Hsp90 as a Buffer of Developmental Eye Defects in Zebrafish

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Declaration

I declare that this thesis was composed by myself. The contributions of others to the work are clearly indicated. This work has not been submitted for any other degree or professional qualification except as specified.

Ruth Bancewicz, 2003

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Abstract

Since studies by Waddington and others in the 1950s it has been widely accepted that strictly Mendelian inheritance is only observed for a minority of mutations. They showed that large amounts of silent genetic variation exists in populations, which can be revealed by changes in the environment or genetic background and dramatically affect the developmental outcomes of mutations. Many human developmental disorders, such as neural tube defects and Microphthalmia, Anophthalmia, and Coloboma (MAC) seem to occur sporadically, but recurrence in families suggests that genetic factors play an important role. Mutations have been identified in some of these families, but the evidence suggests that multiple genetic and environmental factors are important in others for determining the phenotypic outcome.

Recent work in *Drosophila* and *Arabidopsis* has shown that the chaperone protein Hsp90 may have a role in the interaction between genetic and environmental factors during development. A model has been proposed in which Hsp90 suppresses or buffers silent variation under normal conditions, which is only expressed when damaged proteins sequester Hsp90 during environmental stress. Hsp90 structure and function is highly conserved in all organisms studied, and we hypothesised that this buffering mechanism may play an important part in the aetiology of human developmental disease. I conducted studies with zebrafish mutants and pharmacological inhibitors of Hsp90 to determine whether the penetrance or expressivity of the mutant phenotype could be altered.

Zebrafish embryos were treated with low levels of the specific Hsp90 inhibitors, radicicol and geldanamycin. At the concentrations used, a heat shock response was not observed at the protein or RNA level. Homozygotes for the recessive mutations *sunrise* (*sri*) and *dreumes* (*dre*) were treated with radicicol, and the severity of the phenotype was increased in *sri* and marginally decreased in *dreumes*, with respect to control embryos from the same clutch. This effect was also observed with heat shock. Geldanamycin also increased the severity of the sunrise phenotype, but an analogue geldampicin that does not bind Hsp90 had no effect. It can be concluded

from this that Hsp90 can buffer developmental defects in a vertebrate species when a pathway is destabilised by a mutation.

The *sri* mutation was mapped in collaboration with Ralf Dahm and colleagues, Tübingen. A missense mutation was identified in the homeodomain of *Pax6b* that causes an L to P amino acid change. This is predicted to alter the structure or stability of the Pax6b protein. Antisense oligonucleotide knockdown of Pax6b and/or Pax6a function does not phenocopy *sri,* showing that it is not a full loss of function mutation. Preliminary results suggest that injection of WT *Pax6b* mRNA rescues the *sri* phenotype, confirming that the mutation identified in *Pax6b* causes the *sri* phenotype. Pax6b is expressed in the pancreatic islet of WT embryos, and in *sri* the pancreatic islet is severely disorganised, further confirming the causative nature of the *Pax6b* mutation.

This study demonstrates the buffering properties of Hsp90 in a vertebrate system. *Pax6* mutant phenotypes in mammals are notoriously variable, and the fact that the mutation buffered here is in Pax6, in addition to the well documented structural and functional conservation of Hsp90, indicates that this mechanism may be conserved in higher vertebrates.

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Abbreviations

APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
ANOVA	Analysis of Variants
BDCKCHK	Branched-chain α-keto acid dehydrogenase
bp	Base Pairs
BMP4	Bone Morphogenic Protein 4
BSA	Bovine Serum Albumin
CaCl	Calcium Chloride
cDNA	complimentary DNA
CHX10	CHE10 Hox-Containing Homolog
cM	Centimorgan
CNS	Central Nervous System
cR	Centiray
dH_2O	Deionised Water
dpf	Days Post Fertilisation
DMSO	Dimethylsuphoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dre	*dreumes*
DTT	Dithiothreitol
ECL	Electrochemiluminescent
EDTA	Ethylenediamintetraacetate
EMSA	Electrophoretic Mobility Shift Assay
eNOS	Endothelial Nitric Oxide Synthase
ENU	Ethylnitrosourea
ER	Endoplasmic Reticulum
ES cells	Embryonic Stem cells
EtBr	Ethidium Bromide
EtOH	Ethanol
ey	*eyeless*
Fox	Forkhead Box
GA	Geldanamycin
GFP	Green Fluorescent Protein
GHKL family	Gryase, Hsp90, Histidine Kinase, MutL family
gol	*golden*
GRP94	Glucose Regulated Protein 94
h	Hour
HCL	Hydrochloric acid
HD	Homeodomain
het	Heterozygous
hez	*helderziend*
hom	Homozygous

HoxB1	Homeobox b1
HPE	Holoprosencephaly
hpf	Hours Post Fertilisation
HSE	Heat Shock Element
HRP	Horseradish Peroxidase
HSF	Heat Shock Factor
HSP	Heat Shock Protein
IUGR	Intra-Uterine Growth Retardation
JNK	c-jun NH_2-terminal kinase
Kb	Kilobase
KCl	Potassium Chloride
K_d	Dissociation constant
L	Leucine
LiCl	Lithium Chloride
M	Molar
MAC	Microphthalmia, Anophthalmia and Coloboma
MeOH	Methanol
mg	Milligramme
$MgCl_2$	Magnesium Chloride
mic	*microps*
min	Minute
ml	Millilitre
mM	Millimolar
MyoD	Myogenic Differentiation Antigen 1
µl	Microlitre
µg	Microgramme
µM	Micromolar
NaCl	Sodium Chloride
NaOH	Nanogramme
NO	Nitric Oxide
OD	Optical Density
P	Proline
Pax	Paired Box
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Paired Domain
pg	Picogramme
Pitx2	Paired-like Homeodomain Transcription Factor 2
RPE	Retinal Pigmented Epithelium
rne	*rosine*
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RT	Room Temperature
RT-PCR	Reverse Transcription-Polymerase Chain Reaction

sec	Second
SD	Standard Deviation
SE	Standard Error
sdy	*sandy*
SDS	Sodium Dodecyl-Suphate
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
Sey	*Small eye*
SHH	Sonic Hedgehog
SHR	Steroid Hormone Receptor
SHOX	Short Stature Homeobox
Six3	Sine Oculis Homeobox 3
Sox2	SRY Box 2
sri	*sunrise*
SSC	Salt-Sodium Citrate
SSLP	Simple Sequence Length Polymorphism
TAE	Tris-acetate-EDTA
TBST	Tris Buffered Saline-Tween
TEMED	Tetramethylethylenediamine
TESPA	3-aminopropyltriethyloxysilane
TGIF	TG-Interacting Factor
TL	Tup Longfin
Tm	Melting Temperature
TPR	Tetratricopeptide Repeat
TRAP1	Tumour Necrosis Factor Receptor Associated Protein 1
Tü	Tübingen
u	Units
UTR	Untranslated Region
UV	Ultraviolet
V	volts
WT	Wild Type
ZIC2	Zinc-Finger Protein of Cerebellum 2

Chapter 1

Introduction

1.1 Non-Mendelian inheritance of developmental eye defects

There are many familial developmental disorders that do not appear to be inherited in a straightforward Mendelian fashion, such as cleft lip and palate, holoprosencephaly (HPE), and neural tube defects. These often have a heterogeneous genetic component, with causative mutations in different genes. For example, HPE can be caused by mutations in *SHH* (Sonic hedgehog), *TGIF* (TG interacting factor), *SIX3* (Sine oculis homeobox 3) and *ZIC2* (Zinc finger protein of cerebellum 2), among others (Reviewed in Wallis and Meunke, 2000). In some cases oligogenic inheritance seems likely, as mutations have been identified in some affected individuals, but also in unaffected carriers. There are several examples of this among the eye disorders, in which the disease allele is dominant, but does not have full penetrance. Genetic modifiers and environmental factors almost certainly play an important role in the aetiology of these diseases.

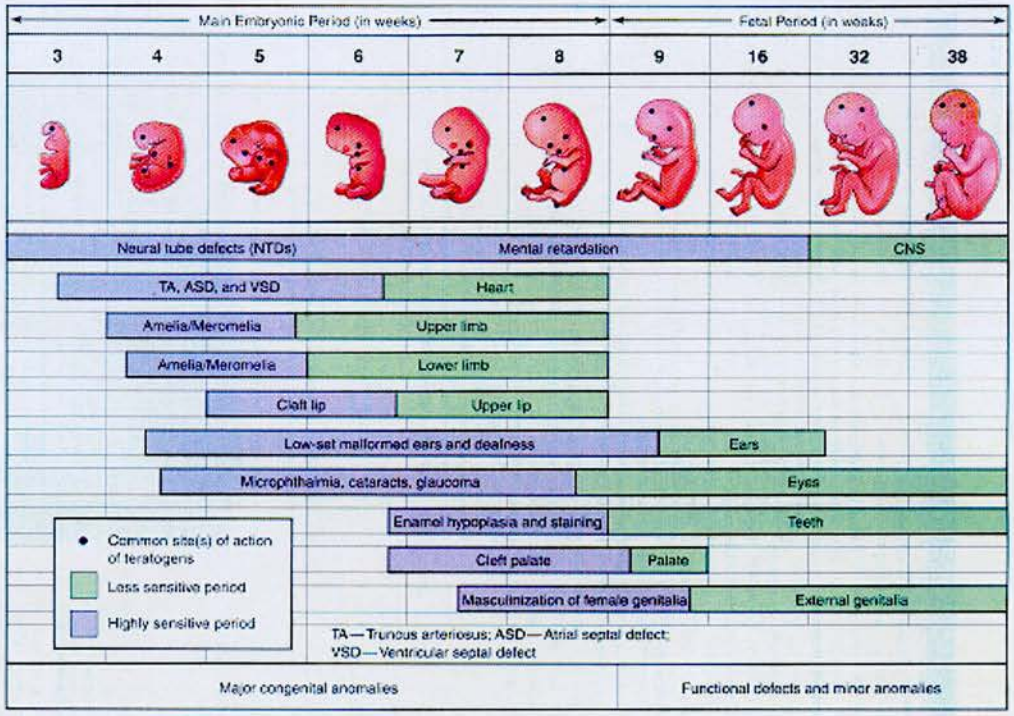
MAC is a developmental disorder characterised by a spectrum of structural eye defects: reduced eye size (Microphthalmia), absence of the eye (Anophthalmia), or non closure of the optic fissure (Coloboma) (Morrison *et al.* 2002). Study of the genetic basis of MAC has shown it to be a good example of a non-Mendelian, genetically heterogeneous disorder that may be susceptible to environmental effects. Causative mutations have been identified in genes as diverse as *Pax2* (Paired box gene 2) (Cunliffe *et al.* 1998), *SOX2* (SRY box 2) (Fantes *et al.* 2003), *CHX10* (CHEH10 hox-containing homolog) (Ferda Percin *et al.* 2000), *SIX3* (Wallis *et al.* 1999), and *SHH* (Schimmenti *et al.* 2003). In some families with recurrent cases of MAC, a single causative mutation has been identified, with a straightforward dominant inheritance pattern (Ferda Percin *et al.* 2000, Fantes *et al.* 2003). In other cases unaffected or extremely mildly affected individuals carry the same mutation as the very severely affected. This suggests that other genetic or environmental factors are important in the aetiology of the disease (Amiel *et al.* 2000, Schimmenti *et al.* 2003). Unilateral malformations often occur, as well as variable severity in each eye for individuals with bilateral defects (Fantes *et al.* 2003, Wallis *et*

al. 1999). This is direct evidence for the importance of environmental factors as well as, or in addition to, stochastic events (Reviewed in McAdams and Arkin, 1999) in the aetiology of these defects.

Clusters of MAC cases in rural areas of the UK were reported by the Observer in 1993, with an alleged link to the teratogenic pesticide Benomyl, leading to significant media interest (Pesticides News, 1997). Teratogens are defined as substances that disrupt normal development (Lawrence, 2000). These pass through the placenta, and can cause intrauterine growth retardation (IUGR), or specific effects on certain organs during especially sensitive periods (Figure 1.1). An epidemiological study of congenital eye malformations in Spain showed that teratogenic effects could account for a high proportion (over 10%) of cases (Bermejo and Martinez-Frias 1998). Suspected teratogens for cases identified in India included agricultural chemicals, abortifacients, vitamin A deficiency, and drug usage (Hornby *et al.* 2002). Also, a recent letter to the Guardian from the chief medical officer to the Gulf war veterans reported an unusually high incidence of anophthalmia in children born in Iraq following the Gulf war, suggesting that this was linked to pollution of the environment as a consequence of the fighting (Hooper, 2002).

Although virtually any chemical can have a teratogenic effect at a high enough dose, some chemicals have a more powerful effect on development. The dose of benomyl required to cause eye defects is so high that that a link between pesticide use and MAC seemed unlikely (Pesticides news, 1997). Also a study of anophthalmia and microphthalmia incidence in the UK did not find enough statistical evidence for the clusters reported by the media, as the numbers of affected individuals are so low (Cuzick, 1998, Dolk *et al.* 1998). Some individuals may however carry genetic factors that increase their sensitivity to a teratogen. In the case of benomyl, it may only be individuals at a vulnerable stage of development, and carrying particular genetic factors that are affected, giving the low numbers of cases reported.

a



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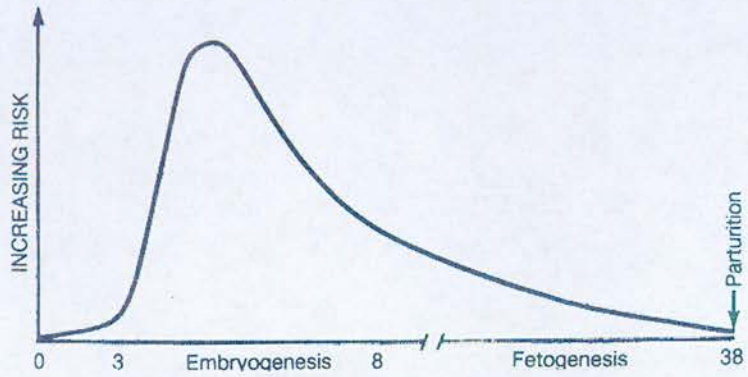


Figure 1.1: Sensitive periods of embryogenesis. (a) Organ-specific periods of sensitivity (Reproduced from Moore, 1998); **(b)** Overall sensitivity to teratogens peaks at approximately day 5 of embryogenesis (Reproduced from Sadler, 2003).

1.2 Hsp90 as a buffer of developmental defects

Studies of genetic disorders in animal models have shown that Mendelian inheritance can be altered on different genetic backgrounds. Waddington showed that a large amount of hidden or "cryptic" genetic variation exists in wild type (WT) *Drosophila* populations, which can be uncovered by environmental changes. The morphological defects that appeared after a heat shock during development, such as loss of cross veins in the wing, could be selected for over successive rounds of breeding and heat shock, until the traits were independent of heat shock (Reviewed in Scharloo, 1991, Rutherford, 2000). This demonstrated that genetic variation for the trait under selection existed in the original population, and was revealed by environmental disturbance. Waddington described the invariance, or robustness of developmental pathways to environmental or genetic disturbance as canalisation, or buffering (Waddington, 1942).

Rutherford and Lindquist (1998) took Waddington's experiments a step further when they observed that *Drosophila* carrying mutations in Heat Shock Protein 83 (*Hsp83*, or *Hsp90*) mutant stocks showed an unusually high rate of morphological defects. Hsp90 is a molecular chaperone with a role in stabilising signalling proteins important in development, as well as a more general role in assisting the folding or degradation of proteins damaged by denaturing conditions (Reviewed in Mayer and Bukau, 1999). It was shown that when *Hsp83* mutant strains were outcrossed to different WT strains, defects arose that were specific to each strain. These results were replicated when geldanamycin was used to compromise Hsp83 function. The morphological defects that were revealed could be selected for, so that the traits became independent of the *Hsp83* mutation. Variation for these traits therefore existed in the original populations, as found by Waddington in his heat shock experiments. The traits selected for by Rutherford and Lindquist were also enhanced at higher temperatures. They proposed a mechanism whereby Hsp90 is important in stabilising many developmental signalling proteins. If Hsp90 function is compromised, or sequestered by general protein damage, these signals are weakened. The simultaneous weakening of several signals important to the same

developmental process can have a major effect on the morphological outcome. The most vulnerable developmental pathways are those that contain cryptic genetic variation, and over many rounds of selection these mutations can accumulate and overcome the buffering effect of Hsp90 on the trait of interest (Rutherford and Lindquist, 1998, Figure 1.2).

Further work in *Arabidopsis* has confirmed these findings, and demonstrated that Hsp90 can buffer potentially useful traits (Queitsch *et al.* 2002). The conclusions drawn from these studies were that Hsp90 can therefore be viewed as an "evolutionary capacitor" that allows the accumulation of hidden genetic variation and releases it upon environmental stress. It is assumed that certain traits that arise under conditions of stress could be useful, and would be selected for if the selective pressure remained (Reviewed in Rutherford, 2000).

Hsp90 has also been suggested to have a major effect on morphological variation *via* an epigenetic mechanism (Sollars *et al.* 2003). A screen for genetic factors affecting a *Drosophila* ectopic outgrowth mutant identified both chromatin remodelling genes, and *Hsp83*, as modifiers of the trait. Sollars *et al.* went on to demonstrate that the effect of *Hsp83* mutations on the trait was epigenetic. Selection for enhanced outgrowth occurred over a short period in an isogenic strain, indicating that genetic variation did not contribute to the phenotype. It was suggested that the effect of Hsp90 on imprinting could be a general mechanism by which inheritance of traits can be affected, and contribute to the effect of Hsp90 on the evolution of new traits under conditions of environmental stress. It appears that Hsp90 can affect developmental outcomes by both genetic and epigenetic mechanisms, although the proportion of morphological variation caused by either mechanism is as yet unknown (Sangster *et al.* 2003).

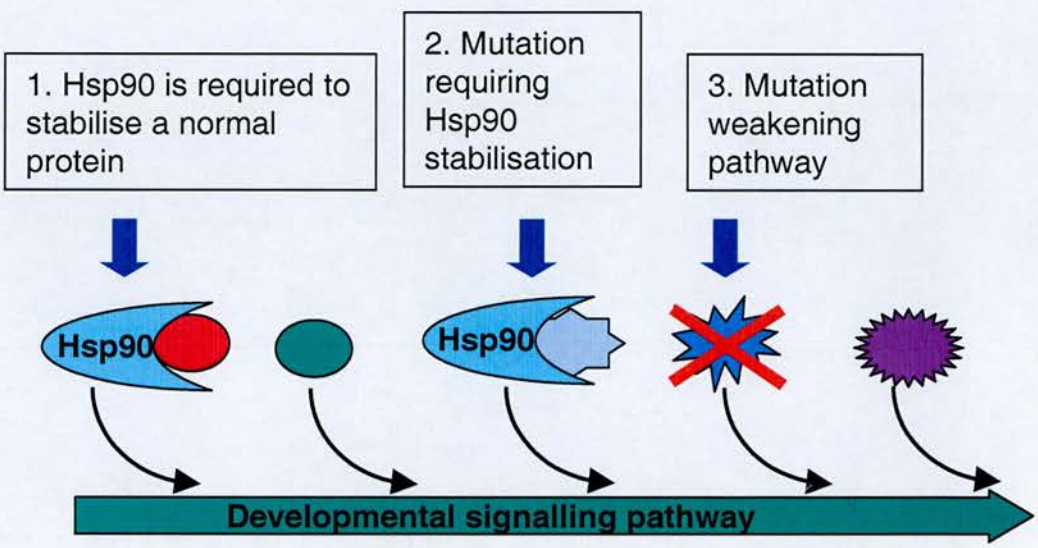


Figure 1.2: Model showing Hsp90 buffering of cryptic mutations. For any developmental pathway there are multiple inputs from different WT and mutant signalling proteins, some of which are stabilised by Hsp90. If Hsp90 function is compromised, a developmental pathway that is dependent on Hsp90 stabilisation of many proteins or that contains other mutations may be affected, and the developmental outcome altered (Rutherford and Lindquist, 1998).

1.3 Heat shock proteins and the stress response

A short period of heat stress during mammalian embryonic development has been shown to protect against a further, more severe heat shock. This protection is concurrent with a reduction in general protein synthesis, and the upregulation of a specific group of proteins. These are defined as Heat Shock Proteins (HSPs) simply by the fact that they are upregulated in response to a temperature elevation, but are also activated by many other factors such as hypoxia, ethanol and glucose deprivation (Walsh *et al.* 1997). HSPs have been identified in all organisms studied. The primary function of many HSPs is to assist in the folding of newly translated or damaged proteins, so that they are often described as "chaperone" proteins. A large body of work has been carried out to investigate almost all aspects of HSP structure, function and regulation (Reviewed in Latchman 1999).

Many of the heat shock proteins have a constitutive form that is dynamically expressed during embryogenesis, suggesting important functions in specific stages of development (Tanguay *et al.* 1993, Vamvakopoulos, 1993, Walsh *et al.* 1997, D'Souza and Brown 1998). Expression of the inducible form is usually relatively restricted under normal conditions, but more ubiquitous during heat shock (Krone *et al.* 1997).

Complex mechanisms exist to ensure a rapid induction of HSP expression, and a downregulation of the translation of all other proteins, in response to stress (Reviewed in Sierra and Zapata, 1994, Reviewed in Morimoto, 1998). HSPs are transcriptionally regulated by heat shock transcription factors (HSFs), which bind heat shock elements (HSEs) in the 5' UTR (untranslated region) or promoter region. HSF1 is the most important of these. Under normal conditions, HSF1 is kept in a monomeric, inactive form by dynamic association with a complex containing Hsp90 and Hsp70 (Guo *et al.* 2001). Upon heat shock, HSF1 forms active trimers and binds HSEs in *HSP* genes, to activate transcription. When the levels of HSPs increase, an HSF1-Hsp70-Hsp40 complex forms which dissociates from the DNA, and HSF1 eventually forms monomers

again, in association with Hsp70 and Hsp90 (Morimoto 1998, Guo *et al.* 2001, Figure 1.3).

Induction of HSP results in thermotolerance that protects sensitive developmental processes from the damaging effects of heat stress, mainly apoptosis due to the build-up of unfolded proteins. Apoptosis in the developing embryo causes malformations, and is the cause of the CNS defects commonly observed after severe heat stress. HSP induction in thermotolerant embryos helps to prevent protein damage, but this survival occurs at a cost, however, as downregulation of normal protein expression causes a developmental delay (Walsh *et al.* 1999).

1.4 Hsp90 structure and function

Hsp90 is unique among the chaperone proteins, in that under normal conditions it interacts with specific kinases and transcription factors important in development, such as the steroid hormone receptors, Raf kinase, v-src, and p53 (Reviewed in Richter and Buchner, 2001, Reviewed in Picard, 2003, Figure 1.4). It is this specificity for a subset of cellular proteins that makes the role of Hsp90 in buffering the effects of mutations so important during development. When Hsp90 function is compromised pharmacologically, or by mutation, the output of developmental signalling pathways is affected (Picard *et al.* 1990). This is analogous to the situation that would occur if Hsp90 was sequestered by unfolded proteins due to environmental stress. Many of the Hsp90 client proteins are important in human disease, as well as ageing and the immune response (Csermely *et al.* 1998, Ishii *et al.* 1999, Smith *et al.* 1998, Harvey *et al.* 2002, Soti and Csermely 2002). This has led to a great deal of interest in Hsp90, and its structure, biochemical function and interactions have been intensively studied.

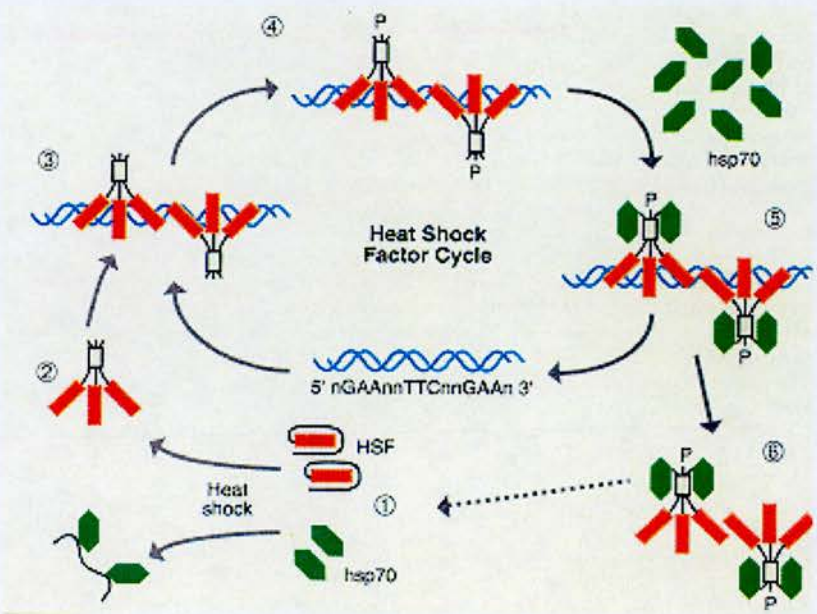


Figure 1.3: Model of *Hsp90* transcriptional regulation. In normal conditions HSF1 is in an inactive form, bound in a complex including Hsp70 (1). Under stress, HSF1 forms a trimer, enters the nucleus (2), binds the HSE (3), and is phosphorylated (4). The *HSP* is transcribed until it accumulates and binds to the HSFs to inhibit transcription (5). The HSF1-Hsp70 complex dissociates and leaves the nucleus (6), and HSF1 returns to its monomeric state (Reproduced from Morimoto 1993).

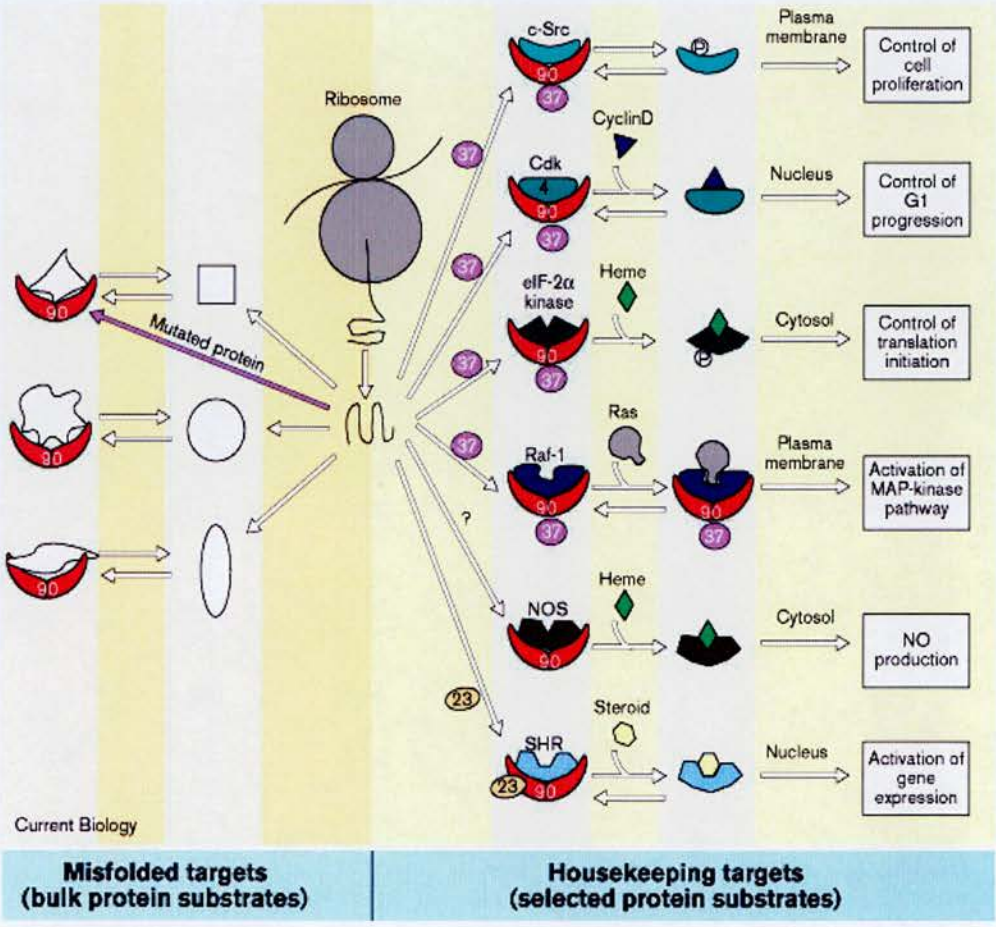


Figure 1.4: The dual function of Hsp90, as a chaperone of a specific set of proteins during normal conditions, and a general chaperone of unfolded proteins during stress (Reproduced from Mayer and Bukau, 1999).

1.4.1 The Hsp90 family

In humans there are two *Hsp90* genes, *HSP90α* and *β* (to be referred to as *Hsp90a* and *b*). Orthologues for these have been identified in other vertebrates, the most studied examples of these being the mouse, chicken and zebrafish. There are also two *Hsp90* genes in yeast, but surprisingly only one form of *Hsp90* (*Hsp83*) has been identified in *Drosophila*. In *Arabidopsis* there are 7 Hsp90 family members (Krishna and Gloor 2001). The Hsp90 protein is highly conserved, with a minimum of 40% amino acid identity between different organisms (Gupta, 1995, Table 1.1). *GRP94* (Glucose Regulated Protein 94) is the endoplasmic reticulum (ER) homologue of Hsp90 (Reviewed in Argon and Simen 1999), while *TRAP1* (Tumour necrosis factor receptor associated protein 1)/*Hsp75* is present in the mitochondria (Felts *et al.* 2000, Pflanz and Hoch, 2000). *Hsp89αΔN*, or *Hsp90N* is thought to be an alternatively spliced form of Hsp90α, but its function is unknown (Schweinfest *et al.* 1998, Grammatikakis *et al.* 2002).

Human	Mouse	Zebrafish	Drosophila	Yeast	E.coli
Hspca/*HSP90α*	***Hsp84-3***/*Hsp86*	*hsp90a/hsp90α*	*Hsp83*	*HSP82*	*HtpG*
Hspcb/*HSP90β*	***Hsp84-2***/*Hsp84*	*hsp90b/hsp90β*		*HSC82*	
Tra1/*GRP94*	*GRP94*				
TRAP1/HSP75			*dtrap-1*		
HSP89αΔN					

Table 1.1: The Hsp90 gene family. Official MGD mammalian nomenclature in bold (http://www.informatics.jax.org/, Blake *et al.* 2003).

The alpha and beta forms share a high level of sequence identity, but seem to have slightly different functions. Each form is more closely related to its corresponding form in other organisms than to its partner in the same animal. The exceptions to this rule are zebrafish and salmon, where both forms are similar to the beta form of other organisms (Gupta 1995, Pepin *et al.* 2001, Figure 1.5). The *Hsp90a* and *b* genes are very similar, with 86% amino acid identity (Zhang *et al.* 1999) and 76% nucleotide identity,

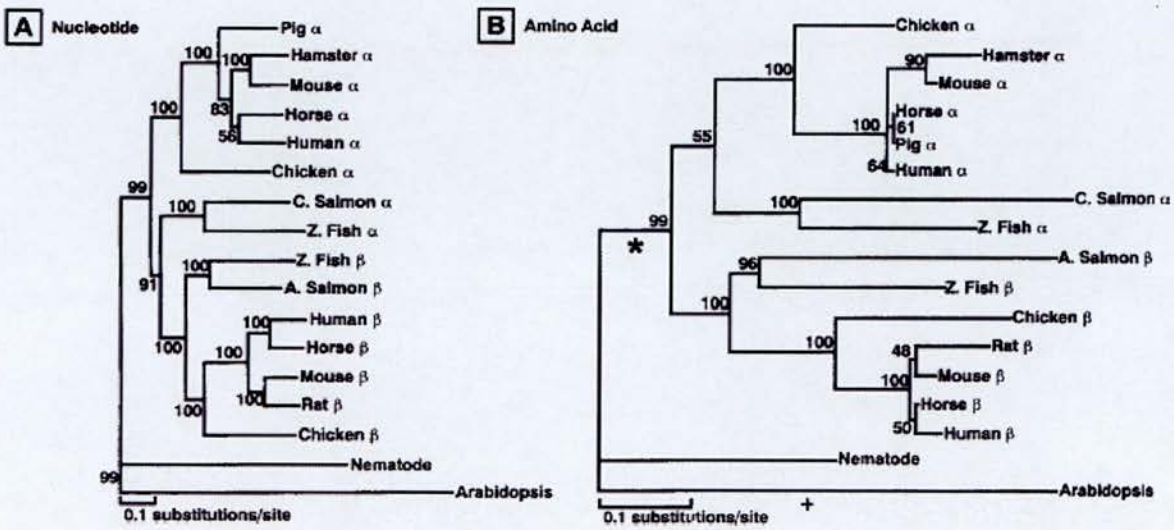


Figure 1.5: Phylogenetic trees showing the relatedness of the *Hsp90alpha* and *beta* forms. (a) Nucleotide; **(b)** Amino acid (Reproduced from Pepin *et al.* 2000).

(Csermely *et al.* 1998). The Hsp90a and b proteins are difficult to distinguish at the protein level, and are often studied jointly, as Hsp90 (Voss *et al.* 2000, Moore *et al.* 1989), although gene specific probes and antibodies to the two forms have been developed (Nemoto and Sato, 1998, Sass and Krone 1997).

1.4.2 Hsp90 protein structure

Nearly all members of the Hsp90 protein family have two highly conserved functional domains, connected by a flexible charged linker (Reviewed in Pearl and Prodromou 2000, Reviewed in Young *et al.* 2001, Figure 1.6). The N-terminal domain binds the target protein, and has an ATP binding site (Prodromou *et al.* 1997, Obermann *et al.* 1998, Panaretou *et al.* 1998, Young and Hartl, 2000). The Hsp90 family is part of the novel GHKL (Gyrase, Hsp90, Histidine Kinase, MutL) ATPase/kinase superfamily, which also includes the Prokaryotic His-Asp signal transduction histidine kinases, the gyrase DNA toposiomerases and the MutL-like DNA mismatch repair proteins. The ATP binding domain of these proteins is unique, in that it contains the Bergerat ATP-binding fold, the significance of which is unclear. This domain is targeted by several drugs that specifically bind Hsp90 in eukaryotes, and which have proved extremely useful in studying the structure and functions of Hsp90 *in vitro* and *in vivo* (Reviewed in Smith *et al.* 1998, Reviewed in Neckers *et al.* 1999). The most widely used of these are geldanamycin and radicicol (Whitesell *et al.* 1994, Schulte *et al.* 1997, Schulte *et al.* 1998, Sharma *et al.* 1998, Schulte *et al.* 1999).

The C-terminal domain of Hsp90 is responsible for dimerisation (Csermely *et al.* 1998), as well as participating in the chaperone function of protein folding (Minami *et al.* 2001, Shaknovitch *et al.* 1992, Scheibel *et al.* 1999, Johnson *et al.* 2000, Nemoto *et al.* 2001). The C-terminal also contains an ATP binding site that is exposed when the N-terminal site is occupied (Marcu *et al.* 2000, Garnier *et al.* 2002, Söti *et al.* 2002b). The charged linker is only present in eukaryotic cells, and may not be essential, as it varies in length and amino acid sequence between species (Pearl and Prodromou, 2000).

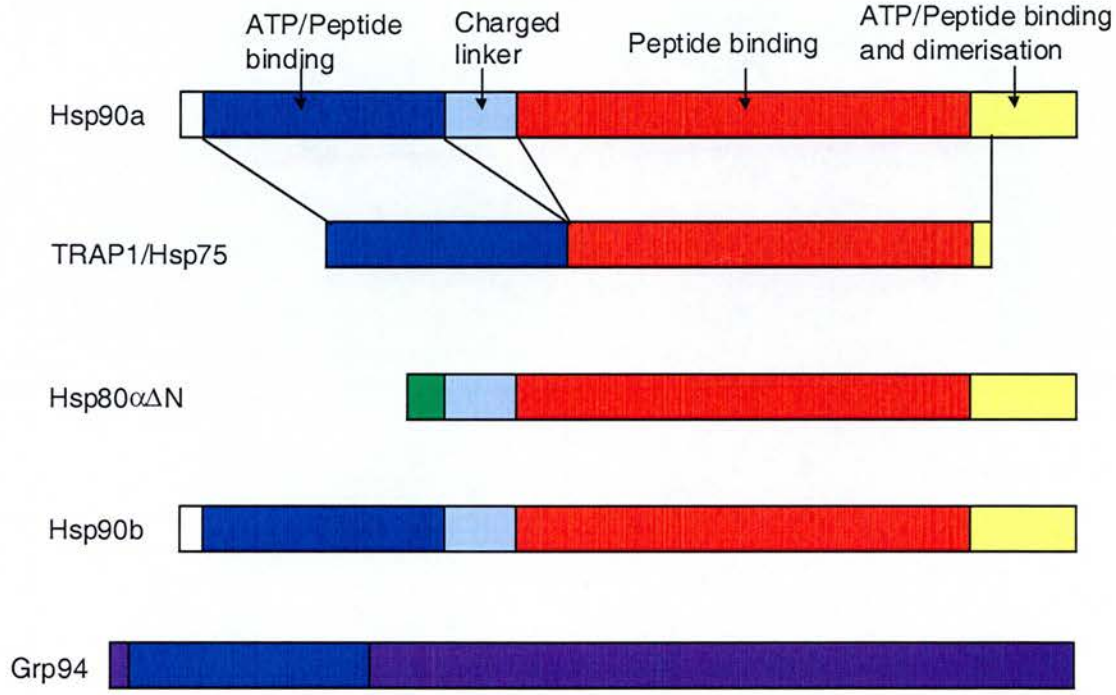


Figure 1.6: Structure of human Hsp90 family proteins. Hsp90αΔN-unique region, green; GRP94-unique regions, purple (Schweinfest *et al.* 1998, Argon and Simen, 1999, Felts *et al.* 2000).

1.4.3 Hsp90 protein function

The a and b forms of *Hsp90* are very similar in sequence, but differ markedly in their regulation. Hsp90b is constitutively expressed (Voss *et al.* 2000) and is only weakly inducible, while Hsp90a is expressed at lower levels under normal conditions but is a great deal more heat inducible (Reviewed in Loones *et al.* 1997). Their expression patterns during development are very different. For example in the mouse they are both ubiquitously expressed at a high level during early embryogenesis, with the a form (Hsp86) at a slightly lower level, and appearing slightly later than b (Hsp84) (Barnier *et al.* 1987, Moore *et al.* 1989, Gruppi *et al.* 1991, Lee, 1990, Walsh *et al.* 1997, Bancewicz, 2000, Figure 1.7). The expression of Hsp86 in the adult is highest in the testis, with moderate levels in some other tissues, while Hsp84 is ubiquitously expressed, with the highest levels in the brain (Tanguay *et al.* 1993). Both forms are also expressed in the eyes at E11.5 to E14.5, with decreasing expression from E15.5 to E18.5, although Hsp86 expression remains high in the retina (Tanaka *et al.* 1995).

Hsp90 protein is present largely in the cytoplasm of HeLa cells, and is one of the most abundant proteins in tissues in which it is present (over 1% total protein in most expressing mouse and human tissues, Lai *et al.* 1984). Nuclear expression has been reported in rat hepatocytes (Schlatter *et al.* 2002). Hsp90a fused to GFP was shown to reproduce this pattern in Swiss 3T3 (mouse embryonic fibroblast) cells under normal conditions, with expression mainly in the cytoplasm, and a higher level of expression in the nucleus after heat shock (Langer *et al.* 2003).

Hsp90 binds "abnormal" proteins. It recognises the structure of folding intermediates (Jakob *et al.* 1995), and can act either as a molecular chaperone, assisting in the refolding and reactivation of denatured proteins, or it can initiate degradation. The interaction that occurs with the target protein is dynamic, with a constant association and dissociation that prevents reactions with the wrong substrates, and allows proper folding

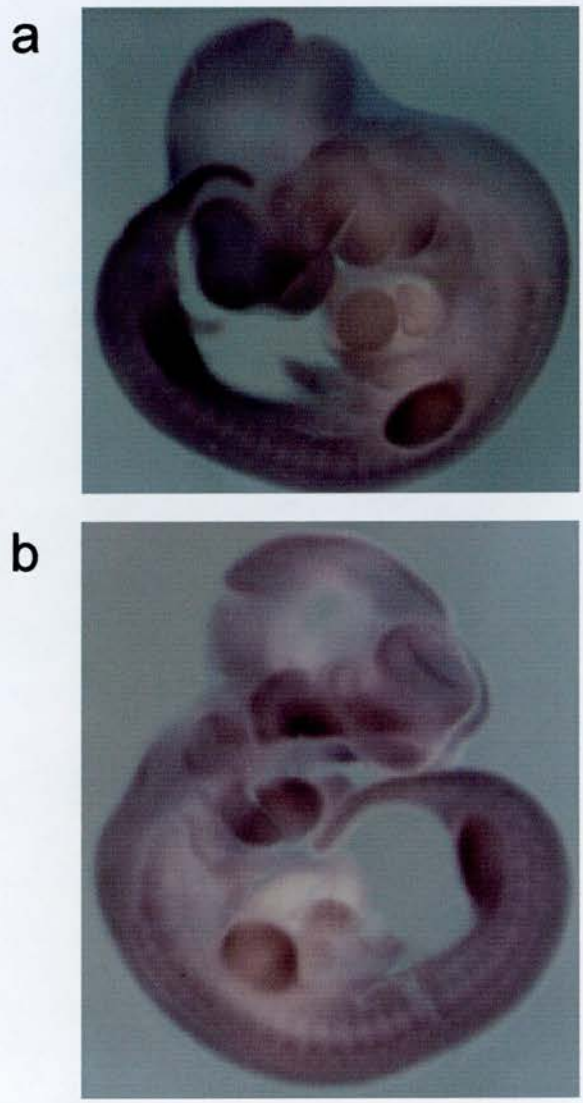


Figure 1.7: Expression of Hsp90 homologues in the mouse at embryonic day 10.5. (a) Hsp86 (Hsp90a); **(b)** Hsp84 (Hsp90b). (Reproduced from Bancewicz, 2000).

to occur. Other chaperones are usually required for the final folding of substrates (Reviewed in Parsell and Lindquist, 1993, Mayer and Bukau, 1999, Caplan, 1999). The interaction of Hsp90 with its substrate is thought to occur usually as a cycle controlled by the binding and release of ATP by the Hsp90 dimer (Reviewed in Young *et al.* 2001). ATP is bound by the Hsp90 dimer, containing a substrate protein. This changes the conformation of the dimer to a closed "molecular clamp" around the substrate, reminiscent of MutL and DNA gyrase. ATP hydrolysis accompanies the release of the substrate. (Grenert *et al.* 1997, Prodromou *et al.* 1997, Obermann *et al.* 1998, Panaretou *et al.* 1998, Prodromou *et al.* 2000, Weikl *et al.* 2000, Young and Hartl, 2000, Richter *et al.* 2001, Meyer *et al.* 2003, Figure 1.8). Although both forms of Hsp90 are sometimes coexpressed, evidence for the existence of heterodimers remains controversial (Perdew *et al.* 1993, Garnier *et al.* 2001).

Hsp90 functions as part of several different multimolecular complexes with many cochaperones. These include Hsp70, p23, and proteins containing the tetratricopeptide repeat (TPR), such as the immunophilins, and p48 and p60 (Hip and Hop) (Reviewed in Neckers *et al.* 1999b). It has been suggested that it is this wide variety of partner proteins that allows Hsp90 to be involved in specific interactions in such a diverse range of cellular processes (Grenert *et al.* 1997).

Most of the proteins that the constitutively expressed Hsp90 interacts with under normal conditions are involved in signal transduction in major cell cycle and developmental regulatory pathways. These are typically large and complex metastable proteins, with regulatory steps involving conformational change (Reviewed in Young *et al.* 2001). They include kinases such as $pp60^{v\text{-}src}$, the src related tyrosine kinases and Bcr-Abl, and also transcription factors, including p53, and the steroid receptors. Other unrelated clients are actin, tubulin, calmodulin, the cystic fibrosis transmembrane receptor, telomerase, and the SV40 large T-antigen (Reviewed in Picard, 2003). Hsp90 is involved in many different stages of their maturation, but does not affect the stability of the mature proteins. This is illustrated by the fact that when Hsp90 is inhibited, the

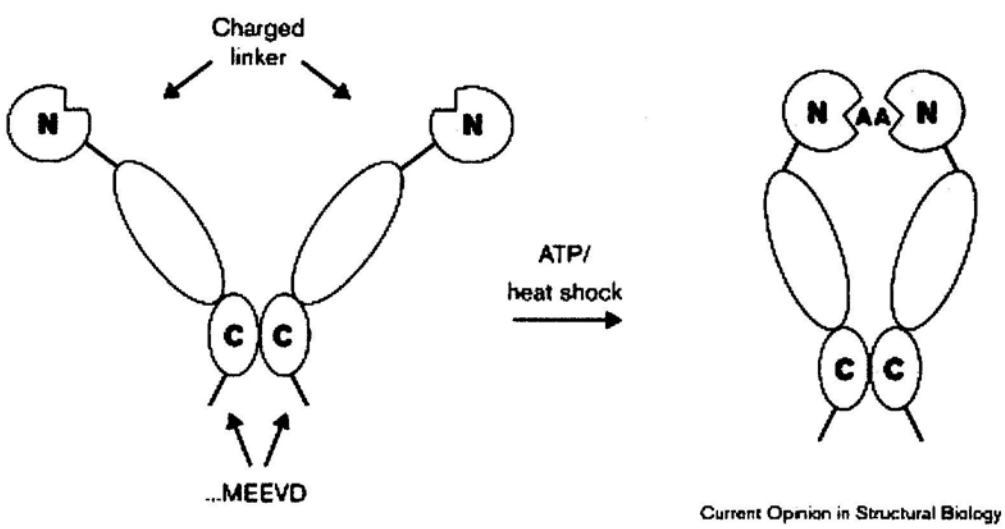


Figure 1.8: The conformational switch of Hsp90. Binding of ATP changes the conformation of Hsp90 to the closed molecular clamp form (Reproduced from Pearl and Prodromou, 2000).

levels of the proteins it interacts with decrease over time and then gradually increase again after inhibition is removed, as only the newly synthesised proteins are affected (Schulte *et al.* 1997, Sakagami *et al.* 1999, Marcu *et al.* 2000b). The most comprehensively studied of the Hsp90 clients are the steroid hormone receptors (SHRs). A model has been proposed in which Hsp90 keeps them in an activatable monomeric state in the cytoplasm. When they contact the hormone they dissociate from Hsp90, dimerise, enter the nucleus, and activate transcription (Nathan and Lindquist, 1995, Ylikomi *et al.* 1998, Reviwed in Buchner, 1999, Reviwed in Mayer and Bukau, 1999).

Hsp90 is essential for development in eukaryotes. Heterozygous *Hsp84* (*Hsp90b*) knockouts in mice are phenotypically normal, while homozygotes have placental abnormalities and die at E10.0-10.5 (Voss *et al.* 2000). Only 45% of embryos cultured in the presence of Hsp90-specific antibodies survive to day 7 of development (Neuer *et al.* 1998). A knockout of both *Saccharomyces cerevisiae* genes prevents growth under any conditions (Borkovitch *et al.* 1989). In *Drosophila*, homozygous Hsp83 mutations are lethal (Rutherford and Lindquist, 1998). The *Hsp90* homologue in *E.coli* (*HtpG*), however, is not essential, and the only effect seen on homologous mutants is a small reduction in growth at increased temperatures (Bardwell and Craig, 1987). The importance of Hsp90 in individual developmental processes has also been demonstrated. It has been shown to be involved somitogenesis (Krone and Sass, 1994, Sass *et al.* 1996, Sass and Krone, 1997, Sass *et al.* 1999), spermatogenesis (Lee, 1990, Gruppi *et al.* 1991, Yue *et al.* 1999), in development of the CNS and brain (Walsh *et al.* 1997, D'Souza and Brown, 1998, Calabrese *et al.* 2002), the placenta (Voss *et al.* 2000), and the eye (Tanaka *et al.* 1995, Kojima *et al.* 1996).

Although a null mutation in *Hsp90b* is lethal in homozygous mice, heterozygotes show no obvious phenotype (Voss *et al.* 2000). Point mutations however, can cause a phenotype in *S. cerevisiae*, and have been useful for *in-vivo* studies of Hsp90 protein function (Nathan and Lindquist 1995). In humans several polymorphisms have been identified, including one heterozygous viable missense mutation that appears to have no

deleterious effect (Passarino *et al.* 2003). A missense mutation has also been identified among infertile men, and may be associated with azoospermia (Yamamoto *et al.* 2002). Point mutations have been identified in *Drosophila* that were lethal in homozygotes but viable in transheterozygotes, causing only fertility defects (Yue *et al.* 1999). Also in *Drosophila*, point mutations have been discovered in two separate studies, which affect the severity of mutant eye phenotypes in heterozygotes (Cutforth and Rubin, 1994, van der Straten *et al.* 1997). The effect of Hsp82 mutations on *Drosophila* eye phenotypes is evidence that Hsp90 may be important for eye development in other organisms, and that a reduction in Hsp90 function may also affect mutant eye phenotypes.

1.5 Zebrafish as a model system to study Hsp90 buffering of developmental defects

The Hsp90 protein is highly conserved, from *Drosophila* to higher organisms. It would be reasonable to expect, therefore, that most of its functions are also conserved. Study of non-Mendelian diseases like MAC, and mouse mutants with variable phenotypes such as *Pax6* (Hill *et al.* 1991) or *Foxc1* (Forkhead box C1)*/Mf1* (Kume *et al.* 1998), show that hidden genetic variation also exists in vertebrates. This variation may also be subject to the same Hsp90 buffering seen in *Drosophila*. Although the buffering mechanism is interesting from an evolutionary point of view, it might also be important for understanding hereditary disease in humans.

1.5.1 Zebrafish as a model organism

Rare developmental disorders are difficult to study in the human population, especially when several factors are involved, and there is a wide variation in phenotype. Model organisms are therefore extremely valuable, as they enable the study of specific genetic and environmental factors in a controlled population. The zebrafish, *Danio rerio* is increasingly used as a model organism for developmental and genetic studies. It is particularly suited to this type of work for several reasons. The life cycle is 2-3 months, and adults can lay approximately 200-300 eggs once a week. The chorion is transparent,

and development can be viewed in detail by light microscopy. The larvae hatch at 2-3 days post fertilisation (dpf), and can feed independently at 5-6 days (Nüsslein-Volhard *et al.* 2002). This allows large scale experiments to be carried out on a regular basis. Additional benefits are low maintenance costs, and the ease of microinjection into the zygote. The fundamental developmental mechanisms of the zebrafish are very similar to other model organisms, so that it can be used to study vertebrate developmental processes, and disease mechanisms (Reviewed in Dooley and Zon, 2000, Kimmel, 1989).

These properties of the zebrafish make it suitable for large scale genetic screens. Hundreds of mutants have been produced by ENU mutagenesis, and are available at low cost from several stockcentres (Haffter *et al.* 1996, Driever *et al.* 1996). These mutants are mostly recessive, and phenotypically characterised, but not mapped. Genetic maps are available, with an increasing number of markers, and the zebrafish genome is currently being sequenced as part of an international effort, so that the mapping and identification of genes should become increasingly easier (Kelly *et al.* 2000, Postlethwait *et al.* 1998). New strategies are being developed to make genetic analysis in zebrafish easier. Insertional mutagenesis allows new mutations to be mapped (Golling *et al.* 2002, Talbot and Hopkins, 2000), while coupled mutagenesis and mapping (sometimes referred to as "tilling") increases the efficiency of chemical mutagenesis screens (Wienholds *et al.* 2002, Rawls *et al.* 2003). Other powerful approaches for the analysis of gene function are the use of pharmacological agents that reproduce certain phenotypes, overexpression studies, and knockdown of gene expression with antisense oligonucleotides (Patton and Zon 2001). Targeted gene knockout has not been achieved in zebrafish, due to difficulties in culturing ES cells. Germ line chimeras, however, have been produced, so that it is reasonable to expect that homologous recombination may be possible in the near future (Ma *et al.* 2001).

One difference between zebrafish and mammalian genetics is the relatively recent genome duplication that appears to have occurred in the zebrafish lineage (Taylor *et al.*

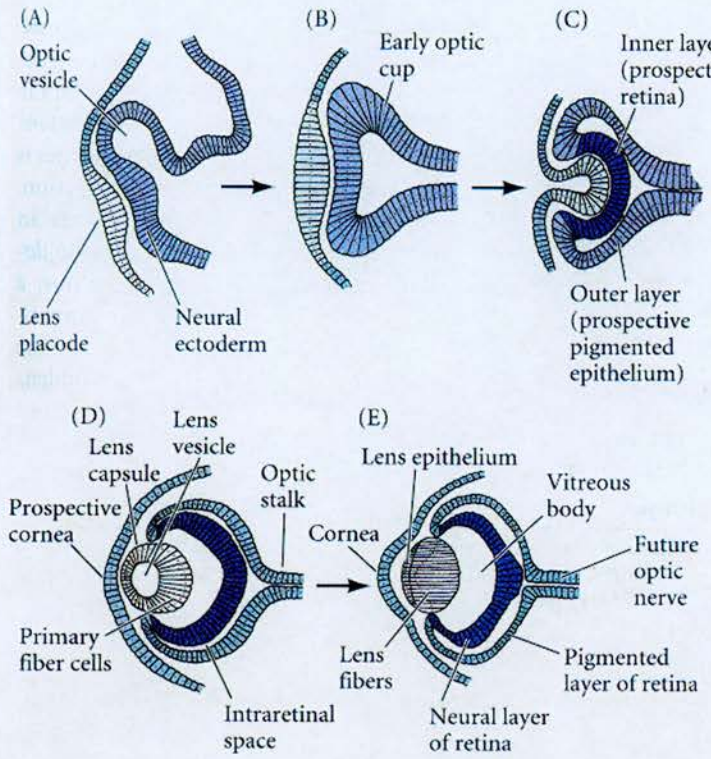
2003). It has been proposed that duplicated genes can be preserved by alterations in their regulatory elements that confer different roles on very similar genes (Force *et al.* 1999). Zebrafish appear to have retained duplicates of many genes. This allows the dissection of the roles of genes that are essential for survival in higher organisms, as viable mutations are more likely to occur.

Fish have already been used to study the effects of toxins in the environment, and work has been carried out to investigate the possibility of using HSPs as an indicator of toxic damage (Bierkens, 2000, Reviewed in Langheinrich, 2003). Notably, a GFP reporter driven by the *Hsp70* promoter has been used to detect toxic levels of cadmium in zebrafish embryos (Blechinger *et al.* 2002). Zebrafish are particularly suited to a study of environmental effects on development because all the variables of their environment can be controlled very easily, large numbers of embryos can be treated, and any defects can be easily observed at any time of development. The increasing characterisation of a large number of mutants also provides an excellent resource for studying different developmental pathways, and the possible involvement of Hsp90 buffering.

1.5.2 Zebrafish eye development

The zebrafish is an excellent model for the study of eye development, as the eye develops rapidly and is functional at just 2 dpf (Appendix A). The development and morphology of the eye is essentially conserved from zebrafish to humans (Easter and Malicki 2002). Development begins with the evagination of the optic vesicles from the future diencephalon. Contact between the optic vesicle and the overlying surface ectoderm stimulates formation of the lens placode, which invaginates to form the lens vesicle. The optic vesicle invaginates to form the optic cup, with the optic fissure that closes around the hyaloid artery and vein. The lens buds off from the ectoderm, while the optic cup differentiates into the different layers of the neural and pigmented retina (Gilbert 2000, Larsen 2001, Figure 1.9).

a



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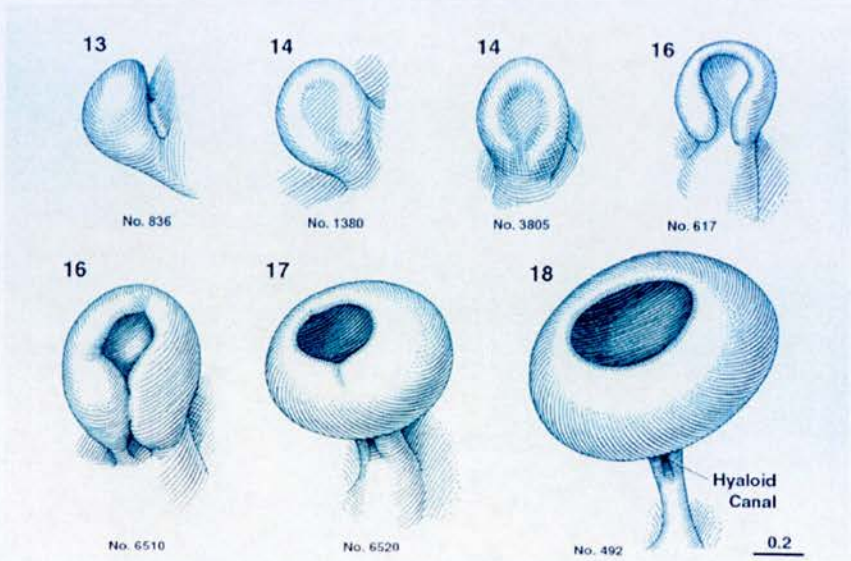


Figure 1.9: Vertebrate eye development. (a) Formation of the retina and lens vesicle (Reproduced from Gilbert 2000); **(b)** Formation and closure of the optic fissure (Reproduced from O'Rahilly and Miller, 1987).

Several screens have been carried out in zebrafish to identify mutations that cause morphological defects in the eye, as well as behavioural defects indicative of a loss or reduction of vision (Fadool *et al.* 1997, Neuhauss *et al.* 1999, Doerre and Malicki, 2002, Vihtelic and Hyde, 2002, http://www.eb.tuebingen.mpg.de/eye-screen/screen.html). These studies have contributed to the detailed analysis of eye development and neurogenesis, as well as providing models of human eye disease (Reviewed in Goldsmith, 2001).

1.5.3 Hsp90 in zebrafish development

In zebrafish, the expression of different HSPs, and the effects of heat shock on development have been well characterised (Reviewed in Krone *et al.* 1997b, Roy *et al.* 1999). The *hsp90a* and *b* genes (referred to as *Hsp90 a* and *b*) have been mapped and cloned in zebrafish (Krone and Sass, 1994), and their expression patterns are known (Table 1.2). Hsp90b is highly expressed at normal temperatures, mainly in the CNS. During heat shock it is expressed at only slightly increased levels, and then still mainly in the CNS (Sass *et al.* 1999, Krone *et al.* 1997, Sass *et al.* 1996, Reviewed in Krone *et al.* 1997b, Figure 1.10). Hsp90a is expressed at relatively low levels at normal temperatures, in select muscle progenitor cell groups during short periods of fast and slow muscle development. During heat shock, it is expressed at very high levels in all tissues. In the chick and mouse Hsp90 is also expressed in somitogenesis, but is present in other tissues. It may be that as both genes in zebrafish appear to have arisen from duplication of the *Hsp90b* form, both forms fulfil the same role as seen in other organisms, but the division of Hsp90 function between them is different.

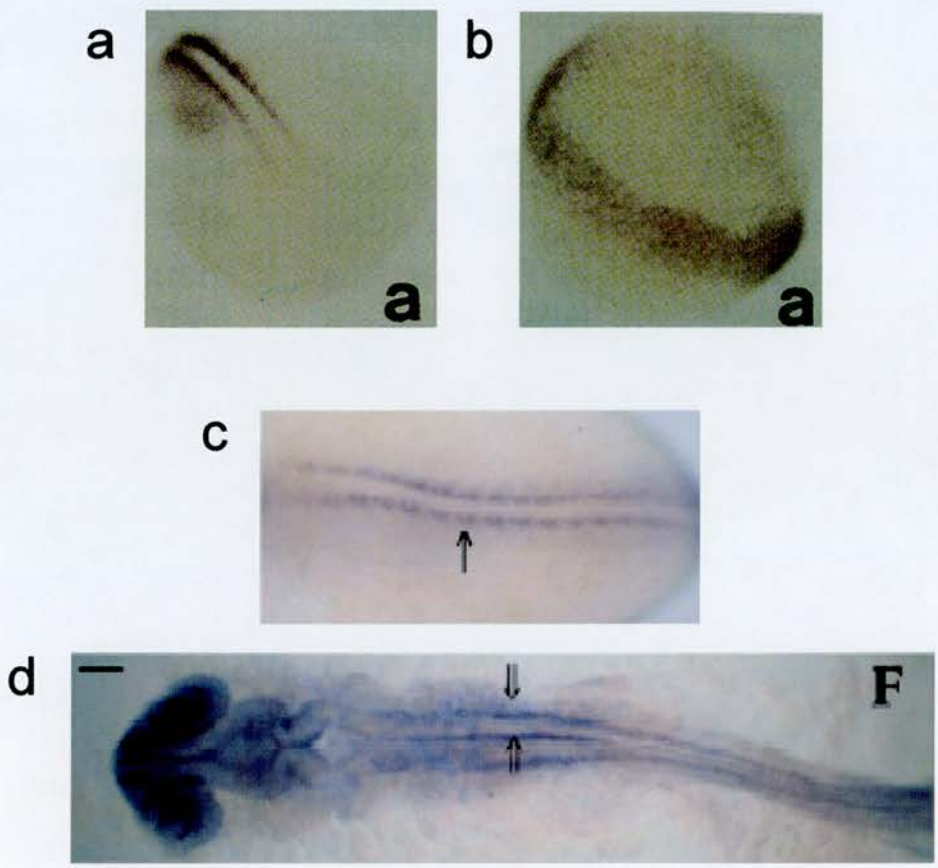


Figure 1.10: Hsp90 expression in zebrafish embryos at normal temperatures. (a) Hsp90a, 16h; (b) Hsp90b, 16h; (c) Hsp90a, 17h; (d) Hsp90b, 20h. a, anterior. (Reproduced from Sass *et al.* 1996, Lele *et al.* 1999).

Protein	Control (28.5°C)	37°C, I hour	4% ethanol	Known tissue-specificity
Hsp90a	+/-	++++	+	Putative myogenic cells
Hsp90b	++	+++	++	CNS, possible other tissues
Hsp70	-	++++	+	Not determined
Hsp47	+/-	++++	+++	Type II collagen expressing cells

Table 1.2: Relative levels and tissue-specificity of zebrafish heat shock protein mRNAs (Reproduced from Krone *et al.* 1997b).

The effect of the Hsp90 inhibitor geldanamycin during early development has been characterised in zebrafish by Lele *et al.* (1999). Geldanamycin inhibits Hsp90 in the same specific manner as in other organisms. The predominant, and lethal effect of treatment before gastrulation, is that some, but not all of the muscle cell groups that express Hsp90a are affected. Other phenotypes are also observed, including reduced pigmentation, small eyes, disrupted development of the fore- and midbrain, and defects of the circulatory system. These are all effects on the tissues that express Hsp90, and therefore may be due to specific inhibition of Hsp90. The fact that certain structures develop normally shows that Hsp90 is only required for the differentiation of specific tissues at specific time points. Inhibition must therefore be carefully timed to disrupt their development.

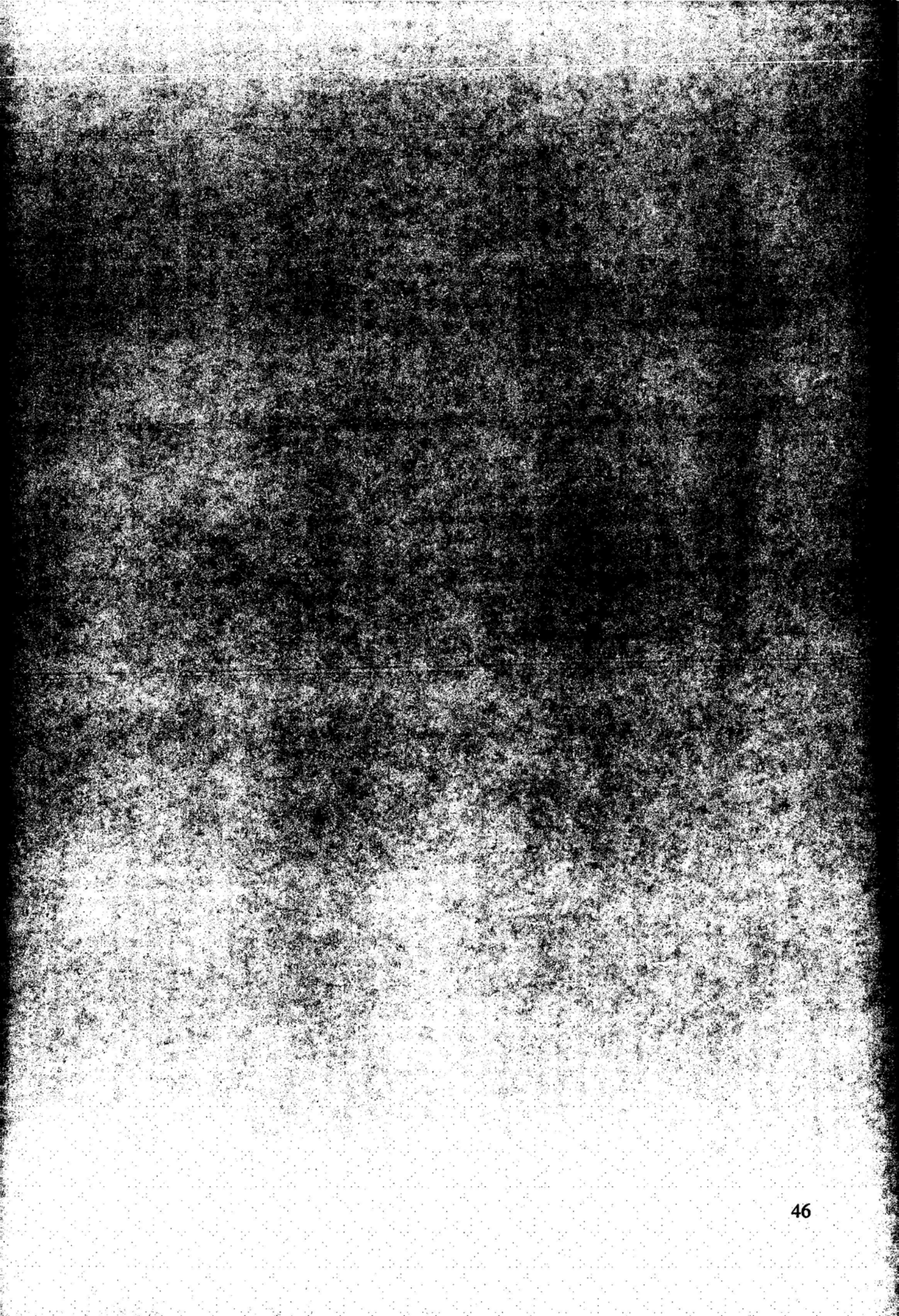
1.6 Aims and objectives

This study was conducted to question whether Hsp90 is a buffer of developmental defects such as MAC in vertebrates, using zebrafish as a model system. This hypothesis is based on the model outlined in Chapter 1.2 (p24): that Hsp90 can buffer the effects of mutations either directly, or indirectly *via* effects on proteins in developmental pathways (Figure 1.2). It was hypothesised that Hsp90 buffering could be identified by compromising Hsp90 function during development in mutant strains, and assessing whether the penetrance or severity of the phenotype was affected. The first aim was to assess the effects of several Hsp90 inhibitors on zebrafish embryos, and to identify the critical time during which developmental defects can be induced. A treatment level

would then be identified for each inhibitor that caused only very mild effects, which might reveal the phenotype of mutations buffered by Hsp90. This dosage and timing would then be used to treat several eye mutants: *sunrise, dreumes* and *microps*. WT embryos were also to be treated, to identify whether any hidden or cryptic variation existed in these strains.

If Hsp90 does buffer variation in mutant zebrafish lines, this mechanism is likely to be conserved in mammals. The mutations that are affected could be mapped, and studied in zebrafish, to identify the Hsp90 clients important in the buffering mechanism. This knowledge could then be applied to the study and treatment of human diseases.

There are two copies of *Hsp90* in vertebrates, including zebrafish, so it may not be practical to attempt to study buffering using mutants, as was done in flies. Using the pharmacological inhibitors, however, allows both forms of Hsp90 to be inhibited at the same time. As vertebrate development is far more complex than that of *Drosophila* or *Arabidopsis*, it was expected that a far higher level of inhibition might be required to compromise Hsp90 buffering. Also the two copies of Hsp90 present in zebrafish could provide an extra layer of buffering, or alter the way in which development is affected by Hsp90 inhibition. This is one reason why it may prove easier to use mutants with a known phenotype, and almost Mendelian inheritance, to look at alterations in inheritance rather than attempting to uncover hidden variation.



Chapter 2

Materials and methods

2.1 Zebrafish stocks and animal husbandry

The zebrafish mutants *sunrise, dreumes* and *microps* were created as part of an ENU mutagenesis screen (Haffter *et al.* 1996), and were obtained from the Tübingen zebrafish stockcentre. The WT lines *golden* (*gol*) and WIK were provided by the Currie Laboratory.

Zebrafish were raised, maintained and bred according to methods outlined in the Zebrafish Book (Westerfield, 1995), and with assistance from HGU technical staff and members of the Currie laboratory. Embryos were staged according to Kimmel *et al.* (1995) (Appendix A).

2.2 Hsp90 inhibitors

Novobiocin (Sigma) was dissolved in water to make a 50 mg/ml stock. A 25 mM stock of radicicol (Sigma) was made in 100% EtOH. Both these stocks were stored at –20°C. Geldanamycin (Sigma, AG scientific, donated by Peter Csermely, Semelweis University, Hungary, and donated by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, USA), and geldampicin (also donated by the NCI) were dissolved in 100% DMSO, to make a 5 mg/ml stock, and stored at –70°C. For treatment of zebrafish embryos, serial dilutions were made using the appropriate solvent, before adding to system water.

Geldanamycin precipitated out when added at a certain concentration to water, with yellow crystals forming on the bottom of the dish, as well as on the surface of the water. The concentration at which precipitation occurred varied between suppliers. Precipitation occurred at 10 μM for geldanamycin supplied by the NCI, and 15 μM for AG Scientific. Yellow crystals did not form with geldanamycin from Sigma, for concentrations up to 20 μM.

2.3 Treatment of zebrafish embryos

2.3.1 Hsp90 inhibitors

Embryos were collected from breeding pairs or as a spawning from a tank of adults. To weaken the chorion embryos were incubated in a 0.025 to 0.1 mg/ml solution of protease in 30 ml of system water in a petri dish for several minutes (timings required varied according to batch of protease), rinsed ten times, and a hole manually torn in the chorion with watchmaker's forceps. Embryos that had reached the correct stage were transferred to 30 ml system water containing the Hsp90 inhibitor, and incubated at 28°C (normal temperature). Controls contained same volume of carrier solvent as experimental plates. After 18 h embryos were rinsed five times in system water containing methylene blue. Larvae were examined between 24 and 144 hpf using a dissection microscope, and the criteria outlined in Haffter *et al.* (1996). To anaesthetise larvae a 1/30 solution of tricaine stock was used.

Protease: Type 1, Sigma, 5 mg/ml stock in dH_2O, store at –20°C in 1 ml aliquots.

Tricaine: Ethyl-3-aminobenzoate methanesulfonate salt, Sigma; 4% in dH_2O, adjusted to pH7 with Tris pH9, freeze or store working stock a 4°C.

At 1 dpf (24-32 hpf) embryos were scored as dead if necrotic and discoloured. Methylene blue is used as a sterilising agent in water containing embryos before 5 dpf (approximately 2 ml of 0.1% methylene blue in 1 litre of system water), as it inhibits the growth of mould (Westerfield, 1995). It was established during initial novobiocin experiments, that system water without methylene blue must be used, as a blue precipitate forms on addition of novobiocin.

Larvae were humanely killed at 5 dpf by immersion in a more concentrated solution of tricaine, and fixed for staining or sectioning as required.

2.3.2 Heat shock

Heat treatment was carried out in sealed petri dishes containing 30 ml system water and floating on a water bath for 30-60 min at 30-40°C, followed by a return to 28°C.

2.4 Quantitation and statistical analysis of severity of eye phenotypes

Quantitative scoring of the *sri* phenotype was carried out by taking digital photographs of each eye at the same magnification for all embryos. Segment analysis was then used to measure the area, major axis, eccentricity (irregularity) and standard deviation of the radius (SD radius, a measure of ellipticity) of the pupil and the outer edge of the retina. This was done by using IPLab software to draw a line around the pupil and outer edge of the retina, and calculate different parameters (Figure 4.2b, p105). Eccentricity is $\sqrt{[(\text{major axis})^2-(\text{minor axis})^2]}/(\text{major axis})$ for the region of interest, where a value of zero is given for a circle, and 1 for an extreme ellipse. A t-test was used to calculate the significance of the difference between the means ((t = mean (difference)/$\sqrt{}$ variance (difference), see Appendix B for an example of the working).

Scoring of the *sri* phenotype was carried out blind wherever possible. *sri* embryos from each treatment group were anaesthetized and sorted into 6 well tissue culture dishes according to the criteria A B and C, outlined in Chapter 4 (Table 4.4, p106). Statistical analysis was carried out as described in Chapter 4 (p104), using the t-test descibed above, and was designed by Andrew Carothers (HGU statistician).

2.5 Microscopy and image analysis

Larvae were anaesthetised and immobilised in 4% methylcellulose (Sigma), in a depression slide, where appropriate.

Zebrafish larvae were photographed using a Leica Fluo III stereomicroscope fitted with an RS Photometrics Coolsnap digital camera, and IPLab software (version 3.2 and 3.6).

Segment analysis was carried out using the IPLab software.

Sections were photographed (brightfield) using a Zeiss Axioplan 2 fitted with an RS Photometrics Coolsnap HQ digital camera and IPLab software. A Netz Power ebq 100 UV light source and filter "GFP2" were used to detect GFP expression.

For immunofluorescence, photographs were taken with a Zeiss Axioskop fitted with a Princeton Instruments digital camera, and a Netz power UV light source.

2.6 Microinjection of zebrafish embryos

Morpholino oligonucleotides were obtained from Gene-Tools, LLC (USA) as lyophilised pellets. Each 300 ng pellet was dissolved in 60 µl dH_2O to make a 5 mM stock, and stored at –20°C. 9 µl of each dilution from this stock was added to 1 µl of 1% phenol red (Sigma, sodium salt) in dH_2O for injection. Microinjection needles were made from 1 mm boro-silicate capillaries (1 mm outer diameter, 0.78 mm inner diameter, Harvard Apparatus) with a CFP micro-electrode puller. A Narishige IM 300 microinjector was used, with a dissecting microscope to inject the morpholinos into the cytoplasm of 2-4 cell stage embryos.

For mRNA injection, 9 µl of RNA was mixed with 1 µl phenol red, and injected as for morpholinos. GFP expression was detected from 24 hpf.

2.7 Histology

2.7.1 Wax embedding and microtome sectioning of zebrafish embryos

Fixing embryos:

1 x 5 min	50% EtOH
1 x 5 min	70% EtOH (Embryos may be kept in 70% EtOH at 4°C until needed)
1 x 20 min	90% EtOH
3 x 10 min	100% EtOH
Into glass bottles	
3 x 20 min	histoclear (last change in 58-60°C oven)
3 x 20 min	hot wax in 50-60°C oven

Embryos were embedded in wax in a 5 cm petri dish coated with glycerol (to prevent sticking and cracking), and cooled quickly on iced water. 5-7 μM microtome sections were cut, and floated onto slides (TESPA (3-aminopropyl-triethyloxysilane) coated or Superfrost Plus electrostatically charged slides (BDH) if slides to be used for immunohistochemistry) in a 45°C water bath. Slides were dried in a 50°C oven overnight, and stored at RT.

Dewaxing:
2 x 5 min xylene
2 x 5 min 100% EtOH
1 x 5 min 90% EtOH
1 x 5 min 70% EtOH
1 x 5 min 50% EtOH
1 x 5 min 30% EtOH
1 x 5 min dH_2O

De-waxed sections were stained with methylene blue and mounted with DePex mounting medium (Gurr, BDH).

2.7.2 Antigen retrieval

Nuclear antigens are often affected by the processing of wax sections. For effective staining with p48, an antigen retrieval step was used.

Make stocks of tri-Sodium citrate (0.1M) and Citric acid (0.1M) (pH 6.0):

41 ml	tri-sodium citrate
9 ml	citric acid
450 ml	dH_2O

Boil buffer in microwave. Put slides in hot buffer and microwave on full power for 30 sec, rest 1 min, then full power again for 30 sec. Allow slides to cool in buffer for 20 min at RT. Wash slides briefly in PBS.

2.7.3 Immunofluorescence

De-waxed sections were circled with a PAP pen, and washed with PBS, before incubation in blocking agent (PBS with 1% BSA and 2% normal serum) for at least 2 h at RT. Sections were then incubated overnight with primary antibody (Table 2.1, p58) in blocking agent at 4°C, followed by 2 hours of 20 min PBS washes. Sections were incubated with a fluorescently labelled secondary antibody (Table 2.2, p58) in blocking agent for 2 h at RT, followed by 2 hours of 20 min PBS washes. Slides were mounted with Vectashield mounting medium (Vector). Slides can be store at 4°C for several weeks. Double staining was carried out by incubating slides with both primary antibodies simultaneously, followed by both secondary antibodies.

TESPA coated slides
Dip 5 racks of 20 glass microscope slides for 20 sec in each of:

Step 1
10% HCl in 70% EtOH
dH_2O
100% acetone Air dry/ 5 min in oven

Step 2
2% TESPA (3-aminopropyltriehoxysilane, Sigma) in acetone
100% acetone
100% acetone Air dry/ 5 min in oven

Store at RT for up to 4 weeks. After this, step 2 must be repeated.

2.8 Western Blotting

2.8.1 Preparation of lysate from zebrafish embryos

(adapted from Westerfield (1995), p9.2)

Decorionate 50-100 embryos and rinse three times in cold Ringer's.

Ringer's:

116 mM	NaCl	11.6 ml 5 M
2.9 mM	KCl	1.45 ml 1 M
1.8 mM	CaCl	0.9 ml 1 M
5 mM	HEPES	25 ml 0.1 M

adjust to pH7.2

dH_20 to 500 ml

Transfer to cold Ringers with EDTA and protease inhibitors in petri dish:

50 ml	Ringers
5 ml	10 mM EDTA, pH7.0
150 µl	Protease inhibitor solution (approx 250 µl dH_20 with 1 tablet Complete mini ETDA free protease inhibitor tablets Roche cat no. 1 836 170)
5 ml	Tricaine stock

De-yolk by pipetting through drawn-out glass pastette with a pipette gun. Rinse twice in cold Ringer's. Spin to pellet embryos and remove liquid. (At this stage embryos can be transferred to a cryotube, quick frozen in dry ice/ethanol and stored at –70°C). Add 200 µl SDS sample buffer, transfer to a small mortar and homogenise (x50 turns). Boil 5 min in a water bath. Centrifuge for 1-2 min at 4°C and transfer supernatant to a new tube (re-homogenise if significant pellet). Freeze at –70°C or run immediately.

SDS sample buffer (10ml):

0.63 ml	1 M Tris-HCl, pH6.8
1.0 ml	glycerol
0.5 ml	B-mercaptoethanol
1.75 ml	20% SDS
6.12 ml	H_20

Store in aliquots at –20°C.

Alternative, 2x (minus B-Me, 5ml):

0.5 ml	1 M Tris-HCl, pH6.8 (or 6.9)
1.0 ml	Glycerol
0.5 ml	2M DTT
2.0 ml	10% SDS
(75 µl	Bromphenol Blue)
0.95 ml	dH_2O

2.8.2 Quantitation of protein samples

A Bradford assay was used to assess the relative amounts of protein in each lysate, and allow equal loading. A Protein assay kit was used (BIO-RAD Protein assay dye reagent, or Pierce Coomassie Plus protein assay reagent). Samples were diluted at least 1/100 in dH_2O if SDS buffer was used. The standard microassay procedure was followed according to the manufacturers instructions, and the OD 595 was measured using a UV spectrophotometer.

2.8.3 SDS-polyacrylamide gel electrophoresis

A denaturing (SDS) polyacrylamide gel was prepared in a BIO-RAD Mini-PROTEAN 3 Cell, or a Hoefer SE 600 Vertical Slab Unit. The appropriate percentage resolving gel was made Sambrook *et al.* (1989) p18.52, poured to allow approximately 1 cm below the comb for the stacking gel, and overlaid with 1 ml Isobutanol saturated with H_2O (mix 1:1 Isobutanol and dH_2O) while setting. Isobutanol was then removed, and the stacking gel was added and allowed to set.

Stacking gel:	1.48 ml	dH_2O
	0.25 ml	1 M Tris pH6.9
	0.26 ml	BIO-RAD 30% Acrylamide/Bis solution 20:1
	10 µl	20% SDS
	10 µl	10% APS (Ammonium Persulphate)
	2 µl	TEMED (N, N, N', N'-tetramethylethylenediamine)

5 x SDS running buffer:	15.1 g	Tris base (Tris(hydroxymethyl)methylamine)
	72 g	Glycine
	25 ml	25% SDS
	Made up to 1 l with dH_2O	
6x SDS loading buffer:	3.5 ml	1 M Tris-HCl pH6.8 (350 mM
	3 ml	glycerol
	1 g	SDS
	0.93 g	DTT (0.6 M)
	1.2 mg	Bromphenol Blue
	10 ml	

Samples were added to loading buffer and denatured by heating for 3 min at 85°C in a PCR block, and immediately returned to ice. The size markers used were Biorad Prestained SDS-PAGE standards (Broad range), or Biorad SDS-PAGE Molecular weight standards (Broad range). Mini-gels were run at 80-100 mA, for 2-3 h, and large gels were run at 50 mA, overnight, or until the dye front reached the bottom of the gel.

2.8.4 Coomassie blue staining

Samples were often loaded in duplicate to determine whether loading was equal. The gel was stained for 30 min in coomassie blue, and de-stained overnight in 30% MeOH, 10% acetic acid.

Coomassie blue stain:	0.25 g	Coomassie brilliant blue
(filter before use)	90 ml	MeOH:H_2O (1:1 v/v)
	10 ml	glacial acetic acid

De-stain:	30% MeOH
	10% glacial acetic acid
	dH_2O

2.8.5 Semi-dry transfer of proteins

Proteins were transferred to a Hybond-P (PVDF, Amersham) membrane with a Biorad Transblot SD Semi-dry transfer cell.

Pre-soak the membrane in MeOH, then in transfer buffer. Cut 6 sheets of Whatman paper to the same size as the membrane and soak in transfer buffer. Layer on the lower electrode: 3 pieces of Whatman paper, membrane, gel, and 3 pieces of Whatman paper. Roll with a pipette to remove trapped air bubbles. Transfer at 1.2 mA/cm^2 or higher, at a maximum of 25 V for 1-2 h.

Transfer buffer:	11.4 g Tris base 54.0 g Glycine 5 ml 20% SDS 800 ml MeOH to 1 l with dH_2O	Alternative:	3.03 g Tris base 14.4 g glycine 200 ml MeOH to 1 l dH_2O

2.8.6 Ponceau S staining

To ensure that transfer was successful, to allow the identification of unstained markers, and to determine if loading was equal, the membrane was stained in Ponceau S solution (Sigma Ponceau S solution) for several minutes, and de-stained in H_2O.

2.8.7 Immunodetection of proteins

Membranes were blocked for 30 min at RT or overnight at 4°C in 5% dried milk in TBST on a rotary shaker, followed by three washes (15, 5, 5 min) in TBST. Membranes were incubated in primary antibody (Table 2.1, p58) in 5% milk in a sealed bag on a test-tube rotator for 1 h at RT followed by three washes in TBST. HRP-conjugated secondary antibody (Table 2.1, p58) was added in the same way, followed by three more washes in TBST.

Detection of the secondary antibody was carried out with ECL Western blotting detection reagents (Amersham Biosciences), according to the manufacturer's instructions, and using Kodak X-Omat AR or Biomax light Scientific imaging film.

2.9 Antibodies

Antigen	Immunogen	Poly/Mono clonal	Host	Cat. no.	Supplier	Use	Conc.
Hsp70	Human	Polyclonal	Rabbit	SPA-812	Bioquote Ltd.	Western blotting	1/2000
Hsp90	Mouse	Polyclonal	Mouse	MA3-010	Affinity Bioreagents	Western blotting	1/500
Myo D	Mouse	Polyclonal	Rabbit	Sc-760	Santa Cruz Biotechnology inc.	Western blotting	1/500
Glucagon	Porcine	Monoclonal	Mouse	G2654	Sigma	Immunofluorescence	1/500
p48			Rabbit			Immunofluorescence	1/500
Insulin	Porcine	Polyclonal	Guinea pig	A0564	Dako Cytomation	Immunofluorescence	1/500

Table 2.1: Primary antibodies used for immunohistochemistry and western blotting.

Antigen	Host	Catalogue no.	Supplier	Detection	Concentration
Rabbit	Goat	170-5046	Biorad	HRP conjugate	1/5000
Mouse	Goat		Amersham	HRP conjugate	1/3000
Rabbit	Goat	A-11012	Molecular Probes	594, Texas red	1/200
Mouse	Goat	O-6380	Molecular Probes	Oregon green	1/200
Guinea pig	Goat		Molecular Probes	594, Texas red	1/200

Table 2.2: Secondary antibodies used for immunohistochemistry and western blotting.

2.10 Quantitative RT-PCR

RNase free reagents and lab ware were used throughout to minimise degradation of the RNA.

2.10.1 Preparation of RNA from zebrafish embryos

Zebrafish embryos were dechorionated and transferred to a 1.5 ml centrifuge tube. RNAlater (Ambion) could be added to this stage, stored at –70°C, and rinsed out with dH_2O when required. Embryos were homogenised (x50 turns) in 200 µl Total RNA Isolation Reagent (ABgene), and transferred to a clean tube on ice for 5 min. 40 µl chloroform (Sigma, 99+%) was added, and tubes were vortexed for 15 sec, followed by 5 min on ice. Samples were centrifuged for 15 min at 4°C at 13,000 rpm. The upper phase was retained, and an equal volume of isopropanol (Sigma, 99+%) and 2 µl glycogen (for molecular biology, Boehringer) were added, and samples left at 4°C for

10 min or at –20°C for several hours or overnight to precipitate the RNA. Tubes were centrifuged for 10 min at 4°C at 13,000 rpm, and the pellet washed twice with 1 ml ice-cold 75% EtOH. Pellets were dried for 10 min at RT with lids off, then resuspended in 50-100 µl dH_2O, and vortexed for 1 min. RNA was stored at –70°C.

2.10.2 RNA check gel

To check the quality of the RNA at least 2 µl was diluted in dH_2O with 1 µl ethidium bromide, and run on an agarose gel at 150V for a short time. In good quality RNA bands representing the 60S and 40S ribosomal subunits should be clearly visible without too much smearing.

2.10.3 DNAse treatment

10 u of RNAse-free DNAseI (1 µl Roche/Boehringer cat no. 776 785, or 10 µl Gibco Amp. grade) was added to RNA, with 10x DNAse buffer to 1x concentration, vortexed and incubated for 30 min at 37°C. Samples were made up to 200 µl with dH_2O, and the enzyme was removed by phenol-chloroform extraction, and the RNA precipitated with 2 µl glycogen, 0.1x vol. 3 M NaAc, pH5.2, 2.5 x vol. 100% EtOH at –20°C for at least one hour or overnight. Centrifuge for 10 min at 4°C at 13,000 rpm, and wash the pellet twice with 500 µl ice-cold 70% EtOH. Pellets were dried for 10 min at RT with lids off, then resuspended in 50-100 µl dH_2O, and vortexed for 1 min. RNA was stored at –70C.

2.10.4 Reverse transcription

RT-PCR was carried out on an MJ Research PTE225 Peltier Thermal Cycler in

0.25 ml tubes.

Reaction mix:	0.5 µl	oligo dT
	5 µl	RNA
	4.5 µl	dH_2O
	10 µl	

Denature at 63°C for 5 min, and return to ice.

Add:	0.5 µl	dNTPs (25 uM, ABgene)
	1 µl	RNase inhibitor (Roche)
	2 µl	glycerol
	4 µl	5x first strand buffer (Gibco)
	2 µl	0.1M DTT (Gibco)
	19.5 µl	

Heat at 45°C for 2 min. Add 1 µl superscript II RT (Gibco). Heat for a further 50 min at 45°C. Add 1 µl *E.coli* RNase H (Gibco), and heat 37°C for 20 min, then 5 min 80°C to kill the enzyme.

2.10.5 Quantitative PCR

Quantitative PCR was carried out by controlling the number of reaction cycles so that the reaction was completed in the linear portion (usually 15-20 cycles). This was done so that differences between the relative amounts of specific cDNAs between different treatment groups could be determined (Figure 2.1). See below for detailed PCR protocol. Control primers for α-actin were used. An initial PCR was carried out with the control primers at 15-20 cycles, to determine the relative amount of cDNA in each sample. The amount of each cDNA was adjusted to give equal products for the control PCR in all samples, and then these amounts were used for the test primers.

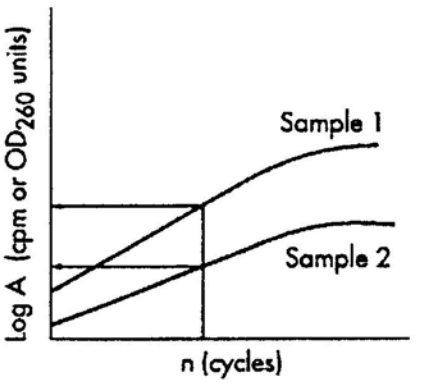


Figure 2.1: Quantitative PCR. Showing progression of a PCR reaction for two samples, and the exponential part of the curve where the difference between the two samples can be measured quantitatively (Reproduced from Siebert, 1999).

2.11 Mutation analysis

2.11.1 Fixing of larvae for mapping

Embryos were fixed in 100% methanol (5 min then replace) and stored at –70°C.

2.11.2 Preparation of DNA from zebrafish

DNA was prepared from either whole embryos or finclips. To take a fin clip, adult zebrafish were anaesthetised in a 1/30 solution of tricaine stock, and a razor blade was used to remove a small piece of the tail fin. Fish were recovered in system water before returning to tanks.

Samples in 1.5 ml centrifuge tubes were covered with 50 µl lysis buffer, and boiled for 10 min in a water bath. 10 µl proteinase K was added, and samples were incubated in a 55°C water bath for several hours, or overnight. Samples were boiled again for 10 min in a water bath to kill the proteinase. DNA was stored at –20°C and 1 µl was used for PCR.

Lysis buffer:	10 mM	Tris pH7.5
	50 mM	KCl
	0.3%	Tween 20
	0.3%	NP40
	1 mM	EDTA

2.11.3 Polymerase chain reaction

Primer design:

The web pages http://www.cybergene.se and Primer3 (Rozen and Skaletsky, 2000) were used to aid design of oligonucleotide primers (supplied as lyophilised pellets by MWG). Stocks were made up to 100 µl with dH_2O. 100 pmol/µl working stocks were made.

Reaction mix:

DNA	0.2-0.4 ng (plasmid), 500-100 ng (genomic DNA)	
PCR buffer	5 µl	GeneAmp 10x buffer (Applied biosystems)
Mg^{2+} buffer	3-4 µl	GeneAmp $MgCl_2$ solution
0.2 mM dNTPs	0.5 µl	of 10 mmol/µl stock (Abgene)
50 pM primers	0.5 µl	of each 100 pmol/µl stock
AmpliTaq	0.3 µl	(1.5u, GeneAmp)
dH_2O	to 50 µl	

PCR was carried out on an MJ Research PTE225 Peltier Thermal Cycler in 0.25 ml tubes.

PCR programme:	5 min	94.5°C denaturing
	Up to 30 cycles:	
	30 sec	94.5°C denaturing
	30 sec/Kb	Tm minus 5°C annealing
	1 min/Kb	72°C extension
	15 min	72°C extension
	hold	15°C

2.11.4 Electrophoresis

To estimate the size and concentration of any DNA fragments, they were separated by agarose gel electrophoresis. An agarose gel (High pure, BioGene or Nuseive GTG Agarose, Biowhittaker Molecular Applications) of the appropriate percentage was made in 1% TAE (Tris-acetate-EDTA) buffer, with 0.2 mg/ml ethidium bromide (EtBr, Sigma or Biorad), and run in TAE buffer at 80-100 V, and the bands viewed on a UV transilluminator. Size markers were used to allow estimation of band size, and the relative DNA concentration. The size markers used routinely were bacteriophage lambda DNA digested with *Hin*dIII ('λ') (Gibco BRL or Invitrogen), DNA marker X, 0.07-12.2 Kbp (Roche) and 100 bp DNA Ladder (Promega).

Fragments required for further manipulation were excised and purified using a gel extraction kit (Qiagen), according to the manufacturer's instructions.

TAE 50X stock:	Tris base	242 g
	Glacial acetic acid	57.1 ml
	0.5 M EDTA	100 ml

Distilled water was added to a final volume of 1 litre. Stock was diluted to 1X with distilled water.

6x Agarose gel loading buffer:	Bromophenol blue	25 μg
	Xylene Cyanol	25 μg
	Glycerol	3 ml
	dH_2O	6.95 ml

2.11.5 Determining the concentration of DNA samples

A 1/100 dilution of the DNA was made, and the optical density was measured at a wavelength of 260 nm (OD^{260}) using a UV spectrophotometer. This was used to calculate the concentration of DNA. An optical density of 1 is equivalent to approximately 50 μg/ml for double stranded DNA. The ratio between the OD^{260} and the OD^{280} gives a measure of the purity of the sample. A ratio of 1.8 indicates that the

sample is free from protein and salt impurities, while a ratio of less than 1.8 shows that the sample is contaminated (Sambrook *et al.* 1989).

2.11.6 Restriction enzyme digestion

Restriction enzymes were supplied by Boehringer Mannheim or New England Biolabs. The amount of each enzyme added was 5% or less of the reaction volume, using the appropriate 10x buffer diluted to 1x concentration with dH_2O. Digests were left at 37°C for several hours or overnight before running on an agarose gel.

A speed vac (DNA 120 Speed Vac, Thermo Savant) could be used to reduce the volume of the digests prior to running.

If digested DNA was required for further manipulation, fragments were purified using a PCR clean-up kit (Qiagen), according to the manufacturer's instructions.

2.11.7 Phenol-chloroform extraction

Add an equal volume of Phenol:chloforom:isoamyl alcohol mixture (Gibco BRL), vortex thoroughly, centrifuge for 5 min at RT. Retain the upper phase for precipitation.

2.11.8 Sequencing

PCR products for sequencing were cleaned using 1 µl Exonuclease I (Exo) and 1 µl Shrimp Alkaline Phosphatase (SAP) per 3-10 µl DNA. Reactions were heated at 37° for 15 min, and denatured for 15 min at 80°C on an MJ Research PTE225 Peltier Thermal Cycler in 0.25 ml tubes. The appropriate amount of DNA was then added to the sequencing reaction mix.

Reaction mix:

DNA	approx 300-500 ng
Primer	1.6 pmol
Dye terminators	4 µl of 1:1 dilution of ABI BigDye version 2 or 3.1
dH_2O	to 10 µl

Programme: 25 cycles:
96°C 30 sec
55°C 15 sec
60°C 4 min

4°C hold

Sequencing products were precipitated with 90 µl 70% EtOH (diluted from 95% EtOH, Fisher) and 1 µl Pellet Paint (Novagen) for 15 min, and centrifuged at 13,000 rpm for 20 min at RT. The supernatant was removed and the pellet washed with 70% EtOH. The pellet was dried for 1 min at 90°C on an MJ Research PTE225 Peltier Thermal Cycler.

Sequencing products were run out using an ABI 3100 by HGU technical staff. Sequencing traces were analysed using Sequencher or Chromas software.

Chapter 3

The Effects of Hsp90 Inhibitors on Zebrafish Development

3.1 Introduction

Before an investigation of Hsp90 buffering in zebrafish mutants could be carried out, it was important to establish the procedure for inhibitor treatment during development. The protocol of Lele *et al.* (1999), describing geldanamycin treatment of very early zebrafish embryos, was used as a starting point to establish the dose and timing of Hsp90 inhibitor treatment required to cause a very low level of developmental defects. It was also necessary to identify the most appropriate Hsp90 inhibitor to use, based on several properties: availability, solubility, stability, cost, toxic side effects in the embryos, and danger of toxicity to the researcher. Treatment was carried out during the temporal "window" in development during which eye development is particularly vulnerable.

Another factor to be taken into account was the activation of a heat shock response. Hsp90 inhibitors are known to activate the heat shock transcription factor, HSF1, which is in complex with Hsp90. On disruption of this complex by geldanamycin, HSF1 is released and activates the heat shock response (Zou *et al.* 1998, Guo *et al.* 2001, Winklhofer *et al.* 2001). It was therefore important to establish that developmental defects arose due to the disruption of processes that are dependant on Hsp90, and not due to a general down-regulation of transcription, and induction of other HSPs, caused by the initiation of a full heat shock response (Yost and Lindquist, 1986).

3.2 Hsp90 inhibitors

A summary of the properties of some Hsp90 inhibitors and their controls is shown in Table 3.1, and Figure 3.1a. The benzoquinone ansamycin drug geldanamycin is the most widely used of these. Initially identified as an antibiotic (DeBoer *et al.* 1970), it was later shown to have anti-tumour properties, reducing the levels of the src protein that is responsible for oncogenic transformation of cells (Uehara *et al.* 1986). Src exists in complex with Hsp90, and when this interaction is disrupted by geldanamycin, src is

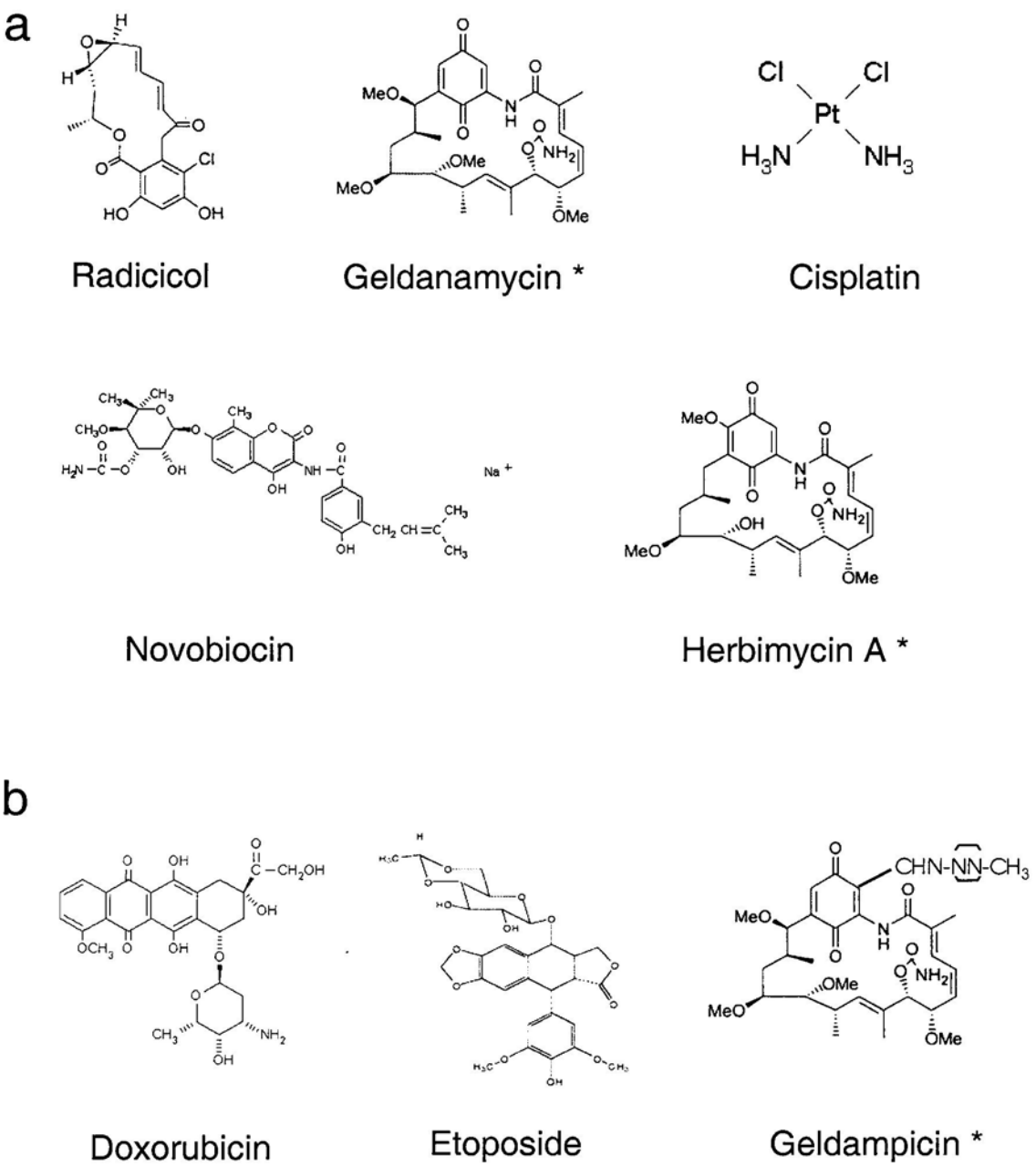


Figure 3.1: (a) Molecular structure of Hsp90 inhibitors. (b) Controls. The names of structurally related compounds are followed by identical symbols (Reproduced from www.sigmaaldrich.com, Chiosis *et al.* 2001).

degraded (Whitesell *et al.* 1994). Many other Hsp90 clients are also degraded on geldanamycin treatment of cultured cells (Schulte *et al.* 1997, Sakagami *et al.* 1999). Geldanamycin competes with ATP for binding of the N-terminal ATPase domain of Hsp90 (Roe *et al.* 1999) and GRP94 (Chavany *et al.* 1996). Geldanamycin also causes the accumulation of free radicals, but for this to occur it must be used at a far higher concentration than is necessary to inhibit cell transformation (Reviewed in Ochel *et al.* 2001, Dikalov *et al.* 2002).

Compound	Solvent	Toxicity (EU)	Cost/mg (Sigma)	Notes
Cisplatin	H_2O	Very toxic	15p	Binds Hsp90 C-terminal, not specific
Geldanamycin	DMSO	Teratogenic, carcinogenic	£252.40	
Herbimycin A	DMSO	Teratogenic	£394.10	Not very stable
Novobiocin	H_2O	Irritant	<1p	Not specific
Radicicol	EtOH	Harmful	£42.02	

Table 3.1: Readily available Hsp90 inhibitors and their properties.

The antibiotic radicicol also binds the N-terminal ATPase domain of Hsp90, but with a higher affinity than geldanamycin (K_d is 1.2 μM and 0.019 μM for geldanamycin and radicicol respectively) (Sharma *et al.* 1998, Roe *et al.* 1999). It also binds the Hsp90 family members, TRAP-1 and GRP94 (Schulte *et al.* 1999). Two other unrelated proteins, ATP citrate lyase, and branched-chain α-keto acid dehydrogenase (BCKDHK) are also bound by radicicol, but with a far lower affinity (Ki *et al.* 2000, Besant *et al.* 2002). Also, ATP citrate lyase is bound by a different part of radicicol to Hsp90 (Ki *et al.* 2000). Herbimycin A is another benzoquinone ansamycin with antitumour activity, which also inhibits Hsp90 binding of client proteins (Uehara *et al.* 1986, Whitesell *et al.* 1994, Sakagami *et al.* 1999). It is extremely unstable, however, which made it seem unsuitable for large-scale studies.

The antibiotic novobiocin is less toxic than the benzoquinone ansamycins. It is particularly useful in biochemical studies of Hsp90 function, as it binds both the N and C-terminal ATPase domains (Marcu *et al.* 2000, Marcu *et al.* 2000b, Söti *et al.* 2002b). Although novobiocin also inhibits topoisomerase II, it can be used as an Hsp90 inhibitor in cell culture, to deplete Hsp90 clients such as Raf1 and v-src (Marcu *et al.* 2000b). Cisplatin is another anti-tumour agent that binds the C-terminal domain of Hsp90 (Itoh *et al.* 1999, Söti *et al.* 2002), but it also acts as a DNA intercalating agent, causing intra-strand DNA breaks (Rang *et al.* 1995). Cisplatin may therefore be too toxic for developmental studies, as well as acting as a mutagen.

Geldanamycin, radicicol and novobiocin were chosen for initial testing *in vivo*. Several compounds, listed in Table 3.2, were also obtained for use as controls (Figure 3.1b). Doxorubicin and etoposide are structurally unrelated topoisomerase II inhibitors with no Hsp90 inhibitory activity, and can be used as negative controls for novobiocin (Marcu *et al.* 2000b). Geldampicin is a benzoquinone ansamycin that differs from geldanamycin by one side chain. It has been shown to compete for Hsp90 binding to geldanamycin beads, but with a 100-fold lower affinity than geldanamycin (Grenert *et al.* 1997). It has been accepted as having negligible Hsp90 inhibitory activity, and is therefore suitable as a negative control (Whitesell *et al.* 1994, Lele *et al.* 1999).

Compound	Solvent	Toxicity (EU)	Cost/mg (Sigma)	Related Inhibitor
Doxorubicin (Adriamycin)	H_2O	Toxic	£10.16	Novobiocin
Etoposide (VP16)	DMSO	Toxic	£8.78	Novobiocin
Geldampicin	DMSO	Unknown	n/a	Geldanamycin

Table 3.2: Control compounds that do not inhibit Hsp90 activity.

3.3 Hsp90 inhibitor treatment of zebrafish embryos

WT embryos were treated with Hsp90 inhibitors according to the protocol outlined in Chapter 2 (p49), to assess the dosage and timing required to cause developmental defects. Novobiocin was used initially, as it is the cheapest, and least toxic of the drugs. Both geldanamycin and radicicol were then tested, and radicicol was chosen as the main inhibitor for further experiments, as it is cheaper, less toxic, and does not require DMSO as a solvent. Geldanamycin was also used, to demonstrate whether the effects seen with radicicol were a result of Hsp90 inhibition, or toxicity due to any particular chemical properties of the drug.

Details of the WT strains used are given in Table 3.3. For initial experiments with novobiocin, *golden* (*gol*) mutants were used. For nearly all the subsequent experiments, to ensure that differences in response to the drug were due to the mutation present and not genetic background, the Tü/AB/TL strain was used. Minor differences in genetic background still exist, however, because if zebrafish are too inbred they fail to thrive (Brand *et al.* 2002). This may be because zebrafish WT strains are not so well established, and deleterious alleles have not been removed. It is possible therefore, that some cryptic mutations could be present.

Strain	Details	Origin	Reference
Tübingen (Tü)	WT laboratory strain	Tübingen pet shop	Haffter *et al.* 1996
AB	WT laboratory strain	Oregon pet shop	Chakrabarti *et al.* 1983
Tübingen long fin (TL)	WT laboratory strain	Fish dealer	Haffter *et al.* 1996
WIK	WT mapping strain, relatively outbred	Wild catch, India	Rauch *et al.* 1997
Tü/AB/TL	Derived from non-carriers of *mic*	1996 ENU screen	Heisenberg *et al.* 1996
mic	Mixed Tü/AB/TL background	1996 ENU screen	Heisenberg *et al.* 1996
sri	Mixed Tü/AB/TL background	1996 ENU screen	Heisenberg *et al.* 1996
dre	Mixed Tü/AB/TL background	1996 ENU screen	Heisenberg *et al.* 1996
golden (gol)	AB background	Fish dealer	Chakrabarti *et al.* 1983

Table 3.3: Zebrafish strains used throughout this study (http://www.zfin.org, Sprague *et al.* 2001).

Embryos must have a hole torn in the chorion before inhibitor treatment, as survival was much lower when the chorion was left intact. The chorion, together with the perivitelline fluid surrounding the embryos may amplify the effects of inhibitors by allowing the drug, or its toxic by-products, to accumulate inside the chorion at a much higher concentration (Bonsignorio *et al.* 1996, Gellert and Heinrichsdorff, 2001).

All three of the Hsp90 inhibitors tested; radicicol, geldanamycin and novobiocin, had a dose-responsive effect on the development and survival of zebrafish embryos, whether treatment was initiated before (30% epiboly to germ ring) or after (60 to 70% epiboly) gastrulation (Figure 3.2, Appendix A). This showed that the defects observed were due to the presence of the inhibitor. The solubility of geldanamycin in water varied between suppliers, and limited the concentration that could be used experimentally (Chapter 2.2, p48). A comparison of the frequency of developmental defects at a concentration that allows over 80% survival (up to 10 μM radicicol at 30-50% epiboly, or 20 μM at 60-70%, 15 μM geldanamycin at 60-70% epiboly, and 50 μM novobiocin at 30-50% epiboly), shows that radicicol and geldanamycin can cause virtually 100% very mild fin defects and less than 20% more serious defects, while novobiocin causes less than 10% serious defects and none that are mild. This indicates that the primary activity of novobiocin results in lethality, and that it may not be suitable for developmental studies.

3.4 Developmental defects observed after treatment with Hsp90 inhibitors

The aim of these experiments was to establish both the timing and level of Hsp90 inhibitor treatment required to cause morphological abnormalities, so that a sub-threshold dose could be used to reveal cryptic mutations.

3.4.1 Novobiocin

When WT embryos were treated with novobiocin, starting from 30% epiboly to germ ring stage, until 24-26 hpf (hours post fertilisation) (18h incubation), developmental

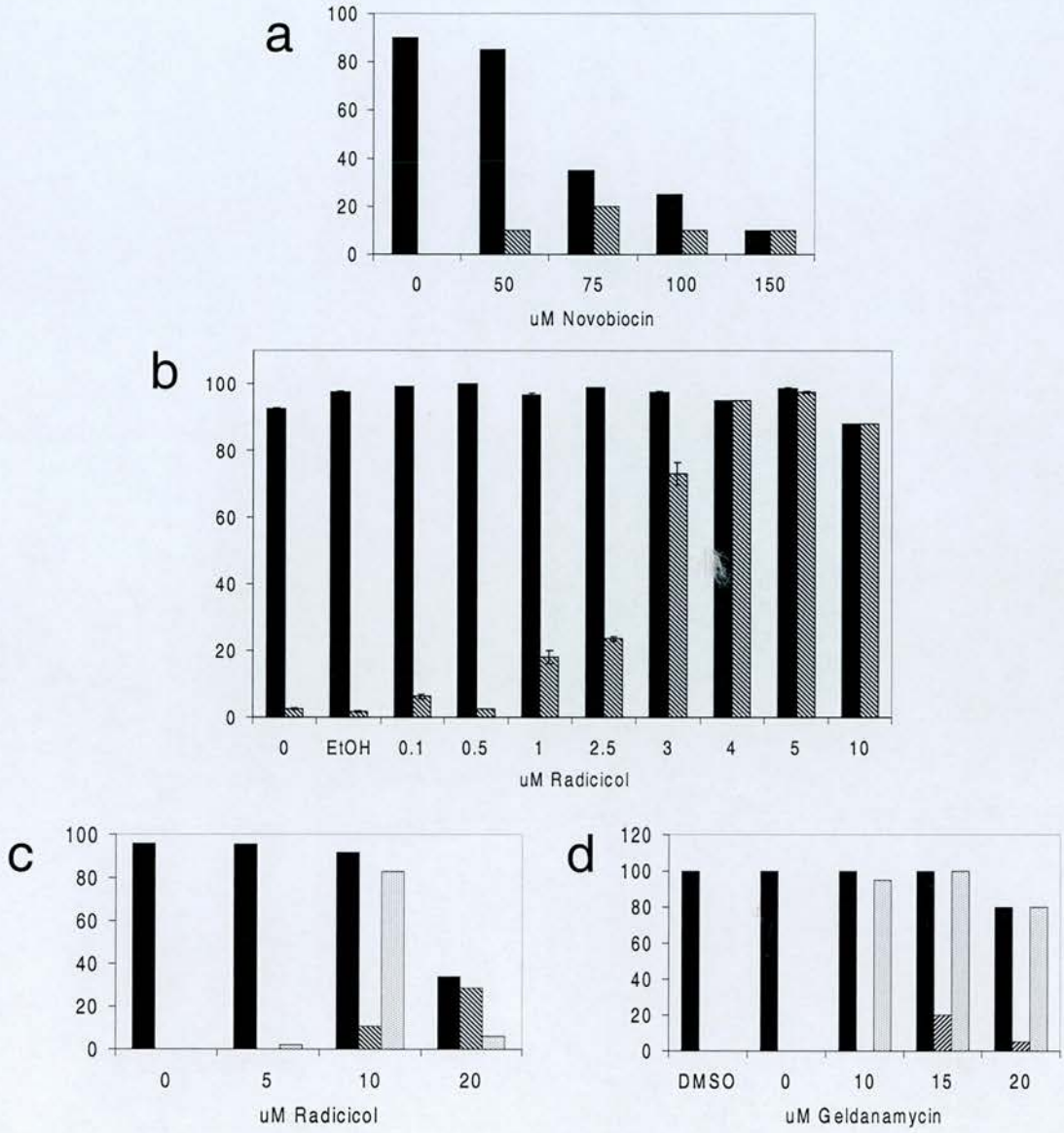


Figure 3.2: Dose-responsive effect of Hsp90 inhibitors on survival at 1 dpf and development at 5 dpf, of WT zebrafish embryos. (a) Novobiocin; **(b,c)** Radicicol; **(d)** Geldanamycin. Survival (black), mild fin defects (spots), major developmental defects (stripes). Treatment was initiated at the 30% epiboly to germ ring stage **(a,b)**, or 60 to 70% epiboly **(c,d)**. Standard error is indicated where bars represent more than one experiment. Y axis, % embryos.

defects were observed at concentrations over approximately 50 μM. A variety of defects were seen in embryos that survived beyond 24h, including heart oedema, very severe oedema with growth retardation and reduced pigmentation, growth retardation, somite defects, microphthalmia, anophthalmia, rachischisis (non-closure of the neural tube, or spina bifida) and other notochord defects (Figure 3.3 and 3.4). Survival rates at these concentrations were however very low. The characteristic shortened embryos seen by Lele *et al.* with geldanamycin were not observed.

In studies of the effects of toxic chemicals on zebrafish embryos, survival and growth were the most sensitive parameters for nearly all compounds tested (Roex *et al.* 2002). Other defects that often develop on exposure to toxic compounds are reduced circulation, reduced pigmentation, and rachischisis (Baumann and Sander, 1984, Nagel, 2002, Schulte and Nagel, 1994). All of these phenotypes are observed on exposure to novobiocin, suggesting that they are due to toxic effects of the drug and not Hsp90 inhibition. Coumarin antibiotics such as novobiocin are known to inhibit eukaryotic topoisomerase II (Fisher *et al.* 1992). Topoisomerase II activity is essential for viability in eukaryotes, as demonstrated by studies in yeast that have shown that topoisomerase II knockouts are lethal, due to defects in chromosome segregation (Rose *et al.* 1990). The topoisomerase II inhibitor etoposide has been shown to cause cell cycle arrest in zebrafish embryos (Ikegami *et al.* 1997).

Although novobiocin is less toxic than geldanamycin or radicicol on a molar basis, a high dose of novobiocin must be used to inhibit Hsp90 in cell culture (1 mM, Marcu *et al.* 2000b), and it was suggested that at this concentration the topoisomerase II inhibitory activity may confound the effects seen on development (personal communication, Leonard Neckers). This is confirmed by the observation that a concentration of only 0.1 mM novobiocin causes a high frequency of lethality in zebrafish embryos. Use of the negative controls etoposide and doxorubicin might have confirmed that the primary effect of novobiocin during development is on topisomerase II. These experiments were not carried out however, as it had already been concluded that survival was too low for

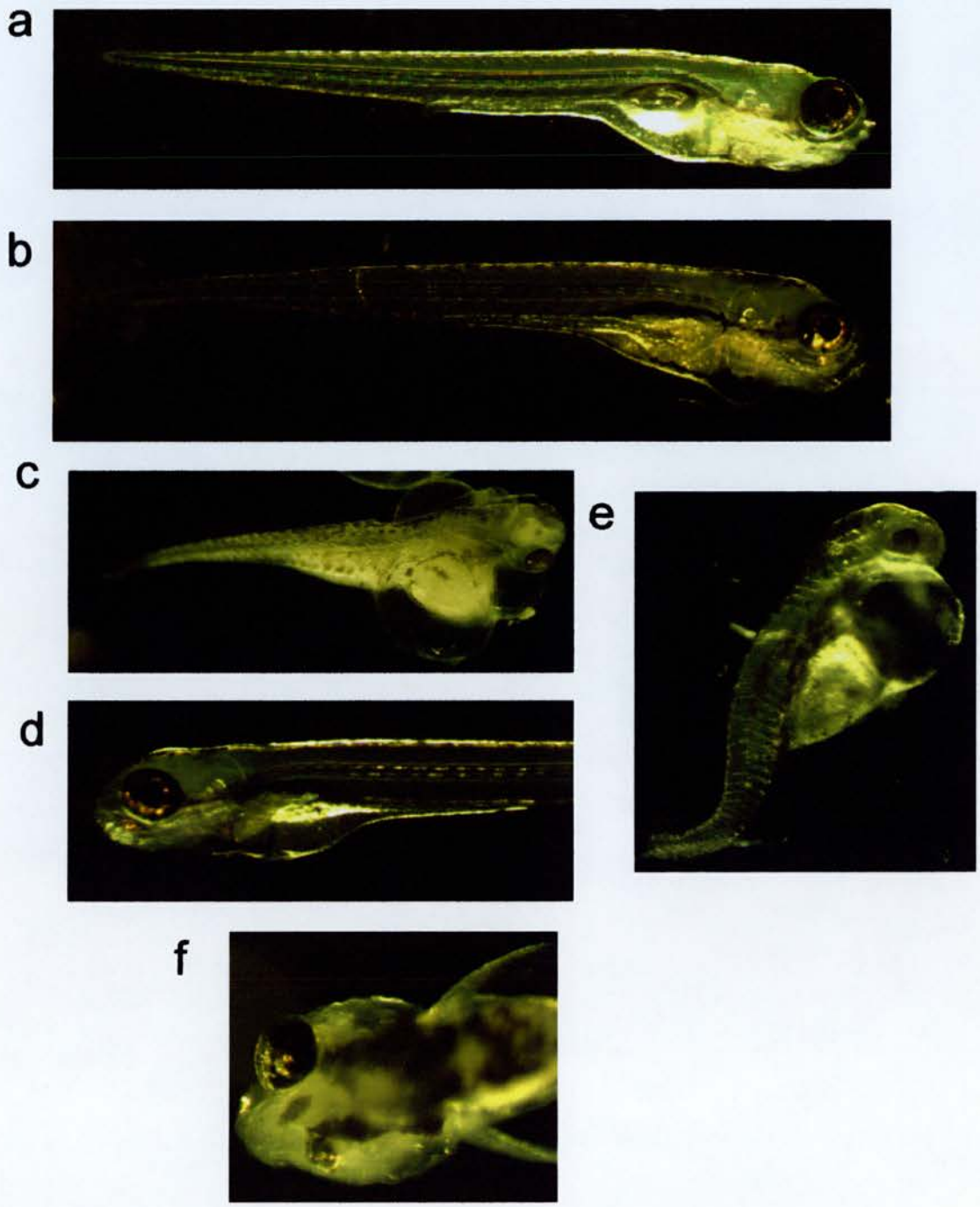


Figure 3.3: Developmental defects caused by novobiocin treatment. **(a)** WT, 6 dpf; **(b)** Heart oedema, 0.025 mM, 5 dpf; **(c)** Very severe oedema and growth retardation, 0.1 mM, 4 dpf; **(d)** Failure of the swim bladder to inflate, 0.05 mM, 4 dpf; **(e)** Somitogenesis defect, 0.3 mM, 3 dpf; **(f)** Bilateral microphthalmia, 0.025 mM, 5 dpf.

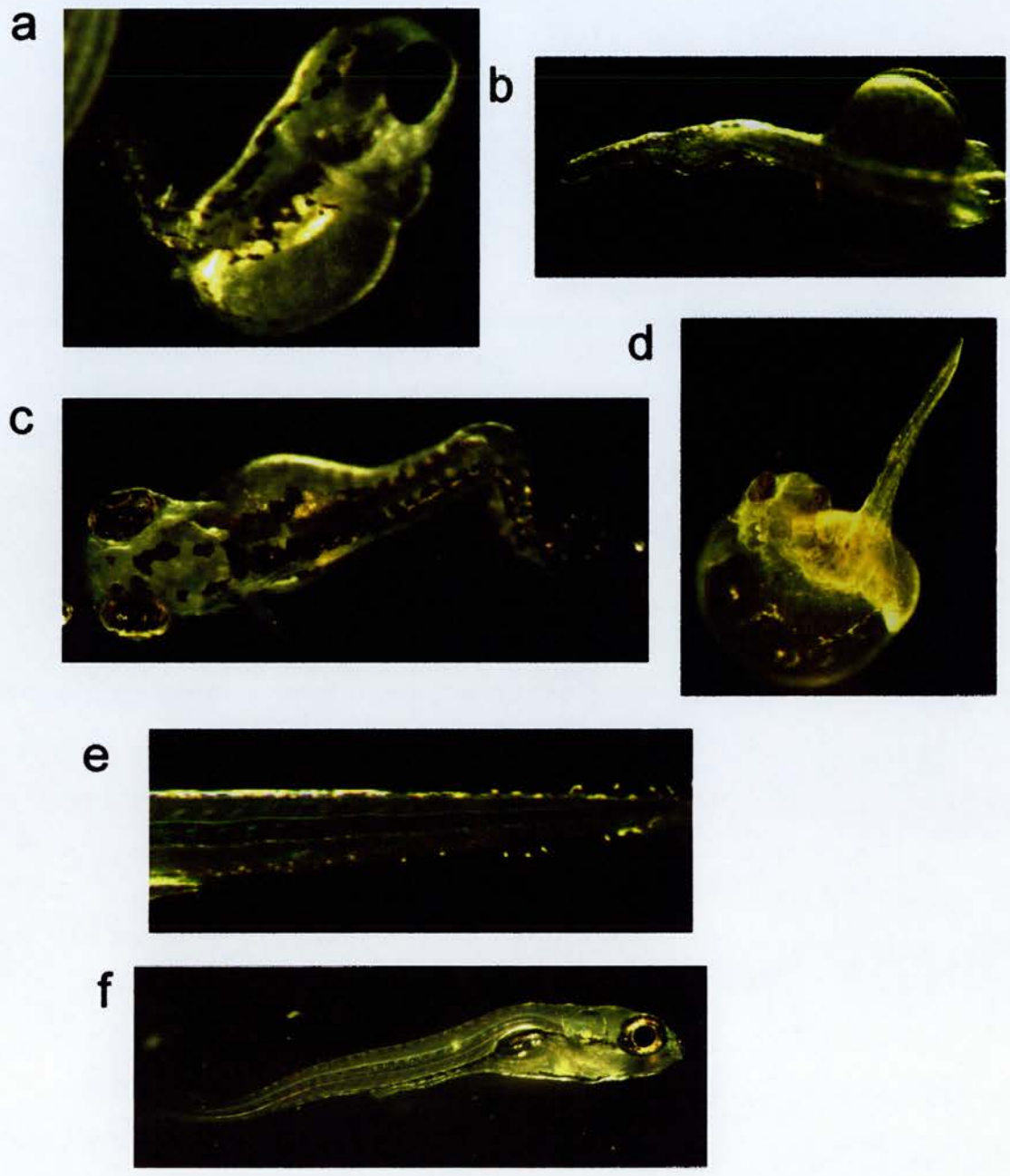


Figure 3.4: Notochord defects caused by novobiocin treatment. **(a)** Truncated tail, 0.13 mM, 4 dpf; **(b)** Rachischisis, 0.02 mM, 1 dpf; **(c)** Bent tail, 0.2 mM, 4 dpf; **(d)** Curled up on yolk sac, 0.2 mM, 4 dpf; **(e)** Slight notochord irregularity, 0.075 mM, 3 dpf; **(f)** Bends in notochord, 0.05 mM.

novobiocin to be used in further experiments. These experiments demonstrate the consequences of exposure to a toxic chemical during development, and can be used as a control in comparison with more specific inhibitors.

3.4.2 Radicicol

In WT embryos treated with radicicol from 30-50% epiboly, at a concentration of 10 μM all the embryos were severely affected, and died between 24 and 32 hpf. These embryos had a similar shortened body axis to those described by Lele *et al.* (1999) for 35 μM geldanamycin (Figure 3.5). Geldanamycin has a far lower affinity for Hsp90 than radicicol, and this difference may be reduced *in vivo* by differences in solubility and metabolism, to give the approximately three-fold difference (on a molar basis) in activity observed. These two lines of evidence, phenotype and concentration, indicate that the defects observed after radicicol treatment under these conditions are due to destabilisation of Hsp90 client proteins by specific inhibition of the Hsp90 protein. A small volume (10-30 μl) of the known teratogen ethanol (Ahlgren *et al.* 2002) was used as a solvent for radicicol, therefore a control containing the same volume of ethanol was always carried out for each experiment.

The severe abnormalities caused by radicicol treatment close to the onset of gastrulation may have masked any eye-specific abnormalities, so treatment was initiated closer to the onset of eye development. The optic primordium becomes visible between 11 and 12 hpf. When radicicol is added between 7 and 8 hpf (60-70% epiboly), survival is markedly improved. At 4-5 dpf developmental defects were scored, including heart oedema, growth retardation, very severe oedema and growth retardation with reduced pigmentation, failure of the swim bladder to inflate (usually inflated by 4 dpf), and notochord defects (Figure 3.6 a-e). Part of the caudal fin was absent in a high proportion of treated embryos (Figure 3.6f). The frequency of the developmental defects described was dose-responsive. A concentration of 10 μM was chosen for subsequent experiments,

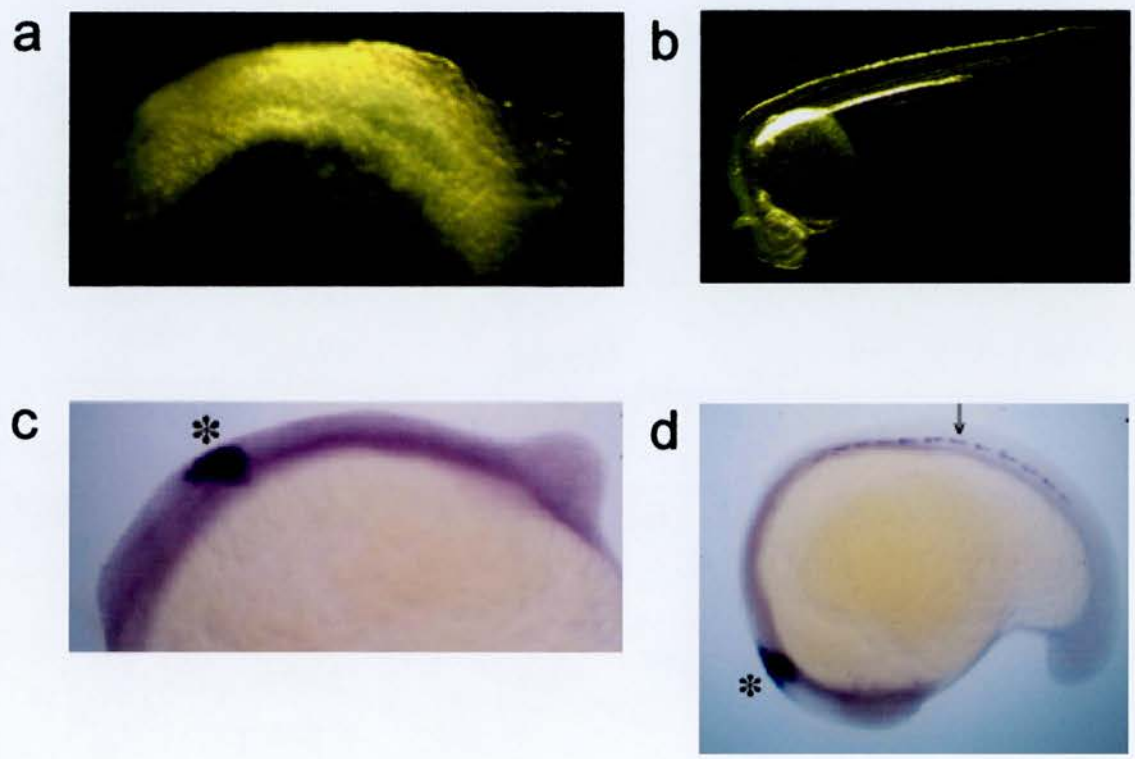


Figure 3.5: WT embryos treated with radicicol have a characteristic phenotype similar to that caused by geldanamycin. (a) 10 μM radicicol, 24 hpf, embryo almost completely necrosed; **(b)** Untreated, 24 hpf; **(c)** 35 μM geldanamycin, 18 hpf; **(d)** Untreated, 18 hpf. (c and d reproduced from Lele *et al*. 1999. * and ↓ indicate *in-situ* staining for *eng-2* mRNA, that is not relevant to this discussion).

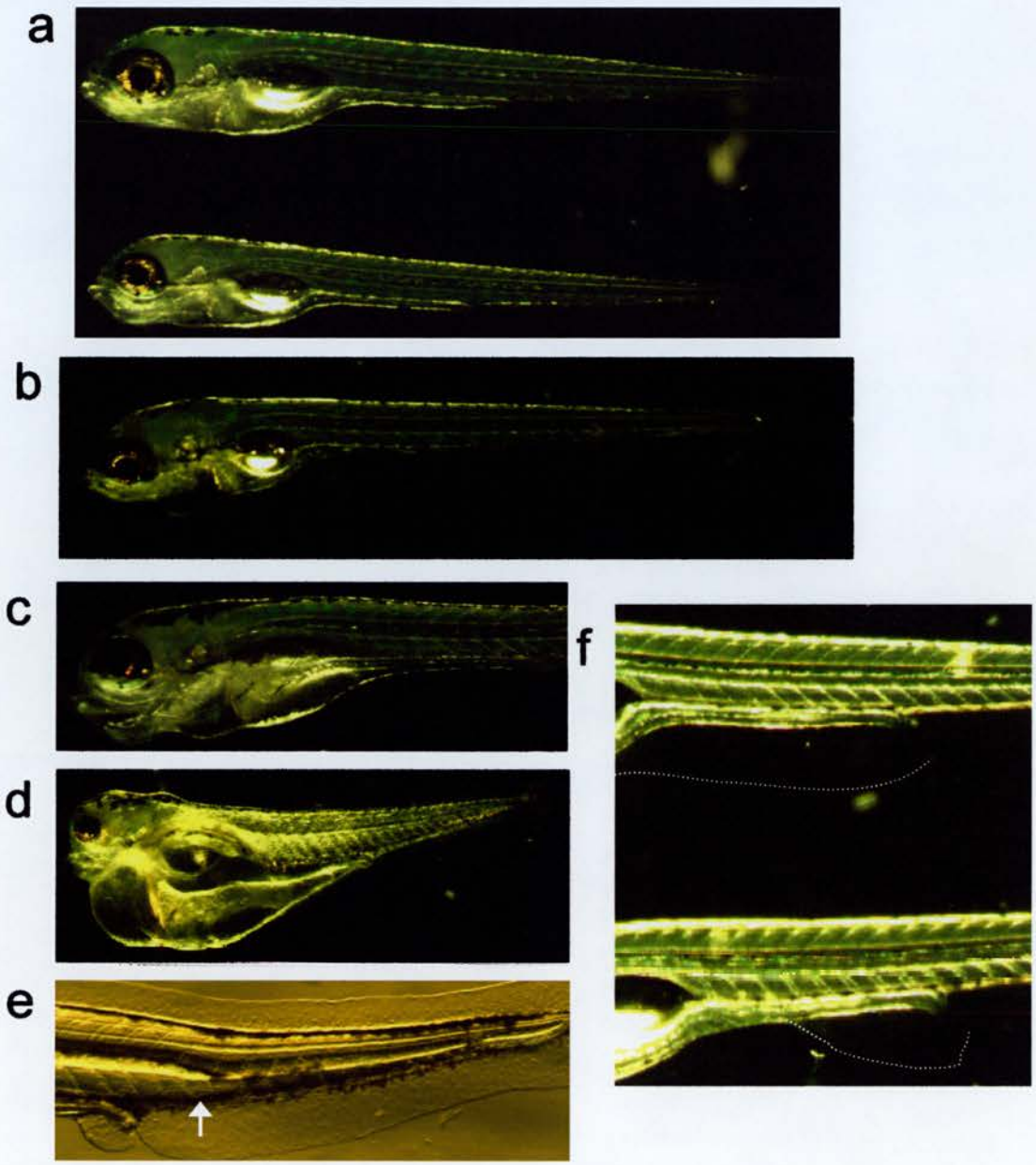


Figure 3.6: Developmental defects caused by radicicol treatment. **(a)** Top WT, bottom growth retardation, 2.5 μM; **(b)** Heart oedema, 1 μM; **(c)** Failure of the swim bladder to inflate, 0.1 μM; **(d)** Very severe oedema and growth retardation, 3 μM; **(e)** Narrowing of the notochord, from the arrow onwards, 14 μM; **(f)** Reduced caudal fin, top WT, bottom affected, 2.5 μM. **(a-d, f)** treated from 30% epiboly-germ ring, **(e)** treated from 60-70% epiboly. All scored at 5 dpf.

as mild fin defects and a low frequency of more severe defects are caused, indicating a low level of Hsp90 inhibition (Figure 3.2).

A general developmental delay was observed after treatment with radicicol or geldanamycin, but not geldampicin (Figure 3.7). This may be due to the destabilisation of ubiquitously expressed Hsp90 clients that are important in growth and differentiation of all tissues, such as p53, and the steroid hormone receptors (Reviewed in Richter and Buchner 2001). Growth recovered after the drug was washed out, and several hours later (approximately 3-6h) treated embryos appeared to be the same size as their untreated sibs.

3.4.3 Geldanamycin

Geldanamycin treatment was also initiated at 60-70% epiboly. After an initial dose-response experiment (Figure 3.2d), a concentration of 10-20 μM was used for all the subsequent experiments. The characteristic shortened phenotype described by Lele *et al.* was never observed, presumably because of the later timing and lower dose that were used. At 5 dpf the range of developmental defects observed included some of those caused by radicicol; growth retardation, heart oedema, very severe oedema and growth retardation with reduced pigmentation, failure of the swim bladder to inflate, and notochord defects. In addition to these, a possible hyperinflation of the swim bladder (although this was very difficult to score), an irregular caudal fin in nearly all larvae (Figure 3.8), and a cloudy appearance in the abdomen or yolk sac were observed. The altered appearance of the abdomen may be a result of disruption of gastrointestinal tract development or physiological function, but these defects were not scored, as they were difficult to quantify.

The caudal fin defects observed after geldanamycin and radicicol treatment did not occur with geldampicin, DMSO, EtOH or novobiocin. The effect on fin development, therefore, appears to be a consequence of Hsp90 inhibition, and a demonstration that the two structurally unrelated inhibitors are affecting the same developmental pathways.

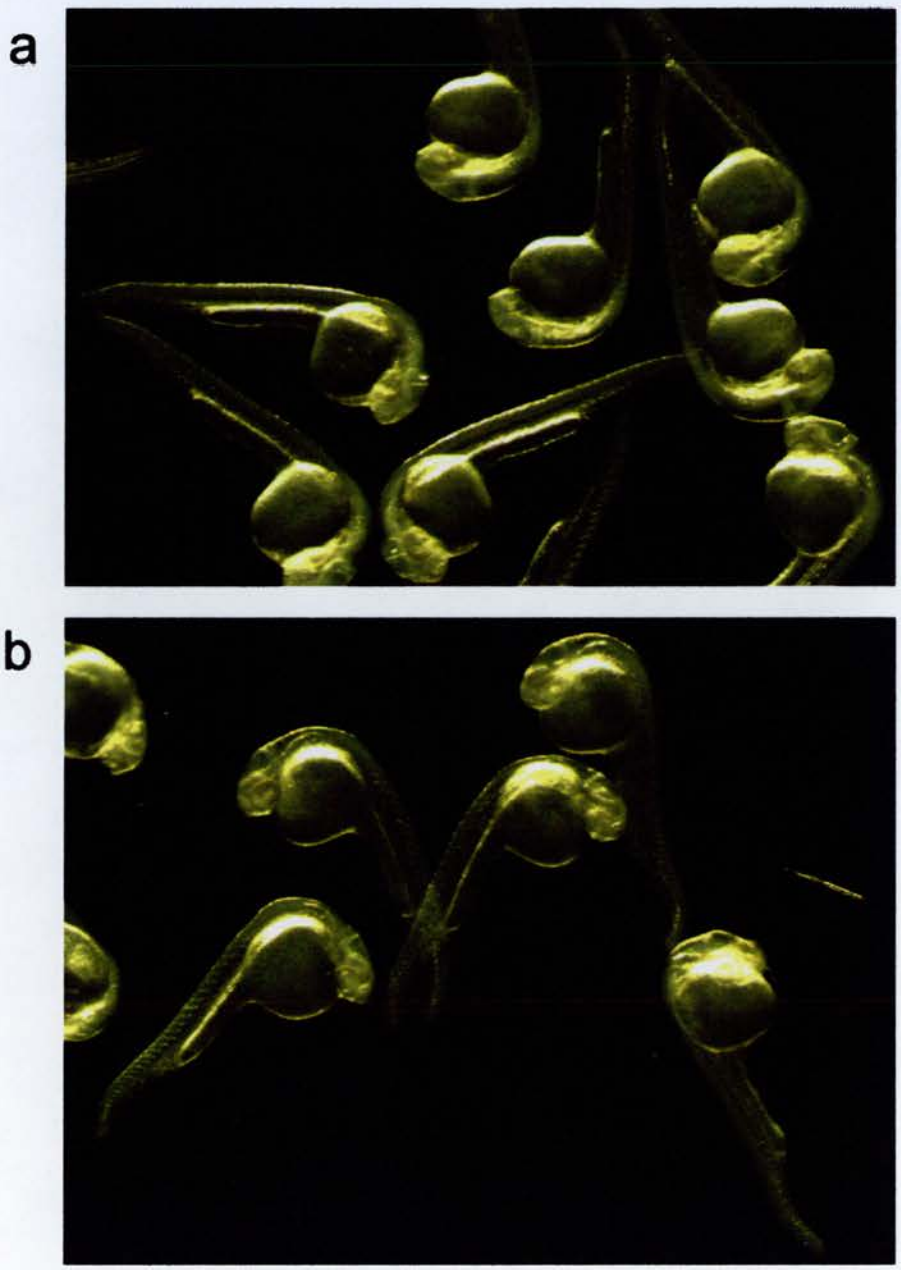


Figure 3.7: Treatment of *sri* homozygous mutant embryos with Hsp90 inhibitors causes a developmental delay that recovers on removal of the drug. (a) Untreated; **(b)** 10 μM radicicol. Treated from 60-70% epiboly.

Figure 3.8: Caudal fin defect caused by geldanamcin treatment. **(a)** Untreated; **(b)** 10 μM geldanamycin. 3 dpf embryos. Treated from 60-70% epiboly.

There is no Hsp90 expression reported in the fins, but there may be factors expressed early in development that affect the regulation or function of other proteins involved in caudal fin development.

Differences in the defects caused by radicicol and geldanamycin may be due to variations in metabolism or toxic side effects caused by the different chemical properties of the compounds, as well as variations in their Hsp90 inhibitory activity. The unrelated proteins, ATP citrate lyase and BCKDHK that are bound by radicicol may cause other side effects when inhibited during development. It is unknown whether geldanamycin binds any proteins outside the Hsp90 family, but for both radicicol and geldanamycin the major substrate is Hsp90, so that other proteins are likely to play a very minor role in their effects on development. Although radicicol and geldanamycin have been shown to interact with most Hsp90 family members, each inhibitor may have different affinities for these, causing different effects in certain tissues. For example, a higher level of inhibition of GRP94 would affect tissues rich in mitochondria such as muscle and brain. Hsp90a and b have distinct dynamic spatial and temporal expression patterns during development (Chapter 1.4.3, p33). If these two forms were inhibited to a different extent by radicicol or geldanamycin, it could explain some of the variation in the types of developmental defects that are caused.

3.4.4 Eye defects

Inhibitor treatment was carried out during the window of development in which the eyes are most vulnerable to teratogens. It was important however, to establish a level of treatment during this period that did not have any effects on eye morphology, so that only embryos carrying eye mutations were likely to be affected. When larvae suffered from severe oedema, the eyes were often small and misshapen, presumably as a result of the oedema and growth retardation already present (WT, 89/517 (17%) 10 μM, 1/282 (0.4%) EtOH, Figure 3.6d). Specific eye defects, however, were rarely observed after geldanamycin or radicicol treatment. In one instance, smaller eyes with an irregular

pupil occurred in one treated, and one untreated larva when Tü WT embryos were treated with 3 μM radicicol from 30-50%. The presence of the defect in an untreated embryo suggests that radicicol is not the primary cause. Only one eye defect, a bilateral anophthalmia, arose among 2002 Tü WT embryos treated with 10 μM radicicol from 60-70% epiboly. A bilateral anophthalmia was also observed for one out of 48 WIK WT embryos treated in the same way. No eye defects were ever observed in geldanamycin treated embryos (146 WT treated at 10-20 μM from 60-70% epiboly).

The black skin pigment cells, known as melanocytes, expand in response to bright light, and retract in darker conditions. This response is linked to visual function, so that blind embryos have fully expanded melanocytes (Goldsmith, 2001, Dahm, 2003). This was not observed in any of the embryos treated, suggesting that blindness did not occur.

3.4.5 Controls: Chemicals and heat shock

It was essential to use the correct controls, to be able to identify specific effects of the inhibitors. It has already been mentioned that some defects occur with all treatment types (heart oedema, growth retardation, very severe oedema and growth retardation with reduced pigmentation, and failure of the swim bladder to inflate), some of which can also occur in untreated embryos. These may be due to toxic effects of the drugs. Geldanamycin is insoluble in water or ethanol, and was dissolved in DMSO, which is a known teratogen (Sharma *et al.* 1985, Larsen and Janners, 1987). DMSO alone occasionally caused the developmental defects common to radicicol and geldanamycin, but at a similar frequency to the controls containing up to 30 μl EtOH, so that these defects may be caused by other environmental effects (Table 3.4).

The similarity in frequency of developmental defects for geldanamycin and geldampicin demonstrates the toxic effect of benzoquinone ansamycins, and suggests that for geldanamycin, and perhaps also radicicol, the majority of the developmental defects observed in all strains treated are likely to be caused by a toxic effect of the drug. A high proportion (over 70%) of embryos however, are unaffected after radicicol and

geldanamycin treatment at 10 μM, indicating that Hsp90 may be inhibited at a dose that causes a low level of developmental abnormalities. The possibility remains that the general developmental defects caused by radicicol treatment are due to specific effects on Hsp90 clients that are important in basic developmental processes.

Treatment	Frequency of defects	% Defects
EtOH	6/252	2.4
10 μM Radicicol	114/386	29.5
Untreated	0/94	0
DMSO	10/679	1.5
10 μM Geldanamycin	9/238	3.8
10 μM Geldampicin	11/365	3

Table 3.4: Frequency of general developmental defects (not including fin defects) in WT embryos after different treatments.

It is also important to consider the defects observed after a heat shock. This allows a distinction to be made between the defects that are caused by Hsp90 inhibition, and those that may be caused as a result of the up-regulation of other HSPs by Hsp90 inhibition. Embryos were subjected to heat stress for 30 min to 1 hour, at the same stage at which an inhibitor would normally be added (See Chapter 2, p50 for details). Although heat shock was carried out right at the start of gastrulation (60-70% epiboly), this is a very severe treatment for such early embryos, and should cause a lasting heat shock response, affecting many aspects of development. All the defects seen after geldanamycin and radicicol treatment also occurred after heat shock, with the exception of caudal fin defects, over inflation of the swim bladder and abdomen defects. In addition, some more severe defects were identified that had not previously been observed for radicicol or geldanamycin (Figure 3.9). These included pronounced notochord defects, coloboma and severe eye malformation. The development of more severe defects after a heat shock than after Hsp90 inhibition, suggests that Hsp90

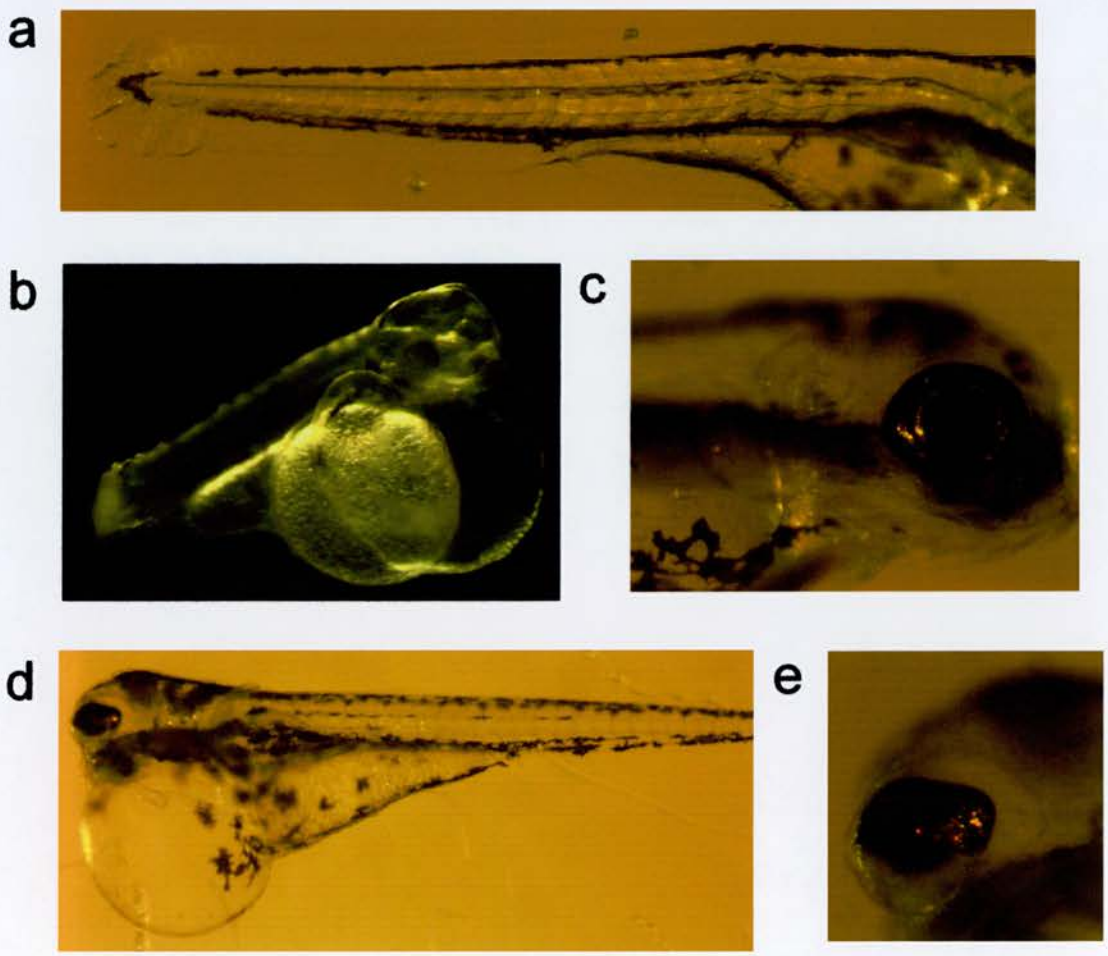


Figure 3.9: Developmental defects that arise in *sri* homozygous embryos after a 37-40°C heat shock. (a) Neural tube defect; **(b)** Severe oedema and growth retardation; **(c)** Coloboma; **(d)** Severe oedema and growth retardation, with eye defect, at higher magnification in **(e)**. Embryos were scored at 4dpf.

inhibitors at the levels used did not induce a generalised stress response, although the possibility remains that a slight induction may have occurred.

The experimental conditions chosen for further testing with mutant zebrafish lines were concentrations of 10-20 μM geldanamycin and 10 μM radicicol, added at 60-70% epiboly. Under these conditions, some mild fin defects were observed, as well as a lower frequency of other defects, survival was over 75% at 5 dpf (compared to over 80% for EtOH), and eye development was not significantly affected.

3.5 A heat shock response is not induced in embryos treated with a low concentration of radicicol or geldanamycin

Experiments were conducted to establish whether the developmental defects caused by the chosen concentrations of radicicol and geldanamycin were a result of Hsp90 inhibition, and not the general stress response that can occur at high levels of Hsp90 inhibition (Zou *et al.* 1998, Bagatell *et al.* 2000). Western blotting and RT-PCR were used to quantify the level of several heat shock proteins that are up-regulated in response to HSF1 activation by heat shock (Yeh and Hsu, 2002, Lele *et al.* 1997). Embryos were treated with inhibitors as described, and harvested after 18h incubation. Where possible, embryos from a single pair mating were used in each experiment, to prevent confounding by alterations in HSP levels due to differences in environmental conditions or developmental stage. To demonstrate the level of induction caused by heat shock, embryos were incubated at temperatures of 32-37°C for 30-60 minutes before harvesting.

Quantitative RT-PCR was carried out with primers for *Hsp70* and *Hsp47*. The skeletal form of actin, α-actin was used as a loading control, as it does not interact with Hsp90, and therefore should be unaffected by heat shock or Hsp90 inhibition. α-actin should not be confused with filamentous, or F-actin that does interact with Hsp90 (Kellermayer and Csermely, 1995). There was no overall difference in the level of *Hsp70* and *Hsp47* mRNA after treatment with 10 μM radicicol or 10-20 μM geldanamycin (Figure 3.10),

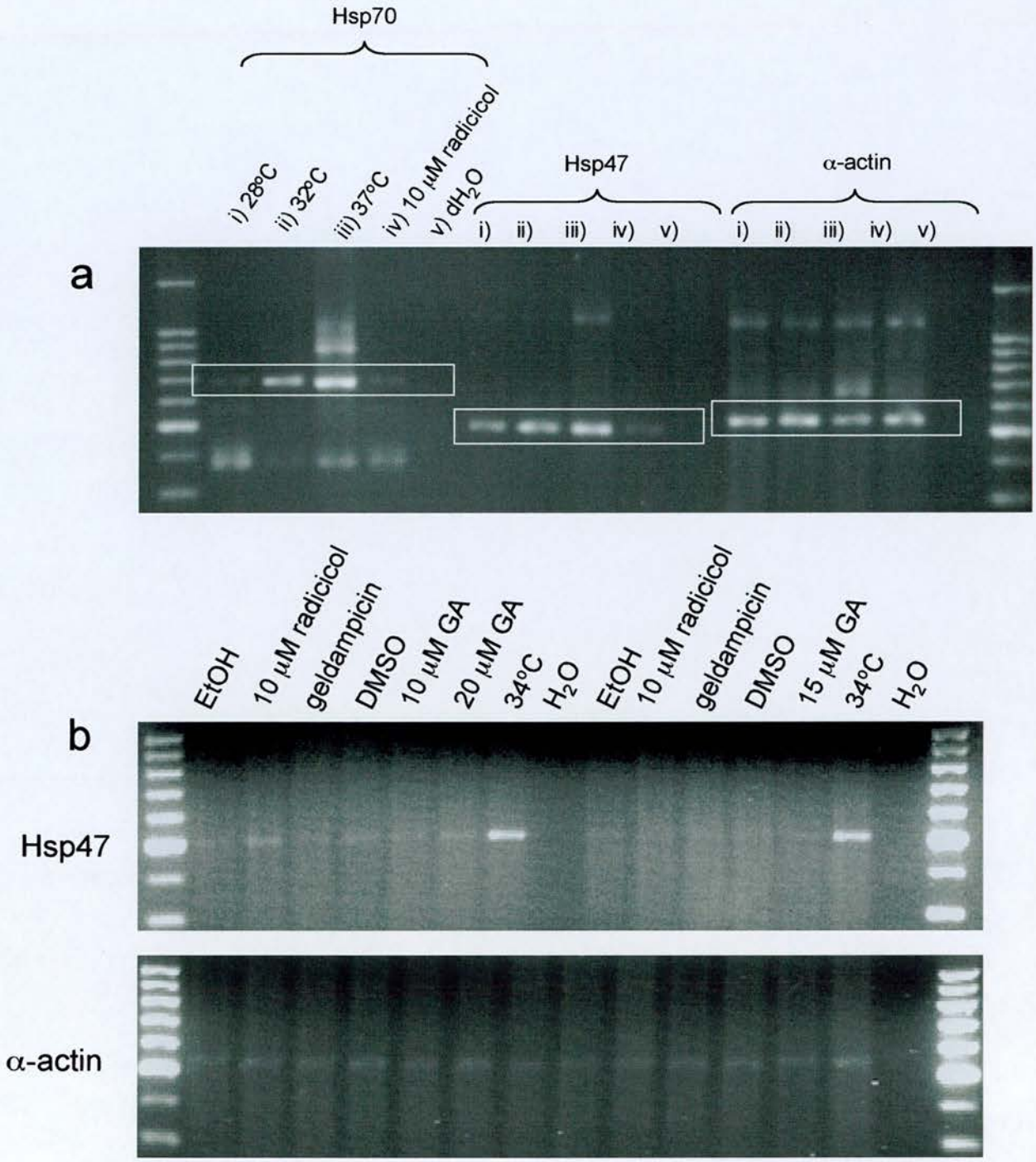


Figure 3.10: No induction of *Hsp70* or *Hsp47* mRNA expression by radicicol or geldanamycin (GA). RT-PCR for **(a)** WT and **(b)** *sri*. DNA marker is 100 bp ladder, brightest band is 500 bp.

when compared to untreated controls. A significant increase in the levels of these proteins could be detected after a heat shock of at least 32°C for 30 min.

Western blotting was carried out using antibodies for Hsp70 (detects inducible form only) and Hsp90 (detects a and b forms). A duplicate gel, stained with coomassie blue was used to demonstrate equal loading (not shown). There was a very slight increase in the level of Hsp90 protein after a 37°C heat shock, and no obvious increase after inhibitor treatment (data not shown). The antibody used for Hsp70 was unable to detect the increase in expression caused by a 32°C heat shock, but there does appear to be very slight increase in Hsp70 levels for both geldanamycin and radicicol compared to a 37°C heat shock (Figure 3.11a,b).

Staining was also carried out with an antibody for MyoD (Moyogenic differentiation antigen 1). The binding activity of MyoD is affected by Hsp90 (Shaknovitch *et al.* 1992, Shue *et al.* 1994). As it has been shown in cell culture that Hsp90 inhibitors can cause the degradation of some Hsp90 client proteins (Marcu *et al.* 2002, Marcu *et al.* 2000b, An *et al.* 2000, Sakagami *et al.* 1999, Soga *et al.* 1998, Schulte *et al.* 1997), it was expected that MyoD levels might be affected by Hsp90 inhibition. No change in the level of MyoD was observed (Figure 3.11c) due either to the stability of MyoD in the absence of Hsp90, or because the level of inhibition was not high enough to disrupt this particular interaction.

It can be concluded from these experiments that 10μM radicicol or geldanamycin does not induce HSP expression at the RNA level. There may be a small induction of HSP translation, as demonstrated by a slight increase in Hsp70 protein levels. A slight induction of the heat shock response may have occurred, increasing translation of Hsp70, but without activating HSF1-mediated transcription of HSPs. Translation of non-HSP RNAs should therefore be unaffected, allowing development to proceed normally, apart from any specific effects of the inhibitors on Hsp90 clients. To determine the exact

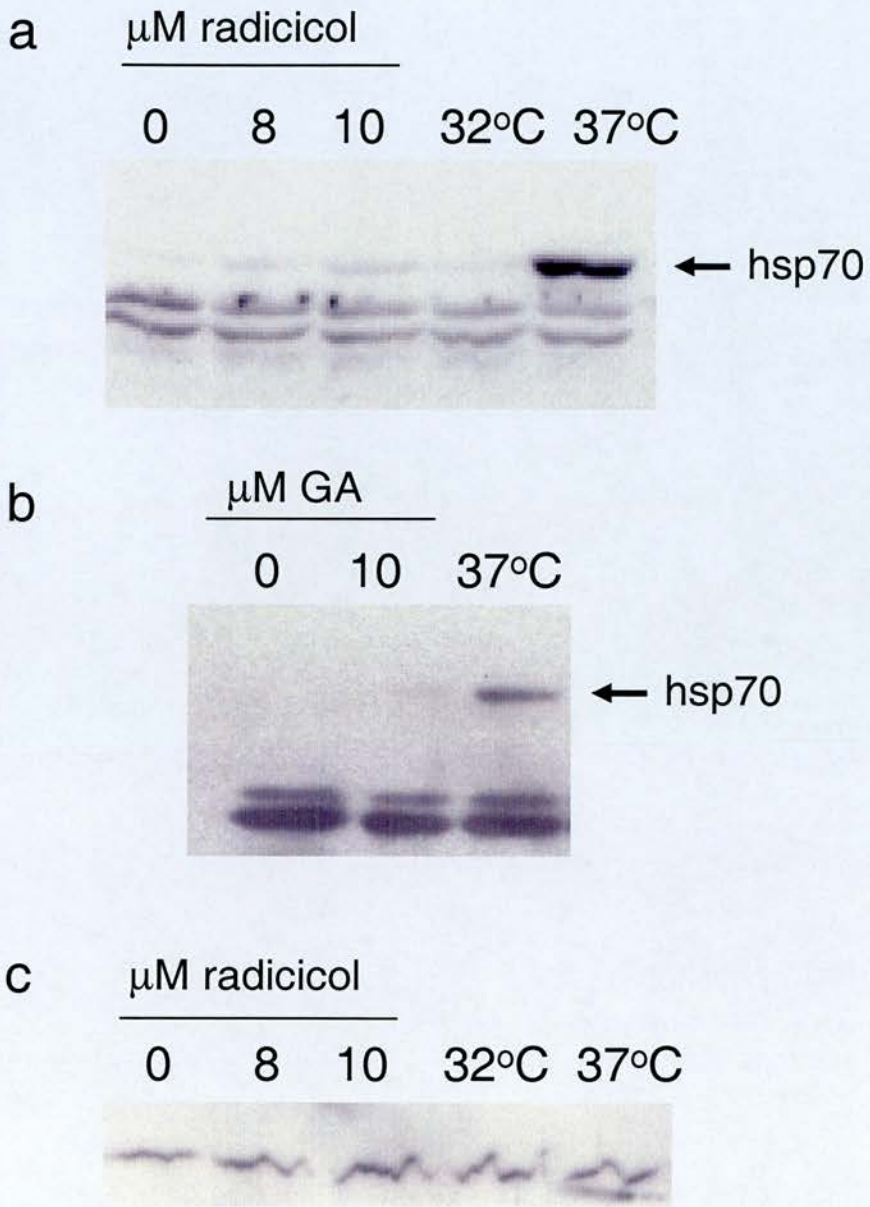


Figure 3.11: A very slight induction in Hsp70 appears to be caused by radicicol and geldanamycin treatment. Western blotting with Hsp70 antibody for treatment of *sri* zebrafish embryos with **(a)** radicicol and **(b)** geldanamycin. **(c)** MyoD.

amount of induction that may be occurring at a protein level, western blotting should be repeated several times with a more sensitive antibody, and densitometry analysis carried out to compare protein levels.

3.6 The frequency of developmental defects caused by Hsp90 inhibition varies between strains

In treating different zebrafish mutant strains with radicicol (described in Chapter 4, p99) it was noticeable that the level of certain developmental defects varied between strains. These results were compiled, and paired analysis of treatment groups and their controls was carried out for each defect. The difference in frequency of the defect for each pair was plotted, and ANOVA was used to determine whether the distribution of these differences varied significantly between strains (Figure 3.12 and 3.13). The difference was significant for heart oedema and very severe oedema and growth retardation with reduced pigmentation (statistical calculations carried out by Andrew Carothers). Both of these phenotypes could be caused by a defect in heart function that causes oedema, which if severe may cause extensive oedema and growth retardation.

It would be impossible to determine whether this difference in response to the inhibitors is due to Hsp90 inhibition or to a general toxicity of the drug, unless these experiments were repeated with a control compound that did not inhibit Hsp90. It would be important to use a structurally similar compound as a control, to minimise any differences in metabolism, stability, diffusion, etc.

Defects such as oedema also arise in untreated embryos, and could be caused by any number of defects affecting the heart or circulation. It would be interesting to identify Hsp90 buffered modifiers of these less specific developmental defects. This would show whether response to drugs or acquired disease could be modified by genetic factors, but only under certain conditions. For example dietary or lifestyle factors might cause a slight stress response that would uncover these variants.

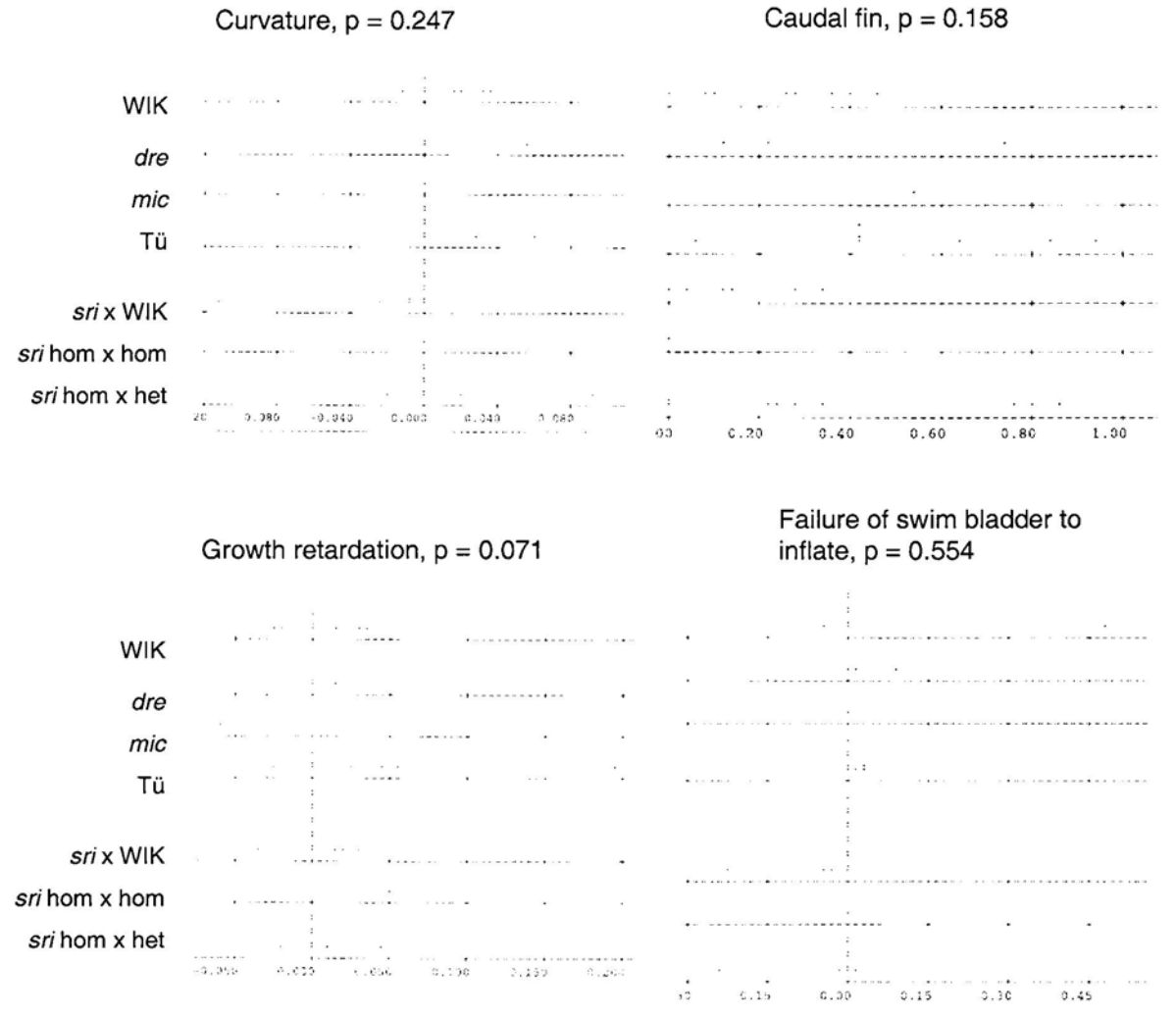


Figure 3.12: Frequency of developmental defects for different strains, treated with radicicol. ANOVA shows whether variation between different strains is larger than within each strain (p of less than 0.05 is considered significant). Difference between the frequency of defects for treated and untreated groups for each experiment is plotted. Data is plotted for each genotype (y axis). The x axis represents the difference between each pair of experiments, represented by a dot. A value of zero indicates no difference between the treated and untreated groups for the experiment.

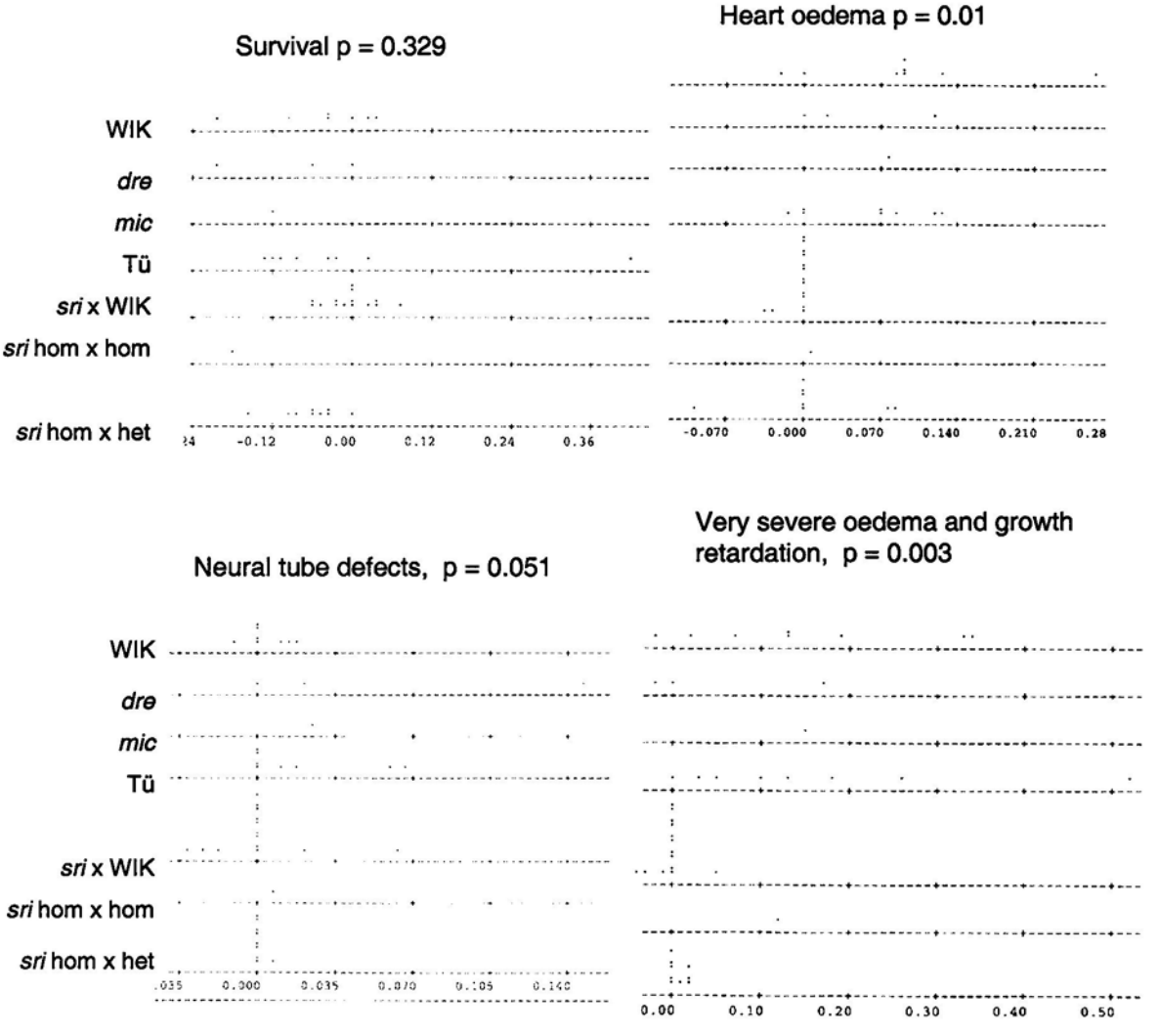


Figure 3.13: Frequency of developmental defects for different strains, treated with radicicol, continued.

It is also worthwhile simply to show that differences in response to drug treatment can be detected between zebrafish strains. This system could be used to identify genetic factors that are important in drug metabolism for pharmacogenomic studies. Breeding of zebrafish is cheap, and uses relatively little space compared to mammalian species, allowing larger experiments to be carried out. This system has already been used to detect toxic levels of certain compounds (Schulte and Nagel 1994, Nagel 2002, Roex *et al.* 2002).

3.7 Summary and Conclusions

Treatment of WT zebrafish embryos with the Hsp90 inhibitor radicicol reproduced the phenotype previously reported by Lele *et al.* for the structurally unrelated inhibitor geldanamycin, suggesting that this effect is due to Hsp90 inhibition. Radicicol and geldanamycin caused lethality and developmental defects in a dose-responsive manner. A concentration of 10 μM radicicol or geldanamycin, for 18h incubations starting from 60-70% epiboly, caused low levels of developmental defects in WT embryos. 10 μM did not cause a significant heat shock response at the protein or RNA level. Also, the developmental defects that arose after heat treatment were more severe than for radicicol or geldanamycin, suggesting that the drugs do not cause a general stress response that damages development. Novobiocin caused a high frequency of lethality and severe defects, due almost certainly to effects on topoisomerase II, and demonstrating the effects of a more toxic drug on development. This work shows that the drugs radicicol and geldanamycin can be used to inhibit Hsp90 in developing embryos, while causing very few non-specific morphological defects.

At the concentration of inhibitors used eye defects were extremely rare, so that in subsequent experiments any eye defects that arise on a mutant background should be due to an involvement of Hsp90 in the mutant phenotype. The use of two structurally

unrelated inhibitors will allow the comparison of effects between them, to confirm that defects observed are due to Hsp90 inhibition and not toxicity. Another important control is geldampicin, which is structurally related to geldanamycin but does not inhibit Hsp90.

In *Drosophila*, when Hsp90 function was compromised, many different morphological defects were observed, which were often specific to certain strains (Rutherford and Lindquist, 1998). In zebrafish however, the same morphological defects were observed in different strains. One notochord defect seemed to arise more frequently in one WT strain (Figure 3.6e). The frequency of this defect was so low that the results were not statistically significant. Some spawnings however, are more severely affected by Hsp90 inhibition, or show a high frequency of one particular defect even without inhibition, suggesting that other environmental and genetic effects may influence the outcome of these experiments. Patricia Yeyati carried out experiments with radicicol in several other strains, and identified two phenotypes, eye and notochord defects, which arose more frequently in one particular mutant background when treated with radicicol, and were enriched in the F2 generation. This shows that Hsp90 can be used to identify cryptic mutations in zebrafish. It may be possible to exploit this system to identify milder mutations in ENU mutagenesis screens.

The differences between morphological defects caused by Hsp90 inhibition in *Drosophila* and zebrafish may be caused by several factors. Zebrafish cannot be completely inbred, as *Drosophila* can. When a new *Drosophila* WT isolate was crossed to *Hsp83* mutants, only 0.8% of flies developed morphological defects, while 1.7% of inbred flies raised on geldanamycin were affected. This suggests that more inbred strains are more dependent on Hsp90 buffering, presumably because they are more likely to be homozygous for polymorphisms or mutations. Other reasons for the difference observed may be due to the relatedness of the strains used, so that although they are not completely inbred, there was no real variation in the buffered mutations. Although the lines used for these experiments have been separate for several generations, they were all on a mixed Tü/AB/TL background.

Another obvious difference between these two systems is that zebrafish have two forms of Hsp90, while *Drosophila* only has one. This may give some redundancy of function, so that it is more difficult to perturb Hsp90 buffering in zebrafish. There may be other layers of redundancy and functional stability among the chaperones in zebrafish. The very nature of *Drosophila* development, with their imaginal discs that can be so easily interfered with, may amplify the effects of Hsp90 buffering. Vertebrate development however, is more complex and there are fewer viable outcomes for each developmental pathway. This is obvious from an observation of human congenital defects; most are lethal very early on. Despite these differences, cryptic mutations in eye and notochord development have been identified, and it may be possible to identify the dose and timing required to reveal cryptic mutations in other developmental pathways.

Chapter 4

The Effects of Hsp90 Inhibitors on Zebrafish Eye Mutants

4.1 Introduction

Investigations were carried out to determine whether Hsp90 buffers developmental eye defects. This was done because it is evident from clinical data (Schimmenti *et al.* 1997, Syagailo *et al.* 1998, Morrison *et al.* 2002) and studies in the mouse (Jordan, 1992, Smith *et al.* 2000, Chang *et al.* 2001), that inherited eye malformations can be affected by multiple environmental and genetic factors. It was hypothesised that among the zebrafish eye mutants available, Hsp90 would buffer the phenotype of some, and that this buffering could be revealed by the use of specific inhibitors during development.

Several recessive eye mutants were selected for their weak or variable phenotype. It was hoped that the homozygotes would be viable and fertile, to allow for easier statistical analysis of crosses. Initially, homozygous adults were to be crossed to wild types, and the embryos treated with Hsp90 inhibitors to test whether the penetrance of the phenotype was altered. Alternatively homozygous embryos could be treated to test for changes in expressivity. This system was used because it was thought that Mendelian inheritance patterns would make statistical analysis of any differences in penetrance or expressivity easier.

4.2 Zebrafish eye mutants

Several zebrafish eye mutants were chosen (by Patricia Yeyati) from among those generated in the 1996 zebrafish ENU mutagenesis screen at the Max-Planck Institute for Developmental Biology, Tübingen (Table 4.1, Development, volume 123, 1996). This screen identified recessive and usually homozygous lethal mutations with defects in specific organs. The mutants chosen were reported as being either viable or semi viable (Haffter *et al.* 1996). All of the mutants selected, except sandy, had previously been complementation tested, and found to be complementary (non-allelic) with respect to each other (Heisenberg *et al.* 1996).

ENU mutagenesis had been carried out on Tübingen (Tü) wild type males that were then crossed to AB, TL, or a Tü:AB hybrid strain (Table 3.2). All the lines ordered were out-crossed to Tü or TL to produce the embryos that were shipped to our fish facility in Edinburgh and raised to adulthood. TL is homozygous for the mutations, *leo*^{tl} and *lof*^{dt2}. *leo*^{tl}, also called *tup* or *Brachydanio frankei*, is recessive and gives spots in the homozygous adults, while *lof*^{dt2} is dominant and gives long fins (Haffter *et al.* 1996). These two mutations are non-deleterious, and are used to aid the identification of adults when carrying out pair mating.

Mutant	Allele	Phenotype	Viability	References	Phenotype visible (dpf)
sunrise (sri)	tq253a	Prominent lens, irregular cornea.	adult viable	Heisenberg *et al.* 1996, Haffter, 1996b.	4
microps (mic)	tm329	Slightly reduced body size, Small eyes, expanded melanopores,	adult viable	Heisenberg *et al.* 1996, Neuhauss *et al.* 1999.	1
rosine (rne)	tm70h	Small lens	adult viable	Heisenberg *et al.* 1996.	4
dreumes (dre)	tm146d	Reduced body size, small lens, ear defect, no unpaired anal fin	adult viable	Heisenberg *et al.* 1996, Whitfield *et al.* 1996, van Eeden *et al.* 1996.	4
helderziend (hez)	tq291	Clear (not green) lens, slightly reduced body size, weak hammerhead phenotype.	adult semi viable	Heisenberg *et al.* 1996.	4
sandy (sdy)	tk20	No melanin in body and eyes	adult semi viable	Kelsh *et al.* 1996, Neuhauss *et al.* 1999.	1

Table 4.1: Phenotypic characteristics of zebrafish mutants chosen from the 1996 ENU mutagenesis screen (http://www.zfin.org).

Heterozygous F1 adults were identified by inter-crossing and comparison of any suspected eye defects with those published for the mutant. The expected phenotypes were observed for *sri, mic,* and *dre*, except the subtle ear defect described for *dre* (Whitfield *et al.* 1996), as the ears were not examined at high magnification (Figure 4.1). Non-closure of the optic fissure, (coloboma) arose at a low frequency for both *sri* and

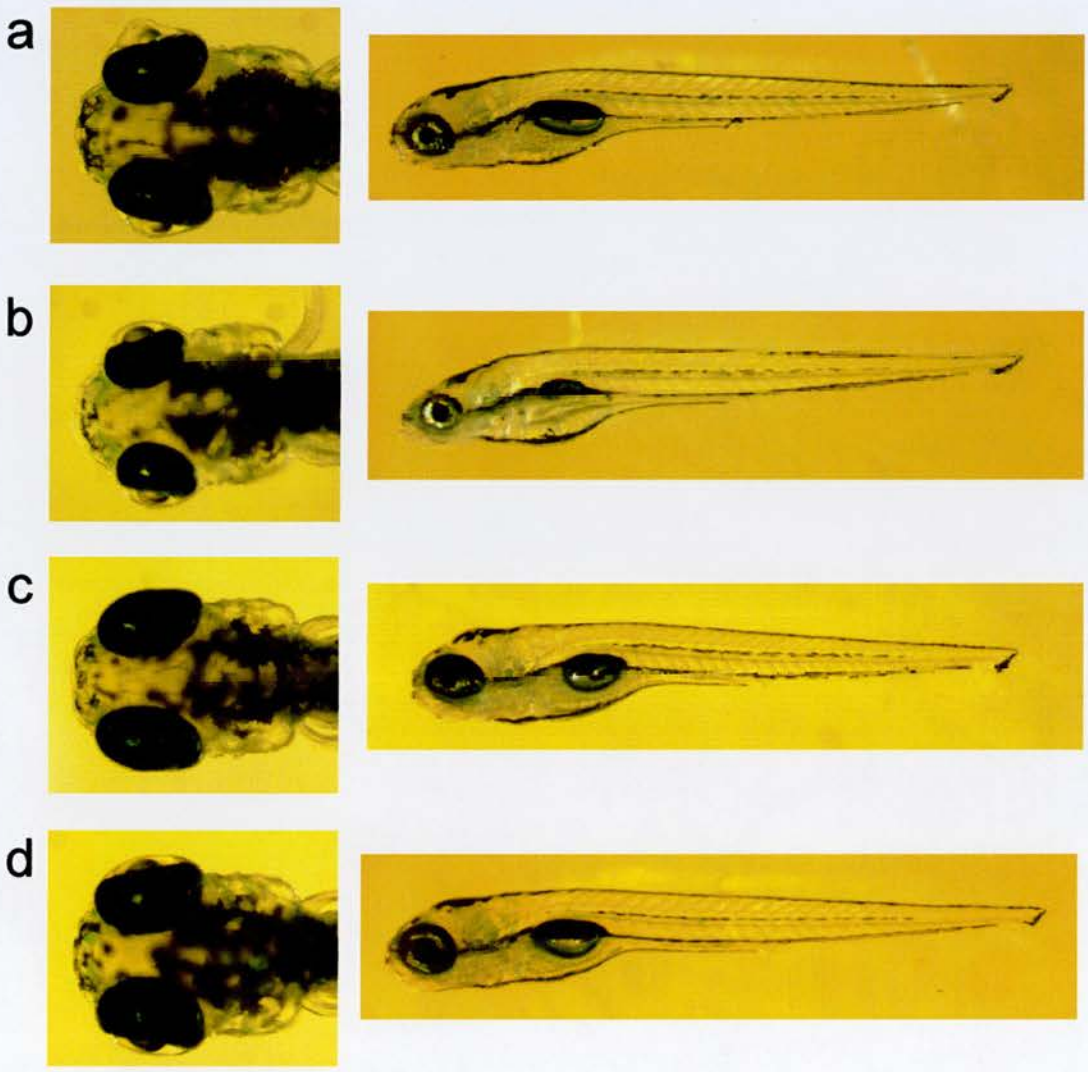


Figure 4.1: The homozygous phenotype observed for the zebrafish eye mutants at 5 dpf. (a) *sri;* **(b)** *mic;* **(c)** *dre;* **(d)** WT.

dre. The frequency of embryos showing the homozygous phenotype should be 25% for a heterozygous cross if the phenotype is fully penetrant. The observed frequency for each mutant was tested for deviation from the expected 25% with a two-tailed Fisher's exact test (Table 4.2). The proportion of *sri* mutants scored was significantly smaller than the 25% expected, showing that the mutation is either not fully penetrant or that the milder defects in some homozygotes cannot be detected by examination of live embryos (Chapter 2.5, p50).

	Embryos				
Mutation	Mutant	WT	Total	**% mutant**	**p value**
sri	131	500	631	20.7	0.0001
mic	262	816	1078	24.3	0.698
dre	162	425	587	27.6	0.3212

Table 4.2: Frequency of the mutant phenotype in heterozygous in-crosses, showing the Fisher's exact test p value. A p value of 0.05 (5%) is considered significant.

A phenotype was not observed for *rne*, either due to difficulty of scoring the subtle defect, lack of carriers in the surviving adults, or silencing by modifiers in the outcross received. *sdy* and *hez* did not survive transportation well, and the phenotype was not observed from crosses of the few adults that remained. These three mutants were not used for further studies.

Homozygous embryos from *sri, mic,* and *dre* were kept and raised. *sri* adults were viable and fertile, while *dre* failed to thrive and were humanely killed. *mic* did not survive beyond two to three weeks, possibly due to a failure of the swim bladder to inflate, or inability to feed due to blindness.

4.3 Treatment of heterozygous zebrafish eye mutants with Hsp90 inhibitors

Experiments were conducted to determine whether a mutant phenotype could be induced by Hsp90 inhibitor treatment of heterozygotes for each of the eye mutants. Heterozygous

embryos were collected from crosses between a WT and a heterozygous or homozygous *mic* or *sri* fish, and treated with radicicol (Chapter 2.3, p49). No eye defects were observed in the treated embryos, apart from the small eyes previously described in oedematous embryos (Chapter 3.4.4, p83). Embryos from *dre* heterozygous crosses were treated in the same way. There was a decrease in the frequency of embryos showing the *dre* phenotype, presumably due to a higher sensitivity of the homozygous embryos to radicicol treatment. There were no mild eye defects among the rest of the treated embryos that could suggest that a slight mutant phenotype had been induced in the heterozygotes.

Mutant	Cross	% Epiboly at start of treatment	μM radicicol	Mutant embryos	
				Treated	Untreated
mic	WT x het	30-50%	2.5-3	0/362	0/27
mic	WT x het	60-70%	10-20	0/80	-
mic	WT x het	80-90%	15-25	0/61	-
sri	WT x het	30-50%	0.1-2.5	0/181	0/98
sri	WT x het	60-70%	10-20	0/91	-
sri	WT x hom	60-70%	10-20	0/279	0/50
dre	het x het	60-70%	10	143/715 (20%)	184/763 (24%)

Table 4.3: Frequency of embryos showing the mutant phenotype in radicicol treated heterozygotes.

It was hypothesised in Chapter 1.6 (p44) that Hsp90 may stabilise or buffer developmental processes, either directly or indirectly. In mutants buffered in this way, reduction of Hsp90 function could be expected to increase the penetrance of the phenotype. For the majority of genes, loss of function of one allele does not cause a dominant, or haploinsufficient phenotype (Wilkie, 1994). It was expected however, that Hsp90 might buffer some developmental regulatory pathways to an extent that in individuals haploinsufficient for one component of the pathway (heterozygous for a recessive mutation), the morphological outcome would be more vulnerable to Hsp90 inhibition. These results show that Hsp90 inhibition, under the conditions used, is not enough reveal the mutant phenotype in embryos heterozygous for the recessive

mutations *sri, dre* and *mic*. It appears, therefore, that a developmental pathway can be relatively robust, despite the presence of a Mendelian mutation.

There may still be several ways to cause a visible eye phenotype in these heterozygotes. Homozygotes for these mutations have a variable phenotype, indicating that other genetic or environmental factors are important in determining the severity of the phenotype. If the most affected embryos were selected over several generations, genetic modifiers that amplify the phenotype may accumulate, so that the pathway containing the mutation might be more vulnerable to Hsp90 inhibition in heterozygotes. Alternatively, if treatment was initiated at 10-11 hpf, immediately before the start of eye development, it might be possible to avoid affecting the more general developmental processes such as gastrulation and the onset of somitogenesis. In humans, eye development is the most vulnerable to teratogens between 3 ½ to 12 weeks. During this time, the eye is continually developing (Moore, 1998). Therefore, if radicicol was added even later, after 11 hpf, this may also have a significant effect on eye development. This later treatment could allow a much higher dose of radicicol to be used, which would be more likely to have an effect on heterozygotes.

4.4 Treatment of homozygous zebrafish eye mutants with Hsp90 inhibitors

Experiments were conducted to determine whether the expressivity of the homozygous mutant phenotype could be affected by Hsp90 inhibition in zebrafish eye mutants. This was done because although the mutations revealed in *Drosophila* and *Arabidopsis* were cryptic (Rutherford and Lindquist, 1998, Queitsch *et al.* 2002), modulation of a fully penetrant but variable mutant phenotype by Hsp90 inhibition would demonstrate that Hsp90 buffers the effects of the mutation. For each mutant, embryos from either a heterozygous or homozygous cross were treated with Hsp90 inhibitors, and scored according the criteria described below. Part of each spawning was treated with the carrier solvent only, as a control (Chapter 2.3, p49).

4.4.1 Measurement of the severity of mutant eye phenotypes

The severity, or expressivity, of the *sri* and *dre* mutant eye phenotype is variable. The most obvious variables for *sri* were the irregularity of the cornea, lens and pupil. The extent of pupil irregularity was also variable for *dre* (4.2a). It was important to establish a method for scoring each phenotype, so that the effect of Hsp90 inhibition on expressivity could be quantified. First, the presence or absence of coloboma was scored. Secondly, for some experiments, digital photographs were taken of each eye, and a line drawn around the pupil and outer edge of the retina. Measurements were then taken of the area, major axis, eccentricity (irregularity) and standard deviation of the radius (SD radius, a measure of ellipticity) of the pupil and the outer edge of the retina (Figure 4.2b). A t-test was used to calculate the significance of the difference between the means (Chapter 2.4, p50)

The *sri* phenotype varied so widely that it was possible to develop a numerical score based on qualitative scoring of the extent of irregularities in the cornea, lens and pupil that could be scored by eye using a dissection microscope. Each eye was scored into one of three categories, and a numerical score was given to the embryo based on the score for both eyes (Table 4.4, Figure 4.2c). Embryos were scored twice, and if a phenotype was on the borderline between two categories it was put into the lower of the two. Embryos with an AC score were given a numerical score of 2. A mean severity score was then calculated for each treatment group (bars in Figure 4.4). Significance of the differences between treatment groups for several experiments was calculated, using the t-test described in Chapter 2.4 (p50, t and p values in Figure 4.4).

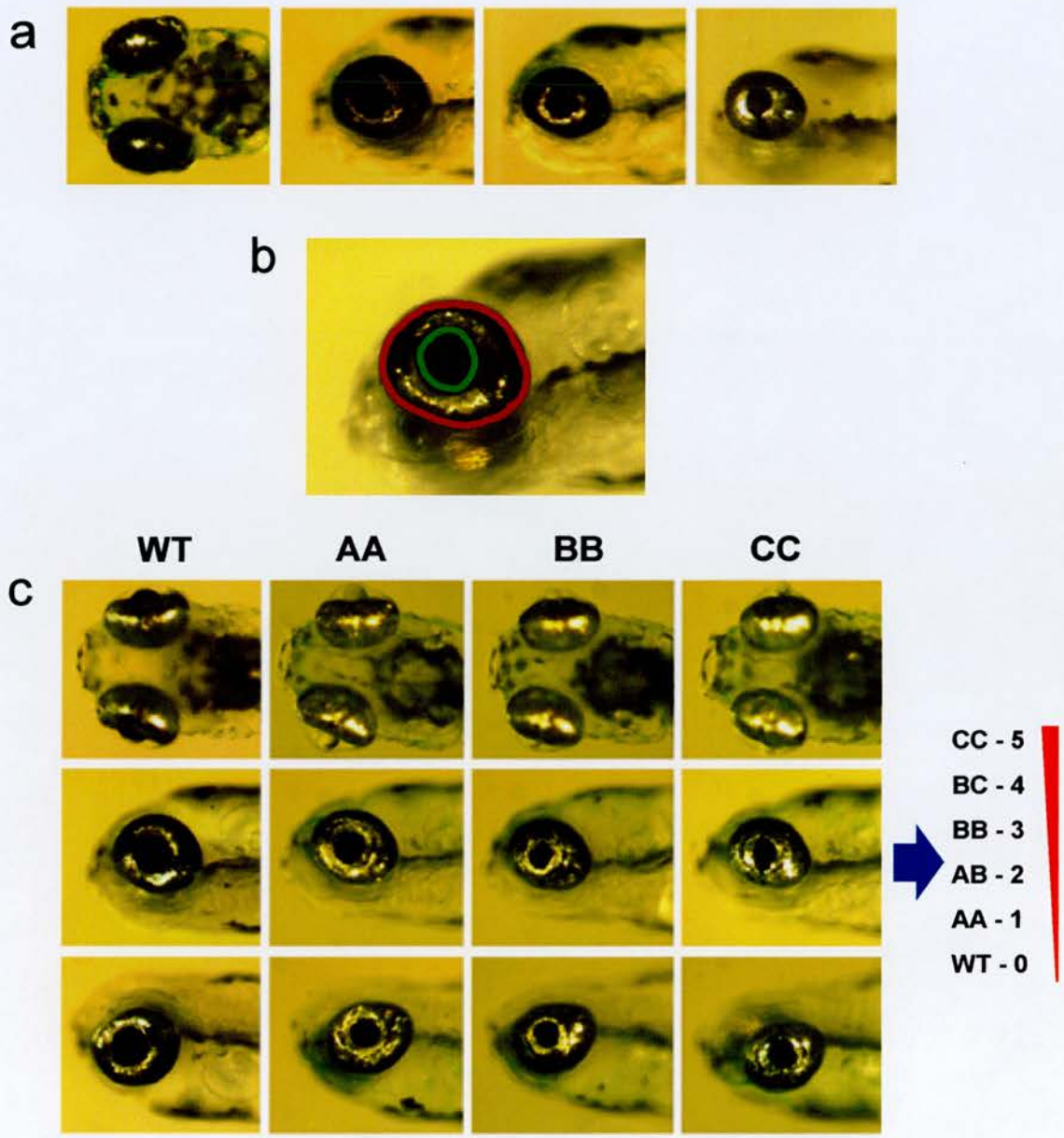


Figure 4.2: Quantification of the severity of zebrafish eye phenotypes. **(a)** Variability of the *dre* phenotype; **(b)** Measurement of the size and shape of the pupil and outer edge of the retina; **(c)** Severity categories for the *sri* phenotype and allocation of a numerical score to each embryo based on the severity in each eye.

	A) Mild	B) Intermediate	C) Severe
Lens	Must be regular and close to normal width, can be slightly protruding	Must be at least half of normal width, must be irregular/smaller/sunken if pupil is regular	Must be less than half normal width if coloboma is absent
Cornea	Must be irregular	Can be extremely irregular	Can be extremely irregular
Pupil	Must be regular, can be slightly smaller	Must be irregular if lens is regular	Coloboma must be present if lens is more than half normal width, or may be almost closed if lens is less than half normal width

Table 4.4: Categories for scoring the *sri* phenotype.

4.4.2 WT

Eye defects were extremely rare in WT embryos treated with 10 μM radicicol or geldanamycin (Chapter 3.4.4, p83). However, segment analysis showed that the eye is slightly smaller in radicicol treated embryos compared to untreated embryos from the same spawning. Both the area and major axis were significantly reduced for the pupil and retina (Table 4.5). A reduction in eye size may be connected to the general developmental retardation observed at 1 dpf. By 5 dpf, there may still be a slight developmental delay in either the whole embryo or just the eye.

a)

			Area		Major axis		SD radius		Eccentricity	
Strain	**Treatment**	**n**	pupil	retina	pupil	retina	pupil	retina	pupil	retina
WT	EtOH	85	6486	16528	182	463	4.47	7.15	0.40	0.50
	radicicol	72	6147	16204	172	458	4.58	7.06	0.40	0.50
sri	EtOH	177	2906	10363	86	292	6.51	6.88	0.44	0.50
	radicicol	180	2465	9669	77	276	9.80	7.48	0.56	0.53
dre	EtOH	158	5599	16405	159	467	7.16	8.46	0.51	0.56
	radicicol	177	5445	16054	154	459	6.85	8.63	0.48	0.56

b)

	Area		Major axis		SD radius		Eccentricity	
Strain	pupil	retina	pupil	retina	pupil	retina	pupil	retina
WT	<0.001	0.021	<0.001	0.1342	0.5946	0.341	0.931	0.746
sri	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
dre	0.023	0.001	0.0046	0.0002	0.1332	0.2802	0.035	0.164

Table 4.5: Segment analysis of the pupil and retina, showing mean values (**a**) and p value for the difference between means for EtOH and radicicol (**b**). A p value of less than 0.05 (5%) is significant.

There may also be a specific effect of radicicol on eye development that causes a very mild reduction in the size of the eye. Under non-stress conditions, Hsp90b is highly expressed in many regions during development including the eye, from at least 16 hpf (Lele *et al.* 1999, Figure 4.3). Studies in mammals have also shown dynamic expression of Hsp90 in different regions of the eye during development (Tanaka *et al.* 1995, Kojima *et al.* 1996, Dean and Tytell, 2001). It is not surprising therefore, that a low level of Hsp90 inhibition may have an adverse effect on eye development. It has already been established however, that at concentrations of 10 μM or less, morphological eye defects are extremely rare, and there was no obvious increase in the pigmentation of embryos that would accompany blindness. Therefore although this reduction in eye size has no apparent deleterious effects on eye development, it should be taken into account when considering the effects of radicicol on eye mutants.

4.4.3 *sunrise*

sri homozygotes were treated with radicicol and scored by all three of the methods described in Chapter 4.4.1 (p104). An attempt was made to carry out the qualitative scoring blind, but this was only possible for geldanamycin, as the particular fin defects caused at a high frequency by radicicol made the treated groups easy to identify.

A significant increase in the mean severity score was observed for 10 μM radicicol, 10 μM geldanamycin, and heat shock (37-40°C, 1 h), but not for geldampicin, compared to untreated controls (Figure 4.4). These results were reproduced for radicicol by segmentation analysis, which showed an increase in eccentricity and SD radius of the pupil and retina. The reduction in area and major axis was also larger than for the WT, showing an additional effect of radicicol on the size of the eye and pupil in *sri* (Table 4.4). There was a significant increase in the frequency of coloboma, from 2.7 to 8.3% (Fisher's exact test, p value 0.0209). This shows that the severity of the *sri* phenotype is increased by Hsp90 inhibitors, and that Hsp90 modulates the expressivity of the *sri* mutation.

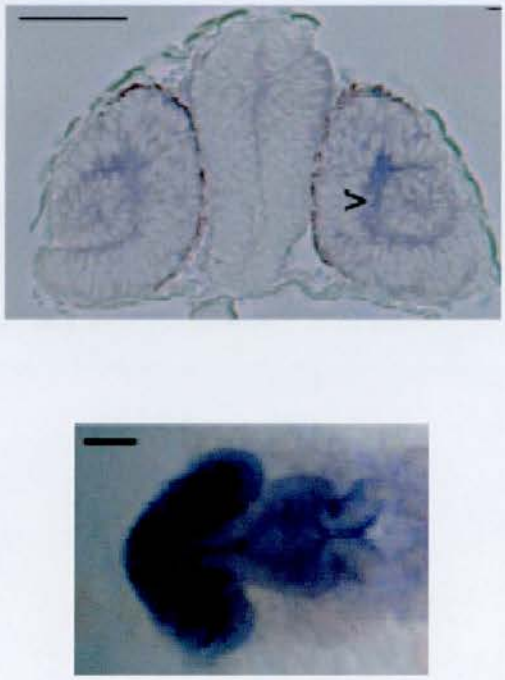


Figure 4.3: Expression of Hsp90 in the zebrafish lens at 20 hpf
(Reproduced from Lele *et al.* 1999).

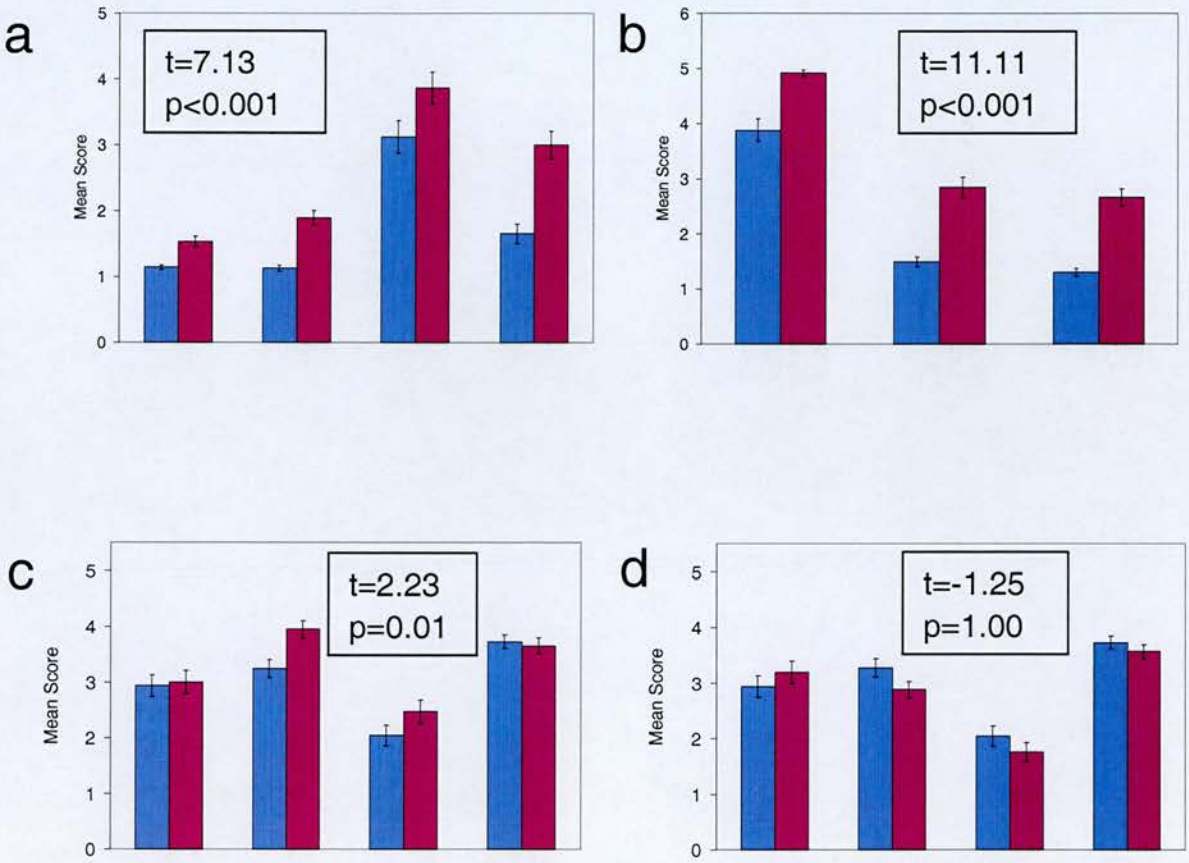


Figure 4.4: Mean severity score for *sri* treated and control groups. **(a-c)** A significant increase in the mean severity score of the *sri* phenotype was observed for **(a)** radicicol, **(b)** heat shock and **(c)** geldanamycin. **(d)** No significant increase was observed for geldampicin. Blue bars (untreated group), red bars (treated group). Each pair of bars represents one experiment. t and p values for a combined significance of the difference between treated and untreated groups for each treatment type are shown.

The effects of radicicol and geldanamycin were dose-responsive, so that a lower concentration of radicicol (4-8 μM) caused a lower increase in severity (t 2.53, p 0.01, by qualitative scoring). A higher dose of geldanamycin (15-20 μM) also gives a larger increase in severity (t 5.58, p 0), but preliminary western blot analysis for concentrations of geldanamycin over 10 μM showed an increase in Hsp70 levels, so that this increase may not be due solely to a decrease in Hsp90 function (data not shown)

The increase in the severity score was more significant for radicicol than geldanamycin when they were both used at 10 μM. This difference is almost certainly due to the difference in the Hsp90 binding affinity of these two drugs (Roe *et al* 1999). The largest increase in severity was observed for heat shock. At this temperature, general protein damage may occur that diverts Hsp90 activity, compromising its normal functions more than pharmacological inhibition alone. Activation of the heat shock response will also have other more general adverse effects on eye development, due to down-regulation of translation and induction of HSPs, especially in embryos made vulnerable by mutation.

The embryos most severely affected with respect to the eye phenotype were also more affected with respect to other defects. The other defects were ranked according to severity (Table 4.6). Severity of the eye trait was significantly correlated to the severity of other defects, with a Pearson correlation value of 0.261, which gives a p value of less than 0.01 (Figure 4.5, statistics carried out by Andrew Carothers). All the embryos in one dish should be subject to the same environmental conditions, but there may be small variations in concentration of the drug or cleanliness of the water that affect the severity of the phenotype. Alternatively there may be genetic factors that affect susceptibility to Hsp90 inhibitors, which affect development as a whole. Genetic factors may include "housekeeping" proteins, such as p53, that interact with Hsp90 (King *et al.* 2001). They may also include proteins involved in the uptake or metabolism of the drug, such as cytochrome 450 (Reviewed in Rogers *et al.* 2002). Variation could also simply be

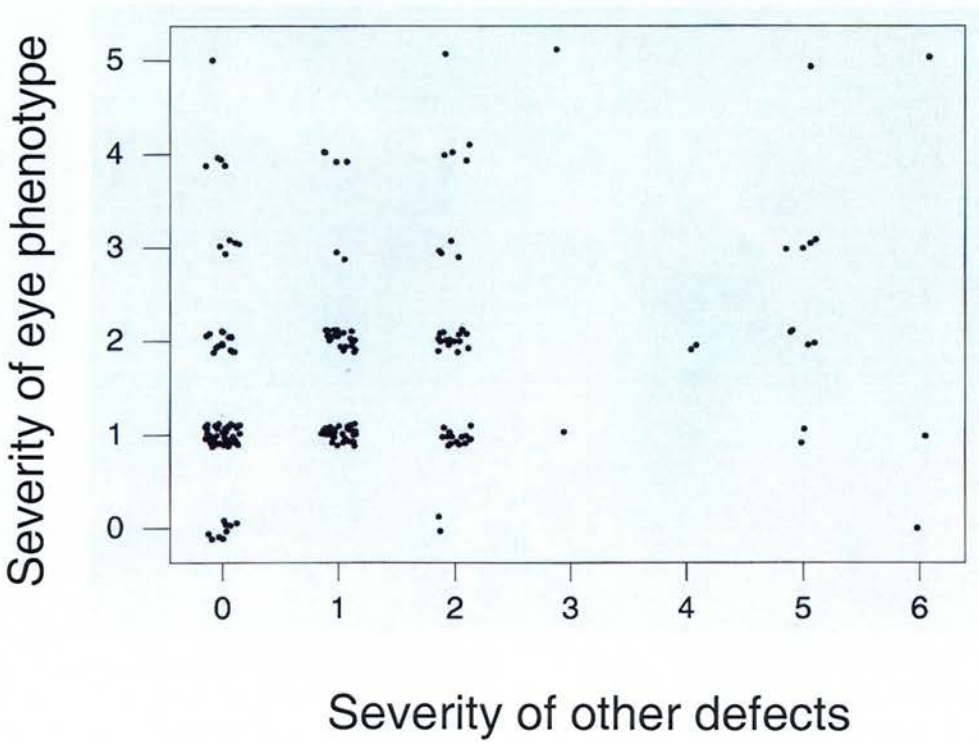


Figure 4.5: The severity of the *sri* phenotype is correlated to the severity of other defects (Andrew Crothers).

"developmental noise" caused by slight random variations in developmental pathways that confer susceptibility to Hsp90 inhibition (Rutherford and Lindquist, 1998, McAdams and Arkin, 1999).

Score	Severity	Defect
0	Unaffected	None detected
1	Slightly affected	Small part of caudal fin missing
2	Very mild	Half of caudal fin missing
3	Mild	Nearly all of caudal fin missing
4	Moderate	Heart oedema
5	Severe	Growth retardation, bends or any other body defect.
6	Very severe	Very severe oedema with growth retardation, reduced eye size and reduced pigmentation.

Table 4.6: Ranking of developmental defects, not including any specific eye phenotypes.

4.4.4 *dreumes*

Treatment of *dre* het x het crosses with radicicol gave no obvious increase in any of the traits measured (Chapter 2.4, p49) in the homozygous embryos, apart from the area and major axis, which are observed in the WT. Statistical analysis however showed that the area of the lens was less significantly reduced than in the WT, while the retina was more affected (Table 4.4). There was also a marginally significant reduction in eccentricity of the pupil. The frequency of coloboma is reduced, although not significantly (3.3% untreated, 0.4% treated, Fisher's exact test, p 0.1437). Frequency of coloboma was also slightly reduced after heat shock (11.1% untreated, 7.1% treated, p 0.2884). Therefore the homozygous phenotype for *dre* appears to be slightly improved, perhaps through effects on the retina. This change is not unexpected, as Hsp90 may buffer any kind of mutation that destabilises protein structure. This could include negative regulators that, when buffering is released, allow the phenotype to improve.

4.4.5 *microps*

mic homozygotes did not survive well after radicicol treatment, so that severity of the phenotype could not easily be scored. From heterozygous crosses, only 16% (27/173) homozygotes survived, while 28% (56/200) homozygotes were present in the untreated

controls. As *mic* embryos do not survive very long even without treatment (up to approximately 14-21 dpf), it is likely that Hsp90-dependent developmental processes that are weakened by the *mic* mutation are further weakened when Hsp90 function is compromised. It would be interesting to treat these embryos at a lower concentration of radicicol to test for effects on severity of the eye phenotype.

4.5 Summary and conclusions

Treatment of the recessive eye mutants *sri, mic* and *dre,* resulted in very different outcomes. It was not possible to induce a mutant phenotype in heterozygotes for any of the mutants. When homozygotes were treated, this resulted in increased lethality of *mic*, a worsening of the phenotype in *sri*, and a slight improvement in the phenotype for *dre*.

The most prominent feature of the *sri* phenotype is the lens, which is reduced even in mildly affected embryos. All of the retinal cell layers appear to be present, but slightly disorganised, perhaps due to a reduction in pressure caused by a smaller lens (Figure 4.6a, Chapter 5.6, p138). It appears, therefore, that the phenotype is primarily caused by the lens. Hsp90 is strongly expressed in the eye, including the lens, so that it could be involved in buffering a lens-specific phenotype. It is unlikely that the mutated protein binds Hsp90, as the Hsp90 client proteins are largely metastable signalling proteins and transcription factors, none of which is likely to give a specific morphological defect when mutated (Reviewed in Picard, 2003). Also, if Hsp90 did interact directly with a mutant protein the effect of Hsp90 inhibition would be expected to be larger. It is possible that the mutation is up or downstream of a factor that does interact with Hsp90, and is in turn affected when Hsp90 function is compromised. It is difficult to predict which proteins could be important, as lens development is complex, and the Hsp90 client proteins are involved in multiple signalling pathways.

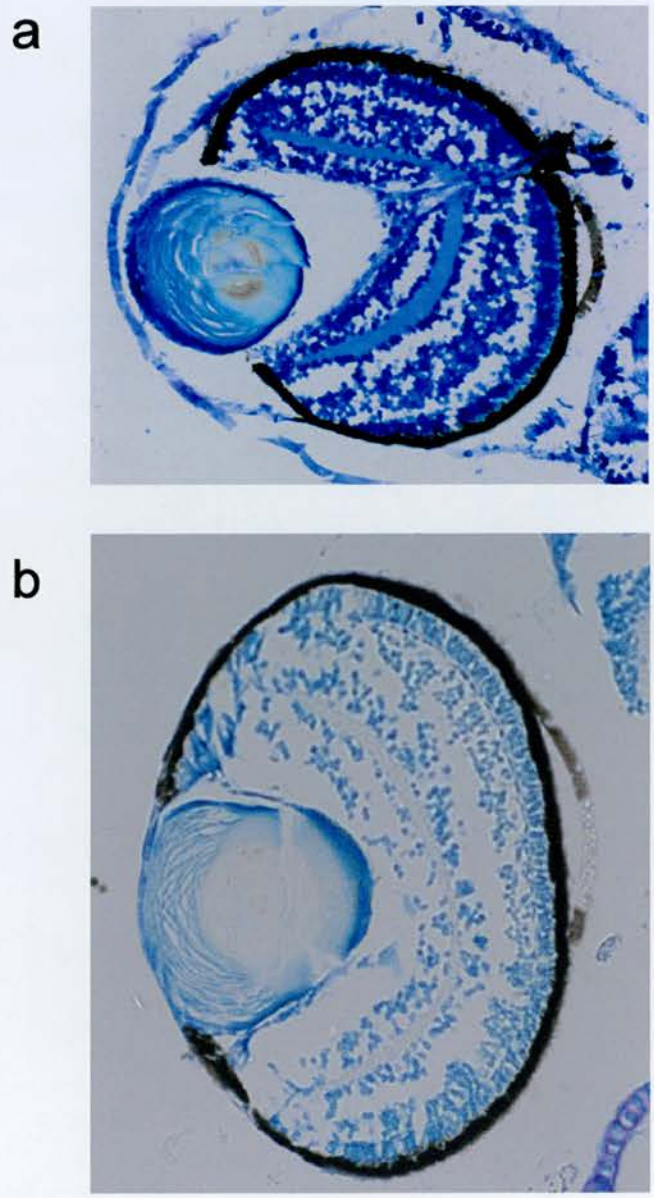


Figure 4.6: Lens defects in zebrafish eye mutants. (a) *sri*; **(b)** *dre*. Untreated embryos were sectioned at 5 dpf.

One obvious candidate Hsp90 client that could affect lens development in *sri* is the inducible form of Hsp70. From investigation of Hsp70 expression in the in chicken lens, it has been suggested to have a role in differentiation of the lens fibres (Dash *et al.* 1994). Hsp70 is highly expressed in the zebrafish lens from 28 to 42 hpf (Blechinger *et al.* 2002b, Halloran *et al.* 2000, Figure 4.7a), during which time the lens starts to differentiate (Easter and Malicki, 2002). Expression of Hsp70 is therefore initiated at a time when the inhibitor was usually rinsed out (from approximately 26-29 hpf), so it may have been affected at a critical stage in the onset of differentiation. Overexpression or increased activity of Hsp70 due to Hsp90 inhibition might have a deleterious effect on lens differentiation. Also, activation of Hsp70 earlier than 28 hpf might have serious effects on the initiation of differentiation. Hsp70 may also be one of the factors that exacerbates the *sri* phenotype after heat shock. Heat treatment could cause protein damage that would sequester Hsp70 and affect lens development, as well as the effects on the timing and level of expression already mentioned.

Another HSP that may be important in the effects on *sri* may be the collagen binding protein, Hsp47, which is also expressed in the developing zebrafish eye (Lele *et al.* 1997b). Several types of collagen are known to be important in lens development (Savontaus *et al.* 1997, Kelley *et al.* 2002). Hsp47 is strongly expressed in the lens from approximately 19-42 hpf (Thisse *et al.* 2001, Figure 4.7b), and timing of expression therefore also coincides with the differentiation of the lens. Hsp47 is strongly upregulated after heat shock, and may play an important role in the effects of heat shock or Hsp90 inhibition on the *sri* phenotype.

The worsening of the *sri* phenotype observed after radicicol treatment might therefore be caused by effects on Hsp70 and Hsp47 translation, due to induction of a heat shock response. It was mentioned in Chapter 3.5 (p87) that Hsp70 is mildly induced by radicicol and geldanamycin, and Hsp47 could be expected to behave in the same way.

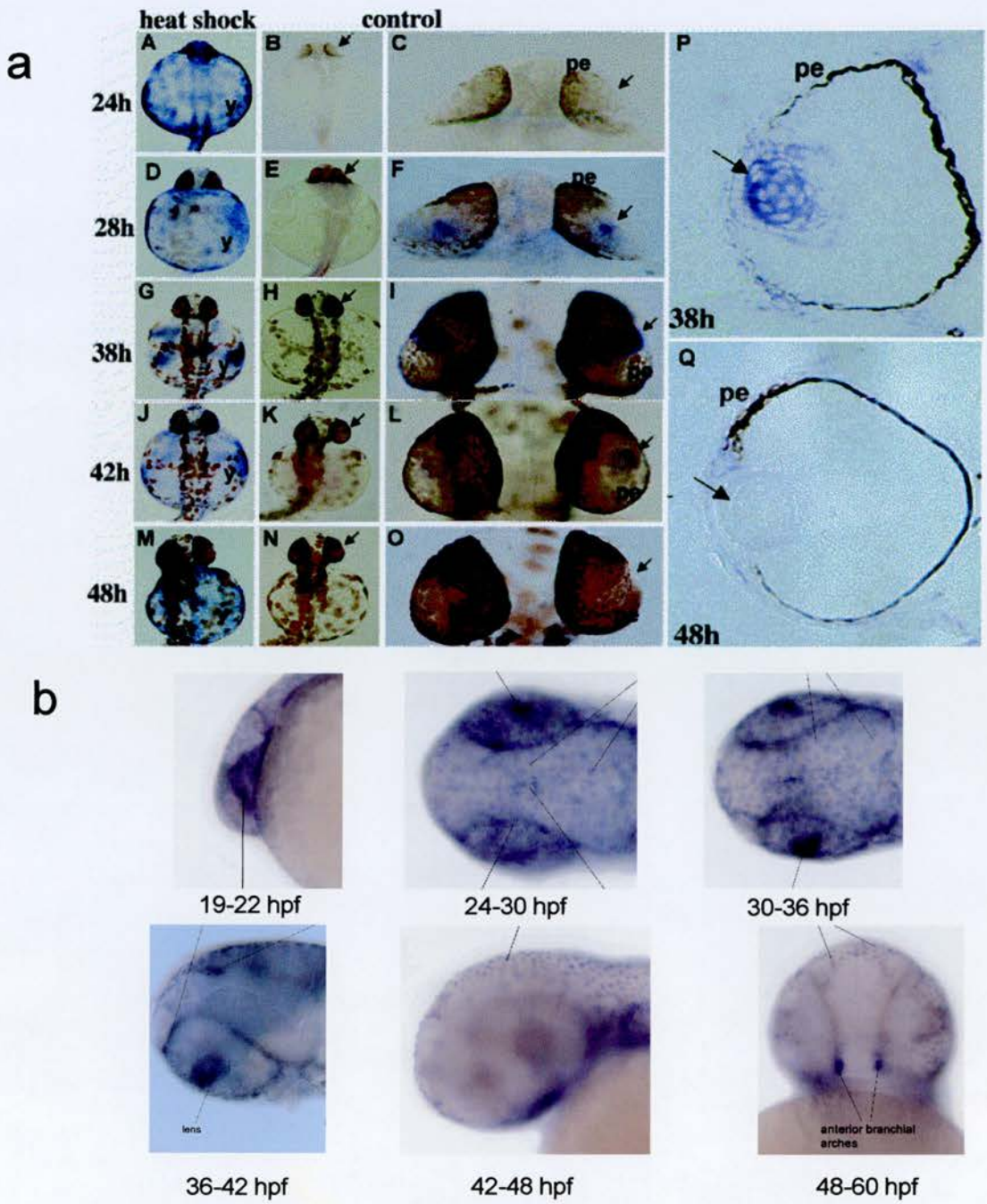


Figure 4.7: Expression of HSP mRNA during zebrafish development.
(a) Whole-mount *in-situ* hybridisation analysis of Hsp70, showing the expression pattern for heat shocked (first column) and control embryos (second two columns), and in sections of control embryos (P and Q). Hsp70 is expressed in the lens from 28-42 hpf under normal (control) conditions, and strongly expressed throughout the embryo after heat shock (Reproduced from Blechinger *et al.* 2002b); **(b)** Whole-mount *in-situ* hybridisation analysis of Hsp47, showing strong expression in the lens from 19-42 hpf. (Reproduced from Thisse *et al.* 2001).

Although Hsp70 and Hsp47 are only expressed in the lens, there is ample evidence that the lens is important for development of the retina and other structures of the eye (Kurita *et al.* 2003, Ashery-Padan *et al.* 2000, Collinson *et al.* 2001). These proteins may also explain the reduction in size of the pupil and retina in radicicol treated WT embryos. Work carried out by Auluck and Bonini (2002) in *Drosophila* has shown that even in the absence of a detectable increase in Hsp70 protein levels, there is evidence for Hsp70 protein activation after geldanamycin treatment. The slight induction observed in translation may therefore be indicative of a larger induction of Hsp70 activity.

Comparison of the *dre* and *sri* phenotypes in more detail however, shows that the effects of Hsp90 inhibition on *sri* is not a general effect on all lens mutants. Both *sri* and *dre* have an irregularity in the anterior part of the lens, and discolouration that is characteristic of cataracts (Figure 4.6). If activation of Hsp70 were the major factor in modulation of the *sri* phenotype by Hsp90 inhibition, it would be expected that *dre* should respond in the same way. Hsp90 inhibition, however, affects these two eye phenotypes in different ways. This shows that the response of mutant phenotypes to Hsp90 inhibition depends upon the specific gene that is mutated, and the developmental processes that are affected by it.

The difference in response of the *sri* and *dre* phenotypes to Hsp90 inhibition suggests several conclusions. The smaller difference caused in *dre* compared to *sri* shows that different mutations rely to differing extents on Hsp90 buffering. During Hsp90 inhibitor treatment, the *sri* phenotype is most likely aggravated by the disruption of a vital developmental process. In *dre* there may be a modifier that regulates the mutated protein, or a factor closely related to it, that is stabilised by Hsp90, and which worsens the mutant phenotype. When Hsp90 is inhibited, degradation or loss of function of this modifier may alleviate some of the damage caused by the mutated protein. An example of this type of mechanism was observed in *Drosophila* eye development.

In *Drosophila* the serine/threonine kinase Raf is required for the signalling pathway that activates the sevenless receptor tyrosine kinase. Sevenless activation is essential for the

development of R7 photoreceptors (Raabe, 2000). Raf interacts with Hsp83 (Hsp90), and mutations in *Hsp83* have been identified in two independent screens for modifiers of an R7 mutant phenotype (Cutforth and Rubin 1994, van der Straten *et al.* 1997). In one screen a mutant was used in which Raf was constitutively activated, causing an increase in the number of R7 photoreceptors. Dominant suppressor mutations were identified, including some hypomorphic alleles of *Hsp83* (van der Straten *et al.* 1997). In another screen, a hypomorphic allele of sevenless was used to identify dominant mutations that further reduced R7 photoreceptor development. In this screen *Hsp83* hypomorphic alleles also impaired signalling by sevenless, but had the opposite effect on R7 differentiation (Cutforth and Rubin 1994). These two experiments together give an elegant example of how a reduction in Hsp90 function can cause opposite effects on the developmental outcome of the same signalling pathway.

Raf signalling is also important in development of the retina and lens in mammals (Hecquet *et al.* 2002, Zatechka *et al.* 2002a, Zatechka *et al.* 2002b), and given the conservation in development observed between zebrafish and higher vertebrates, this is likely to be conserved in zebrafish. Raf may therefore be part of the mechanism involved in the effects of Hsp90 inhibitors on *sri* and *dre*.

These results show that Hsp90 is involved in modulating the expressivity of mutant phenotypes in vertebrates. This has important implications for the human developmental eye diseases with variable expressivity mentioned in Chapter 1.1 (p19). Individuals carrying mutations in genes such as *Pax2* or *Shh* may be affected by environmental factors during development that influence the function of Hsp90. Environmental factors affecting Hsp90 might include dietary intake and other lifestyle factors, or toxic chemicals. Serious eye defects could therefore be prevented by controlling these factors in families with a known history of inherited eye disorders.

Chapter 5

A Missense Mutation in *Pax6b* Causes the Eye Phenotype in *sunrise*

5.1 Introduction

The *sri* mutation was mapped in collaboration with Ralf Dahm and Robert Geisler (Max-Planck Institute for Developmental Biology, Tübingen, Germany). It was undertaken to allow a better understanding of the mechanism by which Hsp90 buffers the *sri* phenotype. It was expected that knowledge of the mutated protein would facilitate identification of factors up and down-stream in the developmental pathway, and show which Hsp90 clients might be important.

5.2 Linkage mapping of the *sri* mutation

sunrise was identified as part of an ENU mutagenesis screen for recessive mutations that affect specific aspects of organogenesis (Haffter *et al.* 1996). The *sri* mutation was mapped by linkage analysis. In this method, PCR analysis with polymorphic markers is carried out on groups of embryos showing the homozygous mutant phenotype, and their unaffected sibs. Primers are used that amplify regions containing microsatellites, or Simple Sequence Length Polymorphisms (SSLPs). SSLPs are CA repeats that vary in length (polymorphic) in populations, but have no deleterious effect. Variation in the length of these repeats can be visualised by agarose gel electrophoresis. In pools of embryos, the intensity of the different size bands relates to the frequency of each polymorphism in the population of embryos. Bands are identified that are more intense for the mutant pool. These markers are closer to the mutated site, with a lower frequency of separation from the mutation by meiotic recombination. This recombination frequency can translated into a genetic distance, known as the linkage distance, in centimorgans (cM) or centirays (cR) (Strachan and Read, 1996, Geisler, 2002). One centimorgan in zebrafish is roughly equivalent to 660 Kb, or approximately 16 cR (Geisler, 2002). Once a rough map position is obtained, single embryos can be used to map the mutation more accurately. The location of the markers on existing genetic maps can be used to locate candidate genes within this region, to be screened for mutations.

To ensure that the fish will be polymorphic for the markers used, the mutant strain to be mapped must be crossed to an unrelated wild-type strain that is relatively out-bred. *sri* homozygotes were crossed to the out-bred wild-type strain, WIK (Rauch *et al.* 1997, Nechiporuk *et al.* 1999, Table 3.3). The F1 was raised, and pools of F2 embryos showing the mutant phenotype and their WT sibs were collected and fixed, before shipping (Figure 5.1a). It was very important to identify the mutant and WT phenotypes correctly, so as to allow calculation of an accurate map position. It was particularly important to avoid scoring WT embryos as mutants (false positives). The variability of the *sri* phenotype made identification of mildly affected embryos difficult, and many embryos showing a very slight mutant phenotype were discarded. DNA extraction and linkage analysis was carried out by Ralf Dahm and Helia Schönthaler using markers for the Boston MGH map (Knapik *et al.* 1998).

The rough map position for the *sri* mutation was between 29.7 and 33.2 cM from the top of linkage group 7 on the MGH map (667 to 723 cR on the T51 radiation hybrid map), between markers z20963 and z11085. The map viewers on the ZFIN and NCBI websites were used to identify genes contained in this region, among which the most promising candidate was *Pax6b* (Figure 5.1b). Intronic primers for exons 1 and 6 of *Pax6b* were designed using the *Pax6b* genomic sequence from the Sanger Centre zebrafish whole-genome shotgun sequencing project (complied and annotated by Philippe Gautier). These were used by Ralf Dahm and Helia Schönthaler to map *Pax6b* onto the T51 radiation hybrid mapping panel that was used as a reference for the mapping markers. The map position of *Pax6b* was closest to the marker fc33g08 at 684 cR on linkage group 7 inside the region containing the *sri* mutation (this marker is not part of the *Pax6b* sequence).

5.3 Identification of a point mutation in the *sri* candidate gene *Pax6b*

Based on the rough map position and what is known about *Pax6* haploinsufficiency phenotypes (Chapter 5.6, p138), it was decided that *Pax6b* was an excellent candidate

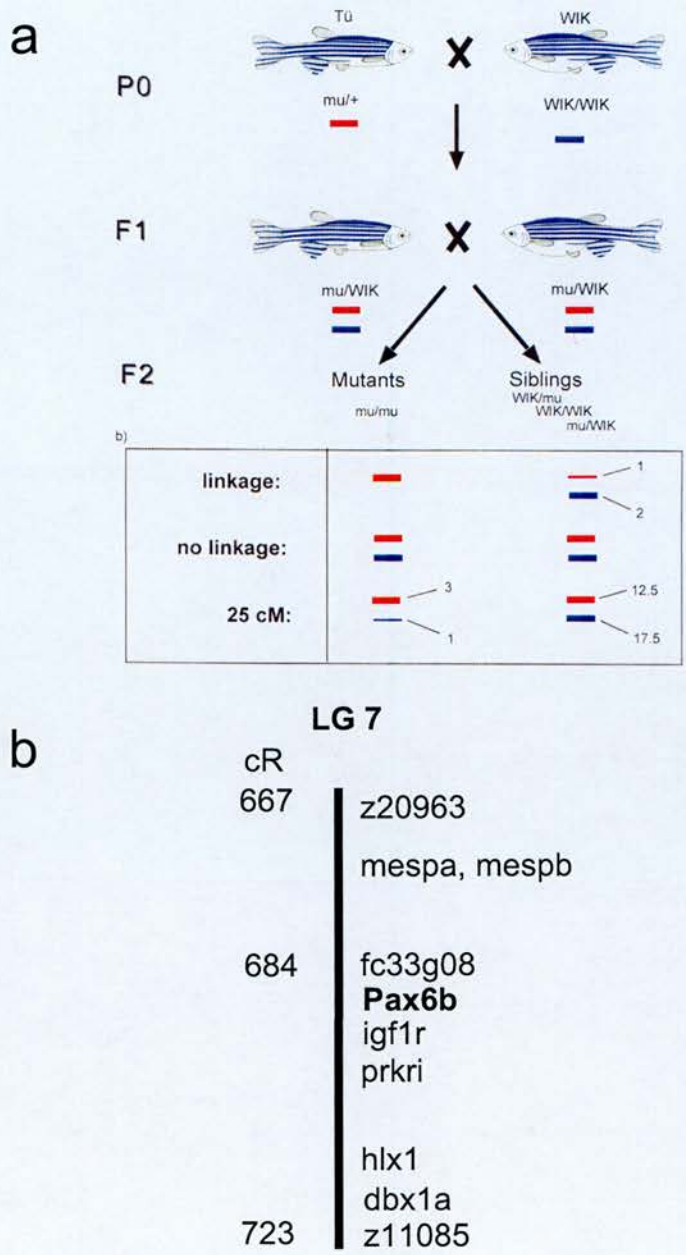


Figure 5.1: (a) Linkage mapping. Adults carrying the mutation are crossed to an unrelated WT strain. The F1 are crossed, and pools of mutant and unaffected sibs are collected. PCR is carried out with polymorphic markers. Markers that give bands which are more intense in the mutant than the sib pools are linked to the mutation (Reproduced from Geisler, 2002); **(b) Rough map location of the *sri* mutation.** Linkage group 7, showing the 56 cR (2.5 cM) region containing the *sri* mutation, and the position of *Pax6b* as confirmed by radiation hybrid mapping.

for the *sri* mutation. ENU usually causes point mutations, the most common of which are AT to TA transversions, and AT to GT transitions (Reviewed in Balling, 2001). These could cause missense or truncation mutations, and possibly splice-site defects. RT-PCR was carried out to identify any differences in the length of the cDNA. The PCR product for *sri* appeared to be the same length as the WT (data not shown). The coding region of *sri Pax6b* was then sequenced to identify any nucleotide changes that could be expected to disrupt the function of the protein.

The *Pax6b* cDNA from *sri* and WT embryos was RT-PCR amplified and sequenced in several sections. The sequence was compared to the published *Pax6b* cDNA (NCBI Genebank reference number NM_131641 at http://www.ncbi.nlm.nih.gov/, Nornes *et al.* 1998). Five changes were identified in the *sri* coding sequence, and four in the 3' untranslated region (UTR) (Figure 5.2, Table 5.1). Three of the nucleotide changes in the coding sequence were synonymous, causing no alteration in the amino acid sequence. These variants were also present in the WT sequence from Tü/AB/TL embryos (Table 3.3).

	Coding				3' UTR			
Variation	429 A>C	989 G>A	991 T>C	1304 A>C	1586 insCTT	1600 G>C	1611 C>G	1650 A>G
Published cDNA	A	G	T	A		G	C	A
sri	C	A	C	C	insCTT	G	G	G
WT	A/C	G/A	T	A/C	insCTT	G/C	C/G	A/G

Table 5.1: Nucleotide variations identified in WT and *sri Pax6b* cDNA, with respect to the published cDNA sequence NM_131641. The mutation is shown in red.

Polymorphisms are relatively rare in mammalian *Pax6*; nearly all nucleotide changes are deleterious (Reviewed in Prosser and van Heyningen, 1998). Only two synonymous changes have been reported in humans, both of which are outside the most conserved regions, the paired domain (PD) and homeodomain (HD) (Davis and Cowell, 1993, Brown *et al.* 1998, Grønskov *et al.* 1999, *Pax6* database at http://pax6.hgu.mrc.ac.uk). It

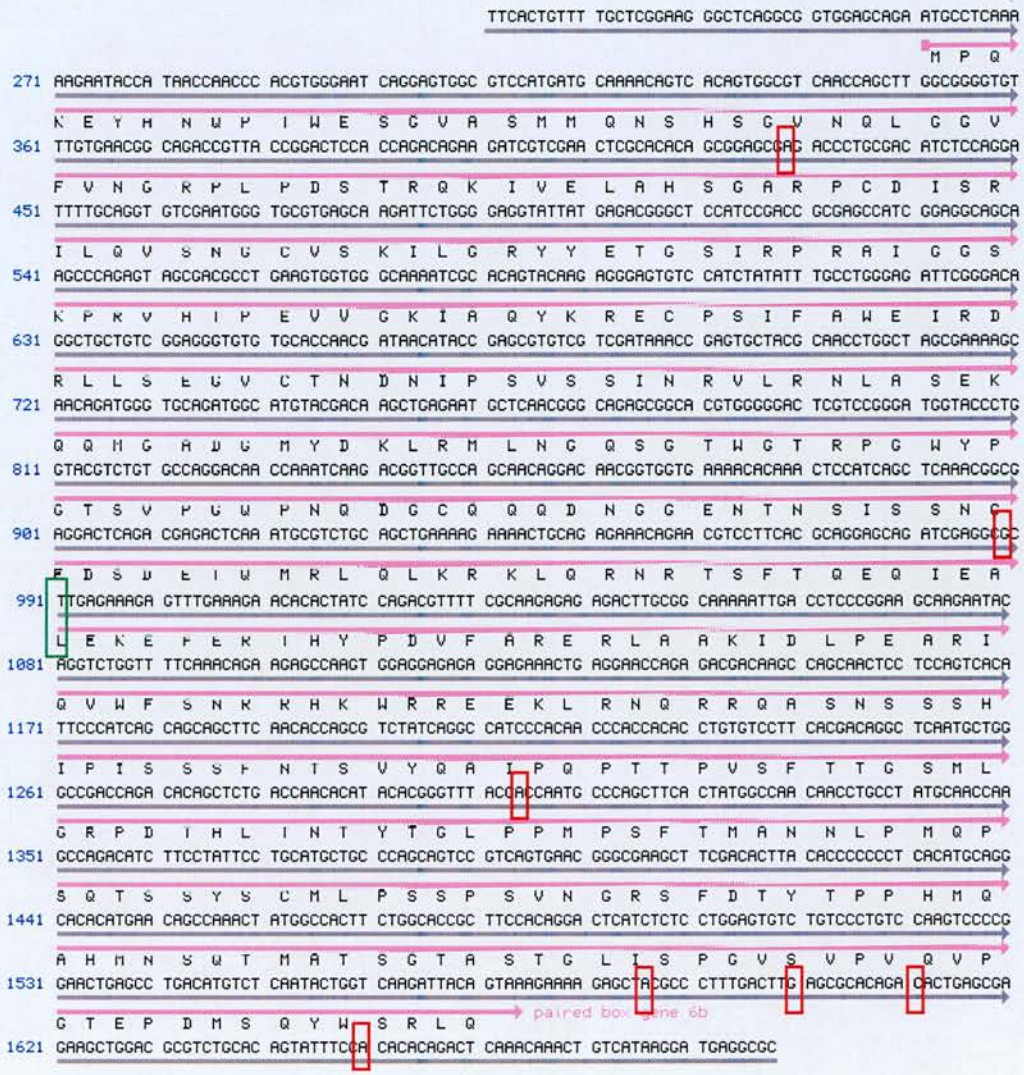


Figure 5.2: *Pax6b* cDNA, showing region amplified by RT-PCR and sequencing (NM_131641 Reproduced from www.ncbi.nlm.nih.gov). Sites of nucleotide polymorphisms are highlighted in red, and the missense mutation in green.

was therefore unexpected to find so many changes in *Pax6b*, especially as one is in the PD, and two are in the HD. The variants described in Table 5.1, as well as two others, were identified in the sequence released from the Sanger Institute (gi 31455425). This confirms that they are not merely sequencing errors on my part. The strain used for the published sequence may be different from those used for *sri* sequencing, and therefore contain different polymorphisms. The WT and *sri* embryos were on a Tü/AB/Tl background, and Tü and AB were used at the Sanger Institute, but the strain used by Nornes *et al.* for the cDNA sequence NM_131641 was not stated. It has been found when assembling the zebrafish genome sequence that the frequency of variants is at least 1/200bp (Sanger centre, 2003). The presence of two copies of many genes may allow sequence changes to occur without causing a reduction in fitness.

One non-synonymous change in the *sri* sequence was identified; a T to C transition in exon 8, at position 991 in the cDNA, that changes the amino acid leucine to a proline in the HD, and is therefore a missense mutation (Figure 5.3). This mutation was also identified when exon 8 was amplified from the genomic DNA. A G to A variant is almost immediately adjacent, at position 989, and the same haplotype is always observed for these two changes, as expected for two such closely linked variants.

The mutation 991 T>C creates a *DdeI* digestion site (Figure 5.4a). This was used to test whether the mutation was present at the expected frequency in individual embryos from a *sri* het x het mating. Genomic DNA was made from individual embryos, and exon 8 was PCR amplified and digested with *DdeI* (Figure 5.4b). The ratio of WT:heterozygous:homozygous did not deviate significantly from the expected frequency of 1:2:1, with a Chi square (Σ^2) value of 1.390, showing that the deviation observed is not significant, with a p value of 0.499 (Table 5.2). A band of approximately 70 bp was identified in WT and heterozygous digests. Sequencing identified two linked polymorphisms in intron 7 that create another *DdeI* digestion site, linked to the WT *Pax6b* allele (Figure 5.4c).

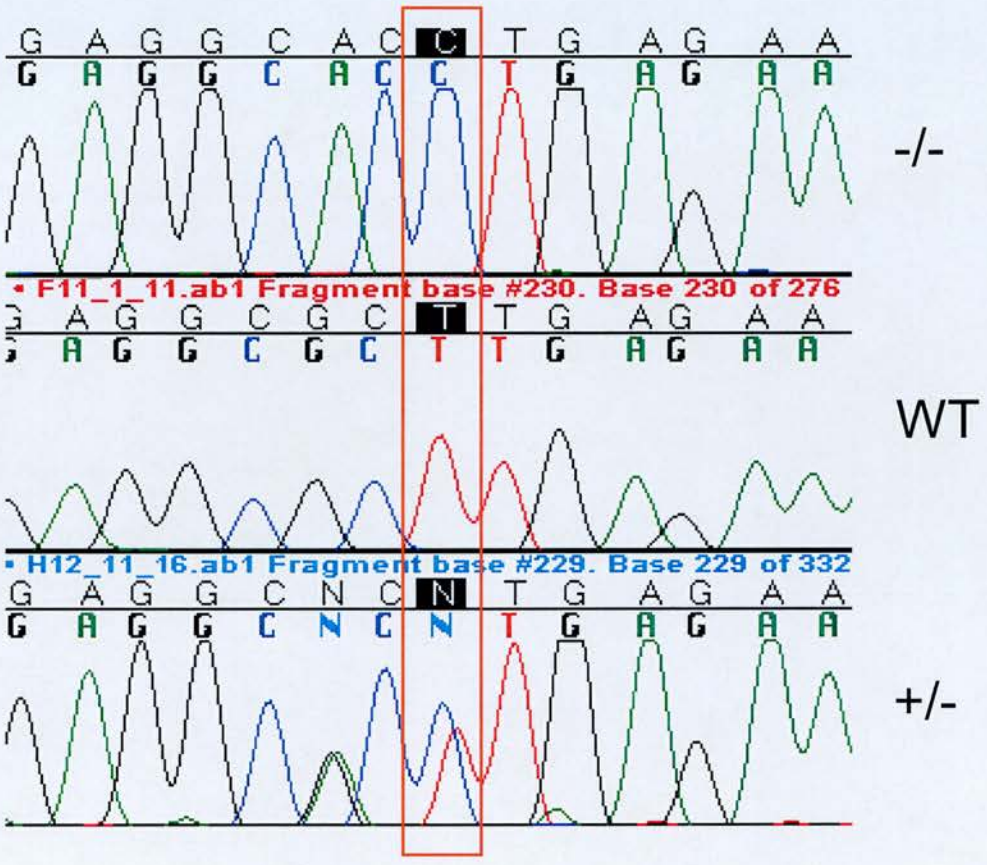


Figure 5.3: A missense mutation in *Pax6b* of *sri*. Sequencing traces, showing the T>C mutation in *Pax6b* genomic DNA from *sri* homozygous, WT, and heterozygous embryos.

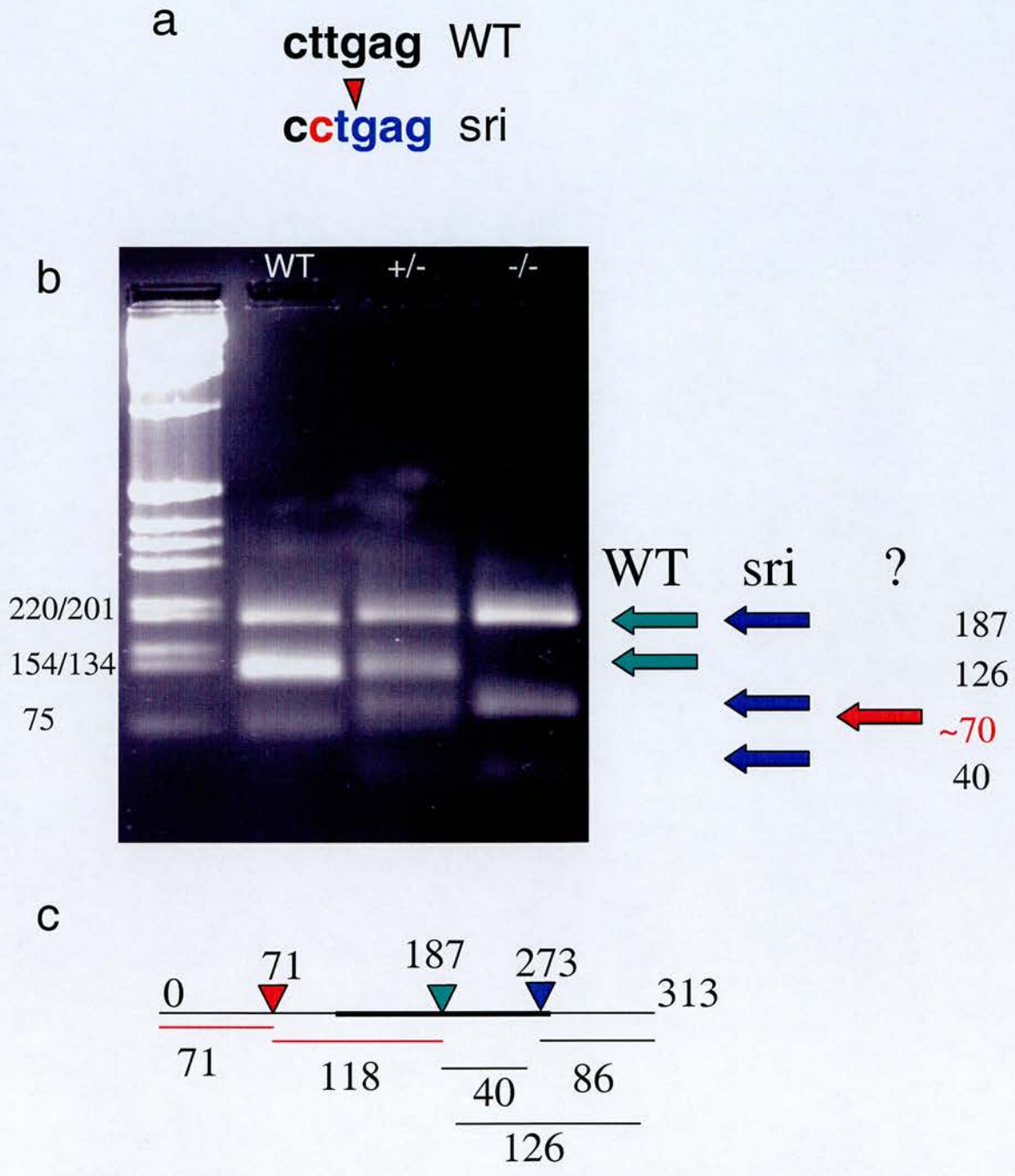


Figure 5.4: (a) A *DdeI* site (ctnag) is created by the 991 T>C mutation (triangle indicates position of incision; **(b) *DdeI* digest,** showing the difference in restriction sites caused by the *sri* mutation, and an additional band at 70 bp in the WT and heterozygote; **(c) Presence of polymorphisms in intron 7**, creating an additional *DdeI* restriction site (red). Thicker line represents exon.

	WT	Heterozygous	Homozygous
Observed	7	23	11
Expected	10.25	20.5	10.25

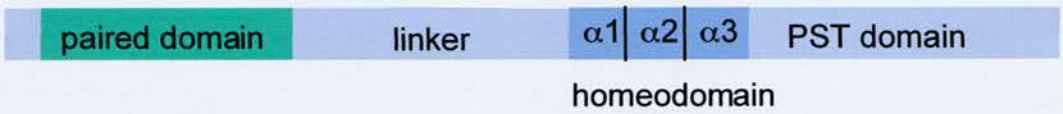
Table 5.2: Ratio of genotypes for *DdeI* digestion of het x het embryos.

5.4 Pax6 structure and function

Pax6b is an orthologue of *Pax6*, a member of the Pax transcription factor family, of which there are nine members in humans and mice. The Pax proteins are defined by the presence of a paired domain (PD), which is involved in sequence-specific DNA binding. The Pax proteins are involved in organogenesis, and mutations in these can cause severe morphological abnormalities (Reviewed in Chi and Epstein 2002, Pichaud and Desplan 2002) Studies of the *Pax6* mutants; *aniridia* in humans, *Small eye* (*Sey*) in mice, and *eyeless* (*ey*) in *Drosophila,* have provided a large body of information regarding the function of Pax6 in development (Simpson and Price 2002). Pax6 has been described as a "master-regulator" of eye development, largely due to its capacity to induce eye development when expressed in *Drosophila* imaginal discs (Halder *et al.* 1995).

Pax6 also contains a paired-like homeodomain (HD), which is linked to the PD by a glycine-rich linker, and a C-terminal transactivation domain, also known as the PST domain because it is rich in proline, serine and threonine (Figure 5.5a). The function of the Pax6 protein is complex, both the PD and HD are thought to be involved in DNA binding, as well as binding to other proteins (Planque *et al.* 2001), and have been shown to act cooperatively (Singh *et al.* 2000, Mishra *et al,* 2002). Regulation of *Pax6* gene expression is also very complex, with alternative splicing and multiple *cis*-regulatory elements (Carriere *et al.* 1993, Reviewed in van Heyningen and Williamson 2002). Notably, an alternatively spliced form exists, containing an additional exon 5a. The exon 5a containing form of Pax6 has altered DNA binding properties, and a mutation that increased the ratio of plus 5a to minus 5a transcription caused eye defects (Epstein *et al.* 1994). Extensive studies have been carried out to identify genes downstream of *Pax6*,

a



b

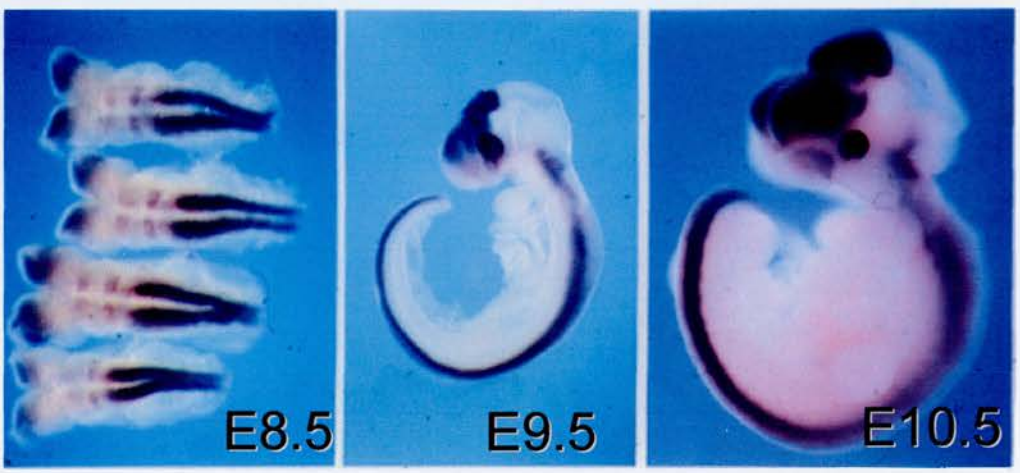


Figure 5.5: (a) Structure of the Pax6 protein; (b) Expression of *Pax6* mRNA in the developing mouse (Reproduced from P. Rashbass).

and have yielded a large and heterogeneous set of genes, many of which may be specific to eye development (Michaut *et al.* 2003, Chauhan *et al.* 2002, Chauhan *et al.* 2002b, Chauhan *et al.* 2002c, Bernier *et al.* 2001).

In the developing mouse, Pax6 is expressed in the eye, forebrain, olfactory bulb and neural tube, indicating an important role in the development of structures other than the eye (Hogan *et al.* 1988, Walther and Gruss, 1991, Grindley *et al.* 1995, Figure 5.5b). This is reflected in the olfactory bulb hypoplasia, and various subtle CNS defects observed in the *Sey* heterozygous mouse (Hill *et al.* 1991, Collinson *et al.* 2000, Collinson *et al.* 2003). Defects in olfaction and minor CNS defects have also been observed in humans heterozygous for Pax6 loss of function (Sisodiya *et al.* 2001).

In humans, homozygous loss of *Pax6* function results in a failure of eye development (anophthalmia) and other CNS defects that cause early neonatal lethality (Glaser *et al.* 1994). Unlike many proteins a reduction in dose (haploinsufficiency) also causes defects. Although aniridia is the most prominent defect in humans heterozygous for a *Pax6* null mutation, the eye phenotype caused is extremely heterogeneous, and can include cataracts, foveal dysplasia, coloboma, lens dislocation, optic nerve hypoplasia, nystagmus and glaucoma (Jordan *et al.* 1992, Online Mendelian In Man record #106210 at http://www.ncbi.nlm.nih.gov/omim). In mouse *Sey* heterozygotes, as well as reduced eye size, a variety of defects occur, including coloboma, Peters' anomaly, cataract, and failure of the eyelids to fuse before birth (Hill *et al.* 1991, Collinson *et al.* 2001, Jordan 1992, Thaung *et al.* 2002). The homozygotes do not develop eyes or nasal structures, and newborns die because they cannot feed (Hill *et al.* 1991). It is interesting to note that overexpression of Pax6 in mice also causes severe eye defects, indicating that eye development is extremely sensitive to *Pax6* dosage (Schedl *et al.* 1996).

Drosophila ey heterozygotes also have reduced eyes, showing that *Pax6* gene dosage is also important in flies (Quiring *et al.* 1994). This is significant, as gene dosage requirements are not always conserved in different model systems. For example, human

anophthalmia can be caused by heterozygous loss of function of the HMG box transcription factor *SOX2* (Fantes *et al.* 2003). In the mouse, however, animals heterozygous for a *Sox2* knockout have normal eyes (Avilion *et al.* 2003). Conservation of the *Pax6* haploinsufficiency phenotype from *Drosophila* to humans is an important clue to the mechanism by which Pax6 regulates eye development. There is evidence that a threshold level of Pax6 protein is required for normal development to proceed (van Raamsdonk and Tilghman, 2000).

There are two orthologs of *Pax6* in zebrafish, termed *Pax6a* and *Pax6b*, or *Pax6.1* and *Pax6.2*. These are thought to originate from a genome duplication that occurred in the zebrafish lineage (Taylor *et al.* 2003). *Pax6a* and *b* are 82% identical at the cDNA level and 95% identical at the amino acid level, while the HD and PD are 100% identical (Nornes *et al.* 1998). The combined expression pattern of these two genes is very similar to that observed in the mouse for *Pax6* (Krauss *et al.* 1991, Krauss *et al.* 1991b, Püschel *et al.* 1992, Macdonald *et al.* 1994, Amirthalingam *et al.* 1995, Thisse *et al.* 2001). Pax6a is strongly expressed in the eyes, forebrain, hindbrain, and neural tube, while Pax6b is only expressed in the eyes, dorsal diencephalon, pancreas, and part of the neural tube (Figure 5.6). Only Pax6b is expressed in the pancreas (Biemar *et al.* 2001) and early lens placode (Nornes *et al.* 1998). The Pax6b protein has been shown to have a higher transactivation activity than Pax6a (Nornes *et al.* 1998). This shows that, although it is likely that they share some functions, the *Pax6a* and *Pax6b* genes are not completely redundant.

Further evidence for divergence of function between the two *Pax6* genes in zebrafish comes from studies of the genomic organisation (Dirk Kleinjan and Philippe Gautier). The exon boundaries in the coding region are conserved, including the alternatively spliced exon 5a. The intronic sequence is highly divergent, apart from several distinct regions that share sequence similarity with the tissue-specific regulatory elements identified in the mouse. (Kleinjan *et al.* 2001, Reviewed in van Heyningen and Williamson, 2002, Griffin *et al.* 2002). Some of these regulatory regions are shared

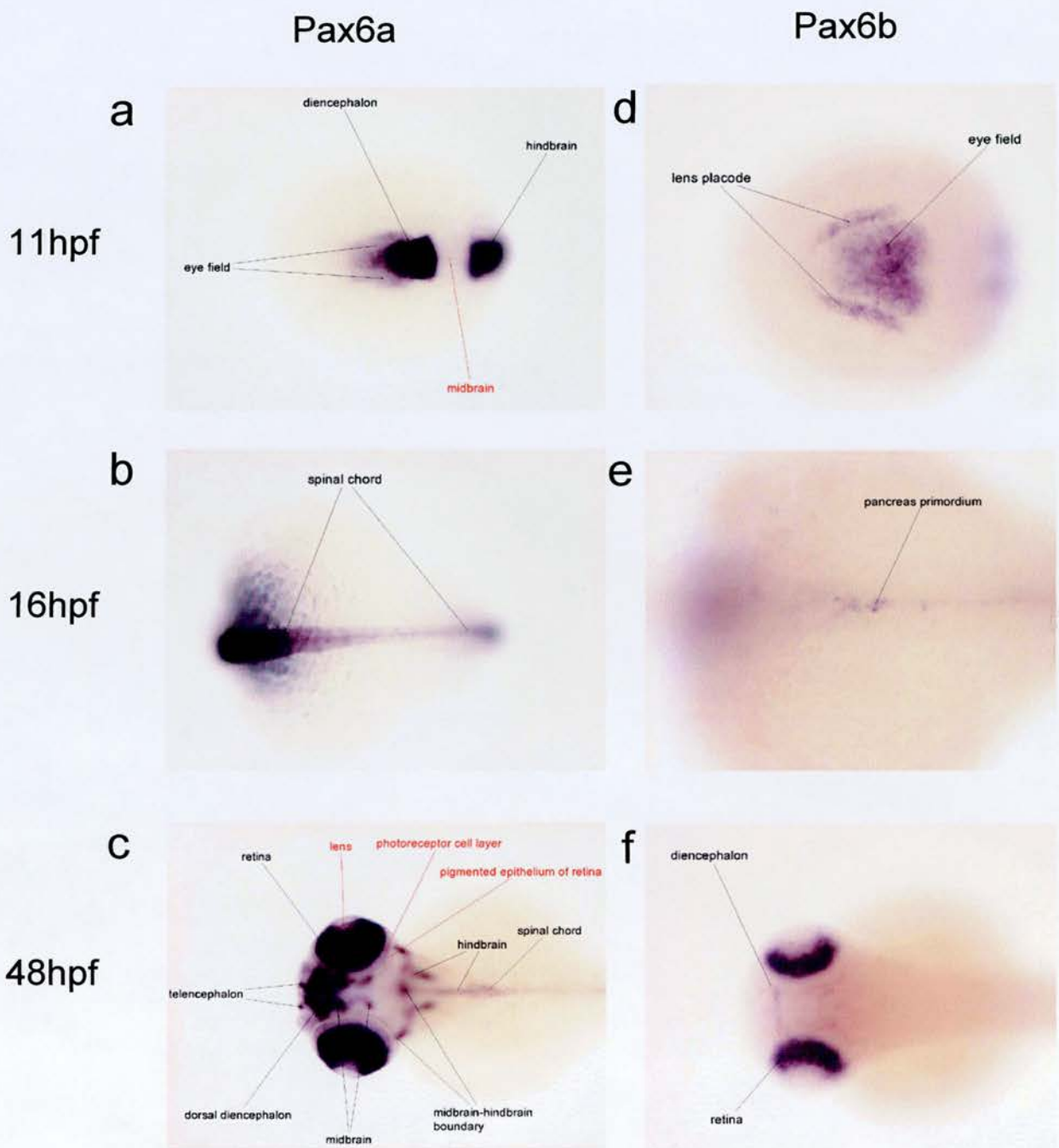


Figure 5.6: Expression of *Pax6* mRNA in zebrafish embryos. (a-c) *Pax6a;* (d-e) *Pax6b* (Reproduced from Thisse *et al.* 2001).

between the zebrafish *Pax6* genes, while others are only present in one of the homologs, indicating that the observed differences in expression may result largely from alterations in regulatory sequence.

Mutations in zebrafish *Pax6a* or *b* have not been reported, despite several screens for mutations that affect eye development (Haffter *et al.* 1996, Driever *et al.* 1996, Fadool *et al.* 1997, Vihtelic *et al.* 2002). It may be that *Pax6a* and *Pax6b* have sufficiently diverged in function so that neither can compensate well for the loss of the other. Complete loss of function mutations, and perhaps most point mutations, may cause very severe phenotypes with dominant or dominant negative inheritance. Mutations of either type are often ignored in mutagenesis screens (Haffter *et al.* 1996). Further evidence for a very early severe phenotype for zebrafish *Pax6* null mutations, comes from a study of gene silencing by double-stranded RNA (dsRNA) injection. Injection of dsRNA for *Pax6a* caused very severe eye and forebrain defects (Li *et al.* 2000).

There may also be regulatory interactions between the two zebrafish *Pax6* genes, similar to the *Pax6* autoregulatory function seen in organisms carrying only one *Pax6* gene (Plaza *et al.* 1993, Grindley *et al.* 1995, Kleinjan *et al.* 2003). Overlapping regulation of *Pax6a* and *b* would amplify the phenotypic effects of a single knockout by affecting the expression of the other *Pax6* orthologue.

5.5 The L224P mutation in *Pax6b* of *sri* is predicted to disrupt protein function

The L224P missense mutation is expected to have a deleterious effect on the function of the Pax6b protein for several reasons. Amino acid 224 is in the first alpha helix of the HD (Figure 5.7a). Homeodomains consists of three alpha helices. Where DNA-binding has been studied, Helix III and the N-terminal arm connect with the major groove of DNA in a sequence-specific manner, while the loop between helices I and II contacts the DNA backbone (Reviewed in D'Elia *et al.* 2001, Figure 5.7b). Any mutation that

a

b

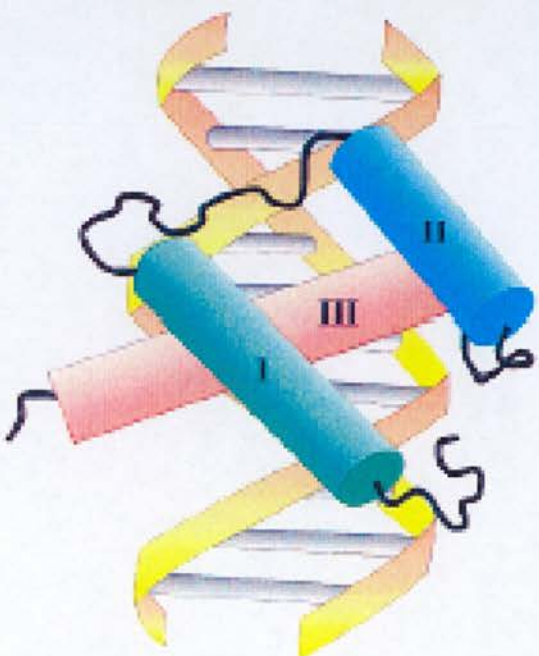


Figure 5.7: (a) Rasmol diagram of the *Drosophila* homeodomain homodimer on DNA. Arrows indicate the lysine that is affected by the *sri* mutation (Philippe Gautier); **(b) Binding of the HD to DNA** (Reproduced from D'Elia *et al.* 2001).

damages the first alpha helix would probably destroy the DNA binding properties of the HD. The L224P mutation is expected to disrupt the first helix of the HD, as proline lacks the ability to form the correct bonds to form an alpha helix, earning it the label of "helix breaker" (Chakrabarti and Chakrabarti 1998). Also L224 is conserved in Pax6 of all species identified to date, and is one of the most highly conserved amino acids in HD containing proteins (analysis by Philippe Gautier). Out of 185 HD proteins analysed, only 11 of the least related proteins have do not have leucine at this position, and none of the substitutions was for a proline. Of the 60 amino acids in the HD, three showed complete conservation, 5 were more conserved than L224, and 54 were less conserved. This amino acid must therefore be very important for HD function.

Missense mutations in *Pax6* are relatively rare (54/302 in the *Pax6* mutation database). This is thought to be due to ascertainment bias, as the phenotype caused by missense mutations is usually distinct from classical aniridia and more heterogeneous than those observed for loss of function mutations (Hanson *et al.* 1993, Azuma *et al.* 2003, Table 5.3). Most of the missense mutations reported are in the PD and PST domain, with only five in the HD (Table 5.3), possibly because the HD is so important for *Pax6* function that most mutations are lethal. It could also be that the phenotype for missense mutations in the HD is not usually in the eye. It is unlikely that no phenotype is caused by amino acid changes in the HD, as it is completely conserved, with no polymorphisms reported to date in mammals.

Mutation	Domain	Phenotype	Species	Reference
R242T	HD, 2nd helix	Partial aniridia, mother unaffected carrier	human	Morrison *et al.* 2002
Q255H	HD, 3rd helix	Aniridia, possibly spicing defect	human	Chao *et al.* 2000
V256E	HD, 3rd helix	Cataracts, mild microphthalmia	mouse	Thaung *et al.* 2002
F258S	HD, 3rd helix	Optic nerve coloboma, growth and mental retardation	human	Azuma *et al.* 2003
S259P	HD, 3rd helix	Slightly reduced eye size, irregular pupil	mouse	Favor *et al.* 2001

Table 5.3: *Pax6* missense mutations in mammals. Amino acid number refers to the human *Pax6* (Genebank reference M93650 at http://www.ncbi.nlm.nih.gov/, Glaser *et al.* 1992).

Although mutations affecting L224 have not been reported for *Pax6*, this amino acid is mutated in two other diseases involving HD containing proteins (Reviewed in D'Elia *et al.* 2001, Figure 5.8). Reiger syndrome is an autosomal dominant disorder characterised by glaucoma, dental hypoplasia and umbilical defects, and is caused by mutations in the paired-class homeobox protein Pitx2 (Paired-like homeodomain transcription facotor 2). One family has been identified with a missense mutation that causes the substitution of glutamine (Q) for leucine. This leucine is at the same position in the first alpha helix of the homeodomain as L224 (Semina *et al.* 1996). Dyschondrosteosis is a skeletal dysplasia syndrome caused by mutations in another paired-class homeobox protein SHOX (Short Stature Homeobox). A patient was identified with a missense mutation causing the substitution L132V (Grigelioniene *et al.* 2000). This substitution is actually conservative, as valine shares several of the biochemical properties of leucine, such as polarity and hydrophobicity (Stryer, 1995), but is still causative for the disease. As the mutation in *sri* is predicted to cause a larger disruption in protein structure than either of these two changes, it is expected that L224P is causative for the *sri* phenotype.

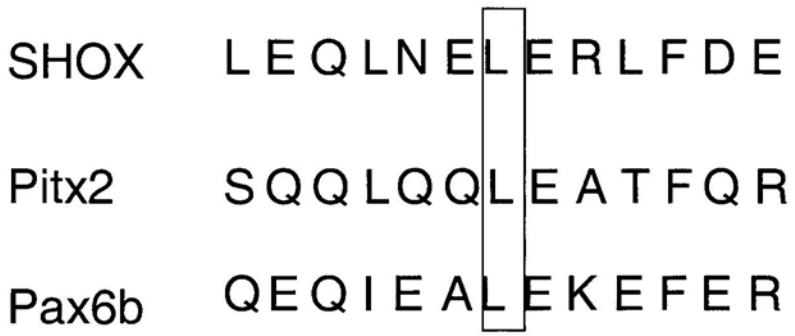


Figure 5.8: Homeodomain proteins, showing the leucine that causes developmental defects when mutated (D'Elia *et al.* 2001). Part of the first helix of the HD is shown. Amino acids shared between all three proteins are shown in red. The leucine that is mutated is highlighted in green.

It is difficult to predict the functional consequences of this mutation, as Pax6 protein function is extremely complex. It is reasonable to assume that at the very least, the function of the HD is impaired. It is known that the paired and homeobox DNA binding domains can act either independently or in cooperation, and that the PD can affect the DNA-binding properties of the HD (Singh *et al.* 2000, Mishra *et al.* 2002). There is no evidence, however, for any direct influence of the HD on PD function, so that the PD in *sri* may be able to function normally. The HD has been shown to be dispensable for eye formation in *Drosophila* (Punzo *et al.* 2001), although this may be compensated for by heterodimerisation (Jun and Desplan, 1996, Mikkola *et al.* 2001) with other Pax homologues (Jang *et al.* 2003). Loss of Pax6 HD function in humans results in aniridia (Azuma and Yamada, 1998, Singh *et al.* 2001). Conversely in quail and *C. elegans* alternatively spliced forms of Pax6 have been identified that lack the PD (Carriere *et al.* 1993, Zhang and Emmons, 1995). The PD-less isoform is still able to interact with other HD proteins such as Chx10 and HoxB1 (homeo box B1) (Mikkola *et al.* 2001). Also there is enough evidence to show that the HD can interact with other proteins, and enhance PD function (Planque *et al.* 2001). Consequently the severity of the effects of losing Pax6b HD function may vary, depending on the specific interactions of Pax6 in different tissues.

5.6 The *sri* phenotype resembles *Pax6* haploinsufficiency phenotypes in mammals

Corroboration for the causative nature of the Pax6b L224 mutation in *sri* comes from detailed examination of the eye phenotype. Nearly all aspects of the *sri* homozygous mutant phenotype are observed in *Sey* heterozygous mice. The reason for the mildness of the *sri* homozygous phenotype must lie either in the effects of the missense mutation on Pax6 protein function, or in the presence of the Pax6a orthologue.

The lens is obviously smaller in both 24h *sri* larvae and *Sey* mice (Figure 5.9a,b) (van Raamsdonk and Tilghman, 2000). A delay in the development of pigmentation in the retinal pigmented epithelium (RPE) can be observed in 72-80 hpf *sri* embryos, suggestive of a delay in differentiation of the retina (Easter and Malicki 2002, Figure 5.9c,d). In studies of mouse *Pax6* -/- chimaeras it was shown that a reduction in *Pax6* gene dosage also delays differentiation of the RPE (Collinson *et al.* 2003). The optic fissure is closed by 72 hpf (2 dpf) in WT zebrafish embryos, whereas in *sri* a fissure below the pupil is still present in 2-3% of embryos at 3 dpf, and recovers in some individuals by 5 dpf (Figure 5.10a,b). Non-closure of the optic fissure, or coloboma, is often found in *Pax6* +/- mice, and has been observed in humans heterozygous for *Pax6* missense mutations (Hill *et al.* 1991, Morrison *et al.* 2002, Chao *et al.* 2003).

During eye development in both zebrafish and mammals, the lens develops from the surface ectoderm, and eventually detaches from it. The zebrafish lens detaches from the cornea by 24 hpf (Easter and Malicki 2002), but in 5 dpf *sri* embryos, an attachment of the lens to the cornea is still visible (Figure 5.10c,d). This defect, known as Peters' anomaly, is also observed in humans heterozygous for *Pax6* missense mutations (Hanson *et al.* 1994), and in *Pax6* +/- mice (Hanson *et al.* 1994, Collinson *et al.* 2001). Irregularity of the cornea is a prominent feature of the *sri* phenotype. Defects in the cornea are also found in *Sey* mice and human *aniridia* (Jordan *et al.* 1992, Collinson *et al.* 2001, Collinson *et al.* 2003). Sections show that the lens in both *sri* and *dre* is

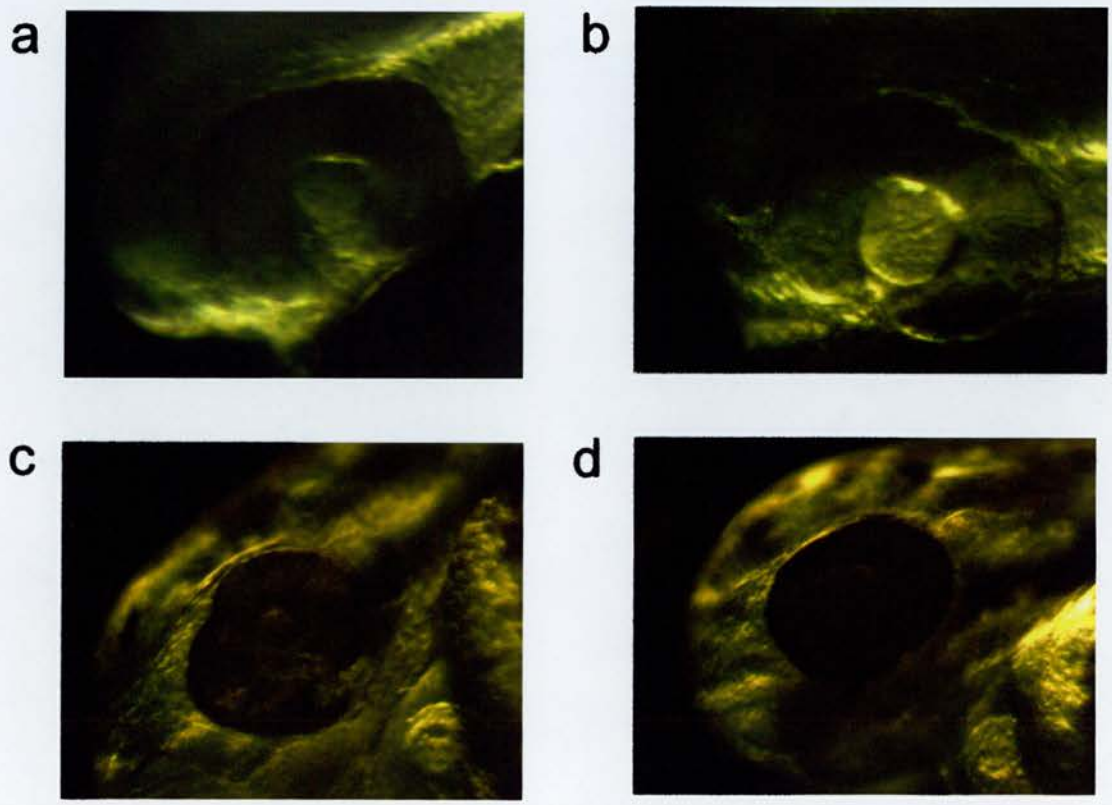


Figure 5.9: Eye defects observed in *sri* embryos. (a) At 24 hpf the lens is reduced; **(b)** WT at 24 hpf; **(c)** Development of pigmentation in the RPE is reduced at 72-80 hpf; **(d)** WT at 72-80 hpf.

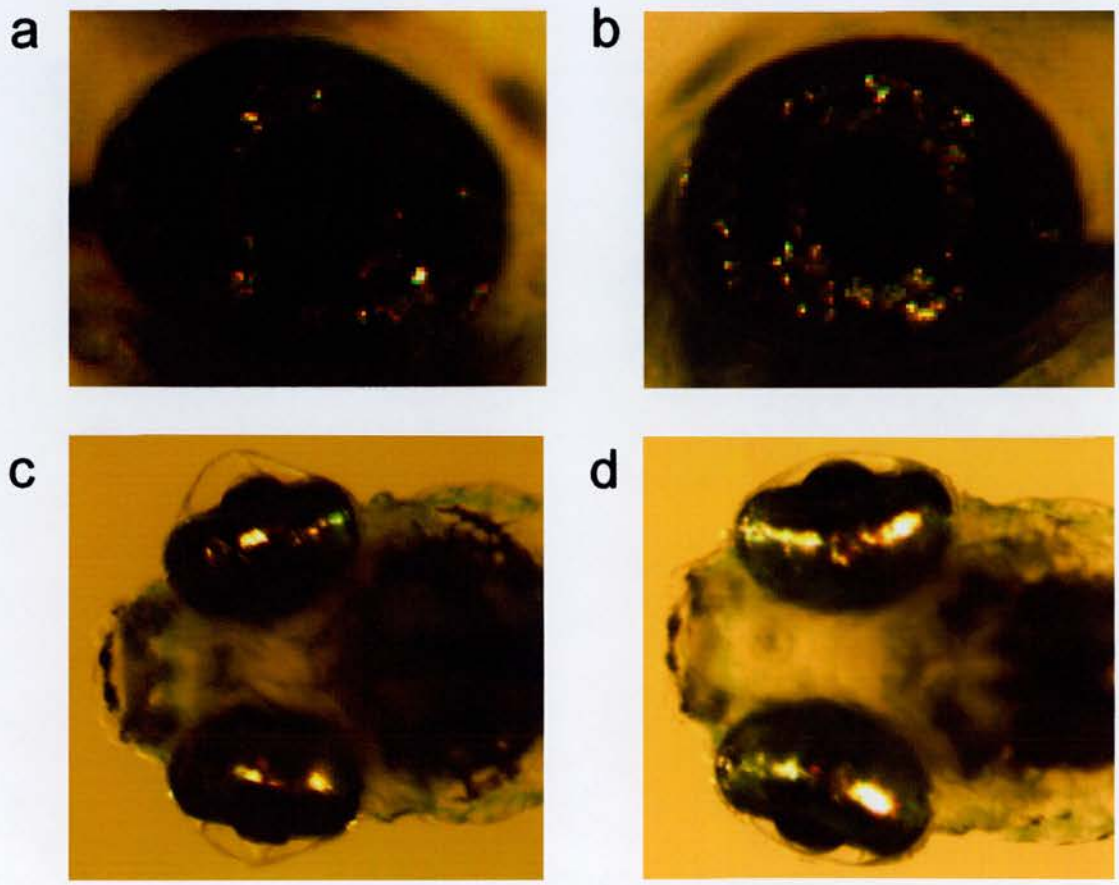


Figure 5.10: Eye defects observed in *sri* embryos. (a) Delayed closure of the optic fissure at 3 dpf; **(b)** WT at 3 dpf; **(c)** Peters' anomaly at 5 dpf; **(d)** WT at 5 dpf.

disorganised towards the anterior, and there is some discolouration at the centre, indicative of a cataract (Figure 4.6). Cataracts are also a common feature of human and mouse *Pax6* heterozygotes (Hanson *et al.* 1994, Hill *et al.* 1991).

The *sri* eye phenotype is most likely to be caused by a reduction in size of the lens, as defects in the cornea and size of the eye can be corrected by rescue of the lens defect in *Pax6* +/- mice (Collinson *et al.* 2001). Sections of the eyes of larvae show that the retina is disorganised, but all the cell types appear to be present in their usual location (Figure 5.11). This disorganisation may be the result of a reduction of pressure in the vitreous fluid caused by lens hypoplasia (Coulombre and Coulombre, 1964). There is no evidence for the aniridia that is a feature of human *Pax6* haploinsufficiency (Hill *et al.* 1991, Prosser and van Heyningen 1998).

A general reduction in the severity of the *sri* phenotype is sometimes observed between 3 and 5 dpf (5/24 with an obvious improvement), indicating that the delay in optic fissure closure may be characteristic of a more general delay in eye development, perhaps due to a delay in lens development (van Raamsdonk and Tilghman 2000). Surviving adults appear to have normal vision, and sections of the eyes (Allyson Ross) show no difference compared to the WT (Figure 5.12).

5.7 The pancreatic islet is affected in *sri* mutant embryos

It has been mentioned that Pax6b is expressed in the pancreas, without Pax6a. Work in mice has shown that Pax6 is required for differentiation of the glucagon producing alpha cells. In mice lacking Pax6 the pancreatic islet was disorganised, and almost entirely lacking in alpha cells, with reduced hormone production in all other islet cells (Sander *et al.* 1997, St-Onge *et al.* 1997). Pax6 has been shown to bind to promoter elements of the glucagon, insulin and somatostatin genes (Sander *et al.* 1997). Studies in humans with aniridia, carrying *Pax6* loss of function mutations, may reveal slight intolerance to glucose (Yasuda *et al.* 2002).

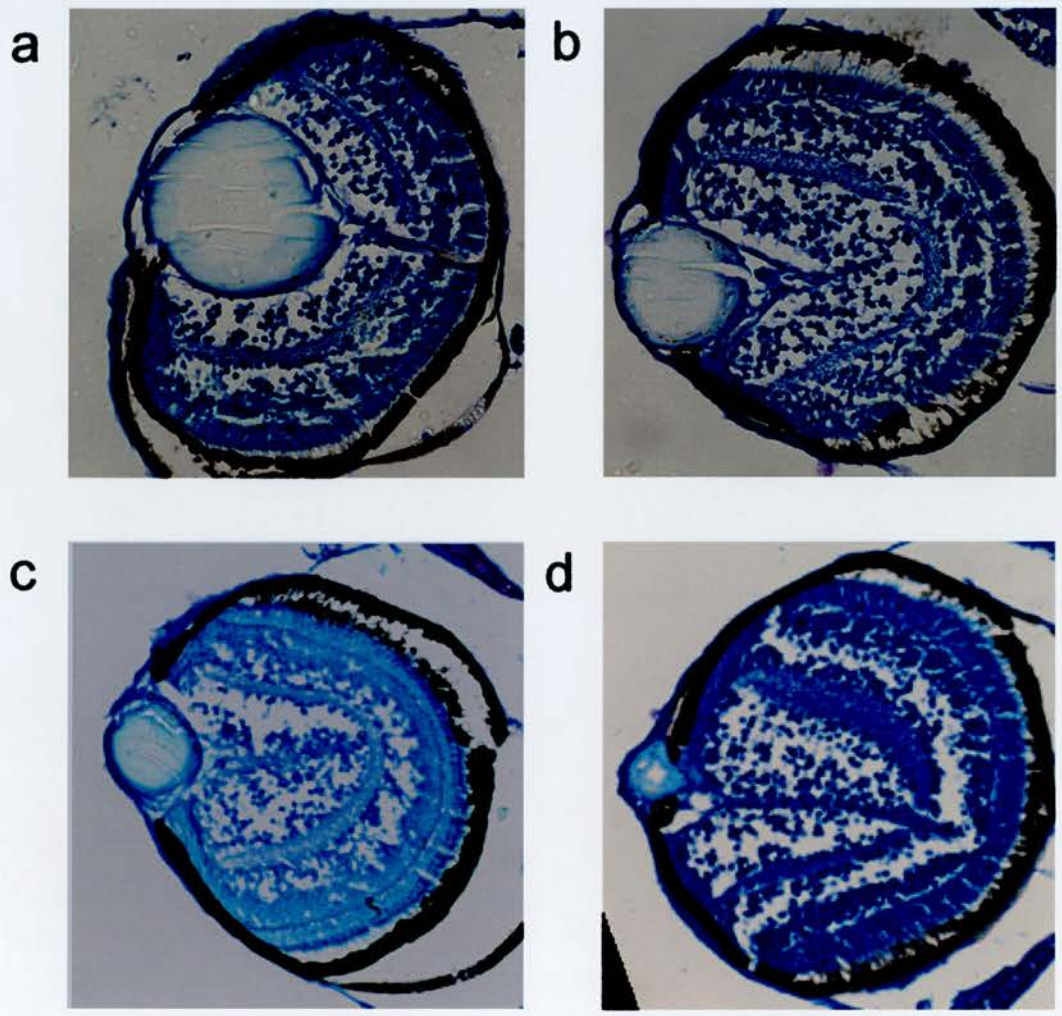


Figure 5.11: Variability of the sri phenotype. (a) WT; **(b)** Mild; **(c)** Intermediate; **(d)** Severe.

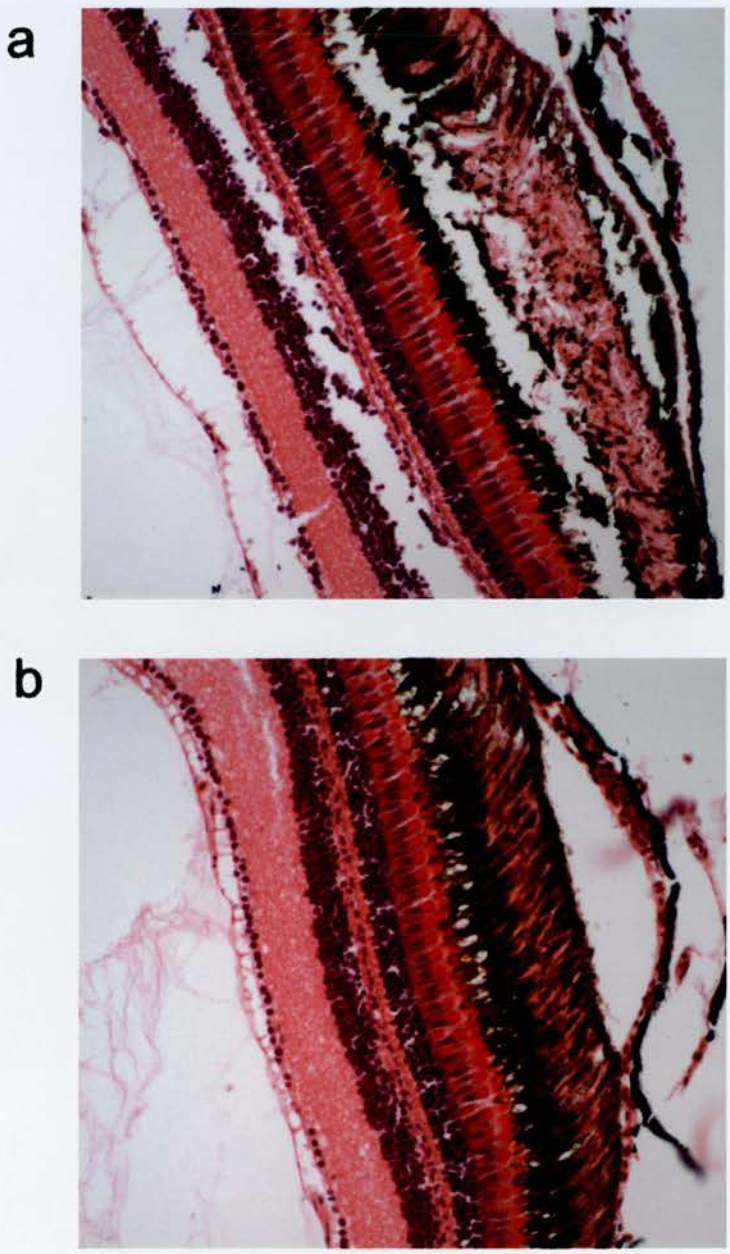


Figure 5.12: (a) Adult *sri* eyes are unaffected; (b) WT.

The zebrafish pancreas contains only one islet of Langerhans, which has the same structure as that seen in mammals, with alpha cells around the outer edge and insulin producing beta cells inside (Argenton *et al.* 1999). Pax6a is not expressed during zebrafish pancreas development, but Pax6b is expressed in the pancreas from 12 somites (approximately 15 hpf) until at least 24 hpf (Biemar *et al.* 2001). Therefore it was expected that loss of *Pax6b* function in *sri* could have a similar effect on pancreas development to that seen in the mouse.

Paraffin wax sections from 5dpf WT and *sri* -/- embryos were stained for glucagon and insulin. The islet of Langerhans was clearly visible by glucagon and insulin staining on the WT (Figure 5.13). The islet can also be clearly recognised by its distinct morphology, as a clump of cells slightly separated from, but inside the pancreas. Both the alpha and beta cell populations were disrupted in *sri* homozygotes, regardless of the severity of the eye phenotype (6 embryos examined). Disruption of this Pax6b expressing cell population in *sri* homozygotes is good evidence that the *sri* phenotype is caused by the L224P mutation, and that this mutation destroys the function of Pax6 in the pancreas, causing a null phenotype.

It is surprising that the *sri* homozygotes should survive, if they lack a pancreatic islet. There may be several factors that allow relatively normal glucose homeostasis to occur in these fish. Strong glucagon staining was observed in oesophagus of every *sri* embryo examined, and the stomach of some (Figure 5.14a,b). The glucagon antibody used is known to cross-react with enteroglucagon in the stomach (Witt *et al.* 1988, Sigma, 2002), so that up-regulation of enteroglucagon expression may partially rescue the absence of glucagon. Morphology of the pancreatic islet was occasionally preserved (in 2/3 embryos examined), and some faint insulin staining was observed in some of the *sri* embryos, (Figure 5.14c) suggesting that only the alpha cells are completely absent, as seen in the *Pax6* -/- mouse. In a fraction of the *sri* homozygotes, enough insulin

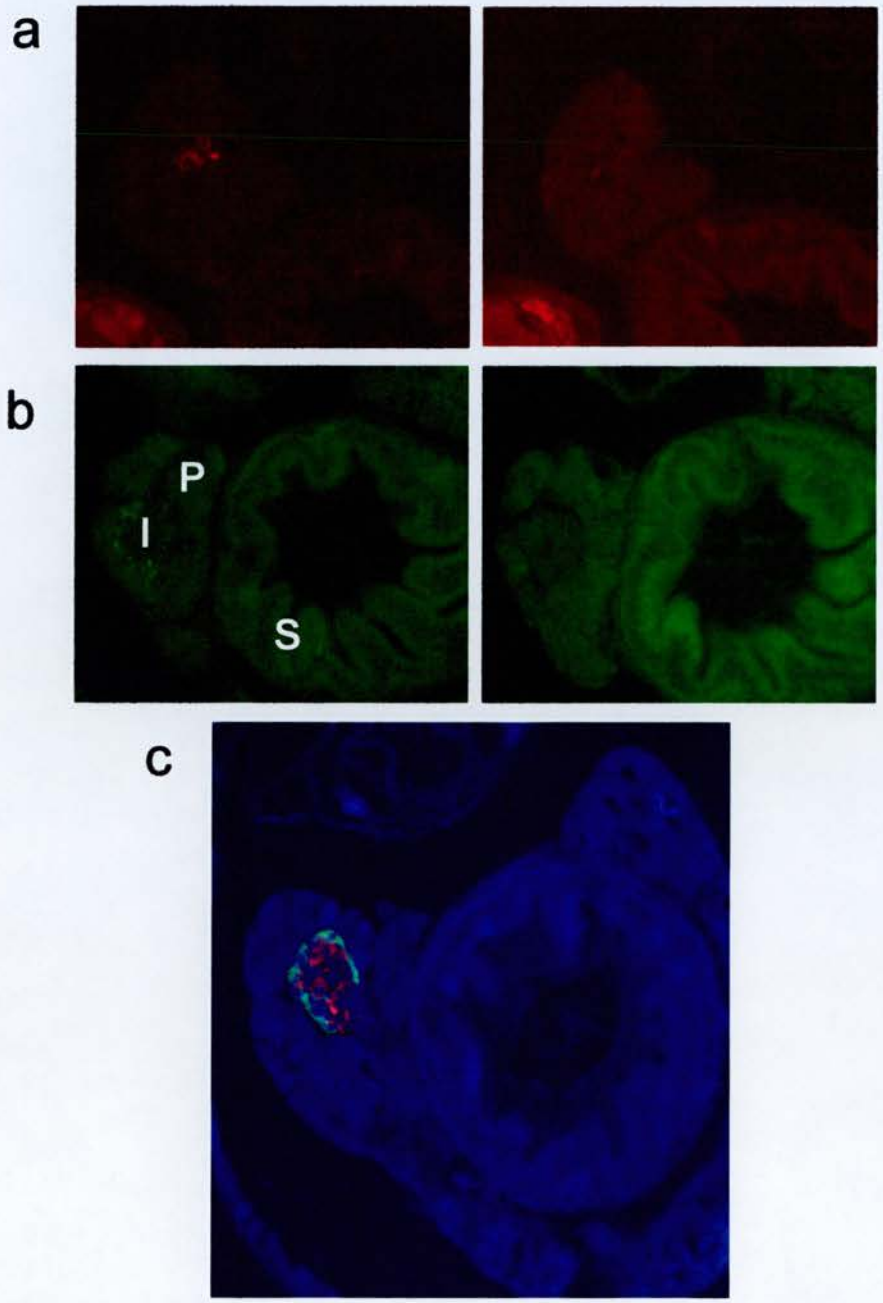


Figure 5.13: Markers of the pancreatic islet of Langerhans in WT embryos. (a) Insulin; **(b)** Glucagon. Right-hand panels show staining with secondary antibody only. (c) Double staining. Green, glucagon; Red, insulin; Blue, autofluorescence. 5 dpf zebrafish embryos. P, exocrine pancreas; I, islet of Langerhans; S. stomach.

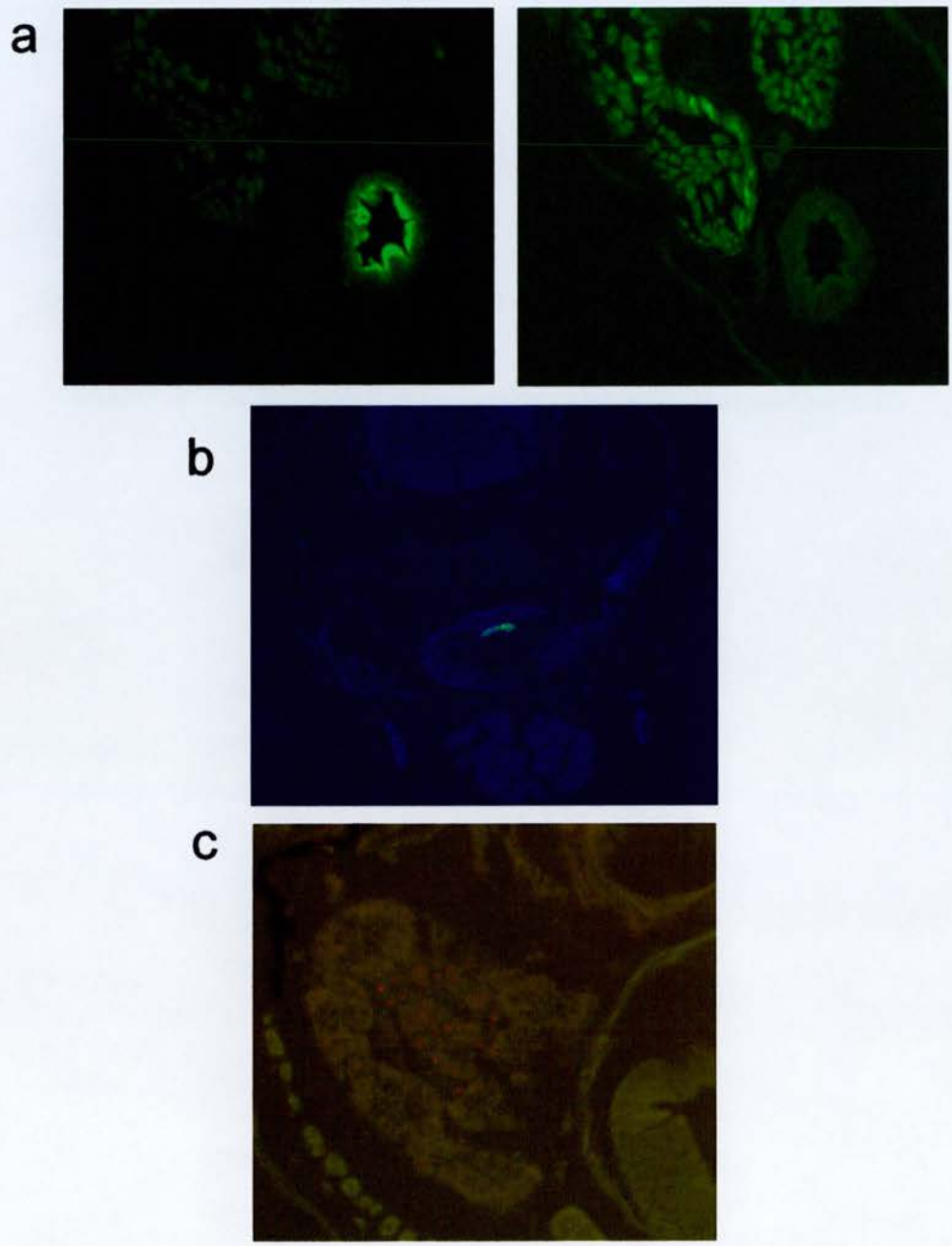


Figure 5.14: Immunohistochemistry of *sri* embryos. (a) Glucagon staining in the stomach. Right-hand panel shows staining with secondary antibody only; **(b) Glucagon staining in the oesophagus.** Green, glucagon; Blue, autofluorescence; **(c)** Double staining showing morphology of the islet of Langerhans and patches of insulin expression in the islet. Green, glucagon; Red, insulin. 5 dpf WT embryos.

expression may remain to allow survival. Somatostatin producing cells may still be present, as seen in the *Pax6* -/- mouse.

Gut and pancreas development in zebrafish is different from that in mammals, so that a major difference in the regulation of glucagon and insulin is not implausible (Field *et al.* 2003, Wallace and Pack, 2003). Further evidence for a major difference in pancreas development in zebrafish, is that a 5 dpf WT embryo stained with an antibody for p48 showed staining only in the islet (Figure 5.15). p48 is a subunit of the PTF1 transcription factor required for development of the exocrine pancreas in the mouse (Krapp *et al.* 1998). It is possible that a related protein is expressed in the developing endocrine pancreas of the zebrafish, while exocrine development is under control of a more diverged transcription factor complex. There may also be other cells that secrete the pancreatic endocrine hormones in fish. Insulin is produced in the yolk sac of rat embryos (Muglia and Locker, 1984), but this would not account for survival of the adult fish. It has been suggested that insulin is produced outside the pancreas in adult rats (Rosenzweig *et al.* 1980), and recently it has been shown in carp that the adipocytes can secrete insulin (Roy *et al.* 2003). It is therefore possible that in fish another source can compensate for the lack of pancreatic insulin.

5.8 Knock-down of Pax6a and Pax6b expression reproduces some aspects of the *sri* phenotype

To confirm whether the *sri* phenotype was caused by the L224P mutation, antisense oligonucleotide "morpholinos" were used to knock down the expression of Pax6a and Pax6b. Morpholinos were designed and synthesised by Gene-tools (http://www.gene-tools.com), to bind the 5'UTR of *Pax6a* and *Pax6b* mRNA, and prevent translation (Figure 5.16). The phenotype caused by the *Pax6b* morpholino did not resemble the *sri* phenotype. For both morpholinos effects were seen in the eyes, with an irregular retina and pupils, cataracts, and flattened cornea. Defects in formation of the CNS were present, including mild cyclopia (eyes closer together but not touching).

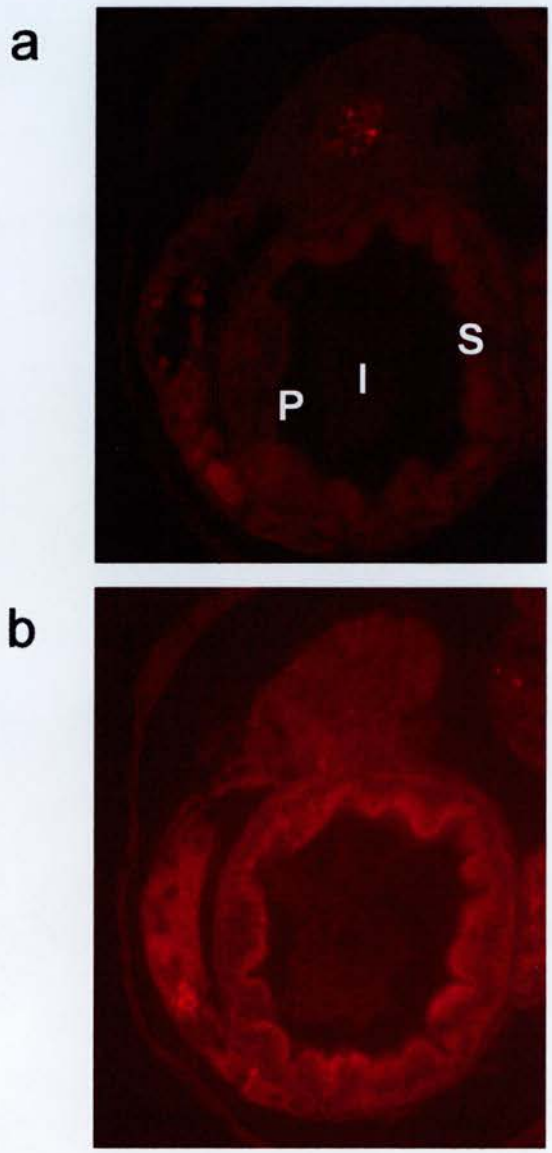


Figure 5.15: (a) p48 staining in the islet of Langerhans of 5 dpf WT embryos; (b) Secondary antibody only.

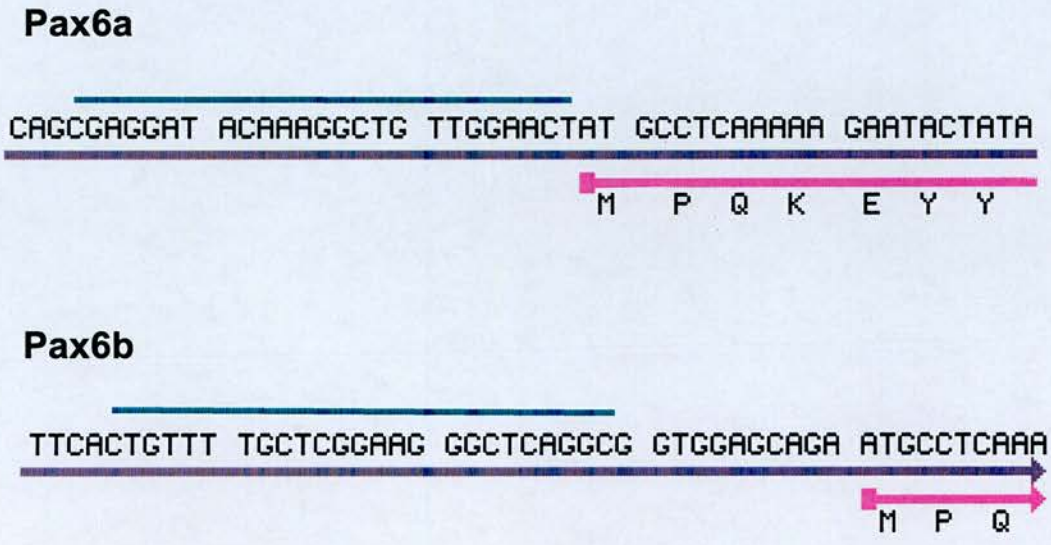


Figure 5.16: Antisense morpholinos (green) were designed (by Gene-tools) to bind the 5'UTR of *Pax6a* and *Pax6b*. (Sequence graphics reproduced from http://www.ncbi.nlm.nih.gov).

Somite defects were also observed, with disruption of the lateral stripe, a curved body, and misshapen somites (Figure 5.17). Although Pax6a and Pax6b are not expressed in the somites, the neural tube is important for the correct induction of somite development (Münsterberg and Lassar, 1995, van Eeden *et al.* 1996). Double knock-down, with injection of the *Pax6a* and *Pax6b* morpholinos together, gave a similar phenotype. The eye phenotype, however, was sometimes more severe, with a protruding lens in the most affected embryos (Figure 5.18). This phenotype roughly resembles the protruding lens of severe *sri* mutants, but does not appear to be a complete phenocopy.

Unexpectedly, ear defects also occurred in the most severely affected embryos. The ears appeared to develop normally until 24 hpf, but defects in the number and position of the otoliths developed by 48 hpf (Figure 5.19a,b). Ear defects may be caused by a lack of inductive signals from the underlying hindbrain that are necessary for ear development (Noramly and Grainger, 2002). This type of defect is found in the zebrafish mutant *valentino* which has defects in the differentiation of rhombomeres 5 and 6 in the hindbrain. In this mutant, early ear development also appears normal, but defects occur after 14 hpf (Mendonsa and Riley, 1999, Figure 5.19c). The ears have not been examined in *Pax6* mutants in mice, and hearing defects have not been reported for human aniridia patients, so if this phenotype occurs in other organisms it may be confined to the homozygotes.

The *Pax6b* morpholino does not appear to be entirely specific. Defects are present in tissues that do not express Pax6b, including the neural tube and hindbrain. The most obvious explanation for this is that Pax6b has a regulatory function on Pax6a. It has been shown in quail and mouse that Pax6 has an autoregulatory function (Plaza *et al.* 1993, Grindley *et al.* 1995), so it is not unreasonable to assume that the two forms of Pax6 in zebrafish may also have a role in regulating each other. The *Pax6b* morpholino therefore, may also downregulate Pax6a function, causing a more severe phenotype than expected.

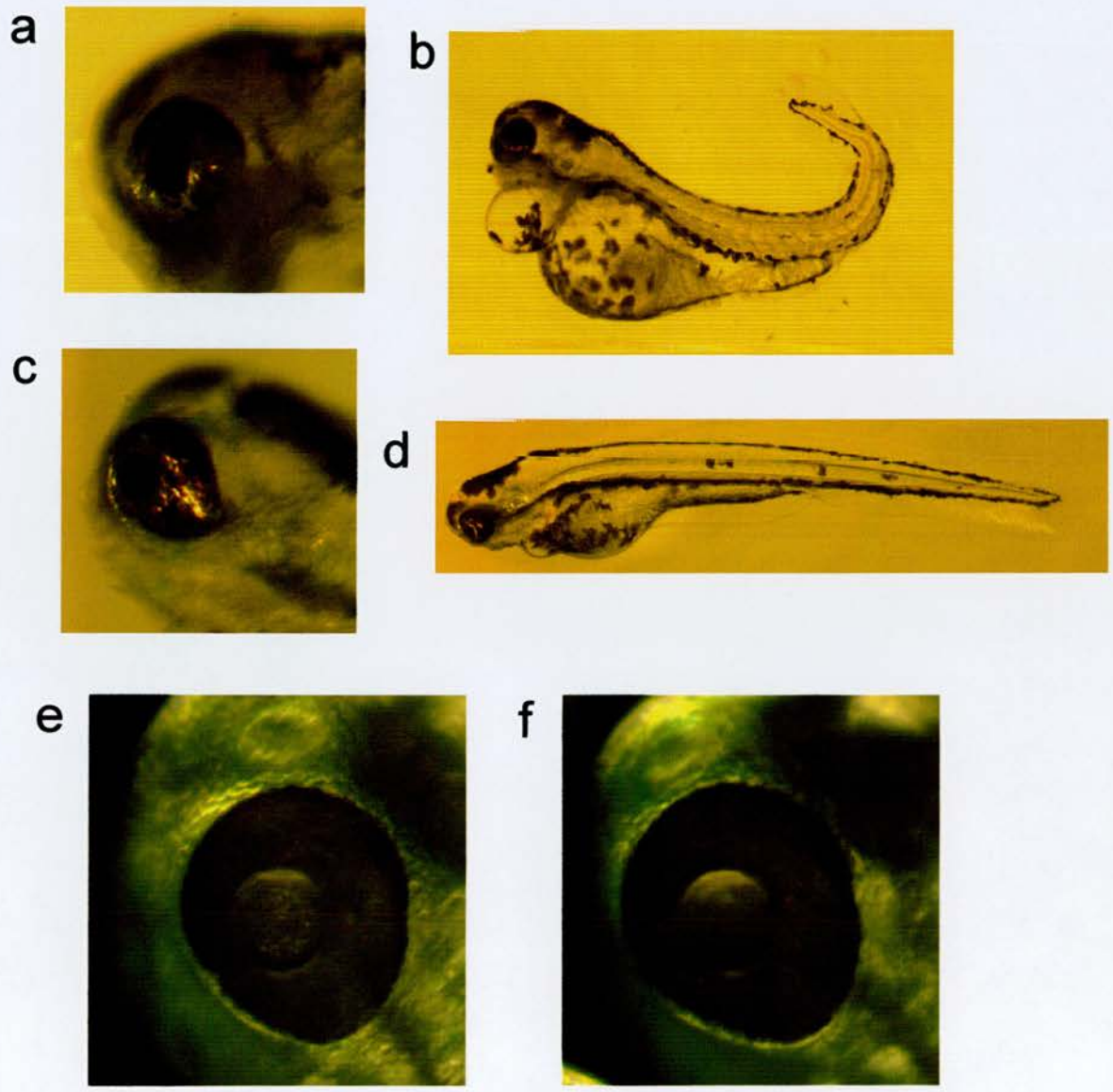


Figure 5.17: Phenotypes caused by morpholino injection. (a) *Pax6a*, 0.5 mM, 5 dpf; **(b)** *Pax6a*, 0.5 mM, 5 dpf; **(c)** *Pax6b*, 1 mM, 6 dpf; **(d)** *Pax6b*, 0.75 mM, 4 dpf; **(e)** Cataracts, 0.5 mM, 3 dpf; **(f)** WT, 3 dpf.

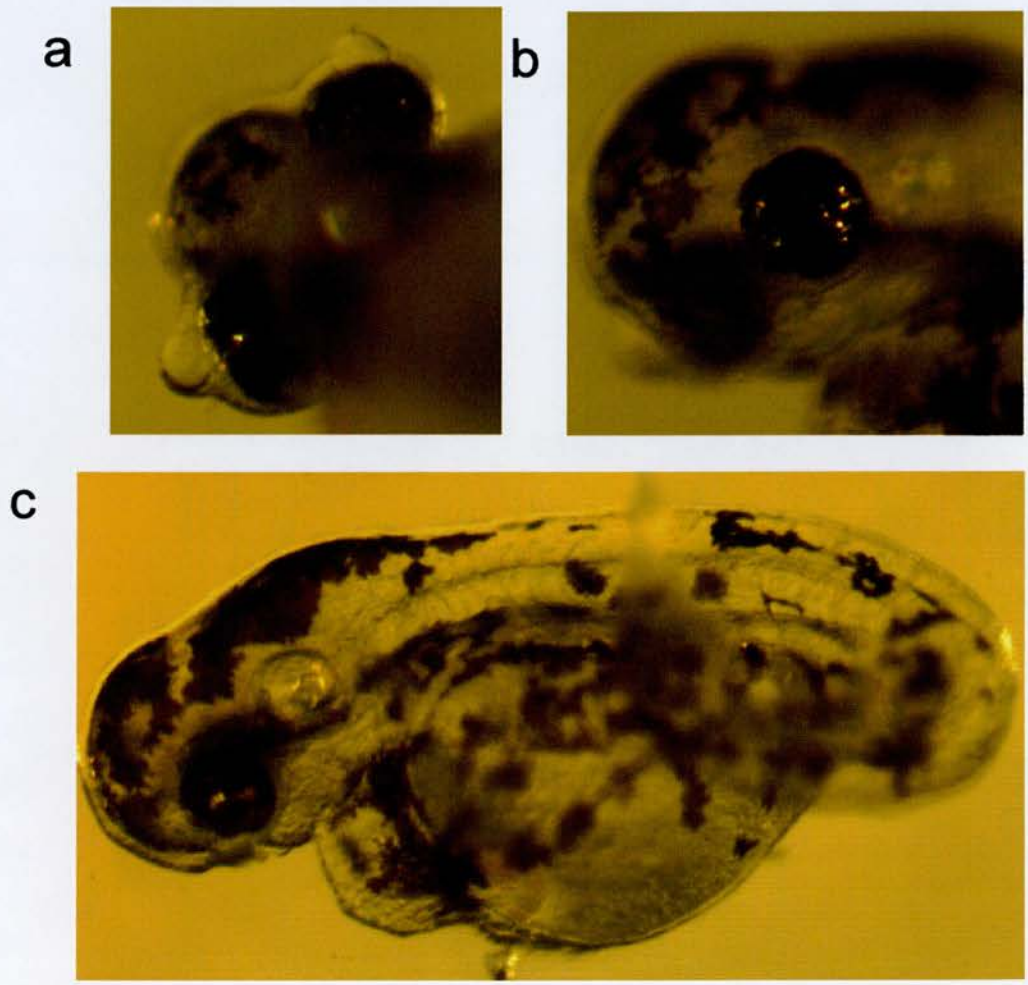


Figure 5.18: Phenotype of *Pax6a/Pax6b* double morphant. (a) Protruding lenses; **(b)** CNS defects; **(c)** Somite defects. All pictures are of the same embryo, 0.25 mM *Pax6a/* 0.75 mM *Pax6b*, 4 dpf.

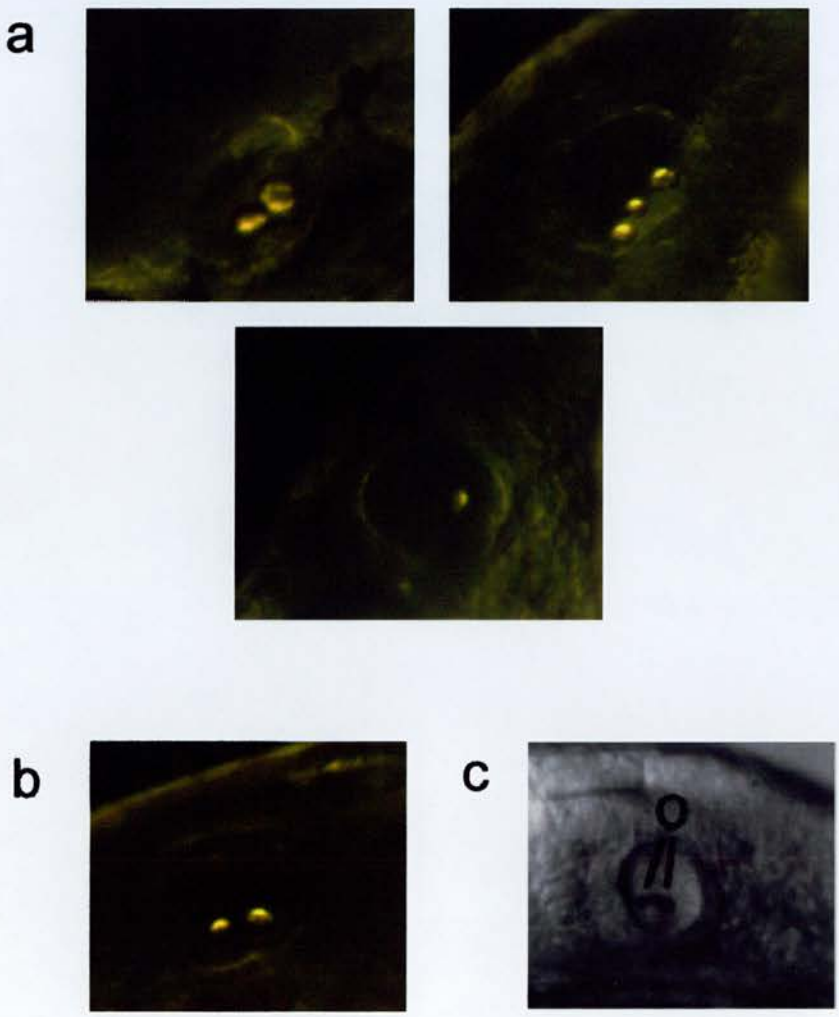


Figure 5.19: Otolith defects. (a) Embryos injected with *Pax6b* morpholinos, 0.5-1 mM, 2 dpf; **(b)** WT, 2 dpf; **(c)** The zebrafish *valentino* mutant also develops otolith abnormalities, as a result of defects in hindbrain differentiation, 1 dpf (Reproduced from Mendonsa and Riley, 1999).

A 25-mer morpholino that should not recognise any mRNA in zebrafish was used as a negative control. This was specific to the *beta-globin* pre-mRNA in reticulocytes from thalassaemic humans that carry a splice-generating mutation at position 705. A high concentration was used (1 mM), but this caused very few defects; 4/51 with a reduced lens and small characteristically shaped eye, 5/51 with somite defects, and several with heart oedema, growth retardation or non-inflation of the swim bladder (Figure 5.20). No defects were observed that resembled those seen with the *Pax6a* and *Pax6b* morpholinos. The fact that the *Pax6a* and *b* morpholinos caused the same phenotype, and have a completely unrelated sequence, shows that the knock-down should be specific to Pax6.

A rough statistical analysis was carried out, where the average concentration of morpholino per embryo was calculated, to allow an approximate comparison of the severity of defects caused by morpholino injection. This showed that for double knock-down embryos, the frequency of survival and other effects was similar to single morphants, while the frequency of eye and neural tube defects was increased. The average concentration for the double morphants is 0.8 if the concentration of both morpholinos is added together, which is twice the average for *Pax6a*, and equal to the average for *Pax6b* (Table 5.4, Figure 5.20c). The frequency of each of the morphological defects for double morphants however, is much more than twice that observed with either morpholino separately. Also the protruding lens phenotype was never observed for either morpholino alone, even though a complete range of concentrations was used. This rough analysis suggests that the combined effect of *Pax6a* and *Pax6b* morpholinos together is more than the two separately, so that there is a synergistic effect when both are injected together. It has already been suggested that there could be regulatory effects by each Pax6 ortholog on the other. The action of the non-targeted protein may therefore partially rescue the phenotype of a single knock-down, by upregulating transcription, so that when both are targeted, the phenotype is more severe. Also, if heterodimerisation is important in zebrafish Pax6

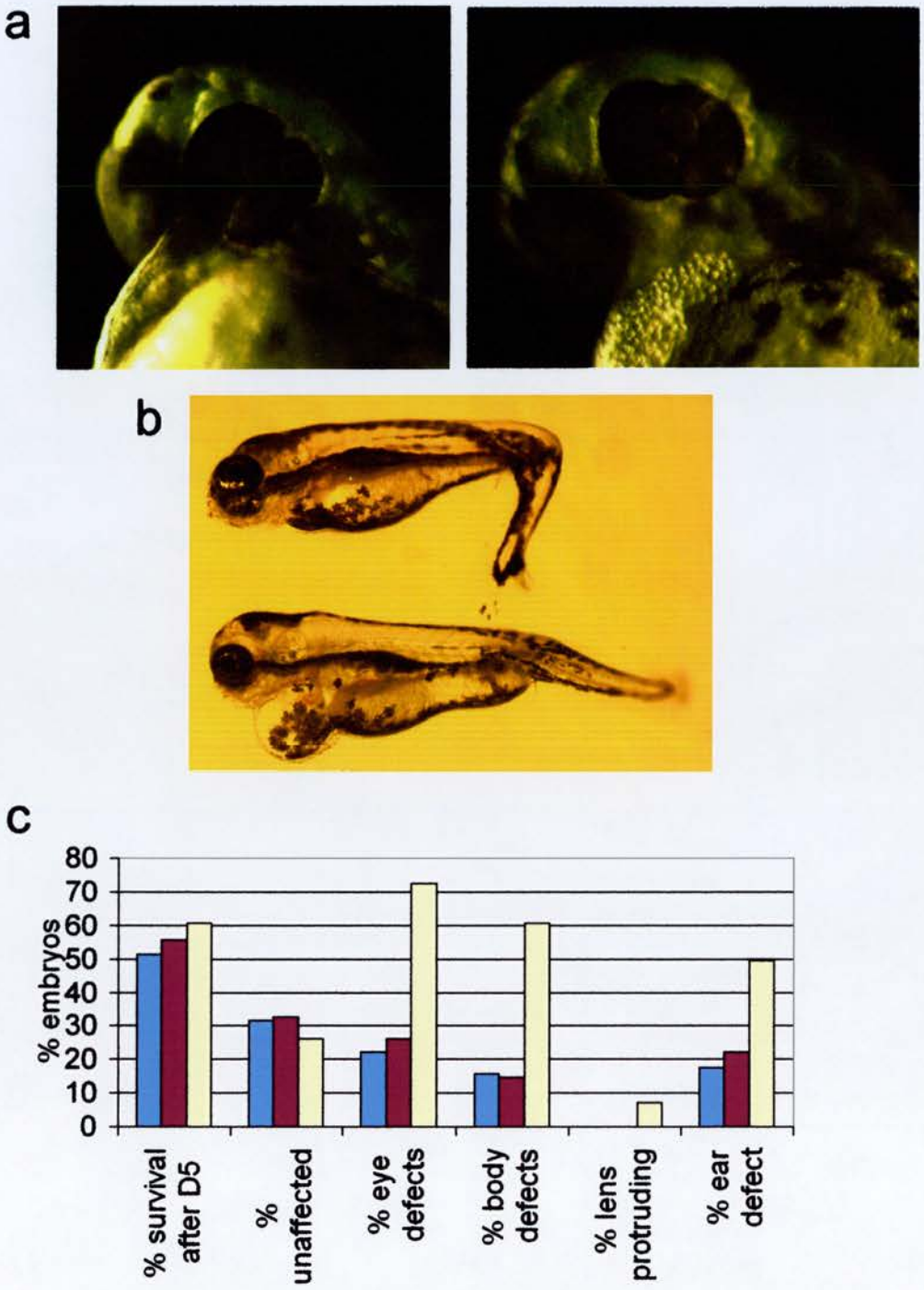


Figure 5.20: Defects caused by a non-specific control morpholino. **(a)** Characteristic eye abnormalities, 1 mM, 2 dpf; **(b)** Somite defects, 1 mM, 5 dpf; **(c) Frequency of developmental defects in Pax6 morpholino injected embryos at 4/5 dpf.** (Purple, *Pax6a*; Red, *Pax6b*; Cream, double).

function, this could cause a more severe phenotype in *Pax6b* morpholino injected embryos.

	n	Average conc. (mM)	% Survival	% Unaffected	% Eye defects	% Body defects	% Ear defects	% Protruding lens
Pax6a	123	0.4	51	32	22	16	18	0
Pax6b	307	0.8	56	33	26	15	22	0
Pax6a/b	252	0.2/0.6	61	26	73	61	50	7

Table 5.4: Frequency of developmental defects in morpholino injected embryos at 4/5 dpf.

The *Pax6a* and *Pax6b* morpholinos appear to have a specific effect, causing a phenotype in tissues that express Pax6. The overlapping phenotypes, however, is suggestive of regulatory influences or heterodimerisation between the two orthologs. The phenotype caused by Pax6b knock-down, however, is unlike that seen for *sri*, suggesting that the L224P mutation is not a complete loss of function.

5.9 Injection of *Pax6b* mRNA rescues the *sri* phenotype

To confirm whether the L224P mutation is causative for the *sri* mutant phenotype, *Pax6b* WT mRNA was injected into *sri* homozygous mutant embryos. mRNA was *in-vitro* transcribed from cloned full-length zebrafish *Pax6b* cDNA, and a GFP transcript was also made for co-injection (Dirk-Jan Kleinjan). Several dilutions of *Pax6b* mRNA were injected, together with GFP mRNA, into 1 to 4 cell stage embryos. Half of the embryos from each clutch were not injected, to be kept as a control group. At 24hpf embryos that did not express GFP were removed. At 3 dpf, each eye was photographed at the same magnification, and measurements were made of eccentricity, SD radius, area and the major axis of the pupil and eye (Chapter 2.4, p50). At 5 dpf the *sri* phenotype was scored for each group, and the mean severity score was calculated.

When approximately 0.1 pg *Pax6b* RNA was injected per embryo, the severity score was 1.1, compared to 1.7 in uninjected embryos, with a t value for the difference of –2.5 (p 0.06, n 19 and 18 respectively). The frequency of coloboma was reduced from 6/38 to 1/36, but not significantly. Measurements at 3 dpf showed a reduction in the major axis, SD radius and eccentricity of the retina (Table 5.5). This is highly suggestive of a

decrease in the severity of the eye phenotype. When 1 pg was injected, the severity score did not change, although the most of the measurements taken at 3 dpf did. The frequency of coloboma was slightly increased from 33/138 (24%) to 23/82 (28%). 1pg therefore caused no real change in the *sri* phenotype. The effects of RNA injection may vary depending on the concentration used, and these experiments must be repeated to confirm these findings.

a

pg RNA	n eyes	Area	p	Major axis	p	SD radius	p	Eccentricity	p
0	36	4540		129.9		8.05		0.498	
0.1	38	4602	0.528	130.4	0.85	6.87	0.156	0.45	0.147
0	138	4799		138.6		8.69		0.514	
1	82	4935	0.042	144.2	0.004	9.88	0.08	0.567	0.008

b

pg RNA	n eyes	Area	p	Major axis	p	SD radius	p	Eccentricity	p
0	36	14742		421.51		7.589		0.5311	
0.1	38	14585	0.134	414	0.001	6.99	0.008	0.5082	0.013
0	138	13613		387.3		8.06		0.5449	
1	82	13917	0	398.7	0	7.63	0.019	0.5344	0.085

Table 5.5: Mean values for measurements taken from 3 dpf injected or control embryos. (a) Lens. (b) Retina.

5.10 Summary and conclusions

The *sri* mutation maps to between 29 and 34 cM from the top of linkage group 7, a region that contains the *Pax6b* gene. A T>C transition was identified in *Pax6b* of *sri* that is predicted to cause an L224P missense mutation. The L224P mutation is in the first α-helix of the homeodomain, and is predicted to disrupt the function of at least the HD, if not the entire Pax6b protein. It is unlikely that the mutation causes a gain of function, as no phenotype is observed in the heterozygotes, even when treated with radicicol.

The *sri* homozygous phenotype includes many aspects of the *Pax6* heterozygous phenotype in mammals; reduced lens, delayed pigmentation of the retina, delayed closure of the optic fissure, irregular cornea, Peters' anomaly, and cataracts. Immunohistochemistry shows that *sri* embryos also have a complete absence of glucagon producing cells. *sri* homozygotes can survive to adulthood and are fertile. It is thought that zebrafish have alternative mechanisms for producing insulin and glucagon that allow the homozygotes to survive.

The only phenotype observed in *sri* is in the lens and pancreas, where Pax6b is expressed without overlapping Pax6a expression. Therefore the absence of defects in regions where Pax6a is also expressed may be due to redundancy of function between Pax6a and Pax6b. Heterodimerisation may also play a role in restoring some of the Pax6b protein function where expression overlaps. Although dominant-negative phenotypes usually occur due to heterodimeristaion of mutant and WT proteins, interactions between WT Pax6a and mutant Pax6b might create a dimer with a relatively normal function. Pax6 is required for lens development in mammals (Ashery-Padan *et al.* 2000) but in *sri* a lens does form, so that some residual Pax6 function must be present. It is possible that the PD can perform part of the Pax6 function alone in the lens, but not in the pancreas, as the PD alone has been shown to rescue *Pax6* mutants in *Drosophila* (Punzo *et al.* 2001).

Morpholino knock-down experiments also point towards a partial reduction of Pax6b function by the L224P mutation. The phenotype for a Pax6b knock-down is far more severe than that observed in *sri* homozygotes, with severe effects on the brain, neural tube and eyes. There may be an additional role for the Pax6b PD in regulating the expression of Pax6a that is unaffected in *sri.* If *sri* is caused by a partial loss of Pax6b function, injection of WT *Pax6b* mRNA into *sri* homozygous embryos should restore the phenotype. Preliminary experiments show that this may be the case. Injection of the *sri* mutant mRNA into WT embryos would also test the presence of any dominant-negative effects.

The buffering of the expressivity of a *Pax6b* mutation by Hsp90 has important implications for human developmental eye disease. It will be possible to test *Sey* mice for Hsp90 buffering by crossing them to Hsp90 mutants (Voss *et al.* 2000), or treatment with inhibitors. It may also be possible to understand the mechanism by which *sri* is buffered by identification of proteins that regulate, or are regulated by Pax6, and that also interact with Hsp90.

Chapter 6

Discussion

6.1 Summary of findings

This study was undertaken to test the hypothesis that the chaperone protein Hsp90 could buffer the penetrance or expressivity of mutant eye phenotypes in zebrafish. Embryos were treated with the Hsp90 inhibitors radicicol and geldanamycin from mid-gastrulation until 1 dpf, by which time the retina and lens have begun to differentiate. A concentration was used that caused only transient developmental retardation and a low level of developmental defects, without induction of a heat shock response. Detailed analysis of the *sri* and *dre* homozygous mutant phenotypes showed that expressivity is altered by Hsp90 inhibition and heat shock; *sri* was noticeably worsened, while *dre* was slightly improved. The *sri* mutation was mapped in collaboration with Ralf Dahm and Robert Geisler (Max Planck Institute for Developmental Biology Tübingen) and the candidate gene, *Pax6b*, was sequenced. An L224P missense mutation was identified in the first alpha helix of the *Pax6b* HD, which is predicted to destroy HD function. Absence of glucagon producing alpha cells, and most insulin producing beta cells in the pancreatic islet of homozygotes, as seen in *Pax6* -/- mice, confirms that Pax6b function is disrupted (Sander *et al.* 1997, St-Onge *et al.* 1997).

6.2 Discussion and future work

6.2.1 The expressivity of a *Pax6b* mutation is buffered by Hsp90

Expressivity of the *Pax6b* mutant *sri* is increased by Hsp90 inhibition. This may occur due to a direct interaction of Hsp90 with the mutant protein, or indirectly through interaction with proteins that play a role in eye development, in pathways that affect Pax6b in some way. Immunoprecipitation of Hsp90 and WT or mutant Pax6 would reveal whether any direct interaction occurs between Hsp90 and Pax6 in WT or *sri* embryos. Identification of any Hsp90 clients with an indirect role will be more challenging. There are many proteins up or downstream of Pax6 in eye development that interact with Hsp90 and may influence the *sri* phenotype if Hsp90 function is

compromised. Hsp70 and Raf1 have already been mentioned (Chapter 4.5, p117). It is unlikely that Hsp70 or other stress induced proteins are involved, as the lens phenotype in *dre* is similar to that seen in *sri*, yet the *dre* phenotype is not worsened by Hsp90 inhibition or heat shock. Raf1 is ubiquitously expressed during development (Storm *et al.* 1990), so that any effects of Hsp90 inhibition would have to be very mild for only the mutant pathway to be affected. The identification of *Hsp90* partial loss of function mutations in two screens for modifiers of Raf1 mediated photoreceptor development in *Drosophila*, however, shows that a slight reduction in Raf1 activity can cause a specific phenotype in a vulnerable developmental pathway. There is no Raf1 mutant in zebrafish, but quantitative analysis may reveal a reduction in Raf1 expression in radicicol treated embryos.

One other possible mechanism for worsening of the *sri* mutation after Hsp90 inhibition is *via* c-jun NH_2-terminal kinase (JNK). It has recently been shown in mice, that JNK regulates a signalling pathway in eye development consisting of BMP4, Shh and Pax2. In JNK mutant mice eye development is disrupted, with defects in the lens and non-closure of the optic fissure. Non-closure of the optic fissure in JNK mutant embryos has been shown to be due to a reduction in Pax2 expression, by a JNK-BMP4-Shh signal cascade. The effects in the lens have not been fully investigated, but are thought to be due to a reduction in c-jun, Pax6 and Sox2 expression (Weston *et al.* 2003).

Both Hsp70 (Park *et al.* 2001) and nitric oxide (NO) (Park *et al.* 2000) negatively regulate JNK. It is possible that Hsp90 could affect Pax2 expression through interactions with both Hsp70 and endothelial nitric oxide synthase (eNOS) (Figure 6.1). Hsp90 sequesters Hsp70 and HSF1 in an inactive complex (Guo *et al.* 2001, Zou *et al.* 1998). A concentration of only 5.3 μM geldanamycin in *Drosophila* larvae is enough to destabilise this complex, and activate Hsp70 (Auluck and Bonini, 2002). Therefore Hsp90 inhibition could activate Hsp70 and cause a reduction of Pax2 expression.

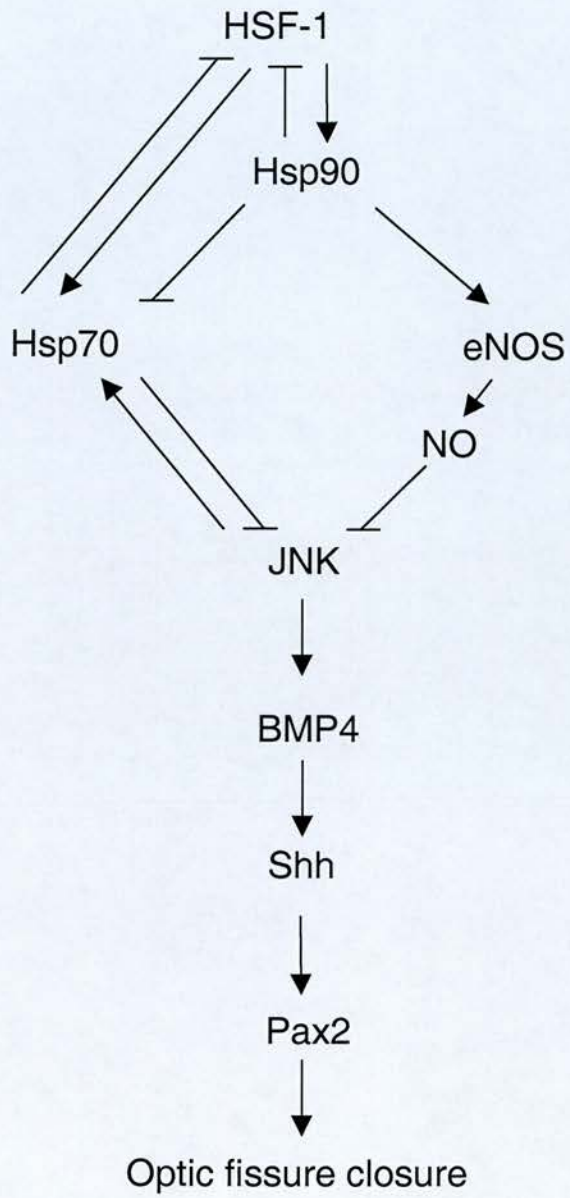


Figure 6.1: Mechanism by which Hsp90 may exacerbate the phenotype caused by the *sri* mutation. Hsp90 sequesters Hsp70 in an inactive complex. Hsp90 stimulates production of NO by eNOS, which also inhibits JNK activity. Hsp90 inhibition has a stronger effect on Hsp70 than eNOS. Hsp90 inhibition *in vivo* could therefore release Hsp70, which in turn would inhibit JNK activity. JNK is required for Pax2 expression in the eye, which causes closure of the optic fissure.

Conversely, Hsp90 has been shown to activate production of NO by direct interaction with eNOS (Garcia-Cardena *et al.* 1998), so that Hsp90 inhibition could decrease NO production and upregulate Pax2 expression. A relatively high concentration of geldanamycin (1.8 mM, cf. 10 μM in our experiments) is required to reduce eNOS activity however, so that the principal effect of Hsp90 inhibition may be to activate Hsp70, with a resultant inhibition of Pax2 expression. A reduction in Pax2 expression in *sri* embryos would be expected to increase the severity of the phenotype. The role of Hsp70 in aggravating the *sri* phenotype could occur independantly of any stress-response activity, or its possible role in lens differentiation.

To test the hypothesis that JNK activity is affected by Hsp90 inhibition, the JNK immunocomplex assay used by Park *et al.* (2000) could be used to assess the effects of radicicol and geldanamycin on different cell types. The effects of Hsp90 inhibition on the expression of Pax2, BMP4 (Bone morphogenic protein 4) and Shh in the eyes of zebrafish embryos could be tested by quantitative analysis. It might also be interesting to look at levels of Pax6 and Sox2. Expression levels in treated WT embryos might also be affected, suggesting a mechanism for the reduction in eye size.

Hsp90 inhibition appears to reduce the severity of the *dre* phenotype. It was expected that Hsp90 is important in different tissues at different stages in development, so that selective effects of Hsp90 inhibition on different structures in a phenotype was not surprising. Hsp90 may stabilise a negative modifier of *dre*, the effect of which is reduced when Hsp90 function is compromised. Alternatively, the mutated protein may be stabilised by Hsp90, and have a gain of function effect while bound to Hsp90, that is lost when that binding is reduced by Hsp90 inhibitor treatment. Mapping the *dre* mutation will allow this mechanism to be investigated more thoroughly. It may also be informative to section *dre* embryos, and determine which structures of the eye are most affected in the embryos with the more severe phenotypes. This could be aided by immunohistochemistry using antibodies that recognise specific cell types in the eye.

Examination of treated embryos might show which structures are affected by Hsp90 inhibition.

Hsp90 inhibition may also influence the *sri* phenotype through modifiers that cause the variation in expressivity that is observed in untreated embryos. There are many modifier genes that influence the phenotype of different disease genes (Reviewed in Nadeau, 2001, Haider *et al.* 2002). It may be possible to select for modifiers that suppress the *sri* phenotype by raising mildly affected embryos separately, and selecting for mild phenotypes for several generations. If the mean severity score decreased with selection, this would show that genetic modifiers of the *sri* phenotype were present. If severity of the mild phenotype line was more affected by Hsp90 inhibition than in unselected lines, this would show that suppressors of *sri* were present that were buffered by Hsp90. Alternatively, selection for a more severe phenotype may cause the accumulation of modifiers that make the *sri* phenotype more vulnerable to Hsp90 inhibition. These modifiers could then be mapped, to allow investigation into the mechanisms by which Hsp90 affects the phenotype.

6.2.2 The *sri* mutant phenotype is caused by a *Pax6b* missense mutation

The L224P missense mutation in *Pax6b* of *sri* is expected to disrupt the function of the HD. Loss of HD function would also affect the PD, which has been shown in some cases to require the presence of the HD to function efficiently (Singh *et al.* 2000, Mishra *et al.* 2002). Lack of a heterozygous phenotype indicates that the mutation is unlikely to have caused a gain of function. The phenotype caused by a *Pax6b* antisense morpholino is more severe than that observed in the *sri* homozygotes. The most likely outcome of the L224P mutation is therefore a partial loss of Pax6b protein function.

The *sri* mutation will add to the study of Pax6b function for several reasons. First, it is a novel mutation; no mutations in the first alpha helix of the HD have been reported. This will allow further investigation into the function of the Pax6 HD, both *in vivo* and *in vitro*. Also, in *sri* homozygotes the retina is relatively unaffected, which will facilitate

studies of the role of Pax6 in lens and cornea development. The major advantage of using zebrafish as a model organism is that development can be followed *in vivo*, and GFP can be used to track the activity of important regulatory elements. Lens transplantation can be carried out in zebrafish (Vihtelic *et al.* 2001), which will allow investigation into the aspects of eye development that require Pax6 expression in the lens.

Viability of the *sri* homozygotes will allow a long-term study of the effects of the mutation on eye development, and the apparent recovery of eye morphology. The mechanism of compensation for loss of glucagon and insulin producing cells in the pancreas is unlikely to be important for human disease, as a reduction of beta cells causes fatal type I diabetes in humans (Berne and Levy, 1998). The duplication of *Pax6* in zebrafish, and the divergence of regulatory elements between *Pax6a* and *Pax6b* have resulted in the sharing of *Pax6* function between the two homologues. The expression of Pax6b, without Pax6a, in the pancreas and early lens placode is evidence for this. Redundancy between Pax6a and Pax6b in areas in which they are co-expressed may contribute to the mildness of the *sri* phenotype. Division of functions between two *Pax6* genes, and redundancy of shared roles, may result in more viable mutations that would allow examination of different aspects of Pax6 function. The possibility of regulation of Pax6a by Pax6b and *vice versa* raises the possibility however, that null mutations in either gene may not be viable.

Further work could now be carried out to identify the effects of the L224P mutation on Pax6 protein function. The complete absence of glucagon producing cells in the pancreas suggests that the HD of Pax6b is essential for the differentiation of these cells. There may be interactors unique to the alpha cells that require the HD to bind Pax6b. Some Pax6 function may be preserved in the lens of *sri* homozygotes, because a lens forms in most embryos, whereas it is absent in *Pax6* -/- mice (Hill *et al.* 1991). The PD alone may be able to carry out most of the functions of Pax6b in the lens, or later expression of Pax6a might suffice to rescue lens development. The small lens in *sri*

resembles that of *Pax6* +/- mice, in which formation of the lens placode is delayed, so that fewer lens cells are specified at the onset differentiation, resulting in a reduced lens (van Raamsdonk and Tilghman, 2000). Markers for the lens placode, such as Sox2 (Kamachi *et al.* 1998, Thisse *et al.* 2001) or FoxE3 (Forkhead box E3) (Blixt *et al.* 2000), could be used to determine whether a similar delay in lens placode formation occurs in *sri* homozygotes.

There may be a residual HD function in *sri* that preserves some of the Pax6b activity in the lens. Evidence for this comes from studies of the $Pax6^{4Neu}$ mutation by Favor *et al.* (2001). $Pax6^{4Neu}$ is an S259P missense mutation in the third alpha helix of the PD, which causes a milder phenotype than that observed for loss of function mutations. Electrophoretic Mobility Shift Assays (EMSAs) using a HD binding sequence showed that while binding of the Pax6 dimer was destroyed by this mutation, the monomer retained a weak binding activity.

EMSAs as well as luciferase reporter assays in cell culture (Chao *et al.* 2003) will reveal whether any residual binding activity remains in the mutated Pax6b protein. Different PD and HD Pax6 binding sites should be tested (Singh *et al.* 2000), to identify whether the function of one or both of the domains is preserved. The DNA elements that Pax6 binds in the lens and pancreas (Kammandel *et al.* 1999) should also be tested, to determine whether binding is more affected in the pancreas than in the lens. Alternatively, reporter assays could be carried out in cell culture, using lens and pancreatic alpha cell derived cell lines, to test whether the mutation has different effects on Pax6 activity in these tissues, due to the presence of different partner proteins. A study of the binding sites for Pax6a and Pax6b in zebrafish using DNA footprinting might reveal whether there was any cross-regulatory activity between the two Pax6 homologues. Finally, co-immunoprecipitation of Pax6a and mutant or WT Pax6b will reveal whether heterodimerisation is important for Pax6 function in WT or *sri* embryos.

6.2.3 Hsp90 as a buffer of developmental defects in vertebrates

Although *sri* could not be described as a cryptic mutation, Hsp90 buffering of a Mendelian mutation with variable expressivity has important implications for human disease. There are many human genetic diseases with an extremely variable expressivity. For example, members of the same family carrying a mutation in *Shh* can suffer from a range of defects, from holoprosencephaly, to a single maxillary central incisor (Roessler *et al.* 1996). It has been shown that mutations in other genes play some part in this variability (Ming and Muenke, 2002). If Hsp90 affected the expressivity of diseases such as holoprosencephaly or MAC under certain environmental conditions, this would represent a considerable influence on the frequency of severe congenital abnormalities in populations. If a population was exposed to a low level of an environmental toxin such as a pesticide, only individuals at a certain stage of development, and carrying predisposing mutations would be likely to be affected (Figure 6.2). This could explain the small clusters of MAC reported in rural areas.

Is Hsp90 a buffer of developmental defects in humans?

Further studies should be carried out to determine whether Hsp90 is involved in modulating mutant phenotypes in humans. To begin with, mouse models of human disease could be used, and treated with Hsp90 inhibitors. For example, it will be important to identify whether *Pax6* missense mutations are affected in mice, in the same way as *sri*. Models of holoprosencephaly could also be used. Also, there are several amino acid changes that have been identified in human Pax6 studies, and do not appear to cause a phenotype (Grønskov *et al.* 1999, Chao *et al.* 2000, Chao *et al.* 2003). These changes may confer susceptibility to Hsp90 inhibition, and cause eye defects only after exposure to certain environmental factors during development. This hypothesis could be tested by creating mice carrying these changes, and treating them with Hsp90 inhibitors. Mice heterozygous for the *Hsp90b* null mutation could also be used for these experiments (Voss *et al.* 2000), but there is a risk that redundancy between Hsp90a and b could rescue the effects of *Hsp90b* haploinsufficiency.

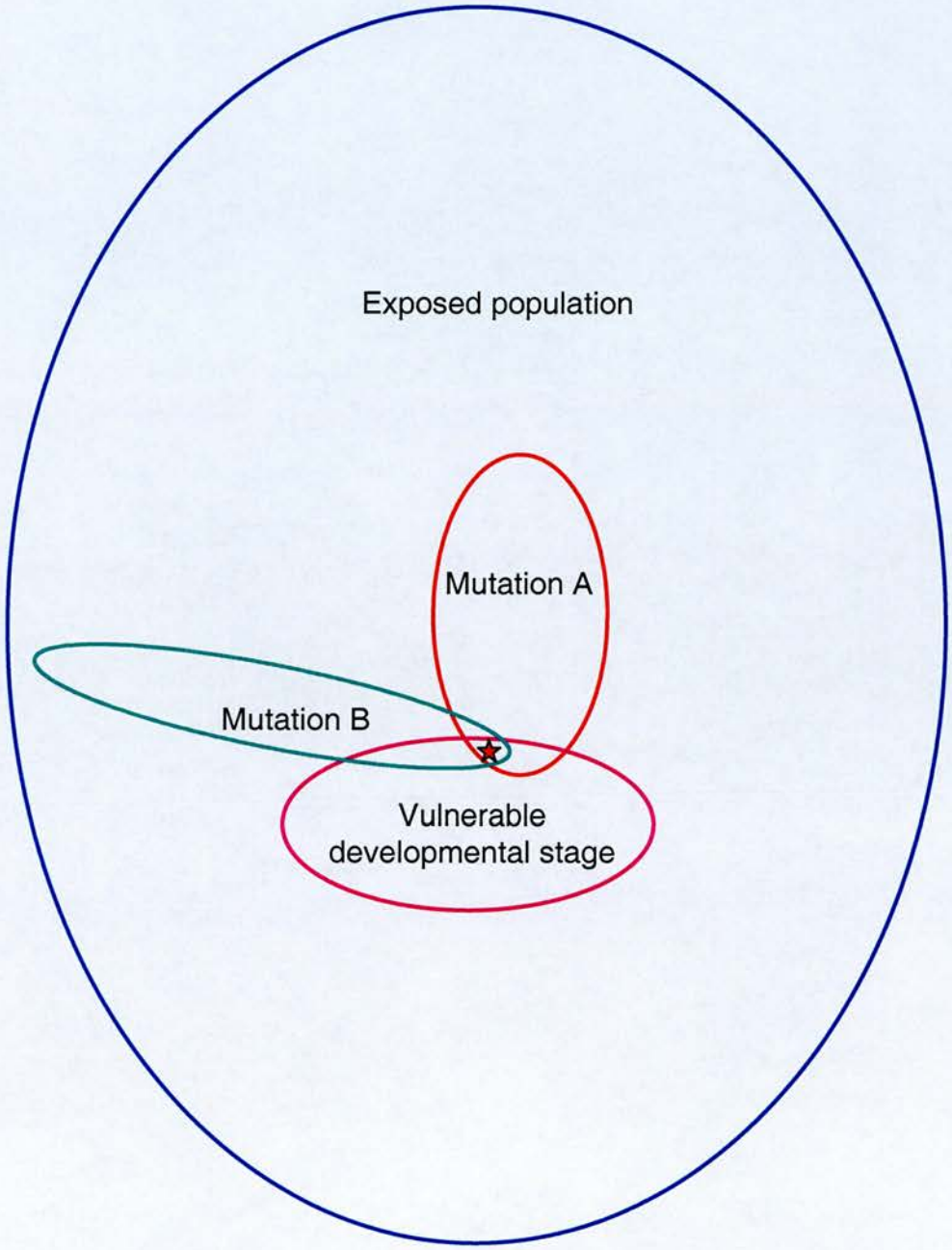


Figure 6.2: Effect of environmental stress on the frequency of developmental defects in the population. If a population was exposed to an environmental stress, such as a pesticide, and this inhibits or sequesters Hsp90, only individuals at a vulnerable stage of development, and carrying certain mutations may develop defects, leading to a low level of affected individuals in the population (represented by a red star).

Once mutations are identified that show a requirement for Hsp90 stabilisation in mice, the effects of environmental factors on these mutant phenotypes could be studied in humans. To identify whether stress has occurred during pregnancy, it would be necessary either to conduct large-scale epidemiological studies based on quantifiable risk factors, or a detailed medical history. It may be possible, however, to conduct retrospective analysis to identify a "memory" of severe stress during early development. Components in the immune system could be identified that remain following this type of stress (van Eden *et al.* 2003). Individuals with a seemingly sporadic occurrence of genetic disease, such as MAC, may be more likely to have been exposed to environmental stress during development. This would suggest that Hsp90 is involved in modulating the phenotypic effects of these mutations.

One other way to study the possible requirement for Hsp90 as a developmental buffer in humans would be to identify *Hsp90* polymorphisms in the population, and measure their effect on development. One study has already identified a nucleotide variant in an adult that has not affected fitness, but which may confer susceptibility to late-onset disease (Passarino *et al.* 2003). Another study has identified *Hsp90* polymorphisms in connection with male sterility (Yamamoto *et al.* 2002). This study confirms that Hsp90 is important in spermatogenesis, but mutations or polymorphisms that compromise the buffering capacity of Hsp90 may be more difficult to identify, as the phenotype may be very pleiotropic.

Prevention of the possible harmful effects of Hsp90 inhibition in humans

If Hsp90 developmental buffering is critical in humans, it will be important to identify the environmental factors that are particularly damaging to Hsp90 function. Chemicals that interact directly with Hsp90, or cause induction of the heat shock response are most likely to compromise Hsp90 buffering. This could be assessed in cell culture or using zebrafish embryos. One way to identify factors that affected Hsp90 activity would be an assay that used an Hsp90 client to activate a reporter gene.

Further investigation of the developmental pathways that are particularly vulnerable to Hsp90 inhibition would suggest other environmental factors that could influence mutant phenotypes. Pesticides, industrial chemicals, medicinal drugs, calorific intake, body temperature and the levels of specific nutrients in the diet may all play a part in modulating Hsp90 function, either through causing a stress response, or direct inhibition of Hsp90 activity. Limiting exposure to chemicals that could affect Hsp90 activity, or regulation of dietary and other lifestyle factors may reduce the risk of developmental disease in individuals carrying mutations in susceptible developmental pathways, or *Hsp90* variants.

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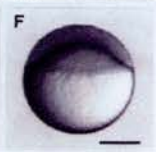
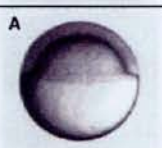
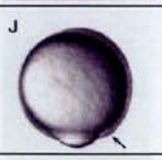
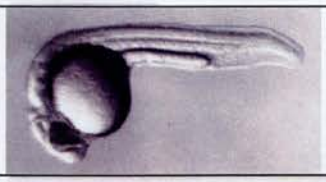
Appendix A: Zebrafish development

(Summarised from Kimmel *et al.* 1995, photographs reproduced from ZFIN developmental staging series).

Time	Stage	Appearance	Comments
4 h 40 min	30% epiboly		
5 h 15 min	50% epiboly		
5 h 40 min	Germ ring		Embryo lies vegetal pole down, germ ring clearly visible
8 h	70% epiboly		
9 h	90% epiboly		
11 h 40 min	5-somite stage		Eye primordium visible
24 h (1 dpf)	Prim-5 stage		Embryo motile, clearly recognisable eye
48 h (2 dpf)	Long-pec stage		

Appendix B: Working for t-test (Chapter 2.4)

EtOH

Experiment	Mean 1	SE 1	SE 1 squared
rad56.1	6062	53	2809
rad56.2	5486	77	5929
rad 56.3	5248	75	5625
Mean	**5598.667**		

Radicicol

Experiment	Mean 2	SE 2	SE 1 squared
rad56.1	5739	100	10000
rad56.2	5534	63	3969
rad 56.3	5062	113	12769
Mean	**5445**		

Experiment	Mean1-2
rad 56.1	323
rad56.2	-48
rad 56.3	186
Mean difference	**461**

Sum SE^2	41101
$\sqrt{Sum\ SE^2}$	202.7338
Mean difference/ $\sqrt{Sum\ SE^2}$	2.273918