Chapter Four

Characterisation of *doc*

4 Characterisation of *doc*

4.1 Introduction

In the previous chapter, the work performed to define the *doc* locus to a genomic region was described. Within this region, three candidate genes were identified: a novel gene, containing multiple WD40 domains, Cadherin-13 and Syntaxin-8. In this chapter, the work undertaken to define which of these genes is responsible for the *doc* phenotype is described, including MO knockdown of mRNA and *insitu* hybridisation to examine the expression profile of genes.

4.2 MO Knock-Down of Candidate Genes

Using previous observations concerning the gene size of cloned mutants and the number of identified alleles of the mutations, from the positional cloning of *sny, gup, sly* and *bal* (Coutelle et al., 2001; Parsons et al., 2002b; Pollard, 2002), the likelihood of each candidate encoding *doc* was examined according to the size of the candidate gene and the observed number of *doc* alleles. The outcome of such consideration was that Cadherin-13 and the multiple WD40 domain protein were considerably more likely to encode *doc* than syntaxin-8. As such, MO's designed against the ATG of the ensemble predicted genes were designed and used to examine the role of each gene in development.

MO's against both the ATG of Cadherin-13 and multiple WD40 domain protein (doc 1) were injected at a concentration of 8ng and 4ng in a volume of 1.4nl into embryos staged between fertilisation and 4-cell stage.

Injection of either 8ng or 4ng of Cadherin-13 MO resulted in no discernable phenotype at 24 hpf. However, injection of 4ng and 8ng of doc 1 MO resulted in a marked reduction of the A-P axis, defects in somite development, with somite blocks forming in a 'U' shape as opposed to the normal 'V' shape and a loss of proper notochord development. Thus, initial evidence suggested that the WD40 domain protein encoded *doc* (**Figure 4.1**).

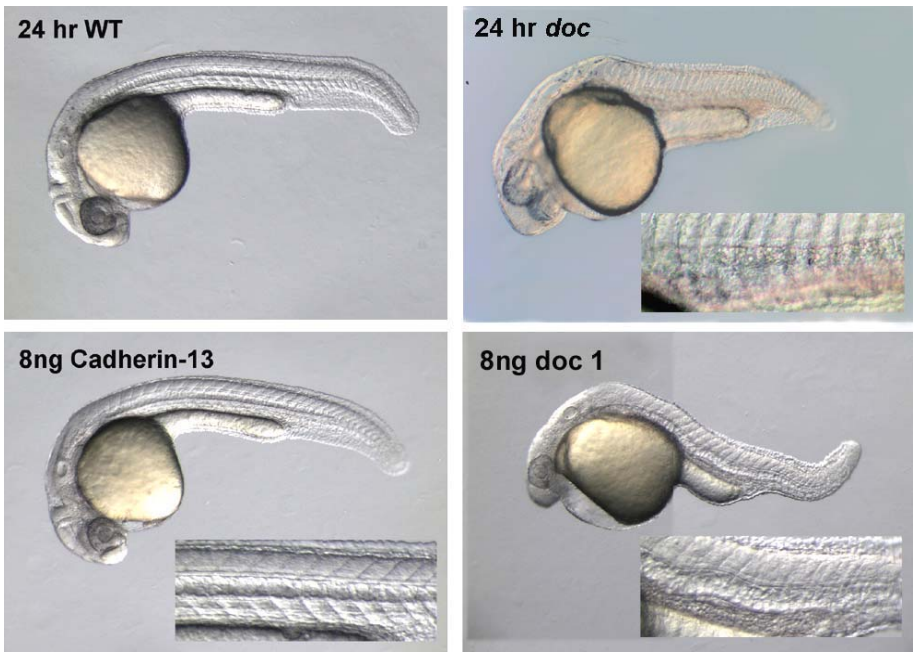

Figure 4.1 MO knock-down of candidate genes.

Lateral view, anterior to the left, ventral to the top, of live 24 hpf embryos. 8ng of Cad-13 shows no observable phenotype as 24 hpf. 8ng doc 1 shows mild phenocopy of *doc* (top right), with a lack of A-P extension, loss of notochord differentiation and 'U'-shaped somites. Boxes show enlarged notochords.

4.3 Analysis of the Novel Multiple WD40 Domain Gene

The ensemble predicted novel WD40 domain gene encoded a cDNA of 1654 bases, which in turn encoded a predicted protein of 447 amino acids. tblastn analysis of the zebrafish gene identified a novel *X. laevis* gene with considerable homology. Comparison of this gene to the zebrafish predicted cDNA demonstrated that the ensemble predicted sequence lacked approximately 500 bases of 5' sequence. By blasting the *X. laevis* protein sequence of this gene against the zebrafish EST database, additional sequence was identified. Compiling of this sequence generated a 2194 base cDNA encoding a protein sequence of 617 amino acids. Analysis of this sequence using Blastp, identified homologous proteins throughout evolution. Related mouse, human and *X. laevis* sequences showed 65%, 64% and 67% identity respectively. **Figure 4.2**.

Figure 4.2 Comparison of doc homologues

Zebrafish, *X. laevis*, Mouse and Human homologues of doc. Shaded bases differ from the zebrafish sequence. Compiled using seqman.

4.4 Additional MO Analysis of doc

Identification of the additional 5' sequence for the novel, WD40 domain protein generated a new initiation ATG, raising queries concerning the previously observed MO result. Analysis of the initial MO suggested that it was binding to an internal region within the mRNA, with the possibility that is was binding to a splice site. As such, a new start of translation ATG targeted MO (doc 2) was designed and injected at 8ng and 4ng in a volume of 1.4nl into embryos staged between fertilisation and 4-cell stage.

The observed phenotype for this new MO was highly similar to the previous phenotype, with embryos displaying shortened A-P axis, abnormal 'U' shaped somites and a lack of distinct notochord development. The only obvious difference between the doc 1 MO and the doc 2 MO was the severity of the phenotype. At 4ng, doc 1 produced a mild phenotype, with defects only becoming obvious at 8ng, whereas the doc 2 MO resulted in an obvious phenotype at 4ng, which became more severe at 8ng (**Figure 4.3**). This secondary doc MO, which binds to the start of translation, as opposed to the internal binding of doc 1 MO, results in a more severe phenotype at lower doses. Despite this, both MOs result in a lack of notochord differentiation, a reduction of AP extension and a loss of proper patterning of surrounding tissues by the notochord.

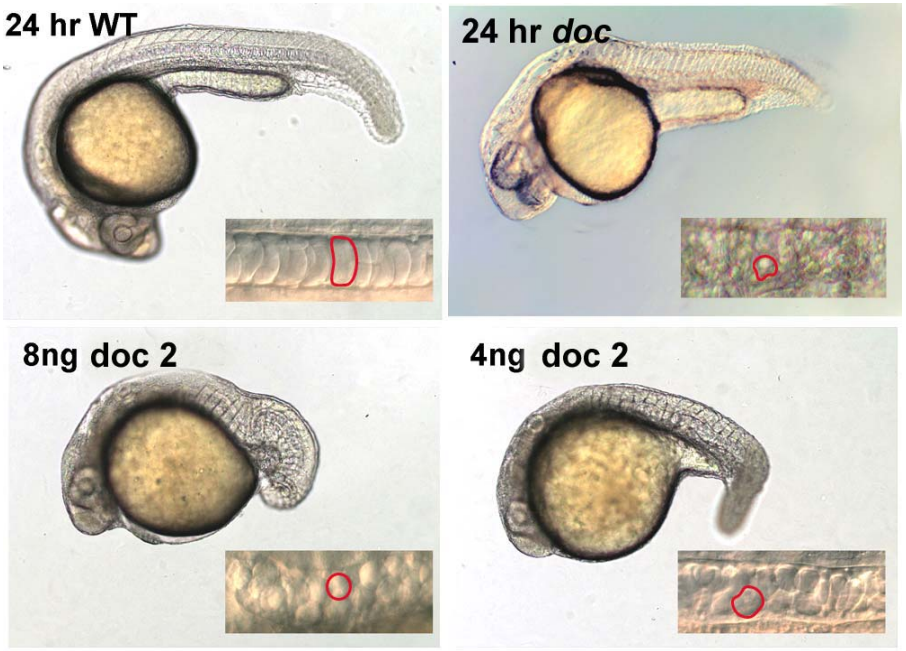

Figure 4.3 MO knock-down with secondary doc MO.

Lateral view, anterior to the left, ventral to the top, of live 24 hpf embryos. Secondary MO knock-down of the WD40 domain protein more accurately phenocopies *doc*. Boxes show enlarged notochords, red boundaries show outline of individual notochord cells. In *doc* mutant and 8ng and 4ng doc 2 MO injected embryos there is a lack of notochord differentiation, 'U'-shaped somites and a lack of A-P extension. At 8ng, the trunk completely fails to extend and the notochord can be seen to buckle as it fails to differentiate.

4.5 Expression of *doc* mRNA

Using the constructed doc protein sequence, primers were designed to amplify an 800 base fragment from wild type cDNA. From this, an *insitu* riboprobe was synthesised. This probe was then used to examine the expression of *doc* mRNA in 4-cell, shield stage, tail-bud stage, 6 somite, 14 somite and 24 hpf embryos.

The lack of expression at 4-cell stage demonstrated that this gene is not maternal. Expression analysis also noted a lack of expression at shield stage, tail-bud stage and 6 somite stage. However, there is observable expression specifically within the notochord at 14 somite stage, which is then extinguished by 24 hpf. Demonstrating that expression of *doc* is confined only to the notochord at 14 somite in the stages observed (**Figure 4.4**).

4.6 Notochord Differentiation in doc MO Injected Embryos

Whole mount *insitu* hybridisation of *echidna hedgehog* (*ehh*) was used to examine the differentiation state of the notochord, since the expression of early markers such as *ehh* is maintained in undifferentiated notochords (Stemple et al., 1996).

In 4ng doc 2 MO injected embryos, the expression of *ehh* is maintained specifically within the notochord of 28 hpf embryos. In 28 hpf wild type embryos, the expression of *ehh* is shut down in all but the most posterior tip of the developing notochord. Thus, is appears that MO knock-down of the muliple WD40 domain protein results in a lack of notochord differentiation, similar to that observed in *doc* mutants (**Figure 4.5**).

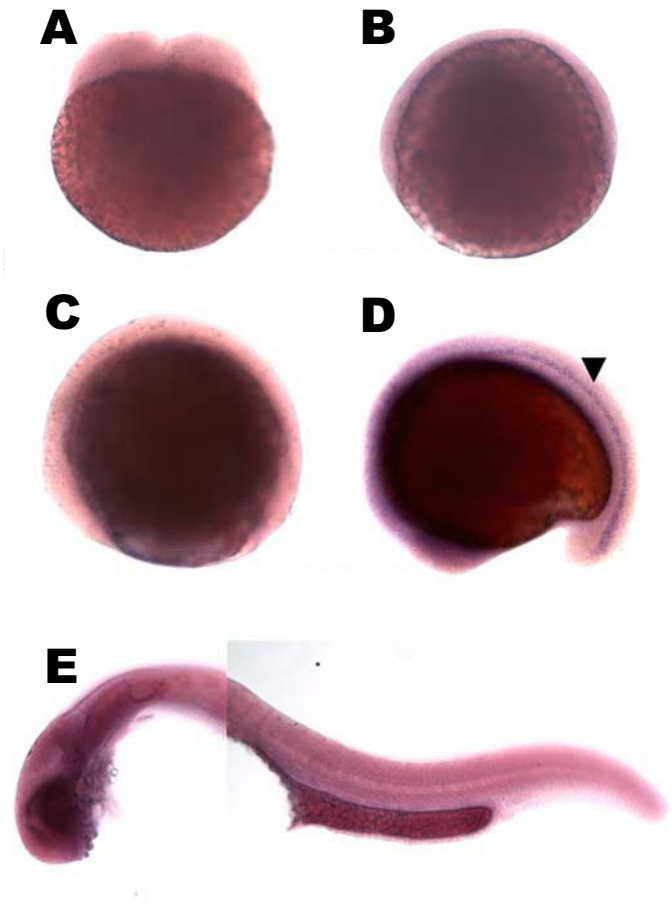

Figure 4.4 Staged expression profile of *doc*.

Lateral views of fixed, staged embryos at (**A**) 2-cell, (**B**) Tail-Bud, (**C**) 6 somite, (**D**) 14 somite and (**E**) 24 hpf. Expression of *doc* is noted only at 14-somite stage specifically within the developing notochord. There is no discernable expression at any other stage.

Figure 4.5 Expression of *ehh* in doc MO injected embryos.

Lateral view of fixed, 24hpf posterior trunk, anterior to the left, dorsal to the top. Expression of *ehh* in 28 hpf wild type embryos (A) is confined to the posterior most developing tip of the notochord. In 4ng doc 2 MO injected embryos (B and C), expression of *ehh* is maintained specifically in the notochord, demonstrating a lack of differentiation.

4.7 Discussion

Positional cloning of *doc* confined the mutation to a 0.5Mb region on linkage group 18. Within this region, several genes were identified from genomic sequence, two of which were deemed likely candidates. The two candidates were Cadherin-13, where cadherins are known interactors to basement membrane, and a novel gene containing multiple WD40 domains. Attempted antisense MO knock-down of Cadherin-13 demonstrated no observable phenotype, though it remains a possibility that the designed MO was unable to fully knock-down the gene product. However, knock-down of *doc* with two separate MOs resulted in embryos bearing significant phenotypic similarity to the *doc* mutants. Such knock-down resulted in 'U' shaped somites, a lack of AP extension and an obvious defect in the differentiation and enlargement of notochord cells. Examination of the this gene demonstrated highly conserved homologues throughout evolution, but was unable to assign function to the protein. Antisense MO knock-down with a secondary MO again phenocopied *doc* and provided stronger evidence that this novel gene encoded *doc*.

To examine the expression profile of *doc* during development an *insitu* riboprobe was generated and used to examine the expression of mRNA in 4-cell, shield, tail-bud, 6 somite, 14 somite and 24 hpf wild type embryos. In the stages examined mRNA was expressed only specifically within the notochord at 14 somite stage. At all other stages expression was absent throughout the entire embryos. So it appears that during the stages of notochord formation and development *doc* is only expressed within the notochord. Though the precise period of notochord expression of *doc* was not defined, the lack of expression in 6 somite and 24 hour embryos suggests that expression is confined to a period between ~11 hpf (6 somite) and 24

hpf. The expression of *doc* specifically within the notochord provides further evidence that it is functioning during notochord development and that mutation of this gene in *doc* embryos is responsible for the specific phenotype of mutant embryos. Since the defining feature of the 'dwarf' mutants is a lack of notochord differentiation, expression of *ehh* within the notochord of doc MO injected embryos was also examined. Expression of *ehh* was noted to be specifically maintained within the notochord of doc 2 MO injected embryos but shut down in wild type embryos of the same stage, thus demonstrating a lack of notochord differentiation in doc 2 MO injected embryos.

The observed cell autonomy of *doc* mutants (Odenthal et al., 1996) and its comparably complete basement membrane has suggested that it may function downstream of BM formation in signalling to the notochord cells and ensuring proper differentiation. Such a role for *doc* fits with the observed expression pattern, where there is an up-regulation prior to the final stages of notochord differentiation and before early markers are extinguished. Further to this, the lack of notochord differentiation, as demonstrated by maintained *ehh* expression, defines a role for doc in proper differentiation of the notochord. However, the novel nature of this gene makes predicting a role or function for this protein in the process of notochord development difficult. The protein demonstrates 14 WD40 domains, and hence may be acting by interacting with other proteins, possibly stabilising BM receptors or may function in organising the cytoskeletal network such that the cells are able to fix sufficiently to the sheath. Defining the precise nature of doc in the process of notochord differentiation is beyond the time scale of this thesis, but how this novel, conserved multiple WD40 domain protein is functioning in the establishment of a fully differentiated notochord remains of great interest.

Though the work presented in these two chapters provides strong evidence that *doc* encodes a novel multiple WD40 domain protein, final proof remains to be provided. Sequencing of the precise mutation from both mutanta cDNA and mutant genomic DNA would provide such evidence. Following that, dCAPS anaylsis can be used to rapidly confirm the presence of the mutation in multiple mutant embryos.

4.8 Summary

- MO knock-down of Cadherin-13 demonstrates no obvious phenotype.
- Antisense MO knock-down with doc1 and doc 1 MO phenocopies *doc* mutants.
- doc encodes a 14 WD40 domain, novel and conserved protein.
- *doc* mRNA is expressed specifically within the notochord at 18 somite stage.
- Antisense MO knock-down of *doc* results in the maintenance of *ehh* in the notochord at 28 hpf.