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PRIMER

Fox transcription factors: from development to disease

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ABSTRACT

Forkhead box (Fox) transcription factors are evolutionarily conserved in organisms ranging from yeast to humans. They regulate diverse biological processes both during development and throughout adult life. Mutations in many Fox genes are associated with human disease and, as such, various animal models have been generated to study the function of these transcription factors in mechanistic detail. In many cases, the absence of even a single Fox transcription factor is lethal. In this Primer, we provide an overview of the Fox family, highlighting several key Fox transcription factor families that are important for mammalian development.

KEY WORDS: Forkhead, Fox, Foregut development, Language acquisition, Pioneer factors, Transcription factors

Introduction

The *forkhead* (*fkh*) gene was originally identified in a random mutagenesis screen performed in *Drosophila melanogaster* (Weigel et al., 1989). This study showed that *fkh* is required for normal gut development, and that its absence results in a characteristic 'forked head' appearance resulting from the homeotic transformation of the foregut into a head structure. Soon after this discovery, a number of related genes – termed Fox genes – were identified in multiple organisms, ranging from yeasts to humans.

The *Drosophila* Fkh protein is characterized by a winged-helix DNA-binding domain ∼100 residues long, termed the 'forkhead box'. All Fox proteins share this distinctive DNA-binding domain but have divergent features and functions. Fox genes control a wide variety of biological functions and are broadly expressed both during development and in adult life. Their roles include, but are not limited to, the regulation of gastrulation (Ang and Rossant, 1994; Weinstein et al., 1994), stem cell and stem cell niche maintenance (Sackett et al., 2009; Aoki et al., 2016), the regulation of metabolism and cell cycle control (Hannenhalli and Kaestner, 2009). Indeed, Fox transcription factors are required for the normal specification, differentiation, maintenance and/or function of tissues such as the trophectoderm, liver, pancreas, ovaries, intestine, lung, kidney, prostate, brain, thyroid, skeletal and heart muscle, skeleton, vascular tissue and immune cells (Zhu, 2016).

Here, we first provide an overview of the Fox gene family and discuss how distinct Fox transcription factors regulate specific stages of development, tissue homeostasis and disease. Owing to their sheer number, we then concentrate on just four families: the FoxA factors and their role in the differentiation and maintenance of multiple cell types; FoxM1 and its control of the cell cycle; the FoxO group in regulating metabolism and longevity; and FoxP for its contribution to speech acquisition.

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An overview of Fox transcription factors

The number of Fox genes currently cataloged varies widely among different organisms. Human and mouse both have 44, *Drosophila* 11, *Caenorhabditis elegans* 15, and *Xenopus* 45, the latter excluding alternate splice forms in all species and pseudogenes that were duplicated along with the rest of the *Xenopus* genome and expressed in exactly the same location as the original genes. Notably, *Xenopus* models contributed greatly to the initial description of Fox expression patterns in early embryogenesis (Pohl and Knöchel, 2005).

In mammals, Fox transcription factors are categorized into subclasses A to S (Fig. 1) based on sequence similarity within and outside of the forkhead box (Hannenhalli and Kaestner, 2009; Kaestner et al., 1999). In many cases, the homozygous deletion of just one Fox gene leads to embryonic or perinatal lethality and, in humans, mutations in or the abnormal regulation of Fox genes are associated with developmental disorders and diseases such as cancer (Halasi and Gartel, 2013; Li et al., 2015a; Wang et al., 2014b; Zhu et al., 2015; DeGraff et al., 2014; Halmos et al., 2004; Ren et al., 2015; Jones et al., 2015; Habashy et al., 2008), Parkinson's disease (Kittappa et al., 2007), autism spectrum disorder (Bowers and Konopka, 2012), ocular abnormalities (Acharya et al., 2011), defects in immune regulation and function (Mercer and Unutmaz, 2009) and deficiencies in language acquisition (Takahashi et al., 2009); see Table 1 for a comprehensive overview of Fox transcription factor expression patterns and their association with developmental disorders and disease.

Distinct protein domains, expression patterns and post-translational modifications contribute to the divergent functions of Fox family members

Fox transcription factors bind a similar DNA sequence, albeit with different affinities, due to their highly conserved DNA-binding motif. How, then, do members of this large gene family have distinct roles? The divergent sequences outside of the conserved DNA-binding domain likely differentiate the function of these proteins, as do their distinct temporal and spatial gene activation patterns (Fig. 2).

The binding domains of FoxA transcription factors, for example, have structural similarity to linker histones H1 and H5 (Clark et al., 1993; Zaret et al., 2010). This feature allows FoxA transcription factors to access closed chromatin (Fig. 3), thus allowing the recruitment of alternative histones and facilitating the subsequent binding of other transcription factors at nearby sites (Cirillo et al., 2002; Li et al., 2012b; Updike and Mango, 2006). Indeed, genome-wide mapping of FoxA binding sites and nucleosome architecture in the mouse liver has shown that FoxA binds both nucleosome-bound and nucleosome-free DNA targets with the same recognition site (Li et al., 2011). For this reason, FoxA family members have been

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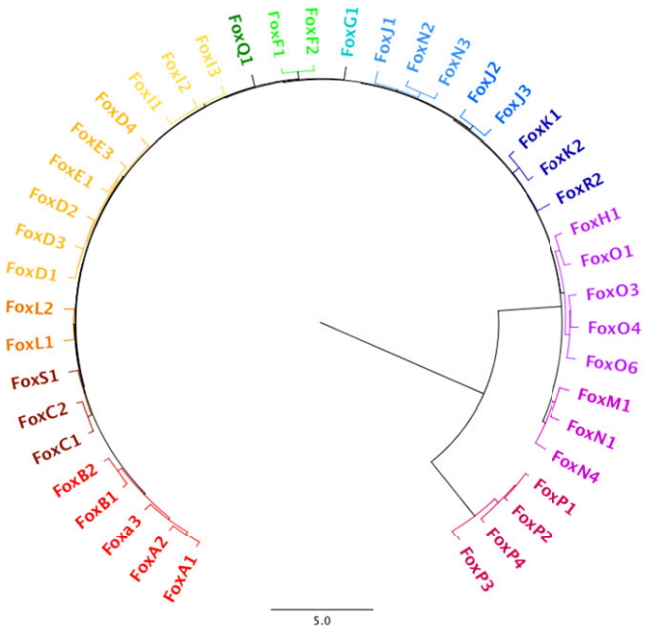

Fig. 1. Phylogenetic tree of mouse Fox family members. The entire sequences of mouse Fox transcription factors were aligned pairwise using Geneious software. The following parameters were employed: global assignment with free end gaps, the Jukes-Cantor genetics distance model, and unweighted pair-group method with arithmetic mean. Differences with other phylogenetic trees of Fox transcription factors are likely the result of grouping by homology to the FKH DNA-binding domain only. Scale indicates the relative number of amino acid changes between proteins.

termed 'pioneer' transcription factors (Cirillo et al., 2002). In this role, they help specify cell types by giving tissue-specific transcription factors access to their binding sites (Iwafuchi-Doi and Zaret, 2016). They also help maintain cell identity by acting as 'place holders' or 'bookmarks' at genes that are normally shut off during cell division; after cytokinesis is complete, cell type-specific genes previously marked by FoxA can be easily reactivated (Caravaca et al., 2013). Accordingly, a number of studies have shown that FoxA transcription factors are expressed in early development and play crucial roles in the development and homeostasis of various cells and tissues (see below).

Because of their actions as pioneer factors, FoxA proteins are required to facilitate the binding of many other transcription factors to their targets. This is especially evident for several nuclear receptors, such as androgen receptor (AR), glucocorticoid receptor (GR) and estrogen receptor (ER). This was first shown for the interaction between FoxA1 and AR in the prostate (Gao et al., 2003), then for ER and FoxA1 (Hurtado et al., 2011; Ross-Innes et al., 2012; Carroll et al., 2005; Lupien et al., 2008) and for FoxA2 and GR (Zhang et al., 2005). The consequences of this cooperative binding, particularly in the context of cancer, are discussed in detail below.

FoxM1 is best known for its role in regulating the cell cycle. Its forkhead DNA-binding domain (FHD) shares only 18% sequence identity with that of FoxA1, and FoxM1 has lower affinity for the forkhead consensus binding site than other Fox proteins (Littler et al., 2010). Some investigators have shown that, instead of directly interacting with the DNA of its proliferative targets, FoxM1 attaches to proteins in the DREAM complex – a larger conglomeration of proteins that prevents the transcription of proliferative targets in the quiescent state, but promotes expression of the same targets during the cell cycle (Chen et al., 2013). However, other reports have demonstrated direct binding of FoxM1 to its targets (Sanders et al., 2013).

Given its crucial function in cell cycle control, FoxM1 expression and activity must be tightly regulated. Indeed, *Foxm1* mRNA and protein are only usually expressed during the cell cycle (Korver et al., 1997). The activity of FoxM1 is also tightly controlled by several post-transcriptional mechanisms (reviewed by Golson et al., 2010): (1) FoxM1 is generally excluded from the nucleus, but phosphorylation by MAPK induces its translocation; (2) an intramolecular repressive domain inhibits the binding of FoxM1 to DNA until phosphorylation by Chek2 (Chk2); (3) FoxM1 only recruits co-factors for gene transactivation when phosphorylated by Cdk1/2; and (4) FoxM1 is targeted to the proteasome when phosphorylated by a presently unknown kinase.

Like FoxM1, the activity of FoxO proteins – a group of proteins that inhibit the cell cycle and regulate lifespan and metabolism – is regulated post-translationally. Phosphorylation by Akt, for example, leads to the nuclear exclusion of FoxO1 and sequestration away from DNA by the 14-3-3 scaffolding protein (Tzivion et al., 2011). Unlike many other Fox transcription factors, FoxO factors contain both a nuclear export signal (NES) and a nuclear localization signal (NLS) (Tzivion et al., 2011). FoxO proteins help to activate transcription of their targets by recruiting the SWI/SNF chromatin remodeling complex (Riedel et al., 2013). These transcriptional targets include antioxidants, cell cycle inhibitors and metabolic genes; in many ways, FoxO proteins act counter to FoxM1.

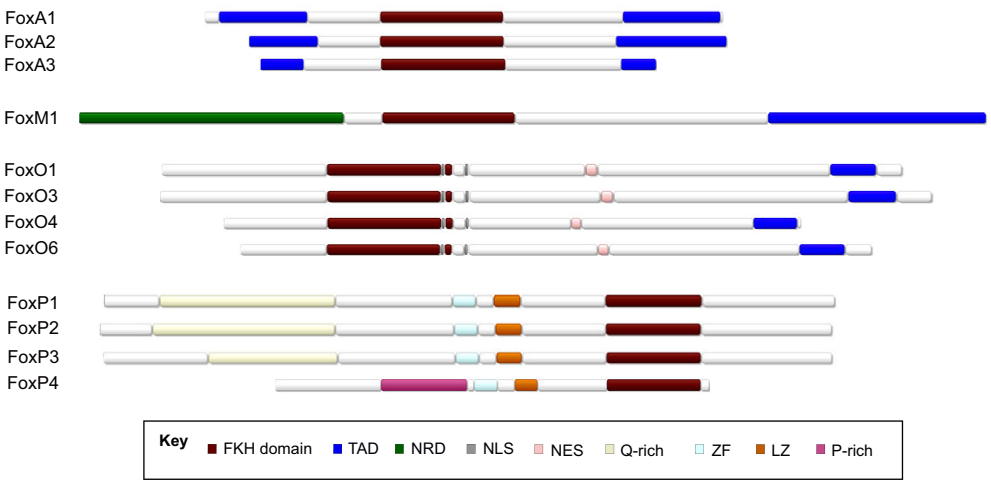

Fig. 2. The domain structure of selected Fox family members. Shown are the domain structures of mouse FoxA1-3, FoxM1, FoxO1, FoxO3, FoxO4, FoxO6 and FoxP1-4. TAD, transactivation domain; NRD, N-terminal repressor domain; NLS, nuclear localization signal; NES, nuclear export signal; ZF, zinc finger; LZ, leucine zipper.

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Table 1. Summary of the functions of Fox family members in mice and roles in human disease

Gene	Null mouse phenotypes	Developmental roles	Role in human disease
Foxa1	Early postnatal (P2-P14) lethality; hypoglycemia; dehydration	Establishes and maintains cellular identity in multiple endodermal-derived tissues including lung, liver, kidney, pancreas and prostate	Mutated or lost in prostate cancer; presence in ER-negative breast cancer correlates with a better prognosis but presence in ER-positive breast cancer correlates with a worse prognosis
Foxa2	Early embryonic lethality (E9-E10) due to lack of node; somite, neural tube, floor plate and motor neuron defects; heterozygote exhibits Parkinson's-like phenotype due to loss of dopaminergic neuron maintenance	Establishes and maintains cellular identity in multiple endodermal-derived tissues	Downregulated or mutated in multiple cancers of epithelial origin
Foxa3	Hypoglycemia after long fast	Liver-specific gene expression maintenance	
Foxb1	Variable embryonic lethality; high penetrance of postnatal lethality among survivors; motor weakness; midbrain abnormalities; lactation defect among female survivors	Neural tube and neuron process development	
Foxb2	Uncharacterized		
Foxc1	Perinatal lethality; hydrocephalus, edema, and eye, skull, cardiovascular system, kidney and skeletal defects	Skull, tooth, eye, cardiovascular, kidney, and hematopoietic stem cell development	Axenfeld-Rieger syndrome type 3; iris hypoplasia
Foxc2	Embryonic or neonatal lethality; thinned myocardium; abnormal spine and skull; distichiasis; increased brown fat; heterozygotes have lymphatic node and vessel hyperplasia	Cardiac muscle, skeletal, iris and lymphatic system development	Lymphedema-distichiasis syndrome; promotes epithelial-to-mesenchyme transition and metastasis in breast, prostate and colon cancer
Foxd1	Embryonic lethality within 24 h of birth; defects in renal system development	Renal and eye development	
Foxd2	Incompletely penetrant renal hypoplasia	Renal development	
Foxd3	Embryonic (E6.5) lethality; lack of primitive streak; failure of gastrulation; expansion of extraembryonic tissue	Neural crest cell development and melanoblast differentiation; embryonic stem cell pluripotency	Autosomal dominant vitiligo
Foxd4	Uncharacterized		
Foxe1	Lethality within 48 h, with cleft palate, absent thyroid, and sparse, kinked hair	Thyroid development	Some mutations associated with thyroid dysgenesis, spiky hair and cleft palate; other mutations associated with thyroid cancer
Foxe3	Fusion of lens and cornea; other lens abnormalities	Lens development; proliferation, cell cycle, and apoptosis in lens	Anterior segment dysgenesis and congenital absence of a lens
Foxf1	Embryonic lethality at ~E9; abnormalities in somite and posterior development as well as in extraembryonic tissues and lateral plate mesoderm; haploinsufficient mice on certain genetic backgrounds die perinatally with pulmonary defects	Pulmonary and gut development; liver stellate cell activation	Misalignment of pulmonary veins; gastrointestinal abnormalities
Foxf2	Lethality within first 2 h of life due to cleft palate		
Foxg1	Perinatal lethality due to lung defects; reduced cerebral size; eye defects	Neuronal differentiation; cell cycle progression	Rett syndrome and other forms of mental retardation; microcephaly and other brain abnormalities
Foxh1	Perinatal lethality with variable patterning defects: variable right isomerism leading to defects in heart, lungs and stomach and asplenia; cyclopia; microcephaly; absent jaw	Regulation of body patterning, including left-right asymmetry	Congenital heart disease and ventricular septal defects
Foxi1	50% perinatal lethality; inner ear and renal defects; 25% of heterozygotes exhibit perinatal lethality	Renal and inner ear development	Pendred syndrome
Foxi2	Decreased circulating glucose		
Foxi3	Lethality starting at E9.5 through perinatal life; lack whiskers and a mouth; absence of inner, middle and outer ear; increased cranial neural crest apoptosis	Differentiation of branchial arch-derived skeletal structures	
Foxj1	Perinatal lethality; defective ciliogenesis and random left-right asymmetry; growth failure	Regulation of left-right asymmetry; T-lymphocyte quiescence	Allergic rhinitis
Foxj2	Uncharacterized		
Foxj3	Decreased muscle mass and recovery after injury	Mitochondrial biogenesis	

Continued

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Table 1. Continued

Gene	Null mouse phenotypes	Developmental roles	Role in human disease
Foxk1	Reduced numbers of myogenic progenitor cells, with cell cycle arrest; reduced muscle recovery after injury	Muscle cell and progenitor proliferation	
Foxk2	Uncharacterized		
Foxl1	Low level of postnatal lethality; gastric mucosa hyperplasia with disorganized glands; decreased acid secretion from stomach parietal cells; abnormal crypt structure with an abnormal distribution of Paneth cells; decreased dextrose uptake in intestine	Gastrointestinal development and function	
Foxl2	Inpenetrant postnatal lethality; female sterility	Ovary differentiation and maintenance, including prevention of transdifferentiation into testis; eye development	Adult granulosa cell tumor; blepharophimosis, ptosis and epicanthus inversus syndrome (BPES; a syndrome that is characterized by the inability to fully open the eyes and in some cases early loss of ovarian function); adult granulosa cell tumor
Foxm1	Lethality beginning as early as E15.5, with some alleles displaying lethality perinatally; reduced proliferation in multiple tissues	Regulates cell cycle progression, karyokinesis and cytokinesis	Upregulated in most cancers
Foxn1	Premature mortality resulting from the lack of a thymus; lack of hair; heterozygotes have enlarged thymus	Thymus development	A form of severe combined immunity deficiency (SCID): T-cell immunodeficiency, congenital alopecia and nail dystrophy
Foxn2	Uncharacterized		
Foxn3		Inhibition of proliferation	
Foxn4	Early embryonic to postnatal lethality with reduced growth	Retinal development; neural development	
Foxo1	Embryonic lethality (E10.5-E11.5) due to incomplete vascular development; heterozygotes have elevated glycogen	Vasculogenesis; suppressor of proliferation and apoptosis; blood vessel, yolk sac and diaphragm development; gluconeogenesis; glycogenolysis; adipogenesis; angiogenesis; osteogenesis; T-cell regulation; embryonic stem cell pluripotency	Rhabdomyosarcoma 2 (RMS2)
Foxo3	Ovarian defect causing progressive female sterility; defects in immune system function and hematopoiesis	Follicular growth activation	Fused to *MLL* gene in acute leukemias
Foxo4	No gross abnormalities	Contributes to myeloid lineage regulation	Fused to *MLL* gene in acute leukemias
Foxo6	Abnormal dendritic spine morphology and defects in memory consolidation	Neuronal development	
Foxp1	Embryonic (E14.5) lethality due to defects in cardiac morphogenesis; edema	Neuronal differentiation; cardiomyocyte proliferation; language acquisition; B-cell differentiation	Mental retardation and language defects with or without autistic features; fusion with *PAX6* in acute lymphoblastic leukemia
Foxp2	Postnatal lethality; reduced vocalization; abnormal neural development; delayed eye opening and ear emergence; impaired motor activity; hypoactivity	Neuronal differentiation; language acquisition	Speech-language disorder 1 (SPCH1)
Foxp3	Hemizygous male and homozygous female lethality at ~P21; scaly skin; reduced T-regulatory cells; reddening and swelling of genitals; micro- and cryptoorchidism	T-regulatory cell development	Multiple autoimmune disorders including type 1 diabetes and immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome
Foxp4	Embryonic (by E12.5) lethality; delay of foregut closure leading to development of two hearts; esophagus and trachea fail to develop	Gastrointestinal development; T-cell response	
Foxq1	Silky coat		
Foxr1	Uncharacterized		
Foxr2	Uncharacterized		
Foxs1	Uncharacterized		

Content was assembled using Mouse Genome Informatics (http://www.informatics.jax.org), Online Mendelian Inheritance in Man (www.omim.org) and the Genetics Home Reference (https://ghr.nlm.nih.gov).

Although most Fox transcription factors are strictly transcriptional activators, some, such as FoxP, have dual activator and repressor functions; indeed, members of the FoxP family are thought to act primarily as repressors (Zuo et al., 2007; Shu et al., 2001; Li et al., 2004a). FoxP family members can bind as homo- or heterodimers through their leucine zipper and zinc finger domains, and this interaction is required for FoxP family members to repress their targets, perhaps because a conformational change occurs upon binding that allows a portion of the N-terminal domain to recruit co-repressors such as histone deacetylases, the lysine acetyltransferase TIP60 (Kat5), and the SMRT complex (Li et al., 2007; Wang et al., 2003; Lal and Bromberg, 2009; Jepsen et al., 2008). The proline-

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rich track within the N-terminus of FoxP3 is essential for its function as a repressor (Xie et al., 2015), and this suggests that the glutamine-rich tracts in FoxP1, 2 and 4 are essential for their function. The structure of FoxP proteins when dimerized prevents them from binding the same strand of DNA on adjacent sites, suggesting that FoxP factors could also act to bring distal chromatin regions together (Stroud et al., 2006). Like FoxM1, recent reports have suggested that FoxP factors act in some cases by binding to other proteins rather than via direct interactions with DNA (Xie et al., 2015).

In addition to divergent protein domains, individual Fox factors have different binding partners and co-factors, which can influence both the specific DNA targets that are contacted and their downstream effects on transcriptional activity (Li et al., 2015b). Finally, many Fox transcription factors are expressed in distinct spatiotemporal patterns, allowing them to carry out distinct functions. However, it should be noted that, despite the well-known distinct roles of various families of Fox transcription factors, it is becoming clear that many of them regulate the same processes.

The FoxA family: regulators of development, differentiation and cell identity

Mammalian FoxA transcription factors were first identified for their DNA-binding properties in rat liver nuclear extracts and were thus originally named hepatocyte nuclear factor 3 (HNF3) α, β and γ (Costa et al., 1989). Of all mammalian Fox genes, *Foxa2* shares the most homology with the original *fkh* gene discovered in *Drosophila*. Its homolog in the nematode *C. elegans* is *pha-4* and, notably, studies of *pha-4* mutants have helped further our understanding of FoxA function by identifying FoxA targets (Gaudet and Mango, 2002), establishing their interactions with nuclear hormone receptors (Ao et al., 2004), demonstrating the recruitment of RNA polymerase II (Hsu et al., 2015) and the histone variant H2A.Z (Updike and Mango, 2006), and characterizing the regulation of PHA-4 by the TOR pathway.

The expression of FoxA factors

In mammals, FoxA1, 2 and 3 exhibit overlapping but distinct expression patterns in a variety of developing and mature tissues (Friedman and Kaestner, 2006). In the mouse, *Foxa2* is the first member of the FoxA family to be expressed. At embryonic day (E) 6.5, *Foxa2* expression can be detected in the primitive streak and in the node, both of which are important for gastrulation. By E7.5, *Foxa2* is expressed in the mesoderm and definitive endoderm (Monaghan et al., 1993; Ang et al., 1993). Its expression is then maintained in endoderm-derived tissues, such as the pancreas, liver, thyroid, prostate and lung, throughout development and in adulthood (Friedman and Kaestner, 2006), and is also observed in ectoderm-derived neural tissues, such as the ventral midbrain, including in dopaminergic neurons and the hypothalamus (Besnard et al., 2004).

During early embryogenesis, the expression of *Foxa1* is similar to that of *Foxa2*, but following a short temporal delay. For example, *Foxa1* can first be detected at E7.0 in the primitive streak, and then later in the notochord, neural plate, floor plate and neural tube (Monaghan et al., 1993). However, the expression of *Foxa1* and of *Foxa2* differ in the adult. Although *Foxa1* is initially highly expressed in the developing pancreas, its expression falls to <10% of *Foxa2* levels in alpha, beta and acinar cells in the adult pancreas (Bramswig et al., 2013). In addition, *Foxa1* is more widely expressed than *Foxa2* in several adult tissues, including the respiratory system, brain and gastrointestinal tract. *Foxa1* is also expressed in some tissues that lack FoxA2 entirely, such as male reproductive organs, the ureter and bladder (Friedman and Kaestner, 2006).

The expression of *Foxa3* is more restricted than that of *Foxa1* or *Foxa2*, and is more specific to the foregut endoderm than other family members. *Foxa3* expression can first be detected at E8.5 and is maintained strongly in the liver but also in the pancreas and intestine throughout adulthood (Monaghan et al., 1993). It is the mostly highly expressed FoxA transcription factor in the adult liver (Kaestner et al., 1994).

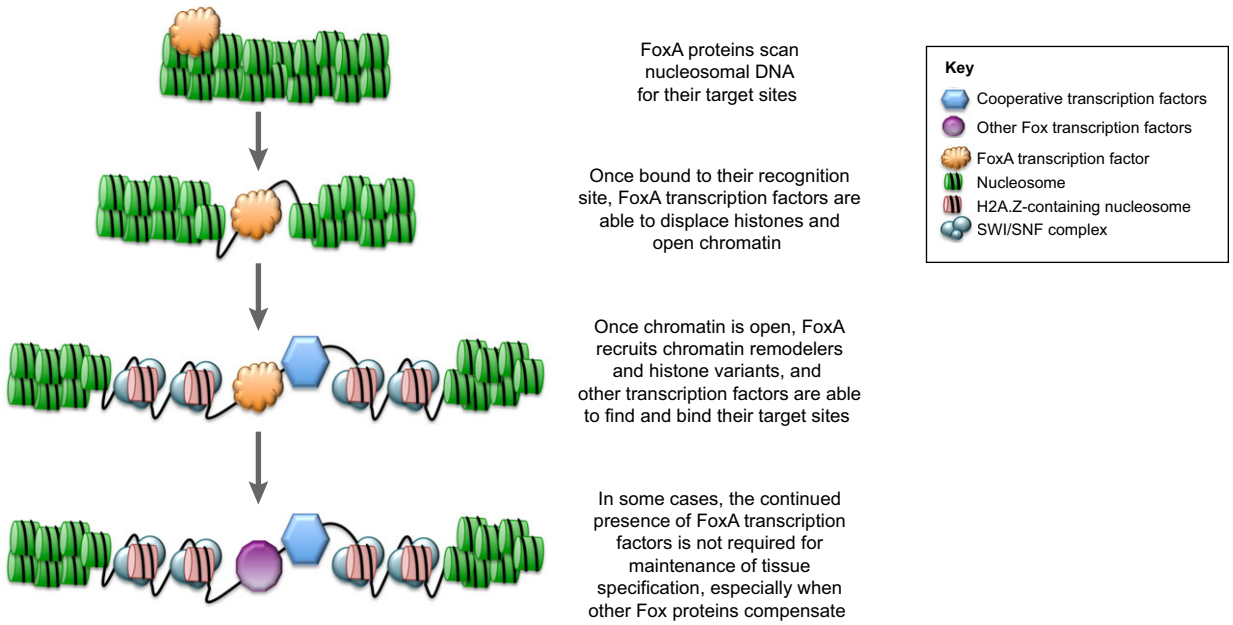

Fig. 3. FoxA proteins function as pioneer factors. The schematic depicts how FoxA transcription factors are able to function as pioneer factors that control gene expression via their interaction with chromatin.

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The role of FoxA transcription factors

The earlier expression of FoxA2 compared with FoxA1 and FoxA3 is reflected in the severity of phenotypes in mice lacking these factors. Mice null for *Foxa2* die between E10 and E11, exhibiting defects in all three germ layers (Weinstein et al., 1994; Ang and Rossant, 1994). Heterozygotes for *Foxa2* are viable but display a Parkinson's-like phenotype following aging (Kittappa et al., 2007). In contrast to *Foxa2* null mice, those lacking *Foxa1* survive until after birth, displaying lethality between postnatal day (P) 2 and 12 due to hypoglycemia and defects in kidney function (Behr et al., 2004; Kaestner et al., 1999); the hypoglycemia is likely to result from deficiencies in glucagon secretion, while the dehydration stems from abnormal kidney development (Behr et al., 2004; Kaestner et al., 1999). These mice also display defects in prostate morphogenesis (Gao et al., 2005). As might be expected from its limited expression pattern, among the FoxA family mice deficient for *Foxa3* have the mildest phenotype. They are viable with a normal lifespan but do, however, display hypoglycemia after a prolonged fast because of defects in hepatic glucose production (Shen et al., 2001).

Since the embryonic lethality of *Foxa2*$^{-/-}$ mice precludes analysis of the role of FoxA2 in the organogenesis and function of many tissues, mice with conditional deletions of *Foxa2* have been derived. Studies of these mutants reveal that FoxA2 on its own is not required for normal liver differentiation (Sund et al., 2000), although defects in lung morphogenesis are seen (Wan et al., 2004). In addition, severe defects in pancreatic islet formation and alpha and beta cell maturation that lead to alterations in glucose homeostasis are observed (Sund et al., 2000; Lantz et al., 2004). Interestingly, mice expressing an activated form of FoxA2 in neurons display an orexigenic (i.e. a stimulated appetite) phenotype (Silva et al., 2009). Although off-target effects of this mutated protein cannot be excluded, this study implicates FoxA2 in yet more aspects of metabolic regulation, namely food intake and energy output.

Cooperativity and compensation among FoxA transcription factors

The conditional deletion of genes encoding individual FoxA transcription factors revealed little requirement for any one FoxA family member in the liver (Lee et al., 2005b; Kaestner et al., 1999; Shen et al., 2001). Gross pancreatic morphology was also unaffected when only one FoxA transcription factor was missing (Lee et al., 2005b; Kaestner et al., 1999). These studies suggest either that FoxA family members are dispensable in these organs or, alternatively, that they can compensate for each other. Both *in vivo* and *in vitro* studies suggest that the latter possibility is likely: both FoxA1 and FoxA2 transcription factors must be suppressed to abolish expression of *Muc2*, which encodes a protein important for intestinal function (van der Sluis et al., 2008), and both *Foxa1* and *Foxa2* are upregulated in *Foxa3* null livers (Shen et al., 2001). In addition, several different analyses using the conditional, simultaneous deletion of *Foxa1* and *Foxa2* demonstrated that these two transcription factors can compensate for each other in a number of tissues. For example, their combined absence results in complete liver agenesis (Lee et al., 2005a), near-complete pancreatic agenesis (Gao et al., 2008), and altered allocation of enteroendocrine cells within the intestine (Ye and Kaestner, 2009), but, as outlined above, deletion of only one FoxA transcription factor leads to a less severe phenotype in these tissues. Finally, mice with a late-gestation deletion of both *Foxa1* and *Foxa2* demonstrate upregulation of *Foxa3*, with FoxA3 also being observed to bind FoxA targets in the double-null animal that were bound only by FoxA1 and FoxA2 in wild-type mice (Iwafuchi-Doi et al., 2016).

FoxM1: a key cell cycle control factor

FoxM1 was identified nearly simultaneously by three groups and hence was originally given three names: Trident (Korver et al., 1997), hepatocyte nuclear factor 3/fork head homolog 11 (Ye et al., 1997) and WIN (winged helix protein in the INS1 cell line) (Yao et al., 1997). All three groups identified FoxM1 by homology to FoxA proteins, but whereas the Clevers group discovered it within the thymus, the Costa lab cloned it from the colon carcinoma cell line Caco-2, and the Wong group identified it in a cell line derived from an insulinoma.

FoxM1 is widely recognized for its role in driving the cell cycle (Fig. 4). Its direct transcriptional targets function at both the G1/S and G2/M transitions, as well as during karyokinesis and cytokinesis (reviewed by Wierstra, 2013; Wierstra and Alves, 2007). Accordingly, FoxM1 expression is mostly confined to actively replicating cells; its expression is thus broad in embryos and declines in differentiated tissue as well as with age (Ye et al., 1997).

FoxM1 exists in various genera and species, including *Xenopus*, *Danio*, *Mus*, *Rattus* and *H. sapiens*, but not in *C. elegans* or *Drosophila*. The role of FoxM1 in *Drosophila* and *C. elegans* might be filled by other forkhead transcription factors, but at this time the only reports associating forkhead with proliferation in these two organisms is the increased replication in the absence of FoxO homologs. In humans, although one *FOXM1* gene exists, splice variants produce three different FOXM1 proteins: FOXM1A, FOXM1B and FOXM1C. Both FOXM1B and FOXM1C are transcriptional activators, whereas FOXM1A is a transcriptional repressor. In mice, only one FoxM1 protein splice variant is produced; it is most similar to human FOXM1B and is likewise a transactivator.

The crucial role of FoxM1 in development is highlighted by studies perturbing its expression. For example, mice lacking FoxM1 display embryonic lethality between E14.5 and E16.5 due to failed liver and heart expansion (Krupczak-Hollis et al., 2004). Mice with conditional deletions of *Foxm1* display profound proliferation defects in tissues such as the liver (Krupczak-Hollis et al., 2004), heart (Bolte et al., 2011), pancreas (Zhang et al., 2006), intestine (Yoshida et al., 2007), lungs (Kalin et al., 2008) and smooth muscle

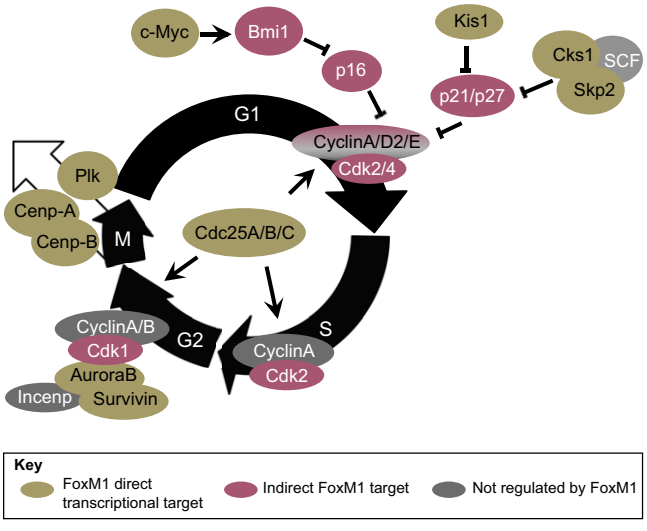

Fig. 4. The role of FoxM1 in cell cycle regulation. FoxM1 regulates various factors involved in cell cycle regulation. No evidence for FoxM1 regulation exists for those cell cycle proteins shown in gray. Cyclin D2, but not A and E, is regulated by FoxM1. p16 is also known as Cdkn2a; SCF as Kitl; p21/p27 as Cdkn1a/Cdkn1b; and survivin as Birc5.

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(Ustiyan et al., 2009). Furthermore, the early deletion of *Foxm1* in either the heart (Bolte et al., 2011) or liver (Krupczak-Hollis et al., 2004) results in embryonic lethality, whereas mice lacking *Foxm1* in smooth muscle cells (Ustiyan et al., 2009) or in the lung epithelium (Kalin et al., 2008) die perinatally.

Interest in FoxM1 was fueled by its prominent role in cancer. FoxM1 is highly expressed in a variety of tumor types and is associated with increased tumor aggressiveness (Laoukili et al., 2007). However, mouse models suggest that FoxM1 elevation by itself is not an initiating event in carcinogenesis. Mice constitutively expressing human FOXM1B in all tissues do not spontaneously develop tumors at least until 12 months of age (Kalinichenko et al., 2003; Wang et al., 2002; Yoshida et al., 2007). However, increased expression of FOXM1B, in addition to initiating mutations, leads to larger, more vascularized and more metastatic tumors (Yoshida et al., 2007; Kalinina et al., 2003). Conversely, mice lacking FoxM1 or with inhibited FoxM1 function in the tissue of interest show a reduced tumor load compared with controls when treated with carcinogens (Yoshida et al., 2007; Gusarova et al., 2007). Similar results have been observed in the lung, where increased FoxM1 activity hastens lung tumor growth in mice with induced Kras expression (Wang et al., 2010), while its absence inhibits lung tumorigenesis mediated by Kras (Wang et al., 2014a). Given this link between FoxM1 and tumor progression, anti-FoxM1 agents are being explored as potential cancer therapeutics (Box 1).

FoxM1 also appears to be implicated in aging. FoxM1 levels decline with age in multiple tissues, and this decrease is accompanied by a decline in both baseline proliferation and replicative potential. The reactivation of FoxM1 activity in aged hepatocytes and pancreatic beta cells was able to rejuvenate replication in these tissues (Golson et al., 2015; Wang et al., 2002). In addition, transplanted aged FoxM1b-expressing hepatocytes were equally successful in repopulating recipient livers as hepatocytes from young mice (Brezillon et al., 2007). Currently, the transplantation of hepatocytes or beta cells can be used to treat liver diseases or type 1 diabetes, respectively; however, a shortage of both of these cell types for transplantation prevents their widespread application. Inducing FoxM1 expression transiently *ex vivo* could, therefore, expand the availability of transplantation-quality donor tissue by rejuvenating older donor tissue. However, as is the case for other proteins involved in the cell cycle, the benefits of activating or attenuating FoxM1 activity must be carefully considered. Drug treatments are currently not targeted to any one tissue, and activating FoxM1 endogenously to rejuvenate the replicative potential in a tissue of interest without targeting it to a specific cell type could potentially cause existing malignancies to become more prone to growth or metastasis. Conversely, inhibiting FoxM1 in cancer therapy could have negative impacts in tissues that are likely to require FoxM1 activity and exhibit high cellular turnover, such as the intestine.

Box 1. Anti-FOXM1 agents as potential cancer therapies

Owing to the association of FOXM1 with aggressive tumors, and because of direct evidence highlighting the importance of FoxM1 in tumor progression in mice, there is intense interest in developing anti-FOXM1 agents as therapies for a variety of cancers. The downregulation of FOXM1 by siRNA *in vitro* decreases DNA replication as well as angiogenic and metastatic potential (Wang and Gartel, 2011; Wu et al., 2010; Halasi and Gartel, 2012; Halasi et al., 2013). A number of proteasome inhibitors have been shown to decrease FOXM1 levels, probably by stabilizing an unidentified negative regulator of FOXM1 (Gartel, 2010), and many of these inhibitors are currently used in cancer treatments; most of their effects are likely to be due, at least in part, to FOXM1 downregulation. However, these drugs are also associated with serious side effects (Adams et al., 1999), so more specific inhibitors of FOXM1 have been sought. Recently, a high-throughput screen identified a small molecule, FDI-6, that makes direct contact with the DNA-binding domain of FOXM1, blocks the interactions of FOXM1 with its transcriptional targets, and suppresses the expression of those targets (Gormally et al., 2014). FDI-6 is specific enough that it does not affect the binding or function of other Fox transcription factors, and it also does not affect proteasomal activity. This novel molecule thus has exciting potential therapeutic applications for a variety of cancers.

FoxO factors: from cell cycle control to metabolism and longevity

FoxO proteins play multiple crucial roles: they regulate the cell cycle, apoptosis, metabolism and lifespan. Recently, interest in FoxO proteins has also been spurred on by their functions in mediating insulin and insulin-like signaling (Fig. 5) (reviewed by Golson et al., 2010). In short, insulin binding to the insulin receptor causes phosphorylation of insulin receptor substrate 1/2 (IRS1/2), resulting in a phosphorylation signaling cascade that involves phosphoinositide 3-kinase and Akt, and ultimately leads to the phosphorylation of FoxO. In the absence of insulin signaling, FoxO proteins reside in the nucleus; however, active insulin signaling causes them to translocate to the cytoplasm, where they are no longer able to bind or transactivate their targets. In addition, Akt-phosphorylated FoxO proteins are stabilized in the cytoplasm through interactions with 14-3-3 scaffolding proteins (reviewed by Tzivion et al., 2011). This stabilization can be disrupted by the phosphorylation of 14-3-3 by Jun kinase and the dephosphorylation of FoxO by protein phosphatase 2A. FoxO protein stability is also regulated by ERK1/2 (Mapk3/1), which phosphorylate FoxO proteins and target them for degradation (Yang et al., 2008).

FoxO in *C. elegans*

The role of FoxO proteins in longevity was first revealed by studies in the nematode *C. elegans*. During periods of high stress, such as a low food supply, *C. elegans* enter a 'dauer' state that allows them to survive and also extend their lifespan (reviewed by Fielenbach and Antebi, 2008). In such times of nutrient shortage, expression of insulin receptor homologs such as DAF-2 is low. The sole *C. elegans* FoxO homolog, DAF-16, is therefore not phosphorylated and is thus able to enter the nucleus and transactivate its targets, a full list of which was recently published (Kaletsky et al., 2016). The effect of DAF-16 on longevity is primarily manifested in the intestine (Murphy et al., 2007; Libina et al., 2003), and its effects on lifespan are likely to be related to the regulation of genes involved in countering oxidative and thermal stress (Nemoto and Finkel, 2002).

DAF-16 also regulates multiple neuronal processes, including many different learning processes, sleep, morphology maintenance and axon regeneration (Kauffman et al., 2010; Tank et al., 2011; Toth et al., 2012; Murakami et al., 2005; Byrne et al., 2014; Nagy et al., 2014). A recent study delineated the neural targets of DAF-16 in *C. elegans* by comparing the transcriptomes of neurons that had been FACS sorted from wild type and insulin signaling-deficient (*daf-16;daf-2*) mutants (Kaletsky et al., 2016). Since many FoxO targets are conserved from *C. elegans* to humans (Webb et al., 2016), it has been proposed that understanding the mechanisms by which FoxO regulates aging in the nematode might provide a basis for extending human lifespan. However, given the fundamental differences in the aging process between these very distantly related species, caution is warranted.

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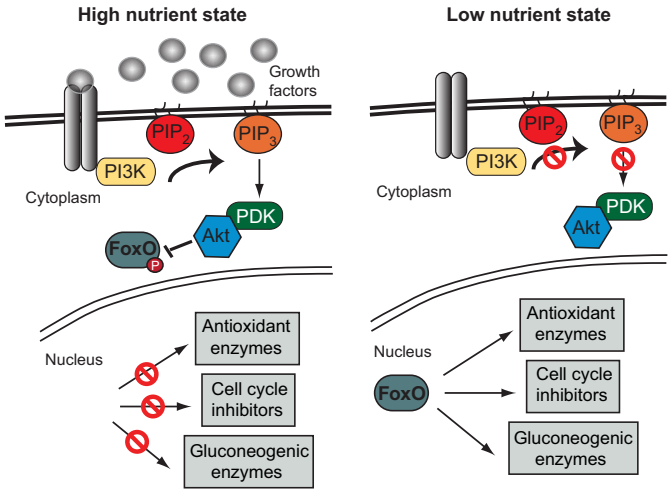

Fig. 5. FoxO regulation in response to insulin signaling in mammals. The binding of growth factors to their receptors triggers a phosphorylation signaling cascade involving phosphoinositide 3-kinase (PI3K), phosphatidylinositol (4,5) bisphosphate (PIP_2), phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), pyruvate dehydrogenase kinase (PDK) and Akt that ultimately results in the phosphorylation of FoxO. This phosphorylation confines FoxO to the cytoplasm. In the absence of growth factor signaling, including that of insulin, FoxO is thus present in the nucleus, where it upregulates anti-stress genes and cell cycle inhibitors. FoxO also regulates gluconeogenic genes in the mammalian liver to protect from hypoglycemia. Modified from Golson et al. (2010).

FoxO in mammals

Mammals have four FoxO proteins: FoxO1, FoxO3, FoxO4 and FoxO6. FoxO6 shares the least sequence homology with other FoxO family members. FoxO1 is the most thoroughly studied of the family but, like FoxO1, FoxO3 and FoxO4 have roles in regulating lifespan and metabolism and in mediating cell cycle arrest.

Regulation of metabolism by FoxO1

FoxO1 is the most highly expressed FoxO family member in insulin-responsive tissues such as the liver, adipose tissue and skeletal muscle (Armoni et al., 2006). Consistent with this broad expression pattern, FoxO1 regulates multiple metabolic processes. In the liver, FoxO1 contributes to the activation of gluconeogenic targets (Nakae et al., 2002; Matsumoto et al., 2007; Titchenell et al., 2015; Haeusler et al., 2010), and deleting one copy of *Foxo1* corrects the insulin resistance phenotype in insulin receptor-deficient mice (Nakae et al., 2002). In skeletal muscle, FoxO1 appears to control whether carbohydrates or lipids are used as fuel by regulating the expression of genes such as those encoding pyruvate dehydrogenase kinase and lipoprotein lipase (Bastie et al., 2005), while in adipose tissue it may protect white fat cells from the dysfunction associated with obesity (Subauste and Burant, 2007). FoxO1 is also a key player in regulating the differentiation of adipose tissue and skeletal muscle (Gross et al., 2008).

In addition to liver, muscle and adipose tissue, FoxO1 is highly expressed in insulin-secreting beta cells, where it may act in a protective capacity in times of stress. Several reports have indicated that FoxO proteins activate the expression of antioxidant enzymes, and FoxO1 protects beta cells against the effects of reactive oxygen species, since it and family members activate the expression of antioxidant enzymes (Kitamura et al., 2005; Kops et al., 2002; Nemoto and Finkel, 2002). FoxO1 has also been implicated in preventing beta cell de-differentiation during stress by suppressing neogenic genes, such as the early endocrine development factor neurogenin 3 (*Neurog3*), which is not normally expressed in the adult endocrine pancreas (Talchai et al., 2012). Protection by FoxO1 occurs in the presence of many metabolic stressors, such as multiple pregnancies, chemical ablation of beta cells, and when insulin resistance is conferred by leptin receptor (Lepr) deficiency. However, a contradictory study of mice deficient for Lepr in all tissues or for FoxO1 specifically in beta cells indicates that FoxO1 promotes the expression of the same genes (Kobayashi et al., 2012). The reason for this discrepancy is not apparent at this time, and both studies demonstrated more severe hyperglycemia in $Lepr^{-/-}$ mice lacking FoxO1 compared with those with FoxO1 expression. Previous work demonstrating the induction of the beta cell maturity marker MafA by FoxO1 also supports a role for FoxO1 in preventing de-differentiation (Kitamura et al., 2005). FoxO1 may thus protect beta cells from dysfunction during the period of insulin resistance that precedes type 2 diabetes.

A second role proposed for FoxO1 is in regulating beta cell mass. FoxO proteins have been shown to upregulate cell cycle inhibitors (Martins et al., 2015). In mice lacking one copy of the insulin receptor in all tissues, beta cell proliferation is reduced compared with that in wild-type mice, but deletion of one copy of *Foxo1* partially rescues beta cell division (Nakae et al., 2002). Conversely, *Foxo1* overexpression attenuates the increased beta cell mass normally observed in mice overexpressing the insulin receptor (Okamoto et al., 2006). However, contradictory reports have called the generality of these observations into question. Mice lacking *Foxo1* either in beta cells or throughout the entire pancreas show no increase in beta cell proliferation either on a chow or high-fat diet (Kobayashi et al., 2012), and mice overexpressing FoxO1 fed a high-fat diet actually showed increased beta cell proliferation (Zhang et al., 2016). These results suggest that FoxO1 might inhibit beta cell replication only when the insulin receptor is completely absent, which is a unique and rare state.

FoxO in mammalian longevity

Like DAF-16 in *C. elegans*, FoxO transcription factors together with calorie restriction and insulin signaling have been reported to regulate lifespan in rodents (Mulvey et al., 2014; Shimokawa et al., 2015). For example, decreased insulin signaling correlates with human longevity (Kojima et al., 2004; Pawlikowska et al., 2009), as do several genetic variants in *FOXO3* (Willcox et al., 2008; Pawlikowska et al., 2009; Flachsbart et al., 2009; Li et al., 2009). In mice, FoxO3, but not FoxO1, is required for an expanded lifespan due to calorie restriction (Shimokawa et al., 2015). However, a meta analysis calls this conclusion for calorie restriction into question (Swindell, 2012). First, in some mouse strains, life expectancy was actually shortened in cohorts with calorie restriction (Liao et al., 2010; Swindell, 2012). Second, *ad libitum* fed controls might not be an ideal comparison for rodents with calorie restriction; *ad libitum* access to normal chow leads to weight gain and associated detrimental health effects (Sohal and Forster, 2014). Mixed results have also been reported for non-human primates (Colman et al., 2009; Bodkin et al., 2003; Mattison et al., 2012), and studies correlating calorie restriction and longevity are hard to perform accurately in humans and thus far have been inconclusive (Heilbronn and Ravussin, 2003).

At present, the mechanisms by which FoxO3 or other FoxO transcription factors influence lifespan, if at all, in humans is still unknown. However, one could envision that the multiple processes in which FoxO plays a role, such as stress resistance, cell cycle regulation and control of apoptosis, could pleiotropically influence healthy aging.

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The FoxP family: language acquisition and cognitive function

In mammals there are four FoxP genes (*Foxp1-4*), and the transcription factors that they encode have been shown to play a role in the development of multiple cell types, including cardiomyocytes (Wang et al., 2004), neurons (Shu et al., 2005), lung epithelial secretory cells (Li et al., 2012a) and regulatory T-cells (Fontenot et al., 2003). Accordingly, FoxP null mice display varying phenotypes. *Foxp1*$^{-/-}$ (Wang et al., 2004) and *Foxp4*$^{-/-}$ (Li et al., 2004b) mice display embryonic lethality by E14.5 due to a thinning of the cardiac ventricular myocardium. *Foxp2*$^{-/-}$ mice display severe cerebellar defects and motor impairment; they usually die by 3 weeks of age (Shu et al., 2005). *Foxp3*$^{-/-}$ mice have a defect in regulatory T-cells and escalated lymphocyte proliferation rates; these combined abnormalities lead to an immune attack on multiple cells and organs, including pancreatic beta cells, resulting in a disease resembling type 1 diabetes (Fontenot et al., 2003), and *Foxp3*$^{-/-}$ mice die by 4 weeks of age (Fontenot et al., 2003). Tissue-specific deletions of FoxP factors also result in developmental defects. For example, mice with a deletion of *Foxp1* in neurons are viable but display a morphological change in the striatum and hippocampus (Bacon et al., 2015).

Members of the FoxP family, notably FoxP1 and FoxP2, have also generated interest because of their roles in regulating cognitive development processes such as speech acquisition. FoxP2 was the first of this transcription factor family to be associated with language deficits (Lai et al., 2001) but, more recently, FoxP1 has also been implicated in speech development (Horn et al., 2010; Hamdan et al., 2010). Mutations in both *FOXP1* and *FOXP2* are associated with other cognitive dysfunctions, such as autism spectrum disorder (ASD) and intellectual disability (Bacon and Rappold, 2012). However, disorders associated with the two genes do not have completely overlapping symptoms, indicating that the two transcription factors might regulate different but related brain functions. For example, language deficits caused by alterations in *Foxp2* are generally accompanied by deficits in orofacial movements, whereas impairments in language acquisition associated with *Foxp1* variants are always accompanied by another cognitive impairment (Bacon and Rappold, 2012). Currently, no known connections between cognitive function and *Foxp3* or *Foxp4* exist.

How FoxP1 and FoxP2 contribute to neural function has also been investigated in animal models. Normally, mouse pups subvocalize when separated from their mother; *Foxp2*$^{-/-}$ pups do not exhibit this behavior, and *Foxp2*$^{+/-}$ pups show a reduction (Shu et al., 2005). No alterations in vocalization were reported for mice with a brain-specific deletion of *Foxp1*; however, these mice exhibit abnormal cognitive and social behavior, echoing phenotypes in patients with *FOXP1* mutations (Bacon et al., 2015).

In a striking example of evolutionary conservation, FoxP1 function has been studied in songbirds. Mouse subvocalization is innate, whereas human speech and birdsong share the necessity that they must be learned from older members of the species. After a *FOXP2* mutation was identified in a family with inherited impaired speech acquisition, the expression patterns of *Foxp1* and *Foxp2* were investigated in zebra finch brains (Teramitsu et al., 2004). Similarities in the expression patterns of *Foxp1* and *Foxp2* were observed between songbird and human brains, and *Foxp2* was upregulated during the period when adolescent finches actively learned song patterns (Haesler et al., 2007). When *Foxp2* gene activity was suppressed during adolescence, the affected birds copied their tutors with less precision than controls. These studies highlight a remarkable conservation of function in Fox transcription factor function between disparate species.

Shared and cooperative roles for Fox transcription factors

Although Fox transcription factors have distinct expression patterns and are known for fulfilling different roles in regulating biological processes, many of these transcription factors often have similar roles. As mentioned above, FoxA and FoxO transcription factors both regulate glucose homeostasis and metabolism. However, FoxM1 (Golson et al., 2015) as well as FoxP1, FoxP2 and FoxP4 (Spaeth et al., 2015) were recently reported to contribute to these processes as well. Another overlap is the action of these proteins as pioneer factors; both FoxO (Hatta and Cirillo, 2007) and FoxE (Cuesta et al., 2007), in addition to members of the FoxA family, can act in this capacity.

Notably, a number of Fox transcription factors have been implicated in regulating cell proliferation. FoxM1 and FoxO, for example, have long been known to have opposing roles in the cell cycle and tumorigenesis. Many other Fox transcription factors, such as FoxC2, FoxE3, FoxF1, FoxF2, FoxN1 and FoxN3, also regulate proliferation (Tuteja and Kaestner, 2007b; Tuteja and Kaestner, 2007a). FoxA2 acts as a tumor suppressor by maintaining a differentiated state and decreasing metastasis in liver, lung and breast tissue (Tang et al., 2011; Wang et al., 2014b; Zhang et al., 2015), while FoxA1 acts as an oncogene in breast and prostate cancer (Yamaguchi et al., 2008; Imamura et al., 2012). Remarkably, in the liver, the FoxA proteins act as both repressors and promoters of hepatic carcinogenesis, dependent on the sex of the animals (Li et al., 2012c). Thus, while male mice deficient for both FoxA1 and FoxA2 develop fewer tumors, the situation is opposite in female mice, demonstrating the key role of FoxA factors in the action of the sex steroid hormone receptors.

Diverse Fox transcription factors also interact with estrogen receptor α (ERα; also known as ESR1) to regulate multiple aspects of tumorigenesis. In the MCF7 human breast cancer cell line, FOXA1 acts as a pioneer transcription factor and opens up chromatin, allowing the nearby binding of ERα (Hurtado et al., 2011). At cell cycle loci, FOXM1 can then displace FOXA1 and transactivate cell cycle genes. FOXM1 binds 70% of the same genes as FOXA1 in this breast cancer line (Sanders et al., 2013). Interestingly, FoxO transcription factors act as ERα status-dependent tumor suppressors in breast cancer. In ERα-positive breast cancer, FOXO3 is associated with less invasiveness, whereas in ERα-negative breast cancer, FOXO3 is associated with more invasive tumors (Sisci et al., 2013). Finally, the FoxA homolog PHA-4 contributes to increased longevity in *C. elegans* (Panowski et al., 2007). As with FoxO proteins, higher nutrient levels lead to the repression of PHA-4 activity and a decreased lifespan, and in this function FoxA is negatively regulated by the TOR pathway (Sheaffer et al., 2008).

Because many Fox transcription factors are required for life, they clearly are unable to compensate completely for each other. However, their highly related consensus binding sites suggest that they can substitute for each other to some degree. It is likely that some of the overlapping roles for Fox proteins reflect differences in expression patterns, such that one Fox protein might perform the same role in the gut that another performs in neural tissues. In other cases, it is likely that distinct Fox proteins act in somewhat divergent ways at the same loci, for example by recruiting different co-factors.

Conclusions

Fox transcription factors are remarkably well conserved DNA-binding proteins and regulators of gene expression. In mammals,

diverse Fox factors share multiple roles. This ancient class of transcription factors contributes to the control of all aspects of development, from before gastrulation to the prevention of adult disease. Indeed, Fox transcription factors constitute a promising drug target considering their involvement in so many diseases. However, because they are transcription factors and hence are not accessible at the cell surface, or even generally in the cytoplasm, they have thus far largely remained unalterable, whether at the expression or activity level. Future studies to target Fox transcription factors should be aimed in the following directions: (1) the cell type-specific delivery of a drug payload that would be internalized; (2) gaining an understanding and then targeting of the regulatory mechanisms of Fox expression; (3) the identification and targeting of Fox target genes. Such approaches will, no doubt, further our understanding of Fox transcription factors in human biology and disease.

Competing interests
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