Chapter 6:

Gene knockdown in zebrafish as an assessment of candidate gene function

CHAPTER 6

GENE KNOCKDOWN IN ZEBRAFISH AS AN ASSESSMENT OF CANDIDATE GENE FUNCTION

6.1 INTRODUCTION

One means of assessing the role of a gene is to somehow prevent it from performing its normal function and observe the outcome. In the mouse this can be a slow and expensive process since knockouts require several generations before a phenotype can be assessed. The zebrafish (*Danio rerio*) offers several advantages in this respect. The fish have a much shorter life cycle and produce large numbers of offspring (about 200 eggs per lay), making them amenable to large-scale screening. Zebrafish eggs are fertilised externally and the embryos are transparent, allowing for continuous study of embryonic stages, impossible in the mouse where development takes place *in utero*. In addition to these practical advantages, the techniques available for causing specific gene knockdown or knockouts in zebrafish are much simpler and faster than those which have been demonstrated as effective in the mouse. Use of antisense technology allows for the screening of a phenotype in the treated individuals without the need for further breeding and the relatively large zebrafish oocytes are well suited for the injection of antisense agents.

Hence the zebrafish represents a rapid and cost efficient model for the assessment of function in a relatively large number of genes, though the success and validity of such an approach is dependent on two factors. The first of these is the capacity to identify homologous genes within the zebrafish genome which show enough similarity to the mouse genes under scrutiny to allow their functions to be assumed to be similar. This question is complicated by the duplication event which the teleost genome underwent

following its divergence from the mammalian lineage (Postlethwait *et al.* 1998; Postlethwait *et al.* 1999). This means that the zebrafish often carries two or more paralogous copies of a particular gene, each of which is homologous to a mammalian counterpart. It is thought that the duplicated genes have been maintained as a result of a division of function between the two copies, with each taking on a more specialised role (Force *et al.* 1999). Despite this, paralogous pairs of genes often retain some shared functions, meaning that zebrafish phenotypes resulting from a single gene defect may be appear to be less severe than in the mammalian ortholog. The implication of this when selecting genes to target in the zebrafish is that when more than one presents as being a possible ortholog, both should be considered suitable candidates and the effects of knocking down both observed. It may also be prudent to knock down both concurrently in order to assess the phenotype in the absence of either function.

The second factor is the ability to recognise a phenotype analogous to the hearing loss and vestibular dysfunction shown by *bronx waltzer* mouse mutants in the fish, a premise which assumes a conservation of function in the ear of the two organisms and demands that fish with a hearing or vestibular defect are discernable from unaffected littermates. These considerations are discussed in the following paragraphs.

6.1.1 The zebrafish ear

Zebrafish possess an inner ear which is roughly analogous to the vestibular systems of other vertebrates. While it does not include a specialised structure for acoustic sound detection, the otic placode contains maculae for the detection of gravity and semicircular canals for detecting dynamic rotation. In addition, zebrafish are members of the Otophysi, a group of ostariophysan fish that possess Weberian ossicles, four small bones which link the swim bladder to the inner ear (Bang *et al.* 2001). These enhance the fish's ability to hear by transferring vibrations to the ear in a similar manner to the ossicles in

mice and humans. Indeed, the otophysans are sometimes known as "hearing specialists" and may be sensitive to a frequency range between 100 and 5000Hz (Fay and Simmons 1999). The sensory patches of the zebrafish ear are remarkably similar to those of humans and mice, with cristae located within each semicircular canal and maculae beneath each of the otoliths (see Figure 6.1d). Each of these sensory patches possesses highly organised stereociliary bundles which detect movement in their surroundings and produce an electrical transduction in response. This conservation of function, alongside the other advantages offered by the zebrafish as a model organism, make it a very attractive system for the study of hearing dysfunction.

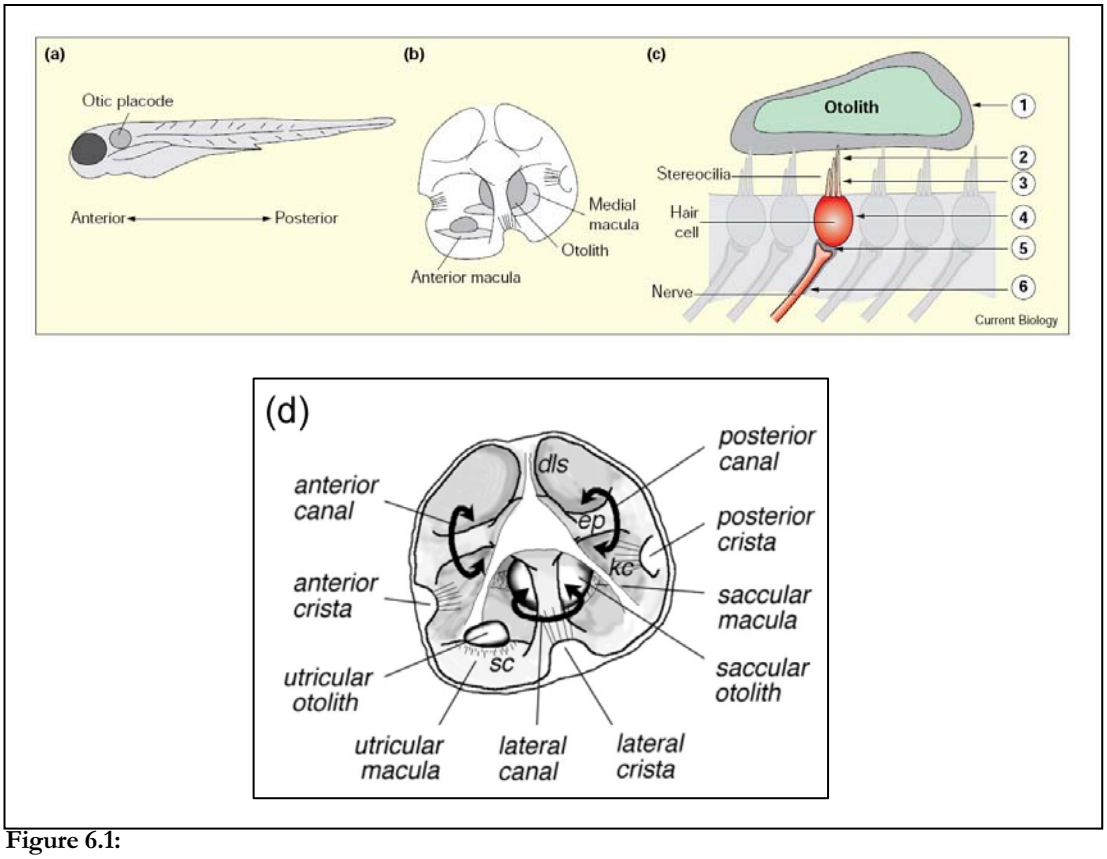

Figure 6.1:
(a) The zebrafish ear. At five days, the embryo is about 3 mm long, with an evident otic placode.
(b) The otic placode, behind the eye, measures about 150 mm across at this stage; it contains hair cell epithelia as maculae and in the semicircular canals.
(c) Sensing by hair cells requires integrity at multiple sites: 1, otoliths; 2, transducer complex in stereocilia; 3, stereocilia; 4, hair cell development; 5, synapse; and 6, auditory pathway (Ashmore 1998)
(d) Detailed representation of the zebrafish ear at 4 days post fertilisation: ep, epithelial pillars; dls, dorsolateral septum; sc, stereociliary bundles; kc, kinocilia of cristae hair cells. (Whitfield *et al.* 2002)

6.1.2 Hearing and vestibular phenotypes in zebrafish

In order to successfully use the zebrafish as a model in the search for the *bronx waltzer* gene, it must be possible to measure hearing and vestibular function in the fish to determine whether the knocked down gene has caused a phenotype similar to that observed in the mouse. The two measures used to assess the phenotype in mice are the Preyer reflex test for auditory function and the observation of circling and head-bobbing behaviour as an indication of vestibular abnormality (see Section 3.1.1.4) and equivalent assessments of both features are possible when working with the zebrafish. In response to a sharp sound such as the tapping of a metal implement on the side of the dish, wild type zebrafish perform a startle response similar to the Preyer reflex which takes the form of a rapid tail flick. This can normally be observed as a brisk darting movement and its absence is immediately apparent, an illustration of this response is shown in Figure 6.2. Behavioural observations of zebrafish with vestibular dysfunction demonstrate striking similarities to those made with deafness and balance mouse mutants. The fish are seen to rest on their sides or upside-down and may swim in circles or loops. Some of these mutants may be unviable beyond approximately 5 days post fertilisation (dpf) since their lack of spatial orientation inhibits them from inflating their swim bladder and engaging in normal feeding behaviour.

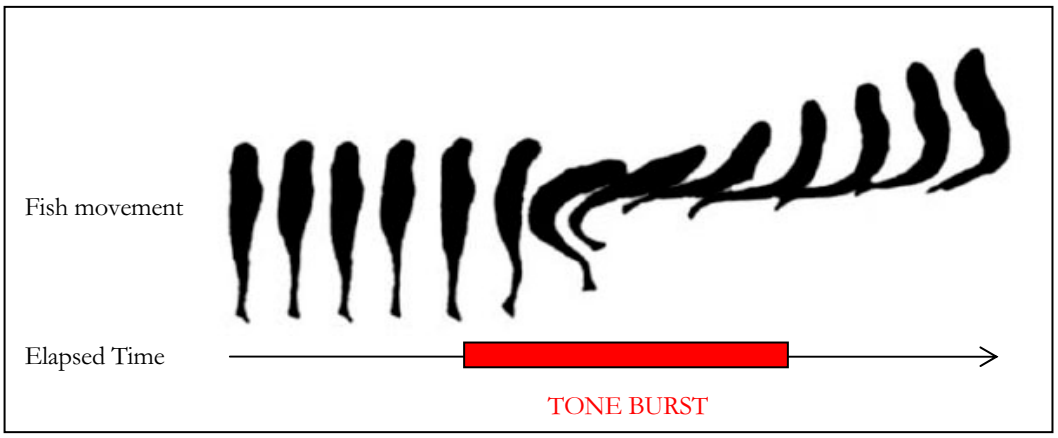

Figure 6.2: An illustration of the startle response in zebrafish adapted from (Bang *et al.* 2002) The drawings show the movement made by a fish when exposed to a short tone burst, with images from left to right showing the position as time elapses. The fish responds to the noise by a sharp flick of the tail which results in a rapid forwards darting movement.

6.1.3 Conservation of function in the mouse and the zebrafish

As described in Section 6.1.1, the zebrafish ear can be considered to be roughly analogous to the vestibular system in the mouse. Since the *bronx walter* mutation affects both the auditory and vestibular systems, it might be expected that a defect in the zebrafish orthologue would give a comparable phenotype, although this expectation is based on the assumption that the functioning of the ear is somewhat conserved between the two species. In considering the validity of such a supposition it is pertinent to examine the group of zebrafish mutants identified in a recent screen which exhibited defective balance yet appeared to possess structurally normal inner ears. It was thus thought that their phenotype may be a result of a disruption to the mechanotransduction apparatus in the ear (Granato *et al.* 1996). Analysis of these circler mutants (Nicolson *et al.* 1998) demonstrated that they fell into 8 discrete linkage groups and showed that the phenotype included hair cell degeneration or bundle defects in three of the mutants, *skylab*, *sputnik* and *mariner*. These mutants are of particular interest here since the *bronx walter* mutation causes degeneration of hair cells in both the cochlea and the

vestibular system and we wish to determine whether the development and functioning of this system is conserved between the mouse and the zebrafish. The cloning of the *mariner* gene by a candidate gene approach (Ernest *et al.* 2000) demonstrated that the five alleles of the mutation, which cause affected fish to circle, all possessed mutations in *Myo7a*. This gene is also responsible for the *shaker1* mutation in mice (Gibson *et al.* 1995) and for Usher syndrome type 1B as well as autosomal dominant and recessive forms of non-syndromic hearing loss in humans (Weil *et al.* 1995; Liu *et al.* 1997; Liu *et al.* 1997; Weil *et al.* 1997). The phenotype of *shaker1* mice includes circling and deafness as a result of stereocilia disorganisation and bundling defects of the hair cells, indicating that the *Myo7a* gene may play a similar role in both organisms. More recently, the defects in *sputnik* fish which show splayed stereocilia and reduced acoustic sensitivity (Nicolson *et al.* 1998) have been demonstrated to be the result of mutations in *cadherin 23* (Söllner *et al.* 2004). Mutations in *cadherin 23* are also responsible for the phenotype of *waltzer* mice (Di Palma *et al.* 2001; Wilson *et al.* 2001) and for Usher syndrome type 1D in humans (Bolz *et al.* 2001; Bork *et al.* 2001). *Waltzer* mice are deaf, exhibit circling behaviour and manifest disorganised stereocilia, suggesting that the function of *cadherin 23* in the inner ear may also be conserved in mice and zebrafish. Whilst the causative agent for the third circler mutant with normal gross ear morphology, *skylab,* has yet to be identified, a number of other genes and pathways have been demonstrated to be important in the development of sensory epithelia in both model organisms. These include members of the Delta-Notch signalling pathway such as *deltaA* (Riley *et al.* 1999) and mind bomb (Itoh *et al.* 2003) in the zebrafish, and *Jagged1* (Kiernan *et al.* 2001; Tsai *et al.* 2001) and *Delta1* (Morrison *et al.* 1999) in the mouse. This pathway is known to be crucial in determining the fate of cells in sensory epithelia, with alterations in the expression of the different ligands being strongly associated with the development and patterning of hair cells and supporting cells, and evidence to support this model is strong in both the mouse (Zine *et al.* 2001) and the zebrafish (Haddon *et al.* 1998). Although many genes involved in

inner ear development are yet to be identified and whilst some have only been characterised in one organism or the other, with such an extensive degree of overlap between those so far described it is possible to imagine that there exists a significant level of conservation of function between the mouse and zebrafish ears, and between the genes encoding them.

6.1.4 Disrupting gene function in zebrafish

Since the publication of much of the sequence of the human genome (Lander *et al.* 2001), as well as the progress made with the sequencing of the genomes of model organisms such as mouse (Waterston *et al.* 2002) and the as yet unfinished zebrafish, much research has focussed on the elucidation of the roles played by genes for which the sequence but not the function is now known. To accomplish this, a variety of forward and reverse genetic approaches have been developed to disrupt individual genes and allow the effects to be observed within a model system.

Both forward and reverse genetic approaches have been successfully demonstrated in the zebrafish. Forward genetic approaches have included large scale mutagenesis screens using ENU (Haffter *et al.* 1996) or insertional mutagenesis (Amsterdam *et al.* 1999) followed by selection for phenotypes of interest and identification of the causative mutation. Meanwhile reverse genetic strategies have included TILLING (Targeting Induced Local Lesions IN Genomes) (Henikoff *et al.* 2004) as well as the extensive use of antisense technologies such as morpholinos (Ekker 2000). Reverse genetics offers the advantage that the identity of the disrupted gene is known and can be specifically targeted, and when carried out using antisense it can also give rapid results since a phenotype is seen in the experimental subject with no requirement for further breeding before the effects of the disruption can be assessed.

6.1.5 Morpholinos

Morpholino oligonucleotides (MO) are non-ionic DNA analogs which possess altered backbone linkages compared with DNA and RNA. In place of a deoxyribose or ribose sugar they possess a morpholine ring and individual subunits are joined by non-ionic phosphorodiamidate linkages. Figure 6.3 illustrates the structure of a morpholino oligo in comparison to a DNA molecule of the same length. They were devised by James Summerton in 1985 and were designed to address the problems encountered with other antisense agents such as sequence specificity and varying activity levels (Summerton and Weller 1997).

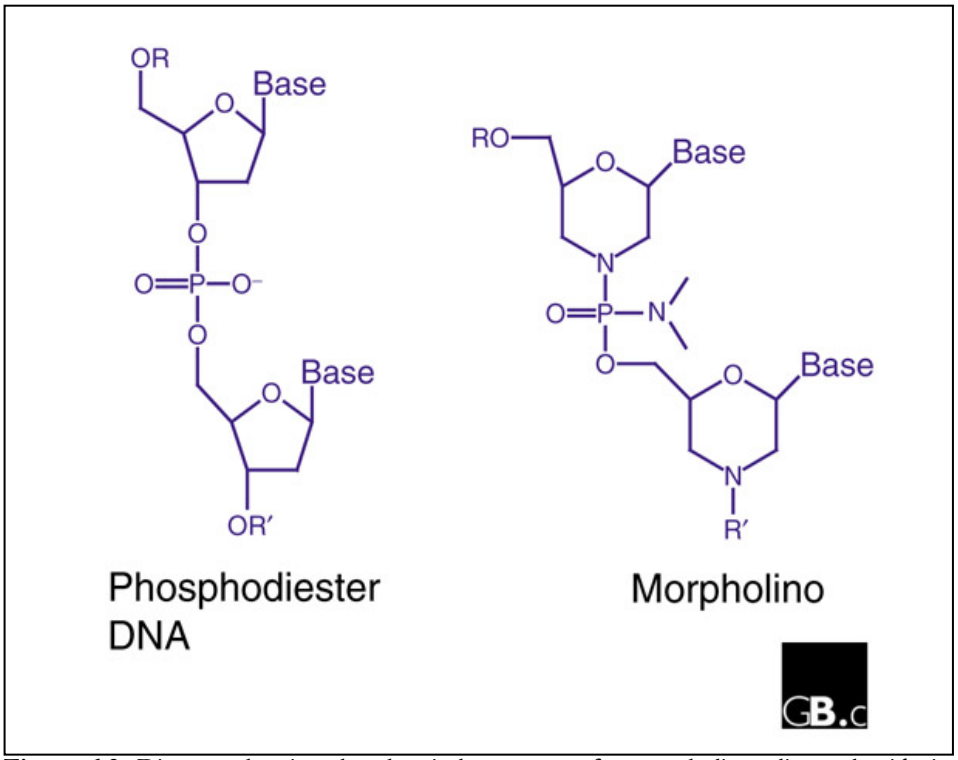

Figure 6.3: Diagram showing the chemical structure of a morpholino oligonucleotide in comparison to DNA. In each example two nucleotide units are joined by an intersubunit bond. R and R' denote continuation of the oligomer chain in the 5' and 3' direction, respectively (from Corey and Abrams, 2001).

Morpholinos bind to complementary nucleic acid sequences via Watson-Crick hydrogen bonding with a similar affinity as for DNA and RNA oligomers and

operate by steric blocking. When designed to block translation they inhibit the binding of the ribosome to an mRNA molecule, but can also be used to target nuclear processing events when designed to bind to splice junctions. The mechanisms involved and factors to be considered when designing morpholinos are discussed in subsequent sections.

6.1.5.1 Advantages of morpholinos

Ideally, a gene targeting technique should be highly specific, straightforward to perform, competent for action in all cell types, efficient at depleting the selected protein, amenable to the targeting of many genes, have little or no non-specific effects, and be reproducible. Most antisense agents fit some of these criteria, with all of them being simpler to administer and more amenable to the targeting of many genes than mutagenesis schemes. Morpholinos have recently become widely used as a result of their many advantages over other antisense technologies. One of the most important of these is that the alteration of the backbone makes them resistant to degradation by nucleases. Most other antisense molecules are targeted for degradation but the morpholine backbone is not recognised by the nucleases and can thus remain present for much longer in the cell (Hudziak *et al.* 1996). This allows the effects of the targeted knockdown to be observed over a much longer time period, thus greatly expanding the possibilities of their application. Another advantage of their altered structure is that the phosphorodiamate linkage which joins individual subunits has no charge and promotes efficient base-stacking, thus minimising any non-specific cellular activity while maintaining good solubility properties (Summerton and Weller 1997).

Morpholinos also offer higher specificity at lower concentration than RNase H-mediated systems such as DNA and S-DNA oligos. Since DNA/RNA and S-DNA/RNA duplexes of only 5 base pairs in length can be cleaved by RNase H (Crouch and Dirksen 1982), there is a high probability that non-target mRNAs will be cleaved, resulting in unpredictable disruption of normal

cellular processes. In addition, the cleavage process releases the antisense oligo in its original form, allowing a single molecule to trigger many non-specific cleavage events. By contrast, the steric blocking mechanism employed by morpholinos requires very close sequence homology in order to be effective, since the oligo will be displaced by the ribosome if it is improperly bound to the target as a result of basepair mis-matches (Hudziak *et al.* 2000). In addition, since morpholinos are effective only when targeted to specific regions of the mRNA molecule (see sections 6.1.4.2 and 6.1.4.3), their potential for causing non-specific effects is greatly reduced. A morpholino need only distinguish its target from the 2%-5% of cellular RNA sequences comprising these targetable regions, as opposed to the full complement of RNA sequence which can be bound to and marked for degradation in an RNase H-mediated approach. An additional advantageous property of morpholinos is their ability to successfully invade and bind to the stable secondary structures sometimes found within leader sequences which can reduce the binding efficiency of other antisense molecules. This point is illustrated in Figure 6.5

While morpholinos have effectively superseded the majority of other antisense approaches, the use of RNA interference (RNAi) has recently become widespread in many organisms (reviewed in Campbell and Choy, 2005). However, Zhao and colleagues (Zhao *et al.* 2001) showed that RNAi was not suitable for use in zebrafish embryos. Using dsRNA designed to block the maternal gene *pouII-1*, the transgene GFP, and an intron of the zebrafish gene *terra*, they showed by *in situ* hybridisation that degradation of both co-injected and endogenous mRNA occurred without sequence specificity. All three targeted sequences gave extensive non-specific effects, while transgenic embryos injected with dsRNA targeted to GFP did not show reduced GFP expression. As a result of this and the other advantages previously discussed, morpholinos have become the antisense tool of choice when studying the zebrafish.

6.1.5.2 Blocking translation (targeting start sites)

Morpholinos designed to block translation operate by displacing the ribosomal complex, preventing it from processing the targeted mRNA. This principal is illustrated in Figure 6.4.

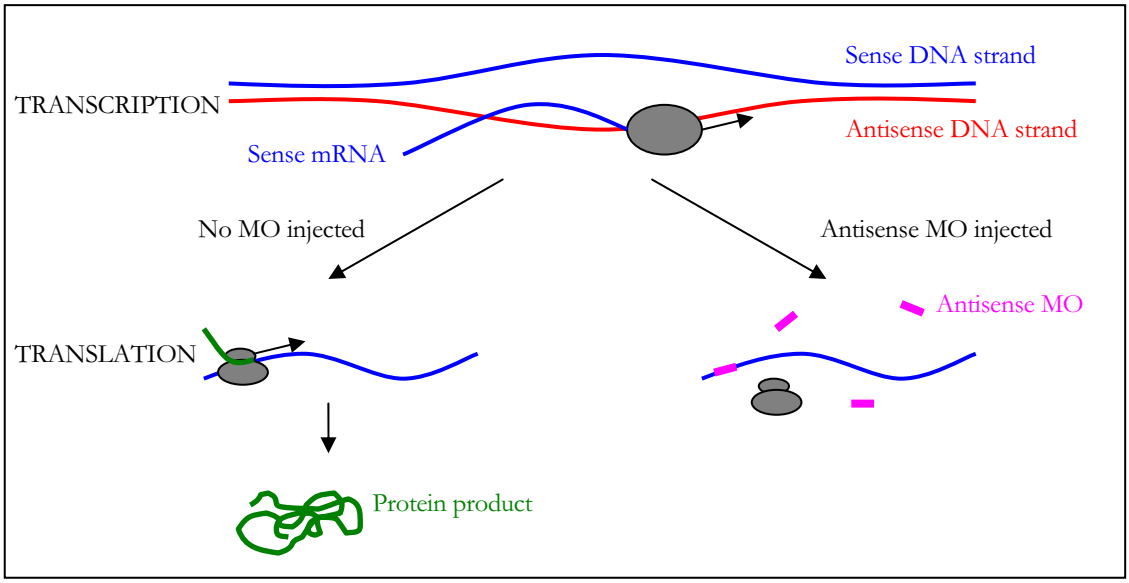

Figure 6.4: Diagram showing the mechanism by which a morpholino (MO) targeted to the start site of a specific mRNA molecule blocks its translation. The complementary binding of the MO to the translational start site inhibits the ribosomal complex from adhering, thus preventing the formation of a protein product.

The most effective translation-blocking morpholinos are those designed at or immediately preceding the AUG translational start site which prevent the initial formation of the ribosomal complex. Those targeted at sequence further downstream show a large reduction in inhibition efficiency which is thought to be a result of the powerful unwindase activity associated with ribosomes following their full assembly at the AUG start site (Nishikura 1992). Figure 6.5 shows the relative inhibition efficiencies of MOs complementary to a series of sections of the leader sequence of Hepatitis B mRNA and illustrates the importance of targeting a morpholino to the correct section of sequence. It also demonstrates the ability of MOs to effectively invade stable secondary structures in mRNA molecules.

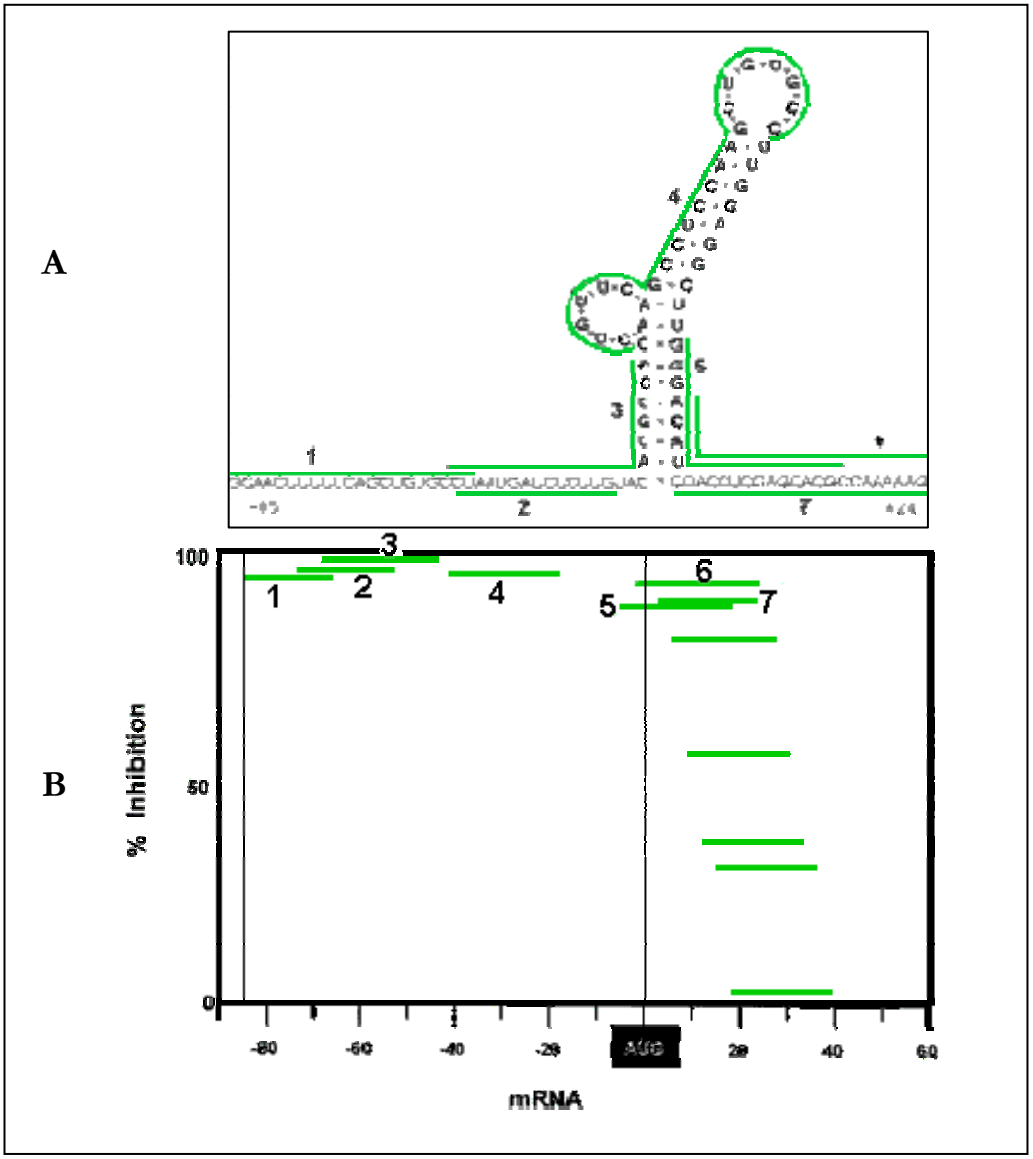

Figure 6.5: Diagram summarising the results of a study to determine the most effective target sites for morpholinos designed to block translation.
The leader sequence of Hepatitis B mRNA is shown in **A**, with the locations of the seven MOs to be tested shown by numbered green lines. In **B**, the inhibition efficiencies of each MO are shown by their position on the y-axis, with the x-axis representing their position on the mRNA molecule relative to the AUG start site. The data demonstrate that MOs are most effective when targeted to sequence either upstream of or immediately subsequent to the AUG start site, with a steep drop in efficiency occurring when the target site is further downstream. This is illustrated by the unnumbered MOs in **B**, which show much lower % inhibition despite being only a few base pairs 3' of MO7. This is thought to be a consequence of the unwindase activity associated with ribosome complex after full assembly at the translational start site. In addition, this experiment illustrates the ability of MOs to bind to mRNA molecules even in the presence of stable secondary structures. (Data and diagrams from Gene Tools – http://www.gene-tools.com)

6.1.5.3 Blocking nuclear processing (targeting splice junctions)

Morpholinos which target mRNA start sites and block their translation can be expected, if achieving efficient inhibition, to cause an almost total absence of the targeted protein product in the developing organism or cell. Many mutations do not have such a dramatic effect since the affected protein may still be partially functional, and thus one may see a different phenotype in an organism carrying a mutation and one which has been injected with a MO targeting the start site of the same gene. A second approach to using morpholinos is to instead target them to splice junctions with the intention of blocking nuclear processing and resulting in a modified but not completely non-functional protein product. Since the purpose of this study is to replicate the *bronx waltzer* phenotype in the zebrafish, and since the nature of the *bv* mutation is unknown, it was thought prudent to also follow this alternative method of gene knockdown using morpholinos.

Morpholinos targeted to splice junctions operate by blocking the progress of the nuclear splicing apparatus and typically cause either the exclusion of a single exon or the activation of a cryptic splice site (AG or GT sites within adjacent exons or introns) which results in the inclusion of a part of the exon or an intron. This principal is illustrated in Figure 6.6

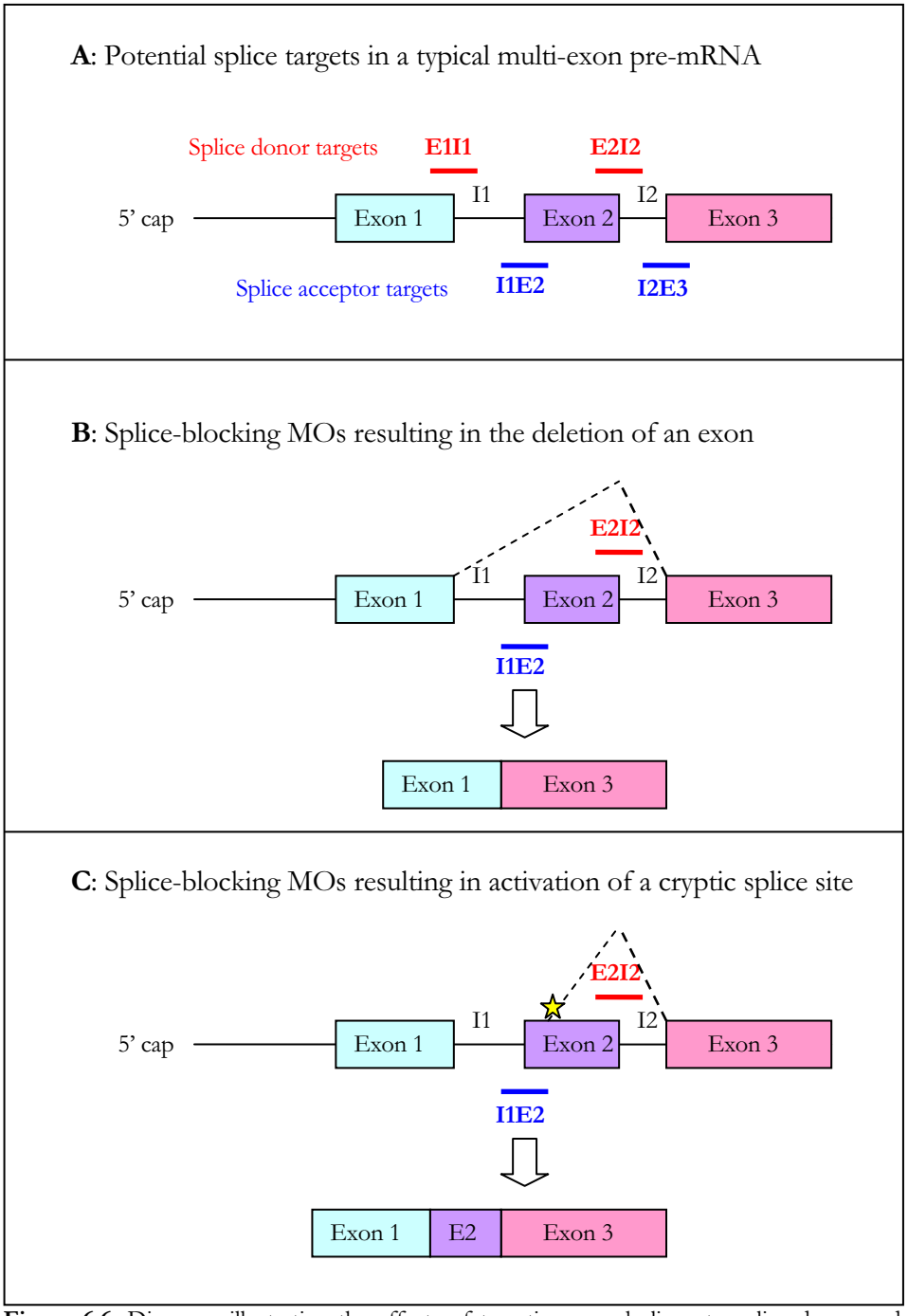

Figure 6.6: Diagrams illustrating the effects of targeting morpholinos to splice donor and acceptor sites on nuclear processing. Figure **A** shows the locations of possible target sites, while **B** and **C** illustrate the potential outcomes of using the most predictive of these sites, the acceptor and donor sites of the exon to be targeted. In **C** the cryptic splice site within exon 2 is represented by a yellow star.

A further property of morpholinos targeted to splice junctions is that they will exert their inhibitory effects only on mRNAs produced by the embryo, since any maternally inherited mRNAs will have already undergone nuclear processing. This can make it possible to observe the more subtle effects of a gene which may result in lethality if its presence is required during early development.

6.2 METHODS

6.2.1 Selection of target genes

The *bronx waltzer* candidate region comprises a total of 53 annotated genes of which 31 have been previously characterised. Even for a relatively rapid method such as the generation of morphants by injection of morpholinos this would represent a significantly larger allocation of time and resources than was available for this project. Therefore it was decided that a subsection of the genes which seemed most promising as candidates for the *bv* causative agent would be investigated using this approach. The criteria used for selecting the most interesting of the candidate genes are described in Chapter 5: Candidate gene assessment. The genes initially chosen for investigation by knockdown using morpholinos targeted to start sites in zebrafish orthologues were *Foxn4, Tcf1, Citron, Ube3b, Myo1h, Cabp1, Paxillin* and *Trpv4*. Later, when designing morpholinos to splice junctions, new published data warranted the additional inclusion of *Git2* and *Kctd10*.

6.2.2 Identification of orthologous genes

The first part of this study, comprising the targeting of morpholinos to the start sites of candidate genes, was carried out several months prior to the second part, the targeting of splice donor sites within the genes. During this time, progress was made in the quality of zebrafish sequence available and also in the accessibility of large-scale computational data relating to predicted orthologs. As such, the approaches followed when selecting zebrafish genes to target were different in each case. In addition, during the interim period a number of new genes emerged as interesting candidates and so were included in the splice donor MO analysis but not in the start site MO study.

6.2.2.1 Morpholinos targeted to start sites

At the time that this study was begun, the sequence data available for the zebrafish consisted mainly of un-annotated public domain sequence reads. Therefore the strategy employed to identify zebrafish genes showing good homology to the mouse genes of interest made use of the sequence alignment and comparison program BLAST (Basic Local Alignment Search Tool).

Peptide sequences of the mouse genes were obtained in FASTA format and used to query the continually updated database of zebrafish ESTs using the WU-BLAST program tblastn (Gish, 1996-2003; http://blast.wustl.edu). This program enables a peptide sequence to be searched against a nucleotide database by first converting it into all the possible nucleotide sequences which might encode it, thus allowing degeneracy in the genetic code to be ignored. Those ESTs which were returned with a score of 90% or greater in respect of similarity to the original sequences were then themselves obtained as FASTA files. These were imported into the genome assembly programme Gap4 (Bonfield *et al.* 1995) and aligned into contigs in order to obtain as large as possible a portion of the expressed sequence and to identify location of the start site. If the start site was not immediately located in this manner then the contiguated sequence was used to search the EST database once again, this time using blastn to identify overlapping sequences and build on the contig until the start site was found. At this stage, the sequence was used to query the full genomic sequence database in order to find the upstream sequence and allow the design of a morpholino to block the start site.

6.2.2.2 Morpholinos targeted to splice junctions

When morpholinos were being designed to target splice junctions, a significant amount of new data regarding the zebrafish genome had recently become available. Within the Ensembl Zebrafish database (http://www.ensembl.org/danio_rerio) many genes had been analysed for

similarity to genes within other genomes and were assigned a score depending on the confidence of the ortholog prediction. In addition, many genes had been annotated with predicted exon and protein structures, making it possible to identify protein domains to disrupt and splice junctions to target.

6.2.3 Morpholino design

6.2.3.1 Designing morpholinos to start sites

In order to achieve optimum efficiency, a morpholino intended to block translation of a particular mRNA should be targeted as closely as possible to the AUG translational start site (see section 6.1.4.2). With this in mind, when designing morpholinos to start sites the first 25 bases of coding sequence (including the start codon) was analysed and then the 25-base window was slid upstream until a 25-base target was found that satisfied the requirements. An optimal MO should have 40-60% GC content and no significant self-complementarity to avoid dimerisation. In addition, it should not contain stretches of 4 or more contiguous G residues which can cause problems with solubility. If no suitable 25-mer was found, the size of the window was reduced until a stretch of sequence was found to satisfy the criteria. Once an appropriate target site was found, its reverse complement was determined and this sequence used as the template for MO synthesis. The sequences of the translation-blocking morpholinos used in this study are given in Table 6.1

Gene	MO sequence
Foxn4	GCAGCGCGAGCCGTGATGAGAGGAG
Myo1h	TCCATAGAAATTCCCCGATTCTTTC
Ube3b	CATCGAGCAGAGATCAATGTAAGTA
Trpv4	GAAGAGCACCTCGTGCCGAAT
Cabp 1	CGATTTAACACAGTTTCCCATAGTA
Cabp 5	GTCTACGTCTCTTCTTTAAGCTGGA
Citron A	TTCTCTCCGCTCCTAGCGACATTTT
Citron B	TTAACTTCCACTCGACCTCAAACTG
Paxillin A	TAAATCGTCCATGTTTCGCGGTCGT
Paxillin B	TCGTCCATGTTTCGCGGTCGTTCTG
Tcf1	CTCTCCTCCGTCCATCCTCCTAGAT
Cdh23	CTCCCGAACCTTCACACCACGACAT

Table 6.1: Sequences of morpholino oligonucleotides designed to block translation of *bronx waltzer* candidate gene orthologs in zebrafish.

6.2.3.2 Designing morpholinos to splice junctions

In designing morpholinos to interfere with nuclear processing, it was first necessary to identify a suitable exon to target. This was achieved by studying

the known or predicted protein domains of each gene and selecting an exon which would disrupt a domain essential for normal protein function. Sequence was then obtained from the splice donor site to include the last 25 bases of the exon and the first 25 of the following intron and this was analysed in a similar manner to that described in Section 6.2.2.1. In this case, targets were selected to span the GT splice donor site rather than the AUG start site but other aspects of MO design remained the same. The sequences of the morpholinos used in this study to target mRNA splicing are given in Table 6.2

Gene	Protein domain targeted	MO position	MO sequence
Foxn4	TF; Forkhead	E2I2	CCCTTACTTCAAGGTGAGGAATTTG
Myo1h	Myosin head	E3I3	TTGGACCAACCTCTAACACAGGGT
Ube3b	HECT domain	E19I19	ATTATTACTGACCTTAATGGAGGTG
Trpv4	Cation channel; TrpL	E11I11	CTTATTGGAATACCTGAAGCATAAC
Cabp1	Calflagin; EF hand	E2I2	CAGACACTGAAACTTACAGTTCATA
Git2	ATP_GTP_A	E6I6	CATCTCCCTCACCTGGCGTAGTCAA
Paxillin A	Paxillin	E4I4	GTCTCTGTGTTTACCTGTAGTGGAG
Paxillin B	Zn-binding LIM	E2I2	GTGTAACGCTTACGTCCAGAATGGG
Citron	Pleckstrin homology	E8I8	GAGTGATGGTGTACCTTCAGTGGGC
Kctd10	K+ channel tetramerisation	E2I2	GTGTGTACTTTACCTGCAATGCTGC
Tcf1	Homeobox; HNF1b_C	E4I4	GTGAATGTATCAGTACCTTGCATCT
Cdh23	Cadherin	E4I4	TGTTTGAGACACTCACCCACTGGTG

Table 6.2: Sequences of morpholino oligonucleotides designed to block splicing of *bronx waltzer* candidate gene orthologs in zebrafish. Cdh23 has not been fully characterised in the zebrafish, the sequence of the splice MO used here was taken from the work of Söllner et *al.*, 2004.

6.2.4 Administration of morpholinos

6.2.4.1 Morpholino preparation

Morpholinos were obtained as 300 nanoMolar aliquots of freeze-dried, lyophilized oligonucleotides from Gene Tools, LLC (Philomath, OR, USA) and on arrival were resuspended in 60µl RNase-free water. Of this stock solution 1µl was diluted in 800µl HCl and its optical density read at λ 265nm. This figure was used to calculate the concentration of the stock using the equation below. The molecular weight and molar absorbance are calculated

from the nucleotide composition of the morpholino and are supplied with the oligonucleotides.

$$\text{Morpholino Concentration} = OD_{\lambda 265} \times \frac{\text{Molecular Weight}}{\text{Molar Absorbance}}$$

The volume of stock solution required for a single dose at the required concentration was calculated using the following equation:

$$\text{Volume of stock solution} = \frac{\text{Required Dose}}{\text{Concentration of stock} \times \text{Dosage volume} \times \text{Total volume required}}$$

The remaining volume was made up with Morpholino Buffer and was calculated thus:

$$\text{Volume of buffer} = \text{Total volume} - \text{Volume of MO stock}$$

Morpholino buffer was composed of one part 25mg/ml phenol red stock and four parts 5mM HEPES (pH7.2) and 200nM KCl. Buffer was filter sterilised before being added to the morpholino stocks. Initially, 7µl of injection aliquots were made up at a dilution of 8ng/1.4nl, with 1.4nl being the calibrated single dosage. These were then serially diluted with 3.5µl being taken and added to a further 3.5µl of Morpholino buffer to give aliquots which would deliver 4ng, 2ng, 1ng and 0.5ng in a 1.4nl dose. Morpholino stocks and injection aliquots were both stored at -20°C.

6.2.4.2 Morpholino injection

Injection needles were prepared from filamented borosilicate capillary tubes (World Precision Instruments, Sarasota, USA) using a Model 720 needle puller (Kopf, Tujunga, USA) with the solenoid set to 3 and heater set to 15. The needle was then opened up using a razor blade to remove the fused tip. This was carried out under a Leica MZ 95 light microscope and needles were cut at an angle in order to provide a sharp point. Prior to being used, morpholino injection aliquots were denatured at 65°C for 5 minutes and immediately cooled on ice. A 1.5µl aliquot was then taken and transferred to an injection needle using a GELoader tip (Eppendorf) and this placed into the pen attachment of a PV820 Pneumatic Picopump (World Precision Instruments, Sarasota, USA). The pen attachment was mounted on a micromanipulator (Narishge, Tokyo) held on a magnetic base (Kanetec, Tokyo) close to the light microscope. To account for the varying size of the needle aperture, a calibration step was performed. The needle was positioned under a light microscope in a small Petri dish filled with mineral oil. Beneath the Petri dish was placed a graticule (Graticules Ltd, Tonbridge, UK). Following a single expulsion to expel any air in the needle, 10 expulsions of morpholino solution were made into the oil and the size of the resulting bubble measured against the graticule. Adjustments were made using the Picopump to control the power of each expulsion and the calibration repeated until the bubble size equalled seven graticules. This had been calculated to be the equivalent of 14nl, hence a single expulsion should give 1.4nl, the required dosage volume.

Zebrafish oocytes were transferred using a glass Pasteur pipette to a glass Petri dish containing a glass slide. The edge of the slide was used to line up the oocytes in a single row, and excess egg water was removed to minimise movement of the eggs. This was placed under the light microscope and the needle positioned against the first in the row. The holding pressure was adjusted using the PicoPump until a small amount of MO leaked out when

the needle came into contact with the surface of an oocyte. This ensures that the contents of the yolk are not drawn up into the needle following injection. The micromanipulator was used to push the needle through the chorion and into the yolk of an oocyte. Injections were made into the streaming part of the yolk using a single depression of the foot pedal and the needle was immediately removed. The presence of phenol red in the buffer made it possible to visualise the location of the injected fluid within the egg, and any where the morpholino had not successfully been delivered into the yolk were discarded. For each morpholino at each dilution 50 oocytes were injected, before being transferred to a labelled Petri dish containing egg water (180mg/L Red Sea Salt; 2mg/L Methylene Blue) and placed in an incubator at 28°C.

6.2.5 Negative and positive controls

As a negative control for the injection process, Morpholino buffer was injected into oocytes in the same manner as described in section 6.2.3.2 and their development monitored. In addition, to control for variability between batches of oocytes from different parental pairs, 50 from each were removed and allowed to develop without intervention.

The positive controls used in this study were morpholinos designed to block the gene *Cadherin23* which is the causative agent for Usher syndrome type 1D in humans and is also mutated in the *waltzer* mice which have a behavioural phenotype similar to *bronx waltzer*. The sequences for these morpholinos were kindly supplied by Christian Söllner and had already been demonstrated to cause disruption to ear function in injected zebrafish larvae (Söllner *et al.* 2004).

6.2.6 Zebrafish

The zebrafish used in this study are maintained in the laboratory of Dr Derek Stemple at the Wellcome Trust Sanger Institute, Hinxton, Cambridge.

General maintenance, collection, and staging of zebrafish were carried out according to the Zebrafish Book (Westerfield 2000); http://zfin.org/zf_info/zfbook/cont.html). The approximate stages are given in hours postfertilization (hpf) or days postfertilisation (dpf) at 28°C.

6.2.6.1 Zebrafish oocytes

One day prior to injections, mating pairs of zebrafish were placed into individual tanks with mesh dividing them from the bottom of the tank to prevent the eggs from being cannibalised. On the day of injections, the fish would begin spawning as soon as their daylight cycle began and oocytes could be collected from the bottom compartment of the tank. These were placed into Petri dishes containing egg water (180mg/L Red Sea Salt; 2mg/L Methylene Blue) and the eggs arising from each mating pair used separately. Injections were carried out using oocytes between the 1-cell and 4-cell stages (Kimmel *et al.* 1995).

6.2.7 Assessment of phenotype

6.2.7.1 Observing development

Approximately five hours after injection when they were between 50% epiboly and shield stages (Kimmel *et al.* 1995), the oocytes were examined under the light microscope. At this stage it was possible to pick out any which were unfertilised and hence failing to divide. In addition, the dye present in the morpholino buffer made it possible to identify those oocytes which had successfully taken up the injected fluid since the cells of these appeared pink and any which did not could be removed. Over the next five days, the developing larvae were observed daily. Notes were made regarding their development and any dead or arrested larvae were counted and removed.

6.2.7.2 Assessing hearing and vestibular defects

Hearing ability was assessed by the administration of a tap test. The Petri dish containing five day old larvae was placed on a hard surface and the edge tapped sharply with the handle of a mounted needle. Those fish which are able to detect the sound and vibration caused by such an action make a sudden and pronounced darting movement, while those which cannot will remain still.

Vestibular function was assessed by observation of behaviour. Fish with vestibular defects are unable to locate the surface in order to inflate their swim bladders so tend to be restricted to the base of the dish. Additionally, when they swim they can be seen to move in circular patterns.

6.2.7.3 Tissue collection

Following assessment of phenotype on the fifth day following injection, zebrafish larvae were culled by overdose of Tricaine anaesthetic (3-aminobenzoic acid ethyl ester). Ten from each sample group were then placed into fix (4% paraformaldehyde in phosphate buffered saline) for use in staining of actin bundles and ten were placed into TRIzol (Invitrogen) for extraction of RNA. These were stored at 4°C until required for further use.

6.2.7.4 Phalloidin staining of actin bundles

Following fixation at five days old, larvae were rinsed and permeabilised by four washes of 30 minutes each in 2% Triton X-100. Staining was carried out with 2.5μg/ml FITC phalloidin (Sigma) in PBS overnight in the dark to prevent leaching of the stain. Samples were then washed with four changes of PBS before being bisected and mounted onto glass slides. Microscopy was carried out using a Biorad Radiance 2100 confocal head attached to a Nikon Eclipse E800 microscope. Images were obtained using a 40X objective lens with a 0.75 numerical aperture.

6.3 RESULTS

6.3.1 Orthologous genes

6.3.1.1 Morpholinos targeted to start sites

In the absence of complete zebrafish genomic sequence or available data regarding orthologues at the time that design of morpholinos targeted to start sites was carried out, transcripts showing significant sequence similarity were identified using the tBLASTn tool to compare mouse peptide sequence to zebrafish EST sequences. Where more than one incomplete coding sequence was identified using this approach, the sequences were assembled and the composite sequence used to query the EST database again until the start site was located. Where more than one distinct zebrafish sequence showed good homology to the mouse peptide, morpholinos were designed to both possible targets as in the cases of *Citron* and *Paxillin* and these were designated A and B. The mouse gene *Calbp1* gave good matches to two zebrafish genes, *Calbp1* and *Calbp5*, both of which were included. The sequences identified in this manner which were used for the design of morpholinos to block transcriptional start sites are shown in Table 6.3

Gene	Mouse Transcript	ESTs	Zebrafish sequence	Description
Foxn4	ENSMUST00000044790	CK236593 AF424786	NP_571174	forkhead box N4; winged helix nude
Myo1h	ENSMUST00000076152	AW422534 AW233228 BM529907	n/a	cDNA clones only
Ube3b	ENSMUST00000072473	BC055184 CD593989	n/a	cDNA clones only
Trpv4	ENSMUST00000071968	BI476124	n/a	cDNA clone only
Cabp1	ENSMUST00000031519	BE015950 BE015751 BG884283	n/a	cDNA clones only
Cabp5	ENSMUST00000031519	CK354975 AL918992	n/a	cDNA clones only
Citron A	ENSMUST00000051704	AF295804	NP_777288	rho-associated, coiled-coil containing protein kinase 2
Citron B	ENSMUST00000051704	BC044428	n/a	cDNA clone only
Paxillin A	ENSMUST00000067268	AW419523	n/a	cDNA clone only
Paxillin B	ENSMUST00000067268	BI673753	n/a	cDNA clone only
Tcf1	ENSMUST00000031535	AF244934	NP_739570	transcription factor 1; hepatocyte nuclear factor 1-alpha

Table 6.3: Zebrafish ESTs identified by BLAST using mouse protein sequences as template sequence. Where multiple ESTs are given, these were aligned into a single sequence fragment using Gap4. Where a single EST is given, this was already a complete coding sequence or contained an identifiable start site to which a morpholino could be designed. Where a single mouse gene matched more than one distinct zebrafish sequence which could not be aligned together, morpholinos were designed to both alternative sequences.

6.3.1.2 Morpholinos targeted to splice junctions

For the design of morpholinos to splice junctions, use was made of the newly available sequence homology-based orthologue predictions in the Zebrafish Ensembl database which also provides predicted exon structures and protein domains, allowing MOs to be targeted to disrupt particular regions of the gene. The orthologues identified by this method, as well as the protein domains and exons to be targeted are given in Table 6.4 Sequence similarity was confirmed using the multiple sequence alignment tool ClustalW (Chenna *et al.* 2003). As an example of the alignment between mouse and zebrafish genes, the Clustal sequence alignment for the mouse gene *Kctd10* and the novel zebrafish gene it shows similarity to is presented in Figure 6.7 The remaining sequence alignments are given in Appendix B.

Mouse Gene	Mouse Ensembl IDs (Gene/Transcript)	Zebrafish Gene	Zebrafish Ensembl IDs (Gene/Transcript)	Alignment score	Target exon	Target protein domain
Foxn4	ENSMUSG00000042002 ENSMUST00000044790	foxn4	ENSDARG00000010591 ENSDART00000008994	1639	2	TF; Forkhead
Myo1h	ENSMUSG00000041972 ENSMUST00000076152	Novel prediction	ENSDARG00000020924 ENSDART00000022921	3270	3	Myosin head
Ube3b	ENSMUSG00000029577 ENSMUST00000072473	Novel prediction	ENSDARG00000020271 ENSDART00000002815	5107	19	HECT domain
Trpv4	ENSMUSG00000014158 ENSMUST00000071968	Novel prediction	ENSDARG00000018242 ENSDART00000021356	3231	11	Cation channel TrpL
Cabp1	ENSMUSG00000029544 ENSMUST00000031519	Novel prediction	ENSDARG00000033411 ENSDART00000041500	854	2	Calflagin; EF hand
Git2	ENSMUSG00000041890 ENSMUST00000019497	Novel prediction	ENSDARG00000016679 ENSDART00000009913	2999	6	ATP_GTP_A
Paxillin (A)	ENSMUSG00000029528 ENSMUST00000067268	Novel prediction	ENSDARG00000017302 ENSDART00000017712	750	4	Paxillin
Paxillin (B)	ENSMUSG00000029528 ENSMUST00000067268	paxillin	ENSDARG00000030659 ENSDART00000040494	1781	2	Zn-binding LIM
Citron	ENSMUSG00000029516 ENSMUST00000051704	Novel prediction	ENSDARG00000011867 ENSDART00000011989	1569	8	Pleckstrin homology
Kctd10	ENSMUSG00000001098 ENSMUST00000001125	Novel prediction	ENSDARG00000017115 ENSDART00000011596	1804	2	K+ channel tetramerisation
Tcf1	ENSMUSG00000029556 ENSMUST00000031535	tcf2	ENSDARG00000006615 ENSDART00000006883	1695	4	Homeobox; HNF1b_C
Cdh23	ENSMUSG00000012819	Not annotated	n/a	n/a	4	n/a

Table 6.4: Mouse genes were matched with predicted zebrafish homologues via large-scale alignment of the two genomes (http://www.ensembl.org). These were verified and an alignment score obtained using ClustalW (http://www.ebi.ac.uk/clustalw/). The structure of the identified ortholog was then examined and a functionally important protein domain selected for disruption. Splice donor site morpholinos were designed to disrupt the processing of an exon within the target region. Where more than one zebrafish gene presented with good homology to the mouse gene, MOs were designed to both candidates as in the case of *Paxillin*.

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CLUSTAL W (1.82) multiple sequence alignment


ENSMUST00000001125      MSGDSVVSSAVPAAATRTTSFKGASPSSKYVKLNVGGALYYTTMQTLTKQDTMLKAMFSG 60
ENSDART00000011596      MSGESVVSSAVPAATTRTTSFKGSSPSSKYVKLNVGGALYYTTMQTLTKQDTMLKAMFSG 60
                        ***:**********:********:************************************

ENSMUST00000001125      RMEVLTDSEGWILIDRCGKHFGTILNYLRDGGVPLPESRREIEELLAEAKYYLVQGLLEE 120
ENSDART00000011596      RMEVLTDSEGWILIDRCGKHFGTILNYLRDGVVPLPESRRETEELLAEAKYYLVQGLVDE 120
                        ****************************** ********* ***************::*

ENSMUST00000001125      CQAALQNKDTYEPFCKVPVITSSKEEQRLIATSNKPAVKLLYNRSNNKYSYTSNSDDNML 180
ENSDART00000011596      CQAALQNKDAYEPFCKVPLVTSSKEEQRLIATANKPTVKLLYNRSNNKYSYTSNSDDNML 180
                        *********:********::************:***:**********************

ENSMUST00000001125      KNIELFDKLSLRFNGRVLFIKDVIGDEICCWSFYGQGRKIAEVCCTSIVYATEKKQTKVE 240
ENSDART00000011596      KNIELFDKLSLRFNGRVLFIKDVIGDEICCWSFYGQGRKIAEVCCTSIVYATEKKQTKVE 240
                        ************************************************************

ENSMUST00000001125      FPEARIYEETLNILLYEAQDGRGPDNALLEATGGAAGRSHHLDEDEERERERIERVRRIH 300
ENSDART00000011596      FPEARIYEETLNILLYESQDGRGPDNALLEATGGAAGRSHHIDEDEERER--HDRVRRIH 298
                        *****************:************************:********   :******

ENSMUST00000001125      IKRPDDRAHLHQ 312
ENSDART00000011596      IKRPDDRTHHHQ 310
                        *******:* **
```

Figure 6.7: Multiple sequence alignment of the mouse gene *Kctd10* (ENSMUST00000001125) and its predicted zebrafish orthologue (ENSDART00000011596) obtained using the software Clustal W (Chenna *et al.* 2003). Residues which match exactly are marked "*", those which are different but have a strong similarity of function are marked ":". Amino acids with weak similarity are shown as "." and those which are functionally dissimilar are indicated by a space.

6.3.2 Morpholino injections

Following injection, the development of the zebrafish larvae was monitored daily. The number of dead or arrested embryos was recorded and these results are shown as survival curves in Figures 6.9 and 6.10 for start site and splice donor MOs respectively. In addition, notes were made regarding their developmental progression and appearance, and on Day 5 following injection their hearing and vestibular phenotype was assessed. In each case, the highest dosage which did not give rise to widespread lethality was used for the assessment of phenotype. For MOs which caused lethality at all concentrations, the results given are for the lowest dosage. These data are presented in Tables 6.5 and 6.6 The data obtained from the negative control experiment where MO buffer only was injected is given in Figure 6.8 and Table 6.5

6.3.2.1 Morpholinos targeted to start sites

By and large, the larvae which were injected with morpholinos targeted to the start sites of candidate genes showed wide-ranging, non-specific developmental defects which made it difficult to identify a phenotype resulting from the disruption of the specific gene. Only those larvae injected with the morpholino targeted to the zebrafish *Cabp1* orthologue developed consistently at a rate comparable to the un-injected wild type controls and appeared phenotypically normal at 5dpf. These fish did not exhibit circling behaviour and gave a positive reaction to the tap test, indicating that their ear function was probably normal. Ooctyes injected with MOs targeted to the start sites of *Citron, Ube3b, Myo1h, Cabp5, Paxillin* and *Trpv4* all gave rise to larvae which were stunted in growth and showed a variety of non-specific defects such as bent tails, poor circulation, pulmonary oedema and fused otoliths. However, none of the fish which were well developed enough at 5dpf to be capable of movement showed either circling behaviour or non-

responsiveness to tapping. It should be noted that some fish with bent tails may appear to circle but this is a direct result of their malformation rather than a vestibular defect. The fish injected with morpholinos directed at the start sites of *Foxn4* and *Tcf1* suffered very retarded development leading ultimately to a widespread failure to hatch.

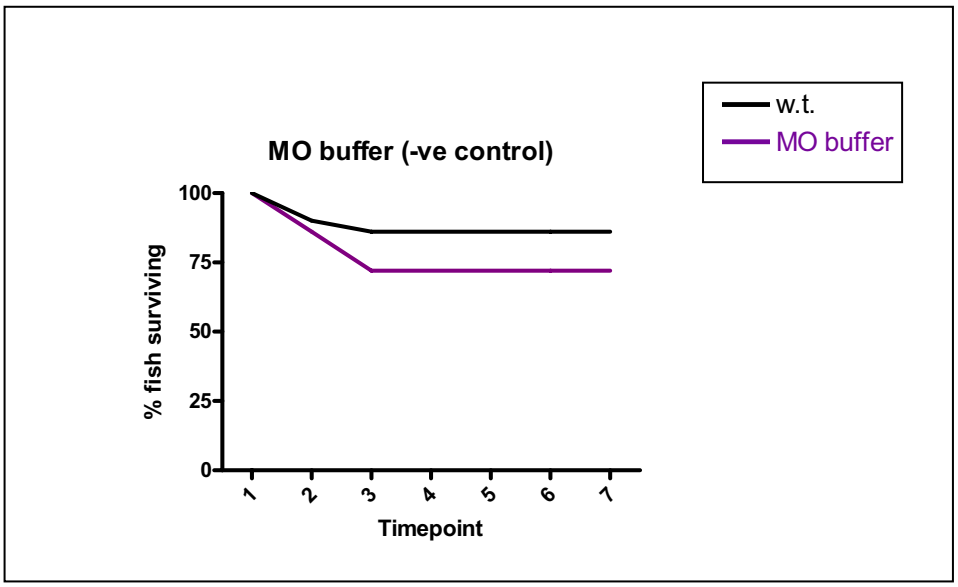

Figure 6.8: Survival curve illustrating the fate of zebrafish embryos injected with MO buffer containing no morpholino oligonucleotide. To control for variability between oocytes from different mating pairs, a wild type group of uninjected embryos was also monitored and is shown by a black line. In both cases the total number of injected oocytes was 50.

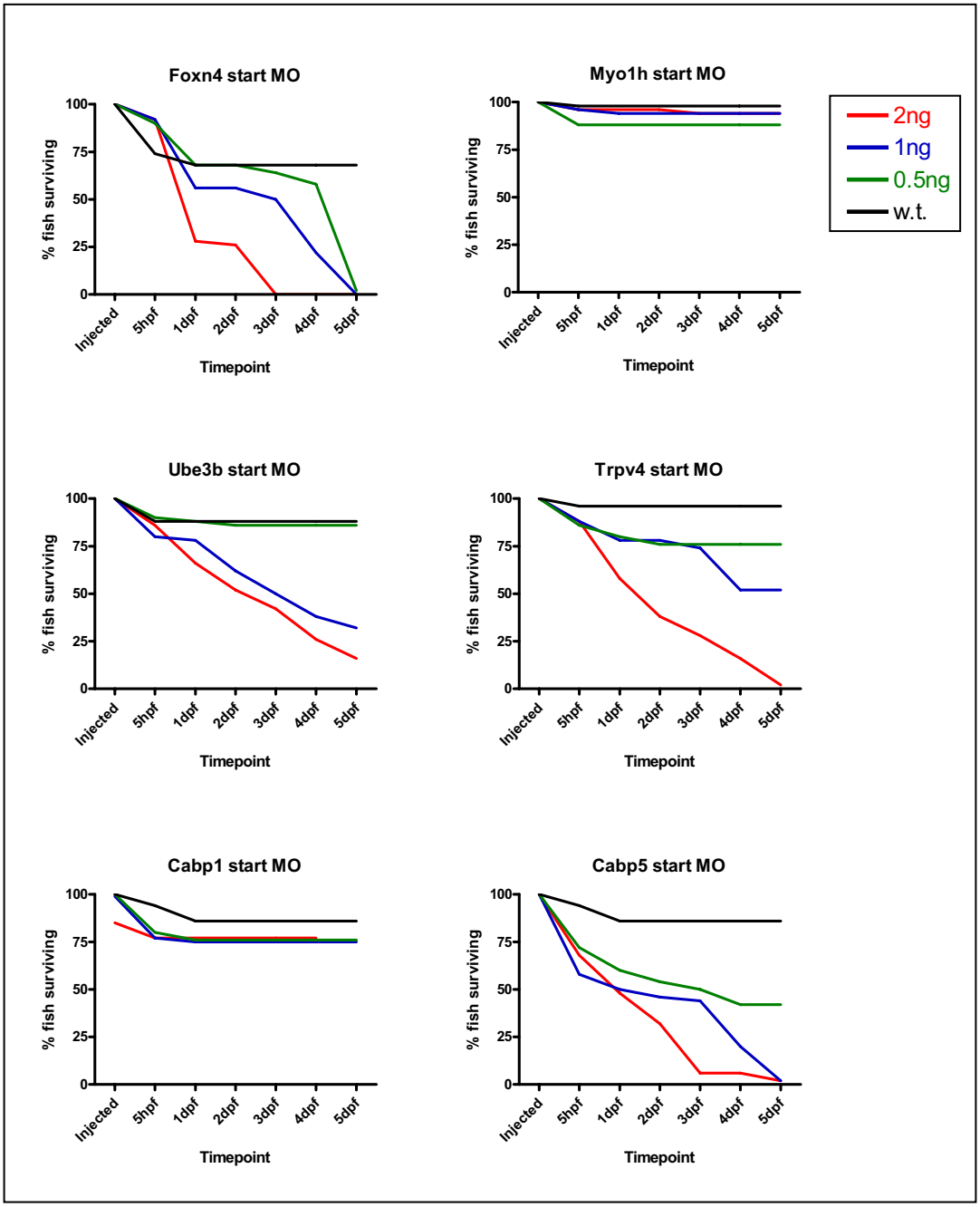

Figure 6.9a: Survival curves illustrating the fate of zebrafish embryos injected with morpholino oligonucleotides designed to block the start sites of *bronx waltzer* candidate genes. Each MO was injected at three different dosages, represented by differently coloured lines. To control for variability between oocytes from different mating pairs, a wild type group of uninjected embryos was also monitored and is shown by a black line. In all cases the total number of injected oocytes was 50.

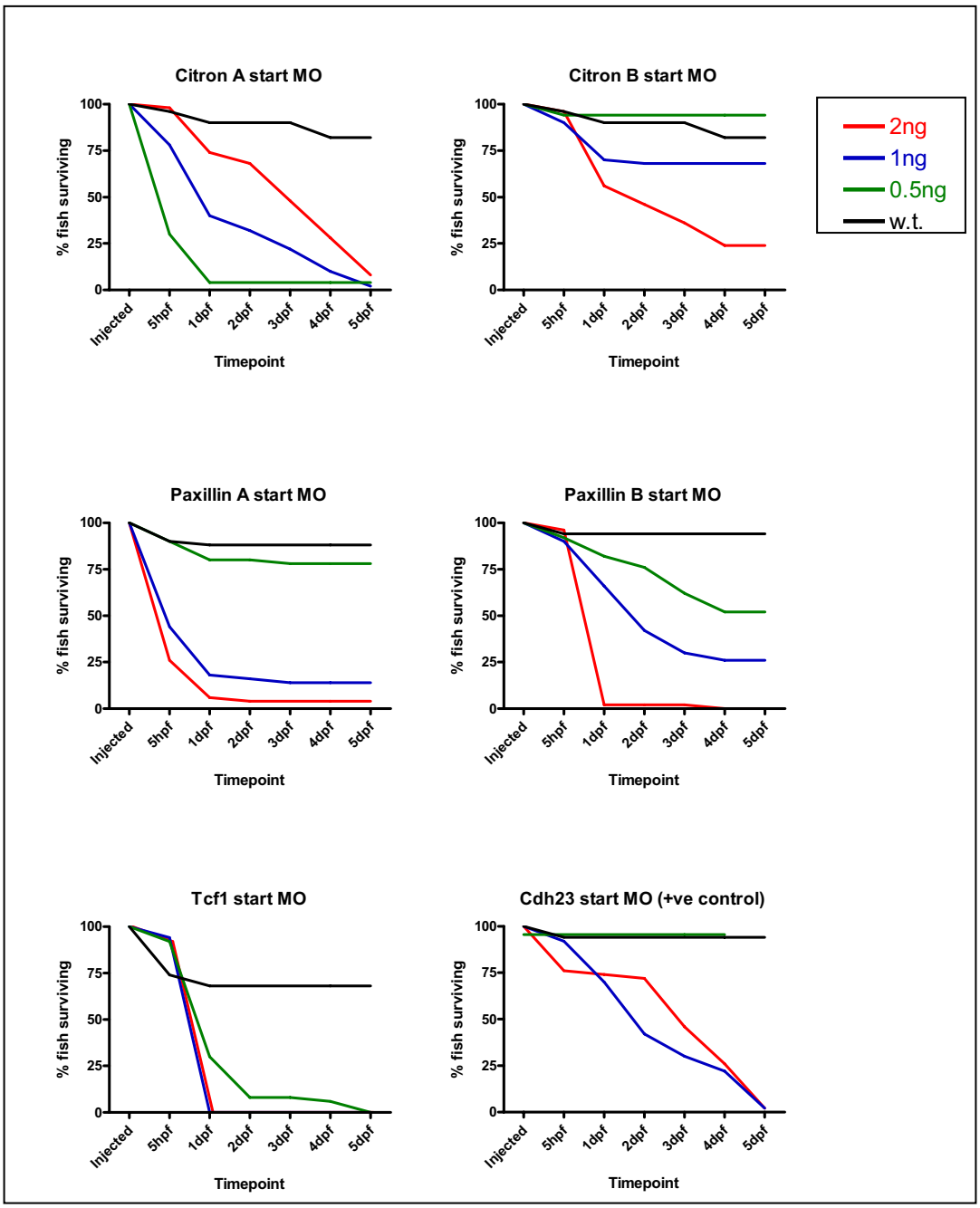

Figure 6.9b: Survival curves illustrating the fate of zebrafish embryos injected with morpholino oligonucleotides designed to block the start sites of *bronx waltzer* candidate genes. Each MO was injected at three different dosages, represented by differently coloured lines. To control for variability between oocytes from different mating pairs, a wild type group of uninjected embryos was also monitored and is shown by a black line. In all cases the total number of injected oocytes was 50

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Gene	MO	Dose	Development notes	5 dpf phenotype	Tap test result
Foxn4	Foxn4	0.5ng	Very retarded development and stunted growth	Only one fish hatched	Positive
Myo1h	Myo1h	2ng	Stunted growth but relatively normal	Small , no circulation. Many have pulmonary oedema	Positive
Ube3b	Ube3b	0.5ng	Stunted growth, many with bent tails	Very small and stunted, many have no circulation	Positive for mobile fish
Trpv4	Trpv4	0.5ng	Variable development rate, some similar to wild type, others small and stunted	Varied – some appear normal, others very small with twisted tails	Positive for mobile fish
Cabp 1	Cabp 1	2ng	Development rate very similar to wild type	Normal in appearance and behaviour	Positive
Cabp 5	Cabp 5	0.5ng	Stunted growth, many with bent tails	Small fish, twisted tails	Positive
Citron	Citron A	0.5ng	Stunted growth, many with bent tails	Small fish, twisted tails, fused otoliths	Fish not moving
Citron	Citron B	0.5ng	Variable development rate, some similar to wild type, others small and stunted	Similar to wild type, a few stunted, fused otoliths	Positive for mobile fish
Paxillin	Paxillin A	0.5ng	Development similar to wild type until 4 dpf, then many fail to fill swim bladders	Many have not filled swim bladders. Circulation appears slow	Positive for mobile fish
Paxillin	Paxillin A	0.5ng	Variable development rate, some similar to wild type, others small and stunted	Varied – some appear normal, others are stunted. Some otoliths fused	Positive for mobile fish
Tcf1	Tcf1	0.5ng	Very retarded development and stunted growth	No fish hatched	n/a
Cdh23	Cdh23	0.5ng	Development retarded, many not hatched by 5 dpf	Hatched fish swim a little and exhibit circling	Negative for most fish
MOBuffer	MOBuffer	n/a	Development rate very similar to wild type	Normal in appearance and behaviour	Positive

Table 6.5: Summary of recorded observations regarding the appearance and behaviour of zebrafish larvae following injection with morpholino oligonucleotides (MO) designed to block the start site of the targeted gene. In each case the observations given are for the lowest dosage which did not result in widespread lethality. Where all doses resulted in lethality, the results are given for the lowest dosage administered. At 5 dpf the fish were examined for circling behaviour which can be a sign of vestibular dysfunction. Their response to the tap test (see Section 6.2.6.2) was also assessed as a measure of their ability to detect and respond to vibration. . A positive result is a sign of normal function in fish which are mobile, whilst a negative one is suggestive of an ear defect.

6.3.2.2 Morpholinos targeted to splice junctions

Of the 12 MOs designed to bind to splice donor sites in order to interfere with nuclear processing, eight consistently resulted in well-developed 5 day old larvae which were sufficiently mobile to allow reliable assessment of their phenotype. This is a significant improvement on the one morpholino targeted to the start site of *Cabp1* which gave useful data in the previous part of this experiment. Since two different sequences were used to target *Paxillin*, the 12 MOs represent 11 genes, of which eight can now be associated with knockdown data in the zebrafish. Only *Citron*, *Tcf1* and *Kctd10* still gave extensive fatalities or non-specific phenotypes at the lowest administered dosage of 0.5ng/cell. The fish injected with morpholino designed to bind at a splice junction within *cadherin23* demonstrated a very clearly abnormal hearing and vestibular phenotype. They rested on their sides, failed to respond to the tap test and swam in tight circling motions. This behaviour is illustrated in Figure 6.11.

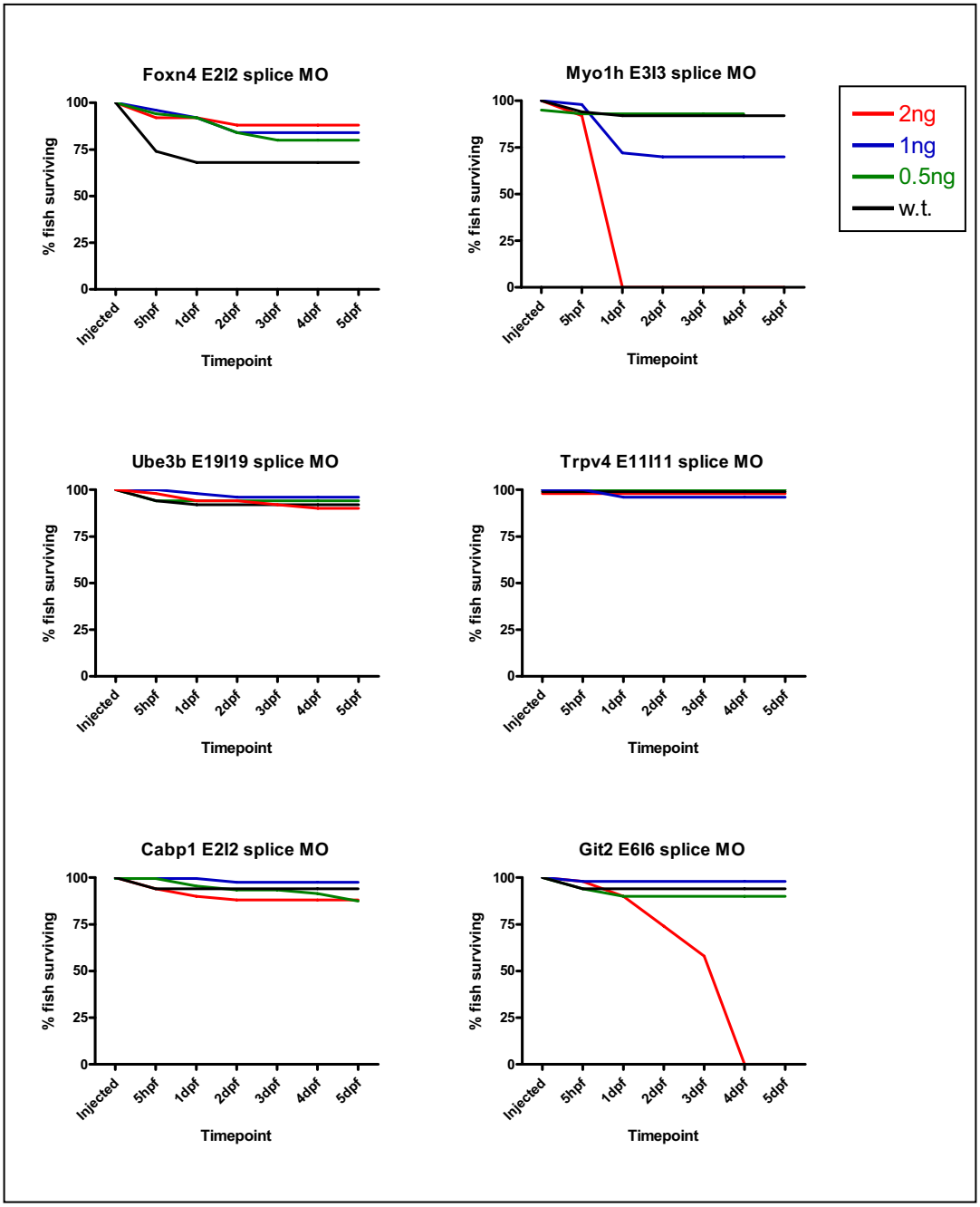

Figure 6.10a: Survival curves illustrating the fate of zebrafish embryos injected with morpholino oligonucleotides designed to block splice donor sites within *bronx waltzer* candidate genes. Each MO was injected at three different dosages, represented by differently coloured lines. To control for variability between oocytes from different mating pairs, a wild type group of uninjected embryos was also monitored and is shown by a black line. In all cases the total number of injected oocytes was 50.

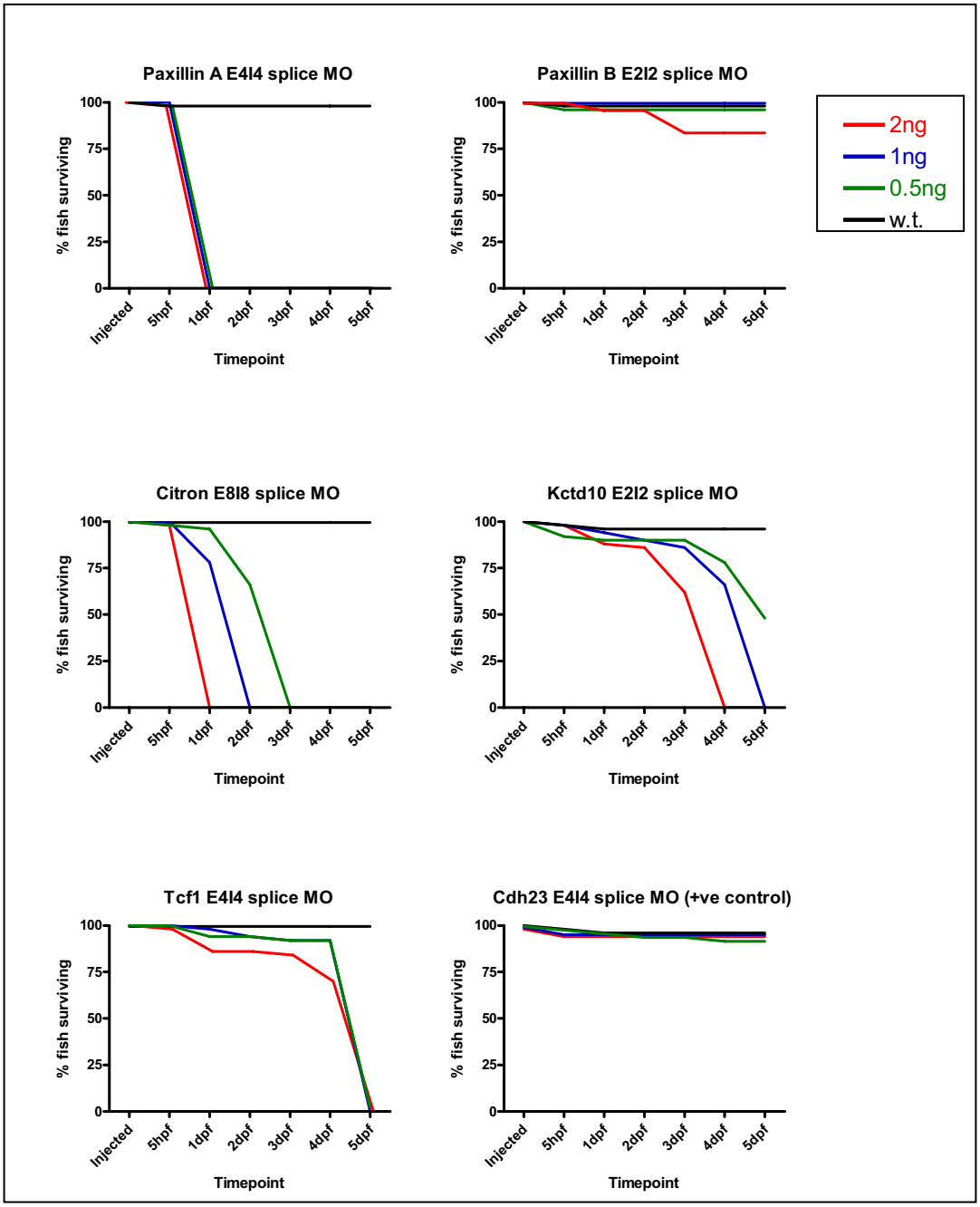

Figure 6.10b: Survival curves illustrating the fate of zebrafish embryos injected with morpholino oligonucleotides designed to block splice donor sites within *bronx waltzer* candidate genes. Each MO was injected at three different dosages, represented by differently coloured lines. To control for variability between oocytes from different mating pairs, a wild type group of uninjected embryos was also monitored and is shown by a black line. In all cases the total number of injected oocytes was 50.

Gene	MO	Dose	Development notes	5dpf phenotype	Tap test result
Foxn4	Foxn4 E2I2	1ng	Development rate mostly similar to wild type	Mostly normal, a few fish are small with bent tails	Positive
Myo1h	Myo1h E3I3	0.5ng	Development rate mostly similar to wild type	Mostly normal, a few fish are small with bent tails	Positive
Ube3b	Ube3b E19I19	2ng	Variable, some similar to wild type, others small and stunted	Varied – some appear normal, others very small with twisted tails	Positive for mobile fish
Trpv4	Trpv4 E11I11	2ng	Development rate very similar to wild type	Normal appearance and behaviour	Positive
Cabp1	Cabp1 E2I2	2ng	Development rate very similar to wild type	Normal appearance and behaviour	Positive
Git2	Git2 E6I6	1ng	Development somewhat retarded	Varied, some appear normal, some have bent tails and pulmonary oedema	Positive for mobile fish
Paxillin	PaxillinA E4I4	0.5ng	Development severely retarded	All fish dead by 1 dpf	n/a
Paxillin	PaxillinB E2I2	1ng	Development rate very similar to wild type	Normal appearance and behaviour	Positive
Citron	Citron E8I8	0.5ng	Development severely retarded	All fish dead by 3 dpf	n/a
Kctd10	Kctd10 E2I2	0.5ng	Very retarded development and stunted growth	All very small and stunted	Fish not mobile
Tcf1	Tcf1 E4I4	0.5ng	Development appears normal until 3 dpf when all fail to hatch	None hatched, all arrested	Fish not mobile
Cdh23	Cdh23 E4I4	2ng	Development rate very similar to wild type	Pronounced circling behaviour, many have unfilled swim bladders	Negative for most fish

Table 6.6: Summary of recorded observations regarding the appearance and behaviour of zebrafish larvae following injection with morpholino oligonucleotides (MO) designed to block a specific splice donor site within the targeted gene. In each case the observations given are for the lowest dosage which did not result in widespread lethality. Where all doses resulted in lethality, the results are given for the lowest dosage administered. At 5 dpf the fish were examined for circling behaviour which can be a sign of vestibular dysfunction. Their response to the tap test (see Section 6.2.6.2) was also assessed as a measure of their ability to detect and respond to vibration. A positive result is a sign of normal function in fish which are mobile, whilst a negative one is suggestive of an ear defect. The negative control for these results was the injection of MO buffer only, the results of which are shown in Table 6.5.

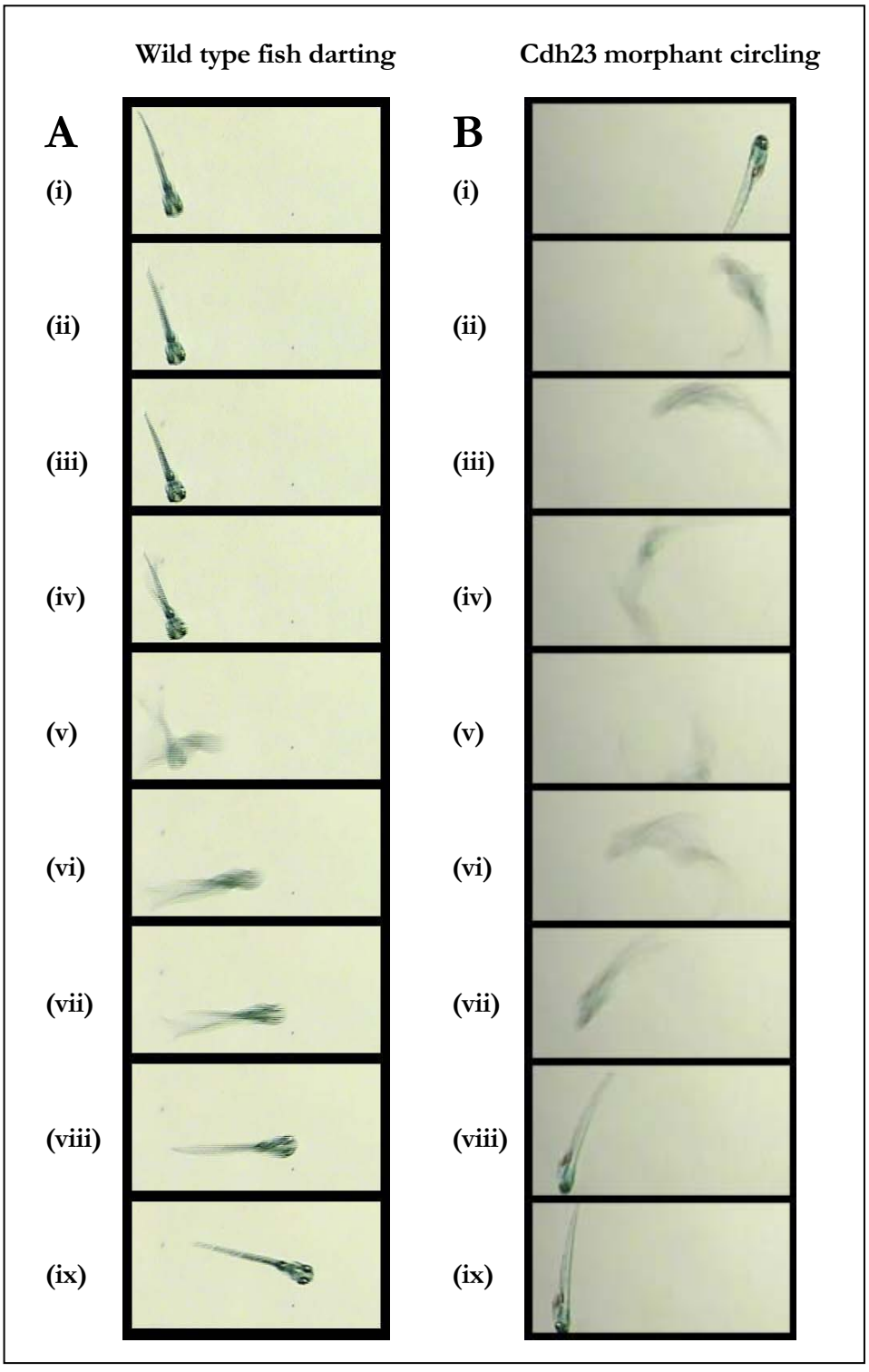

Figure 6.11: Frame-by-frame video stills showing typical fish behaviour observed after 5 days development in **A**) wild type un-injected controls and **B**) larvae injected with 0.5ng of *cdh23* E4I4 morpholino designed to block a splice donor site within the known deafness gene *cadherin 23*. The wild type fish move with a characteristic darting motion which begins with a tail flick as seen in A(iv). By contrast the morphants' movement is much less controlled and often manifests as circling patterns. In addition, their inability to orient themselves or to fill swim bladders results in their resting on their sides at the bottom of the dish (B(i) and (ix)) compared to the normal upright positioning of the controls (A(i) and (ix)).
Both movements take place in less than one second.

6.3.2.3 Phalloidin staining of actin bundles

Initially, samples were processed from wild type control fish and from those injected with 0.5ng of *Cdh23* E4I4 morpholino. These morphants had demonstrated a pronounced circling phenotype and failed to react when the tap test was administered but appeared otherwise normal. Following staining, the hair bundles of these fish were examined using confocal microscopy and examples of the resulting images are shown in Figure 6.12. Splayed stereocilia were observed in all the sensory patches of both the wild type and morphant samples. It should be noted that the varying sizes of the sensory patches in the images is a result of their being from different locations within the ear, rather than being an effect of the MO injection.

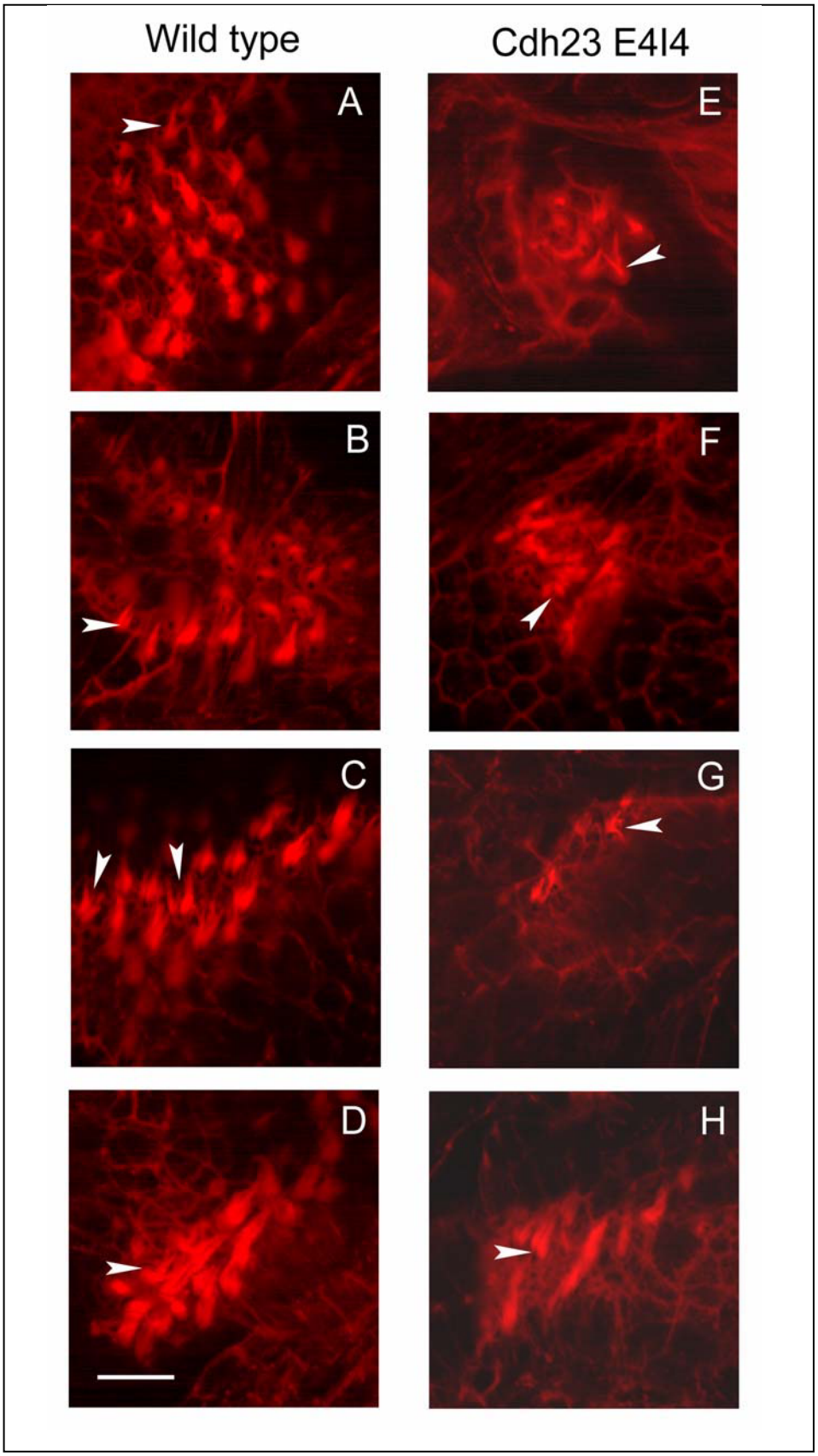

Figure 6.12: Confocal microscope images showing typical inner ear hair cell bundles in wild type un-injected control larvae (A-D) and larvae injected with 0.5ng of *cdh23* E4I4 morpholino designed to block a splice donor site within the known deafness gene *cadherin 23* (E-H). Splayed stereocilia were observed in all the samples examined, some of the bundles showing notable splaying are marked by white arrows. Scale bar = 10μm.

6.4 DISCUSSION

6.4.1 Analysis of negative and positive controls

In order that any interpretation of the results obtained from blocking the *bronx waltzer* candidate genes using morpholinos may be considered valid, it is important that both the negative and positive controls were observed to give the expected results.

6.4.1.1 Negative control: injection of MO buffer

In the course of this experiment, the negative control was to inject zebrafish oocytes with MO buffer containing no morpholino oligonucleotide. This controls for any effects of the manipulation process and the introduction of the dye and other buffer components into the cell. As shown in Figure 6.8 and Table 6.5, the injection of buffer into cells gave rise to a mortality rate slightly higher than that observed in wild type oocytes after the first 24hrs but resulted in no further hindrance to development. After five days the larvae appeared behaviourally normal and responded to tapping with the tail flick characteristic of wild type fish. The fatalities seen after the first day are likely to be due to the invasiveness of the procedure and may be the result of damage caused by the injection process. If the needle is not removed quickly enough from the yolk or if the pressure within it is slightly negative then the injection can cause a small amount of the yolk to leak out of the chorion. This then becomes a target for fungal infection, making it more likely that the oocyte will not survive. However, the normal progression of the buffer-injected eggs supports the premise that any developmental defects or subsequent abnormalities in MO-injected larvae are caused by the presence of the morpholino within the cells.

A more rigorous control would be to inject eggs with a morpholino differing by only one base pair from the target sequence in order to control for the

presence of the oligonucleotide as well as to prove the specificity of the individual morpholino. However, since a large number of morpholinos were already being used in this process, it was decided that this control would not be carried out routinely in order to control the overall cost of the procedure. In the case of any of the morpholinos giving an interesting phenotype, a specific control morpholino would be designed and its effects ascertained.

6.4.1.2 Positive control: injection of cadherin 23 morpholinos

Cadherin 23 (*cdh23*) is a gene whose function within the ear has been well described and has been the subject of previous morpholino studies in the zebrafish. Morpholinos were targeted to both start and splice sites within the gene and both gave rise to larvae exhibiting the circling behaviour and non-responsiveness to sound indicative of an ear abnormality (Söllner *et al.* 2004). In this study the published MO sequences were synthesised and injected in the same manner as those targeted to *bronx waltzer* candidate genes in order to ensure that the methods being employed were capable of detecting an ear abnormality in the fish.

The results of injecting oocytes with the MO targeted to the start site of *cdh23* are shown in Figure 6.9b and Table 6.5. At 0.5ng, the lowest dosage administered, the larvae were relatively retarded in development but those which hatched exhibited circling behaviour and failed to respond with a tail flick when subjected to the tap test. This observation differs from the published data which used dosages of up to 50ng without causing widespread lethality, but the phenotype obtained in those larvae which developed sufficiently correlates with the original findings. This dramatic difference in the response of the fish to higher dosages of the same morpholino is difficult to explain but may result from a difference in experimental design such as the composition of the MO buffer, the particular strain of zebrafish used or the injection procedure.

The injection of oocytes with MO targeted to the splice donor site of *cdh23* exon 4 gave rise to larvae with a pronounced vestibular and balance defect. The data for these experiments are presented in Figure 6.10b and Table 6.6, whilst images depicting their behaviour are shown in Figure 6.11. At all the dosages injected, fish developed relatively normally but showed clear circling behaviour and were observed to rest on their sides rather than remaining upright. They also failed to respond when subjected to the tap test, suggesting a hearing impairment. This confirmation of the phenotype caused by the disruption of *cdh23* carried out under the conditions employed in the present study provides reassurance that should the disruption of one of the candidate genes give rise to an ear abnormality, its presence should be detected using the methods described.

6.4.2 Phalloidin staining of actin filaments

Phalloidin staining of the larvae injected with *Cdh23* E4I4 morpholino which had demonstrated significant vestibular abnormality and circling behaviour seemed to show splaying of stereocilia consistent with the results of the authors who first used this MO sequence (Söllner *et al.* 2004). However, splaying was also observed in the ears of wild type larvae. This suggests that it may be an artefact due to slow fixation, especially as the fix must diffuse through several layers of tissue before reaching hair bundles within the ear. Previous studies of both mice and zebrafish with defective copies of *cadherin23* have given rise to conflicting information regarding the effect of its disruption on stereociliar organisation. It was initially shown to localise to the tip links which connect the stereocilia within a bundle in both mice (Siemens *et al.* 2004) and fish (Söllner *et al.* 2004), leading to the suggestion that its absence may lead to their becoming disorganised and thus prevents normal transduction from occurring. A more recent study by Michel and colleagues (Michel *et al.* 2005) showed *Cdh23* to be a component of lateral links but only during bundle development and also found it to be present in the cuticular plate of adult animals. Meanwhile a similar study by Lagziel *et al.* (Lagziel *et al.*

2005) found *Cdh23* to be present not only in the interstereocilial links but also along the length of sterociliar bundles in vestibular hair cells, the kinocilium and kinocilia/stereocilia links and in the Reisner membrane. Söllner *et al.* (2004) used transmission electron microscopy (TEM) to show that tip links were absent from hair cells in zebrafish carrying mutations in *cadherin 23*. However, since the nature of TEM is such that samples must be looked at in very thin sections, it is possible that the notoriously difficult to visualise tip links were not present in those sections examined. More recent data obtained using field emission scanning electron microscopy (A. Rzadzinska, *pers.comm.*) has shown that not only are tip links present in the stereocilia bundles of *waltzer* mice which carry a *Cdh23* mutation, but that the stereocilia show signs of fusing, rather than of splaying.

Given this uncertainty over the cause of splaying in the hair cell bundles and the evidence of a similar appearance in un-injected control zebrafish larvae, it was considered that phalloidin staining of the larvae injected with morpholinos would not provide informative data concerning the effects on the fine structure of the zebrafish ear of blocking the start or splice sites of candidate genes. Ideally, the hair bundles would be examined using SEM, but this was beyond the scope of the present study.

6.4.3 Comparison of start site and splice site morpholinos

The identification of orthologous genes was carried out in a different manner during the design of morpholinos to start and splice sites. However, in most cases the zebrafish transcripts identified were the same. The use of the additional data available in Ensembl when choosing sequence to target using splice donor MOs allowed the identification of the single best orthologue for both *Citron* and *Cabp1* which previously had two possible candidate transcripts. *Paxillin* still showed a high level of homology to two different zebrafish transcripts, although these matched to different regions of the

mouse gene (see Appendix B), suggesting a division of subunits or possibly a divergence of function.

Many of the oocytes injected with morpholinos directed at the start sites of the candidate genes gave rise to larvae which either arrested early in development or suffered from wide-ranging non-specific defects. For some genes this may be an indication that they play an important role during early developmental stages, with their disruption giving rise to early arrest. However, it is unlikely that so many of the genes investigated in the present study would perform such a function. Non-specific abnormalities are often associated with high dosages but in this experiment even the very low dosages of 0.5ng resulted in very sick fish, while in the literature reports of 10ng doses resulting in specific effects are not unusual (Draper *et al.* 2001; Söllner *et al.* 2004). The normal development of the fish injected with *Cabp1* morpholino at all doses administered makes it unlikely that the experimental method or reagents were at fault since all the fish were subject to the same treatment. It is interesting to note that the fish injected with the control *Cdh23* MO also showed more non-specific abnormalities than reported in the literature from which the sequence was obtained (Söllner *et al.* 2004). This may indicate that the conditions used here varied, although it is not clear how.

In order to address the issues of early arrest and non-specific effects caused by the morpholinos targeted to translational start sites, it was decided to employ splice donor site blocking morpholinos. Since these take effect during nuclear processing of mRNA rather than at the point of translation, the maternal mRNA molecules which are present in eggs when they are laid are unaffected. This allows the embryo to progress through the vital early stages of development before any alterations caused by the MO take effect. Since the *bronx waltzer* phenotype is a subtle one and is not visible in the mouse embryo until E16.5, it is unlikely that such an approach should result in the causative gene being missed. In addition, MOs targeting nuclear processing will tend to cause disruptions to genes such as exon skipping or premature

truncation rather than the effectively total blocking achieved by the use of MOs targeted to start sites. In this way, splice blocking MOs are similar to mutational alterations whereas start blocking MOs mimic the effects of a deletion in the target gene. Of the 11 morpholinos targeted against splice sites of *bv* candidate genes, eight resulted in larvae at 5dpf which were well developed enough to have their hearing and balance phenotype assessed. In contrast, most start site MO produced underdeveloped or grossly abnormal fish are frequently unable or reluctant to move and therefore their swimming behaviour and reactions to the tap test cannot be ascertained. Therefore the splice site MOs offer a clear advantage when searching for an equivalent to subtle phenotypes such as *bv* in the zebrafish.

6.4.4 Confirmation of splice-blocking MOs

An advantage of using splice-blocking MOs is that the products of MO-targeted mRNA processing events can be characterised using RT-PCR. With primers designed within the exons flanking that to be targeted it is possible to establish from the size of product obtained whether the exon has been excised or altered and hence to confirm that the MO has acted effectively. This principle is illustrated in Figure 6.13.

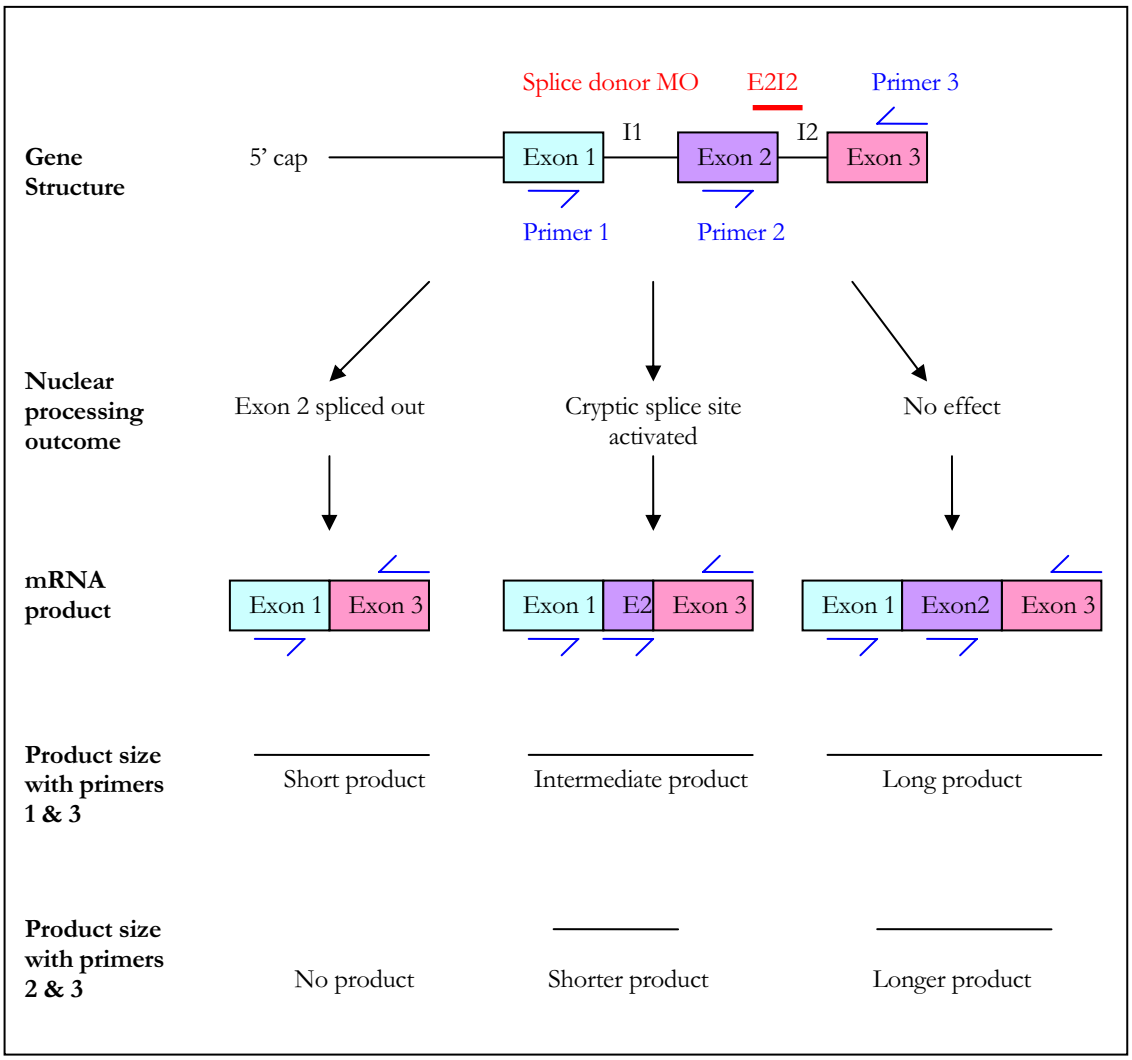

Figure 6.13: Diagram illustrating the strategy for confirmation of the efficacy of morpholino oligos designed to block nuclear processing by binding to splice donor sites. The splice donor MO E2I2 (**red**) is expected to cause the excision of exon 2, or possibly the activation of a cryptic splice site between exons 2 and 3. In order to determine what effect the MO has had on nuclear processing, PCR primers (**blue**) are used to amplify from cDNA samples obtained from injected embryos. The size of the PCR product obtained can be used to infer the structure of the targeted mRNA.

6.4.5 Conclusions

None of the novel morpholinos used in this study gave rise to zebrafish larvae displaying signs of a hearing or balance defect. Most of the start site MOs and four of the splice site MOs caused widespread lethality or non-specific phenotypes, leaving one start MO and eight splice MOs which gave rise to larvae that could be reliably phenotyped. If it is possible to be certain that the MOs caused an effective disruption to the target gene, and that the orthologues identified perform the same function in the zebrafish as they do in the mouse, then these data could represent strong evidence that the included genes do not represent the *bronx waltzer* gene. In the case of the splice MOs, their efficacy at disrupting the target gene should be confirmed by RT-PCR as described in Section 6.4.4, leaving only the question of whether the genes they target represent true functional orthologues of the mouse genes from within the *bv* region. In order to establish this, it would be necessary to carry out further functional analysis. Should any of the genes have given a positive result then this avenue would have been pursued in order to strengthen the case for it representing an orthologue of *bronx waltzer*. In the absence of a good candidate however, further analysis was not considered a priority and it is therefore possible that the genes knocked down in the zebrafish do not represent true orthologues of the mouse genes which map to the critical region. This means that the 11 candidate genes used in this zebrafish study, whilst not being ruled out are less likely to be responsible for the *bv* phenotype.

Although this approach has not yet been of substantial assistance in the search for the *bronx waltzer* gene, it does still hold the potential to contribute to the project. Firstly, a larger selection of candidate genes could be targeted using MOs designed to block splice sites since these proved to be the most successful in producing viable larvae. All of the genes tested here were previously characterized or showed homology to other proteins since their selection relied on a certain level of information being available. However, it is

perfectly possible that the *bv* mutation actually resides within one of the 22 novel genes currently annotated between the flanking markers. Given unlimited time and funds it would be possible to design and inject morpholinos targeted to these, provided that good homology to zebrafish transcripts can be determined, and hopefully one would give fish at 5dpf manifesting behaviour typical of a hearing or vestibular dysfunction like that seen in the larvae injected with *cadherin 23* morpholinos. Secondly, and more realistically, in the event of the discovery of the *bronx waltzer* gene by another means, this method could be use as a verification tool. Since *bv* exists as a single allele, any mutation identified would need to be demonstrated to be causative rather than simply a polymorphism. This approach was taken by Liu and colleagues (Liu *et al.* 2002) who used morpholinos designed to block *Nek8* in order to demonstrate that it was responsible for the cystic kidney disease they had characterised in a single allele mouse model. In these circumstances it would also be possible to generate a mouse knockout but as shown here, knockdowns in the zebrafish are fast, relatively inexpensive and can give recognisable phenotypes when orthologous genes are disrupted. Additionally, the generation of a zebrafish phenocopy would facilitate investigation into the developmental consequences of the mutation, since fish larvae mature externally and can be monitored continuously throughout development.