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Foxn4: A multi-faceted transcriptional regulator of cell fates in vertebrate development

XIANG MengQing[1,2*] & LI ShengGuo[2*]

[1]*State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China;*
[2]*Center for Advanced Biotechnology and Medicine and Department of Pediatrics, Robert Wood Johnson Medical School, Rutgers University, Piscataway, New Jersey 08854, USA*

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Vertebrate development culminates in the generation of proper proportions of a large variety of different cell types and subtypes essential for tissue, organ and system functions in the right place at the right time. Foxn4, a member of the forkhead box/winged-helix transcription factor superfamily, is expressed in mitotic progenitors and/or postmitotic precursors in both neural (e.g., retina and spinal cord) and non-neural tissues (e.g., atrioventricular canal and proximal airway). During development of the central nervous system, Foxn4 is required to specify the amacrine and horizontal cell fates from multipotent retinal progenitors while suppressing the alternative photoreceptor cell fates through activating Dll4-Notch signaling. Moreover, it activates Dll4-Notch signaling to drive commitment of p2 progenitors to the V2b and V2c interneuron fates during spinal cord neurogenesis. In development of non-neural tissues, Foxn4 plays an essential role in the specification of the atrioventricular canal and is indirectly required for patterning the distal airway during lung development. In this review, we highlight current understanding of the structure, expression and developmental functions of Foxn4 with an emphasis on its cell-autonomous and non-cell-autonomous roles in different tissues and animal model systems.

Foxn4, Fox transcription factor, retinal progenitor, amacrine cell, spinal cord, Dll4-Notch

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The Fox (forkhead box) proteins comprise a superfamily of evolutionarily conserved transcriptional regulators. Since discovery of the first Fox gene *fork head* in *Drosophila* in 1989, the Fox family has rapidly expanded to over 2000 members from 108 organisms as a subgroup of the helix-turn-helix family of proteins [1−5]. This protein family is defined by a highly conserved 110-amino-acid Fox DNA-binding domain that can fold into a variant of the helix-turn-helix motif consisting of three α-helices flanked by two large loops (wings), which is termed as a "winged helix" (Figure 1A–C). To date, there are over 40 Fox family members identified in the human that can be grouped into 19 subclasses designated A to S based on sequence phylogenetic analysis. Despite the conserved DNA-binding domain, these Fox transcription factors are highly divergent in their regulatory domains and play pivotal and diverse roles in a large variety of developmental and homeostatic processes including cell proliferation and differentiation, metabolism, speech acquisition, longevity, and tumorigenesis [4,6−10].

Foxn4 is a more recently discovered member of the Fox protein superfamily. It belongs to the N subclass which has four members (Foxn1−4). Although these Foxn factors do share similarities in sequence and structure, their biological functions are not much overlapped. *Foxn1* is most related to *Foxn4* in sequence and is known as the *nude* gene. It is ex-

*Corresponding author (email: xiang@cabm.rutgers.edu; sli@cabm.rutgers.edu)

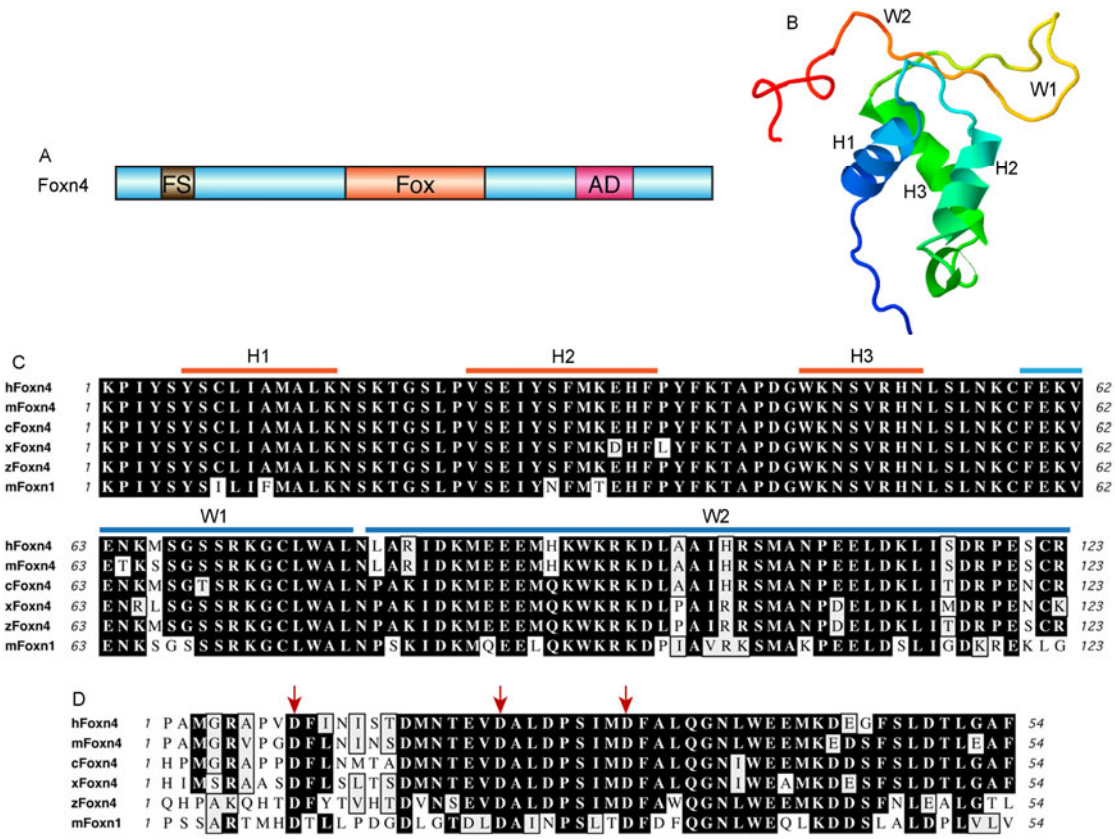

Figure 1 Foxn4 domains and structure. A, Schematic of Foxn4 protein domains. AD, activation domain; Fox, forkhead box/winged-helix DNA-binding domain; FS, Foxn4-specific domain. B, Three-dimensional structure of the Foxd3 winged-helix DNA-binding domain. C, Sequence alignment of Fox domains of human (h), mouse (m), chicken (c), *Xenopus* (x) and zebrafish (z) Foxn4 proteins and mouse Foxn1. Overlined are presumptive positions of the three α-helices (H1-H3) and two large loops (W1 and W2). D, Alignment of activation domain sequences of Foxn4 and Foxn1 proteins. Arrows indicate three critical aspartic acid residues conserved between Foxn4 and Foxn1 proteins.

pressed primarily in the epidermis, hair follicles and thymus. Loss of *Foxn1* function in mice causes hairlessness and dysgenesis of thymus, leading to T-cell related immunodeficiency [11–14]. Foxn3 is involved in craniofacial and eye development while it is currently unclear what is the biological function of Foxn2 [15–17]. Foxn4 has garnered much attention during the past several years because of its important roles in neural and cardiovascular development. In this review, we highlight recent progress toward understanding the structure, expression and biological functions of Foxn4 with an emphasis on its developmental roles in different animal model systems (Figures 1 and 2; Table 1).

1 Foxn4 protein domains and structure

Foxn4 is evolutionarily conserved from the human to zebrafish, presumably in all vertebrate species. Foxn4 proteins from different species are highly homologous in the Fox/winged-helix DNA-binding domain, sharing ⩾89% amino acid sequence identity to each other and ⩾79% sequence identity to the mouse Foxn1 (Figure 1C). Aside from the winged-helix domain located in the middle, a transcriptional activation domain in the C-terminus and a Foxn4-specific domain in the N-terminus have also been identified based on sequence homology [18–20] (Figure 1A). In the transactivation domain, Foxn4 proteins from different species share ⩾56% sequence identity with each other and ⩾39% sequence identity to the mouse Foxn1; more importantly, almost all of the acidic amino acids in this domain, which are supposed to be key to transcriptional activation, are conserved between Foxn4 and Foxn1 proteins [19,20] (Figure 1D). In retinal explants, deletion of the transactivation domain rendered Foxn4 inactive to promote amacrine cell differentiation and activate amacrine cell differentiation genes, or to suppress photoreceptor development and repress photoreceptor specification genes [19]. In cell culture, Foxn4 had transcriptional repression activity but removal of the activation domain relieved this activity [19]. Thus, the activation domain of Foxn4 may act as either a transactivation or trans-repression domain depending on the cellular context and/or available co-regulatory factors.

It has been reported that *Foxn4* transcripts obtained from tissues of embryonic and adult zebrafish encode protein

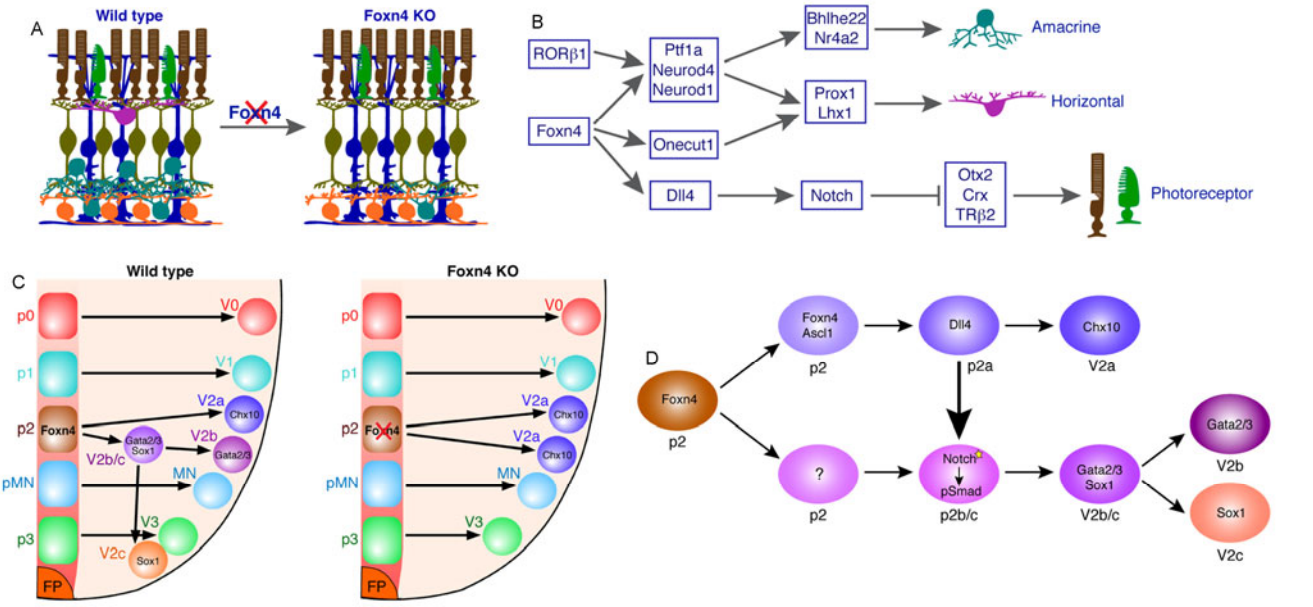

Figure 2 Roles of Foxn4 during retinal and spinal cord development. A, Schematic illustration of retinal phenotype in *Foxn4* null mutant mice. *Foxn4* inactivation results in loss of amacrine and horizontal cells but increased photoreceptors. B, Known network of genes regulated by Foxn4 to specify the amacrine and horizontal cell fates and suppress the alternative photoreceptor fates in early progenitors during retinogenesis. C, Schematic illustration of spinal cord phenotype in *Foxn4* null mutant mice. In developing wild type spinal cords, the Foxn4-expressing p2 progenitor domain generates three subtypes of interneurons: V2a–V2c, whereas in *Foxn4* null spinal cords, only V2a cells are generated. D, The expression of Foxn4 and Ascl1 coupled with lateral inhibition/cis-inhibition leads to the generation of p2a progenitors with high levels of Dll4 ligand and neighboring p2b/c progenitors with high levels of activated Notch (denoted by the asterisk). The activated Notch in turn cell-autonomously activates BMP/TGFβ signaling essential for V2b and V2c fate specification.

Table 1 Foxn4 expression pattern and developmental role

Animal model	Expression pattern		Phenotype/developmental role		Downstream genes
	Stage	Tissue/cells	Gain-of-function	Loss-of-function	
Mouse	E9.5–E13.5	Spinal cord Midbrain	Promotes V2b cells; inhibits V2a cells	Loss of V2b and V2c cells; increased V2a cells	*Ascl1*, *Scl*, *Dll4*, *Gata2*
	E11.5–P6	Retina	Promotes amacrine cells; inhibits photoreceptor, bipolar and Müller cells	Loss of amacrine and horizontal cells; increased photoreceptor cells	*Ptf1a*, *Dll4*, *Prox1*, *Nr4a2*, *Onecut1*, *Neurod1*, *Neurod4*, *Bhlhe22*
	E14.5–P8	Trachea Bronchus Bronchiole Esophagus		Dilated alveoli and impaired septation	*Pdgfa*, *Sftpb*, *α-SMA*
Chicken	st21 and st22	Spinal cord Brain	Ectopic expression of Lhx1, Prox1, Pax6, Lhx3, Tfap2a, Isl1, and Isl2		*Lhx1*, *Prox1*, *Pax6*, *Lhx3*, *Tfap2a*, *Isl1*, *Isl2*
	st12–45	Retina	Promotes amacrine, horizontal, ganglion, and photoreceptor cells		*Lhx1*, *Prox1*, *Pax6*, *Lhx3*, *Isl1*, *Pou4f1*
Zebrafish	14 hpf-adult	Brain			
	20 hpf-day 7	Retina			
	16–24 hpf	Spinal cord			
	6 weeks–5 months	Thymus			
	24–72 hpf	Atrioventricular myocardium		Failure of AV cell differentiation, heart tube looping and electric conduction delay; pericardial edema	*tbx2b*, *bmp4*, *versican*, *Notch1b*
Xenopus	st18–45	Brain			
	st18–36	Spinal cord			
	st16–45	Retina			

isoforms that differ in the N-terminus, and in humans, there are alternative transcripts with and without the Fox domain [21]. The significance of these alternative isoforms is unclear and their presence has yet to be confirmed independently. *FOXN4* has been localized at 12q24.11 in the human chromosome. Unlike some other *FOX* genes [22], *FOXN4* is neither distributed in a cluster nor involved in any gene rearrangement.

2 Developmental expression profiles of Foxn4

2.1 Rodent

F*oxn4* is expressed in both neural and non-neural tissues of the developing mouse. In the nervous system, its expression is detected only in a few selected CNS tissues including the midbrain, hindbrain, spinal cord, and retina while absent from the peripheral nervous system. At about E9.5–E10.5, *Foxn4* commences its expression in the dorsal mesencephalon, ventral rhombencephalon and ventral spinal cord [18,23]. In the spinal cord, Foxn4 protein is found in p2 progenitor cells co-expressing Ascl1, Lhx3 and Pax6, but not in mature V2a ($Chx10^+$) and V2b ($Gata2/3^+$) cells, in other interneuron subtypes, or in motoneurons [23]. Most of the Foxn4-positive cells can be labeled by a short pulse of BrdU and therefore are proliferative progenitor cells rather than differentiated neurons. Consistent with this, Foxn4 expression in the brain and spinal cord becomes weak at E12.5 and disappears by E13.5 [23].

In the mouse eye, Foxn4 expression starts in the central retina at E11.5, then gradually becomes abundant in the entire retinal outer neuroblastic layer by E13.5, but is absent from the inner neuroblastic layer [18,24]. There is no expression of Foxn4 in any other tissues of the eye such as lens and pigmented epithelium [18,24]. Starting from P1 and by P7, the expression of Foxn4 in the retina gradually disappears from the center to the periphery [24]. Thus, both the onset and down-regulation of Foxn4 expression follow a central to peripheral gradient of retinogenesis. Given that Foxn4-positive cells co-express progenitor markers Pax6 and syntaxin in the outer neuroblastic layer and can be labeled by a short pulse of BrdU, it is believed that Foxn4 is expressed only in a subset of dividing progenitors in the retina, just as in the spinal cord [24].

Because Foxn4 was thought to be specific to the nervous system [18], it was somewhat of a surprise later to observe Foxn4 expression in the mouse airway system [25]. At E14.5, Foxn4 expression turns on in a small set of cells in the epithelia of the trachea and esophagus, then spreads to the epithelia of bronchi and bronchioles by E15.5. However, it is undetectable in saccules and alveoli of the emerging lung structure, indicating expression of Foxn4 only in the proximal airway of the developing respiratory system. This expression pattern persists until at least P8. Unlike its expression in the CNS, Foxn4 is expressed only in postmitotic epithelial cells rather than in proliferative progenitors in the proximal airway [25]. This implies that Foxn4 may be involved in different aspects of cell fate determination and differentiation between neural and non-neural tissues.

Thymus may be another non-neural site for *Foxn4* expression. A low-level of *Foxn4* transcripts was detected in the adult rat thymus by qRT-PCR assay [19]. Consistent with this, we observed β-gal expression in a small population of cells in the thymus of postnatal $Foxn4^{lacZ/+}$ mice (Luo H, Li S, Xiang M, unpublished data). Thus, it will be worthwhile to investigate whether Foxn4 has a role in thymus development.

2.2 Chick

In the chick retina, Foxn4 exhibits a similar spatiotemporal expression pattern as in the mouse. Its expression can be detected as early as stage12 (st12) in the medial region of the optic vesicle. As embryogenesis progresses, the *Foxn4* signal spreads from the medial region to the whole retinal neuro-epithelium by st26. There is overlap between Foxn4 and Pax2, Sox2 or Pax6 expression in progenitor cells [26]. Some of the newborn migratory $Lhx1^+$ horizontal progenitors also become Foxn4-positive [27]. From st35 onwards, *Foxn4* expression gradually gets reduced from the medial to peripheral region, and by st45, there are only sparse *Foxn4*-positive cells remaining at the tip of the ciliary marginal zone [26,27].

In the chick spinal cord, Foxn4 becomes detectable in a small number of cells in the rostral region at st13 [28]. These cells increase at later stages and are generated in the ventral p2 domain within the Nkx6.1 expression region but immediately dorsal to the Olig2-positive pMN domain [26,28]. As in the mouse, Foxn4 expression overlaps with Gata2 in the transitional V2b precursors but not with Gata2 or Chx10 in mature V2b or V2a interneurons, respectively [28].

2.3 Xenopus

In *Xenopus*, *Foxn4* expression emerges as early as st16 in the developing eye field at the anterior edge of the neural plate [29]. The *Foxn4*-expressing patch then splits into a pair of symmetrical eye fields as development progresses. During the tailbud stage, *Foxn4* expression becomes restricted to the neural retina within the eye. At st36, *Foxn4* is most strongly expressed in the developing ciliary marginal zone where retinal progenitor cells reside, while largely absent from the rest of the retina. By st45, *Foxn4* expression shrinks to a narrow stripe of the ciliary marginal zone where stem cells are located [29].

In addition to the retina, *Foxn4* is also expressed in the developing brain, spinal cord and pronephros [29]. Its expression in the brain is first seen in the hindbrain and midbrain at st18–19, and by st35–36, it expands to the entire

forebrain including olfactory bulb. This pattern becomes more prominent by the tadpole stage st45 when *Foxn4* is expressed strongly in the olfactory bulb, pallium, prosomeres, hypothalamus, optic tectum, preoptic area, isthmus, reticular formation, and the lining of the ventricle [29].

2.4 Zebrafish

During zebrafish development, *foxn4* is initially expressed ubiquitously from one-cell through gastrula stages [21], presumably largely by maternal transcription. From 10 hours post-fertilization (hpf) onwards, *foxn4* expression is seen in the forebrain which later becomes the olfactory placode, and by the adult stage, it is confined to the basal layer of the olfactory epithelium. Meanwhile, *foxn4* expression becomes detectable in the anterior midbrain at 14 hpf but it subsequently disappears by 22 hpf. From there, *foxn4* expression extends slowly to the periventricular layer of the tectum, isthmus, lateral regions of the tectum, and hindbrain by day 7 [21]. In the adult brain, *foxn4* remains expressed in the periventricular layers, telencephalon, olfactory bulbs, and the pituitary gland.

In the neural tube, *foxn4* is expressed in a pair of dotted lines along the anterior-posterior axis at 14 hpf and this expression lasts only several hours. It is believed that *foxn4* is localized to the region where motoneurons develop [21]. However, in light of the *foxn4* location known in other animal models and discussed above [23,28], zebrafish *foxn4* is more likely expressed by p2 progenitor cells of the ventral neural tube. In the retina, *foxn4* expression begins at 20 hpf from the ventronasal region and then spreads dorsally. By day 7, it is restricted to the neuroepithelial cells of the ciliary marginal zone [21]. Thus, the spatiotemporal expression pattern of *foxn4* in zebrafish brain, spinal cord and retina closely resembles that in *Xenopus*.

Besides the nervous system, *foxn4* is also expressed in the adult thymus as in the rodent [19,21]. In addition, it is found in the atrioventricular myocardium at 24–72 hpf [30]. This site of *foxn4* expression has an important functional implication as discussed below and has yet to be reported in other animal models.

3 Roles of Foxn4 during development

3.1 Foxn4 is required for generation of retinal amacrine and horizontal cells

The function of Foxn4 during retinal development has been studied by both gene targeting and overexpression analyses (Figure 2A). *Foxn4* inactivation in mice leads to retinal dysplasia with thinned inner nuclear and ganglion cell layers as well as outer and inner plexiform layers but thickened outer nuclear layer [24]. This is because loss of *Foxn4* function largely eliminates amacrine neurons and horizontal cells residing in the inner nuclear layer, while causing a fate switch to generate supernumerary photoreceptors located in the outer nuclear layer. When overexpressed in neonatal mouse retinal progenitors, Foxn4 strongly promoted the amacrine cell fate while suppressing the differentiation of photoreceptor, bipolar and Müller glial cells but it had no effect on horizontal cells [24]. These data were thought to imply that Foxn4 may be necessary and sufficient for commitment to amacrine cells by retinal progenitors but only required for competence acquirement during the genesis of horizontal cells. However, recent experiments in earlier chick retinal progenitors have shown that Foxn4 overexpression is able to promote horizontal cell differentiation [19,26]. Thus, Foxn4 may be necessary and sufficient for determining both the amacrine and horizontal cell fates and the discrepancy is probably caused by late progenitors used in the mouse experiment [24], which lack competency for horizontal cell genesis.

As one of the early key factors in the retinogenesis hierarchy, Foxn4 appears to specify amacrine and horizontal cell fates by regulating expression of a series of retinogenic factors and by directly interacting with at least one of them (Figure 2B). *Foxn4* deficiency results in marked downregulation of *Ptf1a*, *Neurod1*, *Neurod4*, *Bhlhe22*, *Nr4a2*, *Neurog2*, and *Lhx1* which are crucial for amacrine and/or horizontal cell genesis [24,31–33]. On the other hand, misexpression of Foxn4 activates expression of retinogenic factor genes including *Ptf1a*, *Prox1*, *Lhx1*, and *NeuroAB* that are required/implicated for amacrine and horizontal cell differentiation [19,26,34,35]. Ptf1a appears to be a key effector that mediates the function of Foxn4 during retinogenesis. It is a bHLH factor expressed in postmitotic retinal precursor cells and its inactivation causes complete elimination of horizontal cells and profound loss of amacrine cells [32,36], a phenocopy of the *Foxn4* mutant retina. Forced Ptf1a expression similarly promotes the amacrine and horizontal cell fates and upregulates expression of *Prox1*, *Lhx1*, *NeuroAB*, and *Pax6* [35]. Unlike the loss of *Ptf1a* expression in *Foxn4* mutant retinas, Foxn4 is expressed prior to Ptf1a and its expression remains normal in the *Ptf1a* mutant retina, indicating that Foxn4 acts upstream of Ptf1a to control the generation of amacrine and horizontal cells [32].

Targeted disruption of the retinoid-related orphan receptor isoform β1 (RORβ1) phenocopies the *Foxn4* mutant in amacrine and horizontal cell development and downregulates the expression of *Ptf1a*, *Prox1*, *Lhx1*, *Neurod1*, *Neurod4*, *Nr4a2*, *Bhlhe22*, *Ebf3*, and *Barhl2* [24,37]. Moreover, overexpression of RORβ1 in neonatal mouse retinas promotes the differentiation of amacrine cells but not horizontal cells. Given the similarity in phenotype and loss of *Ptf1a* expression in *RORβ1* mutant retinas, RORβ1 is believed to be involved in the Foxn4-Ptf1a pathway and acts upstream of Ptf1a. Foxn4 is likely to function in parallel with RORβ1 in this pathway since there is no alteration of *Foxn4* expression in the *RORβ1* null retina [37]. Indeed, Foxn4 and RORβ1 are shown to synergistically activate *Ptf1a* expres-

sion through direct binding to enhancer elements and protein-protein interaction [37]. Thus, the cooperation and coordination between Foxn4 and RORβ1 are critical for amacrine and horizontal cell development (Figure 2B). In a recent study [38], the expression of Onecut1 has been shown to be downregulated in *Foxn4* null retinas but unchanged in *Ptf1a* mutant retinas. Its conditional ablation leads to loss of most horizontal cells while overexpressed Onecut1 acts synergistically with Ptf1a to promote the horizontal cell fate. Therefore, Onecut1 functions downstream of Foxn4 and in parallel with Ptf1a to mediate the function of Foxn4 in horizontal cell specification (Figure 2B).

Early retinal progenitors are multipotent and capable of generating several cell types including ganglion, amacrine, horizontal, and photoreceptor cells. Foxn4 selects the amacrine and horizontal cell fates from the multipotent progenitors not only by promoting these two fates but also by suppressing alternative fates. For instance, there is overt increase of photoreceptor cells and *Crx* expression in *Foxn4* null retinas and overexpressed Foxn4 strongly inhibits photoreceptor differentiation [24], suggesting that Foxn4 normally suppresses the alternative photoreceptor fate of early retinal progenitors. To explore the underlying mechanism, microarray profiling revealed that the Notch ligand gene *Dll4* was significantly downregulated in *Foxn4* null retinas. On the other hand, overexpressed Foxn4 greatly induced *Dll4* expression [33]. Foxn4 colocalizes with Dll4 in a subset of mouse retinal progenitors and can directly bind to a *Dll4* enhancer to activate gene expression [33]. Conditional *Dll4* inactivation results in upregulation of *Otx2*, *Crx*, *Neurod1*, *Thrb*, and *Rxrg* expression followed by an increase of photoreceptor cell differentiation [33]. Thus, Foxn4 suppresses photoreceptor fates in early retinal progenitors by directly activating Dll4-Notch signaling (Figure 2B).

Foxn4 is also involved in axon outgrowth of retinal ganglion cells [39]. In the *Foxn4* null retina, despite no or minimal ganglion cell loss, there is a developmental delay in the distribution of ganglion cell projections to the superior colliculus, and consequently, the axons of ganglion cells fail to penetrate into the retinorecipient layers of the superior colliculus. This effect is most likely indirect and secondary to amacrine cell loss because there is no expression of Foxn4 in either ganglion cells or superior colliculus [39].

3.2 Foxn4 is required for V2 interneuron diversification during spinal cord development

During development of the ventral spinal cord, motor neurons and four classes of interneurons V0–V3 arise respectively from pMN and p0-p3 progenitor domains uniquely positioned along the dorsoventral axis [40] (Figure 2C). The p2 progenitor domain generates at least three subtypes of interneurons: two major subtypes V2a and V2b and a minor subtype V2c. The V2a cells are defined by their expression of Chx10 and Lhx3, V2b cells by expression of Scl, Gata2 and Gata3, and V2c by Sox1 expression [41–44] (Figure 2C). V2b and V2c neurons share common precursors that express Gata3 [44]. By Cre-loxP lineage mapping, Foxn4 appears to be transiently expressed in all p2 progenitors since Foxn4-expressing cells give rise to all neurons of the V2a, V2b and V2c subtypes [45].

Foxn4 plays an essential role in the specification of V2b and V2c interneurons. Loss of *Foxn4* function in mice completely abolishes V2b and V2c cells as indicated by the loss of expression of Scl, Gata2, Gata3 and Sox1 at the V2b and V2c positions, whereas the expression of Chx10 and Lhx3, markers for V2a neurons, is dramatically upregulated [23,28,44]. On the other hand, forced Foxn4 expression induces ectopic expression of V2b markers Gata2, Gata3 and Scl but downregulates the V2a markers Lhx3 and Chx10 [28]. Given that both V2a and V2b neurons are derived from the same p2 progenitor pool, these data indicate that *Foxn4* inactivation leads to a switch of V2b/2c to V2a cell fates in p2 progenitors (Figure 2C).

The function of Foxn4 in V2 subtype specification is mediated in part by Scl and Ascl1. There is complete downregulation of *Scl* and *Ascl1* expression in *Foxn4* null spinal cords [23,28]. Similar to the role of Foxn4 in V2 interneuron diversification, conditional inactivation of *Scl* results in loss of *Gatat2* and *Gata3* expression while its overexpression induces ectopic Gata3-positive V2b cells but inhibits Chx10-positive V2a cells [46]. Similarly, *Ascl1* disruption causes V2b cell loss, albeit to a lesser extent, and an increase of V2a cells [23]. However, unlike Foxn4 and Scl, overexpressed Ascl1 does not promote either V2b or V2a cell differentiation; on the contrary, it suppresses Chx10 expression [23,28]. Thus, both Foxn4 and Scl are necessary and sufficient for V2b cell specification whereas Ascl1 is only necessary but insufficient.

Similar in retinal development, Foxn4 controls subtype diversification in the V2 domain by regulating Dll4-Notch signaling (Figure 2D). Notch signaling has been shown to be essential for generating V2b interneurons as evidenced by the loss of V2b neurons but increased V2a cells in *Notch1* and *Presenilin* mutant spinal cords [28,47]. Dll1-Notch signaling is dispensable for V2b cell specification because conditional ablation of *Dll1* has no effect on V2b cell number and its overexpression induces no ectopic V2b cells [47,48]. In contrast, overexpressed Dll4 upregulates *Scl* expression and increases V2b cells at the expense of V2a neurons [28,47], indicating that V2 cell diversification depends on Dll4-Notch signaling. Dll4 is colocalized with Foxn4 in a subpopulation of p2 progenitors [28]. Inactivating *Foxn4* completely abolishes *Dll4* expression whereas its overexpression induces ectopic *Dll4* expression [28]. Therefore, Foxn4 activates Dll4-Notch signaling to control interneuron fates in the p2 progenitor domain. Recently, we obtained preliminary data to show that activated Notch signaling in turn activates BMP/TGFβ signaling to

specify V2b interneurons from p2 progenitors (Misra K, Li S, Xiang M, unpublished data) (Figure 2D).

It remains to be determined whether Foxn4 directly activates *Dll4* expression in p2 progenitors as in the developing retina [33]. Given the co-expression of both *Ascl1* and *Dll4* with Foxn4 and the dependence of their expression on Foxn4 [23,28], it is conceivable that Foxn4 may activate *Dll4* expression indirectly through regulating *Ascl1* expression. Consistent with this, Ascl1 overexpression can indeed induce ectopic *Dll4* expression [28]. However, *Dll4* expression remains normal in the p2 domain in the absence of *Ascl1*, suggesting that Ascl1 is not required for the initiation of *Dll4* expression and that Foxn4 may activate *Dll4* expression directly and/or via an as-yet unknown mechanism [28].

3.3 Foxn4 is required for specifying the atrioventricular canal

The vertebrate heart is a specialized muscular blood pump that provides oxygen and nutrients to the whole body. Heart development is initiated from a simple heart tube originated from the lateral cardiogenic mesoderm. During the course of cardiogenesis, the heart tube undergoes sequential morphological changes and rearrangements to form distinct chambers that are required for unidirectional blood flow as well as the electrical conduction system critical for proper cardiac rhythm [49,50]. The fish heart represents the simplest form of a chambered heart with two chambers, the atria (inflow) and ventricle (outflow), separated by the atrioventricular (AV) boundary.

In a mutagenesis screen of zebrafish, *foxn4* mutation was identified to be responsible for the failure of myocardial and endothelial cell specification within the AV boundary of the *slipjig* mutant [30]. Consequently, in this mutant, myocardial and endothelial cells in the AV boundary fail to undergo the characteristic morphological changes, AV canal fails to form and heart fails to loop. Moreover, there is no delay in electrical conduction at the AV boundary such that the heart exhibits peristaltic contraction rather than sequential beating [30].

foxn4 disruption in the *slipjig* mutant causes expression pattern changes for several late cardiogenic genes including *bmp4*, *tbx2b*, *versican* and *Notch1b*, but the early ones appear to be normal in expression. In the mutant, the expression of *bmp4* and *versican* is expanded throughout the ventricular myocardium rather than restricted to the AV canal and outflow tract, while *tbx2b* expression is lost from AV cardiomyocytes [30]. Moreover, the endocardial marker *Notch1b* expression in the *slipjig* mutant appears all over the AV canal [30].

tbx2 is known to be responsible for the AV canal and outflow tract formation in mice [51,52]. In zebrafish embryos, both *tbx2b* and *foxn4* are expressed in the AV canal at 48 hpf. Morpholino knockdown of *foxn4* or *tbx2b* recapitulates the phenotype observed in the *slipjig* mutant [30], indicating that *foxn4* is indeed the gene affected by the *slipjig* mutation and that Tbx2 is a downstream effector during AV canal development. There exist evolutionarily conserved Foxn4 and Tbx5 binding sites in the *tbx2* enhancer that can be bound respectively by Foxn4 and Tbx5 in electrophoretic mobility shift assays. In transgenic zebrafish, mutating either *foxn4* or *tbx5* sites eliminates reporter expression from the *tbx2* enhancer in the AV canal [30]. Thus, Foxn4 and Tbx5 function cooperatively to directly bind *tbx2* and activate its expression to specify the AV canal.

In mammals, *Tbx5* functions to activate the chamber myocardial gene program, while *Tbx2* locally represses this program to favor the valvuloseptal and conduction system development [49,53,54]. In humans, several *TBX5* mutations are associated with the Holt-Oram syndrome [55,56], a disease that causes severe limb and heart abnormalities including septal and conduction defects. To date, however, a function for Foxn4 in heart development has yet to be reported in mammals.

3.4 Foxn4 is involved in early alveologenesis during lung development

The respiratory system develops from a pair of primordial lung buds originated from the ventral foregut endoderm followed by subsequent elongation and branching to give rise to a pulmonary tree that ultimately advances into a mature airway and alveoli [57,58]. The mature respiratory epithelium consists of multiple cell types established along the proximodistal axis including ciliated, secretory, basal, and goblet cells in the proximal region, and type I and II cells in the distal alveolar region.

Foxn4 inactivation in mice causes developmental defects not only associated with the nervous system but also in non-neural tissues like the airways. It results in dilated alveoli with thinned wall structure and reduced septa but no defect in proximal airways [25]. Given that Foxn4 is expressed only by a subpopulation of postmitotic cells within the proximal airway including trachea, bronchi and bronchioles, this distal phenotype is thought to result from an indirect non-cell autonomous function of Foxn4 in alveologenesis [25].

Several signaling molecules including Pdgfa, Lfng, Notch, and Rarg/Rxra are required for alveologenesis and their absence results in alveolar defects similar to those seen in the *Foxn4* mutant [59–62]. For instance, inactivating *Pdgfa* in mice leads to a complete loss of alveolar smooth muscle cells followed by a subsequent septation failure in alveologenesis [59,60]. *Foxn4* deficiency causes downregulation of Pdgfa and Sftpb in the alveolar sacs as well as of α-SMA in the septa [25]. Thus, these data suggest that the role of Foxn4 in alveologenesis is mediated at least in part by regulating the expression of Pdgfa and α-SMA.

4 Conclusion

Foxn4, as a relatively new member of the Fox superfamily, has recently garnered much attention because of its essential roles in neuronal fate specification and heart development [50,63,64]. It is expressed in mitotic progenitors and/or postmitotic precursors in neural and non-neural tissues, and has cell-autonomous as well as non-cell-autonomous functions during vertebrate development. During neurogenesis, it appears to promote the amacrine and horizontal cell fates cell-autonomously but suppress photoreceptor cell fates and promote V2b/V2c interneurons via a non-cell-autonomous mechanism through Dll4-Notch signaling [23,24,28,33,47]. In non-neural tissues, it seems to play a cell-autonomous role in heart development but a non-cell-autonomous role in alveologenesis during lung development [25,30]. In addition, Foxn4 is involved in proliferation of early retinal progenitor cells [24]. Thus, Foxn4 has emerged as a key transcriptional regulator of vertebrate development and plays multiple roles in cell fate determination through different cellular and molecular mechanisms. Despite these advances, we do not yet know the complete gene regulatory network and protein interaction network in which Foxn4 participates, the identity of the full set of its transcriptional targets, or whether it has a role in mammalian heart development. Investigation into these and other areas will provide important insight into the complex molecular mechanism by which Foxn4 controls multiple neural and non-neural cell fates during vertebrate development.

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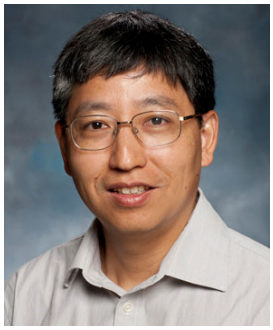

Biographical Sketch

Dr. Xiang MengQing is a Professor at the Center for Advanced Biotechnology and Medicine and Department of Pediatrics, Rutgers University-Robert Wood Johnson Medical School. He received his B.S. degree in plant genetics from Sun Yat-sen University of China in 1985. As a CUSBEA fellow of Class V (1986), he earned his Ph.D. degree in molecular biology and biochemistry from the University of Texas MD Anderson Cancer Center, Houston, in 1991. Before joining Rutgers University as an Assistant Professor in 1996, Dr. Xiang conducted his postdoctoral studies at the Johns Hopkins University School of Medicine. He was promoted to Associate Professor in 2002 and full Professor in 2006, and in 2012, selected into the Program of Global Experts. His research interests center on understanding the molecular mechanisms and regulatory gene networks that govern the determination, differentiation and survival of sensory neurons and cells. Dr. Xiang has received a number of professional awards including the Basil O'Connor Starter Scholar Research Award, Sinsheimer Scholar Award, and the Wolf, Block, Schorr and Solis-Cohen, LLP Award in auditory science.