Characterization of paralogous uncx transcription factor encoding genes in zebrafish

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ABSTRACT

The paired-type homeodomain transcription factor Uncx is involved in multiple processes of embryogenesis in vertebrates. Reasoning that zebrafish genes uncx4.1 and uncx are orthologs of mouse Uncx, we studied their genomic environment and developmental expression. Evolutionary analyses indicate the zebrafish uncx genes as being paralogs deriving from teleost-specific whole-genome duplication. Whole-mount in situ mRNA hybridization of uncx transcripts in zebrafish embryos reveals novel expression domains, confirms those previously known, and suggests sub-functionalization of paralogs. Using genetic mutants and pharmacological inhibitors, we investigate the role of signaling pathways on the expression of zebrafish uncx genes in developing somites. In identifying putative functional role(s) of zebrafish uncx genes, we hypothesized that they encode transcription factors that coordinate growth and innervation of somitic muscles.
1. Introduction

The Uncx gene (also known as Uncx4.1, Phdl and Clox4) encodes a transcription factor containing a paired-type homeodomain homolog to Caenorhabditis elegans UNC-4 homeoprotein (Miller et al., 1992; Rovescalli et al., 1996). The nematode UNC-4 controls synaptic choices of specific motor neurons in the ventral cord by modulating their sensitivity to diffusible Wnt ligands (White et al., 1992; Miller and Niemann, 1995; Schneider et al., 2012). In C. elegans and Drosophila melanogaster, UNC-4 orthologs form a repressor complex with UNC-37, homolog of Groucho/TLE transcriptional co-repressor (Plüg der et al., 1997; Winner et al., 1999; Giot et al., 2003; Von Stetina et al., 2007). Vertebrate Uncx genes are implicated in multiple processes of embryogenesis, as suggested by their expression in olfactory epithelium, telencephalon, mesencephalon, spinal cord, branchial arches, kidney, somites, and forelimb autopod (Saito et al., 1996; Neidhardt et al., 1997).

Many mechanisms underlying the role of Uncx have been proposed, including cell adhesion, axon guidance, cell cycle control, and differentiation process in postmitotic stages (Mansouri et al., 2000; Bussen et al., 2004; Asbreuk et al., 2006; Sewell et al., 2009; Skuntz et al., 2009; Sammeta et al., 2010; Rabe et al., 2012).

Vertebrates, Uncx is transcribed in sclerotomal cells surrounding the notochord, suggesting a conserved role as determinant of axial skeleton morphogenesis (Neidhardt et al., 1997; Mansouri et al., 1997; Koudijs et al., 2008; Sánchez and Sánchez, 2013; Retnoaji et al., 2014). Uncx functions are perhaps best understood in amniotes. Loss-of-function analysis in mice suggests that this gene is required for the maintenance of posterior somite characteristics (Leitges et al., 2000; Mansouri et al., 2000).

Uncx transcription in the presomitic mesoderm (PSM) depends on Delta-like 1 (Dll1) and is independent from signals of the axial structures, such as notochord-floor plate complex, whereas further maintenance requires Uncx itself (Barrantes et al., 1999; Mansouri et al., 2000; Schrögle et al., 2004; Sewell et al., 2009). A central role in the repression of Uncx expression in the anterior somite is played by a complex regulatory network that involves the basic helix-loop-helix transcription factor Mesp2, its downstream co-repressor Ripply, the homeodomain transcription factor MEox1, and a cross-negative regulation with the T-box protein Tbx18 (Takahashi et al., 2000, 2003, 2013; Nakajima et al., 2006; Farin et al., 2008; Skuntz et al., 2009; Yabe et al., 2016). Recently, cell type-specific epigenetic regulation of Uncx gene expression has been associated with axon guidance in C. elegans (Zheng et al., 2013) and with human leukemias (Daniele et al., 2017). It has been proposed that Uncx is implicated in cell cycle progression of neuronal progenitor cells, survival of olfactory epithelium and differentiation of dopaminergic neurons (Sammeta et al., 2010; Rabe et al., 2012).

Although many advances have been made in dissecting the biological significance for development and the mechanisms of action of vertebrate Uncx, other aspects, including molecular evolution and roles in axonal growth, remain poorly defined. To elucidate the cascade of events accomplished by the Uncx proteins the zebrafish (Danio rerio) could be an ideal model due to its amenability to embryological and genetic approaches. However, to date Uncx homologs in zebrafish have not been characterized in detail. In this study, we performed genome and gene expression analyses of the zebrafish genes uncx4.1 and uncx, with a focus on somite formation and innervation. Taken together, our results provide insights into the potential role of zebrafish Uncx genes in the formation of spatially distinct muscle progenitor domains and in axon pathfinding.

2. Materials and methods

2.1. Molecular evolution

The protein sequences used for the evolutionary analysis were retrieved from the NCBI and Ensembl databases. The Homo sapiens Uncx protein was the initial query sequence employed for tBlastn searches (Gertz et al., 2006) in invertebrate and vertebrate genomes, and reciprocal Blasts were carried out on each genome. ClustalW was used to align the proteins selected for phylogenetic analysis with default parameters (Thompson et al., 1994). The phylogenetic tree was built with the Maximum-Likelihood estimation (MLE) using MEGA6 with 1000 replicates; the LG substitution model, with 0.2 as proportion of invariable sites (I) and 4 as gamma distribution parameter (γ), was selected (Tamura et al., 2013). The graphical representation was created with Dendroscope (Huson and Scornavacca, 2012). The synteny analysis between human and zebrafish chromosomes was performed with "Sinteny Database" and a sliding window size of 50 genes (Catchen et al., 2009). The syntenic survey between human and the tunicate Ciona robusta was performed mapping manually the genes on the scaffolds/chromosomes in Ensembl and Genomcics databases. Intron were mapped by using available public resources and designed, with a color code representation, on the protein alignment obtained using ClustalW. The analysis of genomic conservation was performed on ten sequences, employing mVISTA computational tool (Ramere and Dubchak, 2009). To identify conserved non-coding sequences by VISTA, we employed LAGAN (global pair-wise and multiple alignments of finished sequences) with the following parameters: minimum Conservation Width for non-coding sequences (40 bp), minimum conservation identity (50%), and minimum Y value (20%). To improve the comparison of distant homologs, the translated anchoring in LAGAN/Shuffle-LAGAN was used.

2.2. Zebrafish stocks and husbandry

Zebrafish of wild-type AB, sonic-you (syufl392), cyclops (cycb16), acerebellar (acel282b), smoothened (smmed0277), you-too (yotth129), floating head (fh137) and fused-somites (fsms3146) lines were raised and maintained at 28 °C under a reproduction regime (14 h light/10 h dark cycle) at UCL (UK). All embryos were collected after natural spawning and staged in somites (s) and hours post fertilization (hpf) according to Kimmel et al. (1995). Fertilized embryos were kept in Petri dishes containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, 1 × 10−5% Methylene-blue). Ethical approval for zebrafish experiments was obtained from local review panels and from the Home Office UK under the Animal Scientific Procedures Act 1986.

2.3. Actin filament staining

Whole-mount phalloidin staining was performed as described (Whitfield et al., 1996). Embryos were fixed with 4% paraformaldehyde (PFA), followed by permeabilization in 2% Triton X-100/PBS for 1.5 h and incubation in 2.5 μg/ml fluorescein-labeled phalloidin (Sigma) in PBS for 2 h in the dark at 4 °C. Embryos were then rinsed overnight and mounted in 70% glycerol/30% PBS prior to proceed to image acquisition.

2.4. Cloning and probe synthesis

A neurula stage zebrafish cDNA library (kind gift of D. Grunwald) prepared in the λ ZAP II vector (Stratagene) was screened for homeobox-containing genes by PCR with a primer annealing to the cloning site of the plasmid vector and a degenerate primer annealing to a conserved homebox region (TGTACCCCTCCTGTTTGGRAACCA). We cloned a 220 bp cDNA fragment of uncx4.1 that was used as probe to screen 1.2/10^6 recombinant plaques of the same library at moderate density.
stringency. From a fourth screen, a Bluescript phagemid was rescued and its 2 kb insert sequenced, which encoded a full-length Uncx4.1 protein as judged by a BLAST search of GenBank and EMBL databases. To make riboprobes for WISH analysis, a 950 bp uncx4.1-containing pBluescript SK+ plasmid was linearized with EcoRI and transcribed with T7 for the antisense probe, or linearized with Apal and transcribed using T3 polymerase for the sense riboprobe. A 562 bp cDNA fragment of the uncx gene coding sequence was amplified (Fwd: 5′-AGGACCACTCATGTTACAGA-3′ and Rev: 5′-CCGGAGGAGCTTGTGTGGA-3′), cloned into pcR™ II-TOPO® vector following TOPO TA Cloning instruction manual, and sequenced. The TOPO TA plasmid was linearized with HindIII and transcribed using T7 polymerase for the antisense probe or linearized with Apal and transcribed using SP6 polymerase for the sense probe (Suppl. Fig. 1).

2.5. Whole-mount in situ hybridization (WISH)

Zebrafish embryos (n = 20/group) at different stages of development were aneathetized with tricaine MS-222, fixed by immersion in 4% PFA overnight at 4 °C, and eventually de-pigmented using 3% hydrogen peroxide and 1% KOH. Fixed embryos were stored in 100% methanol at −20 °C. Embryos were permeabilized by proteinase K treatment (10 μg/ml). The hybridization was carried out at 65 °C with the specific digoxigenin-labeled probes diluted in hybridization mix (HM: 50% formamide, 1.3 × SSC, 5 mM EDTA, 50 μg/ml yeast RNA, 0.2% Tween 20, 0.5% CHAPS, 100 μg/ml heparin). Embryos were incubated with anti-digoxigenin alkaline phosphate-conjugated antibodies (1:5000; Roche) at 4 °C. Embryos were stained in BM Purple solution (Roche). Additionally, to detect mRNA of other markers (shha, her1, mespa, myod1, egr2b), embryos were incubated in pre-staining buffer (100 mM Tris–HCl, 0.1% Tween 20, 0.1% CHAPS, 100 μg/ml heparin). Embryos were permeabilized with anti-digoxigenin alkaline phosphate-conjugated antibodies (1:5000; Roche) at 4 °C. Embryos were stained in BM Purple solution (Roche). Additionally, to detect mRNA of other markers (shha, her1, mespa, myod1, egr2b), embryos were incubated in pre-staining buffer (100 mM Tris–HCl, 0.1% Tween 20, 0.1% CHAPS, 100 μg/ml heparin).

2.6. Whole-mount immunohistochemistry (WIHC)

Zebrafish embryos were collected and fixed as described for WISH. Embryos were permeabilized by proteinase K treatment (10 μg/ml), incubated with a blocking solution (NGS 3%) for 2 h and incubated with monoclonal primary antibodies (acyethylated α-tubulin, 1:1000 (Sigma–Aldrich); znpl, 1:100 (DSHB); MP20, 1:10 (DSHB); S58, 1:10 (DSHB); F59, 1:10 (DSHB) diluted in PBT containing 3% NGS. After several washes in PBT, embryos were incubated with biotinylated anti-mouse IgG (1:200) or IgA–FITC (1:200) for 2 h. For chromogenic staining, embryos were incubated with avidine-biotine solution (Vectorstain ABC kit, Vector Labs) and, then, with chromogenic substrate 3,3′-diaminobenzidine (DAB) until staining was sufficiently developed. For combined WISH–WIHC experiments, WIHC (znpl or acetylated α-tubulin) was performed subsequently to WISH for uncx4.1 mRNA. Embryos were imaged as described for WISH. WIHC and WISH–WIHC experiments were performed in biological triplicates.

2.7. Microinjections

mRNA: To generate synthetic mRNA for injection, the entire uncx4.1 reading frame (ORF) was cloned into the vector pBUT2 which was made by cloning 5′ and 3′ UTRs of Xenopus β-globin (from pSP64T; Krieg and Melton, 1984) at either side of pBBlueScript (Stratagene) with a synthetic poly linker replacing the BglII site of pSP64T. To clone the uncx4.1 gene ORF in-frame with the Kozak consensus sequence, which increases the efficiency of translation initiation by ribosomes (Kozak, 1986), a PCR on the 2 kb uncx4.1 insert (see Section 2.4) was performed using the following primers: GAGCGAGGATCCACCACTGATGATAG CGGGATC and CCTATTGGTCACAGTGTACATC. The 1340 bp PCR fragment was gel purified, cut with KpnI (introduced into the sequence through the primers), and ligated into the KpnI-digested pBUT2 plasmid. The DNA sequence of the insert was confirmed by sequencing. The plasmid was linearized with EcoRI and the gene was transcribed in vitro with the help of the T3 mMessage mMachine Kit (Ambion) yielding capped RNA for injection. The zebrafish full-length shha-containing pSP64T plasmid for mRNA injection was a kind gift of P. Ingham (Krauss et al., 1993). Synthetic capped shha and uncx4.1 mRNAs were injected repeatedly (n > 3) at concentrations of 400, 200, and 200 pg per embryo, respectively. Injections were carried out on 1- to 2-cell stage embryos.

Morpholino: Gene knockdown was achieved by morpholino (MO) antisense oligonucleotides designed to disrupt splicing of pre-mRNA or inhibit translation of mRNA (Gene Tools). The amount and the sequence for various morpholinos used are as follows: 0.5 mM uncx4.1-utg-MO (blocking translation antisense morpholino), 5′-GATCCCCGA TACGATTTGCATCT-3′; 0.5 mM uncx4.1-utg-mismatch-MO, 5′-GA GGGGATTCGATTGCATCT-3′; 0.5 mM uncx4.1-atg-MO (blocking translation antisense morpholino) 5′-GATCCGATTTGCATCT-3′; 0.5 and 0.8 mM ctrMO (standard control morpholino), 5′-CCT CTACCTGAGTACCTTTATA-3′. All MOs were injected into embryos at one to four cell stages. In total, we analyzed 254 embryos injected with the atg-MO against uncx4.1, and 294 embryos injected with atg-MO against uncx.

2.8. Pharmacological treatments

After partial dechorionation of zebrafish embryos (n = 20/group) at 6 hpf, the following chemical molecules were administered: 50 μM cycloptamine (Sigma–Aldrich), 50 μM SB431542 (Sigma–Aldrich), 40 μM DAPT (N-(N-(3,5-Difluorophenacetyl-L-alanyl))-S)-phenylglycine t-butyl ester; Calbiochem) and 20 μM SUs402 (Calbiochem). Embryos were kept in an incubator set to 28 °C for the duration of exposure until the desired developmental stage. Pharmacological treatments were performed in biological triplicates.

3. Results

3.1. Evolutionary analysis

To decipher the evolutionary history of Uncx genes in metazoans, we performed a ML phylogenetic survey (Fig. 1) employing a collection of 25 manually curated protein sequences (Suppl. File 1) that encompass: nematodes (Caenorhabditis elegans), mollusks (Lottia gigantea, Crassostrea gigas), annelids (Capitella teleta), brachiopods (Lingula anatina), hemichordates (Saccoglossus kowalevskii), echinoderms (Strongylocentrotus purpuratus, Acanthaster planci), cephalochordates (Branchiostoma belcheri, Branchiostoma floridiae), and vertebrates (Cal loricinus milii, Lepisosteus oculatus, Latimeria chalumnae, Danio rerio, Tetraodon nigroviridis, Xenopus tropicalis, Gallus gallus, Gekko japonicus, Mus musculus, Homo sapiens). Selected outgroups were two cephalochordate Hox3 protein sequences from B. belcheri and B. floridiae. We also found Uncx proteins in other genomes but these were excluded from the phylogeny due to their high molecular divergence (e.g., Ciona robusta and Takifugu rubripes) or partial sequence (e.g., Nemastomella vectensis) (Suppl. File 2). Our genome search and phylogeny strongly indicated the existence of a single Uncx gene arisen at the root of bilaterians, as suggested by its presence in the cnidian Nemastomella vectensis genome (Ryan et al., 2006; Suppl. File 2). This gene has been affected by local duplications in invertebrates like C. teleta, Drosophila melanogaster, S. kowalevskii, and Ciona robusta, and lost in the placozoan Trichoplax adhaerens. Among vertebrates, we found a divergent Uncx protein in hagfish (Eptatretus burgeri), while no ortholog was identified
...in the lamprey genome (Petromyzon marinus) (Fig. 1; Suppl. File 2). Instead, a duplication event has been identified in teleosts (D. rerio, T. nigroviridis, Takifugu rubripes), possibly due to the Teleost-Specific Whole-Genome Duplication (TSGD) (Taylor et al., 2001; Taylor et al., 2003; Jaillon et al., 2004; Kuraku and Meyer, 2009). The analysis of gene structure unraveled the preservation of intron positions in Uncx genes, supporting their orthology from invertebrates to vertebrates (Suppl. File 3).

The study of the Uncx genomic locus revealed a high degree of synteny between tetrapods as human (Chr7) and teleosts as zebrafish (Chr1, Chr3), showing the preservation of 11 genes close to Uncx: the conservation of this cluster supports a TSGD-origin for zebrafish Uncx genes. The absence of some of these genes (e.g., gper) in one of the two syntenic clusters suggests the secondary loss of TSGD-derived duplicates (Fig. 2).

Our survey also expanded our understanding of conservation of the Uncx genomic locus (Woolfe and Elgar, 2007), demonstrating high synteny in gnathostomes. In particular, we uncovered the presence of two conserved microsyntenic clusters. First, a gene triplet composed of Uncx, Mical2 and Elf1 genes is present in gnathostomes. Mical...
gene duplet (Suppl. Fig. 4). Then, we analyzed the genomic region of Uncx genes in vertebrates. Horizontal bars represent orthologous genomic regions of human (H. sapiens, Hsa7) and zebrafish (D. rerio, Dre3 and Dre1). Orthologous genes are shown with same colors and are connected by lines. Red lines highlight a conserved microsynteny involving Uncx, Micall2 and Efn1 genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Uncx expression during embryonic development

To generate probes for mRNA in situ hybridization, we first cloned the full-length transcripts of uncx4.1 (NM_001020780) and uncx (XM_005164204.4). In order to have a spatio-temporal overview of uncx gene expression during zebrafish embryogenesis, we performed a whole-mount in situ hybridization (WISH) at various developmental stages until 48 hours post fertilization (hpf).

3.2.1. Uncx4.1 (NM_001020780.2)

Recently, tomographic data based on low-input RNA sequencing and mathematical image reconstruction have revealed early expression of uncx4.1 in the shield at 5 hpf (Junker et al., 2014). In this study, the earliest evidence of uncx4.1 expression was detected in all but the most anterior cells within each somite (11 hpf) (Fig. 3A). During early somitogenesis, uncx4.1 expression is reiterated in newly forming metameric blocks (14.5 hpf) (Fig. 3B–D). Subsequently, expression disappears from the myoseptum to dorsal and ventral margins (19 hpf) (Fig. 3E) to become restricted in few boundary cells positioned in the ventral lateral posterior (VLP) tip of the somite (24–34 hpf) (Fig. 3F–H, J). VLP cells can be easily recognized at the cellular level with differential interference contrast optics, due to the round cell shape compared to the elongated fibroblasts. Phalloidin staining of 34 hpf embryos supports the view that uncx4.1 transcripts mark cells of the future myomere that are morphologically distinct from the main adaxial somite portion (Fig. 3I). We also found uncx4.1-expressing cells on both sides of the notochord, perhaps corresponding to the fish sclerotome, the myogenic contribution to backbone formation (Morin-Kensicki and Eisen, 1997) (Fig. 3K, L).

During neurogenesis, uncx4.1 is expressed in specific regions of the central nervous system (CNS). Early uncx4.1 mRNA signal is found in the second intron (green box). Notably, the upstream peak pattern differs between teleost paralogs (blue box). Altogether, this in-depth study of Uncx evolution defines this gene as an ultra-conserved homeobox gene with a complex evolutionary scenario in vertebrates.
rhombomeres 2–4 at 10 hpf (tailbud stage), as shown by double labeling with egr2k, a marker of early hindbrain rhombomeres 3 and 5 (Figs. 3A, 4A). During development, uncx4.1 expression is visible in the olfactory placodes, telencephalon, and diencephalon, at 18–24 hpf (Fig. 4B–F), and in the ventral thalamus, pre-pectum, cerebellum, pharyngeal arches and pronephric ducts, at 34 hpf (Fig. 4G–L). The position of hindbrain cell bodies expressing uncx4.1 coincided with the ventral-lateral exit roots of branchial motor neurons (Fig. 4H–J). At 48 hpf, we observed a widespread diffuse pattern for uncx4.1 in the developing brain (Fig. 4M).

3.2.2. Uncx (XM_005164204.4)

Initially, weak signal of the uncx transcript is seen in bilateral columns of forebrain cell bodies, in the hindbrain, and in trunk mesodermal cells extending to PSM (12.5 hpf) (Fig. 5A). At 18 hpf, uncx expression is found in the olfactory placodes, telencephalon, diencephalon, hindbrain, spinal cord and ventral somites (Fig. 5B–E). At 22–24 hpf, expression is also present in pre-pectum, tegmentum, and cerebellum (Fig. 5F–H). As development proceeds, cerebral expression remains prominent in cerebellum and hindbrain (34–48 hpf) (Fig. 5I–K) while it was absent in somite structures and spinal cord (data not shown).

3.3. Characterisation of uncx4.1 expression during somite differentiation

To further explore uncx4.1 expression during somitogenesis, we carried out double WISH with her1 and mespaa, which revealed that the earliest sign of uncx4.1 expression occurs in the anterior margin of the PSM and in the anlage of newly forming somite (Fig. 6A, B) (Holley et al., 2000, 2002; Sawada et al., 2000). In paraxial mesoderm, the adaxial cells are the first muscle precursors to express the transcription factor encoding gene myod1. These cells migrate laterally to give rise to slow muscle fibers (Devoto et al., 1996). Double hybridization shows that uncx4.1 mRNA is absent from adaxial cells and is co-expressed with myod1 in non-adaxial muscle precursor cells at early somitogenesis stages (12 hpf, 6 s) (Fig. 6C–E). Co-labeling of uncx4.1 mRNA and a pan-myosin antigen (MF20, myosin heavy chain) illustrated how the expansion of myosin signal in maturing muscle cells occurs at the expenses of uncx4.1 expression in muscle progenitor cells (Fig. 6F–H).

3.4. Uncx4.1 expression and somite patterning

To gain understanding as to whether zebrafish uncx4.1 expression is dependent upon a mechanism of somite antero-posteriorization, we investigated uncx4.1 expression in segmentless fused somite (fss) mutant embryos (tbx24), which fail to develop anterior identities within somitic units (Durbin et al., 2000; Windner et al., 2012). As expected, the posteriorization of fss somites causes the loss of segmental expression of uncx4.1, with uniform transcript distribution throughout the somitic mesoderm (Fig. 6I, L).

As a preliminary assessment of potential roles for Uncx genes in somitogenesis in zebrafish, we used morpholino (MO) mediated knockdown of the zebrafish uncx genes. Unfortunately, injected uncx4.1 and uncx MOs led to various abnormalities including severe defects in body morphology with defective somitogenesis. Consequently we analyzed muscle differentiation only in those MO-microinjected larvae that were not disturbed in their overall morphology (about 50% uncx4.1 morphants, 34% uncx morphants). Analysis of fast and slow muscle fibers in such 34 hpf embryos injected with transcriptional start site MOs and subsequently stained for F59 (fast and slow MyHCs) and S58 (slow MyHC2) immunostaining shows normally differentiated myoblast cell types in chevron-shaped somites (Suppl. Fig. 6). These results do not
3.5. Uncx expression and signaling pathways during somitogenesis

Previous evidence suggests that signaling gradients from the neural tube and notochord-floor plate control the dynamic expression of the Uncx gene during somitogenesis (Schrägle et al., 2004). We thus investigated the potential involvement of the Hedgehog, FGF, Notch/Delta and Nodal pathways in directing the spatial expression of zebrafish Uncx genes.

3.5.1. Hedgehog

Sonic Hedgehog (Shh) is a signaling molecule secreted by the notochord and floor plate which transduces via two transmembrane proteins, Patched 1 (Ptc1) and Smoothened (Smo), and regulates the activity of cubitus interruptus-related (Gli) transcription factors (Borycki et al., 2000). In turn, Gli proteins may act as activators or repressors of Hh signaling (Karlstrom et al., 2003; Tyurina et al., 2005). In zebrafish, the sonic hedgehog-α (shha) gene is expressed in the notochord and floor plate of the neural tube (Krauss et al., 1993).

Our data indicate that uncx4.1 expression ceases in myogenic progenitor cells localized immediately next to shha-expressing tissues (Fig. 7A, B). Furthermore, microinjection of full-length shha mRNA abolishes uncx4.1 expression in zebrafish embryos (Fig. 7C, D), suggesting that Shh may act negatively on uncx4.1 expression. However, the analysis of several zebrafish mutant embryos in the Hh signaling pathway provides a contrasting view of the regulatory role played by Hh signals on uncx4.1 and uncx expression during somitogenesis. The mutant lines studied include sonic-you (syu<sup>shha<sup>−</sup>−</sup>); sonic hedgehog a, shha (Brand et al., 1996), slow-muscle-omitted (smu<sup>ss77</sup>; smoothened) (Varga et al., 2001), you-too (yot<sup>shha<sup>−</sup>−</sup>); gilla2) (Karlstrom et al., 1999), and floating head (fih<sup>ss</sup>; noto) (Melby et al., 1996), the latter mutant lacking shha-expressing tissues, i.e. notochord and most of the floor plate. In syu embryos, uncx4.1 is still expressed in the myoseptum and dorsal somite cells at 19 and 30–34 hpf, respectively (compare Figs. 3E and 7E, and 3H and 7F). The effect of Shh loss in syu embryos seems to be more pronounced in early uncx expression, as documented by diffuse mRNA labeling in the ventral portion of the somites at 19 hpf (compare Fig. 5B and F with Fig. 7G). In smu embryos, the VLP domain of uncx4.1 allow us to make strong conclusions on potential roles for the uncx genes in the somites.
expression is nearly normal (compare Figs. 3H and 7H). The early phase of expression of uncx4.1 is not significantly altered in yet mutant embryos lacking gli2, a dominant repressor of Hh signaling, except for delayed down-regulation in the dorsal domain (Fig. 7I, J). It has already been demonstrated that the level of ptc transcripts, a target of Hh signaling, drops by more than half when using 50 μM cyclopamine, a concentration sufficient to impair slow muscle cell differentiation (Wolff et al., 2003). In our hands, inhibition of Hh signaling with cyclopamine does not alter the spatial and temporal transcriptional dynamics of uncx4.1 and uncx when compared with control EtOH-treated embryos (Fig. 7K–N). Finally, uncx4.1 expression in fth mutant embryos is expanded dorsally and medially along the somite posterior boundary with reference to sibling controls (Fig. 7O, P). Since the fth mutant lacks the entire notochord and most of the floor plate, this expansion may be the manifestation of a synergic action of different hh genes (i.e., shha, shhb, shhh) (Halpern et al., 1995).

3.5.2. FGF

In the zebrafish, Fgf signaling promotes posterior mesoderm development and border positioning (Sawada et al., 2001). Acrerebellar (ace, fgf8a) mutant embryos exhibit only mild somite defects (Reifers et al., 1998; Draper et al., 2003). Groves et al. (2005) have demonstrated that Fgf8a mediates the promotion of a lateral fast muscle fibre population in zebrafish somite. Fgf8a drives myod1 expression in the lateral posterior stripe of immature caudal somites and is required for the lateral terminal differentiation of fast fibers in maturing rostral somites (Groves et al., 2005).

fgf8a mutant embryos (acerebellar, ace<sup>282a</sup>) (Reifers et al., 1998) were thus used to explore the possible contribution of FGF signaling in the regulation of uncx genes during somite development. Both uncx4.1 and uncx fail to confine ventrally in ace homozygote embryos, suggesting that Fgf8a negatively regulates the expression of uncx4.1 (compare Figs. 3H and 8A for uncx4.1; Figs. 5B and 8B for uncx). Accordingly, the expression of uncx in embryos exposed to the Fgf inhibitor SU5402 is disrupted and partially dorsally expanded compared with control embryos treated with DMSO vehicle (compare Fig. 8C with Fig. 8D).

3.5.3. Nodal

A conserved role for Nodal factors, belonging to the TGFβ family, has been proposed in the formation of the mesoderm (Harland and Gerhart, 1997; Hayos and Dougan, 2007). The dynamic expression of uncx4.1 and uncx was largely unchanged during somite formation in the cyclops mutant (cy<sup>216</sup>; nodal-related protein, ndr2) (Rebagliati et al., 1998) (compare Fig. 3F with Fig. 8E, and Fig. 5H with Fig. 8F). Similarly, uncx expression was normal in embryos treated with SB431542, an inhibitor of Nodal signaling (compare Fig. 8C with Fig. 8G).

3.5.4. Notch/Delta

In chia9c, it has been proposed that Notch/Delta signaling induces Unnx transcription in the cranial PSM (Schrögle et al., 2004). We aimed to verify if the regulatory interaction between Notch/Delta driven oscillator activity and Unnx gene expression suggested in birds is conserved in teleosts. Previous data indicate that pharmacological blockade of the Notch/Delta pathway in zebrafish, by using the gamma-secretase inhibitor DAPT, induces somite defects only after long developmental delays, suggesting that Notch/Delta signaling is essential for synchronizing oscillations of neighboring cells in the posterior PSM but not for somite border formation (Mara et al., 2007; Özbudak and Lewis, 2008; Sewell et al., 2009). We found that the expression of uncx4.1 and uncx in zebrafish embryos treated with DAPT is similar to that observed in control DMSO-treated embryos (compare Fig. 8H with Fig. 8I, and compare Fig. 8C with Fig. 8J, respectively).
3.6. Uncx4.1 and axogenesis

C. elegans Unc-4 is well known for its role in axonal connections, acting as a determinant of synaptic choice for motor neurons (Schneider et al., 2012). In this study, we observed ancient syntenic association between Uncx and two genes involved in axogenesis (a Mical gene, mical2) and synaptic choice (Elfn1). Looking for correlations between Uncx gene expression and axon guidance, we first performed double labeling with the primary motor axon marker znp1, finding that the outgrowth of the caudal primary (CaP) motor axons coincides with the progressive down-regulation of uncx4.1 expression during somite development (Suppl. Fig. 7A, B, G). Then, we observed that the netrin-1b (ntn1b) gene, a member of a secreted protein family mediating axon guidance, is expressed in VLP cells at 34 hpf (Suppl. Fig. 7C, D–F, and Fig. 3I with Suppl. Fig. 7F). Finally, uncx4.1 over-expressing embryos display marked up-regulation of ntn1b with stunted and prematurely branching CaP axons, a phenotype possibly caused by surrounding the motor neuron growth cone with cells ectopically expressing the chemotaxtractant ntn1b (Suppl. Fig. 7H–K).

4. Discussion

4.1. Origin and evolution of the Uncx genes

Since Metazoan Uncx proteins are poorly characterized from an evolutionary perspective (Woolfe and Elgar, 2007; Sánchez and Sánchez, 2013), we provided a comprehensive phylogenetic reconstruction showing the orthology of all analyzed genes, which we refer to as Uncx (Fig. 1). The partial protein-coding sequence found in the cnidarian N. vectensis genome (Suppl. File 2) as well as the absence of Uncx in sponges (Porifera), suggest that this homeobox gene was already present in the ancestor of bilaterians, though with instances of gene duplication and/or gene loss (Fig. 2). A common origin for Uncx genes is confirmed by the conservation of intron/exon structure in
Bilateria (Suppl. File 3). We highlighted duplications in unrelated invertebrate taxa (i.e., *D. melanogaster*, *C. teleta*, *S. kowalevskii*, *C. robusta*) and the absence of *Uncx* in early branching metazoans (e.g., Placozoa and Ctenophora), in the agnathan lamprey and in many reptiles, which may underly functional gene diversification, with loss or (re)gain of (ancestral) gene functions (Albalat and Cañestro, 2016). As proposed by Albalat and Cañestro (2016), we report that teleosts have experienced two rounds of whole-genome duplications (WGDs) at the stem of vertebrates (Ohno, 1993; Abi-Rached et al., 2002; Dehal and Boore, 2005) implying the presence of other three *Uncx* members in gnathostome ancestor, which have been secondarily lost during evolution. We sought to provide insights into *Uncx* molecular evolution by analysing its genomic locus from cnidarians to human. It has been reported the presence of almost 800 conserved ancestral microsyntenic pair (CAMP) combinations for several homeobox genes as *Uncx* from cnidarians as *Nematostella* to cephalochordates as *Branchiostoma* (Irimia et al., 2012). We found that the *Uncx* gene forms distinct microsyntenic clusters. An invertebrate CAMP with the transcription factor encoding *Alx/Cart-1* gene is seen in annelids and hemichordates (Suppl. Fig. 3), while in surveyed Olfactores, *Uncx* orthologs are coupled with *Elfn1* (Suppl. Fig. 2). A cluster formed by *Uncx*, *Elfn1*, and *Micall2* genes exists in gnathostomes, which is also duplicated in teleosts (Fig. 2). The conserved chromosomal vicinity of *Uncx* and *Micall2* genes evokes a "hystander interference effect" exerted by one of the two genes, which has been proposed for genes implicated in key developmental mechanisms (Cajiao et al., 2004).

VISTA comparison of *Uncx* loci belonging to mollusks, brachiopods, ascidians, and vertebrates indicates conservation of sequence, consistent with past studies on *Uncx* highlighting the presence of CNEs (conserved non-coding elements) in *Takifugu rubripes* and *Homo sapiens* (Woolfe and Elgar, 2007) (Suppl. Fig. 5). Teleost *Uncx* paralogs lack some of the conserved elements common among coelacanths, spotted gar and human, whose lineages diverged from teleosts before the TSGD (Suppl. Fig. 5). In addition, they exhibit differences in peak patterns as if had undergone an asymmetrical rate of evolution. The expression patterns of the two *Uncx* paralogous genes show unique (*uncx4.1*: pharyngeal arches and kidney; *uncx4.1*: spinal cord) and partially overlapping domains (CNS and somites). These findings are possibly associated with genome duplication producing divergent
regulatory modality, with events of subfunctionalization and/or neo-functionalization. However, the potential for cross-hybridisation needs to be considered when working with paralogous genes. In our work, divergent hybridization patterns with high signal and low background riboprobes were obtained, indicative of high levels of specificity and minimal cross-hybridization between duplicated genes.

The analysis of vertebrate genome environment demonstrated that Uncx4.1 and Unmx genes descend from the same paralogon (Fig. 2); therefore, they derive from the teleost-specific genome duplication (TSGD), which occurred 300–350 million of years ago (Taylor et al., 2001; Taylor et al., 2003; Jaillon et al., 2004; Kuraku and Meyer, 2009). In light of the above, we propose to change the name of teleost Unmx paralogs genes into uncx4 (Unmx4.1; NM_001020780.2) and uncxb (unmx; XM_005164204.4).

4.2. Regulation of the zebrafish uncx genes

In this study, uncx4.1 gene co-expression in the anterior presomitic mesoderm (PSM) with Notch-pathway gene her1, the output of the molecular clock, and with the Mesp1-related factor encoding mespaa (Fig. 6A, B), suggests that zebrafish uncx genes are controlled by players in somite anterior-posterior specification. This observation also indicates that, similarly to what is observed in mouse, zebrafish Unmx genes could be required for maintaining antero-posterior polarity within the somite (Farin et al., 2008; Lee et al., 2009). However, it is worth to note that the murine Unmx gene is expressed in the posterior half of the newly formed somites but, unlike the fish and chick orthologs, it is not active in the PSM (Barrantes et al., 1999; Schräge et al., 2004). Furthermore, we show that, as in the zebrafish mesp quadruple

![Fig. 8. Regulation of uncx gene expression in relation to Fgf, Nodal and Notch/Delta signaling pathways. Whole-mount in situ hybridization of (A, E, H, I) uncx4.1 and (B–D, E, G, J) uncx at (A) 34 hpf, (B–D) 19 h post fertilization (hpf), (E, F, H) 24 hpf, (G) 18 hpf, and (J) 19 hpf. Lateral view, anterior to left. Expression of (A) uncx4.1 and (B) uncx in acerebellar (ace) mutant embryo at (A) 34 hpf and (B) 19 hpf. (C, D) Expression of uncx in embryos treated with (C) DMSO and (D) SU5402 at 19 hpf. (E, F) Expression of (E) uncx4.1 and (F) uncx in cyclops (cyc) mutant embryo at 24 hpf (controls in Fig. 3F and 5H, respectively). (G) Expression of uncx in embryo treated with SB431542 at 18 hpf (control in Fig. 5B). (H–J) Expression of (H, I) uncx4.1 and (J) uncx in embryos treated with (H) DMSO and (I, J) DAPT at (H, I) 24 hpf and (J) 19 hpf. (J) Control in (C).]
mutant, *uncx4.1* expression is extended to the entire somite of fused somite/*tbx6* mutant embryos (Fig. 6I, L) (Yabe et al., 2016). Considering that mouse *Tbx6* is involved in somite boundary positioning together with *Mesp*, and that *Mesp* provides positional information within the somite, a similar mechanism to induce zebrafish *uncx* gene expression in the caudal somite half may occur during the establishment of somite polarity and boundary formation.

In zebrafish, the somite develops into a large myotome, with a smaller group of ventral cells specified as sclerotome (Stickney et al., 2000). Genes encoding myogenic regulatory factors such as *myod1* and *myf5* are expressed early in the most medial presomitic mesoderm adjacent to the notochord (Devoto et al., 1996; Weinberg et al., 1996). Both *myod* and *myf5* control the commitment to the myogenic lineage and are required for the initiation of the *myogenin* gene expression (Pownall et al., 2002). During early somitogenesis in zebrafish, *uncx4.1* expression coincides with that of *myod1* in muscle progenitor cells (Fig. 6C, D), indicating that zebrafish *Uncx* paralogues may function in somites at the onset of muscle differentiation. The absence of *uncx4.1* and *uncx* gene expression in adaxial cell precursors adjacent to the notochord (Fig. 6C-E) suggests that *uncx* genes are not required for the specification and differentiation of slow muscle cells.

During somite formation, the distribution of *uncx4.1* and *uncx* transcripts becomes progressively confined to a small population of undifferentiated myoblasts at the ventral lateral posterior (VLP) margin (Figs. 3 and 6). VLP cells expressing *Uncx* genes are likely connected to an extended ventral monolayer termed growth zone, which is known to contribute to hyperplastic growth of each myotome in marine teleosts (Barron et al., 2000). In this view, *uncx4.1* could inhibit muscle formation via induction of myoblast proliferation at the expenses of muscle differentiation and/or as an antagonist of late differentiation (Fig. 6F-H).

We attempted to place *uncx* genes in the context of signal transduction mechanisms (i.e., Hh, FGF, Notch/Delta, Nodal) already known to play key roles in somite patterning and differentiation in zebrafish. Hh signal transduction is an intricate molecular pathway that acts in a dosage-dependent manner to specify cell fate in the zebrafish myotome (Wolff et al., 2003). The expression of *uncx4.1* is lost in the Hh pathway component *ptc1; ptc2* mutants (Koudijs et al., 2008). However, our data do not clarify whether or not Hedgehog signaling is required to drive expression of *uncx4.1* and *uncx*; and, if so, to which extent. Also, the analysis of the regulatory interactions between *Uncx* genes and the Notch/Delta and Nodal pathways do not provide conclusive results with only changes to *uncx* expression. Accordingly, previous evidence in Notch1 mutant mouse shows that *Uncx* expression is slightly wider than in sibling embryos but essentially unaltered (Barrantes et al., 1999). Finally, somite expression of zebrafish *Uncx* genes in Fgf8a mutant embryos and in embryos treated with the Fgf inhibitor SU5402 is disrupted and dorsally extended, consistent with a negative role played by Fgf signaling in the expression of *Uncx* genes in zebrafish somitogenesis. The relationship between *Uncx* expression and fast muscle fibers warrants more careful examination in zebrafish *fgf8a* mutants. When all our evidence is considered, it suggests a hypothesis whereby *Uncx* gene expression is specifically regulated by Fgf signaling, while Hh, Notch/ Delta and Nodal signals may have more subtle roles in controlling the dynamic pattern of *Uncx* expression during somitogenesis.

### 4.3. A dual role in somitogenesis and axon guidance?

*In silico* analysis of available genome databases revealed the physical co-localization of *Uncx* with genes implicated in synaptic functioning and plasticity, i.e. *Mical1*2 (gnathostome-specific gene duplet). Also, a correlation was observed between the expression patterns of *uncx4.1* and *ntn1b*, a member of a secreted protein family mediating axon guidance, and the trajectory of caudal primary (CaP) motor neuron axons. While Netrin is an attractant cue in *Drosophila* axon guidance (Hiramoto and Hiromi, 2006; Brankatschk and Dickson, 2006), the role of its zebrafish ortholog is not completely resolved, even if a diffuse *ntn1b* expression within the somite is thought to promote ventral elongation of the CaP motor axon (Hale et al., 2011). The ventral restriction of *uncx4.1* and *ntn1b* expression might involve a mechanism comprising the release of positional signals that contribute to the restriction of the CaP axon pathways. This may occur either by attracting CaP axons by diffusion of chemoattractants across inter-somitic boundary epithelia, like in *Drosophila*, or repelling them within each somite through long-range cues (Mitchell et al., 1996). The CaP axon phenotype induced by *uncx4.1* mRNA injection is similar to the effects of ectopically expressed netrins in other systems (*Drosophila*) (Mitchell et al., 1996). We speculate that the zebrafish Uncx4.1 activity in a particular subset of myotomal cells might serve a dual function by interacting with cell-cycle genes in controlling cell divisions during myoblast differentiation, and by activating or maintaining *ntn1b* expression for proper axonal elongation (Fig. 9).
Conflicts of interest

The authors declare that they have no conflict of interest.

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Author contributions

VN, AED, GF, UC, AG, SM, FL, RDP, IP, RM and MF led the investigation and acquired the data. PS, AD, FA, TK, FR, LMC, DD and SW contributed to conceptualization, resources, data analysis and critical revision of the initial draft. UC, VN, GF and PS led the figure and table preparation. PS supervised the project, analyzed the data and drafted the manuscript, further completed by all authors. All authors have read and approved the manuscript.

Appendix A. Supplementary data

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