Characterization of Nuclear Respiratory Factor 1 using transgenic zebrafish

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by

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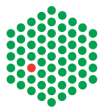

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ABSTRACT

Nuclear respiratory factor 1 (NRF1) is a transcription factor essential for the embryonic development of vertebrates. The zebrafish mutant *nrf* is characterized by apoptotic photoreceptor death during embryogenesis followed by late larval lethality. In zebrafish *nrf1* is initially expressed throughout the CNS at a high level at days 1 and 2 and later fades to levels undetectable by *in situ* hybridization. This study shows that the initial phase of expression is crucial for photoreceptor maintenance, but not for their initial development, as homozygous mutant cells can form normal photoreceptors in a wild type background. By utilizing reporter expression of a marker insertion near the wild type allele of *nrf1* combined with transgenic rescue, I show that a single heat shock induced pulse of *nrf1* expression at any time between 24 and 54 hours post fertilization is sufficient to rescue the mutant phenotype and delay photoreceptor degeneration until larval stages. Moreover, no ectopic defects are detected after ubiquitous expression of the gene, suggesting that Nrf1 serves no detectable instructive role during embryogenesis. These results suggest that Nrf1 plays a permissive role in zebrafish photoreceptor maintenance and is crucial for the formation and survival of the outer nuclear layer, but is not strictly necessary for the initial development of individual photoreceptors. Gene expression comparison analysis identifies several up and downregulated genes in the *nrf* mutant, suggesting that the intraflagellar transport machinery of the photoreceptor connecting cilium might be defect.

1 INTRODUCTION

1.1 Transgenic animals

The ability to transfer exogenous DNA into a model organism, known as transgene technology, has provided biologists with a powerful range of tools. These include gene- and enhancer traps, site directed mutagenesis, overexpression of genes and the rescue of mutants. The genetic techniques available differ for the various model animals, and each animal model system has advantages and disadvantages. Following is an overview of the main techniques available in the three major animal model systems mouse, fly and zebrafish.

1.2 Mouse - the first transgenic animal

The first introduction of exogenous DNA into the mouse germ line was mediated by infecting a preimplantation embryo (4-8 cell stage) with the Moloney murine leukaemia virus (M-MuLV) (Jaenisch, 1976), showing that viral DNA could be inherited through the germline, and hence that exogenous DNA was stably integrated into the host genome after the infection of embryos. In this first report, no transgene expression was observed. The first transgenic mice expressing a gene of interest were generated by microinjection of DNA into the pronucleus of the fertilized oocyte (Palmiter et al., 1982). The foreign DNA was expressed in 15% of the animals, and continued to be expressed in the offspring.

In the case of microinjection of plasmid DNA into the cell, the chromosome suffers random breaks possibly caused by the cells own repair enzymes, and these breaks may serve as integration sites for the foreign DNA (Brinster et al., 1985). Plasmid DNA can be incorporated into the genome as concatemers, multiple head-to-tail arrangements that also can cause deletions of the integrated plasmid sequence and may complicate identification of the insertion. In contrast, retroviral DNA integrates into the genome of the infected cell by a well-defined mechanism, and only a single proviral copy is inserted at a given chromosomal location (Varmus, 1982). No rearrangement of the host genome is induced apart from a short duplication of host sequences on either side of the integration site. The disadvantages of the retroviruses include the small amount of foreign DNA that can be introduced into the vector

(about 7 kb), the possible reduction of expression due to hypermethylation of the virus long terminal repeats (LTR) and the labor intensity of virus production and infection.

1.2.1 Mouse and embryonic stem cells

A breakthrough in mammalian transgene technology was the development of embryonic stem (ES) cell[1] cultures (Gossler et al., 1986; Robertson et al., 1986). In contrast to the hit-and-miss nature of the retroviral approach, this technology enables targeted mutagenesis by homologous recombination (Doetschman et al., 1988), meaning one can knockout, and obtain mutants for any gene of interest. Knockout mouse models are widely used to study human diseases caused by the loss of gene function. Other examples of the application of ES-cell technology are enhancer-, promoter- and gene trapping (Friedrich and Soriano, 1991; Gossler et al., 1989; Korn et al., 1992). The gene trap construct lacking a promoter of its own is inserted into a transcribed gene, and is expressed by means of the promoter of the adjacent gene. The construct is first introduced into ES cells, and gene trap events are detected by expression of a reporter gene in the cells. After successful manipulations of the ES cells they are transferred to a blastocyst and to the uterus where they develop into a chimaeric transgenic embryo (reviewed in Stanford et al., 2001).

Transgene technology in mouse and other mammals is now mainly based on ES cells. The available ES cell technology combined with the close genetic relation to humans, are the main advantages of using the mouse model system. The disadvantages include small litter size, high cost of raising large numbers of animals and development of the embryo *in utero*, not allowing researchers to manipulate and view all stages of the developing embryo.

1.3 *Drosophila* and transposable P-elements

The fruit fly *Drosophila melanogaster* has been the favorite model organism in classic genetic studies for over 100 years. In 1982, a groundbreaking method for gene transfer in *Drosophila* using transposable elements (P-elements) was published (Rubin and Spradling, 1982). After injection of a P-element vector containing a functional *rosy* gene into *rosy* mutant embryos, rescued flies were recovered among

[1] ES cells are derived from the blastocyst, an early embryo containing around 200-250 cells and shaped like a hollow sphere. The stem cells will ultimately develop into an organism as they are unspecialized cells that can produce mature specialized somatic cells and at the same time replicate themselves.

the progeny of the injected individuals, achieving the first rescue of a mutant animal by gene transfer.

Transposable P-elements can be divided into two groups: autonomous elements encoding their own transposase[2] needed for mobilization, and non-autonomous elements that need an external transposase to move. The constructs designed in the laboratory fall into the latter category, and are generally co-injected with a non-moving plasmid producing the transposase. The transposase binds and cuts at the inverted repeats (IRs) surrounding the P-element and integrates it at another locus (figure 1.1). P-element transposition is naturally restricted to the germ line as splicing of intron 3 of the transposase is inhibited in somatic cells by a splicing repression protein (Siebel and Rio, 1990).

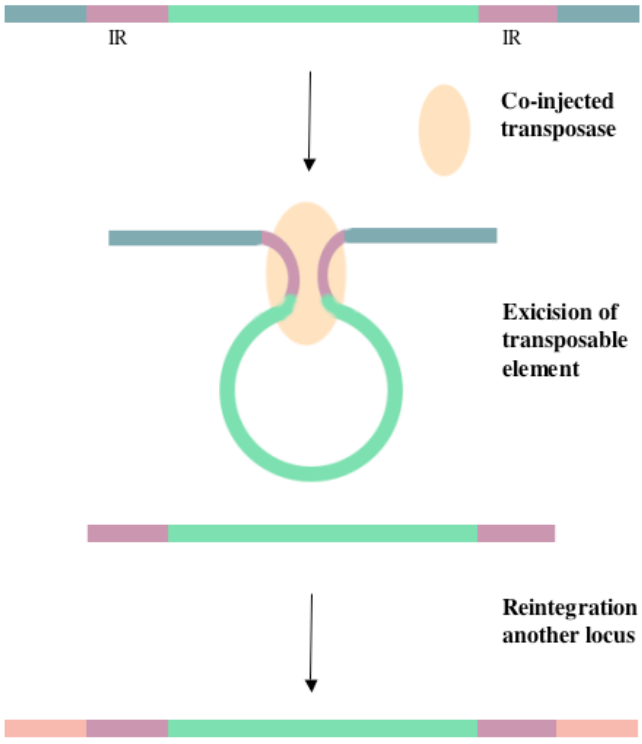

Figure 1.1 Schematic outline of transposition of a P-element.

The versatility of the P-element technology directed the development of several new genetic tools in the fly. One such was the pioneering use of enhancer traps to screen for genes based on their expression pattern (Bellen et al., 1989; O'Kane and Gehring, 1987), leading to both discovery of new genes and analysis of cis-regulatory interactions. In this enhancer trap system the transposon-based vector encodes a detectable reporter gene downstream of a minimal promoter. When the transposon is inserted near an endogenous genomic enhancer the reporter gene is expressed under control of these regulatory sequences, allowing identification of new enhancer sequences. Other tools include large-scale insertional mutagenesis screens with

[2] Transposase is the enzyme that mediates transposition of transposable elements. It seems to have two functions during this process: to recognize the inverted repeats (IR) at the ends of the transposons, and to cleave the target DNA.

engineered transposable P-elements (Cooley et al., 1988) and site-specific recombination using yeast flippase (FLP) recombinase for chromosomal rearrangements (Golic and Lindquist, 1989). In the FLP system, transgenic lines are generated using a combination of P-element vectors containing the FLP recombinase under control of the inducible heat shock protein 70 (hsp70) promoter, and vectors containing FLP recombination target (FRT) sites. The FLP recombinase acts on the FRT sites and induces recombination between these.

1.3.1 Temporal and spatial gene regulation in Drosophila

Another two-component system, the Gal4/UAS variant of the enhancer trap system was also developed in *Drosophila,* introducing spatial and temporal control of transgene expression using two transgenic lines combined, one activator line and one effector line (Brand and Perrimon, 1993; Fischer et al., 1988; Rorth, 1998). A P-element vector, containing the yeast *Saccharomyces cerevisiae* transcriptional activator Gal4 DNA binding protein downstream of a minimal promoter, is transposed to different chromosomal sites in various cell types, where flanking enhancer elements cause tissue-specific expression of the Gal4 protein. When an activator line expressing Gal4 is crossed into an effector line containing the Gal4 target upstream activating sequence (UAS) upstream a gene of interest, Gal4 will bind to the UAS, and initiate transcription of the downstream gene (figure 1.2).

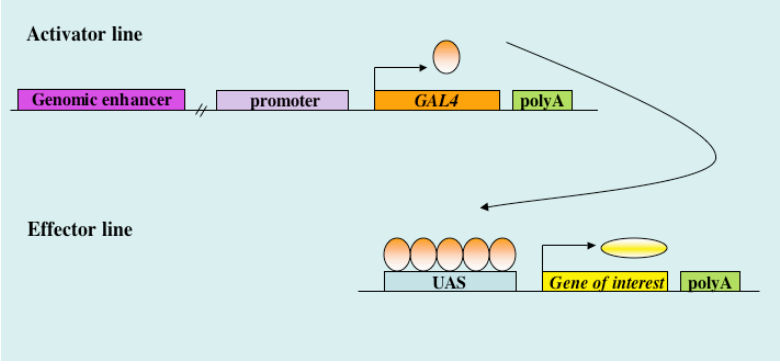

Figure 1.2 Schematic overview of the Gal4/UAS system. The Gal4 DNA binding protein binds the UAS sequences when present in the same cell, and activates transcription of the downstream gene of interest.

Spatially and temporally controlled ectopic expression is thus possible by a mix-and-match of activator and effector lines, combining various enhancers and genes of interest. Another application of this system is targeted cell ablation, where the UAS line carries *ricin A* that is a cytotoxic gene. Ricin A will kill the cell only when Gal4 is present to transactivate the gene (Hidalgo et al., 1995; McNabb et al., 1997). The

Gal4/UAS system has also been used to eliminate synaptic transmission by ectopic expression of the neurotoxin tetanus toxin light chain in nerve cells (Sweeney et al., 1995).

Combining the Gal4/UAS system with a random "tagging" of the genome using P-element technology (Rorth, 1996) a systematic gain-of-function genetic screen was carried out with a Gal4-regulated promoter oriented to transcribe flanking genomic sequences (Rorth et al., 1998). Another variant of this system is the dual-tagging gene trap system that identifies transgenic fly lines whose genes are inactivated by a P-element insertion (Lukacsovich et al., 2001). Upon insertion into the first intron of a gene, two fusion products are made, one containing the 5' part of the gene and Gal4, the other the 3' portion of the gene together with a selectable marker.

Mouse ES cells allowed modifications of the genome to generate or rescue mutations. As previously mentioned this technology is not available in the fly. However, a targeted gene replacement strategy has been developed in *Drosophila* (Rong and Golic, 2000). It takes advantage of both homologous recombination and the endogenous DNA repair machinery of the organism in a three-part system including a transgenic line expressing a site-specific FLP recombinase, a line expressing the site-specific *SceI* endonuclease (The *SceI* functions similarly to restriction endonucleases with the exception that its recognition site is much larger (18 bp) making it a very rare DNA cutter (Colleaux et al., 1988)), and a plasmid vector. Both transgenic lines are under control of the hsp70 promoter, and the vector contains a mutated form of the targeted DNA with incorporated FRT and *SceI* recognition sites. Crossing generates flies containing all three parts of the system, and the two enzymes produce an extra-chromosomal recombinogenic donor DNA molecule from the vector, allowing homology-directed changes in a target locus.

Drosophila has many obvious benefits as a model system, such as easy breeding and maintenance, the many genetic tools available, and a very short generation time. However, it is not a vertebrate and even though many of the signaling pathways are conserved between the fly and vertebrates, the molecular components cannot easily be transferred to vertebrate structures in a straightforward way. But the success, ease and speed of *Drosophila* genomics and the identification of most genes involved in development of the fly embryo, made researchers look for a

vertebrate model system that allows forward genetics and embryology in a high throughput manner.

1.4 Zebrafish as a model system for vertebrate development

To complement the mouse in studies of vertebrate developmental mechanisms, zebrafish was established as a model organism in the 1970s (Streisinger et al., 1981). Development of the embryo *ex utero*, transparency of the embryos, the high number of embryos available from each female and the easy breeding and maintenance are all important factors for the success of *Danio rerio* as a model system. Table 1.1 compares mouse, zebrafish and *Drosophila* as model systems. The ability to produce haploid zebrafish embryos by using UV inactivated sperm or eggs facilitated the detection of recessive mutations (Streisinger et al., 1981; Ungar et al., 1998). Due to its transparency one can observe phenotypic changes at the level of individual cells in the living animal, and cell lineages can be traced at all stages in the living embryo (Kimmel et al., 1990). The similarity of developmental programs, regulation and organogenesis among vertebrates makes zebrafish a good model for studies of human developmental diseases, by offering direct observation of tissue and organ development that neither the mouse, chicken or fly can provide (reviewed in Dodd et al., 2000).

Table 1.1 Comparison of fly, mouse and fish as model systems.

Features	**Fly**	**Mouse**	**Zebrafish**
Species	*Drosophila melanogaster*	*Mus musculus*	*Danio rerio*
Generation time	10 days	Three months including gestation	2-3 months
Tractability	Very low-cost	High –cost	Low-cost
Fecundity	High. Up to 50/day	Low. Litter size 10-12	High. 50-200/week
Development of embryo	10 days into mature fly, via three larval stages	19-21 days gestation period	Hatching after 2-3 days (all organs formed after 72 hours)
Gene knock-out technology	Yes	Yes	Limited
Embryo accessibility	Fair, develop as larva, but organs not visible	Develop *in utero*	Excellent, develop *ex utero*
Embryos translucent	No	No	Yes
Embryonic stem cells	No	Yes	No

The history of germ-line transgenics in zebrafish is similar to the early advancements of transgenesis in mouse. Plasmid injection was primarily used to make transgenic fish for examining promoter regions responsible for regulation of genes important to

embryonic development (Bayer and Campos-Ortega, 1992; Lin et al., 1994; Stuart et al., 1988). The founder fish (F_0) were mosaic and the transgene must be inherited through the germ line to generate stable transgenic lines. Plasmid injections indicated a transgenic frequency around 5% (Stuart et al., 1988; Stuart et al., 1990), though transmission rates as high as 20% was observed (Culp et al., 1991).

By using the *Sce*I meganuclease system one can increase the transgenic frequency of plasmid injections up to 30% in fish (Thermes et al., 2002). The enzyme is co-injected with plasmid DNA containing the transgene of interest flanked by two *Sce*I recognition sites, and induces double stranded breaks in the plasmid. Use of *Sce*I allows an earlier integration event that leads to a higher germline transmission rate.

1.4.1 Large-scale mutagenesis screens in zebrafish

The rapidly developing translucent embryos allowed systematic genome wide mutagenesis screens for visible defects in zebrafish, a method prohibitively costly in mouse. Two different approaches identified mutations affecting wide-ranging aspects of development. One was the effort of two laboratories in a large-scale mutagenesis screen (see the special issue of Development 123: 1996) where male fish were mutagenized with ethyl nitrosourea (ENU), and the F_3 generation screened for visible mutations. In this screen over a thousand mutants with defects in 372 genes were identified and characterized (Haffter et al., 1996). The disadvantage of this approach was that ENU does not provide any molecular "tags" to identify the mutated genes, thus making it a laborious task to clone the genes by position.

Another approach was taken in the laboratory of Nancy Hopkins, where genes were mutagenized using random insertion of retroviral vectors into the genome (Gaiano et al., 1996). The retrovirus was a pseudotyped virus with a genome based on the M-MuLV and an envelope glycoprotein (G protein) from the vesicular stomatitis virus. The G protein gives the virus a broader host–cell range, making it possible to infect zebrafish cells (Burns et al., 1993), reviewed in (Amsterdam and Becker, 2005). This retroviral insertion strategy has the advantage that the mutated gene can easily be identified using inverse PCR, enabling cloning of the gene in as little as two weeks (Golling et al., 2002). This strategy was leading to the mutation and identification of 315 genes essential for embryonic development (Amsterdam et al., 2004).

1.4.2 Transposons in zebrafish

A powerful transposon technology as used in *Drosophila* had not been developed in zebrafish or other vertebrate models, as no active transposons had been found in any vertebrate. To overcome this problem, several approaches were made. One was to derive a synthetic transposon system from ancient remnants of once active transposable elements in fish related to the *Tc1/mariner* superfamily of transposons (reviewed in Plasterk et al., 1999). These sequence elements were used to reconstruct an ancestral fish transposable element, named *Sleeping Beauty (SB)* (Ivics et al., 1997). *SB* can create chromosomal insertions in zebrafish germ cells (Davidson et al., 2003) and can be used as an enhancer trap system (Balciunas et al., 2004).

A second approach came with the identification of the *Tol2* transposable element naturally present in the genome of the freshwater fish medaka (*Oryzias latipes*), encoding a gene for a fully functional transposase capable of catalyzing transposition during embryonic development in medaka (Koga et al., 1996). Using an embryo excision assay where zebrafish embryos were injected with plasmid DNA harboring the *Tol2* element, Kawakami and colleagues showed that the *Tol2* element could be excised from the injected plasmid. This indicated that *Tol2* is an autonomous transposon and is active in the zebrafish germ lineage (Kawakami et al., 1998). It was later shown to be active in mouse ES cells as well (Kawakami and Noda, 2004). *Tol2* can be transposed from an injected plasmid to the germline and be transmitted to the next generation (Kawakami et al., 2000) with a germline transmission frequency of about 50% (Kawakami et al., 2004). A trapping frequency of 8-40% per insertion and 12-23% per injected fish (Kawakami et al., 2004; Parinov et al., 2004) makes *Tol2*-mediated transgenesis better suited for gene and enhancer trapping than *SB* in fish (reviewed in Kawakami, 2005).

Interestingly, *SB* has been used with great success for somatic insertional mutagenesis in mouse. By using this technology no ES work is required, and new mutations can be generated just by breeding mice that are double transgenic for a transposon and the *SB* transposase (reviewed in Carlson and Largaespada, 2005). The system has been shown to have great potential for generating specific mouse models for human cancers and for cancer gene discovery (Collier et al., 2005).

1.4.3 Use of other genetic tools in zebrafish

For zebrafish to be able to compete with mouse and *Drosophila* as genetic model organisms, a broad range of genetic manipulation tools needed to be available in the fish. Application of the Gal4/UAS targeted expression system adapted from *Drosophila* (Brand and Perrimon, 1993; Fischer et al., 1988) was reported in zebrafish (Scheer and Campos-Ortega, 1999). In this system, a stable transgenic activator line expressing Gal4 under control of specific promoters were crossed with an effector line carrying the *Notch1a-intra* gene downstream of UAS resulting in *Notch 1a-intra* expression in cells expressing Gal4 (Scheer and Campos-Ortega, 1999). A slightly different approach was to create transgenics using the activator Gal4-VP16, a fusion of Gal4 DNA-binding domain with the strong transcriptional activation domain VP16 from the herpes simplex virus (Sadowski et al., 1988) to drive expression of a reporter gene in zebrafish through UAS (Koster and Fraser, 2001b). Although mosaic, the expression of the reporter genes was strong, and the tool is well suited for time lapse analysis of behavior and fate of single cells after ectopic expression of a gene of interest (Koster and Fraser, 2001a). In medaka, the hsp70 promoter was used to drive expression of a reporter gene in the Gal4-VP16/UAS system, also combined with the IRs from the *Sleeping Beauty* transposon and the *SceI* meganuclease system which offered to overcome the mosaicism of the former system as integration into the genome is expected to happen earlier than with naked plasmid. 16% of the injected F_0 embryos showed highly uniform expression of reporter gene (Grabher and Wittbrodt, 2004).

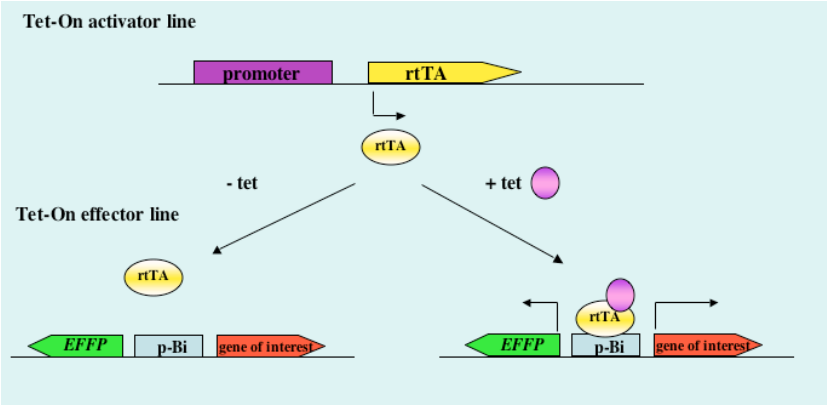

Figure 1.3 Schematic outline of the Tet-On system.

The Tet-On and Tet-Off gene expression systems developed in mammalian cells (Gossen and Bujard, 1992) offer precisely regulated control of transgene expression, using tetracycline (Tet) as an inducer. In this system a chimaeric Tet responsive transactivator (tTA or rtTA) is expressed from a promoter of choice (figure 1.3). In

the Tet-Off system tTA binds to a Tet Responsive Element (TRE) and activates transcription in the absence of the inducer Tet, and conversely in the Tet-On system a mutated form of tTA, rtTA binds TRE and activates transcription in the presence of Tet (Gossen et al., 1995). The transactivator is based on regulatory elements from the Tn10 tetracycline–resistance operon of *E. coli* (Gossen and Bujard, 1992). By titrating the Tet dose, or its more stable derivative doxycycline, tight control of gene expression can be accomplished. When a gene has to be kept silent during developmental studies and gene therapy, the Tet-On system is regarded as more useful than the Tet-Off (Jost et al., 1997). The Tet-On system can drive heart-specific expression in transgene zebrafish lines (Huang et al., 2005), so far the only report of the use of this system in zebrafish.

For the identification of vertebrate gene regulatory sequences the already mentioned transposon based enhancer trap system *Tol2* has been very useful (Parinov et al., 2004). In addition a large-scale enhancer detection screen based on the retroviral M-MuLV has successfully been carried out in zebrafish (Ellingsen et al., 2005). This paper reported the generation of 95 transgenic lines with distinct expression patterns of a reporter gene during embryonic development, and of these, 65 insertions were mapped to zebrafish genomic sequence.

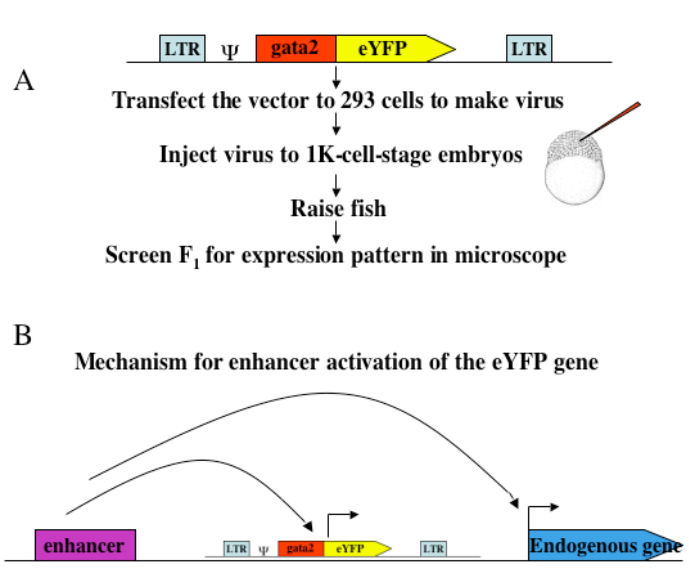

Figure 1.4 (A) Diagram of generation of enhancer trap lines using the CLGY retroviral insertion. (B) A genomic enhancer activates eYFP expression when the retrovirus is integrated in the proximity of the enhancer (after Ellingsen et al., 2005).

The retroviral vector used in this screen contained the zebrafish Gata2 minimal promoter and the fluorescent eYFP reporter gene (figure 1.4). More than 1000 transgenic lines of zebrafish expressing eYFP in tissue specific patterns have been generated so far, and 380 of these insertions have been mapped to the zebrafish genome using the Ensembl database

(www.ensembl.org/Danio_rerio). The average rate of identifying an enhancer detection event in the F_1 progeny in this screen was one in three founders (Ellingsen et al., 2005). As the majority of the activated virus integrations were close to the transcriptional start site or within a gene, the use of these enhancer detection lines as chromosomal marker lines for certain genes could also become an important tool. Having a marker line available for a mutation allows visual identification of a genetic mutant before the phenotype is evident.

1.4 4 Reverse genetics- Morpholinos and TILLING

One of the major goals in genomics is to assign functions to genes, and this can be done by both forward and reverse genetics. Forward genetics is the traditional approach where a phenotype is identified, followed by the sometime laborious mapping of the mutation causing this phenotype. As more and more genomic sequence data is available for the zebrafish[3], sequence information is accumulating faster than functional information about the gene. This has lead to development of reverse genetics, an approach to discover the function of a gene based on information about the sequence of the gene.

The most common reverse genetics method in zebrafish is the use of morpholino (MO) knockout technology (Nasevicius and Ekker, 2000). The MO antisense oligonucleotides are designed to block the translation or splicing of a messenger RNA by binding specifically to the 5'-untranslated region (UTR), the start codon or the splice acceptor sites. In the MO oligonucleotide the ribonucleotide backbone has been converted to a MO moiety, with a phosphorodiamidate intersubunit binding replacing the phosphodiester binding, thus preventing degradation of the oligonucleotide (Summerton and Weller, 1997). Injection of MO antisense into the 1-2 cell stage of fertilized embryos is efficiently blocking transcription of the gene of interest. This rapid, targeted "knockdown" technology has been applied in a wide range of model organisms, including screens to identify novel

[3] The sequencing of the 1.6 -1.7 gb zebrafish genome was initiated in spring 2001 by the Sanger Institute, using both a whole genome shotgun approach and a traditional clone mapping and sequencing technique. After problems emerged posed by the high polymorphism rate in the initial DNA sources, a library from a single double haploid fish (Streisinger et al., 1981) was used as a reference in the mapping and assembly process. The latest assembly Zv6 (as of August 2006) comprises a sequence length of 1.63 gb in 6653 fragments. It is generated on 7,615 clones placed onto the physical map. Remaining gaps were filled with contigs from a 10x whole genome shotgun assembly (www.sanger.ac.uk).

genes with developmental function in both vertebrates and ascidians (Kenwrick et al., 2004; Yamada et al., 2003). However, it has its limitation in that it is a transient method, and mostly suited for early developmental stages.

No method for generating knockouts in zebrafish using ES cells has yet been established, but the TILLING technology (Targeted Induced Local Lesions IN Genomes) has been shown to be efficient for target-selected mutations in zebrafish (Wienholds et al., 2002; Wienholds et al., 2003). TILLING was developed in the plant *Arabidopsis thaliana*, using traditional chemical mutagenesis followed by denaturing high-performance liquid chromatography to detect base pair changes in the offspring (McCallum et al., 2000). Nowadays TILLING technology is mainly based on enzymatic cleavage of heteroduplex DNA followed by direct sequencing of the genome to identify mutations (Wienholds et al., 2003). TILLING can be a very useful tool in the development of zebrafish models for human diseases, as zebrafish homologues of human disease genes easily can be mutated, and the development of the disease can be characterized and studied.

1.5 Application of transgene technology

The knowledge obtained from studies on transgenic organisms has allowed the development of a wide range of medical techniques beneficial to humans, such as gene therapy, genetic testing and molecular medicine. The information gained from this technology is relevant for almost any field of modern biology, including gene regulation, studies of the immune system, organogenesis and the function of oncogenes. As summarized here, using transgenic animals for the study of genes and promoters have become a routine procedure in the laboratory, and from the early start of a plain reporter construct with lacZ or GFP under control of a promoter of interest, the development of new tools have been fast and increasingly more sophisticated. Transgenic animals can also be used as markers, both as developmental markers where specific cell types are expressing a fluorescent protein, and as positional markers on chromosomes, similar to the balancer chromosomes in *Drosophila*.

1.6 NRF1 - Nuclear respiratory factor 1

The nuclear respiratory factor NRF1 was identified as a nuclear transcription factor (TF) regulating transcription of many mitochondrial genes responsible for energy transduction (Evans and Scarpulla, 1989; Virbasius et al., 1993). Simultaneously, α-Pal was discovered as a key TF for the *eIF2 α*-subunit (Jacob et al., 1989). The name α-Pal derives from its binding to a palindromic sequence (Efiok et al., 1994). As NRF1 and α-Pal are products of the same gene, they are referred to as NRF1 hereafter. NRF1 belongs to a class of TFs containing an unusual putative basic leucine zipper (bZIP) DNA binding domain[4] (Efiok et al., 1994). It functions in mitochondrial respiration and cell growth regulation, as well as both insulin regulation (Patti et al., 2003) and central nervous system (CNS) development (Becker et al., 1998; Solecki et al., 2000).

1.6.1 Mitochondrial DNA and respiration

The mitochondrion is involved in several metabolic pathways, including biosynthesis of heme, amino acids, nucleotides, phospholipids and other metabolites, but the best known function of the mitochondrion is to serve as the cells own power supply. The oxidative phosphorylation system is located in the inner membrane of the mitochondrion and produces most of the cellular ATP from a flow of electrons along an electron transport chain. In vertebrates this chain consists of ubiquinone and cytochrome c in addition to four protein complexes (I-IV). ATP synthase uses the electrochemical gradient generated over the membrane from the electron transport chain to synthesize ATP. Each of the components in this chain plays a vital role in the health of the cell and mutations in any one of the proteins that make up these complexes can lead to cell death or stress, which in turn can cause a number of diseases. Mitochondria can also be related to cell type, and can perform specific functions based on the need of their "host" cell. For example mitochondria in brown adipose tissue produce heat needed for adaptive thermoregulation of the body by uncoupling respiration (reviewed in Mozo et al., 2005) and in the liver of tetrapods mitochondria are needed for detoxification of ammonia (reviewed in Campbell,

[4] The bZIP motif consists of a region enriched in basic amino acids adjacent to a leucine zipper characterised by several leucine residues regularly spaced at seven-amino acid intervals. The basic region is mediating sequence specific DNA-binding, while the leucine zipper region is required for protein dimerisation.

1997). This illustrates that dysfunctional mitochondria will not only lead to lower energy production in the cell, but also that cell specific products can be affected.

Mitochondria have their own circular DNA genome (mtDNA). The small mitochondrial genome has a limited coding capacity, encoding the rRNA and tRNAs needed for mitochondrial protein synthesis in addition to 13 of the close to one hundred mRNAs needed for the respiratory chain proteins (reviewed in Enriquez et al., 1999). Nuclear genes provide the remaining respiratory subunits and all of the proteins necessary for mtDNA transcription and replication. As the energy demand of the mitochondrion changes depending on the activity of the cell, the expression of mitochondrial and nuclear encoded subunits of the respiratory chain must be tightly regulated and coordinated. One model for this regulation is that the same TF, or a small set of TFs, can regulate both nuclear genes needed for mitochondrial respiration as well as mitochondrial regulatory genes, linking the nuclear and the mitochondrial genetic system. A support for this model came with the identification of the two TFs, NRF1 and NRF2/GABP (Evans and Scarpulla, 1989).

1.6.2 NRF1 and the mitochondrion

Studies of cytochrome c and cytochrome oxidase subunits led to identification and cloning of *Nrf1* in mouse cells together with a genetically and structurally unrelated partner, *Nrf2/GABP* (Evans and Scarpulla, 1989; Evans and Scarpulla, 1990; Virbasius et al., 1993).

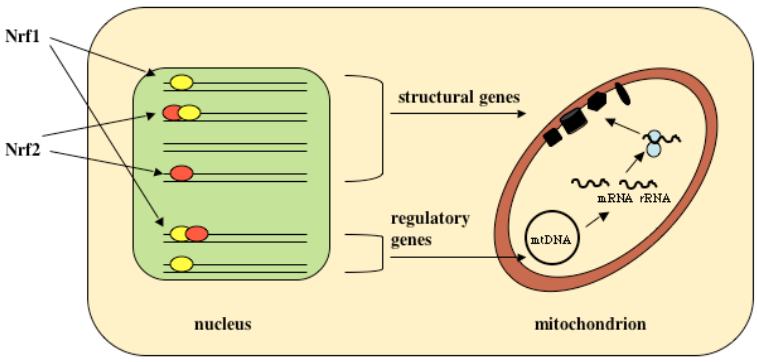

Figure 1.5 Nuclear genes are regulating expression and transcription of mtDNA (regulatory), as well as encoding subunits of the respiratory complexes (structural). (Figure modified from Enriquez et al., 1999).

NRF1 is a nuclear encoded TF acting on nuclear genes encoding both mitochondrial structural genes, that is components of the electron transport chain, and mitochondrial regulatory genes (figure 1.5).

One of these regulatory proteins is the mitochondrial transcription factor A

(mtTFA) required for mtDNA transcription and replication (Fisher and Clayton, 1988; Parisi and Clayton, 1991). MtTFA is synthesized in the cytoplasm and imported into the mitochondrion, and is necessary for mtDNA maintenance and also for embryogenesis in mouse (Larsson et al., 1998). This was supported by the findings that loss-of-function of the yeast mtTFA homolog leads to loss of both mtDNA and respiratory function (Diffley and Stillman, 1991). It has been shown that mtTFA activation is highly dependent on NRF1 and NRF2 binding, although NRF1 appears to be the major determinant of promoter function (Virbasius and Scarpulla, 1994), suggesting that NRF1 is important for a coordinated regulation of nuclear as well as mitochondrial genes. This is further supported with the findings that NRF1 is important for promoter function in the mitochondrial transcription specificity factors TFB1M and TFB2M (Gleyzer et al., 2005).

Another regulatory gene under control of NRF1 is the RNA moiety of the mitochondrial RNA processing RNase (MRP RNase) (Evans and Scarpulla, 1990), participating in the transition between transcription and replication by cleaving transcripts to generate primers for mitochondrial DNA replication (Chang and Clayton, 1987).

NRF1 controls expression of several mitochondrial structural genes. One of the main components of the electron transport chain, cytochrome c, depends on functional NRF1 binding sites within its promoter for maximal activity (Evans and Scarpulla, 1989), and also the gene coding for the cytochrome c oxidase subunit of complex VI has a functional NRF1 binding site in its promoter (Evans and Scarpulla, 1990). NRF1 also acts in transcriptional expression of the gene encoding 5-amino-levulinate synthase (5-ALAS), the rate-limiting enzyme of heme biosynthesis within the mitochondrial matrix (Braidotti et al., 1993). Heme is an essential cofactor for the activity of respiratory cytochromes that are encoded by both the nuclear and mitochondrial genomes, and this control of heme biosynthesis is another indication of a function for NRF1 in intergenomic communication.

One interesting discovery was the physical interaction between NRF1 and PGC1 (PPARγ- coactivator 1), where PGC1 increased the transcriptional activity of NRF1 in the upregulation of mitochondrial genes (Wu et al., 1999). PGC1 has been shown to be important in the adaptive thermoregulation by regulating uncoupling of the electron transport chain in mitochondria (Puigserver et al., 1998), as well as in

controlling β-oxidation of fatty acids (Vega et al., 2000). The process of thermoregulation is complex, and also requires an increase in the number of mitochondria and of the activity of the electron chain transport system (Nicholls et al., 1986), possibly explaining the link between NRF1 and PGC1. In Type 2 diabetes mellitus patients, the expression of both *PGC1* and *NRF1* is reduced, leading to decreased expression of oxidative phosphorylation genes, reduced fatty acid oxidation, and accumulation of lipids in skeletal muscle cells resulting in obesity (Patti et al., 2003).

1.6.3 NRF1 and other genes

In addition to its role in mitochondrial expression, NRF1 is also implicated in coordinating regulation of key metabolic genes in response to changes in the status of the cell, such as the α- subunit of the eIF2 protein (Efiok et al., 1994; Jacob et al., 1989). EIF2 catalyses the binding of Met-tRNA to 40 S ribosomal subunits, a rate limiting step during translational initiation (Evans and Scarpulla, 1990), and the eIF2α subunit is a target for regulation of protein synthesis in response to growth activation and metabolic changes (Cohen et al., 1990).

Many other genes have been reported to have functional NRF1 binding sites in their promoters. As seen from table 1.2 these include the human poliovirus receptor *CD155* (Solecki et al., 2000), the *GluR2* subunit of the neural AMPA receptor in the rat (Myers et al., 1998), the *IAP/CD47* gene (Chang and Huang, 2004), the *CXCR4* receptor involved in chemokine signal transduction (Wegner et al., 1998), and genes involved in purine nucleotide biosynthesis (Chen et al., 1997).

Table 1.2 Overview of genes regulated by Nrf1.

Gene	Function	Pos/neg regulated	Reference
P115	Vesicular docking protein	Positive	(Watanabe, 2003)
CD155	Cell adhesion and motility	Positive	(Solecki et al., 2000)
IAP/CD47	Membrane protein, membrane transport and signal transduction	Positive	(Chang and Huang, 2004)
GluR2 AMPA	Neurotransmitter receptor	Positive	(Myers et al., 1998)
CXCR4 rec	Chemokine receptor at cell surface	Positive	(Wegner et al., 1998)
5'-ALAS	Heme biosynthesis	Positive	(Braidotti et al., 1993)
MtTFA	Mitochondrial transcription factor	Positive	(Virbasius and Scarpulla, 1994)
TFB1M/ TFB2M	Mitochondrial transcription factor	Positive	(Gleyzer et al., 2005)
Cyt c	ATP synthesis in mitochondria	Positive	(Evans and Scarpulla, 1989)

Cyt c oxidase	ATP synthesis in mitochondria	Positive	(Evans and Scarpulla, 1989)
ATP synthase γ-subunit	ATP synthesis in mitochondria	Positive	(Chau et al., 1992)
MRP RNA	MtDNA regulation	Positive	(Evans and Scarpulla, 1990)
Tyrosine aminotransferase	Methionine recycling	Positive	(Chau et al., 1992)
eIF2α	Protein synthesis initiation	Positive	(Chau et al., 1992; Efiok et al., 1994)
E2F1	Transcription factor	Negative	(Efiok and Safer, 2000)
E2F6	Transcription factor	Positive	(Kherrouche et al., 2004)
FMR1	Unknown, mutation causes the Fragile X syndrome with mental retardation	Positive	(Kumari and Usdin, 2001)
GPAT/AIRC	Purine biosynthesis	Positive	(Chen et al., 1997)
Zfp106	Unknown function	Positive	(Grasberger et al., 2005)
Histone 5	Linker histone, component of chromatin structure	Negative	(Gomez-Cuadrado et al., 1995)
CyIIIa	Cytoskeleton actin of aboral ectoderm	Negative	(Zeller et al., 1995)

Proteins active in the mitochondrion
Various receptors
Others

1.6.4 Homologues of Nrf1

Regardless of the name there is only one copy of *Nrf1* in vertebrates, and no other family members have been identified so far (Huo and Scarpulla, 1999). *Nrf1* homologues have been cloned from human (Evans and Scarpulla, 1990), mouse (Schaefer et al., 2000), chick (*ibr*) (Gomez-Cuadrado et al., 1995) and fish (*nrf1*) (Becker et al., 1998), and show strong homology within the vertebrate lineage with 91% amino acid identity between the human and the zebrafish homologue. Two invertebrate homologues were also characterized, sea urchin (*Strongylocentrotus purpuratus*) P3A2 and *Drosophila* erect wing (EWG) (Calzone et al., 1991; DeSimone and White, 1993).

Sequence analysis reveals four conserved regions in all NRF1 homologues, a nuclear localization signal (NLS), a bZIP DNA binding domain, a C-terminal transcription activation domain (Gugneja et al., 1996) and an inhibitory region in the N- terminus (Fazio et al., 2001), indicating that NRF1 can function both as an activator and a repressor (table 1.2). The activation domain has the lowest sequence similarity between the species. The NRF1 protein binds as a homo dimer to a palindromic sequence (T/C)GCGCA(T/C)GCGC(A/G) (figure 1.6) (Efiok et al., 1994; Gomez-Cuadrado et al., 1995; Virbasius et al., 1993), and phosphorylation on serine residues within the amino terminal domain enhances the DNA binding activity

of the dimer (Gugneja and Scarpulla, 1997). Glycosylation of the chicken homolog, IBR, represses transcription of the histone H5 gene, suggesting that posttranslational modifications might be important for NRF1 function.

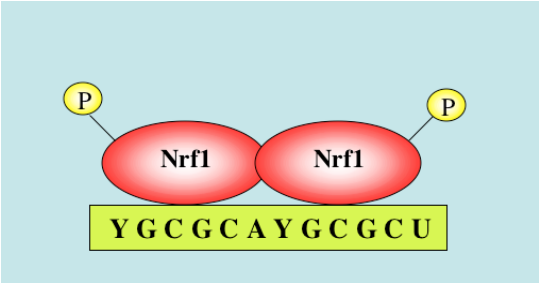

Figure 1.6 Schematic depiction of NRF1 binding to the palindromic DNA recognition sequence. NRF1 binds as a homo dimer. Y = pyrimidine nucleotide, U = purine nucleotide. Figure modified from (Scarpulla, 2002a).

1.6.5 Downregulation of NRF1

Loss of *Nrf1* function leads to different phenotypes in both vertebrates and invertebrates. The sea urchin NRF1 homologue P3A2 represses the expression of the *CyIIIa* cytoskeletal actin gene in the oral ectoderm of the embryo (Hoog et al., 1991; Zeller et al., 1995). Loss of function of *P3A2* affects morphogenesis of the archenteron (a structure later to develop into the lumen of the digestive tract) and leads to embryonic lethality prior to gastrulation (Bogarad et al., 1998). *Drosophila* EWG is involved in indirect flight muscle and CNS development (DeSimone and White, 1993), and certain mutated alleles of the *ewg* locus result in late embryonic or larval lethality, apparently due to defects in the nervous system, while others only cause abnormalities of the indirect flight muscle (DeSimone et al., 1996). Although the protein is localized to the nucleus of virtually all embryonic neurons (DeSimone and White, 1993), its transcriptional activity in the fly has not been demonstrated nor have any target genes been identified.

Transgenic knockout mice were generated where the part of *Nrf1* encoding the NLS and DNA binding and dimerization domain were replaced with a *β-galactosidase* gene by homologous recombination, and this loss of function of *Nrf1* led to embryonic lethality around the time of implantation (Huo and Scarpulla, 2001). Isolated blastocysts from *Nrf1* knockouts lacked the ability to develop further *in vitro* despite having normal morphology, and a reduction of mtDNA in the blastocysts provided the first evidence that NRF1 is needed for mitochondrial maintenance *in vivo* (Huo and Scarpulla, 2001). A common feature of all these loss of function

experiments is that loss of function mutations leads to developmental arrest of the early embryo in both vertebrates and invertebrates.

Down regulation of *Nrf1* in cell culture was reported to result in faster cell cycling, and failure to withdraw from cell cycle in response to absence of growth signal, followed by an increased rate of apoptosis, an effect similar to the overexpression of E2F1 in cells (Efiok and Safer, 2000).

1.7 The zebrafish *nrf* mutant

The zebrafish *nrf* mutant, identified in a retroviral insertional screen (Becker et al., 1998) was an insertion in the first intron of the gene encoding *nrf1*. The mutant phenotype evident at day 5 of development is larval lethal, and homozygous mutant embryos die between 10 and 14 dpf, much later than the mouse, *Drosophila ewg* and sea urchin P3A2 mutants. When compared to wild type fish the mutant is characterized by smaller eyes and a protruding snout (figure 1.7).

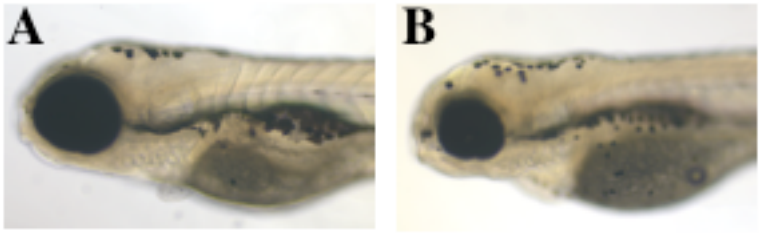

Figure 1.7 (A) Wild type embryo (B) *nrf* mutant embryo at 5 dpf. Note the smaller eyes of the *nrf* mutant.

The eye phenotype of the *nrf* mutant is due to a loss of photoreceptor (PR) cells. *In situ* hybridization shows *nrf1* expression in the developing eyes, ears and CNS at 24 hpf (Becker et al., 1998). At this time *nrf1* expression is observed in every cell in the eye, but at 48 hpf expression is restricted to the ganglion cell layer, the optic nerve and the optic tracts. Very low expression is detected in PR cells. At 5 dpf almost all PRs have undergone apoptosis, but the ganglion cell layer seems to be intact. Nevertheless, apoptotic cells can be found in all neuronal cell layers in the developing eye, but are clearly more pronounced in the PR layer. Some photoreceptors survive, mainly in the central part of the retina (figure 1.8). An optokinetic response test showed that these remaining PRs are functional in allowing movement detection. Elevated levels of apoptosis in the optic tectum, the region of the brain that receives the signals from the eye, have also been detected (Becker et al., 1998).

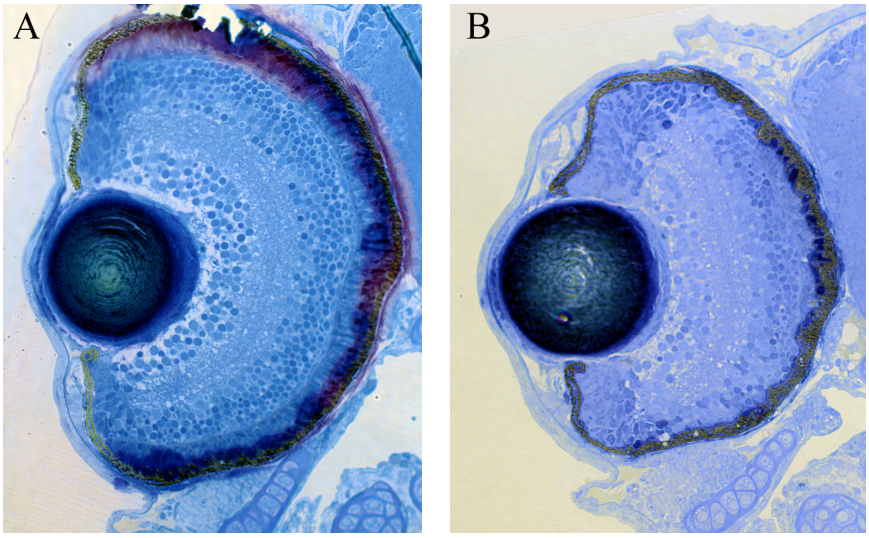

Figure 1.8 Plastic sections of eyes of (A) wild type and (B) *nrf* mutant eye at 6 dpf.

1.7.1 Retina and transport to the PR outer segment

Retinitis pigmentosa (RP) is a group of retinal degenerative diseases and one of the major causes of human blindness due to degeneration of retinal PRs, but the genetic foundation of retinal maintenance remain incompletely understood. There is no treatment to these diseases that are affecting about 1 in 5000 individuals worldwide (Rivolta et al., 2002).

The neural retina of vertebrates develops from an undifferentiated epithelium into a sensory structure containing seven retinal cell types. One of these, the PRs converts light into nerve impulses. They consist of an inner segment (IS) and outer segment (OS), connected by a cilium (figure 1.9). The nucleus as well as all of the cell machinery is located in the IS. The OS consisting of membrane discs with the light receiving opsins inserted has a high turnover rate, shown in rodent cells that renew their outer segments every 10 days (Young, 1967). The OS is therefore dependent on efficient transport of biosynthesis products from the IS. Opsins are synthesized in the proximal region of the IS and transported via the connecting cilium to the OS (Deretic and Papermaster, 1991; Marszalek et al., 2000; Pazour et al., 2002). This transport is mediated via the highly conserved intraflagellar transport (IFT) system of the cilium. This high-speed transport system involving microtubules and specific transport proteins including kinesin II, dynein and IFT particles (reviewed in Scholey, 2003) is thought to be required for the maintenance of all eukaryotic cilia (Baker et al., 2003). The IFT machinery is dependent on energy in the

form of ATP, as ATP runs the kinesin II transport through the connecting cilium (Baker et al., 2003).

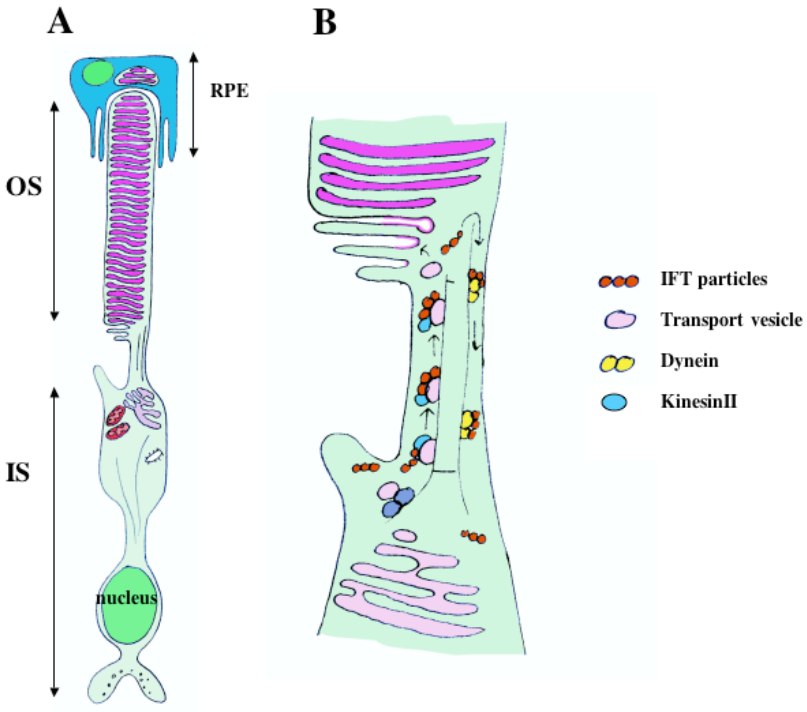

Figure 1.9 (A) The PR is subdivided into an inner (IS) and outer segment (OS), connected by a cilium. The retinal pigmented epithelium (RPE) trims the PRs by phagocytosis of the OS (B) Schematic model of the transport system through the connecting cilium in PRs. Figure adapted from (Williams, 2002) and (Besharse et al., 2003).

Defects in the intraflagellar transport system have been observed in several zebrafish mutants (Doerre and Malicki, 2002; Sun et al., 2004). With its ease of generating transgenic animals and large collections of mutants and enhancer detection lines, zebrafish will undoubtedly aid in understanding vertebrate photoreceptor maintenance in general and retinopathies in particular.

2 AIMS OF THE PRESENT STUDY

When this study was initiated, the *Nrf1* gene had been studied in mammalian cell cultures, but very little was known about the function of NRF1 *in vivo*. It was known that the zebrafish photoreceptors undergo apoptosis when the *nrf1* gene was mutated, and that the mutant fish die at two weeks of age (Becker et al., 1998).

There were two major goals for this thesis. The first was to develop a reliable method for generation of transgenic zebrafish overexpressing *nrf1*. The second aim of this study was to further characterize the *nrf1* gene and the *nrf* mutant in zebrafish, and to learn more about the function of the gene in the maintenance of retinal photoreceptors. As the work proceeded, new technology also allowed us to investigate the gene expression profile of the *nrf* mutant and to identify putative downstream targets for Nrf1.

3 MATERIALS AND METHODS

3.1 Animals

Zebrafish (*Danio rerio*) embryos were obtained from a breeding colony by natural spawning and maintained under standard conditions (www.sars.no/manual.doc). The wild type strains used were either a hybrid of Tüebingen and AB (termed TAB) or TU with tup/tup (spotty) pigmentation. Developmental age is given as hours post fertilization (hpf) or days post fertilization (dpf) at 28.5°C. Embryos were dechorionated chemically with 0.1 mg/ml PronaseE (Sigma) in Holtfreter's solution[5] (<24 hpf) or manually using needles (>24 hpf). After chemical dechorionation embryos were kept in Holtfreter's solution with 1 mM Hepes (pH 7.2), and staged as described (Kimmel et al., 1995).

The zebrafish *nrf* mutant (*nrf*^{hi399}) is due to a retroviral insertion in the first intron of the *nrf1* gene (Becker et al., 1998). At 5 dpf, the eyes of the *nrf* mutant are smaller than the ones of wild-type larvae, and homozygous mutants can be recognized using a dissecting microscope. Adult *nrf* heterozygous fish were identified by PCR using SFG primers as described (Becker et al., 1998).

3.1.1 Zebrafish CLGY298 line

The zebrafish CLGY298 transgenic line is an enhancer detection line expressing the yellow fluorescent protein (eYFP) in a distinct pattern resulting from a single activated retroviral insertion around position 16.98 mb on chromosome 4 (Ellingsen et al., 2005). Fish with the CLGY298 insertion were identified between 24 and 48 hpf using a Nikon TE2000-S inverted microscope, with a 500/20 nm excitation filter and a 515 nm BP emission filter (Chroma)/515 dichroic mirror.

3.2 Microinjections

Injections were accomplished with a pressure injector (Picospritzer® II, Parker Instrumentation) under a dissecting microscope (Nikon, C-D5). Injection needles were prepared from Borosilicate glass capillaries (Sutter Instruments) by using a micropipette puller (Model P-87, Sutter Instruments). Dechorinated embryos were

[5] Holtfreter's solution: 60 mM NaCl, 0.67 mM KCl, 0.9 mM $CaCl_2$

injected at the 1-cell stage directly into the cytoplasm, and the injected embryos were raised in Holtfreter's solution with 1 mM Hepes and 500 μg/ml Penicillin G and 500 μg/ml Streptomycin sulphate (Sigma) to inhibit microbial growth. After 3 days in 24-well plates they were transferred to Petri dishes, and raised as recommended (www.sars.no/manual.doc). For a more thorough description of microinjections, see (Malicki et al., 2002).

3.2.1 DNA preparation and microinjection

Plasmid DNA for injection was prepared using Qiagen Maxi prep Kit (Qiagen). The plasmids were digested with the appropriate endonucleases to remove the fragment from the vector, except when co-injecting with the *Sce*I meganuclease. Linearized DNA was purified via agarose gel electrophoresis and recovered from the gel using gel extraction columns (Qiagen). DNA used for injection was resuspended in dH_2O. The injection solution consisted of 100 ng/μl plasmid DNA, 0.1 M KCl and 0.05% w/v phenol red (Sigma), and was filtered through 0.22 μm micro centrifuge filters (Sigma) before use.

3.2.2 Meganuclease injections

*Sce*I meganuclease (NEB) was co-injected with filtered injection solution (100 ng/μl circular plasmid DNA, 0.2 U/μl *Sce*I 0.5 x commercial *Sce*I meganuclease buffer (NEB), 0.05% w/v phenol red) into 1 –2 cell embryos (Thermes et al., 2002). The enzyme was aliquoted upon arrival and stored at -80°C until use as the efficiency drops dramatically at -20°C (Grabher et al., 2004).

3.2.3 Morpholino injections

The morpholino oligonucleotides (MOs) were designed complementary to the *nrf1* 5'UTR as recommended by the manufacturer (Gene Tools). MOs used were *nrf1* antisense 1.0 and *nrf1* antisense 2.2 (table 3.1). These MOs were also tested for similarity to sequences elsewhere in the genome. Control MO was a standard control from Gene Tools with no target and no significant biological activity with known sequences. Each MO was injected as described above in 1x Danieau solution[6].

[6] Danieau solution: 58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM Ca $(NO_3)_2$, 5 mM HEPES, pH 7.6.).

Effective doses were determined as described (Nasevicius and Ekker, 2000). Embryos were raised to 5 dpf and compared with *nrf* mutant embryos, and with uninjected or control injected siblings.

Table 3. 1 Morpholino sequences

Morpholino name	Sequence	Binds sequence
Antisense 1 (*nrf1* antisense 1.0)	5'-cat cac ccg ata cag ttt cac tca a-3'	Binds *nrf1* ATG start codon and –18 bp upstream
Antisense 2 (*nrf1* antisense 2.2)	5'-act cag cgg ctg cgc tac ttc gg-3'	Binds 5'UTR –27- -51 bp upstream the *nrf1* ATG start codon
Sense (antisense against 1.0)	5'-ttg agt gaa act gta tcg ggg tga tg-3'	

3.2.4 mRNA injections

The mRNA for microinjection was prepared using mCAP RNA Capping Kit (Promega) as recommended by the supplier. The resulting mRNA was run on an agarose gel to determine its quality, and stored at –20°C until use. Concentration was determined spectrophotometrically, and the capped mRNA was injected into 1-cell stage embryos as described earlier. Nothing but mRNA and DEPC treated water was used in the injection solution to avoid contamination with RNase.

3.2.5 Measuring of the injected volume

After each round of injection 10 injections were made into mineral oil to measure the injected volume. The diameter of the injected volume was measured and the volume of the sphere was calculated using the formula $V = 4/3\ \pi\ r^3$ where r is the radius of the sphere.

3.3 DNA constructs

3.3.1 Meganuclease constructs

To generate the meganuclease heat-shock constructs, the 18 bp *Sce*I meganuclease recognition site flanked by *Not*I/*Bam*HI and *Apa*I/*Kpn*I restriction sites was cloned into the pBs-hsp70 plasmid respectively 5' and 3' to the zebrafish hsp70 promoter (a gift from John Kuwada) (Halloran et al., 2000). The 470 bp SV40 polyA signal was PCR cloned from the pBi-*EGFP* plasmid (Clontech) and inserted using the unique sites *Cla*I/*Xho*I. Full-length *nrf1* (GenBank#AF087671) was PCR cloned into the vector using *Hind*III/*Cla*I sites to generate the pBs-*Sce*I-hsp70-*nrf1* plasmid. Full-

length *ibr* cDNA (GenBank#X86013) was removed from the pXeXHM*ibr* plasmid (gift from Adolf Ruiz-Carrillo) and cloned into the vector using *Hind*III/*Cla*I sites to generate the pBs-*Sce*I-hsp70-*ibr* plasmid.

Table 3. 2 Oligonucleotides used for generating meganuclease constructs

Name	Sequence
5'NotI/BamHI (fwd)	5'-ggccgctagggataacagggtaatg-3'
5'NotI/BamHI (rev)	5'-gatccattaccctgttatcccagc-3'
3'ApaI/KpnI (fwd)	5'-ctagggataacagggtaatggtac-3'
3'-ApaI/KpnI (rev)	5'-cattaccctgttatccctagggcc-3'

3.3.2 Gal4-UAS activator and effector lines

The constructs used for generation of these plasmids were the pBs*Gal4*, pBsUAS-*myc-Notch:intra* and pBs2xMAR-UAS-*myc-Notch:intra,* a gift from José Campos-Ortega (Scheer and Campos-Ortega, 1999), and modified as described in the following sections. pBs*Gal4* encodes the full-length *Gal4* sequence, and pBsUAS-*myc-Notch:intra* contains a pentamer array of the Gal4 DNA binding sequence, UAS upstream of the *e1b* minimal promoter of adenovirus. The pBs2xMAR-UAS-*myc-Notch:intra* contains in addition two chicken matrix attachment regions (MARs). The MARs are DNA elements binding specifically to the nuclear matrix, and are thought to minimize positional effects of the integration site on the transgenes (reviewed in Allen et al., 2000). Figure 3.1 illustrates the reporter and activator constructs generated for this study.

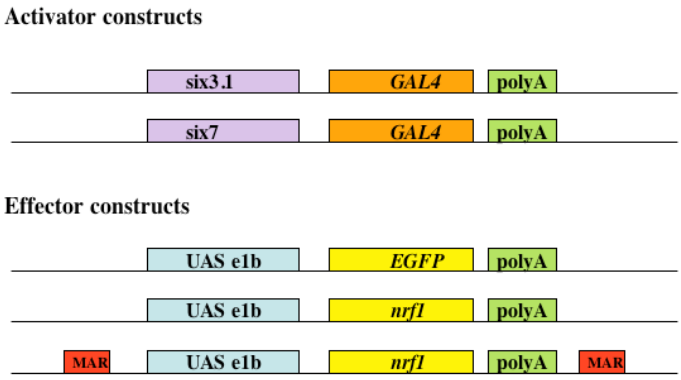

Figure 3.1 Schematic overview of the structure of the Gal4/UAS constructs generated for this study.

3.3.2.1 Construction of activator plasmids

pBs-Six3.1-Gal4

The 3.3 kb *six3.1* promoter with flanking linker sequences on both sides, a gift from H-C. Seo (Wargelius et al., 2003) was cloned into the pBs*Gal4* vector using a unique

*Not*I site upstream of *Gal4*. The expression cassette was purified from the vector sequence by digestion with *Bss*HII before injection.

pBs-Six7-Gal4

The 2.8 kb *six7* promoter, a gift from H–C. Seo (Drivenes et al., 2000) was cloned into the pBs*Gal4* vector using a unique *Eco*RI site upstream of *Gal4*. The expression cassette was purified from the vector sequence by digestion with *Asp*718 and *Not*I before injection.

3.3.2.2 Construction of effector plasmids

pBsUAS-EGFP

The pBsUAS-*myc-Notch:intra* (Scheer and Campos-Ortega, 1999) was used to generate the effector constructs. The *myc-Notch:intra* was removed from the vector using *Sma*I and *Bgl*II digestion, and the 720 bp *EGFP* gene was PCR cloned from the pBi-*EGFP* vector (Clontech) and blunt-end cloned into the vector. The expression cassette was purified from the vector sequence by digestion with *Asp*718 and *Not*I before injection.

pBsUAS-nrf1 and pBs2xMAR-UAS-nrf1

The pBsUAS-*myc-Notch:intra* and pBs2x-MAR-UAS-*myc-Notch:intra* (Scheer and Campos-Ortega, 1999) were used to generate the effector constructs. *Myc-Notch:intra* was removed from the vector using *Sma*I and *Bgl*II digestion, and the 1.6 kb full-length *nrf1* sequence (GenBank#AF087671) was PCR cloned into the vector together with the upstream *Myc* epitope from pBsUAS-*myc-Notch:intra* to distinguish transgenic *nrf1* from the endogenous *nrf1*. The expression cassette was purified from the vector sequence by digestion with *Asp*718 and *Not*I before injection.

3.3.3 Tet activator and effector lines

The Tet-On and Tet-Off gene expression systems are widely used for induction of gene expression in mammalian cells. However, to obtain complete repression of gene expression in the Tet-Off system potentially toxic concentrations of Tet need to be added to the medium (Jost et al., 1997). For this study the Tet-On system was chosen

over Tet-Off so that the embryos were only exposed to tetracycline (Tet) at the time of induction. In this Tet-On system a chimaeric transactivator (rtTA) activates the transcription of a gene of interest from a silent bidirectional promoter (p-Bi) (Clontech). By using a bidirectional promoter one can simultaneously regulate the expression of a gene of interest and a reporter gene. The activator line used in this experiment was a gift from Michael Brand (unpublished). The rtTA transactivator is expressed from the pax2.1 promoter and activates the expression of a reporter gene. To induce Tet dependent expression, 1 µg of Tet (Sigma) was added pr 1 ml Holtfreter's. Figure 3.2 shows the activator and effector constructs used.

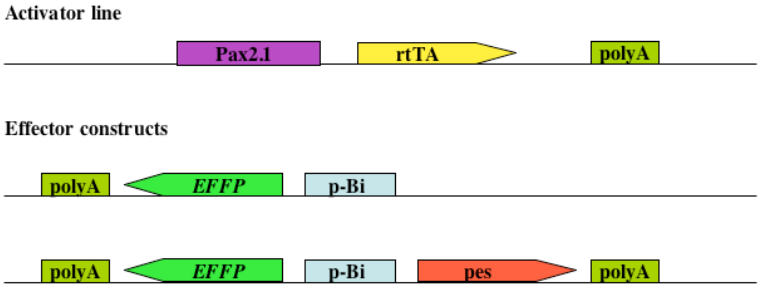

Figure 3.2 Schematic overview of activator and effector lines in the Tet-On system.

3.3.3.1 Construction of effector plasmid

pBi-EGFP-pes

The 2.2 kb *pes* (GenBank#NM131030) sequence was PCR cloned into the pBi-*EGFP* vector (Clontech) using the unique *Pvu*II site.

3.3.4 Plasmid for mRNA injections

The 1.6 kb *nrf1* (GenBank#AF087671) sequence was PCR cloned into the *Eco*RI/*Not*I sites of the pBRN3 vector (Lemaire et al., 1995).

3.4 Screening of transgenic lines

3.4.1 Purification of genomic DNA

Fifty 48 hpf embryos or a tail-clip from one 2-3 month embryo were incubated overnight at 56°C in GNT-K Buffer[7], followed by DNA precipitation with 2.5 volumes 100 % ethanol. The genomic DNA (gDNA) pellet was air-dried and solved in 10% TE buffer (150 μl for tail-cuts and 750 μl for embryo assays).

3.4.2 Screening protocol

Injected F_0 embryos (founders) were raised to sexual maturity and crossed with wild-type fish. gDNA was prepared from pools of F_1 progeny at 48 hpf. A PCR screen for insertion of plasmid DNA into the germ line was performed on gDNA (table 3.3). F_1 from positive founders were raised to maturity, and around 1.5 –2 months they were tail cut to identify heterozygous transgenic fish. The PCR reactions for this second screen were the same as described in table 3.3, except that the number of cycles was reduced to 30. When heterozygous F_1 fish were old enough they were pair mated to give homozygous F_2 offspring.

[7] GNT –K buffer : 50mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.5, 0.01% gelatin, 0.45% Nonidet P-40, 0.45% Tween-20, 100 μg/ml Proteinase K (Sigma)

Table 3.3 Primers and PCR programs. The following oligonucleotides and PCR programs were used in the screen for transgenic fish.

Primer sequence	PCR program[8]	Lines screened
EGFP F7: 5'-acgtaaacggccacaagttc-3' EGFP R5: 5'-gtgttctgctggtagtggtcg-3'	3 minutes at 94°C, 94°C for 25 s, 60°C for 30 s, 68°C for 30 s and 1 cycle at 68°C for 5 min. 30 cycles were performed for tail-cuts and 35 cycles for egg screen. [$MgCl_2$] = 3mM, band length: 495 bp	Bi-*EGFP* Bi-*EGFP-PES* UAS-*EGFP*
Gal4 F1 5'-ttcttctgtcgacgatgtgc-3' Gal4 R2: 5'-gcggtctcgttattctcagc-3'	3 minutes at 94°C, 94°C for 25 s, 57°C for 30 s, 68°C for 45 s and 1 cycle at 68°C for 5 min. 30 cycles were performed for tail-cuts and 35 cycles for egg-screen. [$MgCl_2$] = 2mM, band length: 590 bp	Six7-*Gal4* Six 3.1-*Gal4*
Hsp70 F1: 5'-tgttgtctacaaaagaaaattagcgtt-3' IBR R8: 5-ttctgtttgagtcacgccgt-3'	3 minutes at 94°C, 94°C for 30 s, 53°C for 30 s, 68°C for 30 s and 1 cycle at 68°C for 3 min. 30 cycles were performed for tail-cuts and 35 cycles for egg-screen. [$MgCl_2$] = 3.5 mM, band length: ca 270 bp	Hsp70-*ibr*
Hsp70 F1: 5'-tgttgtctacaaaagaaaattagcgtt-3' NRF R1: 5'-gaggagggcgaatcctcgt-3'	3 minutes at 94°C, 94°C for 30 s, 53°C for 30 s, 68°C for 30 s and 1 cycle at 68°C for 3 min. 30 cycles were performed for tail-cuts and 35 cycles for egg-screen. [$MgCl_2$] = 3.5 mM, band length: ca 310 bp.	Hsp70-*nrf1*
SFG 1: 5'-atcctctagactgccatgg-3' SFG 2: 5'-atcgtaaccgtgcatctg-3'	2 minutes at 94°C, 30 cycles at 94°C for 30 s, 60°C for 30 s, 68°C for 30 s and 1 cycle at 68°C for 5 min. [$MgCl_2$] = 2 mM, band length: ca 340 bp.	*Nrf* mutants
rtTA 1: 5'-tagcttgtcgtaataatggcgg-3' rtTA 2: 5'-aatgaggtcggaatcgaagg-3'	5 minutes at 94°C, 94°C for 45 s, 52°C for 30 s, 68°C for 45 s and 1 cycle at 68°C for 10 min. 30 cycles were performed for tail-cuts. [$MgCl_2$] = 2.5 mM, band length ca 470 bp.	Pax2.1- *rtTA*
e1b2 F: 5'-cgtgtcttgtagttcccgtcatc-3' GFP1: 5'-catcgcgtctcagcctcac-3'	3 minutes at 94°C, 94°C for 25 s, 55°C for 30 s, 68°C for 45 s and 1 cycle at 68°C for 5 min. 30 cycles were performed [$MgCl_2$] = 2.5 mM, band length ca 450 bp.	UAS-*GFP*
e1b2 F: 5'-cgtgtcttgtagttcccgtcatc-3' NRF R1: 5'-gaggagggcgaatcctcgt-3'	3 minutes at 94°C, 94°C for 25 s, 59°C for 30 s, 68°C for 45 s and 1 cycle at 68°C for 5 min. 30 cycles were performed for tail-cuts, 35 cycles for egg screen [$MgCl_2$] = 2.5 mM, band length ca 320 bp.	UAS-*nrf1* (genomic)
Myc F1: 5'-cagatcttcttcagaaataag-3' NRF R1: 5'-gaggagggcgaatcctcgt-3'	3 minutes at 94°C, 94°C for 25 s, 55°C for 30 s, 68°C for 45 s and 1 cycle at 68°C for 5 min. 30 cycles were performed, [$MgCl_2$] = 2.5 mM, band length ca 280 bp.	UAS-*nrf1* (RT-PCR)
e1b2 F: 5'-cgtgtcttgtagttcccgtcatc-3' PES R1 5'-aagttctagcagccgtcgagc-3'	3 minutes at 94°C, 94°C for 25 s, 61°C for 30 s, 68°C for 45 s and 1 cycle at 68°C for 5 min. 30 cycles were performed for tail-cuts, 35 cycles for egg screen [$MgCl_2$] = 2.5 mM, band length ca 380 bp.	UAS-*pes* (genomic)
Myc F1: 5'-cagatcttcttcagaaataag-3' PES R1 5'-aagttctagcagccgtcgagc-3'	3 minutes at 94°C, 94°C for 25 s, 61°C for 30 s, 68°C for 45 s and 1 cycle at 68°C for 5 min. 30 cycles were performed, [$MgCl_2$] = 2.5 mM, band length ca 320 bp.	UAS-*pes* (RT-PCR)
β-actin A4 (+): 5'-gagaagatctggcatcacaccttc-3' β-actin A5 (-): 5'-ggtctcgtggataccgcaagattc-3	Any of the above programs Band length: ca 580 bp	Positive control for gDNA or for RT-PCR

[8] PCR reaction conditions; 0.04U/µl BIOTAQ™ DNA polymerase (Bioline), 300 µM dNTP, 0.3 µM Primers, 1x NH_4 Reaction Buffer (Bioline), 1 µl of gDNA.

3.4 3 Reverse transcription PCR (RT-PCR)

Total RNA was isolated from 20-50 dechorinated embryos at the indicated stages using the SV Total RNA Isolation Kit (Promega) according to the manufacturers instructions with some minor modifications: embryos were homogenized through a 23G needle and RNA was eluted from the column using 30-50 µl dH_2O. RNA was stored at –80°C. The quality of the RNA was evaluated on an agarose gel and the concentration determined by UV spectrophotometry.

cDNA was amplified from 2 µg total RNA using MMLV reverse transcriptase (MMLV RT, Promega). To initiate the reaction 2 µg total RNA and 200 ng random primers (Promega) were denatured at 70°C for 5 minutes and put on ice. To each reaction 5 µl 5x MMLV Buffer, 1.2 µl 10 mM dNTP, 0.6 µl (40 U/µl) RNasin (Promega), and 200 U MMLV RT were added, the total volume adjusted to 25 µl, and the reaction was incubated for 1 hour at 37°C. To rule out the possibility of genomic DNA contamination a reaction without MMLV RT was included.

To identify expression of *Gal4*, *EGFP* and *rtTA*, 2µl cDNA were used in a standard set-up PCR reaction according to table 3.3. An RT-PCR set up with β-actin primers was performed to control the cDNA quality

3.4.4 Quantitative real-time PCR (qRT-PCR)

Primer pairs were designed for each gene using the Primer3 program (Rozen and Skaletsky, 2000). QRT-PCR was performed with QuantiTect™ SYBR Green (Qiagen), according to the manufacturers instructions. Histone H3.3b (zgc:56418) primers were used for normalization. The cDNA samples used for gene analysis were diluted 1:10. The samples were run in triplicates on a LightCycler (Roche) using the LightCycler 3 Run Program, Version 5.32 (Roche). 40 cycles of amplification were run for each sample. The samples were checked via agarose gel electrophoresis to ensure specific amplification of the target gene. A standard curve with different dilutions of *nrf* mutant and wild type cDNA was used for linear regression analysis of the genes of interest. Relative cDNA amounts were calculated using the LightCycler 3 Data Analysis Program (Roche), and the ratios of gene expression in *nrf* mutants versus wild type were calculated. The primer sequences are listed in table 3.4.

Table 3.4. Primers used for qRT-PCR

Gene	Forward primer	Reverse primer
H3.3b	F 5'- accgacgttctttgttttgg,	R 5'- cagacccaccaggtatgctt
eIF1	F 5'-caggacagctggtgggttat	R 5'- gcatcggttatgccagagt
spy1	F 5'- cttgctttctgtgtgccaaa	R 5'- catttcgctgaccacatcac
ppil3	F 5'- agacctcggcgatatgaaaa	R 5'- acaccgcggacattatgttt;
Ldhb	F 5'-atccagtggacgttctgacc	R 5'-catttgcaccactccatacg
myst1	F 5'-acaaaacatggcattcagca	R 5'-ggtccacaagttctcctcca
ift57	F 5'- gatgccaagggttgtctgtt	R 5'- gctccttcaccttgttgagc
nrf1	F 5'- ccgaacagaggagcagaaac,	R 5'- gctccagtgccaacctgtat
dnah9	F 5'- ggaaccgctgataaccgata	R 5'- agtcgagtgctcggactgat
Npl	F 5'- catgaccggtgttgcattag,	R 5'- atatgtgctccccactgctc
Riok	F 5'- gggtgtaacccacaggctaa,	R 5'- cttccctctgcttttgcatc
Neph	F 5'- gctgagactccgtttcctca	R 5'- tcagccagatctgatggatg
Mao	F 5'-accaactcaaaaccgcattc	R 5' gtaggcaaaagggttccaca,
ek1	F 5'- ggactccagcctgtacccta	R 5'- ctggagcagagcccatag
v2rdl	F 5'- ccctcacttagtggcaccat	R 5'- gggatccattgtggatgaag
top1	F 5'- catccaaggctccatcaaat,	R 5'- tcaaagccagcttgtcaatg

All primers were run in the same program with hot start at 95°C for 15 min, 40 cycles of 95°C for 15 seconds, 57°C for 20 seconds and 72°C for 20 seconds. A melting curve analysis step from 65°C to 95°C was included at the end of the run.

3.4.5 Heat shock

Embryos were heat shocked in a waterbath for 1 hour at 38°C. For antibody staining only one heat shock was carried out. For *in situ* hybridization and rescue experiments subsequent heat shocks were carried out as described in the results part with intervals of at least 1.5 hours.

3.5 Plastic Sectioning

For plastic sectioning embryos were fixed on ice for 2 hours in 4% paraformaldehyde/2% glutaraldehyde/PBS, rinsed in cold PBS, postfixed at 4°C overnight in 1% OsO_4/PBS and dehydrated for 5 minutes in increasing concentrations (30%, 50%, 70%, 95%, 100%) of ice cold methanol/PBS. After 30 minutes incubation in ice cold 100% methanol embryos were transferred to cold (1:1) 100% methanol: propylene oxide for 30 minutes. Dehydration was completed with two 30 minutes incubations of propylene oxide at 4°C. Embryos were brought to room temperature and propylene oxide was replaced with a 1:1 mix of propylene oxide: PolyBed mix[9] for > 3 hours. Afterwards the embryos were incubated in a 1:3 mix of propylene: PolyBed mix overnight, and then in 100% Poly Bed mix overnight. Embryos were

[9] PolyBed mix: 12 g PolyBed 812, 24.7 g DDSA. Stir for 15 minutes, add 0.5 ml DMP-30. Stir another 15 minutes and store at -20°C until use. (DDSA: Dodecenylsuccinic anhydride,DMP-30: 2,4,6-tri(dimethylaminomethyl)phenol)

embedded in fresh PolyBed in molding blocks and incubated overnight at 70°C for polymerization. The plastic blocks were sectioned with a glass knife at 1 μm intervals using an ultramicrotome (Leica). The sections were picked up with an eyelash fixed to a toothpick and transferred to water droplets on super frost object slides. After drying the slides on a hotplate, the sections were counterstained with Toluidine Blue Solution[10] for 20 seconds, dried on a hotplate for 15 seconds, rinsed carefully in water and dried. The stained sections were mounted with DPX mounting media (BDH) and a cover slip prior to photographing. Images were recorded using a SPOT digital camera (Diagnostic Instruments) on a Nikon Eclipse E800 microscope.

3.6 Sequencing

Plasmid DNA was purified using the alkaline lysis method or Qiaprep miniprep kit (Qiagen). DNA was sequenced by conventional methods using the ABI PRISM™ Big-Dye Terminator chemistry (Applied Biosystems) and an ABI PRISM 3700 DNA sequencing analyzer (Applied Biosystems). All DNA sequences were compared to those in GenBank databases, using the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul et al., 1990).

3.7 Whole mount *in situ* hybridization

Whole mount *in situ* hybridization with DIG labeled RNA probe was done as described by (Jowett and Lettice, 1994) and (Allende et al., 1996).

3.7.1 Probe synthesis

RNA probes were made according to the manufacturers instructions using the Roche DIG RNA Labeling Kit. *Nrf1* sense and antisense probes were synthesized by *in vitro* transcription of the linearized ZF-*nrf1* plasmid containing the 172-1479 bp region of the zebrafish *nrf1* cDNA. *Ibr* sense and antisense probes were synthesized by *in vitro* transcription of the linearized *ibr*-b7 (rev) and *ibr*-b8 (fwd) plasmids containing the 71-1007 bp region of the chick *ibr* cDNA. Precipitated probes were dissolved in 20 μl DEPC treated water before adding 500 μl hybridization buffer.

[10] Toluidine Blue Solution; 0.1% Toluidine Blue, 0.1% Sodium Borate in dH_2O. Filter solution before use.

3.7.2 Whole mount in situ hybridization protocol

Embryos were fixed overnight in 4% paraformaldehyde/PBS at 4°C, washed in PBST and dechorionated manually before being dehydrated for 5 minutes in increasing concentrations (30%, 50%, 70%, 95% and 100%) of methanol/PBS. Before the *in situ* hybridization the embryos were rehydrated for 5 minutes in 75%, 50% and 25 % methanol/PBS, and finally washed four times for 5 minutes in PBST[11]. 24-48 hpf embryos were permeabilized for 10 minutes in 10 μg/ml Proteinase K (Sigma) in PBST, and subsequently rinsed in PBST. Embryos were re-fixed in 4% paraformaldehyde/PBS for 20 minutes at room temperature, and rinsed 5x 5 minutes in PBST. Embryos were pre- incubated for 10 minutes at 65°C in Hybridization Solution (HS)[12]. The HS was replaced by fresh HS before prehybridization for 3 hrs at 65°C. The probe (1 μg probe/ml HS) was denatured for 5 minutes at 70°C in HS before use. Hybridization was performed overnight at 65°C. The following day embryos were washed for 10 minutes each in decreasing formamide concentrations (75% formamide/2x SSC, 50% formamide/2x SSC, 25% formamide/2x SSC), then washed for 10 minutes in 2x SSC and twice for 30 minutes in 0.2x SSC. All washes were performed at 65°C. The embryos were then incubated for 5 minutes in Maleic Acid Buffer (MAB)[13], for 1 hour in MAB + 2% Boehringer Mannheim Blocking Reagent (BMB), and for 6 hours in MAB + BMB + 20% Inactivated Lamb Serum (LS). All incubations were done at room temperature. Antibody (table 3.5) was added to MAB + BMB + LS to preabsorb while embryos were blocking. MAB + BMB + LS + antibody solution was added to embryos and incubated overnight at room temperature under gentle shaking. The following day embryos were washed five times for 20 minutes in MAB, followed by three 5-minute washes with Alkaline Phosphatase (AP) Buffer[14]. At this stage the embryos were transferred to a 24 well NUNCLON™ plate for staining. Staining was done in the dark with NBT/BCIP solution prepared from ready-to-use tablets (Roche) according to the manufacturers instructions. To stop the staining reaction embryos were washed twice in PBST and refixed for 20 min in 4% paraformaldehyde/PBS at room temperature before

[11] PBST: PBS + 0.1% Tween
[12] Hybridization Solution; 50% Formamide, 5x SSC, 50 μg(ml Heparin, 500 ug/ml tRNA, 0.1% Tween-20
[13] Maleic Acid Buffer (MAB); 100 mM Maleic Acid, 150 mM NaCl, 0.1% Tween-20, pH 7.5
[14] Alkaline Phosphatase Buffer; 100 mM Tris HCl, pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.2% Tween-20, 0.2% Triton-X100

mounting. Hybridized embryos were cleared in glycerol and photographed with a SPOT camera on a Nikon Eclipse E800 microscope.

3.8 Transplantation

For transplantation, donor embryos (*nrf* mutants) were injected with 10% biotin (Vector Laboratories)/ rhodamine-dextran (MW 10,000, Molecular Probes) in 0.2 M KCl at the 1-cell stage. At the blastula stage, cells were transplanted from the animal pole of the injected embryos to the same position in wild type embryos using micropipettes pulled from borosilicate glass capillaries without filament (Sutter Instruments). The transplantation pipette was filled with mineral oil and cells were transplanted using an attached mouth pipette. Embryos were raised individually in 96 well plates in 10% Holtfreter's solution with 1 mM Hepes and 500 μg/ml Penicillin G and 500 μg/ml streptomycin sulphate (Sigma), keeping track of both donor and acceptor fish. At 24 hpf the embryos were screened in the microscope to identify wild type fish with rhodamine positive cells in their retinas. Fish without positive cells in the retina were discarded. After 5 days the *nrf* phenotype was visible, and homozygous donors for the *nrf* mutation were identified. Wild type fish with cells transplanted from homozygous *nrf* mutants were fixed at 3-8 dpf, and stained for biotin using Vectastain ABC Kit according to the manufacturers descriptions (Vector Laboratories). Stained larvae were embedded in plastic resin and sectioned as described in section 3.5.

3.9 Whole mount immunocytochemistry

Dechorionated embryos were fixed in 4% paraformaldehyde for 3 hours at room temperature or overnight at 4°C, and washed four times for 15 minutes in PBST. Embryos were dehydrated for 5 minutes in 50% MeOH/50% PBS, and twice for 5 minutes in 100% MeOH. Embryos were stored in 100% MeOH at –20°C for at least 30 minutes, and rehydrated for 5 minutes in 50% MeOH/50% PBT before washing three times 5 minutes in PBT. Embryos were permeabilized as directed below[15], rinsed in PBST, and post fixed in 4% paraformaldehyde for 20 minutes at room

[15] In 10 μg/ml Proteinase K (Sigma) in PBT: 24 hpf- 20 minutes, 48 hpf – 1 hour, 3 dpf- 2 hours. And later for 1 hour in 20 μg/ml Proteinase K for 4 dpf embryos, 30 μg/ml Proteinase K for 5 dpf embryos, 40 μg/ml Proteinase K for 6 dpf embryos, 50 μg/ml Proteinase K for 6-8 dpf embryos.

temperature. After washing embryos were blocked in IB[16] for at least 1 hour at room temperature on a shaker. The embryos were then incubated in IB containing primary antibody (according to table 3.5) overnight at 4°C on a shaker, then rinsed three times in PBT, and washed four times 30 minutes in PBT on a shaker. A one-hour blocking step in IB at room temperature was followed by an overnight incubation in IB containing the secondary antibody (table 3.5) at 4°C on a shaker. Embryos were rinsed 3 times in PBT, then washed four times 30 minutes in PBT on a shaker at room temperature, washed in DAB[17] solution for 20 minutes, and stained in DAB + 2 µl 0.3% H_2O_2/ml for 20 min - 2 hours. Staining was stopped with three washes in PBS. Immunostained embryos were cleared in glycerol and photographed using a SPOT digital camera (Diagnostic Instruments) on a Nikon Eclipse E800 microscope.

Table 3.5 Antibodies used for *in situ* hybridization and immunostaining

Antibody	Use	Supplier	Dilution	Experiment
Mouse α Na/K ATPase	Primary	Developmental Studies Hybridoma Bank	1:10	Immunostain, kidney
Mouse α-acetylated tubulin	Primary	Sigma	1:200	Immunostain, kidney
Goat α-mouse peroxidase conjugated	Secondary	Jackson Immuno Research Laboratories	1:500	Immunostain, kidney
α-DIG AP conjugated	-	Roche	1:2000	*In situ* hybridization

3.10 Microarray

The 31K zebrafish oligo array consists of two oligo sets; the Compugen set and the MWG set. The Compugen set contains 16512 (60-mer) oligos and the MWG set contains 14240 (50-mer) oligos. The whole set contains several positive and negative control oligos to control the homogeneity and specificity of the hybridization. To update the annotation on both sets, the sequence for each oligo was blasted against 4 major public databases: TIGR, UCSC, Ensembl and RefSeq. Based on the Feb 2005 updates; the zebrafish whole set represent 15,477 unique Ensembl genes, 12,463 unique RefSeq genes and 29,050 unique TIGR EST assemblies. The 5' amine-modified oligos were suspended in 3xSSC and printed on epoxy slides (Corning) and processed following protocols obtained from MWG.

[16] IB; 10% serum, 1% DMSO, 0.8% Triton-X100 in PBS. Serum used in IB depends on the antibodies used for immunostaining. In most cases I used goat serum.
[17] DAB (Diaminobenzidine tetrahydrochloride) solution: 500 µl stock in 30 ml PBS. Stock: 25 mg DAB (Sigma)/500 µl dH_2O, store at –20°C.

3.10.1 cDNA labeling and hybridization

Total RNA from 200 homozygous *nrf* mutants and 200 transgenic embryos (CLGY298) at 26 hpf was extracted with Trizol® (Gibco) reagent using the manufacturers standard protocol. 5 µg of total RNA from each sample were reverse transcribed in the presence of modified aminoallyl dUTP nucleotides. Fluorescent Cy3 or Cy5 molecules were coupled to the appropriate aminoallyl labeled probes using Amersham coupling dye packs and purified with affinity columns (Qiagen). Cy3 and Cy5 labeling were then switched with respect to the probe, and the experiment was repeated.

3.10.2 Scanning and image analysis

A laser confocal scanner (Agilent Technologies, Palo Alto, CA) was used to measure the fluorescence intensities at the target locations on the array using the DEARRAY software (www.scanalytics.com). After background subtraction, average intensities of each clone in the test hybridization were divided by the average intensity of the corresponding clone in the control hybridization. The ratios were normalized on the basis of the distribution of ratios of all targets on the array. Low quality measurements, i.e. copy number data with mean intensity less than 100 fluorescent units and/or with spot size less than 50 units were excluded from the analysis and were treated as missing values.

4 RESULTS

There were two major goals for this thesis, the first was to develop a reliable method for generation of transgenic zebrafish overexpressing *nrf1*, and the second was to use these transgenic zebrafish to further characterize the zebrafish *nrf* mutant. The results from this study are presented in three parts.

- **Section 4.1.** In this part of the thesis three methods for generation of a transgene overexpressing a gene of interest were tested and compared. These were the ubiquitous expression through an inducible heat shock promoter and the two binary expression systems Gal4/UAS and Tet-On.
- **Section 4.2.** In this section a function of the *nrf1* gene in zebrafish photoreceptor development and maintenance is suggested based on the results from the cell transplantation studies, injection of morpholino oligos and transgenic lines overexpressing *nrf1*.
- **Section 4.3.** The final part compares the gene expression profiles of *nrf* mutant and wild type embryos and aims to identify putative downstream targets for Nrf1 by using microarray technology and quantitative Real Time PCR.

4.1 Generation of transgenic lines

Transgenic fish can help to obtain answers on many biological questions, and they are important tools for the study of genes involved in the early development of an organism. Various systems for overexpression of a gene are being used on a routine basis in zebrafish laboratories, while others have not yet been described in detail. In this study the *Sce*I meganuclease system (Thermes et al., 2002) was used in combination with a heat shock construct (Halloran et al., 2000) to generate transgenic zebrafish, allowing temporal control of gene expression. However, his method does not provide spatial control of gene expression, as the hsp70 promoter is ubiquitously expressed upon heat shocking. The two binary systems Gal4/UAS and Tet-On on the other hand offer both temporal and spatial control of gene expression. When this work was started, a report was published about the binary Gal4/UAS system in zebrafish (Scheer and Campos-Ortega, 1999), and I wanted to establish this system in our laboratory. In addition I also sought to test the versatility of the Tet-On system

(Gossen et al., 1995), as nothing had been reported about this system in zebrafish at that time. The following section describes and evaluates the three systems.

4.1.1 The SceI meganuclease system

Upon injection of plasmid DNA into a zebrafish embryo, the injected DNA can remain as non-chromosomal DNA or be incorporated in the genome of somatic cells (transient transgenics), or it can be integrated into the genome of the germ cells (germline transgenics). Plasmid DNA injection is a simple but poorly efficient method for generating transgenic fish (Culp et al., 1991; Stuart et al., 1988), and various factors have been suggested to influence the integration of plasmid DNA into the genome (Collas et al., 1996; Higashijima et al., 1997). The *Sce*I meganuclease system is shown to increase the transgene frequency[18] as well as the germline transmission rate[19] in transgenic fish (Thermes et al., 2002). This system was used in combination with the zebrafish hsp70 promoter to generate stable transgenic lines overexpressing *nrf1* and its avian homologue *ibr*. The promoter can be used to temporally overexpress a gene of interest after heat shocking whole zebrafish embryos at 37°C and the procedure of heat shocking does not interfere with the development of the embryo (Halloran et al., 2000).

4.1.1.1 Hsp70-*nrf1* transgenic lines

After co-injecting the circular pBs-*Sce*I-hsp70-*nrf1* plasmid with *Sce*I meganuclease, a transgenic frequency of 32% was obtained, while germline transmission rates varied from 3-46%, with an average of 19.5% (table 4.1).

[18] Transgenic frequency is the percentage of injected fish (F_0) that integrates the transgene into their germline, e.g. that pass the transgene on to their offspring.
[19] Germline transmission rate is the rate of delivery of the transgene from F_0 to F_1. It reflects how early the integration of the transgene took place in the F_0.

	Positive fish/total tested	% positive
F_0 transgenic rate	18/57	32%
F_1 Transmission rate	-	19.5% (average)
#1	5/161	3.1%
#2	3/54	5.5%
#3	21/46	45.7%
#4	7/155	4.5%
#5	26/113	23%
#6	12/198	6.1%
#7	12/68	17.7%
#8	17/145	11.7%
#9	13/129	10.1%
#10	19/77	24.7%
#11	4/14	28.6%
#12	Not screened	Not screened
#13	10/90	11.1%
#14	1/3	33%
#15	31/81	38.3%
#16	2/21	9.5%
#17	56/189	29.6%
#18	15/51	29.4%
F_1 Expression rate	5/10	50%

Table 4.1 Overview of transgenic hsp70-*nrf1* lines, including transmission rates and *nrf1* expressing lines.

4.1.1.2 Generation of hsp70-*ibr* transgenic lines

Transgenic lines expressing the chick *nrf1* homologue *ibr* under control of the hsp70 promoter were generated using the DNA constructs described in section 3.3.1. The transgenic frequency was 3%, while germline transmission rates varied from 10-33% with an average of 21.7% (table 4.2).

	Positive fish/total tested	% positive
F_0 transgenic rate	4/131	3%
F_1 Transmission rate	-	21.7% (average)
#1	4/12	33.3%
#2	7/70	10%
#3	Not screened	Not screened
#4	13/60	21.7%
F_1 Expression rate	2/2	100%

Table 4.2 Overview of transgenic hsp70-*ibr* lines, including transmission rates and *ibr* expressing lines.

4.1.1.3 *In situ* hybridization screen of expressing lines

After identifying the founders (the positive F_0 individuals), the F_1 generation was raised and screened to identify heterozygous F_1 fish in each line. When these F_1s were mature they were inbred and screened for expression by *in situ* hybridization with *nrf1* or *ibr* probes after two one-hour periods of heat shock. As the reportedly unstable *Gal4* mRNA persists for approximately 60-90 minutes (Scheer et al., 2002), and it was not known how stable the *nrf1* mRNA was, the embryos were allowed to recover

for no more than 30 minutes after heat shock before fixation. As seen in table 4.1, 50% (5 out of 10) of the germline transgenic lines tested expressed ectopic *nrf1* after heat shock, as revealed by ubiquitous staining after *nrf1 in situ* hybridization (figure 4.1). For the hsp70-*ibr* lines, 100% (2 out of 2) of the germline transgenic lines tested expressed *ibr* (table 4.2/figure 4.2). The fact that not all transgenic lines were expressing the transgene could be due to silencing of the transgene, possibly by methylation or by integration into heterochromatin.

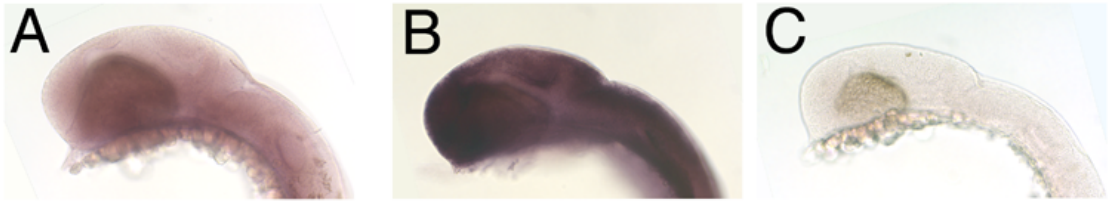

Figure 4.1. *In situ* hybridization illustrating *nrf1* expression in the 26 hpf embryo. (A) Wild type *nrf1* expression pattern, (B) Overexpression of *nrf1* in hsp70-*nrf1*#8 after 2 subsequent heat shocks at 38°C, (C) No *nrf1* expression is detected in *nrf* mutant embryos.

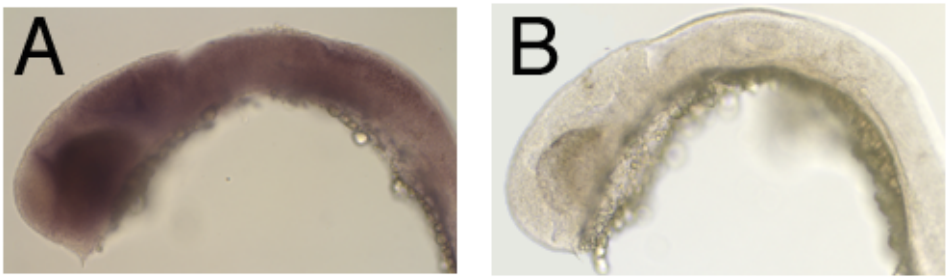

Figure 4.2. *In situ* hybridization illustrating ectopic *ibr* expression after heat shock in 28 hpf embryos. (A) Overexpression of *ibr* after 2 subsequent heat shocks at 38°C. (B) No *ibr* expression is detected in wild type embryos

4.1.2 The Gal4/UAS system

The yeast Gal4/UAS system was developed for targeted gene expression in *Drosophila* (Brand and Perrimon, 1993; Fischer et al., 1988), and therefore appeared to be a good candidate system for targeted gene expression in zebrafish (Scheer and Campos-Ortega, 1999). Neither Gal4 nor UAS sequences are found in *Drosophila* or vertebrate genomes. By generating two different stable transgenic lines carrying, respectively, an activator/driver and an effector construct, and crossing these with each other, the effector gene should be transcribed in the pattern directed by the activator. Due to the combinatorial nature of the Gal4/UAS system, and as more and

more activator and effector lines become available, one can express one gene in a variety of patterns just by crossing a transgenic effector line with various activator lines (Brand and Perrimon, 1993).

The zebrafish *six3.1* promoter was chosen to drive one of the activator lines since it is expressed early in the eye anlagen and subsequently expressed in the retina (Wargelius et al., 2003), while the zebrafish *six7* promoter was chosen because of its early activity in the prospective eye field, starting at around 6 hpf with a transient expression pattern lasting until 16 hpf (Drivenes et al., 2000). By using these promoters I wanted to overexpress genes involved in eye development at an early time point, in an area specific to the prospective eye field, and in the retina. The constructs to generate transgenic fish overexpressing the *nrf1* gene (Becker et al., 1998) were made as described in section 3.3.2.

Gal4/UAS transgenic fish were generated by injection of naked, linearized plasmid DNA as described in Materials and Methods. The results are presented in table 4.3. All the activator lines were tested for expression of the Gal4 transgene by RT-PCR, and positive lines were subsequently crossed with the transgenic effector line UAS-*GFP* to test for transactivation. The effector lines were investigated for expression by RT-PCR after being crossed to the transgenic activator line hsp70-*Gal4* (Scheer et al., 2002). The UAS-*EGFP* lines were also visually screened for fluorescence in the microscope after transactivation.

Table 4.3 Transgenic fish generated in the Gal4/UAS system

Injected constructs	**No of fish tested**	**Transgenic F_0 (% transgenics)**	**Transmission rates**	**Average transm. rate**	**Expression (RT-PCR)**	**Detected trans-activation**
pSix3.1-*Gal4*	185	15 (8.1%)	10 - 42%	20.5%	4 (26.6%)	0
pSix7-*Gal4*	200	7 (3.5%)	3 – 21%	9.8%	2 (28.6%)	0
pUAS-*nrf1*	123	2 (1.6%)	11 – 14%	12.5%	0	0
pUAS-*EGFP*	140	7 (5%)	8 – 33 %	16.3%	1 (14.3%)	1
p2xMAR-UAS-*nrf1*	144	5 (3.5%)	5 – 13%	10%	0	0

Four six3.1-*Gal4* activator lines and two six7-*Gal4* activator lines were expressing *Gal4* when analyzed by RT-PCR, but when crossed to the established UAS-*GFP* line, the expression was not strong enough to detect any transactivation (no fluorescence and no detectable *GFP* expression on RT-PCR). This gives an overall expression rate in the Gal4/UAS system of 19% (7 out of 36 lines).

One UAS-*EGFP* line showed weak fluorescence after transactivation by hsp70-*Gal4,* but this expression was weaker than the already existing UAS-*GFP* line, and thus of no practical use (picture not shown).

4.1.3 The Tet system

The tetracycline inducible expression systems Tet-Off and Tet-On were developed in mammalian cells (Gossen and Bujard, 1992; Gossen et al., 1995). It was a new and undeveloped tool in zebrafish when this study was initiated, and I wanted to test the versatility of the Tet-On system for use in zebrafish in our laboratory. The advantage of the system is that in addition to the spatio-temporal control from the promoter of choice, one has additional temporal regulatory control by the addition of tetracycline. As in the Gal4/UAS system it takes advantage of a two-vector system, with an activator and an effector line. The system can be used with a binary promoter on the effector construct, driving the expression of a reporter gene (*EGFP*) together with a gene of interest that is to be overexpressed (Baron et al., 1995). One binary construct was generated to overexpress a gene of interest *(pes)* under control of this binary promoter. In addition transgenic lines were generated using the "empty" pBi-*EGFP* (Clontech) construct. The Tet-On activator line pax2.1-*rtTA* was used to activate the expression of the genes on the effector constructs. The results are presented in table 4.4. As seen from the table *EGFP* expression could be detected by RT-PCR after transactivation with the pax2.1-*rtTA* line in 1 out of 10 (10%) of the transgenic lines, but the line did not express *EGFP* strong enough to detect it in the microscope. However, all the crosses were RT-PCR positive for *rtTA* expression.

Table 4.4 Transgenic fish harboring Tet constructs

Injected constructs	No of fish tested	Transgenic F_0 (% transgenics)	Transmission rates	Average transm. rate	Transcription of EGFP (RT-PCR)	Expression of EGFP (fluorescence)
pBi-*EGFP*	150	3 (2%)	4 – 21%	13.3%	0	0
pBi-*EGFP-pes*	190	7 (3.7%)	1 – 28%	11.5%	1 (14.3%)	0

4.1.4 Summary of results from transgenesis

As the germline transmission rate is depending on how early the integration of the injected DNA takes place, it was expected that injections with *Sce*I meganuclease would enhance this integration compared to injection of naked plasmid DNA. This

was also found, as average transmission rate of the *Sce*I injected embryos was 20.6%, and for the transgenes injected with naked DNA it was 13.4%. There is a great increase in transgenic frequency when comparing the two injection protocols. The 32% transgenic frequency in the pBS-*Sce*I-hsp70-*nrf1* lines was higher than the transgenic frequencies obtained in both the Gal4/UAS system (1.6-8.1%) and the Tet-On system (2-3.7%). The difference in transgenic frequency (32% for pBS-*Sce*I-hsp70-*nrf1* versus 3% for pBS-*Sce*I-hsp70-*ibr*) in the *Sce*I injected fish is striking in light of the constructs being identical except for the inserted gene, the plasmid preparation was performed in parallel, and injections were done at alternating days.

4.2 Characterization of the zebrafish *nrf* mutant

The zebrafish *nrf* mutant is characterized by its small eyes and protruding snout at 5 dpf (Becker et al., 1998). The smaller eye size is due to neuronal, predominantly PR degradation in the retina, and one important question was whether *nrf1* is necessary for PR development. Another question was at which stage *nrf1* expression is needed for PR development or maintenance. To address these questions the transgenic hsp70-*nrf1* line #8 and hsp70-*ibr* line #2 from the previous part were used (section 4.1.1).

4.2.1 Nrf1 is not necessary in photoreceptors for their initial morphogenesis and differentiation

Loss of function of the transcription factor Nrf1 leads to apoptosis of PR cells in the zebrafish *nrf* mutant starting at 72 hpf (Becker et al., 1998). *Nrf1* mRNA is not detected by *in situ* hybridization in differentiated PR, but rather is highly expressed throughout the entire early retinal neuroepithelium between 24 and 48 hpf. High numbers of apoptotic cells can be detected in clusters in the mutant around 76 hpf, but the retina retains a few PRs in the 5 day old larvae, although the insertional mutation of *nrf1* was determined to be a complete loss of function (Becker et al., 1998), This suggests that either some redundant function led to a partial rescue of these cells, for instance through a duplicated gene, or not all PRs need Nrf1 for their development. The PR escaping apoptosis, though deformed in appearance, were found to be functional by optokinetic response testing (Becker et al., 1998). Thus at least some PRs can develop in the absence of Nrf1 function. This latter finding, along with the observation that apoptosis occurs in large clusters in the *nrf* mutant, suggested that

there might be a non-cell autonomous component to cell death, as has been observed in other zebrafish retinal degradation mutants (Doerre and Malicki, 2002; Goldsmith et al., 2003) as well as in the mouse (Kedzierski et al., 1998). To address this question genetic mosaic experiments were performed by transplanting cells from labeled *nrf* mutant blastulae into wild type recipients. The biotin labeled *nrf* mutant PR cells developed normally after transplantation into a wild type background (figure 4.3c) in 8 out of 8 cases, suggesting that there is no requirement of Nrf1 for their initial development and morphogenesis. Transplantations of mutant cells into mutant embryos and wild type cells into wild type animals were done as a control, and transplantation of wild type cells into mutant embryos led to deformed photoreceptors (figure 4.3a,b&d).

The loss of photoreceptors can be explained either by a defect in one or more cell-autonomous factors (that is within the PR itself) or defects in non-cell autonomous factors (that is defective cell-cell or cell extracellular matrix interactions). As *nrf* mutant donor cells are rescued in a wild type environment, this indicates a non-cell autonomous function. Such a non-cell autonomous PR death has been shown to spread from affected cells to unaffected cells, and this "bystander" effect (reviewed in Ripps, 2002) describes a direct cell-cell mediated communication pathway, through the gap junctions on the neighboring PRs. When *nrf* mutant cells are moved from this possibly "toxic" environment of the mutant into a wild type background, the mutant PRs appear morphologically like wild type, suggesting that the support from the intact photoreceptor matrix in wild type embryos is sufficient for them to survive.

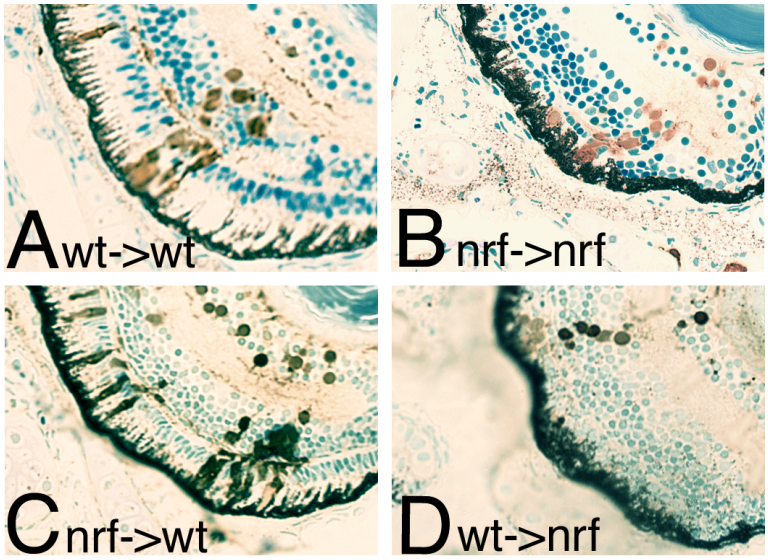

Figure 4.3 Biotin labeled PRs after transplantations. The pictures show plastic cross sections of the outer part of the retinae at 5 dpf, (A) Cells transplanted from wt to wt. (B) Cells transplanted from *nrf* mutant to *nrf* mutant. (C) cells transplanted from *nrf* mutant embryo into wt background. Note that the mutant PRs develop into both cones and rod PRs when transplanted to wild type embryos with IS and OS, as well as other retinal cell types. (D) Cells transplanted from wt to *nrf* background (here wt cells are biotin labeled).

4.2.2 Morpholino knockdown mimics the nrf phenotype

In order to investigate whether an early knockdown of *nrf1* will result in permanent *nrf* mutant phenotype, Nrf1 translation was blocked by injection of *nrf1* antisense morpholinos (MOs). By injecting morpholinos directed against the *nrf1* mRNA, the *nrf* phenotype was faithfully phenocopied (table 4.5). The *nrf1* morphants display smaller eyes than their wild-type siblings, and plastic sections of MO injected larvae revealed that this is due to a reduced number of PRs (figure 4.4). The effect of the MO injections does not wear out over time, evident by the fact that eye size remains reduced, and similarly to the *nrf* mutants the morphants die around 10-15 dpf. These results suggests that although Nrf1 is probably needed throughout life, the effect on PR development is specific for early time points, when the gene is expressed at high levels. Interestingly, the MO also results in larval lethality, and the *nrf1* morphants die at around the same time as the *nrf* mutants, suggesting that the lesions leading to lethality develop early, like the PR defect. By removing *nrf1* expression for a period and then returning to normal *nrf1* expression levels the PRs cannot recover, as the cells have presumably initiated the apoptotic pathway leading to loss of photoreceptors in the *nrf* mutant. *nrf1* MO was also injected into *nrf* mutant embryos, but the phenotype did not become more severe (data not shown).

Table 4.5 Results after MO injections into wt fish

Morpholino	Amount injected (ng)	WT	C1 phenotype	C2 phenotype	N
Uninjected	-	100%	0%	0%	149
nrf1 antisense 1.0	2.3 6.2	51% 42%	45% 48%	4% 10%	147 130
nrf1 antisense 2.2	2.3 6.2	74% 59%	21% 28%	5% 13%	125 125
nrf1 antisense 1.0 + 2.2	1.0 + 1.0 2.3 + 2.3 4.3 + 4.3	37% 7% 3%	57% 76% 65%	6% 17% 32%	134 141 136
Control MO	2.3 6.2	97% 83%	0% 0%	3% 17%	147 150

Injected embryos were scored at 6 dpf using a dissecting microscope, and classified according to the following criteria: Class 1 (C1) embryos were phenotypically identical to *nrf* mutants. Class 2 (C2) shows unspecific background phenotypes, characterized by reduced size and tissue necrosis. n= number of fish injected

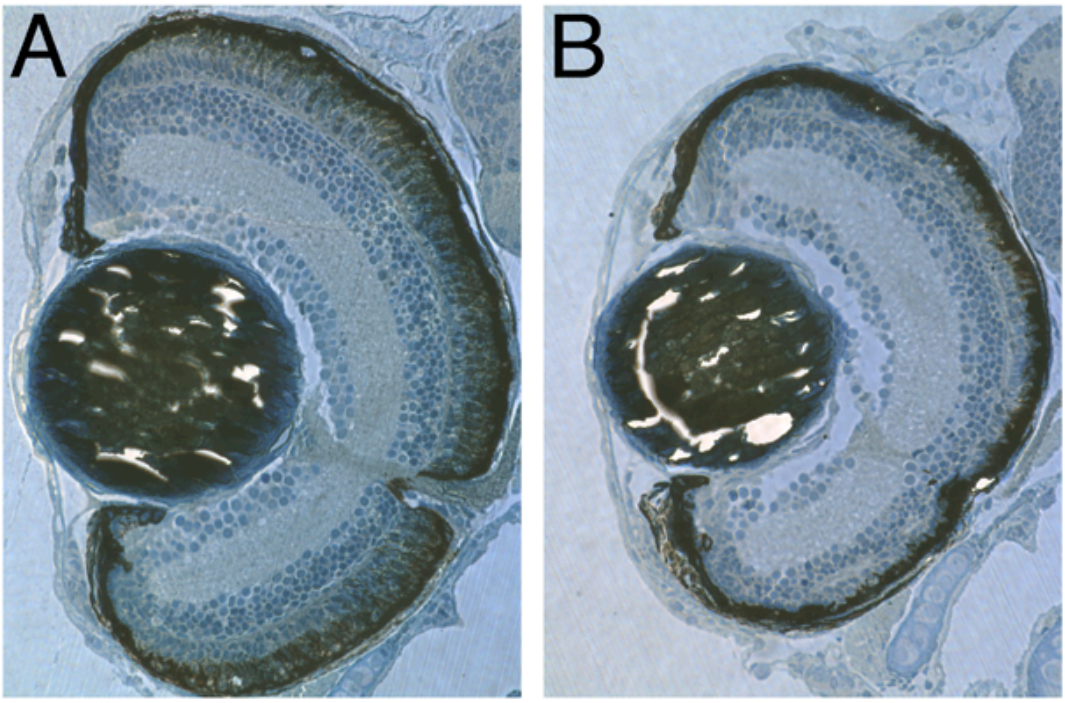

Figure 4.4 Injection of *nrf1* morpholinos phenocopies the *nrf* mutant phenotype. Frontal plastic sections through retinae at 6 dpf larvae. (A) Wild type fish. Note that the PR cell layer is made up of inner (IS) and outer segments (OS). (B) *nrf1* morpholino injected (2.3 ng each MO) larvae. Note the smaller eyes, and the loss of peripheral PRs. The remaining PRs are shorter than the wild type ones (OS is missing). Both fish were raised on same light/dark cycle and fixed at the same time point.

4.2.3 Overexpression of nrf1 has no detectable effect on wild type embryos

In situ hybridization results show strong expression of *nrf1* mRNA between 18-56 hpf. Later on *nrf1* is still being expressed at levels undetectable by *in situ* hybridization, but detectable by RT-PCR (Becker et al., 1998). To address the question whether overexpression of *nrf1* has any effect on the developing embryo,

nrf1 expression was induced at defined time points through development using a homozygous transgenic line carrying *nrf1* cDNA under control of the hsp70 promoter (Halloran et al., 2000). The results do not indicate any developmental effects on the developing wild type embryo (table 4.6).

Fish line	Heat shock hpf	Number of heat shocked embryos	Normal development 5 dpf (%)
Hsp70-*nrf1*	No heat shock	157	154 (98)
	6	39	37 (95)
	24	99	96 (97)
	30	74	73 (99)
	48	76	74 (97)
	72	51	50 (98)
	96	69	68 (99)
TAB (WT)	No heat shock	131	127 (97)
	6	44	42 (95)
	24	83	81 (98)
	30	65	64 (98)
	48	59	59 (100)
	72	48	49 (98)
	96	67	64 (96)

Table 4.6 Overexpression of *nrf1* at various time-points has no apparent effect on wild type embryos.

This corresponds to earlier findings that injection of *nrf1* mRNA did not seem to have any effect on the wild type embryos (table 4.7). The mRNA injection could not rescue the mutants, but these negative results were not conclusive as the mRNA could be degraded earlier than Nrf1 was needed in the cell.

The heat-shocked embryos show no signs of abnormal development, observed until 10 dpf, and the embryos that had overexpressed *nrf1* grew up to adulthood with no obvious defects. Thus strong ubiquitous expression, at least in a few pulses, of *nrf1* in wild type embryos has no detectable effect, suggesting that, while Nrf1 is required for PR development, it serves no additional instructive role in the developing embryo.

Table 4.7 Injection of *nrf1* mRNA does not rescue the *nrf* phenotype, but has no effect on wild type embryos.

Injected	mRNA amount	Injected into	Injected embryos	Surviving embryos (d5)	*nrf* phenotype	WT
dH_2O	-	*nrf* mutant cross	45	42	12 (29%)	30 (71%)
nrf1 mRNA	150 pg	WT	50	47	0	44 (94%)
nrf1 mRNA	200 pg	WT	60	60	0	58 (97%)
nrf1 mRNA	150 pg	*nrf* mutant cross	89	78	21 (27%)	53 (73%)
nrf1 mRNA	200 pg	*nrf* mutant cross	65	57	15 (26%)	42 (74%)

4.2.4 Use of CLGY298 as a live chromosomal marker line

For studies of the *nrf* mutant, one problem is that the mutant phenotype is not visible before a significant number of cells have undergone apoptosis at 4-5 dpf. However, the mRNA is expressed strongly much earlier, between 18 and 56 hpf, and the MO injections suggested that this early peak of expression is necessary for PR integrity. To be able to detect homozygous mutant embryos at early stages, I selected a retroviral insertion, CLGY298, near the *nrf1* locus, as a live chromosomal marker for the *nrf1* wild type allele. The CLGY298 insertion was identified in a large-scale enhancer detection screen (Ellingsen et al., 2005). Expression of the eYFP reporter gene in this line can be seen already at the 10-somite stage in a pattern that is distinct from that of *nrf1* (figure 4.5), but similar to the expression pattern of a microRNA (miRNA) cluster in that genomic region. miRNAs are a class of non-coding RNAs regulating gene expression at the posttranscriptional level (reviewed in Wienholds and Plasterk, 2005).

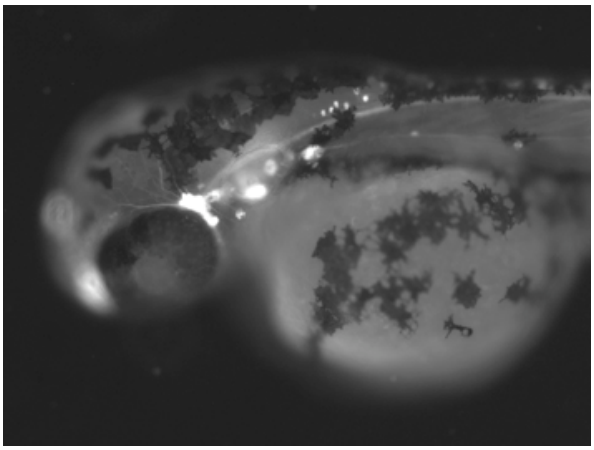

Figure 4.5 Expression pattern of CLGY298 at 48 hpf. Picture by Ståle Ellingsen.

During meiotic recombination two homologous chromosomes can undergo a physical exchange of DNA to produce two germ cells containing a different combination of genes than seen in the parents. The further apart two loci are on a chromosome, the higher the chances of a meiotic recombination. The CLGY298 insertion was mapped to a 15 kb distance downstream of the *nrf1* locus. A centimorgan (cM) is the unit of genetic distance between 2 loci, and 1 cM represents 1% recombination. In zebrafish 1 cM corresponds to 0.74 mb (Shimoda et al., 1999). The 15 kb distance between these two genes equals 0.02 cM, indicating that there is a 0.02% likelihood of a meiotic recombination between these two loci. Through crossing the *nrf* mutant line into the CLGY298 line, a double heterozygote line CLGY298/ nrf^{hi399} was established. F_1 inbreeding of this line resulted in 3/4 fluorescent, phenotypically wild type embryos and 1/4 non-fluorescent *nrf* mutant embryos (table 4.8), allowing easy

identification, by absence of YFP expression, of homozygous *nrf* mutants before 24 hpf.

Table 4.8 F_1 incross of the double transgenic lines CLGY298/ *nrf*^{hi399} x CLGY298/ *nrf*^{hi399}

Observed fluorescence at 48 hpf	% of total (n= 508)	Genotype	Phenotype d5	%
+	73,8%	CLGY298/CLGY298 CLGY298/ *nrf*^{hi399}	WT	100%
			Nrf mutants	0%
-	26,2%	*nrf*^{hi399} /*nrf*^{hi399}	WT	0%
			Nrf mutants	100%

4.2.5 The nrf mutant phenotype is rescued after ubiquitous expression of nrf1

Triple heterozygous fish were generated by crossing the two transgenic lines hsp70-*nrf1* and CLGY298/*nrf*^{hi399}. The resulting triple transgenics hsp70-*nrf1*;CLGY298/*nrf*^{hi399} were selected by fluorescence for inheritance of the enhancer detection insertion, and by PCR for hsp70-*nrf1* and *nrf*^{hi399}. These fish were then pair mated and their offspring was heat shocked at 24 hpf and non-fluorescent *nrf* homozygous mutant fish were identified between 24 and 48 hpf under the fluorescence microscope. Three quarters of the embryos carried the fluorescent marker, and were phenotypically wild type. The other 1/4 was not fluorescent, and thus were genetic *nrf* mutants. But since this was an incross of heterozygous fish only 3/4 of these carried the transgene hsp70-*nrf1*, and could in theory be rescued. This was also found experimentally (table 4.9). When the *nrf* homozygous embryos were analyzed at 5-6 dpf, 3/4 of the embryos did no longer show the *nrf* mutant phenotype, and could not be distinguished from wild type embryos when examined with a dissecting scope. As I was interested in further addressing the rescue effect on the PR cells in particular, plastic sections through the retina of the rescued mutants revealed that the PR cell layer was intact, and the mutant rescued retina was indistinguishable from the wild type retina (figure 4.6).

Table 4.9 Diagram showing the expected phenotype frequencies after incrossing triple heterozygous fish. 3/4 of the embryos has the CLGY298 expression pattern (green) and is genetically wild type. 1/4 is genetically *nrf* mutants, but of these 3/4 has the hsp70-*nrf1* transgene and can be rescued, only 1/4 will not be rescued, and will display the *nrf* phenotype. The ratios are 15:1 of wild-type embryos to *nrf* mutant embryos.

***nrf1*$^{+/-}$; *Hsp70-nrf1*$^{-}$ x ***nrf1*$^{+/-}$; *Hsp70-nrf1*$^{-}$**			
CLGY298		**CLGY298**	
nrf1$^{+/+}$ Hsp70-*nrf1* $^{+/+}$	*nrf1* $^{+/+}$ Hsp70-*nrf1* $^{+/-}$	*nrf1*$^{+/-}$ Hsp70-*nrf1*$^{+/+}$	*nrf1*$^{+/-}$ Hsp70-*nrf1*$^{+/-}$
nrf1$^{+/+}$ Hsp70-*nrf1* $^{+/-}$	*nrf1*$^{+/+}$ Hsp70-*nrf1*$^{-/-}$	*nrf1*$^{+/-}$ Hsp70-*nrf1*$^{+/-}$	*nrf1*$^{+/-}$ Hsp70-*nrf1*$^{-/-}$
nrf1$^{+/-}$ Hsp70-*nrf1* $^{+/+}$	*nrf1*$^{+/-}$ Hsp70-*nrf1*$^{+/+}$	*nrf1*$^{-/-}$ Hsp70-*nrf1*$^{+/+}$	*nrf1*$^{-/-}$ Hsp70-*nrf1*$^{+/-}$
nrf1$^{+/-}$ Hsp70-*nrf1* $^{+/-}$	*nrf1*$^{+/-}$ Hsp70-*nrf1*$^{-/-}$	*nrf1*$^{-/-}$ Hsp70-*nrf1*$^{+/-}$	*nrf1*$^{-/-}$ Hsp70-*nrf1* $^{-/-}$

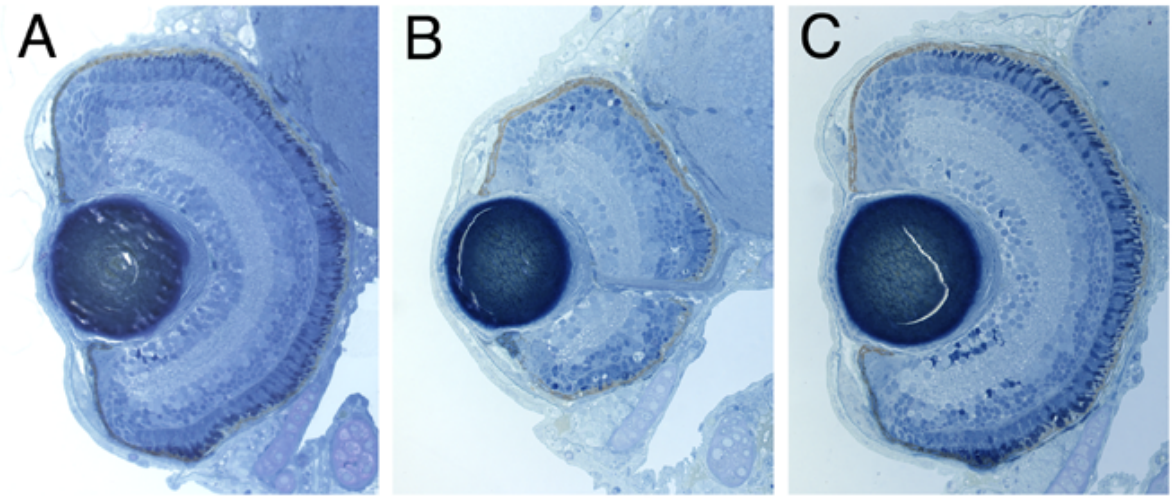

Figure 4.6 Frontal plastic sections through retinae at 6 dpf. (A) The PRs of the wild type embryo are arranged at the outer edge of the retina. (B) In the *nrf* mutant embryo only very few PR cells remains at this stage of development, (C) *nrf* mutant embryo, rescued after one-hour heat shock at 38°C at 24 hpf. The rescued mutant retina looks like a wild type retina, and the PR layer is intact

4.2.6 Conserved function but not expression pattern for nrf1 and ibr

The fact that the phenotypic effects of the mouse *Nrf1* knockout and the zebrafish *nrf* mutation are so different (the mouse knockout dies between embryonic day 3.5 and 6.5 (Huo and Scarpulla, 2001), zebrafish larvae die between 10-14 dpf (Becker et al., 1998)), might reflect that the gene has different functions in these two vertebrates. To analyze this, the expression pattern of *Nrf1* during development was analyzed in both mouse and chick, and functional studies were carried out in zebrafish using the avian *Nrf1* homolog *ibr*.

4.2.6.1 Expression of *Nrf1* in mouse and chicken embryos

The results from whole mount *in situ* hybridization shows that the expression pattern of *Nrf1* in mouse and chick is different than the zebrafish *nrf1* expression pattern (figure 4.7). In both chick and mouse strong expression is detected in the branchial arches, as well as in the optic tectum, the developing eye and the somites. This is distinct from the widespread *nrf1* pattern with strong eye expression observed in zebrafish. Expression is also high in the limb buds in the tetrapods, while in zebrafish there is no detectable expression of *nrf1* in the developing pectoral fins suggesting a common function of *Nrf1* during development of chick and mouse different from zebrafish.

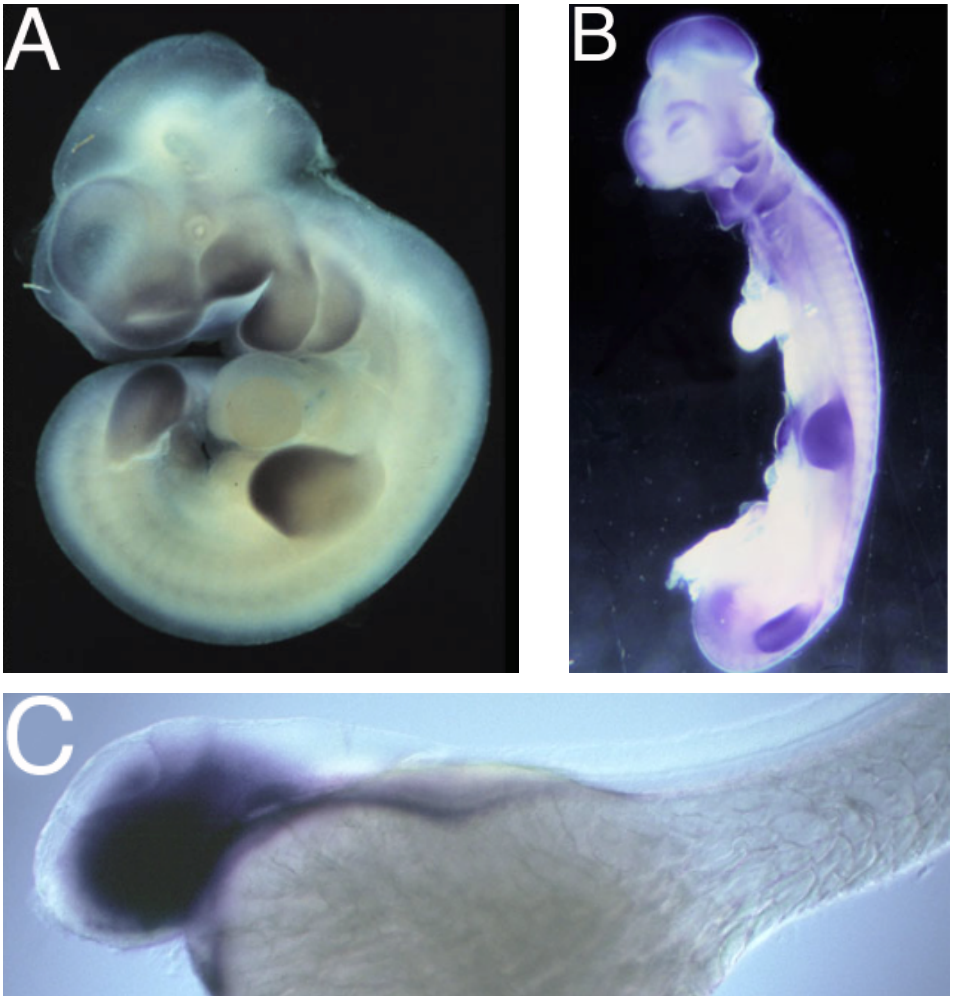

Figure 4.7 Expression pattern of *Nrf1* homologues in vertebrates. (A) Mouse d10.5 embryo. Expression of *Nrf1* can be seen in the optic tectum, eyes, branchial arches, limb buds and somites. (B) Chick stage 23 shows a similar expression pattern as seen in the mouse. (C) *Nrf1* expression in the 48 hpf zebrafish embryo is widespread and can be detected throughout the head. Pictures A and B from T. Becker (unpublished), C from (Becker et al., 1998).

4.2.6.2 Rescue of the *nrf* mutant by ectopic expression of *ibr*

To identify whether chick IBR can rescue the zebrafish *nrf* mutants, triple heterozygous fish were generated through inbreeding of the two afore-mentioned lines, hsp70-*ibr#2* and CLGY298/*nrf*^{hi399}. Embryos were heat shocked at various time points and non-fluorescent *nrf* homozygous fish were identified between 24 and 48 hpf. When the *nrf* homozygous embryos were analyzed at 5-6 dpf, the majority of the embryos did no longer display the *nrf* mutant phenotype, and could not be distinguished from wild type embryos (table 4.10). A section through the retina of the rescued mutant revealed that the PR cell layer was intact, and the mutant retina could be compared to the wild type (figure 4.8). Thus, *ibr* can functionally substitute for *nrf1*, and also serves no additional instructive role when expressed ubiquitously in zebrafish embryos.

Table 4.10 Rescue of *nrf* mutants after overexpression of *ibr*

Heat shock	**+ Fluorescence**	**Phenotype 5 dpf, n (%)**	**- Fluorescence**	**Phenotype 5 dpf, n (%)**
24 hpf	35 (69%)	WT: 35 (100%)	16 (31%)	WT: 12 (75%)
		nrf: 0 (0%)		*nrf:* 4 (25%)
24 + 48 hpf	72 (74%)	WT: 72 (100%)	25 (76%)	WT: 20 (80%)
		nrf: 0 (0%)		*nrf:* 5 (20%)

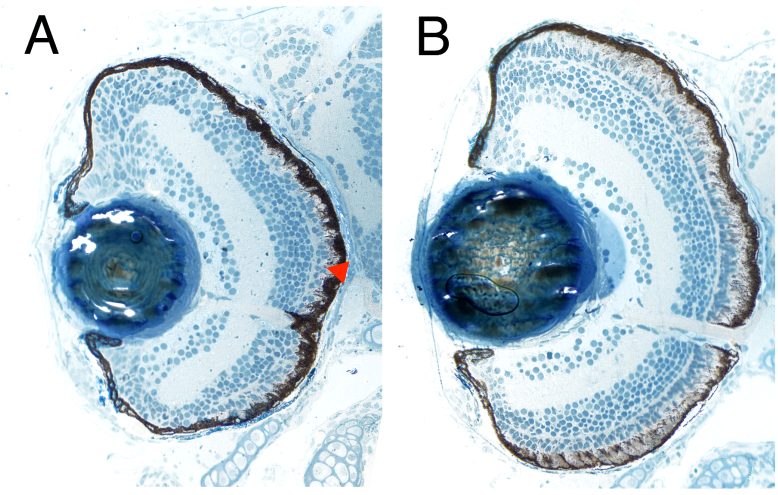

Figure 4.8 Frontal plastic sections through retinae at 6 dpf. (A) Very few PRs remain in the retina of the *nrf* mutant embryo (arrowhead) (B) An *nrf* mutant embryo rescued by overexpression of *ibr* after one-hour heat shock at 38°C at 24 hpf. The retina appears completely wild type, and the PR layer is intact.

4.2.6.3 *Nrf1* syntenic region

The human, chick and zebrafish NRF1 homologues are highly conserved (91%), and also the molecular function of *nrf1* is conserved between fish and chick. However, the expression patterns are divergent between the fish and the tetrapods (figure 4.7). This

difference is likely due to differences in *Nrf1* proximal and/or distal regulatory regions. Searching the genomic areas surrounding *Nrf1* show that it is situated on a chromosomal region syntenic through all vertebrate genomes. Conserved synteny is the presence of two or more orthologous gene pairs on a single chromosome in each of two different species. A genome wide comparison has identified that a large region of chromosome 4 in zebrafish corresponds to chromosome 19 in Tetraodon, and chromosome 7 in human (Woods et al., 2005). The following genes are present in the same syntenic blocks throughout the vertebrate lineages: *Nrf1*, *Ube2h*, *Nipa* and *Slim*. Figure 4.9 shows a schematic view of the order and orientation of these and neighboring genes on the chromosome.

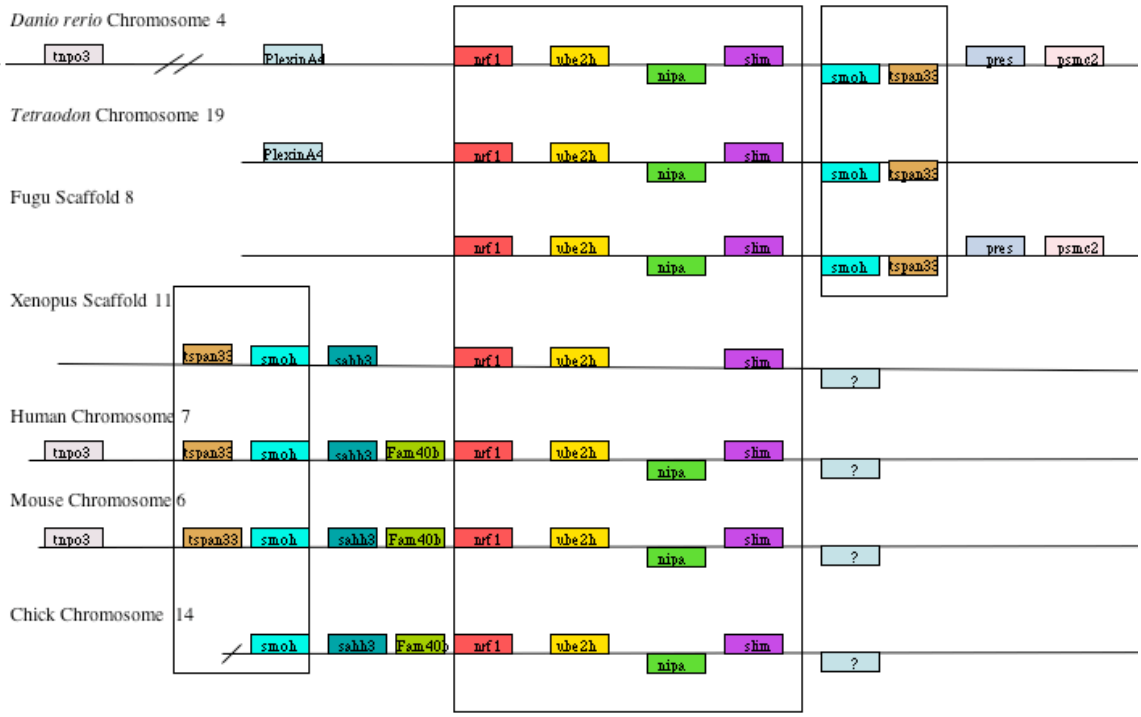

Figure 4.9 Schematic representation of the syntenic relationships of the genomic region surrounding *Nrf1* in vertebrate species. Data obtained from www.ensembl.org.

The differing expression patterns are a product of the chromosomal neighborhood of *Nrf1* in tetrapods vs. teleosts. The expression pattern in the limbs of tetrapods may suggest that Nrf1 serves an additional function in this lineage.

4.2.7 Nrf1 is needed during a critical period for survival of photoreceptor cells

To test whether there was a specific time point at which *nrf1* expression is crucial for survival of developing photoreceptors, the hsp70-*nrf1*;CLGY298/*nrf*^{hi399} embryos

were heat shocked at various time points (table 4.11). At 6 hpf *nrf1* overexpression did not show any rescue effect, indicating that this is too early for an effect of the Nrf1 protein. This is consistent with the negative results after injection of *nrf1* mRNA (section 4.2.3). However, one heat shock between 24 and 55 hpf was sufficient to rescue the mutant embryos, while heat shocks at or later than 72 hpf did not rescue the mutant phenotype (figure 4.10), presumably because many PRs have already undergone apoptosis at this stage.

Table 4.11 The time point of heat shocking is important for the effect of the rescue. The table shows the phenotype displayed at 5 dpf (note that some of the embryos died before 5 dpf, and the phenotype could not be scored).

Time of heat shocking	**+ Fluorescence**	**Phenotype 5 dpf, n (%)**	**- Fluorescence**	**Phenotype 5 dpf, n (%)**
HS 6 hpf	55 (81%)	WT: 55 (100%)	13 (19%)	WT: 0 (0%)
		nrf: 0 (0%)		*nrf*: 12 (100%)
HS 24 hpf	116 (83%)	WT: 114 (100%)	23 (17%)	WT: 18 (85%)
		nrf: 0 (0%)		*nrf*: 3 (14%)
HS 48 hpf	49 (79%)	WT: 49 (100%)	13 (21%)	WT: 10 (83%)
		nrf: 0 (0%)		*nrf*: 2 (17%)
HS 55 hpf	62 (78%)	WT: 62 (100%)	17 (22%)	WT: 14 (82%)
		nrf: 0(0%)		*nrf*: 3 (18%)
HS 72 hpf	62 (83%)	WT: 62 (100%)	13 (17%)	WT: 0 (0%)
		nrf: 0 (0%)		*nrf*: 13 (100%)
HS 96 hpf	49 (80%)	WT: 49 (100%)	12 (20%)	WT: 0 (0%)
		nrf: 0 (0%)		*nrf*: 12 (100%)
No HS	50 (78%)	WT: 50 (100%)	14 (22%)	WT: 0 (0%)
		nrf: 0 (0%)		*nrf*: 11 (100%)

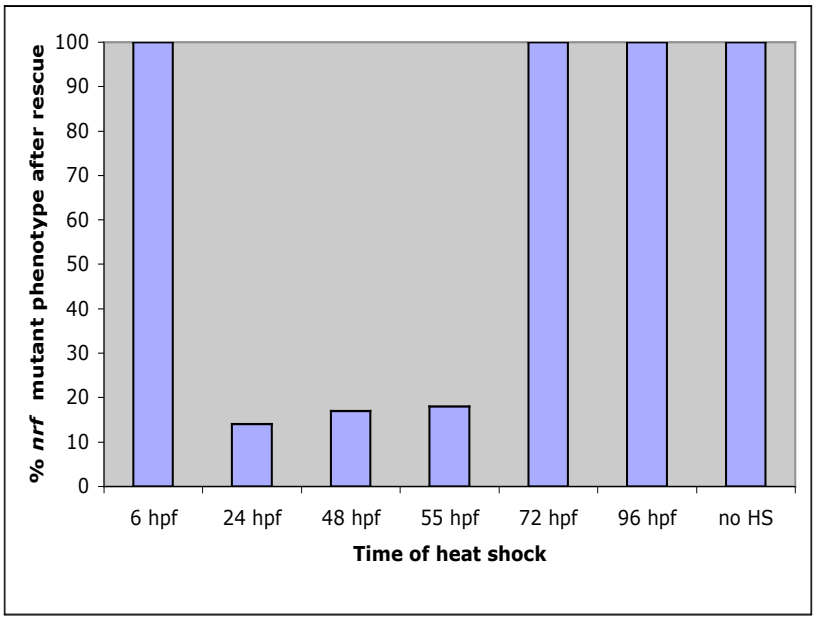

Figure 4.10 Graphics showing the percentage of remaining *nrf* mutant phenotype embryos at 5 dpf after rescue experiments at various time points.

This suggests that Nrf1 is a factor that is needed for the maintenance or survival of the photoreceptors as the PRs can form and differentiate in the absence of Nrf1. After the initial heat shock, the rescued embryos appear normal until around 9 dpf. Then they gradually revert to the mutant phenotype, suggesting that the mutant phenotype is delayed rather than permanently rescued and thus showing that that Nrf1 protein is continually necessary for PR integrity. This result is also in accordance with the earlier findings that PR can form and differentiate in the absence of Nrf1. Examination of retinal sections revealed that apoptosis of PR in the rescued larvae is delayed, and at 13 dpf PRs can no longer be detected in the retina of the rescued mutants (figure 4.11). Note that in all the rescue experiments, the fish were heterozygous for the hsp70-*nrf1* transgene, meaning that 1/4 of the *nrf* mutant embryos could not be rescued, and served as internal controls for the heat shock.

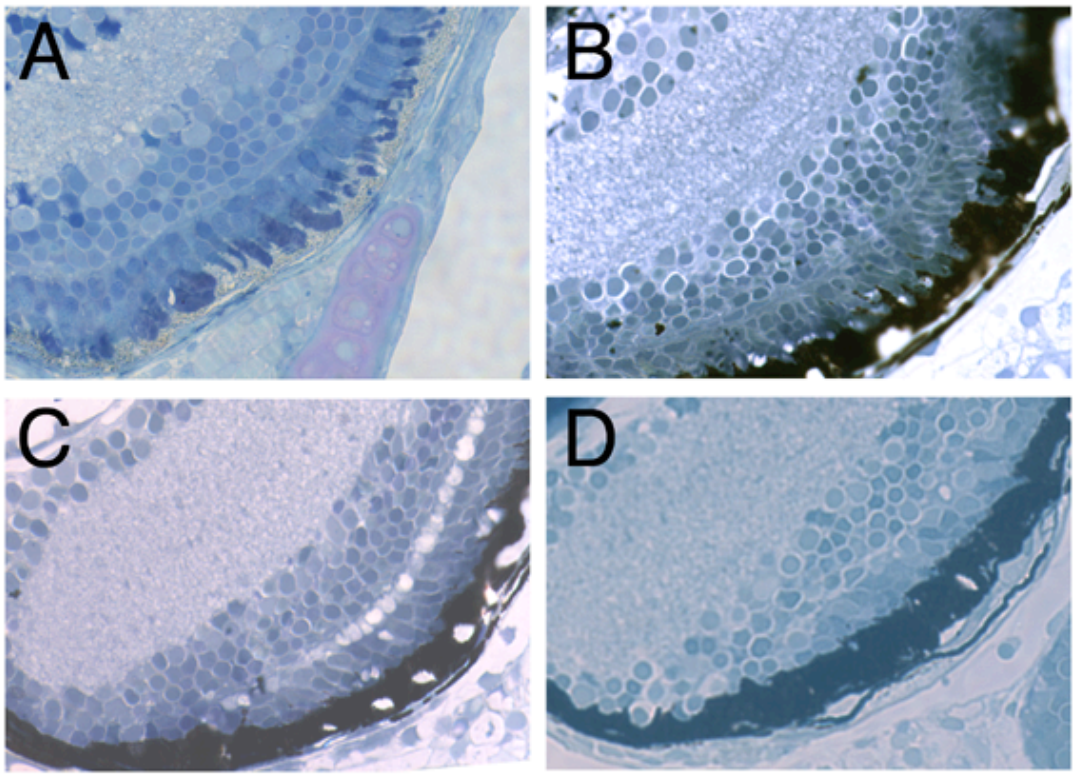

Figure 4.11 Plastic sections of retinae of *nrf1* rescued *nrf* mutant larvae. Embryos were heat shocked for one hour at 24 hpf (A) Larval retina at 6 dpf. Note the PR layer where both inner (IS) and outer segments (OS) are visible. (B) Larval retina at 9 dpf. The PR layer is still evident, with both IS and OS, (C) Larval retina at 11 dpf. The PR layer is thinner than at 9 dpf, and the OS cannot be distinguished any longer, (D) Larval retina at 13 dpf. PR layer is almost degraded. At this stage the eye size is greatly reduced and the larvae display a clear *nrf* phenotype.

When the experiment was repeated using a transgenic line overexpressing *ibr*, the same phenomenon was observed. The effect of *ibr* expression wears off with time, and the *nrf* mutants show an *nrf* phenotype (figure 4.12).

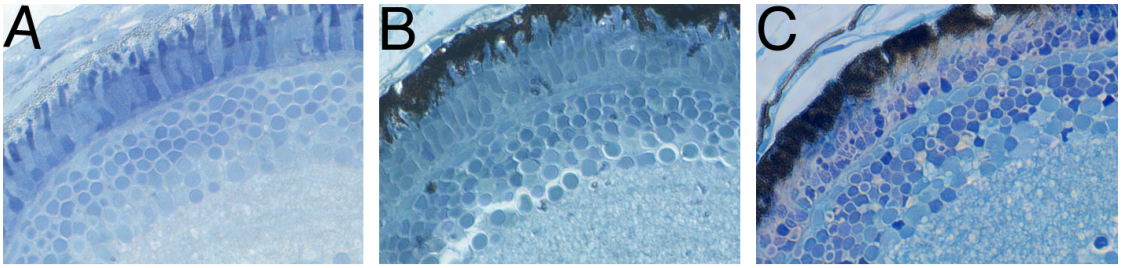

Figure 4.12 Plastic sections of *ibr* rescued *nrf* mutant larvae. Embryos were heat shocked for one hour at 24 hpf (A) Rescued larva at 5 dpf. The layering of the retina appears normal. (B) Rescued larva at 9 dpf. The PR layer is still evident, with both IS and OS. (C) Rescued larva at 13 dpf. PR layer is almost degraded. At this stage the eye size is greatly reduced and the larvae display a clear *nrf* phenotype.

I conclude that, while Nrf1 is necessary for survival of PRs during their development, it plays no role in their specification or morphogenesis, but rather sensitizes these cells for apoptosis, and once apoptosis sets in, there is a bystander effect that leads to loss of these cells in large clusters.

4.3 Identification of Nrf1 downstream target genes

From the rescue experiments it is clear that Nrf1 is needed for maintenance or survival of PR cells between 24 and 55 hpf. To identify possible downstream targets of Nrf1 that might be involved in the retinal phenotype, a gene expression comparison of *nrf* mutants and wild type embryos using microarrays at 26 hpf was performed. This time point was chosen as it was expected that Nrf1 target genes were differentially expressed then, as gathered from the importance of the gene product at this time.

4.3.1 Expression profile comparison reveals new candidate downstream genes for Nrf1

nrf mutant embryos were separated from wild type siblings at 26 hpf using the CLGY298 marker line. RNA from pools of 200 wild type and 200 *nrf* mutant embryos was isolated, reverse transcribed and the cDNA was labeled with fluorescent Cy3 and Cy5 before applied on the microarray chips. The intensity of each fluorescent

dye that hybridized on a single spot was calculated, and spots with an average cal ratio change greater than 1.5 - fold, and intensities >130 were scored as positive. A dye -swap hybridization was performed, meaning that the two arrays were switching color scheme, and clones scoring positive in both experiments were selected as potential differentially expressed targets. A gene was identified as downregulated when the cal ratio for both experiments was lower than 0.67, and upregulated when it was higher than 1.45. In total, 12 potential down-regulated genes were identified (table 4.12), and as expected, *nrf1* was among these. Four of the hits encode novel genes. The other potential down-regulated target genes were *lactate dehydrogenase 4* (*ldhb4*), *eIf1α,* the *vomeronasal receptor v2rdl, myst1*, *spy1*, *PPIL3-cyclophilin-like* and *nephrocystin1*. Deletions in *nephrocystin1* lead to nephronophthisis (NPHP) (Hildebrandt et al., 1997), which is the most common cause of renal failure in children (Hildebrandt and Omram, 2001), and it belongs to a family of 5 genes that all cause kidney failure at an early age. 10% of the NPHP affected individuals also have RP, displaying the renal-retinal Senior-Løken syndrome (SLSN) (Loken et al., 1961; Senior et al., 1961).

9 genes in total were identified as upregulated (table 4.13) with cal ratio >1.45. These include *IFT57* and *dnah9* (axonemal dynein) both involved in intraflagellar transport, and the enzymes *rio kinase 1* (*riok1)*, *ethanolamine kinase (Ek1)*, *monoamine oxidase A (mao)*, *topoisomerase1 (top1)* and *N-acetylneuraminate pyruvate lyase* (*npl*). In addition 2 novel genes were also identified as downregulated. IFT proteins are essential for eukaryotic cilia and flagella assembly, carrying protein vesicles along the ciliary microtubular machinery and moved by kinesin II and dynein. In zebrafish the IFT mutants include *IFT88/oval* (*ovl*) (Tsujikawa and Malicki, 2004b), *IFT81/larry*, *IFT172/moe* and *IFT57/curly* (Sun et al., 2004), all of which develop kidney cysts, but *ovl* and *curly* also display photoreceptor loss (Doerre and Malicki, 2002).

Table 4.12 Overview of putative downregulated target genes

Gene	GenBank ID	Cal ratio		RT-PCR ratio
Nrf1	NM_131680	0.58	0.36	0.86
eIF1α	NM_001006082	0.61	0.53	0.22
eIF1α	NM_001006082	0.57	0.55	0.22
Nephrocystin1	XM_703692	0.66	0.60	0.34
MYST1	BC055629	0.59	0.62	0.31
Spy1	NM_001006091	0.57	0.63	0.42
PPIL3-cyclophilin-like	NM_001002146	0.67	0.61	0.95
V2rdl	NM_001018147	0.66	0.66	0.59
Novel#1	XM_688245	0.61	0.58	-
Novel#2	Fp39h06.xl	0.64	0.64	-
Novel#3	NM_001017780	0.61	0.55	-
Novel#4	BM036100	0.63	0.53	-
Ldhb4	BC044190	0.63	0.66	0.40

Table 4.13 Overview of putative upregulated target genes

Gene	GenBank ID	Cal ratio		RT-PCR ratio
dnah9	NM_200462	1.45	1.46	2.71
Riok1	NM_212995	2.18	1.51	1.90
Novel#5	AI793527	1.54	1.56	-
IFT57/hippi	NM_001001832	1.67	1.45	1.72
Novel#6	AI974135	1.83	1.54	-
Ek1	NM_001013574	1.52	1.69	1.62
Mao	NM_212827	1.45	1.64	-
Top1	NM_001044324	1.52	1.55	1.04
npl	NM_207051	1.89	2.11	1.15

Surprisingly, many of the previously identified downstream targets of NRF1 were not found to be affected in this experiment such as *5'ALAS*, *eIF2α, GPAT*, (Braidotti et al., 1993; Chen et al., 1997; Jacob et al., 1989). In some cases as for example with *E2F6* and *tyrosine aminotransferase* (Chau et al., 1992; Kherrouche et al., 2004), the transcript could not be found on the microarray chip, or the data were not conclusive. Table 4.14 lists NRF1 targets from the literature, relative to the data from the microarray analysis.

Table 4.14 List of genes not affected in *nrf* mutant or not found on microarray chip

Gene	Results from microarray	NRF1 functional binding sites	Pos/neg regulated by NRF1
eIF2α	No change	(Chau et al., 1992)	Positive
P115	Not found	(Watanabe, 2003)	Positive
CD155	Not found	(Solecki et al., 2000)	Positive
IAP/CD47	Not found	(Chang and Huang, 2004)	Positive
5'-ALAS	No change	(Braidotti et al., 1993)	Positive
MtTFA	Not found	(Virbasius and Scarpulla, 1994)	Positive
Tyrosine aminotransferase	No change	(Chau et al., 1992)	Positive
E2F1	No change	(Efiok and Safer, 2000)	negative
E2F6	Not found	(Kherrouche et al., 2004)	positive
FMR1	Not found	(Kumari and Usdin, 2001)	positive
GPAT/AIRC	No change/not found	(Chen et al., 1997)	positive
Zfp106	Not found	(Grasberger et al., 2005)	positive
Histone 5	Not found	(Gomez-Cuadrado et al., 1995)	negative
Cyt c	Not conclusive	(Evans and Scarpulla, 1989)	positive
ATP synthase γ-subunit	No change	(Chau et al., 1992)	positive
MRP RNA	Not found	(Evans and Scarpulla, 1990)	positive

4.3.2 Confirmation of candidate genes using qRT-PCR

To confirm that the selected genes indeed were differentially expressed in the *nrf* mutant, quantitative real time-PCR (qRT-PCR) was performed using RNA samples from 26 hpf *nrf* mutant and wild- type embryos. Histone 3.3b expression was used to normalize the data, as expression levels of this gene were unchanged on the microarray. Primers were designed for all the genes identified in the microarray assay. The qRT-PCR for each gene was performed twice, each with three replicates. Relative cDNA amounts were calculated and the results summarized in tables 4.12 and 4.13 as the ratio of expression in the *nrf* mutant embryos versus their wild type siblings. Eight of 12 of the downregulated genes were confirmed to have reduced expression in *nrf* mutants, among the non-confirmed ones was *PPIL3-cyclophilin like*. Four of 9 were confirmed to have increased expression. Expression of *Top1* and *npl* was not confirmed to be significantly upregulated by qRT-PCR. None of the novel genes could be confirmed as up or downregulated.

4.3.3 There are no apparent defects in the kidneys of nrf mutant embryos

As several genes involved in maintenance of the kidneys were expressed differentially in the *nrf* mutants, kidney malfunction could be a possible explanation of why the *nrf* mutants die. One of the main visible causes of kidney failure in zebrafish is cystic

kidney, the development of fluid filled cysts in the kidneys (Drummond et al., 1998). No kidney cyst formation could be detected in the *nrf* mutant using the light microscope, and no changes in kidney morphology could be detected on plastic sections of whole mount embryos (data not shown). The complete pronephric system can be visualized at 3 dpf by staining with an antibody against renal Na^+/K^+ ATPase (Takeyasu et al., 1988). The Na^+/K^+ ATPase cation pump is needed for maintaining the salt balance in the kidneys, and thus the water balance of the whole animal. After Na^+/K^+ ATPase immunostaining on embryos at 6-8 dpf no staining of kidneys in either mutant nor wild type embryos could be detected (data not shown), most likely because the antibody did not penetrate the tissue well enough at this late point of development. An antibody against acetylated tubulin was used to stain ciliated cells in the embryo (Sun et al., 2004), and was used to examine the presence of cilia in the zebrafish pronephric duct and tubule between 6-8 dpf. No difference in staining of the *nrf* mutant kidneys was observed when compared to wild type (figure 4.13), suggesting that at this time point the epithelial cells in the *nrf* mutant kidneys are ciliated normally.

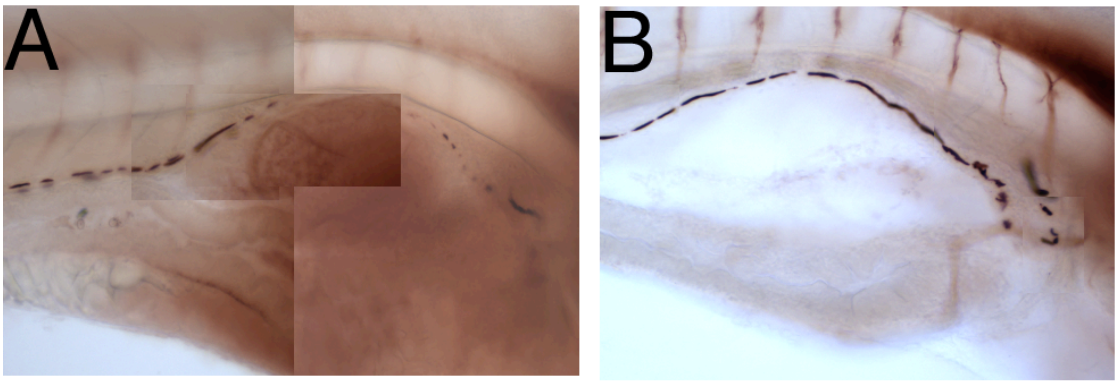

Figure 4.13 Immunostaining of pronephros and pronephric ducts using α-acetylated tubulin in (A) WT embryos and (B) *nrf* mutants at 8 dpf. Note that the mutant does not show less staining than the wild type embryo. The difference in acetylated tubulin staining between the two embryos is presumably due to unequal penetration of the antibody. Dorsal is up and anterior is to the right.

5 DISCUSSION

5.1 Different approaches for generation of transgenic lines

This part of the thesis describes the successful generation of transgenic lines for *nrf1* overexpression under temporal control, and a comparison of varying methods for transgenesis.

By injecting circular or linear plasmid DNA into one-cell stage zebrafish embryos transgenic zebrafish were generated. The transgenic frequency efficiencies for the various vector systems are summarized in table 5.1.

Table 5.1 Summary of results from the generation of transgenic lines

Plasmid injected	Transgenic frequency	Germline transmission rates	Average germline transmission rate
pBs-*Sce*I-hsp70-*nrf1*	32%	3-46%	19.5%
pBs-*Sce*I-hsp70-*ibr*	3%	10-33%	21.7%
pSix3.1-Gal4	8.1%	10-42%	20.5%
pSix7-Gal4	3.5%	3-21%	9.8%
pUAS-*nrf1*	1.6%	11-14%	12.5%
pUAS-*EGFP*	5%	8-33%	16.3%
p2xMAR-UAS-*nrf1*	3.5%	5-13%	10%
pBi-EGFP	2%	4-21%	13.3%
pBi-EGFP-*pes*	3.7%	1-28%	11.5%

As seen from the table, transgenic frequencies after injections of naked plasmid are low. All are in the 2-8% range, a percentage similar to earlier reports on plasmid injections (Stuart et al., 1988; Stuart et al., 1990). However, for the meganuclease co-injected plasmid pBs-*Sce*I-hsp70-*nrf1*, the transgenic frequency was 32%. This is a significant improvement over injection of naked plasmid. For medaka transgenic frequencies as high as 30.5% have previously been reported by using the *Sce*I meganuclease system (Thermes et al., 2002).

The yeast *Sce*I enzyme (Jacquier and Dujon, 1985), which is coinjected with the pBs-*Sce*I-hsp70-*nrf1/ibr* plasmids is an intron encoded transposase (reviewed in (Dujon, 1989). The 18 bp recognition sequence of *Sce*I meganuclease is expected to appear only once in $7x10^{10}$ bp of random sequence, thus it is not likely to find any *Sce*I recognition sites in the 1.6-1.7 $x10^9$ bp zebrafish genome. The mechanism of meganuclease function is not precisely known, but the enzyme remains linked to the longer half of the recognition site after cleavage (Colleaux et al., 1988), possibly protecting the linear DNA from degradation. It has also been hypothesized that the co-injected *Sce*I enzyme counteracts the strong ligase activity present in the cytoplasm of

the fish eggs, and prevents the generation of long concatemers (Thermes et al., 2002). This leads to a higher concentration of free ends available for recombination, which increase the integration efficiency. This has also been suggested to lead to a higher rate of germline transmission as the integration events takes place earlier (Thermes et al., 2002). As suggested from table 5.1, the average transmission rates are slightly higher for the meganuclease co-injected constructs than for injection of naked plasmid.

As the two meganuclease constructs pBs-*Sce*I-hsp70-*nrf1* and pBs-*Sce*I-hsp70-*ibr* are the same except for the different gene inserted, the low numbers of transgenic frequencies of the second meganuclease construct, pBs-*Sce*I-hsp70-*ibr* can be explained by a lower sensitivity of the PCR screen, not allowing the detection of all positive founders.

Three factors are important for the effect of expression of a transgene. One is chromosomal position effect, determined by the site where the transgene insertion takes place and can lead to silencing of the transgene. Silencing can also be induced by generation and insertion of long concatemers (Garrick et al., 1998). The third factor is the choice of promoter used to drive expression. Using transgenes with promoters of zebrafish origin has been suggested to be superior over promoters with heterologous origin (Higashijima et al., 1997). Both the *six3.1* and *six7* promoters used in the Gal4/UAS system are zebrafish promoters with tissue specific but expression patterns (Drivenes et al., 2000; Wargelius et al., 2003), but it is possible that they are not strong enough (*ie*. to produce enough Gal4) to be able to transactivate the effector lines.

However, the main reason why the expression levels in the Gal4/UAS transgenic lines are weak is probably the limited transactivation potential of Gal4 in fish (Grabher and Wittbrodt, 2004). In zebrafish, the Gal4/UAS system could drive expression of a gene of interest, but the expression was not strong enough to give a phenotypic effect (Scheer and Campos-Ortega, 1999). A fusion of the Gal4 DNA-binding domain and the strong VP16 transactivation domain of the herpes simplex virus has successfully been used to enhance transactivation efficacy of the Gal4/UAS system in fish when placed in the same construct (Grabher and Wittbrodt, 2004; Koster and Fraser, 2001b). In a modular misexpression screen in *Drosophila* 14 UAS elements were included in the effector plasmid for high transactivation efficiency (Rorth, 1996). In the constructs used for misexpression in zebrafish, only 5 UAS

sequences were included (Scheer and Campos-Ortega, 1999). This could also be a limiting factor for the transactivation potential of the system.

The Tet transactivation system was originally used with the native Tet repressor blocking transcription when binding to the TRE in presence of tetracycline (Tet-Off) (Gossen and Bujard, 1992). This approach was later replaced with the Tet-On system where transcription is activated in the presence of tetracycline (Gossen et al., 1995). The reason for the change into a positive control system was that active repression of a strong promoter requires a high concentration of repressor, and one needs 100% repression for the effect to be successful, or else there will be background expression. Using the Tet-On system requires lower levels of tetracycline inducer. Various reports have debated the efficiency of the two systems (Mizuguchi and Hayakawa, 2002), but the general view is that the Tet-On system is better suited for transgene animal experiments (Huang et al., 2005).

Huang and colleagues reported that GFP expression could be detected after induction with the tetracycline derivative doxycycline for 7 hours (Huang et al., 2005). I did not detect any differences in transactivation when induction was performed with tetracycline for 6, 12, 24, and 48 hours (data not shown). However, the strength of the promoter and not the induction was probably the limiting factor in this assay. In our case the pax2.1 promoter was used as an activator of expression. Pax2.1 is a tissue specific promoter (Picker et al., 2002), but the promoter used in this transgenic line is not complete as pax2.1 expression is also driven by a large HCNE (highly conserved noncoding element) array (Sandelin et al., 2004). The fact that transactivation was detected in 1 out of 10 lines shows that the assay is functional, but the promoter of choice might not be strong enough or sufficient.

Neither of the binary systems answered to the expectations in zebrafish, as both were expressing poorly. The expression rate of the Gal4/UAS system was 19%, while in the Tet-On system only 10% of the transgenic lines were expressing the transgene. Compared to the expression rates in the *Sce*I injected transgenics (50-100%), this is very low. Although the binary systems with the effector and activator lines promise versatility with a mix and match of various lines, when working with a gene of interest it is still the most time efficient choice to use a transgenic line with the transgene under control of a specific or a heat shock promoter, as these lines are straightforward to generate, and they provide reliable expression.

5.2 Characterization of the zebrafish *nrf* mutant

In this part of the study, I have shown that Nrf1 is needed for maintenance of photoreceptor cells, and that it is a factor needed constantly throughout development and at larval stages. It is needed before 56 hpf in order to rescue the PR cells. Furthermore, the versatility of a fluorescent genetic marker is described. This marker enables the identification of *nrf* mutants before the phenotype is evident by acting as a live chromosomal marker for the *nrf1* wild type allele.

5.2.1 Overexpression of nrf1 has no effect on the developing zebrafish embryo

The finding that overexpression of *nrf1* at various time points through development has no effect on the developing embryos is in contrast to a report suggesting that overexpression of *Nrf1* sensitizes cells in culture to apoptosis (Morrish et al., 2003). However, the findings of Baar and colleagues (Baar et al., 2003) show that an isolated increase in NRF1 is not sufficient to induce a coordinated increase in expression of all the proteins necessary for the assembly of functional mitochondria. This is likely to be due to the requirement for both NRF1 and NRF2/GABP to stimulate mtTFA expression (Scarpulla, 2002b). According to the microarray data in the present study, *nrf2*/*GABP* expression remains unchanged in the *nrf* mutant suggesting that it is not affected by Nrf1 level. MtTFA could not be identified in the sequences in the dataset. Note that the same transgenic lines that gave no effect when overexpressing *nrf1* could transiently rescue the *nrf* mutants during development.

5.2.2 A CLGY enhancer trap line as a live chromosomal marker for the nrf mutant

Homozygous mutant embryos early on are indistinguishable in morphology from their wild type and heterozygous siblings, and the isolation and analysis of large numbers of mutant embryos before the phenotype is evident has been difficult and laborious. Many developmental genes are active before the mutant phenotype is evident, and it is important to be able to identify mutants at an early age. Qian and colleagues have developed a tool using amplified cDNA, for gene expression profiling at the 18-somite stage of a zebrafish mutant, which is indistinguishable in morphology from wild type siblings before 24 hpf (Qian et al., 2005). Another method is the use of a reporter line with a specific promoter to identify mutants at an early stage (Sumanas et al., 2005). The approach described in this study, using an enhancer trap line with a specific pattern and a known insertion site in the genome also allows rescue

experiments and other early morphological analysis on live embryos before the phenotype is visible, as well as gene expression profiling using microarrays. In our lab at the present time more than 1000 enhancer trap lines expressing YFP in a distinct pattern have been generated. Many of these insertions are located in close proximity to a gene, and can be used as marker lines.

5.2.3 Nrf is needed during a critical period for survival of photoreceptor cells

Our results using the hsp70-*nrf1* transgenic line show that *nrf1* does not rescue the mutant PR cells when expressed before and after a certain time window. *Nrf1* is not needed for the initial differentiation and morphogenesis of PR cells as some photoreceptors form and differentiate and are still present at day 5 of development in the *nrf* mutant (Becker et al., 1998). However, our data suggests that Nrf1 is functioning as a maintenance factor for the survival of the differentiated PRs. If Nrf1 is expressed before a certain time point, this has no effect on the survival of the cells, and likewise if it is presented too late the cells have already started the apoptotic pathway. Results from MO injections show that if early Nrf1 translation is blocked, but presented again after a time, the PR cells will nevertheless lose their OS and undergo apoptosis.

Several zebrafish mutants display apoptotic photoreceptor cells between 3-7 dpf, including *ebony*, *ivory*, *brudas, niezerka, ovl* and *mikre oko (mok)* (Doerre and Malicki, 2001; Doerre and Malicki, 2002; Goldsmith et al., 2003). *Ovl* and *mok* show a phenotype similar to *nrf* with loss of OS, leading to apoptotic PRs. Ovl function is not required for the initial formation of PRs, but is necessary for the maintenance of PR OS (Doerre and Malicki, 2002). The PR IS seems to stay intact, but the OS defects are followed by PR degeneration in a central to peripheral pattern. In this case a cause for cell death is suggested to be the ectopically localized opsins (Tsujikawa and Malicki, 2004b). This corresponds to the finding that overexpression of human rod opsin leads to PR cell degeneration similar to the rod opsin mutant phenotype in mouse (Olsson et al., 1992). Interestingly, the loss of OS in *mok* also coincides with mislocalized opsins (Doerre and Malicki, 2001). A suggested model is that when OS are not formed, opsins are localized to other compartments in the cell where their activity can interfere with other signaling pathways, causing cell death (Tsujikawa and Malicki, 2004a). The light pigments are present in the *nrf* mutant (Becker et al., 1998), but it remains to be seen if they are mislocalized in apoptotic PRs.

5.3 Downstream candidates for Nrf1

In this part of the thesis I identified potential downstream targets of Nrf1, and suggested that the retinal phenotype of the *nrf* mutant might be due to a defect in the PR connecting cilium.

5.3.1 Genes potentially regulated by Nrf1

The availability of zebrafish cDNA microarrays provides a valuable tool to identify downstream target genes affected in the *nrf* mutant. Our study has identified 21 potentially down- or up-regulated genes in the *nrf* mutant, of which *nrf1* was one and thus serves as a positive control for the quality of the microarray data. 17 of these genes were confirmed using qRT-PCR. To identify primary Nrf1 target genes the microarray analysis was performed on 26 hpf embryos, a time point within a critical period in which Nrf1 was needed for photoreceptor maintenance. *Nephrocystin1*, *IFT57/Hippi* and *dnah9* are particularly interesting candidate genes as they are all involved in ciliary defects in eukaryotes (Essner et al., 2005; Wolf et al., 2005).

Nephrocystin1 localizes exclusively to sensory cilia in *C. elegans* (Jauregui and Barr, 2005), but is more widely expressed in the mouse, including the limb buds, branchial arches and retina, and primary cilia of renal epithelial cells (Otto et al., 2000)Schermer et al., 2005). Interestingly, this expression pattern corresponds to the *Nrf1* expression pattern in mouse (Schaefer et al., 2000). The paralogue Nephrocystin5 is localized to the connecting cilia and outer segments of mouse PR cilia (Otto et al., 2005). Mutations in *Nephrocystin1* are responsible for juvenile NPHP (Saunier et al., 1997), and the protein interacts with Nephroretinin (Nephrocystin4), Inversin and β-tubulin (Mollet et al., 2002; Otto et al., 2003), as well as a series of cell-cell and cell-matrix signaling proteins (Benzing et al., 2001; Donaldson et al., 2002).

Also intraflagellar transport proteins have been shown to be important for ciliary function. The mouse *IFT88/tg737* mutant has reduced production of the ciliary transport protein IFT88 (Moyer et al., 1994; Pazour et al., 2002), causing shorter photoreceptor OS and subsequently leading to retinal degradation and to polycystic kidney disease. It is suggested that both phenotypes are caused by ciliary dysfunction. The zebrafish *ovl/IFT88* mutant shows a similar phenotype (Doerre and Malicki,

2002). Two other zebrafish mutants *elipsa* and *fleer* have the same defects as *ovl*, and may share a common genetic pathway (Doerre and Malicki, 2002). Three IFT mutants (*ift57/curly*, *ift81/larry* and *ift172/moe*) were identified in an insertional mutagenesis screen for cystic kidney mutants (Sun et al., 2004). Additional defects of the *ift57/curly* mutant are curved body and PR degradation, but maternal contributions complicated the interpretations of photoreceptor OS formation. However, morpholino knockdowns demonstrate that Ift57 is not necessary for generation of cilia, but for their maintenance, and that PRs degenerate in the absence of Ift57 (Tsujikawa and Malicki, 2004b). It is not known whether overexpression of IFT proteins can be harmful to the cell, but deflagellation in *Chlamydomonas* is characterized by stimulation of IFT particle trafficking, indicated by an upregulation of IFT proteins in the shortening flagella (Pan and Snell, 2005). In the *nrf* mutant upregulation of the IFT protein IFT57/Hippi could be detected.

Upregulation of the dynein heavy chain gene *dnah9/lrdr1* is interesting as this is a conserved ciliary dynein. In zebrafish it is required for normal left right development (Essner et al., 2005), but no signs of left right asymmetry defects were found in the *nrf* mutants. An interesting feature is that both mouse NRF1 and the *Drosophila* homolog EWG interact specifically with a dynein light chain (Herzig et al., 2000), and this dynein light chain subunit is thought to be associated with the intermediate chains in both axonemal and cytoplasmic dyneins (King and Patel-King, 1995).

The cell cycle regulators Spy1 and MYST1 were found to be downregulated in the mutant. MYST1 is a histone acetyltransferase that is ubiquitously expressed in the zebrafish embryo (Thiesse, 2004). Spy1 interacts with Cdk2, an essential regulator of the eukaryotic cell division cycle, promoting the transition from G1 to S phase in human cells (Porter et al., 2002), and from G2 to M phase in *Xenopus* oocytes (Lenormand et al., 1999). In addition Spy1 can suppress apoptosis after DNA damage by preventing caspase activation (Gastwirt et al., 2006) indicating that Spy1 could be a factor involved in the photoreceptor apoptosis in the *nrf* mutant.

3 of the 12 confirmed differentially expressed genes were metabolic enzymes. The main function known for NRF1 is in regulation of respiration and other biosynthetic pathways such as translational initiation and purine biosynthesis (Chen et al., 1997; Efiok et al., 1994; Virbasius and Scarpulla, 1994). It is also likely that

NRF1 regulates other metabolic pathways. This is suggested from the downregulation of the metabolic enzyme *ldhb4* and upregulation of *Riok1* and *Ek1*.

I also found 6 novel genes to be differentially expressed in the *nrf* mutant on the microarray chip, but was not able to confirm any of these on qRT-PCR.

5.3.2 Genes not found to be differentially regulated in the mutant

Most of the NRF1 target genes known from the literature were not found to be differentially expressed in the zebrafish *nrf* mutant. This can in some of the cases be explained by the fact that the genes have not been cloned in zebrafish, and/or were not found in the microarray data set.

A loss of mitochondrial genes could to some extent explain the *nrf* phenotype. A minor loss of mitochondrial function will affect the ATP level in the PR cells. The loss of ATP can lead to a slower rate of ATP dependent transport of important photo-transducing molecules through the connecting cilium in mutant zebrafish. This can slow the overall turnover rate of the outer segment. As it is known that 9 billion opsin molecules are synthesized and transported to the OS every second in each vertebrate retinae (reviewed in Williams, 2002), this can cause great damage. An arrest in transport of newly synthesized rod opsin molecules has been shown to cause loss of OS and subsequent PR cell death (Marszalek et al., 2000).

The *Nrf1* expression pattern in tetrapods is different from the expression pattern in zebrafish, and an explanation of this gain of function in tetrapods could be a change in regulatory elements upstream of *Nrf1*. As illustrated in figure 4.9 there has been a shuffling of the genomic regions upstream *Nrf1* between the tetrapods and fish lineages. The syntenic block conserved throughout the vertebrate lineages consists of *Nrf1*, *Ube2h*, *Nipa*, and *Slim,* and also includes a miRNA cluster (mir-96, mir-182 and mir-183) between *Nrf1* and *Ube2h*. The expression pattern of the marker line CLGY298 is caused by a retroviral insertion near this cluster. In zebrafish, the genes *nrf1*, *ube2h* and *slim* all show a similar widespread expression pattern (www.zfin.org). Zebrafish *nipa* expression pattern is not published, but its role in cell cycle regulation suggests that also this gene is widely expressed. In addition *smo* located 3' to this syntenic block displays a widespread expression pattern (Chen et al., 2001).

6 CONCLUSION

In this study various combinations of transgenic fish have been generated in order to identify live *nrf* mutant embryos before the onset of the mutant phenotype. This has enabled rescue experiments on the *nrf* mutant and gene expression comparison of an *nrf* mutant versus wild type prior to the onset of the *nrf* phenotype. I have shown that *nrf1* is a gene necessary for the development of the outer retina though it has no direct role in the development of photoreceptors it is rather a maintenance signal. Finally I have suggested that the photoreceptor degradation observed in the *nrf* mutant is a result of intraflagellar transport machinery failure in the connecting cilium of the photoreceptors.

7 FUTURE PERSPECTIVES

The future perspectives in characterizing the *nrf* mutant should mainly be to confirm and analyze the mechanism of photoreceptor degradation suggested here based on the microarray data. The hypothesis of ciliary transport defects can be analyzed by electron microscopy of the *nrf* mutant photoreceptors, and determine if the transport machinery is indeed defect. Many of the genes identified as up or down regulated in the *nrf* mutant have not yet been cloned in zebrafish, and the expression pattern of these should also be determined. Knockdown studies of Nrf1 downstream target genes using the MO technique can tell us more about the pathways involved in the *nrf* phenotype.

As NRF1 is a transcription factor and binds DNA at known binding sites (Gomez-Cuadrado et al., 1995; Virbasius et al., 1993), it will be interesting to look for these binding sites up and downstream of the Nrf1 regulated genes identified in this work. However, just searching the upstream and downstream sequences of the genes gives many hits that are not necessarily real binding sites. Transcription factor binding sites are expected to be conserved to a certain degree, and in order to increase the selectivity and specificity an alignment search between the zebrafish, Tetraodon and Fugu genome should be done. If we can find conserved binding sites it is likely that they are functional and that Nrf1 in fact directly regulates the gene. The genomic sequences can be searched for Nrf1 binding sites by using Position Weight Matrix (PWM), which is a transformation of Position Frequency Matrix (PFM). A PFM has already been generated for IBR (Gomez-Cuadrado et al., 1995), and since I have shown that *IBR* can replace *nrf1* in zebrafish the IBR PFM can be used.

Another important question to be answered is what is the cause of death of the *nrf* mutant. The brain size of the *nrf* mutant is clearly reduced (Becker et al., 1998), and this should be further looked in to. Almost all the cells in the vertebrate body are ciliated, including the brain cells (reviewed in Pan et al., 2005), and if Nrf1 is indeed affecting maintenance of the cilium it is possible that this can have an effect on brain development. Disruption of cilia after *ift88/ift57* MO injections in zebrafish leads to loss of fluid flow followed by fluid accumulation in the brain (Kramer-Zucker et al., 2005).

Several reports have demonstrated that NRF1 is important for regulation of mitochondrial genes in cell culture (Evans and Scarpulla, 1990; Gleyzer et al., 2005;

Virbasius and Scarpulla, 1994). However, mouse embryos with disruption in the *Nrf1* gene survive until embryonic days 3.5 - 6.5 (Huo and Scarpulla, 2001) illustrating that normal expression of the mitochondrial genome is not absolutely required for embryonic development until the blastocyst stage in mouse, consistent with earlier findings (Piko and Chase, 1973). Mitochondrial function in the zebrafish *nrf* mutants should also be examined, as a comparison of fish and mammalian early development is not straightforward due to the different embryonic mechanisms. Preliminary data using Mitotracker®, a membrane potential-sensing dye that will stain active, respiring mitochondria indicates that there is no apparent difference in mitochondrial activity between *nrf* mutant and wild type larvae in the retina at 4 dpf (data not shown). These results have to be repeated and confirmed, and other stages also have to be examined before any conclusion can be made.

So far I have not been able to rescue mutant fish further than 21 days, but this seems to be due to the heat shocking method rather than toxicity of *nrf1* overexpression as the control embryos also failed to survive the treatment. An interesting question is whether it is possible to rescue the *nrf* mutant by supplying pulses of *nrf1* throughout development and how low Nrf1 dose is needed for proper development.

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NOMENCLATURE

Organism	Gene	Protein
Human	*NRF1*	NRF1
Mouse, other vertebrates	*Nrf1*	NRF1
Zebrafish	*nrf1*	Nrf1

Source www.zfin.org
In cases where the type of organism is not clear from the text, the mouse nomenclature has been used as standard

SYMBOLS AND ABBREVIATIONS

Symbol	Explanation
5-ALAS	5-amino-levulinate synthase
α-Pal	α-palindromic sequence binding protein (=NRF1)
ATP	adenosine tri phosphate
bp	base pair
bZIP	basic leucine zipper
Chlamydomonas	*Chlamydomonas reinhardtii*
cM	centimorgan
CNS	central nervous system
dpf	days post fertilization
DIG	digoxigenin
dnah9	*axonemal dynein, heavy polypeptide 9*
EGFP	enhanced green fluorescent protein
eIF1α-	elongation initiation factor 1 α- subunit
eIF2α-	elongation initiation factor 2 α- subunit
Ek1	Ethanolamine kinase1
ENU	ethyl nitrosourea
ES cells	embryonic stem cells
ewg	*erect wing* (*Drosophila Nrf1* homolog)
F_0	founder generation (transient transgenics)
F_1	first generation (germ line transgenics)
FLP	flippase recombinase
FRT	FLP recombination target
Fugu	*Takifugu rubripes*
Gb	giga bases
gDNA	genomic DNA
G protein	glycoprotein
HCNE	highly conserved noncoding elements
hpf	hours post fertilization
hsp70	heat shock protein 70
IBR	Initiation binding repressor
IFT	intraflagellar transport
IR	inverted repeats
IS	inner segment
kb	kilo base
ldhb4,	lactate dehydrogenase b4
LTR	long terminal repeats
mao	*monoamine oxidase A*
MAR	matrix attachment region
mb	mega base
miRNA	micro RNA
M-MuLV	Moloney murine leukaemia virus

MO	morpholino oligonucleotides
mok	*mikre oko*
MRP RNase	mitochondrial RNA processing RNase
mtDNA	mitochondrial DNA
mtTFA	mitochondrial transcription factor A
MYST1	*MYST histone acetyltransferase 1*
nipa	*nuclear interacting partner of ALK*
NLS	nuclear localization signal
nm	nanometer
NPHP	nephronophthisis
npl	*N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)*
Nrf1	Nuclear respiratory factor 1
Nrf2/GABP	Nuclear respiratory factor 2/ GA binding protein
OS	outer segment
ovl	*oval*
PGC1	PPARγ- coactivator 1
PR	photoreceptor
QRT-PCR	quantitative real time PCR
riok1	*RIO kinase1*
RP	retinitis pigmentosa
rtTA	reverse tetracycline responsive transcriptional activator
SB	Sleeping Beauty
SceI	I- *SceI* restriction endonuclease (meganuclease)
slim	*scruin like at the midline*
SLSN	Senior-Løken syndrome
Spy1	*Speedy1* homolog
Tet	tetracycline
Tetraodon	*Tetraodon nigroviridis*
TF	transcription factor
TILLING	Targeted Induced Local Lesions IN Genomes
top1	*topoisomerase (DNA) I*
TRE	Tet responsive element
tTA	tetracycline responsive transcriptional activator
UAS	upstream activating sequence
ube2h	*ubiquitin-conjugating enzyme E2 H*
UTR	untranslated region
v2rdl	*vomeronasal 2 receptor, d1*
VP16	herpes simplex virus transcriptional activation domain
YFP	yellow fluorescent protein
Zf	zebrafish