively studied FoxO subclass, after which similar properties have been progressively uncovered in other forkheads.

Transactivation domains

Forkhead factors differ in their transcriptional regulatory properties in that they can act as either activators or repressors of target gene expression. In the last few years, data have accumulated suggesting that several forkhead factors may direct gene expression either way, depending on recruitment and interaction with cofactors or co-repressors. Table 1 lists current knowledge on transactivational properties of most forkhead proteins. In general, members of the same subfamily share similar activating or repressing functions, although there are several clear exceptions to this rule. The inconsistencies in transactivational properties between forkhead factors are reflected by the lack of conserved conventional activation or repression domains, such as basic or acidic stretches or regions enriched for specific amino acid residues. Between subfamily members, however, non-canonical sequence similarities have been observed in the case of the FoxA and FoxF families [19–21]. In Foxd2 and Foxd3, an acidic patch has been identified N-terminal to the forkhead domain. Such acidic domains, however, have been identified as regions required for transactivation, whereas Foxd2 and Foxd3 generally function as repressors of gene expression. Other repressors, particularly Foxp1 and Foxp2, share a similar repressor domain N-terminal relative to the forkhead domain, which includes a zinc-finger motif [22]. They also contain a typical polyglutamine stretch that influences repressor activity and is lacking in a particular splice variant [23].

Table 1 Transcription regulatory properties of forkhead transcription factors

Listed are forkhead factors for which evidence for transcriptional activation (+) or repression (−) is available (third column). Note that for some family members, both activation and repression have been reported (+/−). FoxH1 reportedly lacks any transcriptional activation properties and depends on interactions with Smad factors for target gene expression [24].

Forkhead	Activation (+) or repression (−)	Reference(s)
FoxA	+	[22,200,201]
FoxC1	+	[28]
FoxC2	+/−	[202,203]
FoxD1	+	[204]
FoxD2	+/−	[137,202]
FoxD3	−	[205]
FoxE1	−	[206]
FoxF1–FoxF2	+	[19,20]
FoxG1	−	[207]
FoxH1	+	[208]
FoxI1	+	[209]
FoxJ1–FoxJ2	+	[29,210]
FoxK1	−	[211]
FoxK2	+	[212]
FoxL2	−	[213]
FoxM1	+	[85]
FoxN1	+	[214]
FoxN3	−	[84]
FoxO1–FoxO6	+/−	[30,215,216]
FoxP1–FoxP4	−	[17,22,217]
FoxQ1	−	[218]

NLSs (nuclear localization signals)

For transcription factors to exert their gene-regulatory function, they must be located in the nucleus. NLSs have been identified in most gene family members, all confined to regions of the forkhead domain. A well-characterized bipartite NLS is shared by all members of the FoxO subfamily, located in the C-terminal end of the forkhead domain [25,26], whereas an additional NLS is present in FOXO1 [27]. Interestingly, a PKB (protein kinase B or Akt) phosphorylation motif located within the conserved NLS has a major impact on its functional abilities, acting in concert with other post-translational modifications to drive FoxO factors out of the nucleus (see below). In the case of FoxA2, FOXC1, FOXF2 and FOXJ2, nuclear localization is dependent on two amino acid stretches at both N-terminal (α1) and C-terminal (W2) ends of the forkhead domain ([19,21,28,29], and see Figure 2). Considering the high degree of sequence similarity within the forkhead domain, it is tempting to speculate that NLSs may be similarly arranged in other forkhead family members.

Phosphorylation motifs

Forkhead activity can be controlled by post-translational modifications in addition to gene expression levels. The most extensively studied modification is phosphorylation of FoxO proteins by PKB upon stimulation with insulin-like growth factors (reviewed in [26]). Three conserved PKB-recognition sites exist in FoxO subfamily members (Figure 2), one of which is lacking in FoxO6 [30]. A single PKB site is present in the N-terminus of the forkhead domain, which upon phosphorylation affects DNA binding and transactivational potential [31]. Phosphorylation of all three PKB motifs, in combination with SGK (serum- and glucocorticoid-induced protein kinase), CK1 and DYRK1A (dual-specificity tyrosine-phosphorylated and -regulated kinase 1A) phosphorylation of serine residues adjacent to the C-terminal PKB site [32–34], results in nuclear exclusion and sequestration in the
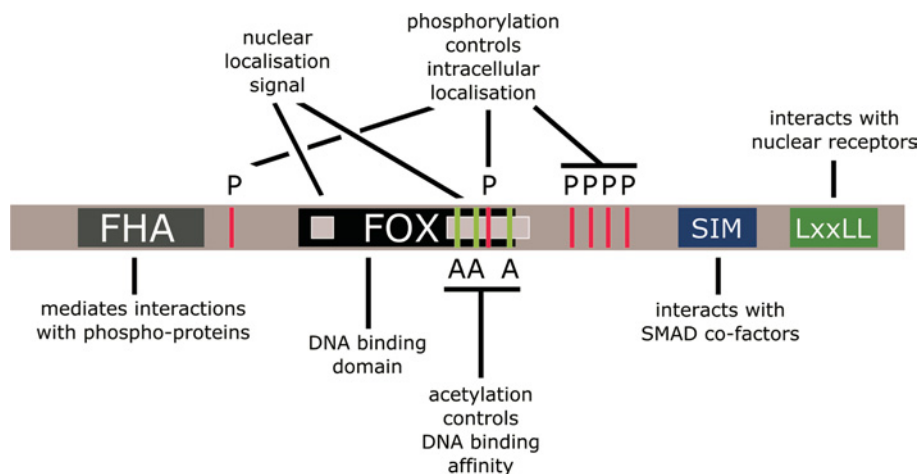
Figure 2   Domains and motifs in forkhead transcription factors

A graphical display of the relative positions and functions of known regulatory elements in the primary protein structure of forkhead factors. FHA recognizes and binds phosphorylated threonine residues on partner proteins. FOX binds target DNA sequences in regulatory regions of target genes. In addition, it is involved in interactions with homeodomain proteins, although the subdomains responsible are as yet unknown. SIM binds Smad transcription factors at target promoters. LXXLL, lysine-rich amino acid motif (where X denotes any residue), implicated in the interaction with nuclear receptor proteins. White boxes within the forkhead domain reflect the presence of a bipartite NLS within the forkhead domain. P above the red lines refers to residues that can be phosphorylated by PKB, IKKβ, SGK, CK1 or DYRK1A, resulting in nuclear export of the protein. A below the green lines indicates the positions of lysine residues that can be acetylated, thereby attenuating DNA-binding affinity. Note that this is a schematic overview, and that these domains and motifs are not all present in any one particular forkhead protein. See text for details and references.

cytosol, thereby terminating FoxO functional activity [35–37]. Additionally, a C-terminally located serine residue in FOXO3a can be phosphorylated by IKKβ [IκB (inhibitory κB) kinase β] [38], a factor known to activate NF-κB (nuclear factor κB) signalling through inhibition of IκB. Subsequently, phosphorylated FoxO factors can be ubiquitinylated by Skp2 [39], a subunit of the SCF (Skp2/cullin/F-box) E3 ubiquitin ligase complex, targeting them for proteasomal degradation [40,41]. FoxA2 is the only forkhead factor not in the FoxO subfamily that is also regulated by such insulin-responsive, PKB-mediated translocation control [42], albeit by phosphorylation of a non-canonical PKB motif. Still, the consensus RXRXX{S/T} motif that is recognized by PKB [43] can also be found in other members, raising the possibility of PKB control of other forkhead factors.

Another potentially interesting feature consists of post-translational modifications under the control of the Ras–Ral pathway. Ral-dependent phosphorylation of C-terminal threonine residues by JNK (c-Jun N-terminal kinase) has been reported for FOXO4, which affects transcriptional competence independently of nuclear shuttling [44,45]. Whether such a regulatory pathway applies to all FoxO and other family members remains to be determined, especially since these phosphorylation sites do not appear to be highly conserved.

Acetylation motifs

In addition to regulation by PKB phosphorylation, a second pathway has been identified that regulates FoxO transcriptional activity. Direct acetylation of FoxO factors by CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300 or deacetylation by Sir2 can influence transcriptional regulation of downstream target genes [46–49]. The mechanisms whereby CBP/p300 and Sir2 bind and modify FoxO factors and how this influences FoxO functioning are still not entirely clear [50]. It has been proposed, however, that acetylation of positively charged lysine residues in the forkhead domain (Figure 2) attenuates the ability of FoxO proteins to bind target DNA, thereby reducing their transcriptional activity [51]. The same authors have also shown that the initial acetylation sensitizes FoxO factors

to phosphorylation by PKB [51]. The widespread occurrence of consensus acetylation sites in forkhead proteins suggests the possible existence of a common regulatory pathway involving acetylation.

FHA (forkhead-associated domain)

Alignment of a subset of forkhead transcription factors from multiple species led to the identification of a second conserved domain, designated the FHA domain ([52]; see Figure 2). With the cloning of additional forkhead genes, however, it became clear that it is not a common feature for forkhead factors to contain such an FHA domain, and none have been identified outside the FoxK subfamily. Although the FHA domain is a highly conserved structural module, this does not apply to the amino acid sequence, which is highly divergent. It is found in proteins in a wide variety of species, from bacteria to humans, and its presence in *Arabidopsis* suggests that the FHA domain stems from earlier periods in ancient evolutionary history than the forkhead DNA-binding domain. It specifically recognizes and binds phosphorylated threonine residues on target proteins, thereby mediating protein–protein interactions. FHA domains have been identified in proteins ranging from kinases to ubiquitin ligases, implicated in divergent processes such as protein degradation and vesicular trafficking, but the majority are involved in checkpoint and cell-cycle control [53,54]. An important role for the FHA domain has been postulated in expression timing of a whole set of genes referred to as the *Clb2* cluster in *S. cerevisiae* (reviewed in [55]). Here, the forkhead transcription factor Fkh2 is dependent on its FHA domain for interactions with the phosphorylated Ndd1 cofactor and subsequent transcriptional activation [56,57], providing a very elegant mechanism for tightly controlled transcriptional regulation.

SIDs [Smad (similar to mothers against decapentaplegic)-interaction domains]

Forkhead transcription factors have been implicated in signalling pathways of the TGFβ (transforming growth factor β)

superfamily. Activation of TGFβ receptors leads to phosphorylation and formation of Smad protein complexes which subsequently translocate to the nucleus where they mediate transcriptional regulation of downstream target genes [58]. FoxH1 was identified as a cofactor for SMAD complexes, through direct binding of its C-terminal SID [59,60]. Comparison of the FoxH1 SID with other Smad-interacting proteins resulted in the identification of two Smad-interaction motifs, PPNK [SIM (Smad-interaction motif)] and LPTSY or PN{V/A}V{A/M}P{L/P} [FM (FoxH1 motif)] in the C-terminus of FoxH1 (Figure 2), which contact the MH2 (mothers against decapentaplegic homology 2) domain of Smad2 [61,62]. Recently, FoxO factors were shown to interact directly with Smad complexes at the *p21^Cip1* locus [63]. This connection is achieved through binding of the FoxO DNA-binding domain to the MH1 domain in Smad3 and Smad4. Interestingly, the transcriptional repressor FoxG1 can bind directly to the FoxO–Smad complex through a C-terminal domain, thereby inhibiting the transactivation of *p21^Cip1* [63]. Similarly, FoxG1 can inhibit TGFβ signalling through comparable interactions with FoxH1 [64]. To our knowledge, no SID nor FM sequences have been identified in forkhead factors other than FoxH1, suggesting the existence of other interaction domains.

Nuclear receptor interaction motifs

Forkhead transcription factors are known to interact with members of nuclear receptor families. Members of the FoxO subfamily in particular are known to associate with multiple nuclear receptors [50], including the androgen receptor [65], the glucocorticoid receptor [66] and the retinoic acid receptor [66]. These interactions are thought to be mediated by a short motif containing the amino acid sequence LXXLL [26], located in the C-terminal region of all FoxO factors (Figure 2). Interestingly, this motif is also found in other forkhead proteins, including Foxm1, Foxk1 and all members of the Foxp subfamily. However, interactions with nuclear receptors have also been demonstrated for other forkheads lacking the LXXLL motif, such as FoxA members and FoxH1 [67,68], suggesting that other structural attributes may be involved. The general consequence of forkhead interactions with nuclear receptors is highly inconsistent, as they can act both to enhance or interfere with each other's function [67,69,70]. Receptor type, nuclear receptor ligand binding, phosphorylation status and cell type all determine the final outcome of a particular interaction.

Homeodomain interactions

As forkhead factors generally recognize and bind similar DNA elements, interactions with members of other transcription factor families could underlie specificity for gene activation of particular downstream targets. This view is supported by observations of direct protein–protein interactions between forkhead factors and homeodomain-containing transcription factors. FOXC1, FoxD3 and, in particular, Foxa2 have been shown to interact with several homeodomain proteins, including PITX2 (paired-like homeodomain transcription factor 2), Oct-4 (octamer 4), Pdx-1 (pancreas duodenum homeobox 1) and HOXA10 (homeobox A10) and engrailed [71–74]. Similar to nuclear receptor interactions, the result of interactions between these factors can vary, and is dependent on issues such as cell type and the presence of DNA-binding elements for both factors in target gene promoters.

Strikingly, the interaction between forkhead and homeodomain factors appears to be mediated by their DNA-binding domains. As the forkhead domain is the most conserved domain in forkhead factors, this suggests a general mechanism of protein–protein

interactions between members of these two transcription factor families. Not surprisingly, Foxa2 is able to bind to most of the aforementioned and other homeodomain proteins [72]. If target specificity was to benefit from this interaction, however, one would expect that some degree of selectivity would be coded for in the forkhead domain. It will therefore be interesting to learn which specific components of this domain are involved in this interaction.

MECHANISMS OF TRANSCRIPTIONAL REGULATION

Despite the expanding list of forkhead genes and knowledge of *in vitro* and *in vivo* functions, relatively little is known about the mechanisms of activation or repression of gene expression by forkhead transcription factors. Direct interactions with components of the basic transcriptional machinery [e.g. TBP (TATA-box-binding protein) and TFIIB (transcription factor IIB)] have been described for FOXF2 and FoxO family members [19,75]. As mentioned above, acetylation of FoxO factors attenuates their transactivational properties. In contrast, binding of CBP/p300 to FoxO proteins and its acetylation activity are also required for transcriptional activation by FoxO factors [47,49], suggestive of an additional regulatory role for CPB/p300 as a cofactor of FoxO factors. It is therefore tempting to speculate that CBP/p300 must be recruited by FoxO factors to target genes for histone acetylation, resulting in local changes in chromatin conformation and access to the promoter for general transcription factors, providing a second mechanism by which FoxO members regulate transactivation [50]. Interestingly, FoxO mutants that are unable to bind DNA were shown to still localize to sets of target gene promoters [76], suggesting that, at specific sets of genes, FoxO factors can influence transcription via a mechanism independent of DNA binding. Although the exact mechanism is still unclear, it has been suggested that acetylation could influence the balance between DNA-dependent and -independent transcriptional regulation, thereby switching the function of FoxO transcription factors to specific sets of target genes that do or do not require FoxO binding [50,76].

FoxA1, and possibly other FoxA members, determines nucleosome positioning of enhancer sites in transcriptionally competent cells, as has been established for the albumin enhancer [77]. Similar to linker histones H1 and H5, which both contain a winged-helix domain but lack a second wing [78,79], FoxA factors can bind to nucleosomal core histones, thereby replacing linker histones and relieving chromatin compaction at target enhancer and/or promoter sites. In addition, FoxA binding causes a sharp bend of the DNA helix, resulting in widening of the minor groove [7,16] and increased DNaseI sensitivity [80]. Together, this creates an open chromatin configuration, enabling other transcriptional activators to induce target gene expression. Consistent with this, a recent study on long-range gene regulation by ER (oestrogen receptor) binding revealed the presence of FoxA1 in the proximity of sites where ERs can subsequently bind. This binding of FoxA1 appeared to be essential for gene expression of ER target genes, since the removal of FoxA1 resulted in the inability of ER to associate with its binding elements [81]. Although essential for gene activation, FoxA binding is not necessarily sufficient for nucleosomal positioning at the MMTV (murine mammary tumour virus) LTR (long terminal repeat), depending on hormone-induced glucocorticoid receptor binding for nucleosomal rearrangements. Initial changes in chromatin structure, however, are induced by binding and activity of FoxA in both the absence and presence of glucocorticoid receptors, hence the name pioneer factor [82].

In the case of the transcriptional repressor Foxk1, interactions with the scaffolding protein Sin3b have been described [83]. This large protein is part of a co-repressor complex that also includes HDACs (histone deacetylases) and/or nucleosomal remodelling factors. Since this complex lacks DNA-binding activity, recruitment by Foxk1 could direct a reversal of open chromatin conformation and specific repression of downstream target genes. Similarly, the transcriptional repressor Foxn3 associates with SKIP (Ski-interacting protein) [84], a transcriptional adaptor protein known to interact with repressor complexes that involve Sin3a and HDACs. It is currently not known whether other transcriptional repressors, such as Foxd3 and Foxp1, could operate through a similar mechanism.

FUNCTIONS OF FORKHEAD TRANSCRIPTION FACTORS

Cell-cycle regulation

In yeast, progression through the different stages of the cell cycle is co-ordinated by waves of expression of a large number of genes. The controlled periodicity of G_2/M-phase-specific expression of > 30 genes collectively known as the *Clb2* cluster is controlled by the forkhead transcription factor Fkh2 (reviewed in [55]). In mammalian cells too, expression of multiple G_2/M-phase-related genes is regulated by a forkhead transcription factor. *Foxm1* gene expression is restricted to proliferating cells, and FoxM1 activity is cell-cycle-regulated [85]. Dividing cells from *Foxm1*-deficient mice exhibit mitotic defects, resulting from deregulated expression of genes required for mitotic entry and progression, including *CCNB* (cyclin B), *Plk1* (Polo-like kinase 1), *cdc25* (cell division cycle 25) and the kinetochore component *CENP-F* (centromere protein F) [86,87]. In addition, FoxM1 controls progression into S-phase by up-regulating expression of the CDK2 (cyclin-dependent kinase 2)-activating phosphatase *Cdc25A*, while down-regulating protein levels of the CKIs (CDK inhibitors) $p21^{Cip1}$ and $p27^{Kip1}$ [88,89]. These CKIs are targeted by the SCF ubiquitin ligase complex, subunits of which are transcriptionally regulated by Foxm1 [87]. Together, defects in S- and M-phase by loss of FoxM1 result in chromosomal instability and polyploidy [86].

Cell division is influenced by FoxO factors at two separate levels. First, FoxO transcription factors prevent cells from entering the cell cycle. This quiescent state is maintained through regulation of a whole set of cell-cycle mediators. Induction of $p27^{Kip1}$ and $p21^{Cip1}$ results in inhibition of cyclin E–CDK2 and cyclin D–CDK4 complexes, involved in entry into S-phase [63,90,91]. Up-regulation of the retinoblastoma protein family member *p130* by FoxO factors prevents transcriptional activation of an array of cell-cycle-entry inducers by E2F4 (E2F transcription factor 4) [92]. In addition, stimulation of *CCNG2* (cyclin G2) expression, specifically expressed in quiescent cells, and down-regulation of *CCND* family members, involved in regulation of cell-cycle entry, assist further in preventing cell-cycle entry [76,93,94]. Secondly, FoxO factors seem to have contrasting roles in the regulation of G_2/M-phase regulation. During G_2-phase, they increase the expression of *CCNB* and *Plk1* genes, which are required for mitotic entry and completion [95]. In contrast, FoxO factors were also found to induce a delay in G_2-phase and entry of mitosis, possibly through regulation of *GADD45a* (growth arrest and DNA-damage-inducible protein 45α), a component of the G_2/M-checkpoint system [86,96]. Precisely how FoxO factors regulate the transition from G_2- to M-phase clearly has not yet been established, and requires further investigation.

Interestingly, members of the CIP (CDK-interacting protein)/KIP (kinase inhibitor protein) family of CKIs appear to be common target genes for forkhead transcription factors. In addition to FoxO factors and FoxM1, additional forkheads such as FOXA1, Foxg1 and Foxk1 can also act upon $p21^{Cip1}$ or $p27^{Kip1}$ expression [63,97,98], although they can differ in their activating or repressive effect.

Cellular survival

A first indication that forkhead transcription factors are implicated in regulation of cell survival came from observations in *Caenorhabditis elegans*, where the forkhead DAF-16 is an important regulator of longevity [99,100]. Subsequently, lifespan regulation by DAF-16 appeared to be controlled by Akt-mediated insulin-responsive signalling pathways [101]. Interestingly, these pathways are highly conserved in other species, and in mammals the DAF-16 orthologues FoxO1, FoxO3, FoxO4 and FoxO6 are all regulated by PKB/Akt (see above). Mammalian PKB, induced by insulin-like growth signals, actively suppresses apoptosis through inhibition of FoxO transcription factors [35], but cannot prevent apoptosis triggered by expression of PKB-resistant FoxO mutants [102]. *In vivo*, a wealth of data point to a role for FoxO factors in mediating haematopoietic and neuronal apoptosis [35,103,104], and they have recently been linked to amyloid β toxicity in Alzheimer's disease [105]. Interestingly, other cell types generally respond to FoxO activation by going into cell-cycle arrest [91,92]. Such a quiescent state has a more favourable metabolic rate and can promote cell survival under stressful conditions, analogous to increased stress resistance and longevity during periods of nutrient starvation in *C. elegans* Dauer formation. This was shown recently to be dependent, at least in part, on interactions with β-catenin, a central mediator of the Wnt signalling pathway [106].

The mechanisms that underlie FoxO-mediated cell-cycle arrest and apoptosis involve transcriptional regulation of pro-apoptotic target genes, such as the *FasL* and *Bim* genes [35,103,107]. Alternative pathways that result in a similar cell fate include FoxO regulation of $p27^{Kip1}$, which prevents cell-cycle entry and induces cell death in certain cell types [90,108]. Exactly why FoxO factors induce cell-cycle arrest in some cell types but cause apoptosis in others is not entirely clear. A major clue could come from studies that have distinguished between DNA-binding-dependent and -independent FoxO target genes, and found that cell-death induction, but not cell-cycle arrest, requires FoxO DNA-binding ability [76].

Longevity (and therefore aging) is inextricably linked to stressful stimuli, mainly in the form of reactive oxidative species. Similar to DAF-16 in *C. elegans*, FoxO factors provide cells with stress-resistance strategies. Protection of quiescent cells against oxidative stress involves FoxO-mediated up-regulation of MnSOD (manganese superoxide dismutase) and catalase, two enzymes with antioxidant activity [109,110]. Under low-stress conditions, any DNA damage that may result from oxidative stress is overcome by FoxO induction of the *GADD45a* DNA-repair gene [96]. These stress-response mechanisms suggest that, in mammals too, FoxO factors may have the ability to regulate lifespan. Figure 3 shows the forkhead transcription factors involved in cell cycle and cellular survival.

Metabolism

At both the cellular and organismal levels, forkhead transcription factors have been implicated in regulation of metabolic processes. To date, metabolic regulation by forkheads is confined to members of the FoxA, FoxC and FoxO subfamilies. Evidence for involvement of FoxA1 and FoxA3 in metabolic processes comes from findings of hypoglycaemia in *Foxa1*- and *Foxa3*-null mutant
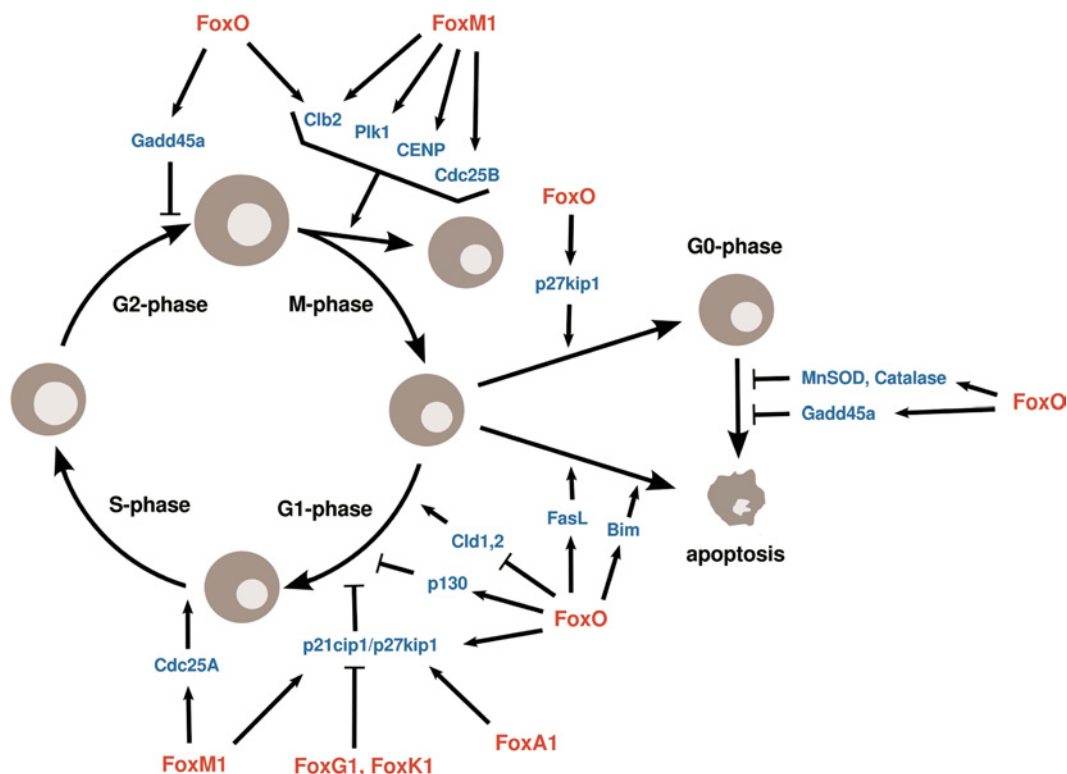
Figure 3   Forkhead transcription factors in cell cycle and cellular survival

Schematic overview of known roles for forkhead transcription factors (displayed in red) in regulating expression of genes implicated in the process of proliferation, survival and/or apoptosis (blue). Note that particular forkhead factors can have contrasting roles in balancing cell cycle and survival depending on the type (e.g. neuronal or haematopoietic) and status (e.g. cycling or quiescent) of the cell. See text for details and references. CENP, centromere protein; Clb, cyclin B; Cld, cyclin D.

mice [111–113]. *Foxa2*-deficient mice are embryonic lethal, but tissue-specific deletion in pancreatic β-cells also results in hypoglycaemia and disorganized islet arrangements [114]. The underlying causes for hypoglycaemia can differ between FoxA family members, however, with glucagon levels affected in *Foxa1*$^{-/-}$ mice and the GLUT2 glucose transporter down-regulated in *Foxa3*-deficient mice, whereas subunits of a β-cell-specific potassium channel were identified as Foxa2 target genes. For Foxa2, multiple additional target genes have been identified in pathways controlling insulin secretion and glucose homoeostasis in the pancreas [115,116]. In the liver, Foxa2 has recently been linked to fat metabolism, as it was shown to be responsible for regulation of fatty-acid burning upon fasting [117].

FoxC2 has an important role in regulating energy storage and fat metabolism. Analysis of *Foxc2*$^{+/-}$ mice (homozygous animals die *in utero*), in which changes in brown adipose tissue mass were observed, suggests that FoxC2 is involved in regulating energy storage [118]. Upon overexpression of *Foxc2* in adipocytes, there is a decrease in total body fat mass, and these transgenic mice are protected against diet-induced insulin resistance [118,119]. FoxC2 in adipocytes therefore seems to influence overall glucose metabolism in transgenic mice, and *Foxc2* overexpression seems to prevent diet-induced obesity and insulin resistance.

Members of the FoxO family also occupy important roles in regulation of metabolism (reviewed in [18,120]). A principal feature that links FoxO factors with metabolism is the insulin-dependent regulation by PKB, as described above. Additional links include the fact that FoxO target genes are often implicated in metabolic processes in liver, muscle and pancreas. Examples

include *G6PC* (glucose-6-phosphatase), involved in gluconeogenesis in the liver [121] and *PDK4* (pyruvate dehydrogenase kinase 4), implicated in glucose saving in liver and muscle [122]. Related to their function in cell survival, FoxO factors protect β-cells from glucose-induced oxidative stress through up-regulation of transcription factors that induce expression of Ins2 [123]. Other clues come from *in vivo* models using heterozygous *Foxo1*-null mice (homozygous *Foxo1*-null mutants are embryonic lethal [124]), which are protected against diabetes, whereas mice transgenic for a dominant active Foxo1 show opposite effects and develop diabetes [125,126]. Overall, FoxO factors appear to be important in controlling the effects of insulin signalling on gluconeogenesis and other metabolic processes.

Immunoregulation

Multiple forkhead transcription factors are expressed in immune cells, and hence are implicated in regulation of the immune system [127,128]. Foxp3 is essential for specification and function of regulatory (CD4$^+$ CD25$^+$) T-cells, which are important for regulating T-cell reactivity and preventing autoimmunity [129–131]. Similar prevention of autoimmunity additionally requires Foxj1, which is thought to act by transcriptional activation of *IκBβ*, a potent inhibitor of NF-κB. Since NF-κB can cause T-cell activation, inhibition of this factor results in reduced autoimmunity [132]. Also, Foxj1 is thought to control the release of T-cells into the periphery [133]. The gene responsible for the nude phenotype, *Foxn1*, is known for its function in the regulation of proliferation and differentiation of epithelial cell populations

[134]. The same holds true for the thymus, where Foxn1 is required for development and differentiation of thymic epithelial cells, which are in turn essential for development and selection of T-cells [135]. As described above, FoxO transcription factors are known regulators of the cell cycle. Similar functions have been proposed in thymic cells, where FoxO factors induce T-cell quiescence [136]. Finally, roles for other forkheads such as Foxd2 and Foxp1 have been proposed [137,138], but additional work is required to clarify their functions in immunoregulation.

Embryonic development

Similar to most other transcription factor families, forkhead factors are commonly involved in embryonic development, in roles often conserved across different species. There are striking parallels between the number of forkhead genes in different species and their anatomical complexity. Not surprisingly, forkhead transcription factors are often implicated in the regulation of differentiation processes during embryonic development.

Already during early embryonic patterning, forkhead factors are implicated in gastrulation and the formation of midline structures required for establishing the body plan. The severe phenotype of $Foxh1^{-/-}$ mice reflects the importance of Foxh1 in nodal signalling, as primitive streak, node, notochord and prechordal plate mesoderm are all severely affected in null mutants [139,140]. $Foxa2$ is also expressed in notochord, floorplate and primitive streak, and, similar to $Foxh1$, $Foxa2$-deficient mice do not develop a proper notochord and other midline structures [141,142], and, as a result, mice deficient in these two forkheads display severe secondary defects. Another forkhead, Foxj1, is expressed specifically in ciliated cells, including ciliated cells in the node at presomite stages, and is essential for establishing left–right asymmetry during early development [143].

During the very early stages of organogenesis, forkhead factors are also heavily involved in patterning of the germ layers. In the absence of Foxa2, patterning defects in the definitive endoderm result in malformation of the foregut [141,142], in analogy to the original forkhead gene in *Drosophila* [1], and similar defects can be observed in $Foxh1$-deficient mice [139]. In the mesoderm, forkhead factors are involved in specification of developmental fate, in which Foxc factors induce a paraxial mesodermal fate from which somites will subsequently develop [144]. In contrast with Foxc factors, Foxf1 promotes differentiation into lateral plate mesoderm, and subsequently into splanchnic mesoderm, which gives rise to mesenchymal tissues and vasculature. Additionally, Foxf1 is implicated in development of extra-embryonic mesoderm tissues including placenta, amnion and yolk sac [145,146].

During later stages of organogenesis, the cellular role of forkheads in the regulation of differentiation becomes apparent. Many forkheads induce tissue-specific gene expression, and mice mutant for particular forkheads often display differentiation-related developmental defects. Foxa1 and Foxa2, often with complementary activity, are important for regulation of tissue-specific gene-expression programmes during morphogenesis and differentiation of tissues such as prostate, pancreas, lung and liver [147–151]. Foxd1 deficiency leads to impaired branching morphogenesis, nephron patterning and differentiation [152]. Development of the inner ear is severely affected in mice deficient in Foxi1, resulting in hearing and balance impairments [153]. Additionally, $Foxi1^{-/-}$ mice suffer from distal renal tubular acidosis [154], and parallels between cellular defects in inner ear and kidney development suggest a function for Foxi1 in specification of an intercalated cell fate. Foxj1 is a central player in ciliogenesis during development, and Foxj1 deficiency results in the absence of cilia in respiratory, reproductive and central nervous systems [143]. Mice deficient

in Foxl2 are characterized by infertility because of premature ovarian failure, and analysis of the mouse mutant suggests that Foxl2 is a key factor in granulosa cell differentiation, which in turn regulates the delicate timing of follicle activation [155,156]. In addition, Foxl2 is a required commitment of an adult ovarian phenotype, while suppressing the testis-determination pathway in granulosa cells in postnatal ovaries [157]. The role of FoxM1 in cell-cycle regulation is well established (see section 'Cell-cycle regulation'), and $Foxm1^{-/-}$ mice display severe proliferation-related defects. In addition, however, Foxm1 directs hepatoblast differentiation towards a biliary epithelial cell lineage fate [158]. FoxN factors are also implicated in directing differentiation, with Foxn1 regulating differentiation of post-mitotic cells in hair follicles and epithelial cells in thymus, nails and nasal cavity [159,160], whereas Foxn4 controls cell-fate decisions in both retina and spinal cord [161,162]. Homozygous deletion of $Foxp1$ results in increased proliferation in combination with an aberrant myocardial organization, suggesting a role for Foxp1 in myocyte maturation [163].

In addition to the direct induction of tissue-specific gene expression during development, several forkhead factors regulate differentiation by controlling the decision of cells to commence differentiation. FoxD3, for one, prevents differentiation of embryonic stem cells and trophoblast progenitor cells and maintains them in a pluripotent state [164,165]. Several other forkhead factors, notably Foxe3 in the lens and Foxg1 in the neuroepithelium, prevent differentiation of proliferating cells by stimulating them to keep dividing [166,167].

Are all forkhead transcription factors implicated in regulating differentiation during development? Some factors may serve other roles in addition to their role in differentiation. For example, in addition to its role in differentiation of neural crest cells, FoxD3 also induces neural crest cells to migrate away from the neural tube, and regulates the expression of cell-adhesion molecules such as N-cadherin in migrating neural crest cells [168]. *In vitro*, FoxO factors have been studied extensively, and, although implicated in differentiation of adipocytes and myoblasts [126,169], they are better known for their role as downstream signal transduction components affecting metabolism, cell-cycle regulation and survival (see above). However, mice mutant for $FoxO$ genes have only recently become available [124,170,171], and future research may eventually disclose some cellular role for FoxO factors in differentiation processes after all. $Foxk1$ mutant mice that make it to term show a significant growth deficit and muscle atrophy, owing to myogenic progenitor cells having lost the ability to proliferate and provide in novel myocyte populations in repair of muscular injury [172]. However, mice with targeted mutation of $Foxk1$ only develop in 6 % of cases, suggesting that Foxk1 is vital for embryonic development. Not much is known, however, about its function in Foxk1-positive tissues such as somites, myocardium and brain during development.

Mice mutant for other forkhead transcription factors have only been analysed morphologically, and conclusive information on a cellular function is not yet available. $Foxb1$-deficient mice, for example, display brain defects, growth retardation and muscle weakness after birth [173,174], but the underlying mechanisms remain unclear. Most of what we know about FoxP2 comes from studies in humans, as heterozygous mutations in the $FOXP2$ gene were identified in patients with a severe speech and language disorder (KE-family), establishing an exciting link between developmental genetics and the human ability to speak [175]. The recent creation of $Foxp2$-null mutant mice [176] will help in elucidating the function of FoxP2, as well as the molecular cascades involved in FoxP2-mediated development of the neural circuitry that governs speech.

CLINICAL IMPLICATIONS

Several human phenotypes can be attributed to mutation of members of the forkhead family of transcription factors (see also [177]). Often, functional conservation is apparent between species, with many phenotypes of forkhead mutations in mice resembling human anomalies caused by orthologues. Strikingly, a substantial fraction of these mutations are implicated in ocular phenotypes. These include mutations in the *FOXC1* gene in cases of congenital glaucoma and Axenfeld–Rieger anomaly [178,179], *FOXC2* mutations in distichiasis in addition to lymphoedema [180], *FOXE3* mutations in ocular anterior segment anomalies and cataract [181,182] and *FOXL2* mutations in BPES (blepharophimosis–ptosis–epicanthus inversus syndrome) [183,184]. Other forkhead-related phenotypes include thyroid agenesis, cleft palate and choanal atresia as a result of mutations in the *FOXE1* gene [185] and T-cell immunodeficiency in combination with alopecia and nail dystrophy caused by *FOXN1* mutations [186], whereas members of the FOXO subfamily have been implicated in cancers such as rhabdomyosarcoma and leukaemia [187,188]. The identification of mutations in the human *FOXP2* gene in a family with a severe speech and language disorder has provided an interesting link between neurodevelopment and development of speech [175]. Finally, mutations in the X-linked *FOXP3* gene can result in IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome [189,190]. Additionally, striking similarities have been observed between human anomalies and murine phenotypes caused by mutation or deletion of forkhead genes. Examples include *Foxi1*$^{-/-}$ mice, which suffer from severely impaired hearing, and developmental defects in the inner ear resemble phenotypes in certain congenital human auditory conditions [153]. However, disease-causing mutations in the human orthologue have not yet been identified. To date, the vast majority of known mutations affect the DNA-binding domain, either by local mutations or as a result of missense mutations N-terminal to the forkhead domain. Although mutations identified so far are mainly homozygous, it remains to be determined whether heterozygous mutations or single nucleotide polymorphisms can cause similar or less severe phenotypes.

Recent studies have provided the first indications that forkhead factors can be exploited therapeutically. Regulatory (CD4$^+$ CD25$^+$) T-cells are indispensable for suppression of immune system activation, thereby maintaining immune system homoeostasis and preventing autoimmunity (see above). As Foxp3 is crucial for development and function of regulatory T-cells [129–131], modulation of Foxp3 expression could be used in immunotherapy in autoimmune diseases. Loser et al. [191] showed that *in vitro*-generated T-cells transduced with Foxp3 can be used in mice to protect against autoimmune dermatitis. Similarly, such cells were able to reverse disease in a mouse model for autoimmune diabetes [192]. In inflammatory arthritis, overactivated neutrophils often persist in the affected joints. This was recently shown to result from an impairment of transactivation of the apoptosis inducer *FasL*, due to active suppression by Foxo3a [193], making Foxo3a an attractive target for targeted gene therapy in autoimmune diseases.

Signalling pathways involving insulin/PKB, neurotrophin and Wnt have been shown to be important in molecular mechanisms underlying cancer, neurodegenerative diseases and aging, with FoxO factors as important mediators [194,195]. Interestingly, induction of FoxO3a is able to sensitize breast cancer cells to chemotherapy-induced apoptosis [196]. Since much is known about post-translational modifications that influence FoxO function, members of the FoxO subfamily could prove to be useful candidates for the development of gene therapy strategies. In treating metabolic diseases such as diabetes, pharmacological regulation of phosphorylation could theoretically be used to target FoxO factors as well as Foxa2 [197]. Although targeting forkheads in disease treatment sounds promising in theory, this clearly requires additional investigation.

In addition to their possible use in disease therapy, forkhead expression can be useful for the prognosis of the clinical outcome of particular diseases. Consistent with the role of Foxp3 in suppressing autoimmunity, expression levels of Foxp3 appear to be predictive of the clinical outcome of renal transplantation, with higher levels associated with a more favourable outcome [198]. In contrast, increased Foxp3 expression appears to be a warning sign for patients suffering from ovarian carcinoma, whereas decreased expression is associated with a more favourable prognosis [199].

CONCLUDING REMARKS

Forkhead transcription factors are increasingly being recognized for their importance in mammalian development and physiology, acting downstream of multiple conserved signalling pathways, involving phosphoinositide 3-kinase/PKB, Wnt and TGFβ, among others. Still relatively little is known, however, about the mechanisms that underlie target specificity and how forkheads can function in such a large variety of cellular processes.

Existing functional protein domains and interactions are important for the functionality of forkhead factors. However, most of what we know stems from studies on a limited number of family members. As particular regulatory motifs are often absent in most family members, it will be vital to identify structural elements in other forkheads. The increased availability of techniques such as microarrays and MS will help to understand how forkheads are targeted to specific loci, and how binding of forkheads leads to transcriptional activation or repression.

In vitro studies have mainly benefitted research on biochemical properties of forkhead proteins, as functional aspects of forkhead proteins may be highly dependent on cell type and physiological context. Most functional studies on forkhead factors have therefore been limited to regulation of cell cycle, survival and metabolism, which are analysed relatively easily. During development, however, processes such as proliferation, differentiation and migration are tightly regulated and are context-dependent. Useful *in vitro* models are therefore not commonly available, which is reflected by the low number of studies that have provided evidence for involvement of forkheads in developmental and physiological processes *in vivo*. However, for the majority of murine forkheads, knockout or transgenic mice are now available and are analysed phenotypically. In the coming years, more elaborate analysis of these and other *in vivo* models should elucidate a detailed function for each forkhead transcription factor.

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