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Ret signalling integrates a craniofacial muscle module during development

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SUMMARY

An appropriate organisation of muscles is crucial for their function, yet it is not known how functionally related muscles are coordinated with each other during development. In this study, we show that the development of a subset of functionally related head muscles in the zebrafish is regulated by Ret tyrosine kinase signalling. Three genes in the Ret pathway (*gfra3*, *artemin2* and *ret*) are required specifically for the development of muscles attaching to the opercular bone (gill cover), but not other adjacent muscles. In animals lacking Ret or Gfra3 function, myogenic gene expression is reduced in forming opercular muscles, but not in non-opercular muscles derived from the same muscle anlagen. These animals have a normal skeleton with small or missing opercular muscles and tightly closed mouths. Myogenic defects correlate with a highly restricted expression of *artn2*, *gfra3* and *ret* in mesenchymal cells in and around the forming opercular muscles. *ret*$^{+}$ cells become restricted to the forming opercular muscles and a loss of Ret signalling results in reductions of only these, but not adjacent, muscles, revealing a specific role of Ret in a subset of head muscles. We propose that Ret signalling regulates myogenesis in head muscles in a modular manner and that this is achieved by restricting Ret function to a subset of muscle precursors.

KEY WORDS: Zebrafish, GDNF, Head muscle, Musculoskeletal

INTRODUCTION

Movement, eating and vocalisation require the action of functionally related muscles. In order to generate functional musculoskeletal systems different populations of cells need to be coordinated in their development. In the head, functionally interacting muscles arise from different pharyngeal arches and it is not known whether muscles possess specific identities that relate to their subsequent function. The basis for establishing differences between muscles derived from the same pharyngeal arch is not understood, as they arise from common anlagen. The muscles of mastication and facial expression in mammals are derived from mesoderm of the first and second pharyngeal arches, respectively. In fish, mesoderm from the first and second arches form muscles required for jaw opening, jaw closing and respiration (Edgeworth, 1935). Differences in the shape and function of muscles in mammals and fish reflect extensive changes to the head skeleton and associated muscles following their divergence. Skeletal perturbations result in a disruption of the associated muscles, suggesting that morphogenesis of these two tissues is co-dependent, but how this relates to muscle shape and size is not clear. Head muscles do not appear to possess distinct identities during development that are important for their subsequent shape or function. Engrailed 2 (En2; Eng2a – Zebrafish Information Network) is a homeodomain protein expressed specifically in muscle precursors in the dorsal part of the first pharyngeal arch (Hatta et al., 1990), but does not appear to confer specificity to the muscle (Degenhardt and Sassoon, 2001). The basic helix-loop-helix genes capsulin (*Caps*; *Tcf21* – Mouse Genome Informatics) and musculin are expressed in all pharyngeal arch muscles, but double mutant mice lack masticatory muscles derived only from the first arch (Lu et al., 2002). Caps and Musculin are repressors of transcription and are believed to inhibit myogenic regulatory factor (MRF) genes that regulate differentiation (Buckingham, 2006). In mouse, the MRFs Myf5 and Myod (Myod1 – Mouse Genome Informatics) function redundantly to promote pharyngeal arch myogenesis (Kassar-Duchossoy et al., 2004), whereas in zebrafish, only Myod (Myod1 – Zebrafish Information Network) is required (Hinits et al., 2009). Muscle differentiation involves expression of muscle actin and myosin genes; individual head muscles express different myosin type genes, but this does not correspond to the pharyngeal arch from which they originate (Marcucio and Noden, 1999; Hernandez et al., 2005; Elworthy et al., 2008). This diversity in myosin gene expression suggests that head muscles do possess distinct identities, but how these identities are specified is not understood.

The Ret tyrosine kinase signalling pathway is crucial for the development of several neuronal and neural crest lineages, but has not previously been implicated in muscle development (Enomoto, 2005). Ret receptors activate a number of intracellular signalling pathways in response to signalling from glial derived neurotrophic factor (GDNF) family ligands (GFL), mediated through specific receptors (Gfra). In mammals, the Gfra receptors are bound specifically by GDNF, neutrophin, artemin (Artn) and persephin (Airaksinen and Saarma, 2002). Gfra3 is specifically activated by Artn and not by other family ligands (Baloh et al., 1998). Binding of Gfra receptors by the appropriate GFL induces Gfra dimerisation and the activated GFL-Gfra complex then promotes Ret dimerisation and, hence, activation (Airaksinen and Saarma, 2002).

In this work, we identify a novel role for Ret tyrosine kinase signalling during the development of a functionally related set of head muscles in the zebrafish. We show that Ret signalling acts to

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specifically regulate myogenesis in muscles attaching to the opercular bone. Specific activation of Ret signalling is achieved through the restricted expression of both *ret* (*ret1* – Zebrafish Information Network), *gfra3* and an activating ligand, *artemin2* (*artn2*), in cells associated with opercular muscles. The specificity of the muscle phenotype in Ret signalling mutants reveals an unexpected modularity of head muscle development that does not correspond to the origin of the muscles from a particular pharyngeal arch, but instead reflects the subsequent function of the muscles.

MATERIALS AND METHODS

All protocols used were as described previously (Nusslein-Volhard and Dahm, 2002; Westerfield, 2007) except those described below.

Generation and identification of mutants

The *stumm* (*stm*) mutant (allele *hy024*) was identified in a large-scale N-ethyl-N-nitrosourea (ENU) mutagenesis screen (Tübingen 2000 Screen Consortium). *stm*^{hy024} was mapped to linkage group 14 by bulk segregation analysis and fine mapped using 1000 embryos to a 0.2 cM interval between z1812 and z28688. Mapping was confirmed with a single-nucleotide polymorphism (SNP; 1204a) to position the locus 0.2cM towards the centrosome from z1812 at 38.1 (MGH mapping panel); the map position of *gfra3* was confirmed on the T51 radiation hybrid panel. *stm*^{hy024} mutants and wild-type (WT) siblings were identified by sequencing a 532 bp region of exon 4 from *gfra3* amplified with primers gfra3-1 (GTACCGGG-TTGGCTTAGATTC) and gfra3-2 (GTAGGAACAGGAGGGCACAA). The *ret* mutant (*ret*^{hu2846}) was identified by TILLING in the Sanger Zebrafish Mutagenesis Project and has a predicted stop codon at amino acid position 229 in the extracellular domain of the protein (www.sanger.ac.uk/resources/zebrafish/). *ret*^{hu2846} mutants were genotyped by PCR to amplify a 542 bp fragment then tested for the presence of a new *Hpy*118*I* using primers Ret3-5 (TCGACGACTCCACTTCTGA) and Ret3-6 (GATGTTCACATGTAAAGATCTG).

Morpholino injections

Antisense oligonucleotide morpholinos directed against *gfra3*, *ret*, *artn2*, *tfap2a* and *tfap2c* were manufactured by GeneTools LLC (Oregon, USA). Morpholinos against *gfra3* were designed to the exon 2 donor site (gfra3-2: AGCTCGCTCTTACCCTGTGGAAAGC) and to the exon 4 splice donor site (gfra3-4: GAGGCAGATTTTCTGACCTGCACAG). Perturbation of *gfra3* RNA splicing was achieved by co-injection of 1 nl of gfra3-4 (1 mM) and gfra3-2 (0.4 mM) to single-cell stage embryos. Efficacy of morpholino knockdown was assessed by PCR amplification of a 380 bp fragment of *gfra3* from cDNA extracted from morphants and control animals, using primers directed to exon 2 and to exon 4 (gfra3-3: GATGCCCAGAACACCTCATG, gfra3-4: AACCTCCGCAGGGCTCGG). Morpholinos against *artn2* were designed against sequence immediately 5′ to the start of the open reading frame (artn2-2: TACTCGGC-CACCACCAAACTCCCAC). To knock down Artn2 translation, 1.5 nl of artn2-2 (0.2 mM) was injected at the single-cell stage. Ret function was knocked down by injecting 1 nl morpholino ret-1 (0.5 mM) as described previously (Heanue and Pachnis, 2008). Tfap2a and Tfap2c function were knocked down as described by Hoffman et al. (Hoffman et al., 2007).

Cloning and phylogenetic analysis

Full-length sequence for *gfra3* (GenBank: HQ401362) was obtained by PCR amplification from cDNA and extended by RACE (BD Biosystems). Zebrafish *artn1* and *artn2* genes were amplified using published sequence (Hatinen et al., 2007) with the following primers: artn1-f, CATCC-CTGGCAGAGGAAGGTGA; artn1-r, AGTCATCGAATCCGTTGGCA; artn2-f, TGAAGATGACCAGCAGCCAGC; arnt2-r, TTCAGCACAC-CTCATCCCACGC. Sequence comparisons were performed using BLAST; sequences were aligned using CLUSTALX (Thompson et al., 2002) and phylogenetic tree reconstruction performed by Quartet Puzzling using Puzzle (Strimmer and von Haeseler, 1997) or by Bayesian probability using PAUP (Huelsenbeck and Ronquist, 2001).

In situ hybridisation and immunohistochemistry

Riboprobes were synthesised for *gfra3*, *artn1*, *artn2*, *ret* (Bisgrove et al., 1997; Marcos-Gutierrez et al., 1997), *myod* (Weinberg et al., 1996), *myf5* (Groves et al., 2005), *tbx1* (Piotrowski et al., 2003) and *capsulin* (*tcf21*) (Knight et al., 2008). Antibodies used for immunohistochemistry were: anti-Myosin (MF20), anti-Engrailed (4D9), anti-Col2a1 (II-II6B3) [all from the Developmental Studies Hybridoma Bank (DSHB), University of Iowa, USA]; anti-GFP (Torrey Pines Laboratories, CA, USA); anti-Phosphohistone-H3 (Merck Biosciences); zns-5 (Zebrafish International Resource Centre, University of Oregon, OR, USA). TUNEL labelling was performed using a POD-conjugated secondary antibody with DAB staining (Roche). Nuclear labelling of cells was achieved using DAPI (Vector Laboratories), muscle was labelled with Alexa-conjugated phalloidin (Invitrogen), cartilage labelled by Alcian Blue and bone detected by Alizarin Red or calcein.

Quantitation of muscle fibre, opercular bone size and cell number

Muscle fibres were labelled by immunohistochemistry and fibres for each dorsal arch muscle counted using Nomarski optics in ten animals for each condition. Standard error of the mean (s.e.m.) was calculated for each dataset and plotted in Excel (Microsoft). TUNEL positive cells and En2-expressing cells were counted in ten mutants and ten wild-type siblings for each stage examined. The area of the opercular bone was measured from light microscopy images using ImageJ (National Institutes of Health). Statistical significance of difference was assessed by applying the Student's *t*-test using an unpaired two-tailed distribution.

RESULTS

stm^{hy024} mutants show a highly specific head muscle phenotype

No genes are known to regulate specificity between head muscles during development, as it has proved difficult to separate muscle development from that of the associated skeleton. To identify genes important for regulating head muscle development, we screened for mutants with specific cranial muscle defects that show no cranial skeleton perturbation. *stm*^{hy024} mutants had smaller pharyngeal arch muscles, a tightly closed mouth with the lower jaw pointing upwards, but normal cranial skeletal elements (Fig. 1A-F; see Fig. S1A,B in the supplementary material). The muscle phenotype in *stm*^{hy024} involves a specific reduction of muscles that attach to the opercular bone (Fig. 1G,H), but the skeleton at the origin (otic skeleton) or insertion (opercular bone) points of these muscles were normal and the opercular bone was not significantly reduced in size (see Table S1 in the supplementary material). To quantify the progression of this opercular muscle phenotype, fibres were counted from dorsal first arch muscles levator arcus palatini (lap) and dilator operculi (do) and from the second arch muscles adductor hyoideous (ah), adductor operculi (ao) and levator operculi (lo), as they are flat and readily amenable to quantification (Edgeworth, 1935). All the dorsal arch muscles showed a normal orientation relative to their insertion or origin and the fibres in the muscles were aligned normally in *stm*^{hy024} mutants. However, a specific subset of muscles, including the do, ao and lo, had fewer muscle fibres than the adjacent lap or ah muscles (Fig. 1I; see Table S2 in the supplementary material). The affected muscles (do, ao and lo) all attach to the dorsal aspect of the opercular bone. The superior hyohyoideus (hh sup) muscle attaches to the opercular bone from the ventral second arch and was always absent in *stm*^{hy024} mutants (Fig. 1C,D). Muscle defects in *stm*^{hy024} could arise as a result of changes in the identity of the muscle type. In *stm*^{hy024} mutants slow and fast myosin distribution appeared normal, despite a reduction of fibre number in the opercular muscles (see Fig. S1C-J in the supplementary material). Early muscle identity is likewise unaffected in *stm*^{hy024} as expression of En2 in dorsal first arch

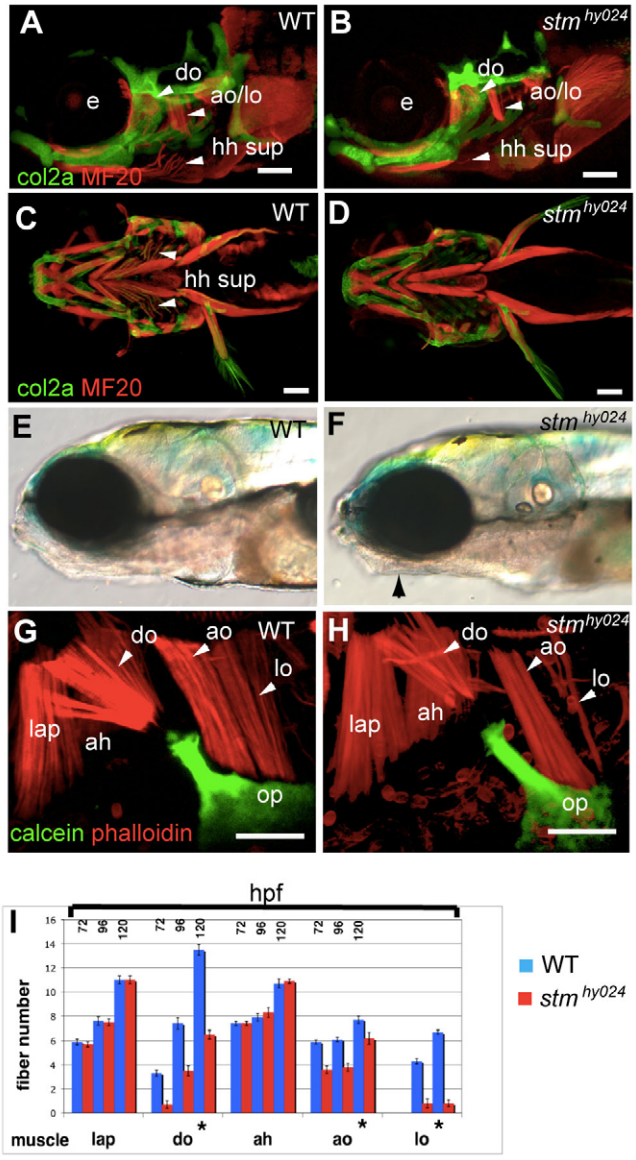

Fig. 1. Musculoskeletal labelling of *stm*^{hy024} zebrafish mutants reveals specific muscle defects. (**A-D**) Immunolabelling of muscle (red) and cartilage (green) reveals that *stm*^{hy024} mutants (B,D) have reduced dilator operculi (do), adductor operculi (ao), levator operculi (lo) and hyohyoideus superiores (hh sup) muscles (arrowheads) relative to wild type (WT; A,C). e, eye. (**E**,**F**) *stm*^{hy024} mutants (F) have tightly closed mouths (arrow) relative to WT (E). (**G**,**H**) Higher magnification views of dorsal pharyngeal arch muscles reveal that the do, ao and lo attaching to the opercular bone (op, green) are reduced in *stm*^{hy024}; the non-opercular muscles levator arcus palatini (lap) and adductor hyoideous (ah) are unaffected. (**I**) Quantification of fibre number in dorsal arch muscles (mean ± s.e.m.) reveals a specific and significant reduction (*P<0.0001) of the opercular muscles in *stm*^{hy024} relative to WT (n=10), during muscle development at 72, 96 and 120 hours post-fertilisation (hpf). Scale bars: 100 µm in A-D; 50 µm in G,H.

muscle precursors was unaffected prior to muscle formation (Fig. 3K,L; see Fig. S1K,L in the supplementary material). Therefore, the specific opercular muscle phenotype in *stm*^{hy024} is not due to skeletal abnormalities or alterations to muscle identity and reveals that head muscle development is regulated in a modular manner, independent of muscle origin from a particular pharyngeal arch.

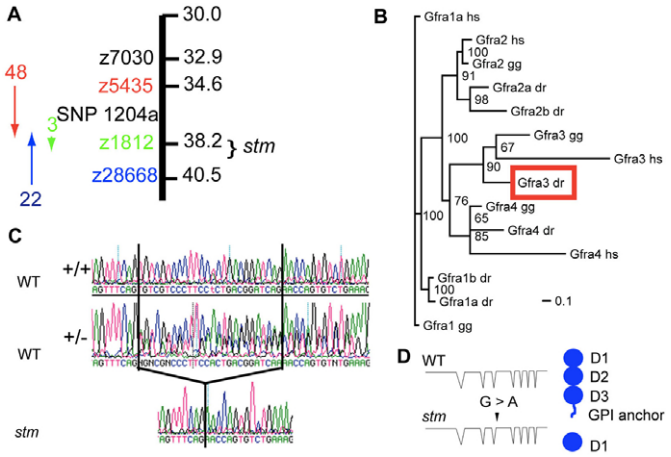

Fig. 2. Positional cloning of *stm*^{hy024}. (**A**) *stm*^{hy024} maps to a 0.2 cM region on chromosome 14:32.1 between simple sequence length polymorphisms (SSLPs) z1812 and z28668. SSLPs used for mapping are coloured with arrows indicating orientation relative to the *stm*^{hy024} locus; coloured numbers show the number of recombinants from 1000 meioses. (**B**) A maximum likelihood tree of vertebrate Gfra genes reveals that *stm*^{hy024} encodes a zebrafish *gfra3* orthologue (red box). Branch lengths represent maximum likelihood distances and values at node indicate support. Trees generated by Bayesian probability produced a similar topology (not shown). Protein sequences used for tree construction include Gfra1, Gfra2, Gfra3 and Gfra4 from human (hs), chicken (gg) and zebrafish (dr). (**C**,**D**) Sequence traces from *gfra3* transcripts amplified from homozygote wild type (WT+/+), heterozygote (WT+/–) and *stm*^{hy024} reveal that a 26 bp deletion occurs at the exon 4 splice acceptor site (C). This causes a frameshift and results in a truncated protein possessing domain D1 but lacking domains D2 and D3 plus the GPI anchor signal (D).

A mutation in a GDNF family receptor causes the *stm* phenotype

Positional cloning was used to map *stm*^{hy024} to a region of chromosome 14 containing a Gfra gene (Fig. 2A) and confirmed using a radiation hybrid panel (see Fig. S2A in the supplementary material). Phylogenetic analysis revealed that it is an orthologue of amniote *Gfra3* genes (Fig. 2B). Sequencing of *gfra3* transcripts from *stm*^{hy024} mutants revealed a 26 bp deletion in the open reading frame, causing a frame shift and premature stop codon (Fig. 2C). This deletion is due to a guanosine-to-adenosine transition at the intron 3-exon 4 splice site, resulting in loss of the canonical splice acceptor with a cryptic splice site in exon 4 used instead. The mutated Gfra3 protein in *stm*^{hy024} mutants possesses only extracellular domain 1 but lacks domains D2 and D3 necessary for binding GDNF family ligands (Fig. 2D). To confirm that a loss of Gfra3 function caused the muscle phenotype, *gfra3* splicing was disrupted by injection of antisense morpholino oligonucleotides. *gfra3* morphants had tightly closed mouths at 5 days post-fertilisation (dpf) and showed statistically significant reductions of the do, ao and lo muscles relative to uninjected control animals at 80 hpf (P<0.0001), but other adjacent head muscles were not affected (see Fig. S2B; Table S3 in the supplementary material). Interestingly, the muscle phenotype in *gfra3* morphants started to recover by later stages of development (144 hpf) and RT-PCR of *gfra3* from morphants and controls at different stages showed that an attenuation of morpholino function occurred at this point (see Fig. S2C in the supplementary material). This recovery of muscle development in *gfra3* morphants contrasts

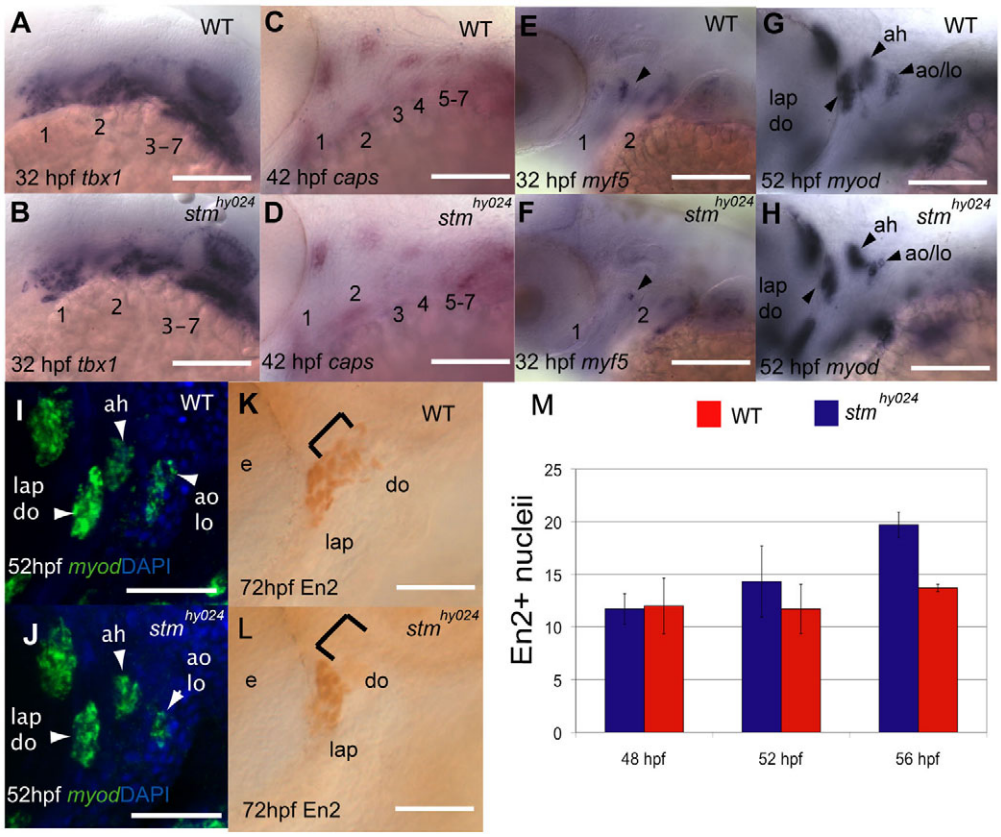

Fig. 3. *stm*^{hy024} mutants show myogenic defects specifically in opercular muscles. (**A-H**) In situ hybridisation with *tbx1* (A,B) and *capsulin* (C,D) probes reveals that muscle specification is unaffected in the pharyngeal arch muscle precursors of *stm*^{hy024}. However, both *myf5* (E,F) and *myod* (G,H) expression are reduced in *stm*^{hy024} relative to wild type (WT). Arrowheads indicate muscle primordia. (**I-L**) Fluorescent in situ reveals that *myod* expression is reduced specifically in the precursors of the lo and ao at 52 hours post-fertilisation (hpf; I,J) but is unaffected in the adjacent ah. At later stages, there are fewer En2-expressing (En2$^+$) muscle cells specifically in the do but not the lap of *stm*^{hy024} (K,L). Brackets indicate do muscle cell nuclei. (**M**) Quantification of En2$^+$ muscle precursors in *stm*^{hy024} (blue) and WT (red) at 48, 52 and 56 hpf reveals that *stm*^{hy024} mutants have fewer muscle precursor cells than WT in the lap or do primordia from 56 hpf (mean ± s.e.m.). Pharyngeal arches 1-7 are shown by numbering in A-F. ah, adductor hyoideous; ao, adductor operculi; do, dilator operculi; e, eye; lap, levator arcus palatini; lo, levator operculi. Scale bars: 50 μm in A-H,K-L; 20 μm in I,J.

with *stm*^{hy024} mutants that never recovered to the same extent and highlights a constant requirement for Gfra3 function during opercular muscle development.

Gfra3 function is required for myogenesis but not for muscle specification

The specific opercular muscle defect in *stm*^{hy024} mutants might result from a perturbation of myogenic specification. The earliest specifiers of head muscle identity (*tbx1*, *capsulin*) are unaffected in *stm*^{hy024} mutants (Fig. 3A-D). By contrast, expression of the MRF gene *myf5* was reduced in the second arch of *stm*^{hy024} at similar stages (Fig. 3E,F). At later stages of head muscle development, *myf5* was no longer expressed in the pharyngeal arches of WT animals or *stm*^{hy024} mutants (data not shown). Another MRF, *myod*, is expressed at stages when pharyngeal arch muscles start differentiating and express muscle structural proteins (Lin et al., 2006). *myod* was specifically reduced in the dorsal first and second arches of *stm*^{hy024} (Fig. 3G,H) and labelling of *myod* transcripts revealed an obvious reduction in the muscle precursors of the ao and lo, but not in the ah, a non-opercular dorsal second arch muscle (Fig. 3I,J). This specific reduction of *myod* in ao and lo, but not ah, precursors reflects subsequent reductions of these muscles and suggests that Gfra3 is needed for normal muscle differentiation in opercular muscles, but not in adjacent non-opercular muscles. Prior to muscle differentiation, En2, a marker of dorsal first arch muscle precursors, was expressed normally in *stm*^{hy024} mutants, but by 72 hpf it was reduced specifically in the do and not the lap (Fig. 3K,L). In order to quantify myogenic defects in *stm*^{hy024}, the number of muscle precursors expressing En2 (En2$^+$) was evaluated throughout head muscle development. There was a significant reduction in En2$^+$ myoblasts in *stm*^{hy024} compared with WT from 56 hpf, but not at earlier stages (Fig. 3M). This reduction of En2$^+$ muscle precursors corresponds to the stage at which *myod* expression was obviously reduced in muscle anlagen of *stm*^{hy024}. Changes to the expression of *myod* and En2 could be due to cell death or changes to cell proliferation in muscle cells. TUNEL labelling of apoptotic cells revealed no statistically significant increase of cell death in the pharyngeal arches of *stm*^{hy024} at any stage of muscle differentiation (see Fig. S2D in the supplementary material). Phosphohistone-H3 protein labelling of cells in the G2 to mitosis transition showed that En2$^+$ muscle precursors of the lap and do rarely proliferated at 48 hpf, when differentiation starts, and that this did not change in *stm*^{hy024} despite a reduction in the number of En2$^+$ cells (see Fig. S2E-J in the

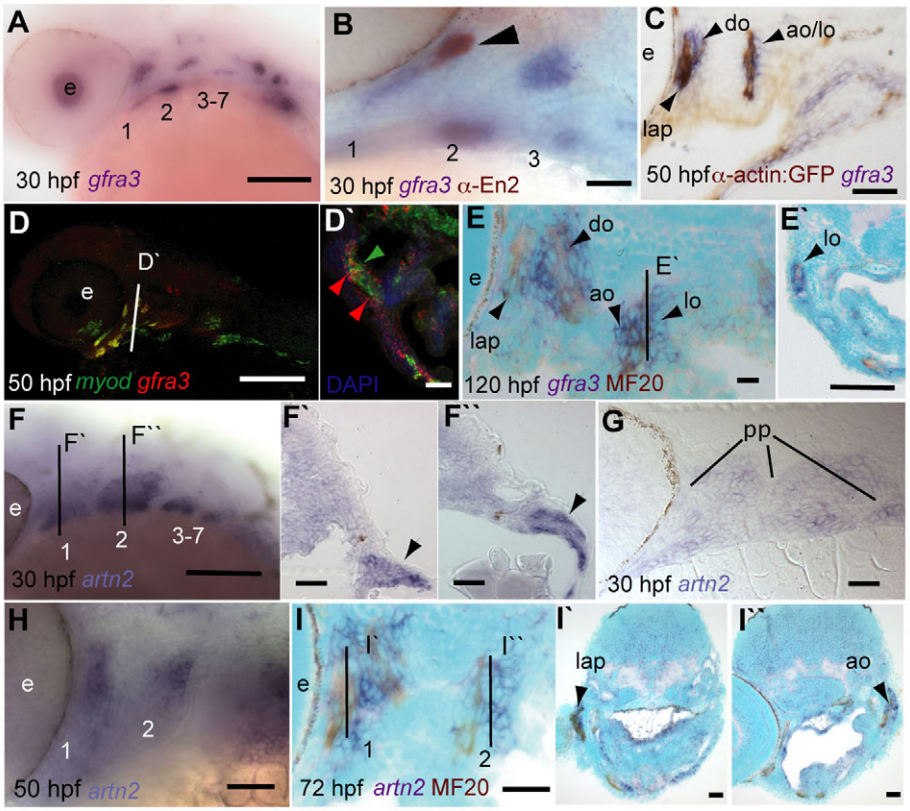

Fig. 4. *gfra3* and *artn2* show tissue-specific expression in an opercular complex. (**A**,**B**) *gfra3* (purple) is expressed in pharyngeal mesoderm at 30 hours post-fertilisation (hpf; A) and in muscle precursors (arrowhead) with En2 (brown, B). (**C**) At 50 hpf, *gfra3* is expressed in forming muscle fibres expressing GFP (brown) in *alpha-actin:GFP* animals and associated cells. (**D**,**D′**) Fluorescent in situ of *myod* (green) and *gfra3* (red) reveals colocalisation (yellow) in all forming pharyngeal arch muscles, but not in fin or ocular muscles. A transverse section (indicated by the line in D) through the second arch reveals *gfra3* expression in *myod*$^{-}$ cells (D′). Red and green arrowheads indicate *gfra3*+*myod*– and *gfra3*+*myod*+ cells, respectively. (**E**,**E′**) At stages when primary muscle fibres have formed (120 hpf), sagittal sections show that *gfra3* is expressed in mesenchymal cells adjacent to muscle fibres (brown, E) that completely surround the forming lo muscle (E′). Line in E indicates level of section in E′. (**F-I″**) In situ hybridisation shows that *artn2* is expressed in mesenchyme of the pharyngeal arches from 30 hpf. Transverse (F′,F″) and sagittal (G) sections of the arches (1,2), reveal that *artn2* expression is restricted to mesenchymal cells (arrowheads) and is excluded from the pharyngeal pouches (pp). Subsequently, *artn2* becomes restricted to discrete domains in the dorsal first and second arches at 50 hpf (H). Sagittal (I) and transverse (I′,I″) sections reveal that as muscle fibres form (brown), *artn2* expression is adjacent to the forming opercular muscles. Lines in I indicate level of sections in I′ and I″. Pharyngeal arches 1-7 are shown by numbering. ao, adductor operculi; do, dilator operculi; e, eye; lap, levator arcus palatini; lo, levator operculi. Scale bars: 100 µm in A,D,E′,F,I′,I″; 20 µm in B,C,D′,E,F′-I.

supplementary material). Gfra3 function, therefore, appears to be necessary for myogenic differentiation, as cell death or proliferation did not change in the arches of *stm*^{hy024}, the number of En2^{+} muscle cells failed to increase and *myod* expression was reduced in forming opercular muscles.

gfra3 is expressed in pharyngeal arch muscles

To understand how Gfra3 functions to promote opercular muscle differentiation, its expression was characterised relative to head muscle development. *gfra3* expression was observed in the pharyngeal arches from 20 hpf (data not shown) and colocalised with En2^{+} muscle precursors of the lap and do at 30 hpf (Fig. 4A,B). Expression persisted in the arches at the onset of muscle differentiation and colocalised with an *alpha-actin*:GFP transgene (Higashijima et al., 1997) expressed in differentiating muscle cells (Fig. 4C) and with *myod* in all forming pharyngeal arch muscles, including the lateral rectus (Fig. 4D). Transverse sections showed that adjacent non-*myod*-expressing cells in the second pharyngeal arch express *gfra3*, revealing that *gfra3* is in both differentiating muscle cells and other non-differentiating cells of the pharyngeal arches (Fig. 4D′). As muscle cells differentiate and form fibres, *gfra3* persisted in cells surrounding the maturing muscle fibres (Fig. 4E,E′). Many of these *gfra3*$^{+}$ cells showed an elongated morphology characteristic of immature myofibres and expressed *gfra3* at stages when the muscles start to function in opening and closing of the jaw. In *stm*^{hy024} mutants, *gfra3* expression was mostly lost in the pharyngeal arches, potentially owing to nonsense-mediated decay (see Fig. S2K,L in the supplementary material). Similarly, *gfra3* expression was reduced in the arches of *gfra3* morphants (see Fig. S2M,N in the supplementary material). *gfra3* is, therefore, expressed in both muscle precursors and surrounding mesenchymal cells from early stages and continues to be expressed as muscle differentiation occurs.

A Gfra3 ligand, Artn2, is required for opercular muscle development

Mammalian Gfra3 receptors are specifically bound and activated by Artn ligands (Baloh et al., 1998). The ligand binding site of zebrafish Gfra3 possesses the same conserved residues for Artn binding as mammalian orthologues, suggesting that it shows similar specificity

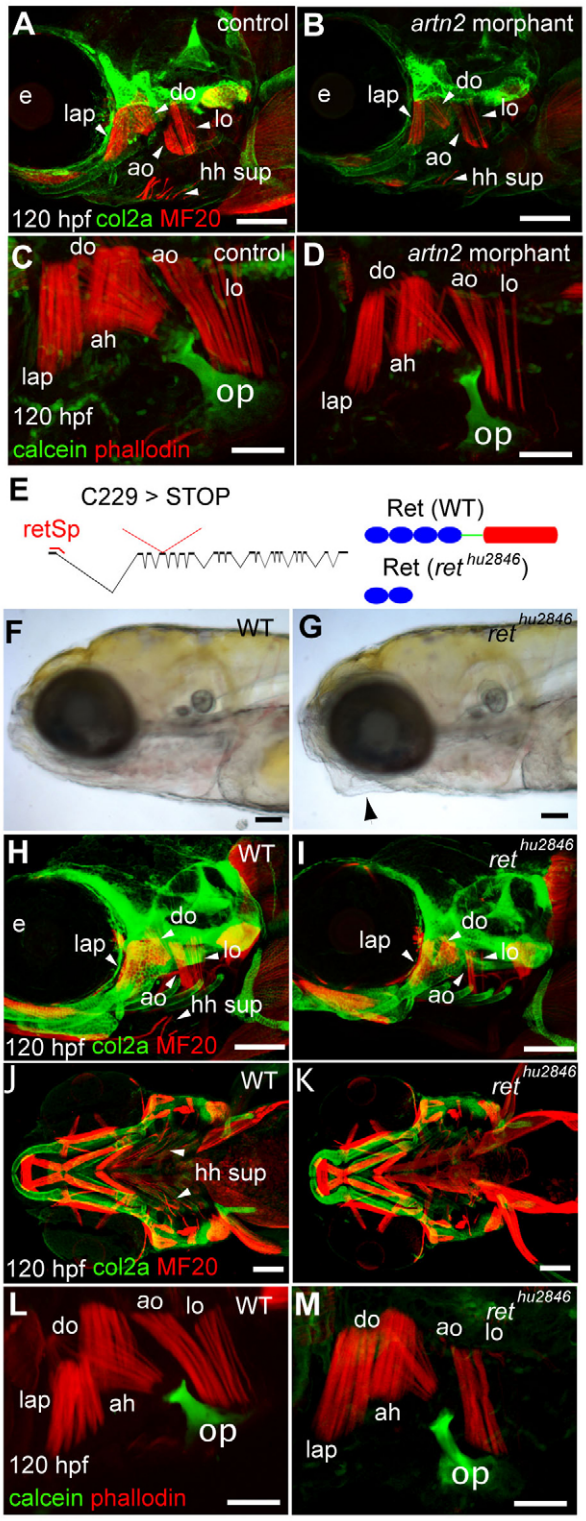

Fig. 5. Loss of Ret or Artn2 function results in specific opercular muscle defects. (**A-D**,**H-M**) Immunolabelling of cartilage, bone (green) and muscles (red) of uninjected controls (A,C), *artn2* morphants (B,D), wild type (WT; H,J,L) and *ret*^{hu2846} mutants (I,K,M) at 120 hours post-fertilisation (hpf). Specific reductions of the do, ao, lo and hh sup muscles occur in *artn2* morphants and *ret*^{hu2846} mutants, but the lap and ah muscles are unaffected. (**E-G**) *ret*^{hu2846} mutants have a tightly closed mouth (G, arrowhead) relative to WT (F) and have a point mutation in codon 229 (T>A) of exon 4 in the *ret* gene that changes a cysteine to a stop codon in the coding sequence (E). This results in a truncated protein lacking the tyrosine kinase domain (red), the transmembrane region (green) and part of the extracellular domain (blue). A morpholino directed to the exon 1 splice donor site (retSp) causes aberrant splicing of *ret*. ah, adductor hyoideous; ao, adductor operculi; do, dilator operculi; e, eye; hh sup, hyohyoideus superiores; lap, levator arcus palatini; lo, levator operculi. Scale bars: 100 μm in A,B,H-K; 50 μm in C,D,L,M.

during ligand binding (Wang et al., 2006; Hatinen et al., 2007). Zebrafish have two *artn* genes and the residues necessary for Gfra3 binding are conserved with mammalian Artn proteins (Hatinen et al., 2007). Expression of *artn1* and *artn2* was assessed to determine whether they could be potential activators of Gfra3 during opercular muscle development. *artn1* was never expressed in cells of the pharyngeal arches (data not shown). By contrast, *artn2* was restricted to mesenchymal cells of the pharyngeal arches (Fig. 4F) and was present in ventral cells of the arches where cells derived from the cranial neural crest (CNC) lie (Fig. 4F′,F″), but expression was absent from the pharyngeal pouches (Fig. 4G). At the onset of myogenic differentiation, *artn2* was expressed in cells overlying the do and overlying the ao and lo muscles (Fig. 4H) and persisted in mesenchymal cells adjacent to forming opercular muscles (Fig. 4I-I″). *artn2* expression in cells adjacent to forming *gfra3*$^{+}$ opercular muscles make it a good candidate for activating Gfra3 during head muscle development. Knockdown of *artn2* function resulted in specific reductions of the opercular muscles do, ao and lo, but adjacent non-opercular muscles lap and ah were not significantly affected (see Table S4 in the supplementary material). The associated skeleton and opercular bone were not affected in *artn2* morphants, revealing a requirement for Artn2 function during opercular muscle development but not for skeletal development (Fig. 5A-D).

Ret is required for opercular muscle development

Many of the biological actions of the mammalian Gfra receptors are mediated through the Ret tyrosine kinase receptor (Airaksinen and Saarma, 2002). The specific requirements for Gfra3 and Artn2 during opercular muscle development might reflect a general requirement for Ret function during head muscle development. To test whether Ret function is required for head muscle development, a zebrafish *ret* mutant was identified through a TILLING screen. The *ret*^{hu2846} mutant has a stop codon in exon 4 of *ret* that results in a prematurely truncated protein possessing part of the extracellular domain, but lacking the transmembrane domain or the intracellular tyrosine kinase domain essential for signalling (Fig. 5E). *ret*^{hu2846} mutants had a tightly closed mouth but showed no obvious defects in their cranial skeleton (Fig. 5F-M). Quantification of muscle fibres in *ret*^{hu2846} mutants revealed that there is a specific reduction of opercular muscles, but not of adjacent non-opercular muscles (see Table S5 in the supplementary material). To confirm that the specific opercular muscle defects are due to a loss of Ret function, animals were injected by a morpholino (retSp) to knock down expression of *ret* mRNA (Heanue and Pachnis, 2008). *ret* morphants showed the same specific opercular muscle reductions as *ret*^{hu2846} mutants (see Table S6 in the supplementary material).

Ret colocalises with Gfra3 in opercular muscle precursor cells

Zebrafish *ret* was expressed in the pharyngeal arches from 20 hpf in cells that lie medially within the arch (Fig. 6A-B). To determine whether *ret*+ cells in the forming opercular muscles are of CNC

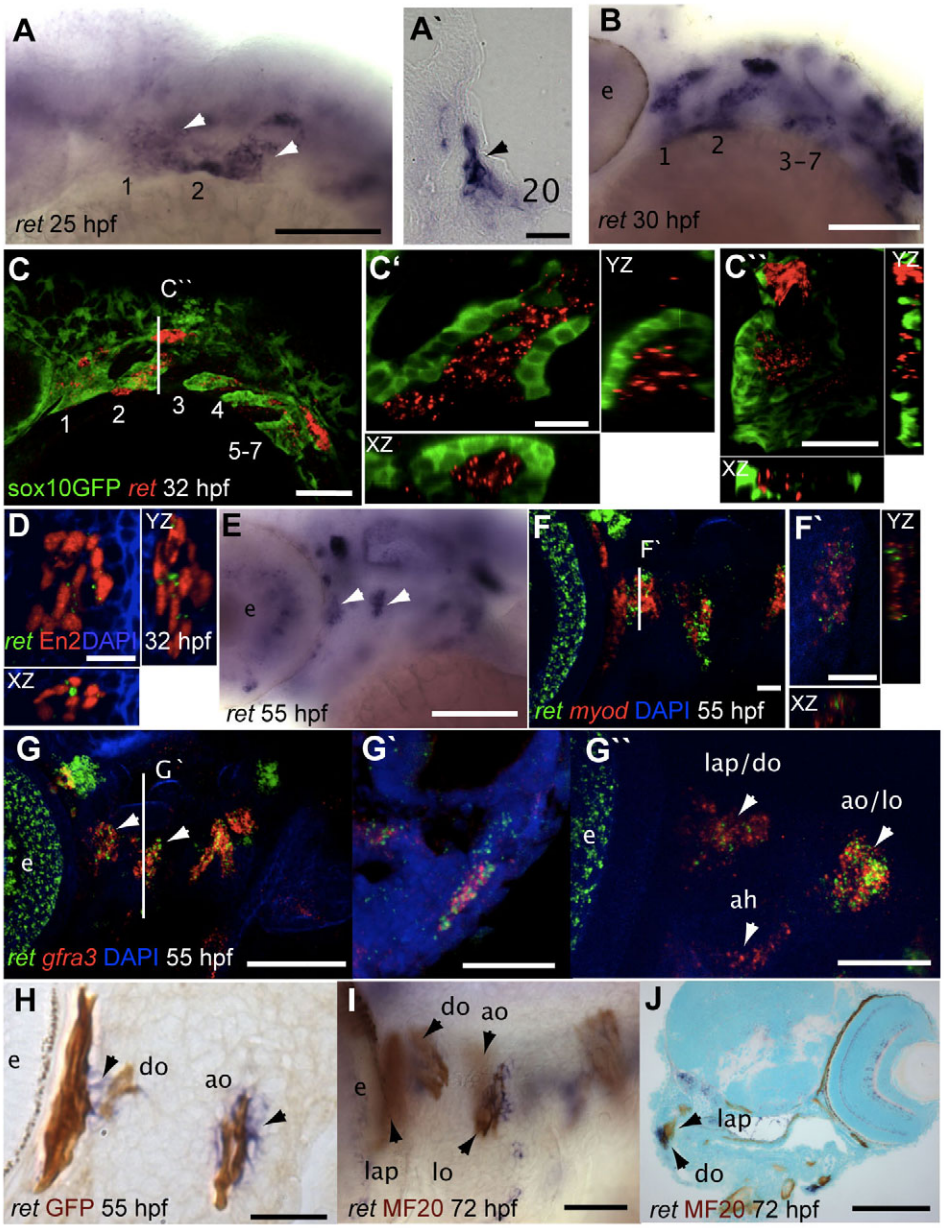

Fig. 6. *ret* expression becomes restricted to cells associated with opercular muscles. (**A-B**) *ret* is expressed in the first (1) and second (2) arches at 25 hours post-fertilisation (hpf; A, white arrowheads); transverse section through the arch (A′) reveals that *ret*$^+$ cells (black arrowhead) lie medially. *ret* becomes restricted to medial cells of the arches at 30 hpf (B) relative to GFP$^+$ cells in *sox10:GFP* animals (C). (**C-D**) Confocal sections reveal that *ret*$^+$ cells are surrounded by GFP$^+$ cells (C′) and transverse sections fail to show colocalisation of *ret* and GFP (C″). At similar stages, *ret* expression is detected in myoblasts of the first arch that show En2 immunoreactivity (D). (**E**) *ret* expression in the arches becomes restricted to discrete populations of cells, including some associated with the developing opercular muscles (arrowheads). (**F**,**F′**) *ret* and *myod* do not appear to be expressed in the same cells of the opercular muscles (F) and transverse sections fail to show colocalisation (F′). (**G-G″**) *ret* and *gfra3* are both expressed in opercular muscles at 55 hpf (G) and transverse sections (G′) and ventral views (G″) show that they colocalise in the ao and lo, but not in the ah. (**H**) Differentiating muscle fibres (brown) in *alpha-actin:GFP* fish do not express *ret* (blue) at 55 hpf, but *ret*$^+$ cells lie adjacent to fibres (arrowheads). (**I**,**J**) Lateral view of the arches in 72 hpf embryos following in situ hybridisation and immunolabelling (I) shows that *ret* expressing cells (blue) are associated with the forming do, ao and lo muscles (brown). Transverse sections reveal that *ret* is expressed in cells of the do but not the lap (J) at this stage. Lines in C, F and G indicate the level of section in C″, F′ and G′, respectively. *yz* and *xz* planes are shown for C′, C″, D and F′. ah, adductor hyoideous; ao, adductor operculi; do, dilator operculi; e, eye; lap, levator arcus palatini; lo, levator operculi. Pharyngeal arches 1-7 are shown by numbering in A-C. Scale bars: 100 µm in A-C,E,G,J; 20 µm in A′,C′,C″,F,F′,G′-I; 10 µm in D.

origin, *ret* expression at 32 hpf was compared with GFP$^+$ CNC cells in the arches of *sox10:GFP* transgenic fish (Wada et al., 2005). There was no co-expression of *ret* with GFP in the arches; rather the *ret* expression was located in a central core of cells surrounded by GFP$^+$ CNC-derived cells (Fig. 6C-C″). Mesoderm becomes surrounded by CNC in the arches in a similar manner, suggesting that the *ret*$^+$ cells are mesodermal (Kimmel et al., 2001). *ret* was expressed in myoblasts of the dorsal first arch expressing

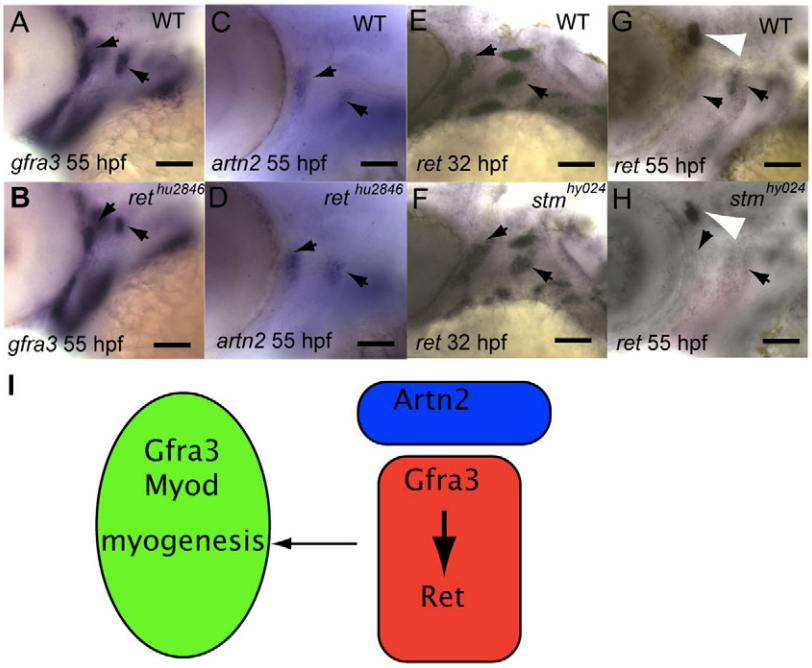

Fig. 7. Gfra3 function is required for maintaining *ret* expression in opercular associated cells. (**A-H**) Expression of *gfra3* (A,B), *artn2* (C,D) and *ret* (E-H) in *ret*^{hu2846} mutants (B,D) and wild-type (WT) siblings (A,C), *stm*^{hy024} mutants (F,H) and WT siblings (E,G) at 32 hours post-fertilisation (hpf; E,F) or 55 hpf (A-D,G,H). At 55 hpf, there are fewer *gfra3*-expressing cells in the forming adductor operculi (ao) and levator operculi (lo) (arrowheads) of *ret*^{hu2846} mutants (B) compared with WT (A). By contrast, *artn2* expression in the dorsal pharyngeal arches is unaffected in *ret*^{hu2846} mutants (D) compared with WT (C). At 32 hpf, there are fewer *ret*$^{+}$ cells (black arrowheads) in the dorsal second arch of *stm*^{hy024} mutants (F) relative to WT (E). Later, at 55 hpf *ret* expression in the opercular muscles is lost, but is unaffected in the anterior lateral line ganglia (white arrowhead, H). (**I**) A model for Ret signalling during opercular muscle development. Ret receptor and the Gfra3 co-receptor are present on myoblast cells (red). Artn2 from adjacent mesenchymal cells (blue) binds the Gfra3 co-receptor in Ret^{+} cells and muscle cells. Activation of Ret signalling by Gfra3 and Artn2 is required to maintain *ret* expression in myoblast cells and for myogenic differentiation to occur, involving *myod* expression and a loss of *ret* expression. Scale bars: 100 μm.

En2, revealing these *ret*$^{+}$ cells are myogenic precursor cells (Fig. 6D). To determine whether *ret* is expressed in CNC at stages when head muscle differentiation occurs, *ret* expression was compared with GFP in *fli1:GFP* transgenic fish, where it is strongly expressed in CNC and its skeletogenic derivatives in the pharyngeal arches (Crump et al., 2006). There was no *ret* expression in CNC-derived GFP^{+} cells of the pharyngeal arches in *fli1:GFP* fish at 55 hpf, when myogenic differentiation occurs, suggesting that the *ret*$^{+}$ cells in the muscle are not CNC-derived (see Fig. S3C,C′ in the supplementary material). Potentially, *ret* is expressed in CNC-derived cells that do not express GFP in either *fli1:GFP* or *sox10:GFP*; therefore, *ret* expression was assessed in animals lacking CNC caused by knockdown of Tfap2a and Tfap2c function (Hoffman et al., 2007). In *tfap2a tfapc* morphants *ret* was still expressed in the pharyngeal arches, revealing that the majority of *ret*$^{+}$ cells in the arches were not CNC (see Fig. S3A,B in the supplementary material). As development proceeded, *ret* expression became more restricted to the forming opercular muscles (Fig. 6E). The majority of *ret* expressing cells did not express *myod* and were restricted to the periphery of the muscle, suggesting that most *ret*$^{+}$ cells were not differentiating (Fig. 6F,F′). *gfra3* was expressed in both *myod*$^{+}$ differentiating muscle cells and *myod*$^{-}$ cells in the arches (Fig. 4D′). *gfra3* and *ret* colocalised in cells adjacent to the forming opercular muscles, with cells interspersed within the muscles of the ao, lo and do (Fig. 6G,G′). Colocalisation of *ret* and *gfra3* was strongest in the precursors of the ao and lo opercular muscles, but was absent from the non-opercular ah muscle (Fig. 6G″). Differentiating muscle fibres expressing *alpha-actin:GFP* did not express *ret*, but *ret*$^{+}$ cells were closely associated with them (Fig. 6H). As muscle formation proceeded, *ret*$^{+}$ cells became localised to the opercular muscles (do, ao, lo and hyohyoideus), but not the non-opercular muscles lap or ah (Fig. 6I). Transverse sections revealed that these *ret*$^{+}$ cells were closely associated with fibres in the forming opercular muscles, but *ret* was not expressed in muscle fibres (Fig. 6J). In summary, *ret* was expressed in mesodermal cells and not CNC of the arches, was then excluded from differentiating myoblasts and became restricted to cells in the forming opercular muscles. During muscle differentiation *ret* colocalised with an activating Gfra3 co-receptor and Ret loss of function resulted in the same specific reduction of opercular muscles as in animals lacking Gfra3 and Artn2 function. It is the opercular muscles that contain *ret*$^{+}$ *gfra3*$^{+}$ cells and not adjacent non-opercular muscles, highlighting a potential manner in which Ret can act specifically during myogenesis.

Ret signalling is mediated through Gfra3 in the pharyngeal arches

The same highly specific muscle phenotypes observed after loss of Gfra3, Artn2 and Ret function implies that they are functioning in the same pathway. To determine whether this is due to regulation of one gene by another, their expression was analysed in animals lacking functional Gfra3, Ret or Artn2. At early stages of myogenesis *gfra3* expression was unaffected in *ret*^{hu2846} mutants (data not shown); during muscle differentiation, *gfra3* was expressed at similar levels as in WT, but there appeared to be slightly fewer *gfra3*$^{+}$ cells in the ao and lo muscles (Fig. 7A,B). *artn2* expression was normal in *ret*^{hu2846} mutants at both early (data not shown) and later stages of myogenesis (Fig. 7C,D). Ret signalling is, therefore, not required for regulating the level of *gfra3* or *artn2* expression during myogenesis, but might be necessary for ensuring an appropriate number of *gfra3*$^{+}$ cells. In *stm*^{hy024} mutants, early *ret* expression in the dorsal second arch was reduced at 32 hpf (Fig. 7E,F) and expression was lost in the opercular-associated cells during muscle differentiation; by contrast, expression was unaffected in the nearby anterior and posterior lateral line ganglia (Fig. 7G,H). Potentially, this could be due to a loss of muscle-associated cells, such as connective cells. Labelling of bone and connective cells revealed no obvious perturbations in *ret*^{hu2846} mutants, and two connective cell markers, *tenascin C* and *tenascin W*, continued to be expressed in cells connecting the ao and lo muscles to the opercular bone and other opercular-associated cells in *ret*^{hu2846} and *stm*^{hy024} mutants (see Fig. S3D-M in the supplementary material). Thus, *ret* expression in the arches is dependent on

Gfra3 at later stages of myogenesis and loss of *ret* expression in *stm*^{hy024} mutants is not due to a failure of connective cells to form at the opercular bone.

DISCUSSION

The molecular mechanisms that dictate specific muscle shape and size during development remain elusive. In this study, we identify a highly specific requirement for Ret signalling during the formation of a subset of functionally related head muscles. Expression of *ret*, *artn2* and *gfra3* is coordinated in cells associated with opercular muscles and differentiation of these muscles requires function of all three genes. Strikingly, only opercular muscles and not adjacent non-opercular muscles require Ret signalling for their development and this correlates with the restriction of *ret*$^{+}$ cells to opercular muscles. This suggests that muscles and their associated tissues are coordinated as discrete modules during musculoskeletal development and that Ret signalling is needed for the development of a specific subset of muscles.

Coordinated activation of Ret drives the development of a subset of head muscles

Animals lacking Artn2, Gfra3 and Ret function all show the same, highly specific opercular muscle defect, implying that they are acting in the same pathway. The muscle defects correlate with the highly restricted expression of *artn2* and *ret* in cells that form a complex involving the opercular muscles. Gfra3 loss of function results only in opercular muscle defects despite expression of *gfra3* in other muscles. As *gfra3* is the only Gfra receptor expressed in cells adjacent to and in head muscles during muscle differentiation (data not shown), it is likely that Ret activation requires Gfra3 function. The highly restricted expression of *ret* in differentiating opercular muscles correlates directly with the specific nature of the muscle phenotype caused by loss of Ret function. This suggests that Ret signalling is activated specifically only in the opercular muscles and occurs by a restriction of Ret to a subset of cells in them. The modular nature of the muscle phenotype in Ret mutants is unexpected, as it involves muscles derived from different pharyngeal arches that connect to the opercular bone. Adjacent muscles derived from the same muscle anlagen, appear normal in Ret signalling mutants. This reflects the restriction of *ret*$^{+}$ cells to opercular muscles and suggests that these muscles have a distinct identity involving Ret signalling that is independent of their origin from a particular arch.

In the head, muscle connective cells are derived from CNC (Noden, 1983; Couly et al., 1992) and perturbations to the CNC can cause both skeletal and muscle defects (Rinon et al., 2007). The *ret*$^{+}$ opercular cells might be CNC-derived, suggesting that Ret is functioning in muscle connective cells. However, *ret*$^{+}$ cells in the forming muscles do not co-express GFP in *fli1*:GFP or *sox10:*GFP transgenic fish, suggesting that they are not CNC-derived. Ablation of CNC did not result in a loss of *ret* expression, and *ret* is expressed in En2^{+} myoblasts of the dorsal first arch. *ret* appears to be expressed in mesodermal cells, and subsequently myoblasts, in the arches. Subsequently, the majority of *ret*$^{+}$ cells do not express *myod* or *alpha-actin*:GFP, markers for differentiating muscle cells, but *ret* is expressed with *gfra3* in cells of the ao, lo and do. Potentially the *ret*$^{+}$ *gfra3*$^{+}$ cells are a secondary population of muscle cells that contribute to growth of the muscles after the primary muscle fibres have formed, similar to that in the dermomyotome (Devoto et al., 2006). These possibilities could be resolved by lineage analysis of the *ret*$^{+}$ opercular-associated cells.

Ret signalling promotes muscle differentiation

All muscles require MRF function for their differentiation (Buckingham, 2006). Our findings suggest that Ret is able to regulate differentiation specifically in opercular muscles through a regulation of MRF genes. Muscle specification is normal in an absence of Ret signalling as myoblasts express *tbx1* and *caps* as normal. Both *myf5* and *myod* expression are reduced in the pharyngeal arches of *ret*^{hu2846} and *stm*^{hy024} mutants, revealing a role for Ret during myogenesis. As muscle differentiation commences, Ret is required specifically for normal *myod* expression and muscle fibre formation in opercular muscles, but not in adjacent non-opercular muscles, despite their origin from the same precursors in the arch. How Ret signalling can regulate myogenesis in opercular muscle cells is not clear. However, given that *ret* is expressed in myoblasts at earlier stages, Ret signalling might act to regulate myogenic progression. The reduction of early *myf5* expression in *stm*^{hy024} mutants suggests that Ret signalling is needed continuously for myogenesis; the recovery of muscle in *gfra3* morphants supports a late role of Ret signalling during muscle formation. Based on our results, we propose a model for how Ret signalling can regulate myogenesis in opercular muscles (Fig. 7I). Artn2 binding to Gfra3 causes activation of Ret in myoblast precursors. Active Ret signalling is required for maintaining *ret* expression in these cells and for regulating subsequent myogenesis. As the majority of myoblasts do not co-express *ret* and *myod*, one implication of this model is that *ret* expression is lost in differentiating myoblasts. The role of Ret signalling in modulating myogenesis in precursor cells for a subset of muscles thus provides a mechanism for coordinating the size and development of a certain muscles, but not adjacent muscles.

Evolution of an opercular muscle scaffold

It is intriguing that all the muscles requiring Ret signalling for their development attach to the opercular bone and so are functionally related. In zebrafish, opercular muscles function during respiration and act to open the mouth by moving the opercular bone (Lauder, 1982). One consequence of having the development of functionally interacting muscles regulated by one signalling pathway is that all muscles will be affected, simply by modulating this pathway. Such a mechanism might allow for coordinated changes to muscle systems during evolution, thus ensuring that their function is maintained. Interestingly, African seed finches that have evolved to eat tougher seeds show an increase in the size of muscles needed for jaw closing but not for jaw opening (Clabaut et al., 2009), revealing that differential changes to functional muscle systems do occur during evolution. If the opercular muscles are developing as a module, it is intriguing to consider how they might have changed during evolution and whether Ret signalling plays a conserved role in their development. The do and lo opercular muscles are not found in basal ray-finned fish, indicating that some aspects of the zebrafish opercular are derived features. By contrast, the opercular bone and two of the muscles (ao, hh sup) were present in the last common ancestor of fish and amniotes (Diogo et al., 2008; Zhu et al., 2009). In the lineage leading to mammals and birds, the opercular bone and associated muscles were lost and it is not clear what the homologues of opercular muscles are in mammals. We have shown that in zebrafish both the ao and hh sup require Ret signalling for their development and these muscles contain *ret*$^{+}$ cells. The ao and hh arise from mesoderm of the second pharyngeal arch and in mammals this mesoderm forms the muscles of facial expression and platysma (Edgeworth, 1935). If Ret signalling has a role in patterning mammalian head muscles, it is likely to be

required for the development of these muscles. We note that in mouse, *Ret* expression has been described in cells of the second pharyngeal arch (Pachnis et al., 1993). It is not clear whether these cells are present in muscle, but if Ret signalling has a conserved role in opercular muscle development, we would predict that it is needed for facial muscle development in mammals. No muscle perturbations have been described in mouse *Ret*-null mutants but, as they die owing to kidney defects at birth, head muscle defects might not have been apparent (Schuchardt et al., 1994). Likewise, it would be intriguing to assess whether altered Ret signalling in the head is associated with muscle perturbations in human craniofacial syndromes.

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Competing interests statement
The authors declare no competing financial interests.

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