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Transcription Factor Networks Specify Sympathetic and Adrenal Chromaffin Cell Differentiation

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ABSTRACT

The identification of mechanisms leading to the restriction of lineage potential and cell fate specification of multipotential progenitor cells falls within the purview of the developmental biologist. In specific, neural crest (NC) cell differentiation has long been a favored model process to examine how environmental cues cooperate with cell intrinsic factors to specify the birth of multiple cell lineages, including sympathetic and adrenal chromaffin (SA) cells. Over the years, a handful of genes (MASH-1, Phox2a/b, Hand2, GATA-2/3) have been identified that, when their expression patterns are perturbed, lead to a variable degree of disruption in SA cell development, function and tissue-specific gene expression profiles. These genes have historically been thought to act in a monotonous, linear fashion (e.g. gene product A regulates gene B, whose product in turn regulates gene C). Recent genetic studies in mice and other model organisms provide substantial evidence to indicate that these regulatory effectors may interact in a non-linear, self-sustaining feedback network. This review summarizes our current knowledge of the five principal players that partake in the transcriptional regulatory circuitry that is employed during SA cell development.

Keywords: neural crest, sympathetic and adrenal chromaffin cell, transcription factors, MASH-1, Phox2a/b, Hand2, GATA-2/3, TH, DBH

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SYMPATHETIC AND ADRENAL CHROMAFFIN CELL DEVELOPMENT: LINEAGE SPECIFICATION

During early embryogenesis, NC cells delaminate from the dorsal surface of the neural tube beginning at about the 6-somite stage in mice (e8.0~8.5) or around HH stage 9 (e2.0) in chick embryos (Gammill *et al*. 2006). The NC cells migrate ventrally or dorsolaterally to arrive at diverse final destinations within the embryo where they differentiate into cell lineages that include sympathetic neurons, sensory neurons, melanocytes, parafollicular cells and adrenal chromaffin cells as well as much of the bone, cartilage and connective tissue of the head and neck (le Douarin 1982). To date, we have only an incomplete mechanistic understanding of how multipotential NC cells are eventually induced toward a given lineage commitment decision and their choice of migration paths, but it is widely accepted that the combinatorial effects of extrinsic environmental cues encountered by migrating NC cells, together with their cell intrinsic programs, determine their final cell fate decisions (for details, see Harris and Erickson 2007).

In order to pattern SA cell lineages, trunk-derived NC cells migrate ventrally, passing through the anterior somitic mesoderm of the developing embryo to arrive in the vicinity of the dorsal aorta (DA) at around e10 in the mouse or at e2.5 in the chick (**Fig. 1**; Loring and Erickson 1987; Goridis and Rohrer 2002). Bone morphogenetic proteins (BMPs) secreted from the wall of the DA have been shown to be essential for the specification of SA cell lineages. Definitive evidence demonstrating that BMPs can augment sympathetic neuronal differentiation comes from experiments showing that administration of exogenous BMP (-2, -4 or -7), or forcible expression of a constitutively active BMP type I receptor, increases the number of tyrosine hydroxylase (TH) -expressing cells within a NC cell population (Varley *et al*. 1995; Reissmann *et al*. 1996; Shah *et al*. 1996; Varley *et al*. 1996, 1998; Schneider *et al*. 1999; Bilodeau *et al*. 2001). Furthermore, *in ovo* forced expression of BMP-4 in the vicinity of the developing sympathetic ganglia results in the generation of ectopic TH-positive cells (Reissmann *et al*. 1996). Several reports have documented that the mRNAs encoding BMP-4/7 and their receptors (BMPR-IA and BMPR-IB) accumulate in the DA wall and in NC cells that aggregated nearby, respectively (Reissmann *et al*. 1996; Shah *et al*. 1996; McPherson *et al*. 2000). Finally, it has been shown that after physically implanting beads that release noggin, a potent and specific inhibitor of BMP-4/7, adjacent to the DA, the expression of noradrenergic and neuronal markers in NC cells that had coalesced nearby is abolished (Schneider *et al*. 1999).

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Invited Mini-Review

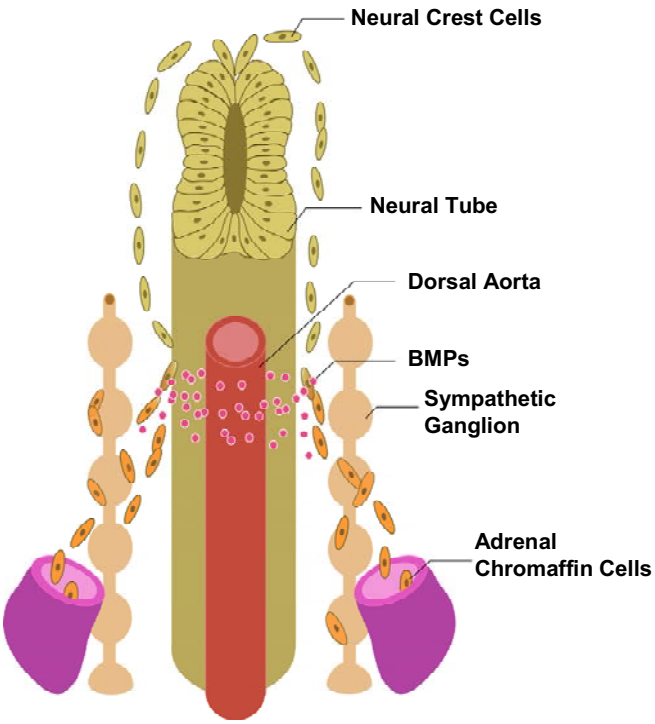

Fig. 1 Sympathetic and adrenal chromaffin cells arise from neural crest cells, which emerge from the dorsal surface of the neural tube. Once the trunk-derived neural crest cells have arrived in the vicinity of the dorsal aorta (DA), they begin to form the primary sympathetic ganglia in response to Bone Morphogenetic Protein (BMP) signals derived from the wall of the DA. These primary sympathetic progenitors subsequently embark on a second migratory route to their final destination, i.e. the definite sympathetic ganglia, the adrenal gland and the extra-adrenal chromaffin tissue, where they finally become sympathetic neurons or chromaffin cells, respectively.

However, genetic proof for BMP involvement *in vivo* in SA cell development is lacking since homozygous mutant embryos carrying ablative mutations in the genes encoding BMP-2 and BMP-4 die early during gestation, between e6.5 and e10.5 (Winnier *et al*. 1995; Zhang and Bradley 1996). *bmp7* homozygous mutants expire perinatally with evidence of skeletal, renal and eye defects but with no reported SA cell deficiency (Luo *et al*. 1995; Dudley *et al*. 1995). The BMPs bind to heterodimeric complexes that consist of type I (BMPR-IA, BMPR-IB and ALK2) and type II (BMPR-II, ActR-II and ActR-IIB) serine/threonine kinase receptors (reviewed in Kawabata *et al*. 1998). While conventional germ line mutagenesis of all six receptors has been performed, only disruption of the BMPR-IA, ALK2 and BMPR-II genes results in embryonic lethality between e6.5 and e9.5 (Mishina *et al*. 1995; Gu *et al*. 1999; Mishina *et al*. 1999; Beppu *et al*. 2000). Thus, a definitive test for the role of BMPs and their receptors in SA cell commitment and differentiation *in vivo* must await the generation and analyses of conditional gene-targeted alleles.

In response to BMP signals emanating from the DA wall, NC cells undergo differentiation to form the primary sympathetic ganglia and express transcription factors MASH-1 (Mammalian Achaete-Scute Homolog 1)/CASH-1 (the chick homologue of MASH-1), Phox2a/b, Hand2 (heart and neural-crest derivatives-expressed 2) and GATA-2/3, which are known to impact SA cell differentiation in zebrafish, chick and mouse studies (Guillemot *et al*. 1993a, 1993b; Pattyn *et al*. 1997, 1999; Howard *et al*. 2000; Lim *et al*. 2000; Tsarovina *et al*. 2004; Lucas *et al*. 2006; Moriguchi *et al*. 2006; Pattyn *et al*. 2006). Secondarily, these SA cell precursors then adopt neuronal and catecholaminergic characteristics, which are typified by the expression of neurofilament, SCG10, neuron-specific tubulin, TH and dopamine β-hydroxylase (DBH) (**Fig. 2**; Cochard *et al*. 1978; Cochard and Paulin 1984; Groves *et al*. 1995; Sommer *et al*. 1995; Ernsberger *et al*. 1995; Schneider *et al*. 1999; Ernsberger *et al*. 2000; Flatmark 2000). These differentiated SA cell precursors then embark on a second migratory passage from the vicinity of the DA to their final destinations where they generate sympathetic ganglia, adrenal chromaffin cells or extra-sympathoadrenal lineage derivatives such as the transient embryonic organ of Zukerkandl (**Fig. 1**; Anderson and Axel 1986; Anderson *et al*. 1991). While sympathetic neurons and adrenal chromaffin cells have historically been postulated to share a common SA cell precursor (Anderson and Axel 1986; Anderson *et al*. 1991), the observation that there exist subtle differences in the effects attributed to gene-ablative mutations in the sympathetic neurons or the adrenomedullary chromaffin cells has lead many in the field to speculate that perhaps there are alternate, distinct NC progenitor cells that are committed exclusively to either one or the other lineage (Unsicker *et al*. 2005; Huber 2006).

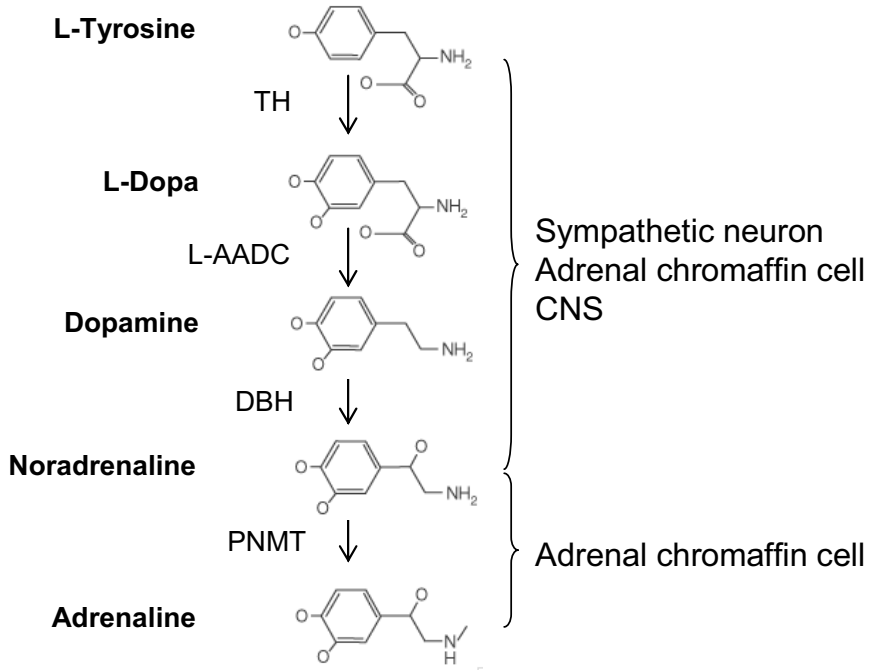

Fig. 2 Anabolic pathway of Noradrenaline and Adrenaline. L-Tyrosine is converted to L-Dopa by the rate-limiting enzyme Tyrosine Hydroxylase (TH). L-Dopa is converted to Dopamine by the ubiquitous enzyme L-Aromatic Amino Acid Decarboxylase (L-AADC). Dopamine is converted to Noradrenaline by Dopamine β-Hydroxylase (DBH). In adrenal chromaffin cells, Noradrenaline is N-methylated by tissue-restricted enzyme Phenylethanolamine N-Methyltransferase (PNMT) (for review, see Flatmark 2000).

SYMPATHETIC AND ADRENAL CHROMAFFIN CELL DEVELOPMENT: A PARADIGMATIC TRANSCRIPTION FACTOR REGULATORY NETWORK?

Much of our understanding of the role of transcription factors in SA cell specification and function derives from loss- and gain-of-function experiments in cell-based or organismal systems whereby the expression levels of individual genes has been altered. The interpretation of the observed SA cell developmental deficiencies following these experimental manipulations relies principally on the expression analysis of putative downstream target genes using *in situ* hybridization or immunocytochemical localization techniques. There are a number of potential limitations in such studies, as the precise expression levels of target genes in the regulation of developmental events cannot be easily evaluated using either of these qualitative assays. Another theoretical shortcoming in the application of these techniques or their interpretation is that reduction of a putative downstream gene expression in a given mouse/chick/zebrafish mutant does not always indicate that it is directly regulated by the upstream regulator, as the gene ablation might autonomously or non-autonomously affect the survival and/or organization of cell populations expressing the gene under study (Boyle and de Caestecker 2006). To circumvent some of these issues, we enriched adrenal chromaffin cells by flow cytometry to quantitatively examine gene expression levels in pure SA cell populations (Moriguchi *et al*. 2006).

In avian primary sympathetic ganglia, the basic helix-loop-helix (bHLH) transcription factor CASH-1 (the chick homologue of MASH-1) is expressed immediately prior to the paired/homeodomain transcription factor Phox2b (at the 29/32-somite stage), which is then followed by expression of the bHLH transcription factor Hand2 and Phox2a (at the 31/32-somite stage) and the zinc finger protein GATA-2 (the functional counterpart of mouse GATA-3 in the developing chick SA system at the 33/34-somite stage). Finally at the end of this temporal gene expression cascade, TH and DBH, both of which are required for noradrenalin anabolic production, can be detected in 35-somite chick embryos (Ernsberger *et al*. 1995, 2000; Howard *et al*. 2000; Tsarovina *et al*. 2004). Through the analyses of gene loss- and gain-of-function studies in model organisms, our current understanding is that SA cell differentiation is governed by this handful of key transcription factors that are (directly or indirectly) activated by BMPs. Loss-of-function mutations in any of these five transcription factors in the mouse leads to lethality of homozygous mutant animals, which suffer variable deficits in SA and non-SA tissue development (Guillemot *et al*. 1993a; Pattyn *et al*. 1999; Lim *et al*. 2000; Tsarovina *et al*. 2004; Moriguchi *et al*. 2006). For simplicity, we will restrict this discussion to deficiencies in SA cell lineages.

MASH-1 is induced by the administration of BMPs to primary NC cells in culture (Lo *et al*. 1998). In murine embryos, the SA progenitor cells begin to express MASH-1 when they first aggregate near the DA to form the primary sympathetic chain (Huber *et al*. 2002; Morikawa *et al*. 2005). MASH-1 expression in the sympathetic ganglia remains high until e13, followed by rapid down-regulation from e14.5 (Morikawa *et al*. 2005). In adrenal chromaffin cells, MASH-1 expression seems to be down-regulated two days later at e16.5 (Huber *et al*. 2002). The biological significance and mechanisms governing MASH-1 down regulation in these cells is unknown, although our recent data are consistent with the possibility that GATA-3 may play a role in the silencing of MASH-1 (Moriguchi *et al*. 2006).

Initial studies indicated that MASH-1 was essential *in vivo* for the development of the autonomic nervous system lineages, including SA cells. MASH-1-deficient NC cells arrived normally at the DA of e10.5 embryos to form the primary sympathetic ganglia and could express some (e.g. neurofilament 68 and 160, neuron-specific tubulin, Phox2b and c-Ret), but not other (e.g. Phox2a, TH and DBH) SA cell markers before ensuing atrophy (Guillemot *et al*. 1993a; Sommer *et al*. 1995; Hirsch *et al*. 1998; Huber *et al*. 2002). However, a more recent study indicated that in MASH-1 homozygous mutant embryos, a complete SA cell differentiation program lagged behind that of wild-type animals by 1-2 days (Pattyn *et al.* 2006). From these observations, one can only conclude that MASH-1 is not essential for migrating NC cells to acquire a SA cell fate, and that perhaps together with other partner regulatory molecules, it co-operatively promotes the differentiation and survival of SA cell precursors.

SA cells also express Phox2a, a homeodomain transcription factor closely related to Phox2b (Valarche *et al*. 1993; Morin *et al*. 1997; Pattyn *et al*. 1997). Gain-of-function experiments have indicated that Phox2a is sufficient to promote autonomic neurogenesis by supporting the expression of MASH-1, Phox2b, TH and DBH in chick and zebrafish embryos, although Phox2a expression largely depends on both Phox2b and MASH-1 (Guo *et al*. 1999; Stanke *et al*. 1999). Nevertheless, loss of *Phox2a* function did not significantly impair SA cell development (Morin *et al*. 1997).

The homeodomain transcription factor Phox2b is expressed in all noradrenergic neurons of the central and the peripheral nervous systems (Pattyn *et al*. 1997, 1999, 2000; Dauger *et al*. 2003). In the sympathetic primordium, Phox2b expression is followed by Phox2a and is induced by BMPs independently of MASH-1 (Hirsch *et al*. 1998; Huber *et al*. 2002). Interestingly, recent reciprocal gene replacement experiments between the *Phox2* genes have clearly revealed that the Phox2a and Phox2b proteins act in a functionally distinct, non-reciprocal manner. During the development of SA cells, Phox2a, which acts downstream of Phox2b, could not fully complement Phox2b function even if it was expressed from within the endogenous *Phox2b* locus (Coppola *et al*. 2005), definitively demonstrating that, regardless of possible differences in their transcriptional regulation, the Phox2a and -2b proteins are functionally distinct.

Although it has been shown that gain-of-Phox2a or -2b function confers the capacity to induce sympathetic neuron-like traits in chick embryos, only Phox2b is required for sympathetic development in the mouse. Phox2b-deficient mice die at midgestational stages, but they can be rescued to birth by the administration of noradrenaline intermediates in the drinking water of heterozygous intercrossed *Phox2b* dams (Pattyn *et al*. 2000). In the absence of Phox2b, murine NC cells that assemble at the DA at e10.5 and colonize the adrenal gland at e13.5 lack noradrenergic markers (such as TH and DBH; Pattyn *et al*. 1999; Huber *et al*. 2005). Homozygous *Phox2b* mutants preserve initial MASH-1 expression at e10.5, but the expression is prematurely down-regulated at e11.5 in sympathetic ganglion cells, indicating that Phox2b is required for the maintenance of MASH-1 expression. The expression of neuron-specific tubulin, neurofilament 68, c-Ret and Hand2 are significantly suppressed in the absence of Phox2b (Pattyn *et al*. 1999; Howard *et al*. 2000; Pattyn *et al*. 2000; Huber *et al*. 2005). These data are consistent with the interpretation that Phox2b plays a proximal and vital role in SA cell differentiation.

The *Dbh* promoter has been shown to be directly regulated by Phox2. Both Phox2a and Phox2b bind to regulatory elements in, and stimulate transcription from, the *Dbh* promoter in conjunction with cyclic-AMP pathway activation (Yang *et al*. 1998; Kim *et al*. 1998; Swanson *et al*. 2000; Adachi *et al*. 2002). Phox2a can also *trans*-activate the *Th* promoter (at -175 to -158 bp) *via* direct DNA binding (Zellmer *et al*. 1995).

The bHLH transcription factor Hand2 (aka dHand) is expressed in the sympathetic ganglion primordium, and promotes noradrenergic differentiation of NC cells *in vitro* and *in ovo*. Hand2 cooperates with Phox2a in activating transcription of *Dbh* promoter constructs in cell-based co-transfection assays (McFadden *et al*. 2002; Firulli *et al*, 2003; Rychlik *et al*. 2003; Xu *et al*. 2003). *Hand2* transcrip-

tion, which can be induced by BMPs, is initiated after CASH-1 and Phox2b, but before Phox2a, GATA-2, TH and DBH in chick embryos (Howard *et al*. 2000; Tsarovina *et al*. 2004). In several independent studies, Hand2 expression in sympathetic ganglia has consistently been shown to depend on *Phox2b* activation, but is independent of MASH-1 (Howard *et al*. 2000; Huber *et al*. 2002, 2005; Morikawa *et al*. 2005), suggesting an epistatic regulatory relationship between *Phox2b* and *Hand2* for SA cell differentiation. Thus, it was probably surprising and unexpected that forced expression of Hand2 would lead to reciprocal induction of Phox2b expression, as well as to the expression of noradrenergic and pan-neuronal markers, in NC and P19 embryonal carcinoma cells (Howard *et al*. 2000; Morikawa *et al*. 2005).

Constitutive *Hand2* germ line mutation leads to early embryonic lethality (Srivastava *et al*. 1997). A recent analysis of *Hand2* conditional deletion mutation specifically in neural crest descendents, achieved by the use of a Wnt1/Cre-expressing transgene, indicated that, except for TH and DBH, Hand2 is dispensable for the expression of the usual protein repertoire characteristic of SA cells (Morikawa *et al*. 2007). Similar gene expression effects were also detected in the zebrafish *Hand2* deletion mutant, *hands off*, in which sympathetic precursor cells aggregated to form sympathetic ganglia expressing Phox2a/b and Zash1 (the zebrafish counterpart of Mash1), while GATA-2, TH and DBH levels were strongly reduced (Lucas *et al*. 2006). Given the plethora of *in vitro* and *in vivo* data that implicate Hand2 as a key player in SA cell differentiation, these recent observations are indeed surprising and perhaps hint at broader than anticipated genetic redundancy amongst bHLH proneural genes in sympathetic neuronal development (Howard *et al*. 2000; Morikawa *et al*. 2005, 2007).

The zinc finger transcription factors GATA-2 and GATA-3 are both expressed in SA cell lineage of mouse embryos, while only GATA-2 is expressed in the chick (George *et al*. 1994; Groves *et al*. 1995; Lim *et al*. 2000; Tsarovina *et al*. 2004). GATA-2 expression in chick embryos may also be modulated by BMPs since it was strongly suppressed by the BMP antagonist, noggin (Tsarovina *et al*. 2004). Gain-of-function studies in chick embryos revealed that, unlike Phox2a/b and Hand2, forced expression of GATA-2 induced ectopic neurons lacking noradrenergic traits in chick peripheral nerve precursors (Gordis and Rohrer 2002; Tsarovina *et al*. 2004). However, transgenic GATA-3 complementation of *Gata3*-deficient SA cells significantly restored a population of highly TH-positive sympathetic neurons, suggesting that the effect on noradrenergic cell induction by GATA-2/3 depends on the cellular context (Moriguchi *et al*. 2006). Interestingly, GATA-3 deficiency results in loss of GATA-2 expression in e10.5 primary sympathetic ganglion cells (Tsarovina *et al*. 2004) and in chromaffin cells (Moriguchi, unpublished data), suggesting that GATA-2 expression in SA tissues depends on GATA-3.

Gata3 constitutively mutant mice expire of noradrenergic deficiency by e10.5, and can be rescued either pharmacologically or by complementation with a SA cell lineage-specific GATA-3 transgene (Lim *et al*. 2000; Moriguchi *et al*. 2006). Inactivation of *Gata3* in mice leads to a significant loss of TH and DBH expression, as well as to a general deficiency in SA cell development, both in sympathetic neurons and adrenal chromaffin cells (Lim *et al*. 2000; Tsarovina *et al*. 2004; Moriguchi *et al*. 2006). Although the primary sympathetic ganglia form normally in e10.5 *Gata3* mutant embryos, at later embryonic stages, enhanced apoptosis is evident such that by e18.5, *Gata3* null mutants develop abnormally small thoracic paravertebral sympathetic ganglia (35% smaller in size compared to wild-type littermates) with reduced number of neurons (Tsarovina *et al*. 2004; Moriguchi *et al*. 2006). Similarly, adrenomedullary chromaffin cells in e18.5 mice lacking GATA-3 also amounted to only 30% of controls, and these cells lack TH and DBH expression (Moriguchi *et al*. 2006). Furthermore, Phox2b (which hitherto had been thought to act upstream of GATA-3) and Hand2 expression were both found to be significantly reduced, implicating GATA-3 as a positive regulator for the maintenance of Phox2b and Hand2 transcription at least in the remaining GATA-3-deficient chromaffin cells (Moriguchi *et al*. 2006). Curiously, MASH-1 was not appropriately down-regulated during differentiation of the remaining GATA-3-deficient chromaffin cells in e18.5 *Gata3* mutants. Thus, it appears that GATA-3 may be a negative regulator of MASH-1 expression during late embryogenesis (Moriguchi *et al*. 2006). Insofar as terminally differentiated target gene regulation is concerned, GATA-3 has been reported to activate the *Th* promoter, but remarkably without any requirement for its DNA binding activity as it apparently acts by tethering to the CREB protein bound to a CRE site in the *Th* promoter (Hong *et al*. 2006).

SYMPATHETIC AND ADRENAL CHROMAFFIN CELL DEVELOPMENT: ENIGMATIC CROSS-REGULATORY GENE INTERACTIONS

Taken together, although some of the transcription factors discussed here seem to function sequentially in what could be considered to be a classical linear developmental hierarchy, the data are actually more consistent with the possibility that SA cell differentiation is controlled by mutually reinforcing feedback transcriptional interactions between GATA-3, MASH-1, Hand2, Phox2a and Phox2b (**Fig. 3**). Unfortunately, the current literature sheds little additional light on whether any of these transcription factors are mutually (directly or indirectly) regulated by one another. The *Phox2a* and *Phox2b* promoters have been characterized, but without specific reference to any SA cell lineage-restricted activity (Flora *et al*. 2001; Hong *et al*. 2001; Samad *et al*. 2004). Thus, the data published to date characterizing Phox2a and Phox2b expression provide an inadequate foundation on which to base further regulatory analysis. Our *Gata3 cis*-regulation studies have revealed that even a 662 kbp *Gata3* YAC, containing approximately 451 kbp and 211 kbp of 5′ and 3′ flanking sequence information, respectively, is missing the regulatory element(s) that confers expression in SA system organs (Lakshmanan *et al*. 1999; Hasegawa *et al*. 2007). Hence, (at least to date) any formal proof supporting a hierarchy proposed based upon gene ex-

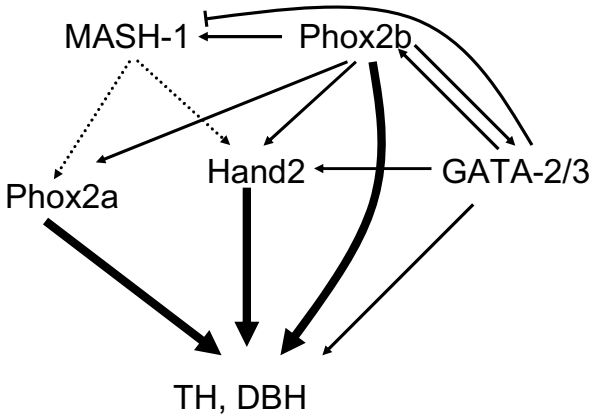

Fig. 3 Crosstalk in regulatory gene networks in SA cell development. MASH-1-deficient SA cells fail to express Phox2a and Hand2 at e10.5, although their expression is only temporally delayed (stippled arrows; see text). Phox2a and Hand2 expression largely depend on Phox2b. *Dbh* is directly regulated by Phox2a and 2b. Phox2a *trans*-activates the *Th* promotor *via* direct binding. Phox2b is required for the maintenance of MASH-1 expression from e10.5 onwards. Hand2 and GATA-3 expression are suppressed in the absence of Phox2b, whereas Phox2b and Hand2 are suppressed in GATA-3-deficient chromaffin cells at e18.5. MASH-1 expression is de-repressed in GATA-3-deficient chromaffin cells. Hand2 is dispensable for other SA-specific transcription factor expression, although it is essential for TH and DBH expression. Hand2 cooperates with Phox2a in activating the *Dbh* promoter. Thick arrows indicate direct regulation, whereas thin arrows indicate direct or indirect regulation.

pression analyses in *Mash1*, *Phox2*, *dHand* or *Gata3*-ablative mutant animals remains absent (**Fig. 3**). Until the regulatory elements governing the SA tissue specificity of each of these transcription factors are identified, how and which transcription factors directly regulate which of the multiple regulatory genes discussed here is a major question that remains outstanding.

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