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Isolation and characterisation of the chick orthologue of the Opitz syndrome gene, *Mid1*, supports a conserved role in vertebrate development

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ABSTRACT The X-linked form of Opitz syndrome (OS) is caused by loss of function of the microtubule-associated MID1 protein. The phenotype of OS includes defects along the central body axis, namely hypertelorism, cleft lip and palate, hypospadias and cardiac structural anomalies. Here we describe the isolation and characterisation of full-length cDNA clones representing the chick *Mid1* gene and the detailed profile of its expression in stage 7 to 28 chick embryos. Consistent with the remarkable sequence conservation of MID1 between human and chick was the good correlation of the pattern of *cMid1* expression with the tissues affected in OS. In stage 10 embryos, transcripts were concentrated in the head mesenchyme which includes migratory neural crest cells. However, the incomplete overlap with a neural crest marker, *Sox10,* suggests that *Mid1* is a marker for somitomeric mesoderm and potentially for a subset of neural crest cells. Consistent with this, *cMid1* expression was also detected at later stages in neural crest-derived facial mesenchyme, in the myotome and in the condensing muscle blocks of the limb. Expression of *cMid1* was observed in the neural epithelium of the forebrain beginning at stage 7 with increased signal in presumptive rhombomeres 2/3. By stage 15, expression is highest in the diencephalon. Other areas with high expression are certain facial epithelia and the midgut that will give rise to the oesophagus and trachea. These data indicate that *Mid1* plays an evolutionarily conserved developmental function in vertebrates that may involve effects on cellular proliferation, tissue interactions and morphogenesis.

KEY WORDS: *Mid1, Sox10, MF-20, mesoderm, Opitz GBBB syndrome*

Introduction

Opitz GBBB syndrome (OS; Opitz syndrome) is a classic example of a defect of the primary midline developmental field. Despite marked interindividual variability in clinical presentation, the disorder can be recognised by characteristic facies (ocular hypertelorism and variably cleft lip with or without cleft palate [CL/P] and laryngotracheo-oesophageal [LTE] clefting) in combination with difficulty in swallowing (dysphagia), structural heart defects as well as anal and genital anomalies (Opitz, 1987; Robin *et al.*, 1996). Part of this complexity may be explained by the possible interaction between the multiple genetic loci independently implicated in causation of OS: an X-linked form caused by mutations in the *MID1* gene at Xp22.3 (Quaderi *et al.*, 1997; Gaudenz *et al.*, 1998; Schweiger *et al.*, 1999; Cox *et al.*, 2000) which comprises at least 50% of all OS cases (Cox *et al.*, 2000), and at least one autosomal form at chromosome 22q11.2 for which the gene has not been identified (McDonald-McGinn *et al.*, 1995; Robin *et al.*, 1995; Fryburg *et al.*, 1996). Significantly, this 22q11 interval is also implicated in a group of disorders collectively known as the 22q11 deletion syndrome (which encompasses DiGeorge and Velocardiofacial syndromes) that shows some phenotypic overlap with OS (McDonald-McGinn *et al.*, 1995; Robin *et al.*, 1995). The 22q11 deletion syndrome (also known as CATCH22) represents one of the most common genetic causes of malformations (Glover, 1995).

The X-linked *MID1* gene encodes a protein of 667 amino acids that is the defining member of a new subclass of the RBCC (RING, B-box, Coiled-Coil) family of proteins. This subclass is characterised by the combination of both a fibronectin type III motif and a recently

Abbreviations used in this paper: CL/P, cleft lip with or without cleft palate; LTE, laryngotracheo-oesophageal; OS, Opitz syndrome; PP2, protein phosphatase 2; RBCC, Ring, B-box, Coiled-Coil.

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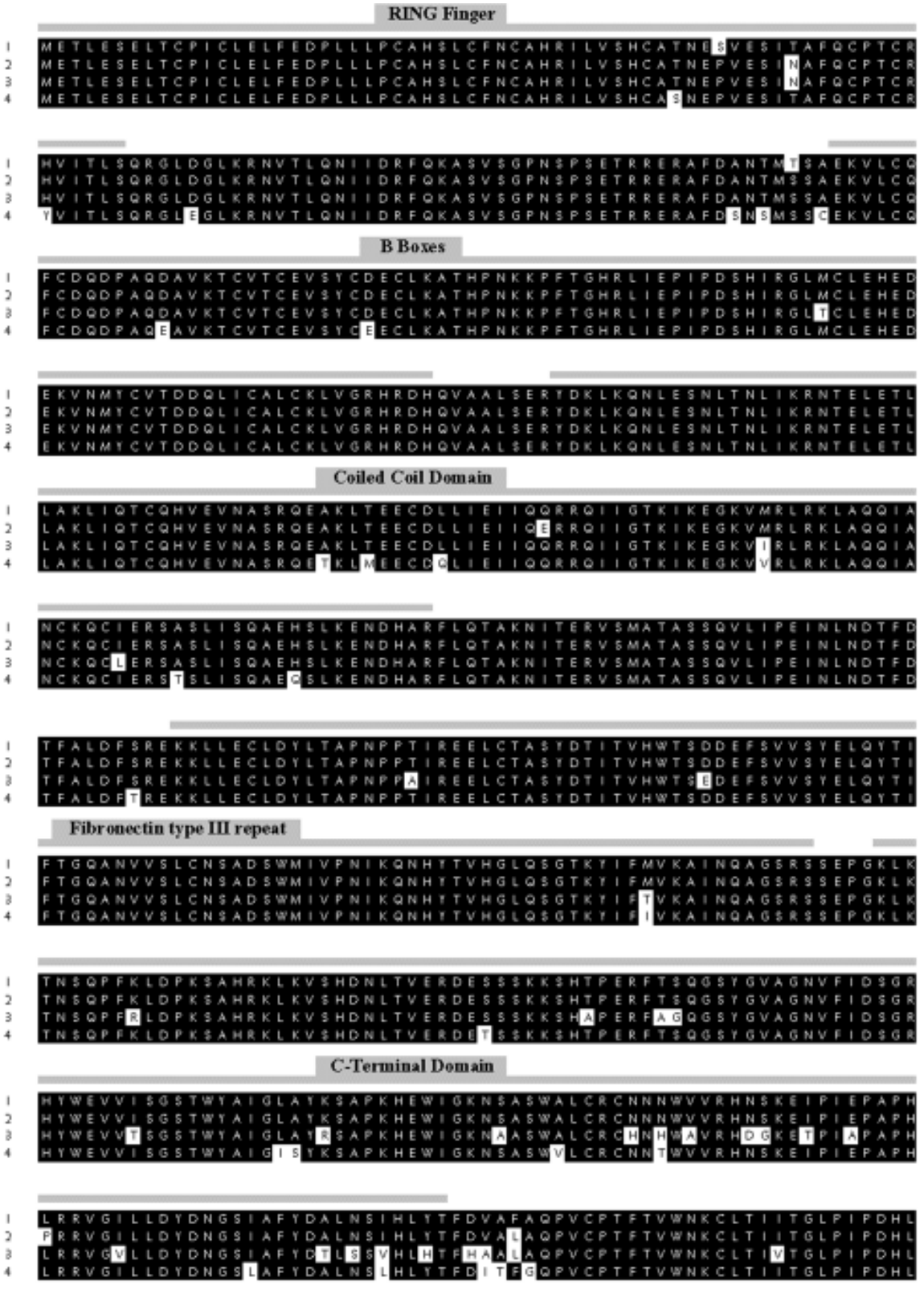

Fig. 1. Amino acid alignment of chick Mid1 with human, rat and mouse MID1. (1) *Human MID1 - Genbank accession #AF269101;* **(2)** *Rat* (R. norvegicus) *MID1 - Genbank accession #AF186461;* **(3)** *Mouse* (M. musculus) *Mid1 (Genbank accession #AF026565);* **(4)** *Chick* (G. gallus) *Mid1 - Genbank accession #AF269102. Black boxes indicate identical amino acid residues. Human versus rat, 99.1% identity; human versus mouse, 94.9% identity; human versus chick, 95.4% identity. The RING, B-boxes, coiled-coil, FNIII and C-terminal domains are highlighted.*

defined C-terminal (B30.2-like or SPRY) domain (Short *et al.,* in preparation). Despite the fact that many RBCC proteins function within the nucleus (for example, the products of the proto-oncogenes, PML and RFP, and those with developmental roles, such as *Pleurodeles waltls*, PwA33, and *Xenopus laevis*, XNF7), recent studies have shown that MID1 associates with cytoplasmic microtubules throughout the cell cycle (Cainarca *et al.,* 1999; Schweiger *et al.,* 1999).

The majority of mutations in *MID1* that cause OS are truncating mutations with many, but not all, directly affecting the C-terminal half of the protein (Cox *et al.,* 2000). Significantly, all examined MID1 mutations disrupt the normal microtubule-associated distribution although not all in the same manner or to the same degree (Cainarca *et al.,* 1999; Schweiger *et al.,* 1999; Cox *et al.,* 2000), leading to the belief that the X-linked form of OS is caused by loss of function of MID1 (Cox *et al.,* 2000).

Although the actual function of MID1 is not known, cell fractionation experiments have demonstrated that MID1 is found as part of large multiprotein complexes on microtubules (Cainarca *et al.,* 1999). The recent identification of the Alpha 4 protein, which has a well-characterised role in regulating PP2-type phosphatases (Schmelzle and Hall 2000), as a strong interacting partner of microtubule-bound MID1 may help elucidate the function of MID1 (Liu *et al.,* 2001; Short *et al.,* 2002). The signalling pathway in which Alpha 4 functions appears to be conserved from yeast through to mammals and affects a number of aspects of cell-cycle progression, including the regulation of microtubule structure and function as well as protein biosynthesis (Goldberg, 1999). It is believed that the disruption of this Alpha 4-regulated protein phosphatase action from microtubules may, at least in part, underlie the complex and variable clinical phenotype of OS.

Studies are currently underway to address the role of the *Mid1* gene in mammalian development through the use of cell culture and gene targeting experiments in mice. The latter is based on preliminary analysis of the expression of the murine *Mid1* gene over a limited developmental time period, which indicated that its pattern of expression was largely consistent with the tissues affected in patients with Opitz syndrome (Dal Zotto *et al.,* 1998). However, the chick has long been the model system of choice for studies of neural crest, craniofacial and muscle development due to the accessibility and manipulability of the embryos. In fact, the resurgence in interest in the chick as a system in which to undertake molecular studies has come with the advent of antisense technology, the use of growth factor-soaked beads, and *in vivo* electroporation. In order to determine whether the chick could be utilised as a complementary system in which to investigate the role of MID1 in craniofacial development, we sought to isolate the *MID1* orthologue from chick and characterise in detail its pattern of expression throughout the relevant stages of development.

Results

Cloning of Chicken Mid1

Of nearly 50 positively hybridising plaques, eight (clones 5B1, 7B1, 7B2, 8B1, 9B1, 12B1, 16B1 and 16B2) were initially chosen and successfully purified through additional dilution and plating series. Insert sizes of the isolated phage ranged from 1.0 kb (7B1) to 2.0 kb (8B1 and 9B1). Assembly of the sequences revealed an open reading frame encoding a protein of 667 amino acids. Comparison of this chick sequence with that of human, rat and mouse *MID1* revealed a striking level of conservation: for example, the comparison with human MID1 showed 84.4% and 95.4% identity at the nucleotide (over the complete open reading frame) and amino acid levels, respectively (see Fig. 1). Even the 88 bp of 5' untranslated region obtained from these chick cDNAs showed >80% identity with the human sequence (data not shown). Analysis of the predicted amino acid sequence using the program Pfam demonstrated, as expected, that the RBCC, FNIII and C-terminal domains were each present in the chick protein (Fig. 1). This level of conservation indicated that this chick sequence was likely to represent the orthologue of human MID1.

Expression of cMid1 *at Embryonic Stages 7 through 28*

Preliminary whole mount *in situ* hybridisation carried out on stage 19 - 21 embryos showed strong expression in the craniofacial region (Fig. 2 A,C). At stage 19, expression in the frontonasal mass was strong although this decreased slightly by stage 21, whereas expression remained strong in the maxillary and mandibular processes and the second branchial arch at both stages (Fig. 2C). Weaker expression was also evident in the caudal

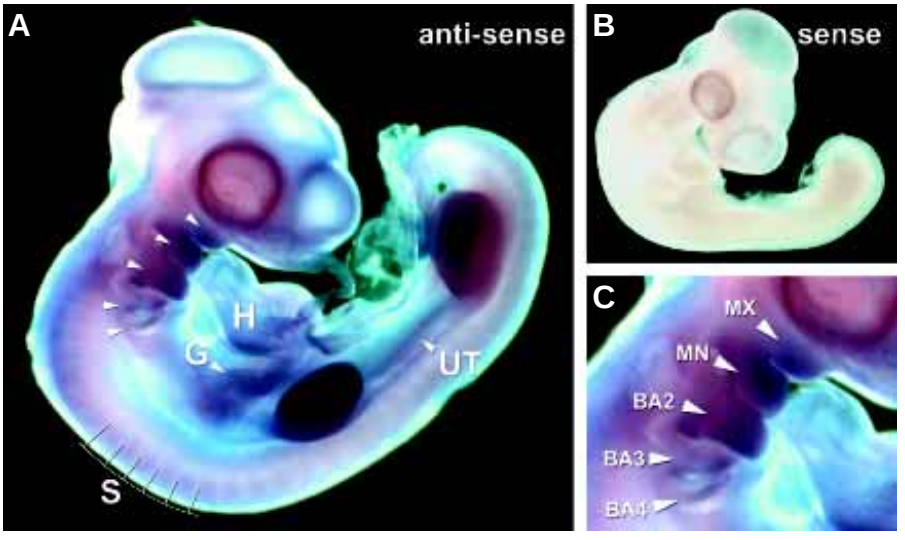

Fig. 2. Whole mount *in situ* hybridisation using *cMid1* on stage 21 chick embryos. *Whole mount* in situ *hybridisation using an antisense* **(A)** *or sense* **(B)** cMid1 *probe. Using the antisense probe, a high level of* cMid1 *expression was detected in the craniofacial region (arrowheads) and both the forelimb and hindlimb buds. Weaker expression can also be seen in the urogenital tract, somites and the gut.* **(C)** *In the craniofacial region, expression was highest in the maxillary and mandibular prominences as well as branchial arch 2. Branchial arches 3 and 4, in addition to the branchial clefts, also express* cMid1 *but in these tissues expression was considerably lower and appeared to be restricted to the caudal half of each arch. No expression was seen when using the* cMid1 *sense probe (B). Abbreviations: BA2-4, branchial arches 2-4; G, gut; H, heart; MX, maxillary prominence; MN, mandibular prominence; S, somites; UT, urogenital tract.*

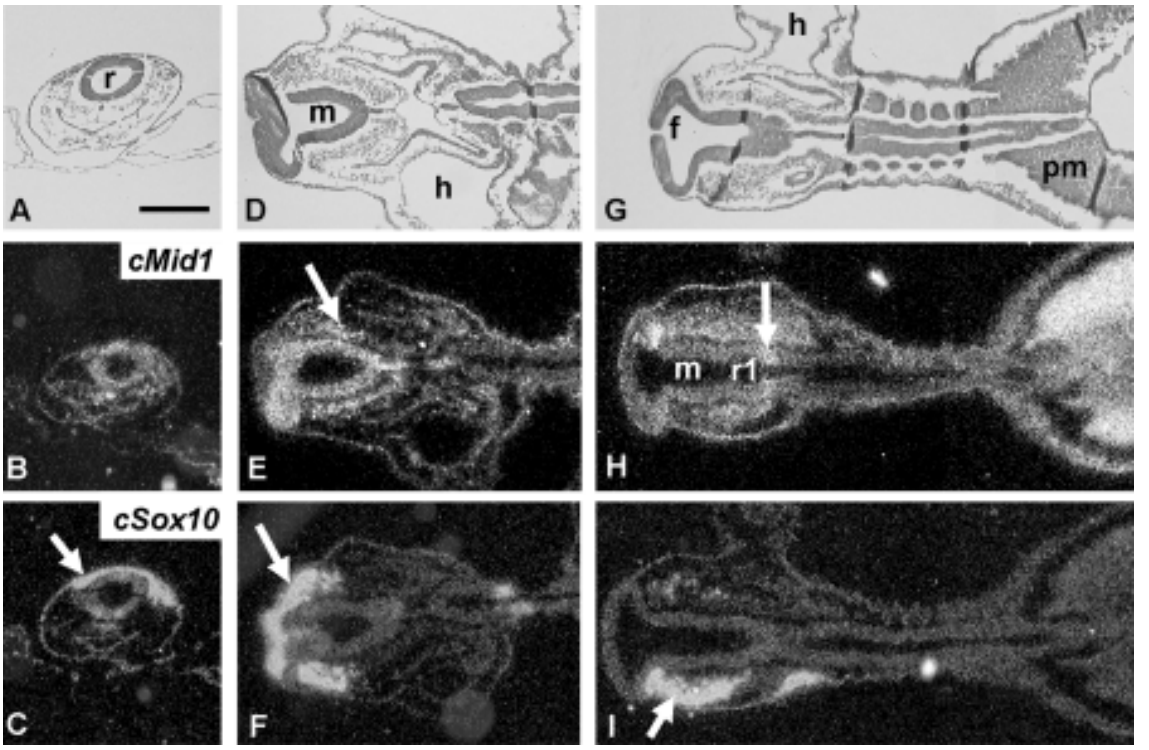

Fig. 3. Expression of *cMid1* in the mesoderm, hindbrain and head mesenchyme of stage 10 embryos. (A-C) *Coronal sections through hindbrain.* **(D-I)** *Frontal sections stained with toluidine blue adjacent to those used for in situ hybridisation.* **(B,E,H)** *Sections to which cMid1 cRNA was hybridised and* **(C,F,I)** *are sections to which* cSox10 *cRNA, a marker for neural crest cells, was hybridised. Signal is seen as white silver grains on a black background.* **(B,C)** *Note expression of* cMid1 *in the head mesenchyme compared to the more dorsally expressed* Sox10 *(arrow in (C)).* **(E,F)** *Expression of* cMid1 *in the head mesenchyme (arrow in (E)) does not overlap regions expressing* Sox10 *(arrow in (F)).* **(H,I)** *Expression of* cMid1 *in the head mesenchyme partly overlaps* Sox10 *expression (arrow in (I)). The section in (I) is more dorsal than the one in (H). There is also expression of* cMid1 *in the r2/r3 region (arrow in (H)) and in the neural epithelium at lower levels. Scale bar, 250 μm. Abbreviations: f, forebrain; h, heart; m, mesencephalon; pm, presomitic mesoderm; r1, rhombomere 1.*

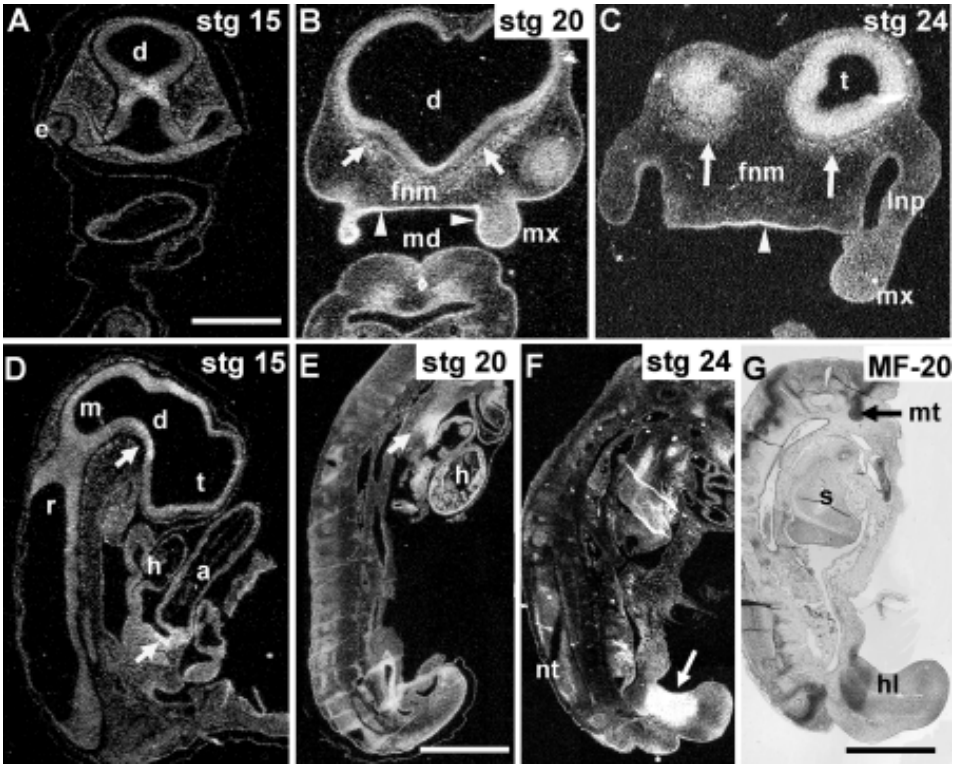

Fig. 4. Expression of *cMid1* in the diencephalon, myotome, facial prominences and midgut. (A-C) *Frontal sections;* **(D-G)** *sagittal sections.* **(A-F)** *are hybridised to and* **(G)** *is immunocytochemistry to the MF-20 antibody.* **(A,D)** *Expression of* Mid1 *in the ventral and dorsal diencephalon and base of the allantois.* **(B,C)** *Expression in frontonasal mass mesenchyme under brain (arrows) and in maxillary prominences. There is also strong expression in the epithelium (arrowheads) and signal in the midline of the mandible (mandible not in section in (C)).* **(E)** *Section of body with head removed. Expression in the midgut (arrow) and heart.* **(F,G)** *near-adjacent sections through the body with head removed showing strong expression of* Mid1 *in the muscle of the limb bud (arrow in (F)).* **(G)** *The trunk myotome expresses MF-20 and the condensing muscle in the hindlimb. Scale bar, 500 μm for (A-D); 1 mm for (E-G). Abbreviations: a, allantois stalk; d, diencephalon; e, eye; fnm, frontonasal mass; h, heart; hl, hindlimb; m, mesencephalon; md, mandibular prominence; mx, maxillary prominence; nt, neural tube; r, rhombencephalon; s, stomach; mt, myotome.*

portion of branchial arches 3 and 4 (Fig. 2C). Other tissues in which expression was also detectable included the midgut, urogenital tract, eyes, heart, the anterior CNS and in a regularly spaced pattern either side of the neural tube which was likely to be somitic tissue. Somewhat surprising was the very strong expression in the developing forelimb and hindlimb buds (Fig. 2A).

Using *in situ* hybridisation on serial sections of stage 7-10 chick embryos, we observed expression of *cMid* in the mesenchyme adjacent to the mesencephalon and hindbrain (Fig. 3 E,H) however, in stage 10 embryos, this did not completely overlap with expression of *cSox10*, a marker for neural crest cells (Fig. 3 F,I; Cheng *et al.,* 2000). In coronal sections it was obvious that *cSox10* is located at a more dorsal position than the *cMid1* expressing regions (Fig. 3 B,C). In order to see whether these regions of expression remained separate at older stages, we compared expression of the two genes in stage 28 embryos. In these sections it was clear that *cSox10* labelled neural crest derived glial cells (data not shown) and that the early differences in expression of *cMid1* and *cSox10* were maintained. In other sections of stage 7-10 embryos (Fig. 3H), strong *cMid1* expression was also seen in the presomitic mesoderm. Therefore mesodermal expression was present outside the cranial region of the embryo. There were also transcripts in neuroepithelium of the forebrain and presumptive rhombomeres at stage 7. By stage 10 the expression in the hindbrain was largely restricted to the r2/r3 region.

We observed expression of *cMid1* throughout the anterior neuroepithelium but with higher levels of expression particularly apparent in the diencephalon at stage 15 (Fig. 4 A,D). At stage 20, all the facial prominences have formed and there was increased expression of *cMid1* in the central frontonasal mass mesenchyme and underlying the ventral surface of the brain (Fig. 4B). There was also expression throughout the maxillary mesenchyme. Epithelial expression was present in the caudal edge of the frontonasal mass and medial maxillary prominence. Similar patterns of expression were maintained at stage 24 (Fig. 4C) and at stage 28 (Fig. 5B). However at stage 28 there was increased expression at the lateral corners of the frontonasal mass where fusion with the maxillary prominences will take place (Will and Meller, 1981; Sun *et al.,* 2000).

The expression in early mesoderm suggested that later in development *cMid1* might be expressed in mesodermal derivatives such as muscle. Beginning at stage 24 and continuing at stage 28 we observed strong *cMid1* expression in the limb bud (Figs. 4E, 5F). In order to determine whether the expression was within muscle, we stained adjacent sections with MF-20, an antibody that recognises myosin heavy chain (Bader *et al.,* 1982). We found that MF-20 staining overlapped regions of *cMid1*expression in the limbs and also in the myotome (Fig. 5 C,D,G,H). The dorsal myotome expresses higher levels of *cMid1* than do other regions of the myotome (compare Fig. 5 C,D). In addition to skeletal muscle, *Mid1* was expressed in cardiac muscle (Fig. 5 I,J). *cMid1* was also expressed in the dermis covering the brain, in mesenchyme ventral to the brain and within the facial prominences. Thus *cMid1* was clearly expressed in other mesenchymal cell types that were not of the muscle lineage.

Some of the most consistent features of OS are the abnormalities of the larynx, trachea and oesophagus. We were therefore not surprised to see that *cMid1* was expressed in the midgut, which gives rise to the tracheal region (Fig. 4E) and later, at stage 28, in the mesenchyme surrounding the trachea and oesophagus (Fig.

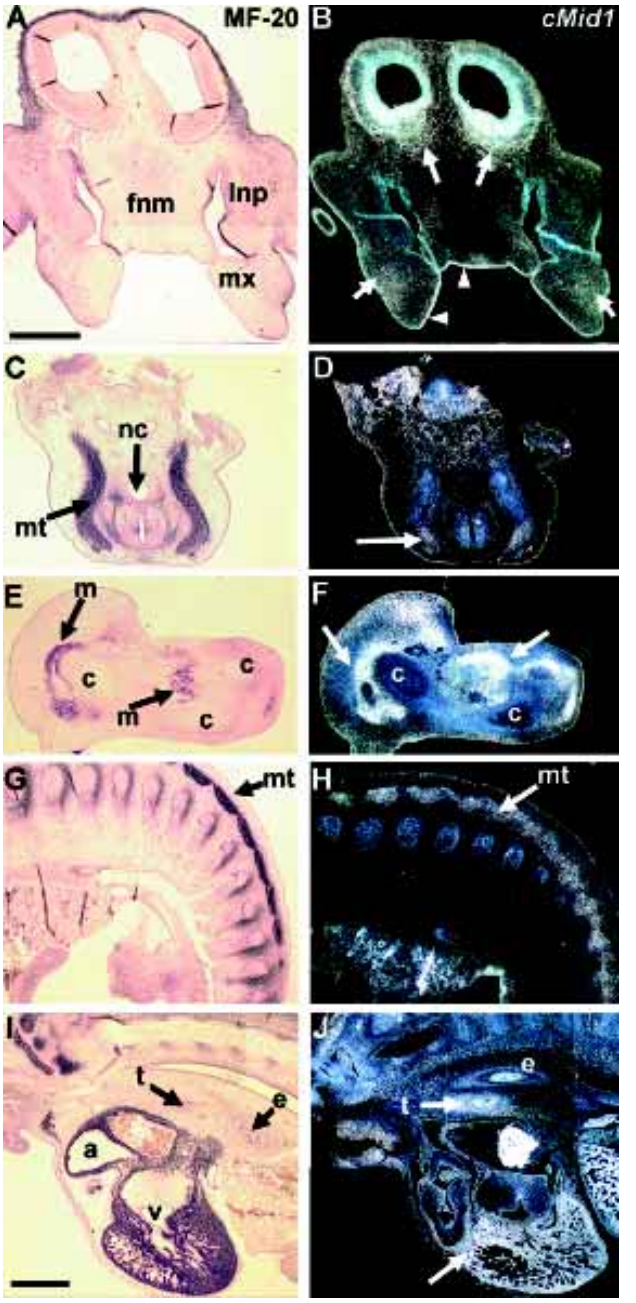

Fig. 5. Expression of *MF-20* and *cMid1* in the stage 28 embryo. (A,B) *Frontal sections through the facial region showing little* MF-20 *staining but localised expression of* cMid1 *in the mesenchyme (arrows) and in the frontonasal mass and maxillary epithelium (arrowheads).* **(C,D)** *Sections through the neck showing expression of* MF-20 *in the myotome and overlap of* cMid1 *transcripts in the dorsal myotome (arrow in (D)).* **(E,F)** *Sagittal sections through the hindlimb showing that* MF-20 *labels condensing muscle blocks and this overlaps the areas expressing* cMid1. **(G,H)** *Sagittal sections through the trunk illustrating* MF-20 *expression in the myotome overlapping expresion of* cMid1. **(I,J)** *Sagittal sections through the mid-trunk and heart showing strong* MF-20 *staining in the heart. The trachea, oesophagus and heart express* cMid1. *Blood in the heart shows dense silvergrains. Scale bar, 500 μm for all panels. Abbreviations: a, atrium; c, cartilage elements; e, oesophagus; fnm, frontonasal mass; lnp, lateral nasal prominence; m, muscle blocks; mt, myotome; mx, maxillary prominence; nc, notochord; t, trachea; v, ventricle.*

5J). Anomalies of the umbilicus, although less frequent, have also been reported in OS. Consistent with this, we also saw strong expression in the allantoic bud, a region analogous to the umbilical cord in mammals (Fig. 4D).

Discussion

Mid1 from Chicks and Humans exhibits a High Level of Sequence Conservation

The classic presentation of Opitz syndrome includes as its diagnostic features: a characteristic facial appearance (most notably ocular hypertelorism), dysphagia (difficulty in swallowing), and genital anomalies such as hypospadias in males. However, there are frequently other associated malformations, such as cardiac septation defects, labiopalatine and laryngo-tracheo-oesophageal anomalies, and an imperforate or anteriorly displaced anus. This array of features indicates that the disorder likely arises as a result of a disturbance to the midline developmental field, whereby the proper formation of structures along the midline body axis is compromised (Opitz, 1987; Robin *et al.,* 1996). In addition to the phenotypic variability in Opitz syndrome, there is genetic heterogeneity with an indistinguishable clinical presentation resulting from abnormalities of either the X-linked gene, *MID1* (Quaderi *et al.,* 1997; Gaudenz *et al.,* 1998; Schweiger *et al.,* 1999; Cox *et al.,* 2000), or an as yet unidentified autosomal gene residing at 22q11.2 (Robin *et al.,* 1995). Studies are ongoing in a number of laboratories including our own using the mouse as a model system. However, the chicken system represents a more accessible and widely used system to address mechanisms of craniofacial development. Therefore, in order to investigate whether the chick system would be suitable for, and amenable to, investigations of MID1 function during development, we undertook to identify the chick *Mid1* gene and compared its profile of expression during development to that of murine *Mid1* and its human counterpart by inference from the clinical features of the disease.

Numerous cDNA clones were isolated that covered a full open reading frame of 2001 bp. The protein encoded by this open reading frame is 667 amino acids in length and shows over 95% identity (99.3% similarity) with human MID1, indicating that these clones likely represent the orthologous chick *Mid1* sequence. Using *in situ* hybridisation initially on whole mount chick embryos and then on serial sections of embryos from stage 7 through 28, we have performed a detailed analysis of *cMid1* expression. We have shown that the pattern of *cMid1* gene expression is largely consistent with the clinical features seen in Opitz syndrome although a few differences between the chick and the previously reported mouse *Mid1* expression patterns were observed (see below).

cMid1 *Expression correlates well with the Tissues Affected in Opitz Syndrome*

The combination of features in Opitz syndrome such as the characteristic facial abnormalities and cardiac septation defects shows some overlap with the other disorders (for example, DiGeorge and Velocardiofacial syndromes) collectively referred to as the 22q11.2 deletion syndrome. These latter disorders have long been considered to arise as a result of defects in neural crest cell contribution to various regions of the body (Scambler, 2000). It is, therefore, conceivable that the Opitz syndrome phenotype could also result from a defect in contribution from the neural crest. Indeed, we observed expression of *cMid1* in neuroepithelium but found there was only partial overlap in expression with a neural crest-specific marker, *cSox10*, suggesting that *cMid1* is not expressed in the majority of migrating and proliferating neural crest cell populations. However, these observations do not exclude the possibility that a disruption to microtubule-linked functions, as a result of MID1 mutation, could delay or perturb neural crest cell release from the dorsal neural tube. In this regard, one intriguing possibility is that mutation (ie. loss of function) of MID1 perturbs the epithelial-mesenchyme transition (EMT) that occurs concomitant with release of neural crest cells from the neural folds. A similar effect on EMT processes could also be envisaged during fusion of the paired craniofacial primordia, lateral and medial nasal processes and frontonasal mass. This process of fusion is critical for the formation of the lip and palate (Sun *et al.,* 2000), and when perturbed could be expected to result in cleft lip with or without cleft palate that is seen in many cases of Opitz syndrome.

From their preliminary studies in mice, Dal Zotto and colleagues concluded that the murine *Mid1* gene was expressed predominantly in undifferentiated cell populations and therefore the OS phenotype was likely due to abnormal cellular proliferation (Dal Zotto *et al.,* 1998). Consistent with this, we did observe expression of chick *Mid1* at the lateral edges of the frontonasal mass mesenchyme and throughout the maxillary mesenchyme, which are both areas of high cellular proliferation (Peterka and Jelinek, 1983; McGonnell *et al.,* 1998). Therefore, *Mid1* may have some role in outgrowth of the corners of the frontonasal prominence as they meet and fuse with the maxillary prominences (Will and Meller, 1981). Moreover, the high expression of *cMid1* in the facial epithelia suggests that the gene may be involved in epithelial-mesenchymal interactions, as facial epithelium is required for outgrowth of underlying mesenchyme (Wedden, 1987; Richman and Tickle, 1989). Whatever the underlying cellular or tissue problem in OS, *Mid1* clearly plays an important role during craniofacial, midgut and muscle morphogenesis and further studies are needed to clarify the developmental role(s) of this RBCC protein. In this regard, it will be of interest to see whether factors such as Sonic hedgehog (Shh), which controls the width of the frontonasal mass (Hu and Helms 1999), and has overlapping expression in the frontonasal mass epithelium (Helms *et al.,* 1997), either affects, or is associated with, expression of *cMid1*.

Significantly, the correlation with the array of anomalies in Opitz syndrome extends also to other embryological tissues in which the chick *Mid1* gene is expressed. For example, we observed strong *cMid1* expression in the allantoic bud, a region analogous to the umbilical cord in mammals. This allantoic expression would therefore correlate with the occasional finding of umbilical hernias in patients with Opitz syndrome. Notably, however, there are some apparent differences between the expression pattern we have observed for chick *Mid1* and that reported for the mouse *Mid1* by Quaderi and colleagues (Quaderi *et al.,* 1997; Dal Zotto *et al.,* 1998). Most striking was that we detected expression of *cMid1* in the myotome as well as very prominent expression in the muscle blocks of the limbs, whereas neither was mentioned for *mMid1*. Although it is not known whether the human *MID1* is expressed in muscle, one classic feature of the OS phenotype (i.e. dysphagia) could be explained by abnormal muscle morphogenesis or function. Limb anomalies, however, are uncommon in Opitz syndrome with upper limb dystonia and limb / digit shortening representing the few examples (Williams and Frias, 1987; Cox *et al.,* 2000). One other notable difference between chicks and mice is that we have

observed expression of *cMid1* in the heart, whereas Quaderi and colleagues highlight that the fetal mouse heart is devoid of *mMid1* expression (Quaderi *et al.,* 1997; Dal Zotto *et al.,* 1998). Finally, one of the characteristic clinical features of male OS patients is the genital anomaly of hypospadias. We have not been able to report whether *cMid1* is expressed in analogous developing tissue as, unlike mammals, the chicken embryo does not develop a genital tubercle until stage 30 and the genital tubercle is rudimentary until after hatching. For these reasons the chicken is not the best model system for studying the role of *cMid1* in formation of the various anomalies in the genitourinary system seen in Opitz syndrome.

The array of anomalies seen in patients harbouring loss of function mutations in MID1 highlights the critical role that this microtubule-associated protein plays in development of the craniofacial complex and other organ systems. Although we and others have recently implicated MID1 in the regulation of PP2-type serine/threonine phosphatase activity (i.e. the modulation of signal transduction events) and hence in the regulation of processes such as cell cycle progression and protein biosynthesis (Liu *et al.,* 2001; Short *et al.,* 2002), there are likely numerous other roles for MID1 which may become apparent with the identification of the other factors present in the MID1 macromolecular complexes. Our isolation of the highly conserved chick *Mid1* gene and demonstration that its developmental pattern of expression correlates well with the tissues affected in Opitz syndrome will provide new opportunities by which to investigate the role of this gene and the pathophysiological consequences of its mutation.

Materials and Methods

Isolation and Characterisation of Chicken Mid1 *Clones*

Full-length cDNA clones representing chicken *Mid1* were isolated by screening 10^6 pfu from an E4.5-5 whole chick embryo cDNA library. As a probe, a 1.4kb human *MID1* cDNA fragment was employed that was generated by PCR using the full-length human *MID1* cDNA as template with the 5'MID-fusion (5'-GT**G AAT TC**C TGA AGA TGG AAA CAC TGG AGT C-3'; position –7 to +17) and MIDdelCTD (5'-CTG **CTC GAG** CCC GCC TAG TTG ATG GCC TTS ACC-3'; position +1410 to +1386) primers. These primers were designed to incorporate either an *Eco*RI and *Xho*I restriction site (indicated in bold type in the relevant primer sequences) to facilitate cloning of the product. Positively hybridising plaques were purified through additional dilution and plating series before phage DNA was isolated. Automated DNA sequencing was performed by cycle sequencing with Applied Biosystems Dye Terminator chemistry and analysed on an Applied Biosystems 377 sequencer (Institute for Medical and Veterinary Science Sequencing Service, Adelaide). The entire sequence of selected positive clones was determined by a combined end-sequencing and primer walking strategy and assembled in DNAsis v2.0 (Hitachi). cDNA sequence analysis and searches were performed either locally using DNAsis v2.0 or via the internet using BLAST at the NCBI (http://www.ncbi.nlm.nih.gov/). The predicted amino acid sequence was also analysed using the web-based program Pfam.

In Situ *Hybridisation on Whole Mount and Serially Sectioned Chick Embryos*

For whole mount *in situ* hybridisation, we generated both digoxygenin-labelled sense and anti-sense RNA probes by *in vitro* transcription from templates derived by either *Not*I or *Nsi*I linearisation of clone 7B2 in pGEM7 (for the sense and anti-sense probes respectively). Hybridisation, washes and detection were performed on stage 19 - 21 chick embryos according to published protocols (Shen *et al.,* 1997). Appropriately stained embryos were prepared and, following passage through an increasing PBTX / glycerol concentration series, stored in 100% glycerol for photography.

For *in situ* hybridisation of serial sections, three different clones of the *cMid1* coding sequence were used to generate ^{35}S-labelled anti-sense RNA probes: a full-length 2.0 kb clone (9B1); the 1.4kb clone (7B2) representing the 3' half of the open reading frame linearised with *Nsi*I, and; a 350bp fragment from the 3' end encompassing nucleotides 1906 to 2255. This 350bp PCR fragment was synthesised using clone 7B2 as template with the following primers: 5'-CCG **GAA TTC** TCC CAA TAG AGC CT-3' and 5'-CCG **GAA TTC** TTT TCA GTT CTG GC-3' (*Eco*RI restriction sites incorporated into the primer sequences are in bold). The resultant fragment was isolated and cloned into the *Eco*RI site of pBluescript KS+. All three probes gave similar results. Chicken *Sox10* was generously provided by P. Scotting (Cheng *et al.,* 2000). MF-20 monoclonal antibody supernatant (Developmental Studies Hybridoma Bank, Iowa) was used undiluted. All embryos were fixed in 4% paraformaldehyde in phosphate buffered saline at 4°C overnight and then embedded in wax. Sections were dewaxed and alternate slides used either for *in situ* hybridisation or for immunocytochemistry. *In situ* hybridisation was done as described (Rowe *et al.,* 1991). MF-20 staining was detected using an alkaline phosphatase conjugated anti-mouse secondary antibody (The Jackson Laboratory, Bar Harbor, Maine) and immersion in 5-bromo-4-chloro-3-indolyl phosphate, *p* toluidine salt (BCIP), nitro blue tetrazoliumchloride (NBT) substrate.

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