A hindbrain-repressive Wnt3a/Meis3/Tsh1 circuit promotes neuronal differentiation and coordinates tissue maturation

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SUMMARY
During development, early inducing programs must later be counterbalanced for coordinated tissue maturation. In Xenopus laevis embryos, activation of the Meis3 transcription factor by a mesodermal Wnt3a signal lies at the core of the hindbrain developmental program. We now identify a hindbrain restricting circuit, surprisingly comprising the hindbrain inducers Wnt3a and Meis3, and Tsh1 protein. Functional and biochemical analyses show that upon Tsh1 induction by strong Wnt3a/Meis3 feedback loop activity, the Meis3-Tsh1 transcription complex represses the Meis3 promoter, allowing cell cycle exit and neuron differentiation. Meis3 protein exhibits a conserved dual-role in hindbrain development, both inducing neural progenitors and maintaining their proliferative state. In this regulatory circuit, the Tsh1 co-repressor controls transcription factor gene expression that modulates cell cycle exit, morphogenesis and differentiation, thus coordinating neural tissue maturation. This newly identified Wnt/Meis3/Tsh1 circuit could play an important role in diverse developmental and disease processes.

INTRODUCTION
Early posterior neural development has been extensively studied in vertebrates. BMP antagonism induces neural tissue, whereas posterior specification is driven by caudalizing signaling pathways, such as Wnt/β-catenin, retinoic acid (RA) and FGF (Elkouby and Frank, 2010; White and Schilling, 2008; Dorey and Amaya, 2010). The combined action of these factors induces the midbrain, hindbrain and spinal cord regions. In the anterior, caudalizing antagonists are expressed; a battery of Wnt inhibitors, as well as the RA-degrading enzyme CyP26 (Elkouby and Frank, 2010; White and Schilling, 2008), prevent forebrain caudalization. Much is known about neural caudalization and its antagonism in the anterior, but little is known about the intrinsic negative regulatory pathways that act within the posterior neural tissue. Negative regulation within the hindbrain and spinal cord domains could regulate the transition of induced proliferating progenitor populations to differentiation, thus controlling cell number, cell fate, tissue size, morphology and function.

In early Xenopus and zebrafish development, the Meis3 TALE-family transcription factor induces the hindbrain, including primary neurons and neural crest (Dibner et al., 2001; Vlachakis et al., 2001; Waskiewicz et al., 2001; Gutkovich et al., 2010). Hindbrain induction by a mesodermal Wnt ligand is a conserved vertebrate feature, and mesodermal Wnt3a directly activates neural Meis3 expression to trigger the hindbrain developmental program, regulating expression of Hox paralogous group 1-4 genes (Dibner et al., 2004; Elkouby et al., 2010).

Wnt3a is also expressed in the neural plate (McGrew et al., 1997), but a thorough investigation of later neural Wnt3a function in the hindbrain is lacking. We now show that early hindbrain-inducing components act later to repress the hindbrain developmental program. Contrasting early mesodermal Wnt3a activity, later neural Wnt3a plays a negative role in hindbrain development. Neural Wnt3a and Meis3 proteins act by a positive-feedback mechanism. The resulting accumulation of Meis3 protein above a threshold level triggers auto-repression of Meis3 gene expression. At high levels, Meis3 induces expression of the co-repressor protein, Teashirt1 (Tsh1). Initially, Meis3 protein directly activates its own gene expression; later, Tsh1 is recruited to the Meis3 promoter by Meis3 protein to repress transcription. In the hindbrain, Tsh1 protein acts as a switch, shifting the cellular response to the Wnt signal from activation to repression of Meis3 gene expression. We further show that Meis3 protein plays a dual role in the hindbrain, both inducing neural progenitors and maintaining their proliferative state. The role of the repressive Wnt/Meis3/Tsh1 circuit is to restrain Meis3 protein to levels that enable cell cycle exit and neuron differentiation. In this circuit, Tsh1 protein coordinates neural tissue maturation. This newly identified Wnt/Meis3/Tsh1 circuit could have an important role in regulating a diverse range of developmental and disease processes.

MATERIALS AND METHODS
Xenopus embryos
Ovulation, in vitro fertilization, embryo culture, dissections and explant culture were as described previously (Re’em-Kalma et al., 1995; Bonstein et al., 1998).

Plasmid constructs
Meis3-HA
The HA tag fragment was cut (ClaI/EcoRI) from a pcDNA3 plasmid (Clontech). A Meis3 full-length fragment was cut (EcoRI/NorI) from pCS107 and subcloned into pGEM T-Easy. The HA tag was subcloned into pGEM-T Easy-Meis3 (Clontech). The HA-Meis3 was then cut (ClaI/NorI) and subcloned back into the pCS107 vector.

Meis3 5′UTR probe plasmid
A fragment of the Meis3 5′UTR was PCR amplified by Meis3 5′UTR primers (supplementary material Table S1), from a pGL3 plasmid containing Meis3 5′ genomic sequence, the entire 5′UTR, the 1st exon and part of the 1st intron (Elkouby et al., 2010). The Meis3 5′UTR fragment was cloned into pGEM-T Easy.
RNA, DNA and morpholino oligonucleotide (MO) injections
Capped sense in vitro transcribed full-length mRNA, BMPR1A dominant-negative receptor (DNR), Meis3 (Salzberg et al., 1999), Meis-Myc, VP16-Meis3 (Dibner et al., 2001), Meis3-GF (Dibner et al., 2004), Tsh1, Myc-Tsh1 (Koebernick et al., 2006), Dkk1 (Glinka et al., 1998) and THVR (Wu et al., 2005) were injected into one-cell embryos. Xenopus Wnt3a (pCS107) plasmid (Elkouby et al., 2010) was also injected in CMV-driven zygotic expression assays. MOs (Gene Tools) used were Meis3-MO (Dibner et al., 2001), Wnt3a-MO (Elkouby et al., 2010) and Tsh1-MO (Koebernick et al., 2006).

In situ hybridization
Whole-mount in situ hybridization was performed with digoxigenin-labeled probes (Harland, 1991) Meis3, EphA2 (Dibner et al., 2001), Wnt3a(Fonar et al., 2011). N-Tubulin (N-Tub), Slag (Gutovich et al., 2010), Tsh1 (Koebernick et al., 2006), Iro3 (Gomez-Skarmeta et al., 1998) and Sox3 (Hardcastle and Papalopulu, 2000).

Semi-quantitative (sq) RT-PCR analysis
sqRT-PCR was performed (Snir et al., 2006). In all sqRT-PCR experiments, three to six independent experiment repeats were typically performed. In all experiments, each sample was routinely assayed a minimum of two times for each marker. See supplementary material Table S1 for primer sequences.

Chromatin immunoprecipitation (ChIP)
One-cell embryos were injected with Meis3-Myc (0.8 ng) (Dibner et al., 2001) or Myc-Tsh1 (0.5 ng) (Koebernick et al., 2006) encoding RNAs and/or the Meis3-MO (30 ng). For each group, ~30 stage 17 embryos were crosslinked by 1% formaldehyde/PBS for 35 minutes. Crosslinking was quenched by 0.125 M glycine/PBS, followed by three washes in PBS and freezing at ~80°C. ChIP was performed with either rabbit IgG (control; Pierce) or anti-Myc antibody (rabbit polyclonal, Millipore) as described previously (Blythe et al., 2009; Elkouby et al., 2010), except that DNA shearing was performed by Bioruptor sonicator (Diagenode) for 11 cycles of 30 seconds ON, 30 seconds OFF on high power output, and after immunoprecipitation, beads were washed five times in wash buffer I (Blythe et al., 2009) and once in TE prior to elution. Quantitative PCR (QPCR) was performed using a Stratagene Mx3000P device and SYBR Premix Ex Taq II (Takara). QPCR conditions were: 2 minutes at 95°C (QPCR) was performed using a Stratagene Mx3000P device and SYBR

RESULTS
Meis3 and Wnt3a act in a negative-feedback loop within the neural plate
In Xenopus, a mesodermal-derived Wnt3a signal induces the hindbrain at mid-gastrula stages (Elkouby et al., 2010), yet the later role of neural-specific Wnt3a in this process is unknown. We examined Meis3 and Wnt3a activity interactions at later neural plate stages. Meis3-deficient embryos do not express Wnt3a in the neural plate at late-gastrula nor mid-neurula stages (Fig. 1Ab,e), whereas Wnt3a expression is robustly expanded in embryos over-expressing Meis3 protein (Fig. 1Ac,f). Meis3 is necessary and sufficient for both the onset and maintenance of Wnt3a expression in the neural plate (Fig. 1Ag). Cyclohexamide (CHX) assays in animal cap (AC) explants suggest that Meis3-mediated regulation of Wnt3a expression is direct (not shown). Thus, early mesodermal Wnt3a induces neural Meis3 expression and Meis3 protein activates later Wnt3a expression in the neural plate.

We examined the effects of ectopic Wnt3a activity on Meis3 expression at later neural stages. Meis3 expression is activated at gastrula stages by the inducible constitutively active Tcf protein (THVR; Fig. 1Bb) (Elkouby et al., 2010), but at later stages THVR suppresses Meis3 expression (Fig. 1Be). This phenomenon is Meis3 specific, as expression of other regional neural markers, such as En2 (isthmus) and EphA2 (r4) is clearly detected (Fig. 1Bi); the hindbrain region has formed, but it no longer expresses Meis3. In this experiment, THVR robustly induced expanded Meis3 expression at gastrula stages (Fig. 1Bb). Wnt3a caudalizing activity is strictly Meis3 dependent (Elkouby et al., 2010), so in these experiments the early increase in Meis3 protein caudalized the neural plate, as En2 and EphA2 expression is expanded anteriorly (Fig. 1Bb). Only later does this ectopic Wnt activity repress Meis3 expression (Fig. 1Be). Like THVR, zygotic-driven Wnt3a overexpression also represses later Meis3 expression (Fig. 2Bb). These results suggest that Meis3 repression by Wnt3a/β-catenin is part of a hindbrain negative-feedback loop.

We determined the timing of this shift in Meis3 transcriptional response to Wnt/β-catenin activity. Similar to gastrula stages (Fig. 1Bb), mid-neurula stage THVR activation also suppressed later Meis3 expression (Fig. 1Bf), but contrastingly, this later THVR activation cannot caudalize; En2 and EphA2 expression is unaffected (Fig. 1Bb). Unactivated THVR does not affect Meis3 expression or neural caudalization (insets of Fig. 1Be). Consistent with negative-feedback activity, Wnt/β-catenin signaling suppresses Meis3 expression only after hindbrain induction (Fig. 1Bb). Reciprocally, overexpression of the zygotic Wnt antagonist Dkk1 eliminates Meis3 expression at gastrula stages (Elkouby et al., 2010; Fig. 1Bc), but at later-neurula stages, Meis3 expression...
is restored to fairly high levels (Fig. 1Bg). We also show that this Meis3 repression is dependent on Meis3-induced, neural Wnt3a activity (supplementary material Fig. S1A,B).

Our previous experiments showed that Meis3 induced by mesodermal Wnt3a protein caudalizes neural cell fates and rescues posterior neural cell fates in Wnt-deficient embryos (Elkouby et al., 2010). These results suggest that neural-specific Wnt3a does not act downstream to Meis3 to caudalize. Alternatively, neural-Wnt3a activity may control a negative-feedback loop that represses later Meis3 expression, optimizing Meis3 protein levels in the neural plate.

To elucidate neural Wnt3a function, we performed recombinant explant assays. Dorsolateral marginal zone (DLMZ) explants induce posterior neural cell fates in adjacent recombinant neural ectoderm (NE) via secretion of Wnt3a (Elkouby et al., 2010). We now performed these experiments by recombining wild-type (WT) DLMZs with Wnt3a-deficient NE explants (Fig. 1C). The DLMZ robustly induced posterior neural marker expression in Wnt3a-deficient NE. Moreover, expression of the EphA2 hindbrain (r4) marker was not inhibited, but dramatically expanded in nearly 70% of the explants (Fig. 1Cd-f). However, 60% of the explants showed either a strong increase or a moderate decrease in expression, implying a later Wnt3a modulation of neuron differentiation (Fig. 1Cd-f). Expression of Slug (neural crest) was not affected in 80% of the explants (Fig. 1Cg-i). These results suggest that neural-specific Wnt3a is not required for neural caudalization per se, but plays a role to fine-tune cell fate distribution. Furthermore, by mid-neurula stages, neural tissue has already lost its competence to Wnt/β-catenin caudalizing activity (Fig. 1Bj). Further elucidating this point, in isolated and recombinant AC explants, Meis3 induction of hindbrain markers such as EphA2, Krox20, HoxA2 and Gbx2 and HoxB3 is not inhibited, but even somewhat stimulated by simultaneous disruption of Wnt activity, whereas the spinal cord marker HoxB9 was inhibited (supplementary material Fig. S1C-E).

Supporting this observation that later Wnt-signalizing represses hindbrain fates, Meis3 rescue of Dkk1-injected/Wnt-depleted embryos consistently led to highly ectopically disorganized Krox20 and EphA2 expression levels (Elkouby et al., 2010). Although mesodermal Wnt3a drives the hindbrain developmental program, neural-expressed Wnt3a could play a negative regulatory role in the hindbrain. As Wnt3a is traditionally considered a strong neural caudalizer, we addressed its potential counterintuitive negative regulatory role in the hindbrain.

Repression of Meis3 gene expression by Wnt3a/β-catenin is mediated by Meis3 protein

Given the importance of Meis3 protein in hindbrain development, we investigated its repression mechanism. We overexpressed Meis3 about twofold higher than the typical overexpression concentration that caudalizes embryos. These higher Meis3 levels repress endogenous Meis3 gene expression as detected by a Meis3 5’ UTR probe that specifically recognizes endogenous, but not exogenously injected Meis3 mRNA (Fig. 2Aa,b). As a control, a Meis3 ORF probe detects the ectopically injected Meis3 mRNA (Fig. 2Ac,d). Ectopic Meis3 levels repress endogenous Meis3 expression at high Meis3 levels (Fig. 3G), suggesting that Meis3 protein must accumulate over a concentration threshold to initiate auto-repression. Accumulation of Meis3 protein in the neural tissue of normal embryos probably controls the exact timing of endogenous auto-repression. Indeed, at mid-neurula stages, when THVGR suppresses Meis3 expression, Meis3 is already expressed at high levels (Fig. 1Ba,d).
We next examined the epistatic relationship between Wnt3a/β-catenin and Meis3 activities in the context of Meis3 repression. Similar to THVGR overexpression, zygotic Wnt3a overexpression strongly represses Meis3 expression (Fig. 2Bb). Further confirming Meis3 auto-repression, endogenous Meis3 expression is expanded in Meis3-deficient embryos (Fig. 2Bc). Wnt3a overexpression in Meis3-deficient embryos, however, cannot repress Meis3 gene expression; this combination of activating an inducer while withdrawing a repressor, strongly expands Meis3 expression (Fig. 2Bd). This upregulated expanded pattern strikingly mimics the earlier, broader, Meis3 expression pattern seen in late-gastrula stages. We validated these results by overexpressing THVGR in wild-type versus Meis3-deficient AC explants. THVGR reduction of basal Meis3 expression in ACs is Meis3 dependent (Fig. 2C). Apparently, Meis3 represses its own expression more directly, whereas Wnt3a/β-catenin activates Meis3 gene expression, probably increasing cellular Meis3 protein levels above the threshold level required for auto-repression (Fig. 2E). Indeed, CHX assays in AC explants suggest that the Meis3 gene is a direct-target of the Meis3 protein (Fig. 2D), and this early positive auto-regulation was also observed in vivo (supplementary material Fig. S2).

**Tsh1 protein is a repressor of Meis3**

Meis family proteins typically act as transcriptional activators (Dibner et al., 2001; Inbal et al., 2001). Thus, Meis3 autorepression activity may require interactions with transcription repressor partners. During *Drosophila* development, Tsh and Hth (Meis) proteins interact to repress gene expression (Casares and Mann, 2000; Bessa et al., 2002). In *Drosophila* gut development, Tsh expression is induced by high Wg levels, and Tsh protein then acts to repress Wg target gene expression (Wallzer et al., 2001). In the hindbrain, Tsh proteins could act to restrain the Wnt/β-catenin response, fitting the Meis3 repression scenario we observe. In Xenopus hindbrain development, both Tsh1 gain and loss of function inhibit expression of hindbrain markers (Koebernick et al., 2006), suggesting a negative-feedback effect. Tsh1 protein is thus a good candidate for interacting with Meis3 to mediate its auto-repression.

Ectopic Tsh1 levels indeed repress Meis3 expression in neurula embryos (Fig. 3A). In Tsh1-deficient embryos, Meis3 expression is upregulated and expanded. This expanded expression pattern strikingly resembles the normal broader Meis3 pattern seen in late gastrula and early neurula stages (Fig. 3C and inset). For Tsh1 deficiency, we used the Tsh1-MO that specifically knocks-down Tsh1 protein (Koebernick et al., 2006). Verifying Tsh1-MO specificity in our system, Tsh1 mRNA overexpression restores the normal Meis3 expression pattern in Tsh1-morphant embryos (supplementary material Fig. S3A). Thus, Tsh1 protein is an endogenous repressor of Meis3 gene expression. Indeed, Tsh1 and Meis3 transcripts overlap in the hindbrain (supplementary material Fig. S3B). Tsh1 protein probably represses the Meis3 gene cell autonomously, as ectopic Tsh1 protein sharply inhibits Meis3 expression levels in dissociated AC explants (supplementary material Fig. S3C).

Tsh1 transcripts are absent in gastrulae; zygotic expression only initiates at early neurula stages (Koebernick et al., 2006). These expression dynamics correlate with the negative shift in Meis3 transcriptional response to Wnt/β-catenin. Tsh1 protein could serve as a switch, in which Tsh1-negative cells continue to upregulate Meis3 in response to Wnt3a/β-catenin, whereas Tsh1-positive cells suppress Meis3 expression. Wnt/β-catenin activity in Tsh1-negative hindbrain cells of gastrula to early neurula stages upregulates Meis3 expression (Fig. 1Ba,b), while the same activity in Tsh1-positive hindbrain cells of mid-neurula onwards stages downregulates it (Fig. 1Bd-f). To test whether Tsh1 serves a switch, Meis3 expression was examined in Tsh1 overexpressing embryos at mid- and late-gastrula stages, when Wnt3a/β-catenin activates Meis3 expression, but before endogenous Tsh1 protein is expressed (Koebernick et al., 2006). Indeed, precocious ectopic Tsh1 levels repress Meis3 expression (Fig. 3B).

We determined that later Meis3 expression by Wnt3a/β-catenin is indeed Tsh1 dependent. THVGR strongly represses Meis3 expression (Fig. 3Cb), and in Tsh1-deficient embryos Meis3 expression is significantly expanded (Fig. 3Cc). Strikingly, in Tsh1-deficient embryos, THVGR continues to robustly induce and expand Meis3 expression (Fig. 3Cd), mimicking its early Meis3 inducing activity (Fig. 3D, inset). Therefore, in Tsh1-negative cells, Wnt3a/β-catenin signaling perpetuates Meis3 expression, failing to shift to the later repression program. Tsh1 switches the cellular response of the Wnt3a/β-catenin signal from activation (Fig. 3Cd) to repression (Fig. 3Cf) of Meis3 gene expression.

**Tsh1 expression is regulated by Meis3**

Tsh1 expression dynamics and the threshold levels of Meis3 activity required to repress Meis3 expression suggest that Tsh1 expression initiation at neurula stages is regulated by Meis3 and
Wnt3a/β-catenin. Tsh1 expression is eliminated in Meis3-deficient mid-late neurula stage embryos (Fig. 3Db), and induced and expanded in Meis3 overexpressing embryos (Fig. 3Dc). Meis3 is both necessary and sufficient for Tsh1 gene expression. At early neurula stages, either Meis3 or Wnt3a proteins are required for the induction of Tsh1 expression (Fig. 3De,f). We concluded that Wnt3a/Meis3 epistasis controls Tsh1 expression. THVGR induction of Tsh1 expression in AC explants is Meis3 dependent (Fig. 3E) and Meis3 rescues Tsh1 expression in Dkk1 overexpressing embryos (Fig. 3F). Consistent with Meis3 protein being directly required for Meis3 gene repression, it also acts downstream of Wnt3a/β-catenin to regulate Tsh1 expression.

Activation of Tsh1 expression by Meis3 activity may not necessarily be cell autonomous (not shown) and may require additional non-autonomous Wnt activity. Whereas Meis3 rescues Tsh1 expression to control levels in Dkk1 embryos, Meis3 alone induces more robust Tsh1 expression (Fig. 3F). Meis3 seems to serve as the limiting factor, but Wnt activity may also contribute to Tsh1 expression. Furthermore, we measured Meis3 and Tsh1 expression in response to Meis3 activity. There was a strong correlation between Meis3 repression and Tsh1 induction; only high Meis3 levels that induce Tsh1 can repress Meis3 expression (Fig. 3G). Lower Meis3 levels neither induce Tsh1 nor repress Meis3 expression (Fig. 3G). These lower Meis3 levels still caudalize, as they strongly induce expression of the Meis3-responsive hindbrain marker Krox20 (not shown). Thus, induction of Tsh1 expression requires high levels of Meis3 activity. In this scenario, Wnt3a/β-catenin activity induces Meis3 levels over a critical cellular threshold; Meis3 activates Tsh1 expression, which then represses Meis3 gene expression.

Meis3 and Tsh1 proteins act in a complex to repress Meis3 gene expression directly

As Wnt3a/β-catenin repression of Meis3 expression is dependent on both Meis3 and Tsh1 proteins, we examined epistasis between these two proteins. We monitored repression of Meis3 expression by overexpressing one protein while simultaneously knocking down the other. Each of these proteins is sufficient to strongly repress Meis3 expression (Fig. 4A,B), but they mutually require the activity of one another to do so. Neither overexpression of Meis3 on a Tsh1-deficient background, nor reciprocal overexpression of Tsh1 on Meis3-deficient background represses Meis3 expression (Fig. 4Ac,Bc). These embryos more closely resembled the Tsh1- or Meis3-deficient phenotypes, respectively (Fig. 4Ad,Bd). Moreover, the combined knock down of these proteins synergistically expands Meis3 expression to its broader earlier-like pattern (supplementary material Fig. S4). Therefore, Meis3 and Tsh1 are not epistatic to one another, but jointly act to repress Meis3 expression.

We previously identified a 3 kb region of the *Xenopus laevis* Meis3 promoter that drives Meis3-like, hindbrain-specific, reporter gene expression in transgenic animals (Elkouby et al., 2010). We examined whether Meis3-Tsh1 proteins could directly repress Meis3 expression on the Meis3 promoter. There are two putative Meis consensus binding sites in the *Xenopus laevis* Meis3 promoter region: MeisA, −486 bp upstream to the transcription start site; MeisB, +356 bp downstream, in the 5′ UTR (Fig. 4C). By in vivo ChIP analysis, we determined whether these sites bound Meis3 protein at mid-late neurula stages in Meis3-Myc-expressing embryos. Both the MeisA and MeisB sites were specifically enriched in the Meis3-IP sample, and not in the IgG-IP control
As negative controls, we used a third region of Meis3 promoter, which lacks a Meis-binding site (MeisC, –2640 bp; Fig. 4C), and a fragment of the Mlc2 promoter. These control fragments were not enriched in the Meis3-IP sample; their levels did not exceed the IgG-IP control background levels (Fig. 4D). During sample preparation, we sheared the cross-linked DNA to fragments ≤1000 bp, with the vast majority ranging between 100 and 500 bp. We cannot completely eliminate the possibility of one site hitchhiking on the other, but our ChIP resolution suggests that both the MeisA and MeisB sites (~850 bp apart) are each bound by Meis3 protein. As Meis3 is a direct-target gene of itself (Fig. 2D), Meis3 protein probably auto-activates or auto-represses its expression by binding these two consensus sites within its promoter.

Meis3 auto-activation occurs prior to Tsh1 expression (Fig. 2D) and Tsh1 protein could be recruited to the Meis3 promoter by Meis3 protein to repress gene expression. Thus, we determined by ChIP, whether Tsh1 also occupies the MeisA and MeisB sites. In Myc-Tsh1 expressing embryos, both MeisA and MeisB sites were specifically bound in the Tsh1-IP sample and not in the IgG-IP

**Fig. 4. Meis3 and Tsh1 act cooperatively on the Meis3 promoter; recruited Tsh1 represses transcription.** (A) Meis3-5’UTR expression analysis in mid-neurula embryos injected at the one-cell stage with Meis3 mRNA (1 ng; b) or Tsh1-MO (5 pmol; a), or both (c). (B) Meis3 expression in mid-neurula embryos injected at the one-cell stage with Tsh1 mRNA (0.4 ng; b) or Meis3-MO (28 ng; d), or both (c). (C) A schematic representation of the Meis3 proximal promoter region. Arrow indicates start of transcription; green ovals indicate two Meis consensus sites (A and B), indicated in light-blue text; white oval indicates ChIP-negative control site (C); numbers indicate positions relative to the transcription start site (+1); ATG (+585) is the translation initiation site. (D) ChIP-QPCR analysis on the Meis3 promoter in Meis3-Myc injected embryos (0.8 ng) at mid-neurula stages. MeisA and MeisB are Meis consensus site ampiclons, MeisC and Mlc2 are negative control sites. IgG ChIP is a negative IP control. Pooled data from three independent experiments are represented as the percentage of input chromatin. Error bars are s.e.m. from three independent experiments. For amplicon primers, see supplementary material Table S2. (E) ChIP-QPCR analysis on the Meis3 promoter in Myc-Tsh1-injected embryos (0.4 ng), either wild type or Meis3-MO (28 ng) co-injected, at mid-neurula stages. IgG-IP, MeisC and Mlc2 are negative controls as in D. Pooled data from three independent experiments are represented as the percentage of input chromatin. Error bars are s.e.m. from three independent experiments. Tsh1-ChIP on Meis3-MO embryos (yellow) completely abolished the Tsh1-ChIP signal seen in wild-type embryos (dark blue). Tsh1-ChIP to both wild-type and Meis3-MO embryos were performed together in the same experiment and ChIP run; thus, Tsh1-ChIP on wild-type embryos is a positive control for Tsh1-ChIP on Meis3-MO embryos. (F) In vitro co-IP of Tsh1 and Meis3 proteins. In vitro synthesized HA-Meis3, S35-Met-Tsh1 and S35-Met-Cyp26 were mixed. HA-Meis3 IP was performed. IgG-IP and Cyp26 are a negative controls. (G) Kinetics of Tsh1 and Meis3 transcription in response to increasing levels of a forced Meis3 transcriptional activator. RT-PCR was performed on pools of 18 mid-neurula AC explants from VP16-Meis3 mRNA-injected embryos (0.25-1 ng). Results were quantitated, normalized to the Ef1 control and plotted as fold change. Bars are s.e.m. from three independent experiments. −RT samples showed no DNA contamination. A ‘0’ x-value is the non-injected controls, with a ‘1’ y-value as a basal expression level. (H) RT-PCR to Meis3-5’UTR and Krox20 in mid-neurula embryos injected with VP16-Meis3 mRNA (250 pg). Robust Krox20 expression controls for a strong Meis3 protein activity.
control (Fig. 4E). The control fragments were not enriched in the Tsh1-IP sample (Fig. 4E). Thus, Tsh1 binds the Meis3 promoter via the two Meis sites. We determined whether Tsh1 occupancy is Meis3 dependent. Tsh1 ChIP analysis was performed as described, but in Meis3-morphant deficient embryos. Meis3 protein is absolutely required for Tsh1-Meis3 promoter binding, as the Tsh1 ChIP signal on the MeisA and MeisB sites is abolished in Meis3-depleted embryos (Fig. 4E). The Tsh1 ChIP to both control and Meis3-deficient embryos were performed in the same experiment, and processed together, so that Tsh1 WT ChIP serves as a positive control for the Tsh1/Meis3-deficient ChIP. The experimental conditions were identical to Fig. 4B, thus coupling Tsh1 occupancy and function on the Meis3 promoter. These results reveal a recruitment mechanism, in which Tsh1 protein requires Meis3 protein to bind the Meis3 promoter.

Tsh1 binding the Meis3 promoter via Meis3 protein should require a physical interaction, and Drosophila Hth and Tsh proteins do interact (Bessa et al., 2002). We also detected specific physical interactions between the Xenopus Meis3 and Tsh1 proteins by co-IP in vitro (Fig. 4F). We used the Cyp26 protein as a negative control; Cyp26 and Meis3 expression do not overlap in the embryo, and are not predicted to physically interact (Fig. 4F). Thus, Meis3 and Tsh1 proteins probably physically interact to repress Meis3 promoter transcription.

To verify that Tsh1 recruitment provides repressor function, we used a forced transcriptional-activator Meis3, VP16-Meis3 (Dibner et al., 2001). We monitored Meis3 and Tsh1 expression in response to increasing levels of ectopic VP16-Meis3 protein in AC explants (Fig. 4G). VP16-Meis3 strongly induces Tsh1 expression (Fig. 4G), but is unable to repress Meis3 expression. In contrast to the tenfold decrease in Meis3 expression seen in AC explants expressing wild-type Meis3 protein (Fig. 3E), VP16-Meis3 induces a two-fold expression increase (Fig. 4G). This effect was also observed in vivo. VP16-Meis3 strongly induces Meis3 and Krox20 expression (Fig. 4H). Increased Krox20 expression is the typical readout for strong wild-type Meis3 activity, which consistently downregulates endogenous Meis3 expression (Fig. 2Ab). Unlike the wild-type Meis3 protein, VP16-Meis3 seems refractory to Wnt3a/Meis3/Tsh1 repression. When Tsh1 activity is overridden in the transcription complex on the Meis3 promoter, there is no repression.

Meis3 plays a dual-role in hindbrain development and the Wnt3a/Meis3/Tsh1 circuit acts to promote neuron differentiation

We addressed the physiological role of Meis3 suppression by the Wnt3a/Meis3/Tsh1 circuit. Whereas Meis3 is necessary for specification of primary neurons, strong Meis3 activity that induces hindbrain, typically inhibits terminal primary neuron differentiation (Gutkovich et al., 2010). Meis3 could play a dual role in hindbrain development: first, inducing cell types within the hindbrain, but later maintaining these cells in a proliferating progenitor state and preventing further differentiation. If so, Meis3 downregulation could stop proliferation, allow cell cycle exit and enhance differentiation. Indeed, in Drosophila eye and vertebrate retinal neuron development, Meis proteins induce eye/neuronal fate and proliferation, but their downregulation enables differentiation (Bessa et al., 2002; Bessa et al., 2008; Heine et al., 2008). Our observations that mid-neurula induced THVGR activity repressed Meis3 expression without perturbing hindbrain marker expression (Fig. 1Bf-j), whereas neural-specific Wnt3a-deficiency modulated n-Tub expression in explants (Fig. 1C-d), support a later role for this Wnt3a/Meis3/Tsh1 circuit in primary neuron differentiation.

To address this issue, three experimental strategies were used to mis-express Meis3 protein at mid-late neurula stages, when it should be endogenously repressed. An inducible Meis3 (Meis3-GR) was overexpressed, and activated only prior to mid-neurula stages. VP16-Meis3 was also overexpressed; VP16-Meis3 is refractory to Wnt3a/Meis3/Tsh1 repression and constitutively upregulates Meis3 expression (Fig. 4G,H). Finally, we used Tsh1-deficient embryos, in which endogenous Meis3 expression is never repressed (Fig. 3Cc, Fig. 4Ad). In Xenopus, early neural progenitors express the Iroquois 3 (Iro3) gene (Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998). Iro3 expression is followed by Ngn1 expression, which induces NeuroD expression and promotes primary neuron differentiation as marked by n-Tub expression (Sasai, 1998). Ngnr1 simultaneously represses Iro3 expression, thus expression of Iro3 in neural progenitors and n-Tub in fully differentiated primary neurons is mutually exclusive (Bellefroid et al., 1998). We examined Meis3-GR, VP16-Meis3-overexpressing and Tsh1-deficient embryos for Iro3 versus n-Tub expression levels. Their proliferative state was determined by phospho-Histone H3 (PH3) immunostaining (Fig. 5A). For PH3 analysis, we used stacked images of multiple focal planes in cleared neural tubes, to allow for efficient visualization of proliferating cells. Meis3-GR was activated at mid-neurula stage; unactivated Meis3-GR had very weak activity in 15% of the embryos, whereas 85% developed normally (Fig. 5Ae-g).

All three Meis3 mis-expressing strategies cause expanded Iro3 expression, repression of n-Tub expression, and a marked increase in proliferating neural tube cells (Fig. 5Aa-p). Similar to Meis3 in Tsh1-deficient embryos (Fig. 3Cc, Fig. 4Ad), Iro3 expression is also expanded to its broader early-neurula stage pattern (Fig. 5An, inset). Furthermore, embryos injected into one blastomere in the two-cell stage with VP16-Meis3 consistently expressed ectopic levels of Iro3 and Sox3. Sox3 specifically marks proliferating neural progenitors and not n-Tub-expressing primary neurons; like Iro3 and n-Tub expression, Sox3 and n-Tub expression are mutually exclusive (Bourgugnon et al., 1998; Hardcastle and Papalopulu, 2000; Bonev et al., 2011). Double in situ hybridization shows that ectopic Iro3 expression is restricted to Sox3 expanded zones of expression on the injected-side (supplementary material Fig. 5SA). These results show that, at neurula stages, Meis3 maintains the proliferative progenitor state of neurons, preventing their terminal differentiation. This later Meis3 activity is uncoupled from its earlier caudalizing activity (supplementary material Fig. 5SB).

Reciprocally, we injected embryos with low concentrations of the Meis3-MO. This low Meis3-MO concentration causes a moderate reduction of Meis3 protein levels (Fig. 5Ay), resulting in a hypomorph (Meis3-MOhyp) phenotype. Meis3-MOhyp embryos could unmask the later effects of Meis3 protein depletion, without severely compromising its early functions in hindbrain formation, mimicking Meis3 reduction by the Wnt3a/Meis3/Tsh1 circuit. In Meis3 hypomorphants, Iro3 expression is unaffected or moderately decreased (Fig. 5Aq-t). Expression of n-Tub is expanded specifically in the hindbrain region, with ectopic neuron differentiation foci (Fig. 5Ab-r,u,v), which are also observed in the enlarged hindbrain-derived trigeminal neural tube (Fig. 5Av-x). Proliferation is also reduced in Meis3-MOhyp embryos (Fig. 5As). This moderate decrease in Meis3 protein levels permits more cell cycle exit of neuron progenitors, enhancing differentiation. We used the VP16-Meis3 tool to analyze the transcripational profile of Meis3-maintained neural progenitors. These cells fail to express the pro-neural marker genes Ngn1, Myt1, NeuroD and n-Tub, while expressing high levels of Iro3 (Fig. 5B), thus Meis3 maintains early Iro3+
proliferating neural progenitors that cannot differentiate. Further
supporting a role for Meis3 protein as a cell-cycle regulator, ectopic
VP16-Meis3 downregulated expression of the neural cell-cycle
inhibitors p27Xic1 and Gadd45γ, the activities of which are required
for primary neuron differentiation (Vernon et al., 2003; de la Calle-
Mustienes, 2003). VP16-Meis3 represses their expression cell
autonomously, in both dissociated and intact AC explants (Fig. 5C).
In the developing neural plate, the Wnt3a/Meis3/Tsh1 circuit is
required to downregulate Meis3 expression at the appropriate stage
to promote neuron differentiation, presumably after a crucial mass
of progenitors has formed. Tsh1 loss-of-function also perturbs
neural tube closure, resulting in an early-like open neural plate (Fig.
3Cc, Fig. 4Ad, Fig. 5Ao,p). As Tsh1 loss of function causes an
early-like upregulated expression of Meis3 and Iro3, a stalling of
primary neurons in a proliferative progenitor state, and an open
neural plate morphology, we conclude that Tsh1 acts as a neural
tissue maturation coordinating factor.

DISCUSSION
This study reveals a novel genetic circuit of Wnt3a, Meis3 and Tsh1
proteins that orchestrates downregulation of Meis3 expression,
restrickind hindbrain induction and shifting neuronal progenitors to
differentiation (Fig. 6A,C). We showed that early Wnt3a activates
Meis3 gene expression directly, as well as indirectly via Meis3 auto-
induction. At later stages, high Meis3 protein levels activate Tsh1
gene expression, leading to Tsh1 and Meis3 co-binding on the Meis3
promoter (Fig. 6B). These delicate checks and balances between
Wnt3a, Meis3 and Tsh1 protein activities are crucial for proper cell
fate decisions in the maturing neural plate.

Tsh1 loss of function causes a compound phenotype. First,
primary neurons remain in their proliferating progenitor state,
falling to differentiate. Next, the neural transcription factors Meis3
and Iro3, maintain their early broad expression patterns, and are not
downregulated to their later confined patterns. Finally,
morphological cellular convergent extension (CE) movements
required for proper neural tube closure are inhibited. CE
movements are controlled by the Wnt planar cell polarity (PCP)
pathway (Tissir and Goffinet, 2010). CE and active PCP are
incompatible with cycling cells (Ciruna et al., 2006; Devenport et
al., 2011); therefore, Tsh1 could induce cell cycle arrest in the
neural plate, permitting PCP activity that drives neural tube closure.
Immature neural tissue transforms from an open field of
proliferating neural progenitors to a closed neural tube of
differentiating cells. Neural tissue lacking Tsh1 exhibits immature
characteristics. Tsh1 protein acts as a maturation factor that
coordinates cell cycle exit, primary neuron differentiation and
distinct cell movements in the neural plate.

Meis proteins form transcription complexes with Pbx and Hox
proteins to activate target genes (Sagerstrom, 2004; Moens and
Selleri, 2006). In the zebrafish hindbrain, Pbx4 protein recruits the
transcriptional repressors CBP and HDAC to chromatin. Meis3
protein competes with Pbx4 to reduce promoter accessibility of
HDAC and CBP to activate transcription (Choe et al., 2009).
This is consistent with Meis3 hindbrain function as a transcriptional
activator (Dibner et al., 2001). Supporting a conserved role for Tsh
family proteins in hindbrain development, a recent genetic study in
zebrafish showed that Tsh3b protein negatively regulated hindbrain
Hox paralogous 1-4 gene function (Erickson et al., 2011).
In a human neuroblastoma cell line, Tsh3 binds the FE65 adaptor protein recruiting HDAC to chromatin, suppressing caspase 4 gene expression; in Alzheimer’s brain postmortem samples, reduced Tsh3/FE65 gene expression triggered an increase in caspase 4 levels, suggesting a causative role in disease progression (Kajiwara et al., 2009). Tsh3 also recruited repressive SWI/SNF proteins to chromatin in cultured myoblasts (Faralli et al., 2011). These studies support our observation of joint Meis3/Tsh1 repression of the Meis3 promoter in the vertebrate hindbrain. Meis3 probably prevents HDAC recruitment, promoting Meis3 transcription; later, Meis3-recruited Tsh1 directs transcriptional repression components to the complex. The Meis3 promoter undergoes rapid stage-dependent changes in chromatin dynamics to modulate its expression; this flexibility allows fine-tuned regulation of neural cell fate decisions.

β-Catenin shuttles proteins into the nucleus (Fagotto et al., 1998) and Xenopus Tsh and Drosophila Tsh proteins undergo nuclear translocation via physical interaction with β-catenin (Gallet et al., 1998; Gallet et al., 1999; Onai et al., 2007). This challenges our observation that Tsh proteins inhibit Wnt/β-catenin activity. However, the coupled import of Tsh and β-catenin could serve as a potent cell-sensing mechanism, suggesting that Tsh nuclear shuttling occurs only under Wnt-activated conditions, when β-catenin enters the nucleus, ensuring Tsh-mediated repression of Meis3 expression in the appropriate space and time. High cellular Meis3 levels are prerequisite for Tsh1 expression. This regulatory mechanism triggers Tsh1 inhibitory activity exclusively in cells where Meis3/Wnt/β-catenin neural caudalizing activity is high, thus explaining why early Wnt/β-catenin activity induces Meis3 expression, but later suppresses it. This novel mechanism of Wnt modulation induces expression of a second protein, Tsh1, which then piggy-backs onto β-catenin to the nucleus to modify the transcriptional response of the pathway.

Wnt acts at multiple stages in the induction, proliferation and differentiation of neurons (Megason and McMahon, 2002; Amoyel et al., 2005; Nordstrom et al., 2006; Elkouby et al., 2010). We suggest a mechanism in which Meis3-Tsh1 interplay explain this signaling integration. At gastrula stages, Wnt/β-catenin activates Meis3 expression, which induces primary neuron precursors. At neurula stages, Wnt/β-catenin and Meis3 activities are both required to induce Tsh1 expression, subsequently repressing Meis3 expression, shifting progenitor cells from a proliferative determined state to terminal differentiation.

Meis3 protein has a dual role in the developing hindbrain. Although Meis3 is required for early hindbrain formation, including primary neuron induction (Gutkovich et al., 2010), we have unmasked a later role controlling primary neuron differentiation. Meis3 protein maintains early-induced primary neurons in a proliferative progenitor state, preventing differentiation (Fig. 6C). A model describing the Wnt3a/Meis3/Tsh1 circuits role in controlling gene expression and cell cycle at different stages of primary neuron development.

**Fig. 6.** A hindbrain repressive Wnt3a/Meis3/Tsh1 circuit promotes neuron differentiation. (A) The Wnt3a/Meis3/Tsh1 circuit (red arrows and blocks) represses the hindbrain developmental program (blue arrows). (B) Upper panel: β-catenin and Meis3 bind Tcf and Meis sites on the Meis3 promoter to activate transcription. Lower panel: high Meis3 activity induces Tsh1 expression. Tsh1 protein recruited to Meis sites represses Meis3 transcription. (C) A model describing the Wnt3a/Meis3/Tsh1 circuits role in controlling gene expression and cell cycle at different stages of primary neuron development.

**Fig. 6A**

- Tsh1
- Meis3
- FGF
- PG1-4
- Hox
- Specification of Posterior Neural Cells

**Fig. 6B**

- β-catenin
- Meis3
- Tcf
- Meis3 promoter
- ON
- OFF
- Meis3 site
- Tsh1 site

**Fig. 6C**

- Neural precursor
- Neural progenitor
- Terminal differentiation
- Neurogenin → NeuroD → n-Tub
- Sox2
- Sox2
- Meis3
- Tsh1
- Wnt3a
- Meis3
- β-catenin
- Tcf
- CyclinD1
- CyclinD3
- BM hematopoietic cells

**RESEARCH ARTICLE**

**DEVELOPMENT**
proliferation and inhibits neuron differentiation in Xenopus (Richard-Parpaillon et al., 2004). It would be interesting to examine the potential regulation of hindbrain cyclin/cdk expression by Meis3. Interestingly, we show that Meis3 strongly downregulates p27Xic1 and Gadd45g expression. These cell cycle inhibitors promote cell cycle exit required for primary neuron differentiation (Vernon et al., 2003; de la Calle-Mustienes, 2003). Plant TALE homologues exhibit this duality during compound leaf development (Shani et al., 2009). Our work supports this dual developmental strategy for Meis/KNOX proteins as a general evolutionary trait.

Meis3-induced progenitors are stalled as Iro3+ neurons, consistent with our findings that Meis3 induces primary neurons upstream of Ngnr1 activity (Gutkovich et al., 2010). Furthermore, Ngnr1-induced primary neuron differentiation in Meis3-depleted embryos was even stronger than in wild-type embryos (Gutkovich et al., 2010), suggesting that in order to maintain the Iro3+ state, Meis3 could potentially inhibit neuron differentiation by repressing p27Xic1 and Gadd45g gene expression. Meis3 inhibition released the unregulated full capacity of Ngnr1 neuron-inducing activity. In Meis3-induced progenitors, upregulated cell cycle activators and downregulated cell cycle inhibitors could prevent cell cycle exit, regardless of pro-neural gene expression. Such uncoupled regulation was shown for Meis1 in the retina (Bessa et al., 2005; Heine et al., 2008). Iro3 expression is strongly induced by Meis3, and Iro protein positively regulates pro-neural gene expression (Bellfroid et al., 1998; Gomez-Skarmeta et al., 1998). Iro3 neural progenitor maintenance was suggested to require interactions with other factors, and its downregulation was required for neuron differentiation (Bellfroid et al., 1998). Ihh, Iro and Tsh proteins form differential transcriptional complexes in Drosophila (Pichaud and Casares, 2000; Bessa et al., 2002), and their expression overlaps in the vertebrate hindbrain. Expression of Iro3, cyclin genes, cdk inhibitors and pro-neural genes could be differentially regulated by distinct Meis3/Iro3/Tsh1 complexes. Indeed, Meis3-expressing/Tsh1-deficient progenitors do not express pro-neural genes but show strong Iro3 expression. We show that Tsh1 suppresses Iro3 expression and activity, suggesting a similar Tsh1 repressive recruitment to a potential Meis3-Iro3 complex.

Both Meis proteins and Wnt/β-catenin signaling act as acute myeloid leukemia (AML)-promoting factors (Eklund, 2007; Mikesch et al., 2007). Wnt pathway components and Meis proteins are overexpressed and activated in AML cells (Eklund, 2007; Mikesch et al., 2007). Analogous to the dual-role of Meis3, Meis1 and Meis3 proteins induce both AML transformation and malignant progression acceleration, which are the rate limiting regulators of AML (Thorsteinssottid et al., 2001; Wang et al., 2006; Wong et al., 2007). In AML cells, Flt3 is a Meis1 direct-target gene that induces Wnt/β-catenin activity (Wang et al., 2006; Mikesch et al., 2007). Recently, Tsh proteins were implicated as tumor suppressors in breast and prostate cancer (Yamamoto et al., 2011). Perhaps Tsh gene expression is downregulated in AML, which triggers de-regulated Meis and Wnt protein activities. Tsh transcriptional repressor function was suggested to regulate target genes in Alzheimer’s disease progression (Kajiwara et al., 2009). We show that Meis3 and Tsh1 protein interplay converges on Meis3 gene expression to coordinate the induction, proliferation and differentiation of developing neurons. Further investigation will determine whether other Wnt/Meis1/Tsh genetic circuits have analogous roles in regulating diverse developmental processes, as well as cancer and neurodegenerative disease.

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