HYPOXIC GENE REGULATION AND HIGH-THROUGHPUT GENETIC MAPPING

by

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Activation of Heat shock proteins (Hsps) is critical to adaptation to low oxygen levels (hypoxia) and enduring the oxidative stress of reoxygenation. Hsps are known to be regulated by Heat shock factor (Hsf), but my results demonstrate an unexpected regulatory link between the oxygen sensing and heat shock pathways. *Hsf* transcription is up-regulated during hypoxia due to direct binding by Hypoxia-inducible Factor-1 (HIF-1) to HIF-1 response elements in an *Hsf* intron. This increase in Hsf transcripts is necessary for full *Hsp* induction during hypoxia and reoxygenation. The HIF-1-dependent increase in Hsps has a functional impact, as reduced production of Hsps decreases viability of adult flies exposed to hypoxia and reoxygenation. Thus, HIF-1 control of *Hsf* transcriptional levels is a regulatory mechanism for sensitizing heat shock pathway activity in order to maximize production of protective Hsps. This cross-regulation represents a mechanism by

which the low oxygen response pathway has assimilated complex new functions by regulating the heat shock pathway's key transcriptional activator.

Beyond studying the regulation of specific genes, I have also developed a method to identify small, yet important, changes within entire genomes. Genetic variation is the foundation of phenotypic traits, as well as many disease states. Variation can be caused by inversions, insertions, deletions, duplications, or single nucleotide polymorphisms (SNPs) within a genome. However, identifying a genetic change that is the cause of a specific phenotype or disease has been a difficult and laborious task for researchers. I developed a technique to quickly and accurately map genetic changes due to natural phenotypic variation or produced by genetic screens. I utilized massively parallel, high-throughput sequencing and restriction site associated DNA (RAD) markers, which are short tags of DNA adjacent to the restriction sites. These RAD markers generate a genome-wide signature of fragments for any restriction enzyme. Taken together with the fact that the vast majority of organisms have SNPs that disrupt restriction site sequences, the differences in the restriction fragment profiles between individuals can be compared. In addition, by using bulk segregant analysis, RAD tags can be used as high-density genetic markers to identify a genetic region that corresponds to a trait of interest. This dissertation includes both previously published and unpublished co-authored materials.

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I dedicate this document to my parents, who always cultivated a thirst for learning.

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CHAPTER I

INTRODUCTION TO HYPOXIA AND GENOMICS

Background of the hypoxia pathway

At low levels of oxygen (hypoxia) most eukaryotes must respond in a manner that maximizes oxygen delivery and conservation. The key complex that mediates the response to hypoxia is the transcription factor Hypoxia-Inducible Factor-1 (HIF-1). The heterodimeric HIF-1 complex is composed of HIF-1α and HIF-1β. When this complex is formed it transcriptionally activates a wide array of genes involved in metabolism, growth, proliferation, angiogenesis, and cell survival or death. Both HIF-1α and HIF-1β are constitutively expressed during normal oxygen levels (normoxia) but HIF-1α protein is quickly degraded before dimerization can occur with HIF-1β (Wang et al. 1995). In contrast, HIF-1β is stable during normoxia and hypoxia. Therefore, it is apparent that regulation of HIF-1α is the key step in controlling the cellular response to hypoxia.

HIF-1α contains a basic helix-loop-helix and PAS domain at the N-terminal end, which are both required for dimerization with HIF-1β and DNA binding. The HIF-1 complex binds to the hypoxia response element (HRE) of promoter DNA to

transcriptionally activate downstream genes. The core recognition sequence HREs is 5'-TRCGTG-3'. Research now also shows that genes need more than just an HRE to recruit the HIF-1 complex and be transcribed. A nearby cyclic-AMP response element (CRE) is also necessary for hypoxic induction of most HIF-1 targets (Firth et al. 1995). A nuclear localization signal is located near the C-terminal end of the HIF-1α protein, as well as a transactivation domain. Near the center of the protein is the oxygen-dependent degradation domain (ODDD) that contains PEST-like motifs consisting of sequence rich in proline, glutamic acid, serine, and threonine amino acids. The PEST motif is present in many proteins with a very short half-life and HIF-1α is no exception, having a half-life of less than 10 seconds in normoxia.

HIF-1α is ubiquitinated during normoxia and targeted by the proteasome for degradation (Salceda and Caro 1997). HIF-1α is ubiquitinated by the von Hippel-Lindau (VHL) tumor suppressor protein as part of an E3 ubiquitin ligase protein complex (Hon et al. 2002; Kamura et al. 2000). VHL causes HIF-1α to be ubiquitinated at both the N- and C-terminal ends of the ODDD. If HIF-1α is not ubiquitinated, it remains stable and can associate with HIF-1β.

The key step in HIF-1α degradation is the hydroxylation of two prolines at either end of the ODDD. Without these hydroxylations, VHL does not recognize HIF-1α and therefore is unable to flag HIF-1α for proteasomal degradation. A family of prolyl hydroxylases were found to be the proteins that hydroxylate HIF-1α (Berra et al. 2003; Bruick and McKnight 2001; Epstein et al. 2001). The fact that the hydroxylation step of

these HIF-1α prolyl hydroxylases (HPHs) is oxygen-dependent was the first evidence of how cells sense oxygen and initiate the HIF-1 pathway.

When oxygen is present the HPHs hydroxylate HIF-1α, which allows for VHL mediated ubiquitination and proteasomal degradation of HIF-1α. When oxygen is limited, hydroxylation does not occur and HIF-1α remains stable and forms the HIF-1 complex with HIF-1β. HIF-1 then associates with co-activators p300/CBP and transcriptionally activates genes with HREs and CREs.

An additional form of HIF-1α regulation also utilizes oxygen-dependent hydroxylation. Factor Inhibiting HIF-1 (FIH-1) hydroxylates an asparagine residue at the carboxy end of HIF-1α, with a mechanism similar to the hydroxylation of the prolyl residues in the ODDD. The asparagine that is hydroxylated by FIH-1 is in the transactivation domain of HIF-1α and when hydroxylated, HIF-1α cannot interact p300/CBP (Lando et al. 2002a; Lando et al. 2002b; Mahon et al. 2001). Therefore FIH-1 suppresses the activity HIF-1α without affecting its stability in an oxygen-dependent manner.

A final form of oxygen-dependent HIF-1α regulation is has been proposed but is still considered controversial. The interaction of HIF-1α with VHL is possibly enhanced by the acetylation of a lysine residue in the ODDD, which is mediated by the ARD1 (Jeong et al. 2002). At this point it is most likely that this mechanism is a method for differential regulation and is not sufficient to target HIF-1α for degradation alone.

In addition to oxygen-dependent regulation, it is becoming apparent that many other pathways can control HIF-1α. Various receptor-mediated factors such as insulin,

certain growth factors, nitric oxide and a number of oncogenes are capable of increasing HIF-1α protein levels or enhanced transcriptional activity (Zelzer et al. 1998). The two main pathways utilized by oxygen-independent regulation of HIF-1α are phosphatidylinositol 3-kinase (PI3K)/Akt or mitogen activated protein kinase (MAPK) (Mottet et al. 2003). Akt activation increases HIF-1α protein synthesis in a post-transcriptional manner. MAPK also causes more HIF-1α protein to be translated and may also somehow increase its transcriptional activity (Bilton and Booker 2003) in a manner that has not been elucidated. To date, the understanding of how HIF-1α is regulated in an oxygen-independent manner is just beginning to be analyzed and many other facets of this control are most likely yet to be discovered.

HIF-1α plays a major role in tumor growth and is critical during and after a heart attack or stroke. Activation of the HIF-1 complex up-regulates many genes involved in anaerobic metabolism, growth, proliferation, angiogenesis, and cell death (Huang and Bunn 2003; Pugh and Ratcliffe 2003). Tumors exploit this control of cellular and organismal physiological pathways via the natural hypoxic environment caused by rapid growth see in cancer or by genetic alterations which stabilize HIF-1α itself (Vogelstein and Kinzler 2004). Overexpression or activation of HIF-1α is often seen in a wide array of cancers and is correlated with patient survival (Semenza 2002), and it has been shown that targeting the HIF-1 pathway is a potential avenue for cancer therapy (Pouyssegur et al. 2006; Semenza 2003). Thus, HIF-1 is a central regulator of normal and pathological changes in response to low oxygen. For these reasons HIF-1α has been a focus for much research and complete understanding of the intricacies of HIF-1α regulation is of great

importance. I utilized the fruit fly model organism, *Drosophila melanogaster*, to study facets of the hypoxia pathway. The fly HIF-1α homologue, *Similar*, has been shown to be conserved in structure in all of the critical domains and much of the overall HIF-1 regulation has been shown to be identical to other organisms, including humans (Lavista-Llanos et al. 2002). There are also many benefits of using *Drosophila* for research purposes, such as the easy husbandry, short developmental time, genetic malleability, as well as the ease of RNA interference (RNAi) in cell cultures derived from fly tissue. Another reason to use a cell line is that it simplified the analysis because the regulators of HIF-1 are autonomous at a cellular level. Therefore only the cellular pathway is being assayed and will not be affected by outside factors. Additionally, because of the fact that the HIF-1 pathway is fully conserved in *Drosophila*, the findings of this research can be immediately applied to mammalian HIF-1 control.

Background of genomic variation

With the advent of DNA sequencing more than 30 years ago, researchers have been analyzing how information is stored in a series of nucleotides. Over time, the technology to identify the sequences of entire genomes has been established, leading to published sequences of many organisms, including humans in 2001 (Venter et al. 2001). However, newer sequencing methods are continuously being created, allowing for an explosion of data regarding DNA in countless organisms. This data has only started to be mined for useful information concerning disease, development, and diversity, among

others. One interesting feature that has become apparent with the wealth of sequence information is the importance of genomic variation.

There are many different types of changes that can occur the cause genomic variation between individual. Sections of a chromosome can duplicate themselves, be inverted, or deleted all together. These modifications can be larges sections of a chromosome or just a few base pairs (bp) of DNA. Also, single nucleotide polymorphisms (SNPs), or a change in a single bp of DNA, occur throughout almost all organisms. The rates of these SNPs differ amongst organisms, but these small changes are often the cause of phenotypic variation in a species. If there is a beneficial alteration in DNA there is selective pressure to maintain that change in future generation. As first outlined by Charles Darwin (Darwin 1859), this selective pressure can greatly influence phenotypic variation. However, much as been learned since the 19th century with regards to the mechanism of natural variation within a species. While Darwin referred to the pressure of the natural world, we now know that at the heart of phenotypic variation is genetic variation.

While SNPs can be the cause of a phenotypic change, the vast majority of SNPs are not seen in a physical change of the organism. Yet it is these DNA changes that can be analyzed to determine a wide array of information. When chromosomes recombine, whole regions of the chromosome are passed to the new progeny. This region will contain SNPs that will always be present if that specific chromosomal region is present. Therefore, researchers can assay for specific SNPs that identify particular regions of a genome.

Thus, examining many SNPs at once can lead to a clear picture of an individual's ancestry. Furthermore, by grouping a collection of individuals that all have a common phenotype, researchers can then look for SNPs that are shared by the entire pool. I utilized this bulk segregant analysis to devise a technique that will be able to map induced genomic changes or natural variation within a species. To do this I adapted the previously established restriction-site associated DNA (RAD) marker technique for rapid genotyping with new, massively parallel, high-throughput Solexa sequencing.

Bridge to Chapter II

In the preceding chapter I have outlined the hypoxia pathway, including the regulation of HIF-1, as well as downstream target genes of HIF-1 that are up-regulated during hypoxia. In Chapter II, I will use my previously published co-authored data to describe a novel target of HIF-1 that I uncovered. I will also show how this regulation has a global effect on a cell's response to low oxygen and discuss the complex interactions of stress response pathways.

CHAPTER II

HEAT SHOCK PATHWAY IS ACTIVATED BY THE DIRECT REGULATION OF HEAT SHOCK FACTOR BY HIF-1 DURING HYPOXIA

In order to endure oxygen deprivation, most eukaryotes utilize a conserved set of cellular adaptations (Semenza 1999). Many of these changes are brought about by the activation of the transcription factor HIF-1, a heterodimeric complex composed of HIF-1α and HIF-1β subunits. When this complex is formed it binds to specific DNA enhancer sequences and regulates the activity of target genes. Both HIF-1α and HIF-1β are constitutively expressed in normal oxygen conditions (normoxia), but HIF-1α protein is quickly degraded before dimerization can occur with HIF-1β (Wang et al. 1995). Normoxic HIF-1α degradation is mediated by a series of hydroxylations and ubiquitinations, which tag HIF-1α for disposal through the proteasome (Hon et al. 2002; Huang et al. 1998; Ivan et al. 2001; Jaakkola et al. 2001).

The HIF-1 complex transcriptionally regulates a wide array of genes involved in anaerobic metabolism, growth, proliferation, angiogenesis, and cell death (Huang and Bunn 2003; Pugh and Ratcliffe 2003). This multifaceted control of cellular and organismal physiological pathways is exploited by solid tumors through the natural hypoxic environment caused by rapid growth or genetic alterations which stabilize HIF-1α (Vogelstein and Kinzler 2004). Overexpression or activation of HIF-1α is often seen in a wide array of cancers and is correlated with patient survival (Semenza 2002), and studies have shown that targeting the HIF-1 pathway is a promising means of cancer therapy (Pouyssegur et al. 2006; Semenza 2003). Thus, HIF-1 is a central regulator of normal and pathological changes in response to low oxygen.

While many genes that are up-regulated during hypoxia are known to be regulated by HIF-1, there are also diverse sets of genes up-regulated that have not been linked to the actions of HIF-1. Among these are the highly conserved heat shock proteins (Hsps) that are highly up-regulated during hypoxia but have not been linked to HIF-1 regulation (Shen et al. 2005). Hsps are known to act as cellular chaperones for proteins that are misfolded by cellular stresses (Welch 1993). Heat shock factor (Hsf) was one of the first studied transcription factors and its activation by stresses that promote the unfolding of proteins has been well characterized. When cells are unstressed Hsf is in a monomeric state, but cellular stress induces trimerization of the protein (Westwood et al. 1991; Wu 1995). The trimeric form of Hsf activates transcription of downstream genes, such as *Hsps* (Orosz et al. 1996; Pelham 1982). However, this study identifies a novel mode of regulation of heat shock pathway activity during hypoxia through a HIF-1-dependent

increase in Hsf transcript levels. This up-regulation of *Hsf* is necessary for the full increase of Hsp transcripts normally observed during hypoxia and also during reoxygenation. These findings establish a novel regulatory link between two stress pathways previously thought to be independent in responding to hypoxia.

Results

Hsf transcript levels increase during hypoxia in a HIF-1α-dependent manner

Drosophila melanogaster Kc_{167} cells were treated with GFP control double-stranded RNA (dsRNA) or dsRNA directed to eliminate transcripts of the *Drosophila* HIF-1α homologue, *similar* (Lavista-Llanos et al. 2002; Nambu et al. 1996), through RNAi. After exposure of the treated cells to normoxic or hypoxic conditions, total RNA was isolated for semi-quantitative reverse transcription-PCR in order to characterize the transcript levels of HIF-1α, Hsf, and Actin5c as a control (Fig. 1A). Interestingly, I found that transcript levels of Hsf increased during hypoxia, and that this up-regulation was HIF-1α-dependent. Cells lacking HIF-1α due to RNAi did not display a hypoxic increase in Hsf, instead maintaining Hsf levels more similar to control normoxic cells. Real-time PCR was then used to more accurately characterize these results (Fig. 1B) and further corroborated that Hsf transcripts increase under hypoxic conditions in a HIF-1α-dependent manner. As an additional control I repeated the RNAi with an alternate dsRNA sequence targeting another area of HIF-1α, which also showed a HIF-1α-

dependent hypoxic increase of Hsf (Fig. 1C). This control experiment confirms our results were not due to off-target effects of the original RNAi.

The increase in Hsf transcript levels during hypoxia is directly regulated by HIF-1α

The DNA recognition element to which HIF-1 binds during hypoxia contains a core 5'-RCGTG sequence (Semenza et al. 1996). I had identified multiple instances of a related motif, 5'-TACGTGC, in the intron of the known HIF-1 target gene (Aprelikova et al. 2004) HIF-1 *prolyl hydroxylase* (PHD), and searched for this motif in the *Hsf* gene region. I identified two of these putative hypoxia response elements (HREs) in close proximity to one another in the second intron of *Hsf*. The two sites were 923 and 992 base pairs (bps) downstream from the transcriptional start site of *Hsf* respectively. When this genomic region was aligned (Bray and Pachter 2004) with seven other *Drosophila* species these potential HREs were perfectly conserved (Fig. 2A). The sequence conservation of the two HRE motifs strongly suggests that there is evolutionary pressure to maintain these specific sequences.

I next tested if these conserved HRE motifs had a regulatory function during hypoxia. A portion of the second intron of *Hsf* containing the potential HREs was cloned upstream of a minimal promoter driving GFP in the Green H Pelican reporter vector (Barolo et al. 2000). This reporter construct was then transfected into the Kc_{167} cell line and put under normoxic or hypoxic conditions. The hypoxic cells showed a dramatic increase in GFP fluorescence compared to the normoxic cells (Fig. 2B). The hypoxic increase in GFP expression was eliminated by HIF-1α RNAi treatment. The original

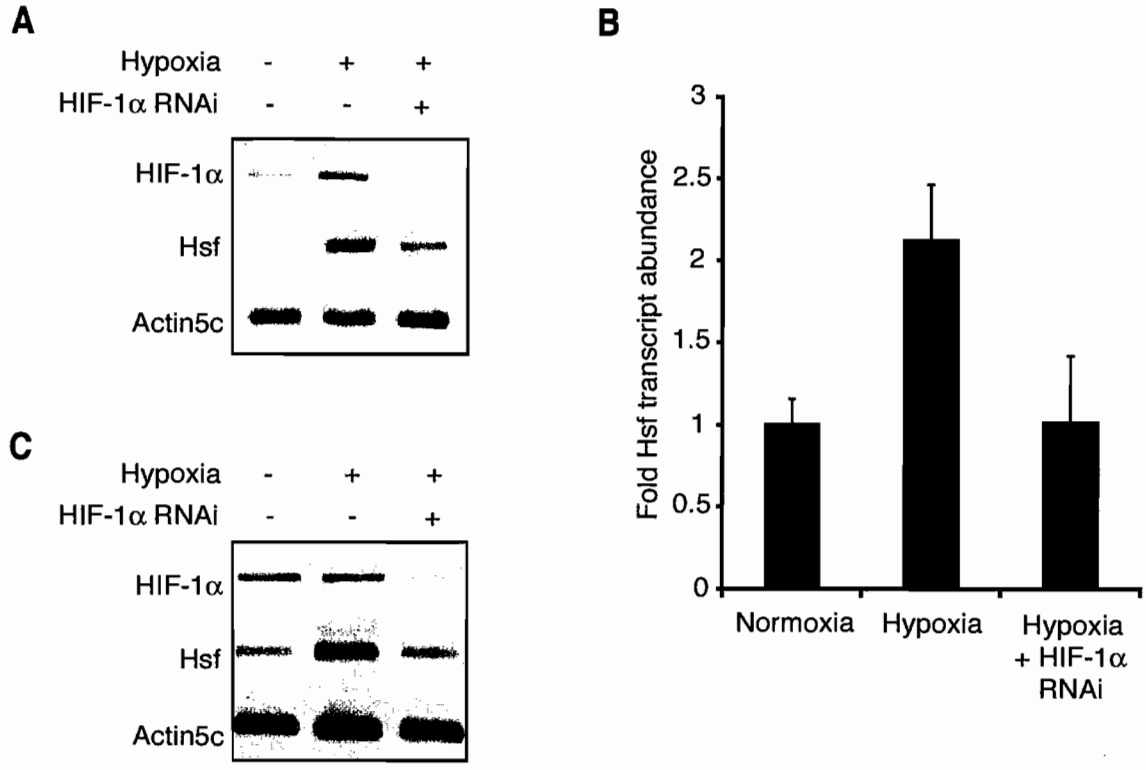

Figure 1. *Hsf* transcript levels are increased in a HIF-1α-dependent manner. A) RT-PCR analysis of the abundance of transcripts encoding HIF-1α, Hsf and Actin5c (control) during normoxia or hypoxia in Kc_{167} cells. *Hsf* is up-regulated after hypoxia and RNAi inactivation of HIF-1α eliminates this up-regulation. B) Real-time PCR experiments confirm that RNAi inactivation of HIF-1α reduces the up-regulation of *Hsf* after hypoxia. Standard error of the mean is shown. Transcript changes in each condition are significantly different ($p < 0.05$). C) RT-PCR analysis of the abundance of transcripts during normoxia or hypoxia. An alternate dsRNA sequence targeting HIF-1α for RNAi showed similar results as in (A) reducing the possibility that results from the original RNAi were due to non-specific effects.

reporter vector lacking the *Hsf* intron showed no hypoxic activation of GFP (data not shown), confirming that it was the cloned intronic region of *Hsf* that was leading to the HIF-1-dependent induction of the reporter during hypoxia.

The HRE-containing region was also tested for direct binding by HIF-1 by chromatin immunoprecipitation. I transfected Kc_{167} cells with a epitope-tagged HIF-1α expression vector (Gorr et al. 2004). DNA bound to HIF-1 protein during hypoxia was immunoprecipitated and the *Hsf* intron genomic region as well as control genomic regions were PCR amplified to test for enrichment compared to DNA immunoprecipitated from a mock transfection. I found distinct enrichment of a 260-bp fragment encompassing the two HREs of the *Hsf* intron (Fig. 2C). As a positive control I showed an enrichment of a genomic region containing an HRE within the intron of the known HIF-1 target *Hph*. A negative control fragment located in an *Actin5c* intron showed no enrichment between the HIF-1α pull-down and the untransfected pull-down. These data indicate that *Hsf* is a direct target of HIF-1α through the binding of an intronic region containing two HREs that act as an enhancer of transcription during hypoxia.

Full induction of Hsps during hypoxia is dependent on HIF-1α regulation of Hsf

The functional impact of the up-regulation of *Hsf* by HIF-1α on Hsp induction during hypoxia was then assayed. Kc_{167} cells were exposed to normoxia and hypoxia after treatment with control and HIF-1α RNAi and reverse transcription-PCR assayed transcript levels of various Hsps. All *Hsps* examined were dramatically up-regulated under hypoxia and this increase was partly HIF-1α-dependent (Fig. 3A). Hsp transcripts

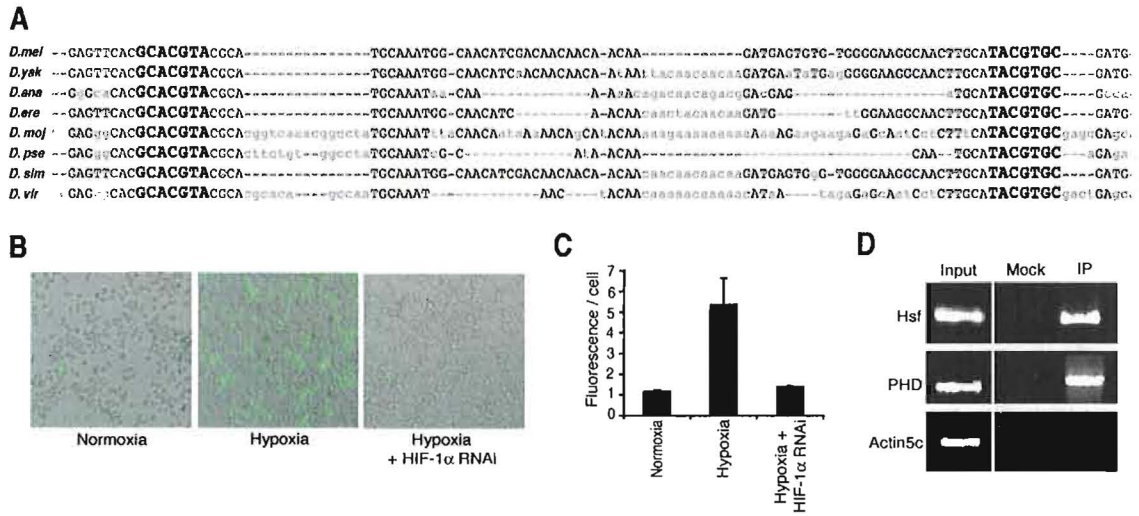

Figure 2. The *Hsf* gene region has conserved HREs and is a direct target of HIF-1. A) An alignment of the nucleotide sequence of the second intron the *Hsf* gene in eight *Drosophila* species shows two HREs are fully conserved. B) The genomic sequence containing the two HREs in the *Hsf* intron were cloned up-stream of the minimal promoter of the Green H Pelican reporter vector. This construct was transfected into Kc_{167} cells split into three conditions: normoxia, hypoxia or hypoxia with HIF-1α RNAi. The reporter was not activated by normoxia but hypoxia induced expression of the GFP reporter. The hypoxia activation of the reporter was eliminated by the addition of HIF-1α RNAi. C) Fluorescence was measured using an ISS PC1 spectrofluorometer and normalized by cell number. Quantification confirmed a significant increase in fluorescence during hypoxia and a significant decrease from the hypoxic induction when HIF-1α RNAi was added to hypoxic cells. D) Chromatin immunoprecipitation and PCR showing enrichment of the genomic region containing the two HREs within the *Hsf* gene in epitope-tagged HIF-1α transfected versus untransfected Kc_{167} cells. The *Hph* gene and *Actin5c* genes were used as positive and negative controls respectively.

were not completely eliminated in hypoxic cells treated with HIF-1α dsRNA, presumably because the hypoxic stress activated the basal (normoxic) levels of Hsf protein already present in the cells. No HREs were found near any of the *Hsp* genes, therefore it is unlikely that HIF-1 was directly up-regulating these genes during hypoxia.

I tested if the up-regulation of *Hsps* during hypoxia was dependent on Hsf. Cells were treated with control or Hsf RNAi and placed in normoxic and hypoxic conditions. When Hsf was removed through RNAi, Hsp transcripts were eliminated completely, compared to the strong induction seen in cells treated with control dsRNA (Fig. 3B). Real-time PCR was used to more accurately quantify the results from both of the RNAi experiments. HIF-1α RNAi reduced the up-regulation of Hsps during hypoxia, yet Hsf RNAi completely removed Hsp transcripts (Fig. 3C). From these results, I can discern that Hsf regulates *Hsps*, while HIF-1 regulates *Hsf*.

The lack of strong *Hsp* up-regulation in hypoxic HIF-1 knockdown cells suggests that the HIF-1-mediated increase in Hsf transcript levels is an important step in regulating the sensitivity and activity of the heat shock response pathway. The functional impact of an increase in Hsf transcript levels in hypoxia was tested by assaying the response to hypoxia of a fly heterozygous for the null Hsf^{1} mutation (Jedlicka et al. 1997), and therefore containing only a single wild-type copy of *Hsf*. After exposure to hypoxia, these flies had reduced levels of Hsf transcripts compared to wild-type Oregon R flies as measured by real-time PCR (Fig. 4). The heterozygous flies with a reduction in Hsf transcripts also showed a strong reduction in Hsp26, Hsp27 and Hsp68 transcript levels compared to the control flies, although two *Hsp70* genes had normal levels of induction.

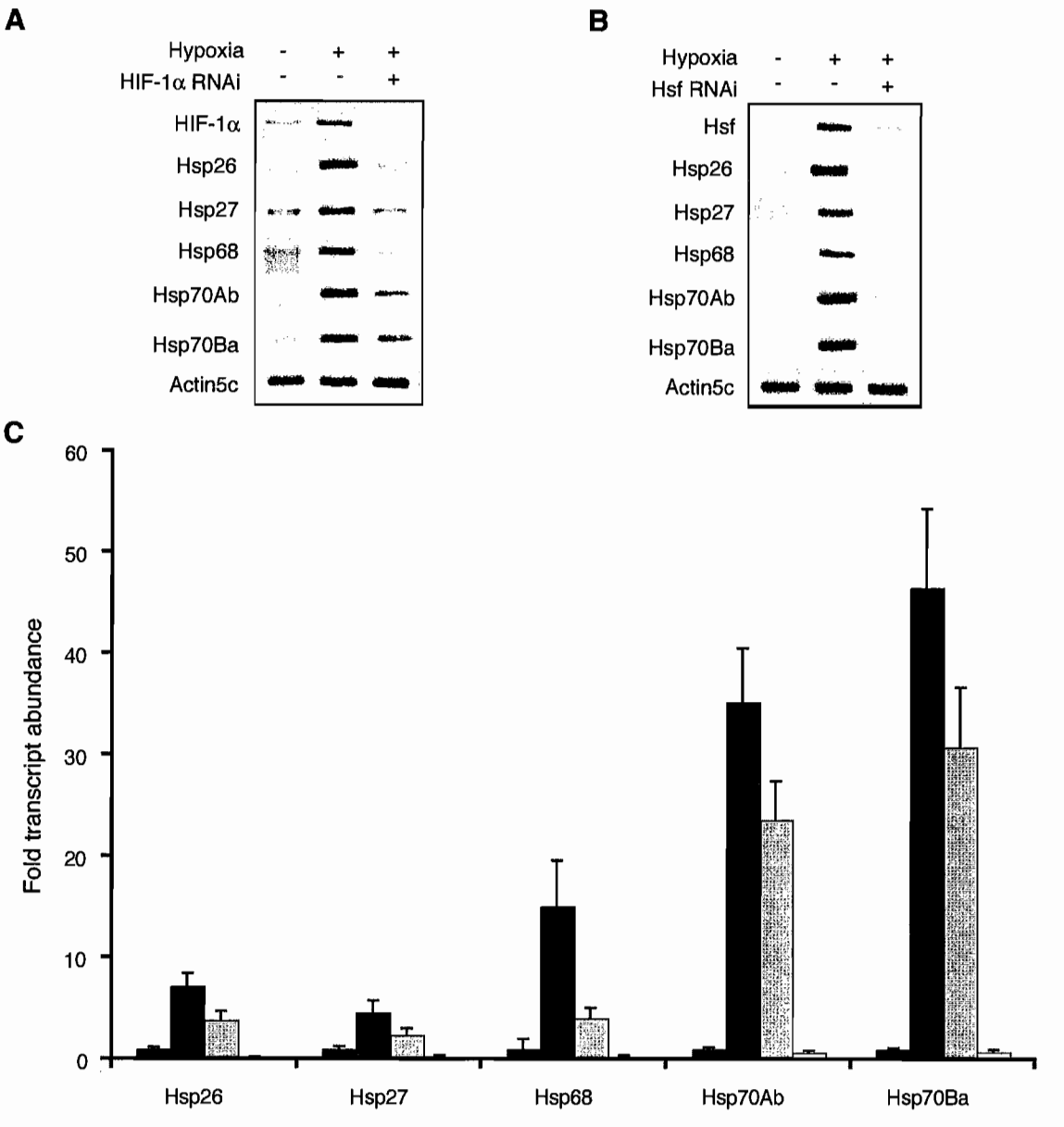

Figure 3. *Hsp* transcript levels are increased in an Hsf and HIF-1α-dependent manner during hypoxia. A) RT-PCRs of transcripts involved in the heat shock pathway are up-regulated after hypoxia. Inactivation of HIF-1α reduces the increase in Hsp transcripts. Actin5c is used as a control. B) RNAi of Hsf eliminates up-regulation of *Hsps* completely. C) Real-time PCR analysis of transcripts from normoxic cells (black bars), hypoxic cells (dark grey bars), hypoxic cells treated with HIF-1α RNAi (light grey bars) and hypoxic cells treated with Hsf RNAi (white bars). Hsp transcripts were normalized to normoxic levels. RNAi treatments reduced the transcripts Hsps compared to hypoxia alone ($p < 0.05$).

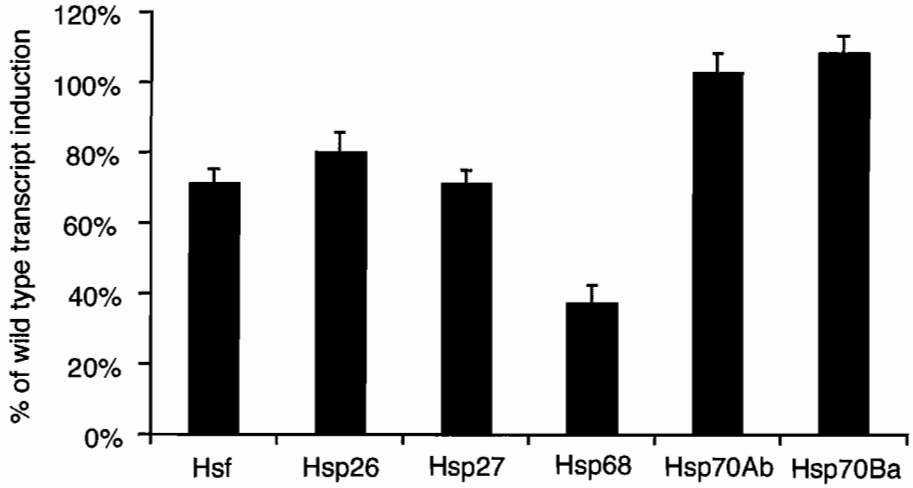

Figure 4. Up-regulation of *Hsps* is Hsf dosage-dependent. Real-time PCR of flies heterozygous for a null *Hsf* mutation show a significant reduction in Hsf, Hsp26, Hsp27 and Hsp68 transcript abundance compared to wild-type flies after hypoxia ($p < 0.05$). This demonstrates that Hsf transcript abundance is critical to the magnitude of Hsp production. Standard error of the mean is shown.

These findings suggest that Hsf abundance impacts the up-regulation of some *Hsps* in a dose-dependent manner during hypoxia. Lower Hsf transcript abundance than the levels normally achieved during hypoxia are insufficient for the full up-regulation of *Hsps*.

Full induction of Hsps and viability during reoxygenation is dependent on increased Hsf levels

During the return to normal oxygen conditions, Hsp levels remain high and are critical to tissue survival during this reoxygenation (Donnelly et al. 1992; Kabakov et al. 2003). The effect of the HIF-1-dependent increase in Hsf level on Hsp expression persists during reoxygenation. Kc_{167} tissue culture cells with HIF-1α knocked down by RNAi had little increase in Hsp expression after hypoxia treatment and a reoxygenation period (Fig. 5A). Thus, the up-regulation of *Hsf* during hypoxia is critical to the high levels of Hsp transcripts during reoxygenation, as well as hypoxia.

Furthermore, I examined the functional importance *in vivo* of increased Hsf transcript abundance by assaying larval survival under hypoxia and reoxygenation stress. First instar larvae were reared in a regime of alternating hypoxia and reoxygenation. The Hsf^1 heterozygotes had greatly reduced survival compared to larvae reared in normoxia (Fig. 5B). Control wild-type larvae showed no significant difference in survival between normoxia and the hypoxia and reoxygenation environments. These findings demonstrate the dosage importance of Hsf transcript levels for coping with hypoxia and reoxygenation at the organismal level.

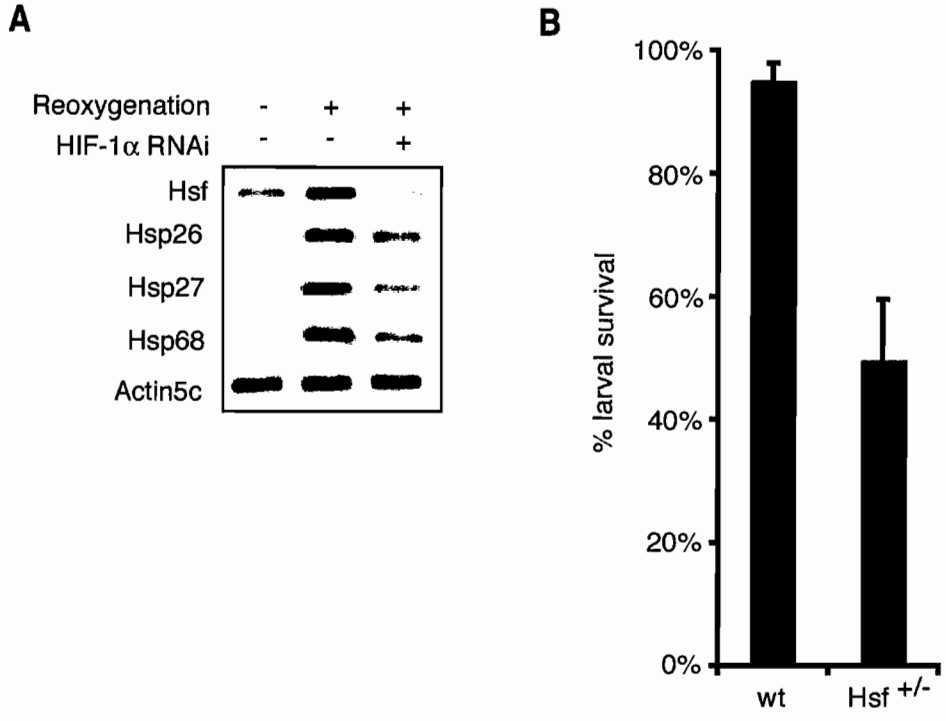

Figure 5. Up-regulation of *Hsps* after reoxygenation is HIF-1α-dependent and critical to survival. A) RT-PCR of transcripts involved in the heat shock pathway are up-regulated after hypoxia. HIF-1α RNAi reduces the increase in Hsp transcripts. Actin5c used as a control. B) Larvae reared in either normoxia or hypoxia with a reoxygenation period each day were allowed to develop into pupae. Development of wild-type larvae was minimally affected by the hypoxic and reoxygenation stress. However, half as many *Hsf*$^{+/-}$ larvae reached the pupal stage when faced with repetitive hypoxia and reoxygenation compared to normoxic larvae. The reduction in survival was significant ($p < 0.05$). Standard error of the mean is shown.

Taken together these experiments show the sequential order and importance of the hypoxia response. During hypoxia, HIF-1 directly up-regulates *Hsf*, which in turn up-regulates the whole family of *Hsps*. Without the HIF-1 regulated increase in Hsf, Hsps transcript levels never reach full induction during hypoxia or reoxygenation, and organismal viability is reduced.

Discussion

Up-regulation of *Hsps* during hypoxia is part of the canonical low-oxygen stress response seen in *Drosophila* (Liu et al. 2006), *C. elegans* (Shen et al. 2005), and mammalian tissues (Morimoto 1993). This study provides evidence that the up-regulation of *Hsf* during hypoxia surprisingly requires the activity of HIF-1, the effector of the low oxygen response. The transcriptional control of *Hsf* by HIF-1 has a functional impact on the activity of the heat shock response during hypoxia and the return to normal oxygen levels. Cells lacking HIF-1 or with reduced dosage of Hsf only increase Hsp transcript production slightly during low oxygen exposure and reoxygenation. The decreased production of Hsps reduces viability in flies experiencing hypoxia and reoxygenation, demonstrating that the full induction of the heat shock response is essential to counter the diverse physiological stresses associated with low oxygen.

Thus, I propose a model where HIF-1 directly up-regulates *Hsf* during hypoxia, and the increased Hsf abundance in turn allows Hsf to further up-regulate *Hsps* during low oxygen exposure and also after the return to normal oxygen levels. The regulation of

Hsf by HIF-1 provides a clear example of how cross-regulation between physiological stress response pathways can allow one pathway to sensitize the second and elicit a response under conditions where normally it would not be activated.

Complex regulation of physiological response pathways

Cross-regulation between physiological pathways appears to be a feature of the low oxygen response. It has been shown that the insulin pathway can dramatically affect the HIF-1 pathway (Zelzer et al. 1998). Through the actions of the phosphatidylinositol 3-kinase/Akt pathway, HIF-1α translation is increased in a manner that outpaces the naturally normoxic degradation of HIF-1α (Mottet et al. 2003). This leads to HIF-1 activation even when oxygen is present and up-regulating its downstream targets. Recently, it has been shown that transforming growth factor-β1 activates the HIF-1 pathway by reducing the levels of prolyl hydroxylases that tag HIF-1α for degradation. Interestingly, it is also known that Hsp90 plays are role in stabilizing HIF-1α (Isaacs et al. 2002; Minet et al. 1999). This mechanism is independent of the canonical oxygen-dependent regulation of HIF-1α and was the first evidence of any link between the heat shock and hypoxia stress pathways.

The cross-regulation between HIF-1 and Hsf found here is a new type of control, where the transcriptional effector of the low oxygen response directly regulates the transcript level of the effector of the heat shock response in order to sensitize the pathway. Interestingly, it has been already shown that HIF-1 and Hsf pathways have regulatory interactions, but in response to heat. Studies using *C. elegans* and rats showed

that HIF-1 activity was essential for heat acclimation (Maloyan et al. 2005; Treinin et al. 2003). Our findings may explain the mechanism behind this phenomenon, in that the increase in metabolic activity during high temperature may cause oxygen scarcity, thus stabilizing HIF-1 and increasing Hsf transcript levels.

Transcriptional control of the heat shock response

The activity of the heat shock pathway has been shown to be controlled by the trimerization and post-translational modification of Hsf protein subunits (Orosz et al. 1996). Our results indicate that transcriptional control of *Hsf* is a means of further regulation of heat shock pathway activity. This transcriptional regulatory step is controlled by HIF-1, supporting a model in which the HIF-1 pathway causes increased *Hsf* transcription during hypoxia as a means to increase the cellular abundance of Hsf and increase the sensitivity of the heat shock pathway. In addition, the control of heat shock response sensitivity by HIF-1, the regulator of the low oxygen response, suggests that stress response pathways can assimilate complex new functions by regulating the transcriptional activators of other stress pathways.

Disease implications

It has been shown that the increase in Hsp levels are critical for cell survival during hypoxia and the subsequent reoxygenation (Kabakov et al. 2003; Nakano et al. 1997). Our results indicate that it is through the HIF-1 pathway that the cell achieves this Hsp increase and is a means to protect against the stress of hypoxia. HIF-1 accumulation

and activity have been linked to tumor progression and various Hsps have also been shown to be crucial to cancer survival (Ciocca and Calderwood 2005), thus, the hypoxic and heat shock response pathways play important roles in the pathophysiology of cancer. My finding that the activity of HIF-1 controls the output of the heat shock pathway offers possible therapeutic approaches for mitigating hypoxic tissue damage and tumor growth by targeting this novel regulatory link.

Methods

Cell culture and hypoxia treatments

Drosophila melanogaster Kc_{167} tissue culture cells were obtained from the *Drosophila* Genomics Resource Center. Cells were maintained in Schneider's *Drosophila* Medium (Gibco), supplemented with 5% heat-inactivated Fetal Bovine Serum (Gibco). For hypoxia experiments, cells were incubated for 6 hours in chambers flushed with 0.5% O_2 gas. The reoxygenation step consisted of a 15 minute return to normal oxygen levels.

RNA interference

RNAi was performed as previously reported (Clemens et al. 2000). The following primer pairs were used to generate template DNA: control Green Fluorescent Protein (GFP) (5′-GCCACAAGTTCAGCGTGTCC and 5′-GCTTCTCGTTGGGGTCTTTC), HIF-1α (sima) (5′-

CTGCGGGACTATCATAACAACC and 5′- AGGCTCAAAATCAATCTTTTGG), alternate HIF-1α (5′-GCATCACATCAAAGAGTCCCGAG and 5′-TCCGCAACCGTAACACCACTAC) and Hsf (5′-TGCCAAACAGTCCGCCTTATTAC and 5′-TGCTTTCCAAGTGCCCGTG). The T7 promoter sequence (5′-TAATACGACTCACTATAGGGAGA) was added 5′ to all above primers when ordered (IDT).

Reverse Transcription-PCR

Total RNA was isolated using standard Trizol protocols. Instructions from Superscript III One-Step RT-PCR System with Platinum Taq (Invitrogen) were followed using 1 μg of total RNA and 21 cycles of amplification were used for each test. The following primer pairs were used: HIF-1α (5′-CGAACTCGGTACTAAAGAACCTGC and 5′-GGGTCCTACTTTCACGCAAGG), Hsf (5′-ATCTGCTGCGTGGCGATG and 5′-CGTCCGTGTCCAAAATGTCG), Hsp26 (5′-ATGGCGTGCTCACCGTCAGTATTC and 5′-GGATGATGTTGGATGATGATGGCTC), Hsp27 (5′-AGGAGGAAGAAGACGACGAGATTCG and 5′-CATTGGGTGTGTTGTGGTGTGTCC), Hsp68 (5′-TTCACCACCTATGCCGACAACCAG and 5′-TCACATTCAGGATACCGTTTGCGTC), Hsp70Ab (5′-TCCATTCAGGTGTATGAGGGCG and 5′-CGTTCAGGATTCCATTGGCGTC), Hsp70Ba (5′-ACGATGCCAAGATGGACAAGGG and 5′-

CGTCTGGGTTGATGGATAGGTTGAG) and Actin5c (5′-GGATGGTCTTGATTCTGCTGG and 5′-AGGTGGTTCCGCTCTTTTC).

Real-time PCR

Total RNA was isolated using standard Trizol protocols. cDNA was synthesized following the SuperScript III Reverse Transcriptase protocol (Invitrogen). Real-time PCR was performed using the Sybr Green PCR Master Mix (Applied Biosystems) and an ABI PRISM 7900HT detection system (Applied Biosystems). The supplied analysis software was used for data interpretation. The following primer pairs were used: Hsf (5′-ACACCGCAGCCTCACATTATGACC and 5′-ATTTCCCTGGAGCAGCAAGTCCTC), Hsp27 (5′-AGGAGGAAGAAGACGACGAGATTCG and 5′-CATTGGGTGTGTTGTGGTGTGTCC), Hsp68 (5′-TTCACCACCTATGCCGACAACCAG and 5′-TCACATTCAGGATACCGTTTGCGTC), Hsp70Ab (5′-TCCATTCAGGTGTATGAGGGCG and 5′-CGTTCAGGATTCCATTGGCGTC), Hsp70Ba (5′-ACGATGCCAAGATGGACAAGGG and 5′-CGTCTGGGTTGATGGATAGGTTGAG) and Actin5c (5′-TGCTGGAGGAGGAGGAGGAGAAGTC and 5′-GCAGGTGGTTCGCTCTTTTCATC).

Hypoxia reporter construction

A small region of the *Hsf* intron containing the possible hypoxia regulatory elements was cloned into the Green H Pelican reporter vector. Kc_{167} cells were transfected using the Effectene kit (Qiagen) with this reporter and put in normoxia, hypoxia or hypoxia with HIF-1α RNAi. Images were taken using a Nikon Eclipse TE2000-U microscope and MetaVue image capture software.

Chromatin immunoprecipitation

Kc_{167} cells were transfected using the Effectene kit (Qiagen) with a pAc5.1/Sima plasmid, which contains the full-length HIF-1α (sima) cDNA sequence with a c-terminal V5 epitope tag under the control of the Actin5c promoter. An equal quantity of mock transfected cells was used as a control and all purification steps were carried out in parallel with the control and experimental cells. 24 hours after transfection, control and experimental cells were incubated in hypoxia at room temperature for 24 hours. DNA isolation and purification procedures followed standard V5 protocol.

PCR was used to detect *Hsf*, *Hph*, and *Actin5c* genomic regions in each of the samples of isolated DNA. 35 cycles were used to amplify 2 μl of template from each sample using the following primers: Hsf (5′-CTCCCACCACATACCGCTAATC and 5′-AAAAGCCAACTGAATGACCAAGG), Hph (5′-CCTTCTCACACTCCCTTCGCTG and 5′-CACTCTCTGCCAAGCCAAACC), Actin5c (5′-TGTGTGTGAGAGAGCGAAAGCC and 5′-CTGGAATAAACCGACTGAAAGTGG).

Larval survival

First instar larvae of wild-type or flies with only one copy of the *Hsf* gene were counted and placed 10 per vial of food. These vials were split and placed into groups for normoxic or hypoxia and reoxygenation stresses. This experimental group was maintained at 0.5% oxygen for 23 hours, then placed in ambient oxygen for one hour, before returning to hypoxia. This cycle was repeated daily and after two weeks the vials were scored for survival.

Bridge to Chapter III

By utilizing a genomic approach to study the hypoxia pathway I was able to uncover a previously unseen aspect of HIF-1 regulation, in addition to discovering a novel cross-regulation of two separate stress response pathways. In Chapter III, I continue to use a genomic approach in my studies. Chapter III will discuss the findings of my work sequencing genetic markers to facilitate mapping of phenotypic differences between individuals, using zebrafish (*Danio rerio*), the filamentous fungi *Neurospora crassa*, and the threespine stickleback (*Gasterosteus aculeatus*).

CHAPTER III

MASSIVELY PARALLEL HIGH-THROUGHPUT GENETIC MAPPING

P.D. Etter contributed to the experimental design and protocol development described in this chapter. The core scientific ideas where mine, as well as the majority of labor.

SNPs have been proven to be a valuable tool for genetic mapping. The rate of SNPs can vary from one change in every 100 bp up to once in over 1000 bp depending on the organism (Sachidanandam et al. 2001). These small changes have been harnessed to map the inheritance of specific regions of a chromosome. SNPs can be used to map the location of mutations caused by genetic screens in recombinant progeny, as well as identify natural phenotypic modifiers (Berger et al. 2001; Martin et al. 2001; Stickney et al. 2002; Wicks et al. 2001) In humans, disease alleles and phenotypic modifiers have also been mapped using SNPs (Bader 2001; Pharoah et al. 2004).

A common mapping technique is to identify SNPs that disrupt restriction endonuclease recognition sites. Disruption of these restriction sites by a SNP leads to differential digestion between individuals. Therefore the restriction profile of the entire genome can be characterized for any individual. However, until recently, it has been

difficult to simultaneously assess all of the fragments created from digestion of the genome with a specific restriction enzyme. Recent studies have used microarrays to help identify many more SNPs that affect the restriction profile of an individual (Matsuzaki et al. 2004). This has increased the number of SNPs that can be worked with at once and therefore increased the accuracy of genetic mapping studies.

RAD marker genotyping, an array-based representation of a given restriction site in the genome allows parallel screening of thousands of polymorphic markers, and has shown it can be used to map natural variation and induced mutations in diverse organisms (Lewis et al. 2007; Miller et al. 2007a; Miller et al. 2007b). RAD marker microarray studies to map genetic traits have the advantages of not requiring a sequenced genome and the relative speed and low costs involved compared to other methods. Despite these advantages, the array technology has some disadvantages. Its development and implementation for a new organism involves the labor-intensive and fairly time consuming step of creating subtracted RAD marker libraries and microarray construction. Another major drawback of array-based genotyping is restricted flexibility of the experiments. Every comparison between samples must be hybridized to a single microarray; therefore mapping tests cannot be done in parallel.

Newly developed sequencing techniques open a new avenue for massively parallel high-throughput genetic mapping studies. Here, utilizing Solexa sequencing methods, I have designed an approach that allows for quick and inexpensive next-generation RAD marker mapping without the need for laborious microarray construction and multiple hybridizations. It will provide a useful tool for researchers and allow rapid

phenotypic mapping without necessitating prior sequencing or library construction. Flexibility in the number of individuals combined in each channel during the sequencing run allows for over- and under-sequencing of RAD markers from different individuals and/or experiments, which provides flexibility to the depth of sequencing depending on the needs of each experiment. I, along with others in the lab, have begun to demonstrate the utility of this approach by performing fine mapping of a previously mapped locus in the threespine stickleback and bulk segregant analysis of new phenotypic variants in zebrafish. In addition, I have now used this technique to identify polymorphic markers between two lines of zebrafish. This is the first step of genetically mapping genomic variation.

In the rest of this chapter I will describe how to generate tags for RAD marker sequencing and how analyze the large amounts of data to map phenotypic variation. I will also demonstrate how I have used this technique to date, including how experiments have been started to map variations in zebrafish, stickleback and *N. crassa*, pending the acquisition of sequencing data.

Results

RAD marker generation for sequencing

There is a successful history of assaying restriction sites that are disrupted by SNPs (Botstein et al. 1980; Vos et al. 1995). A common drawback of these techniques is the difficulty of analyzing all the restriction sites in a genome. The RAD marker array

technology has dramatically increased the ability to assay a large number of restriction site polymorphisms. However, not all researchers have the proper microarray resources and the construction of the library is time consuming and must be done for every organism of interest. I have adapted the RAD marker technology with the massively parallel, high-throughput Solexa sequencing to greatly improve genetic mapping.

Genomic DNA is digested with a particular restriction enzyme and an adapter (P1) is ligated to the overhanging "sticky" ends of these fragments. The P1 adapter contains a P1 amplification primer, a Solexa sequencing primer, and a barcode. The adapted fragments are then randomly sheared and ligated to a second adapter (P2). The P2 adapter is a "Y" adapter that has divergent ends designed to contain a site for amplification that only binds the P2 reverse amplification primer after the correct sequence from the divergent end is filled in by complementary strand synthesis originating from the P1 primer. Therefore, only fragments that were ligated to the first P1 adapter at the restriction cut site (RAD markers) will be amplified during the final PCR amplification step. This enriches for markers since only they will contain a P1 adapter at one end and P2 at the other (Fig. 1).

Barcoded RAD sequencing

An advantage of this technique is the use of barcodes, which are unique strings of four to five nucleotides placed after the Solexa sequencing primer and preceding the restriction cut site overhang in the P1 adapter. Each DNA source is digested individually

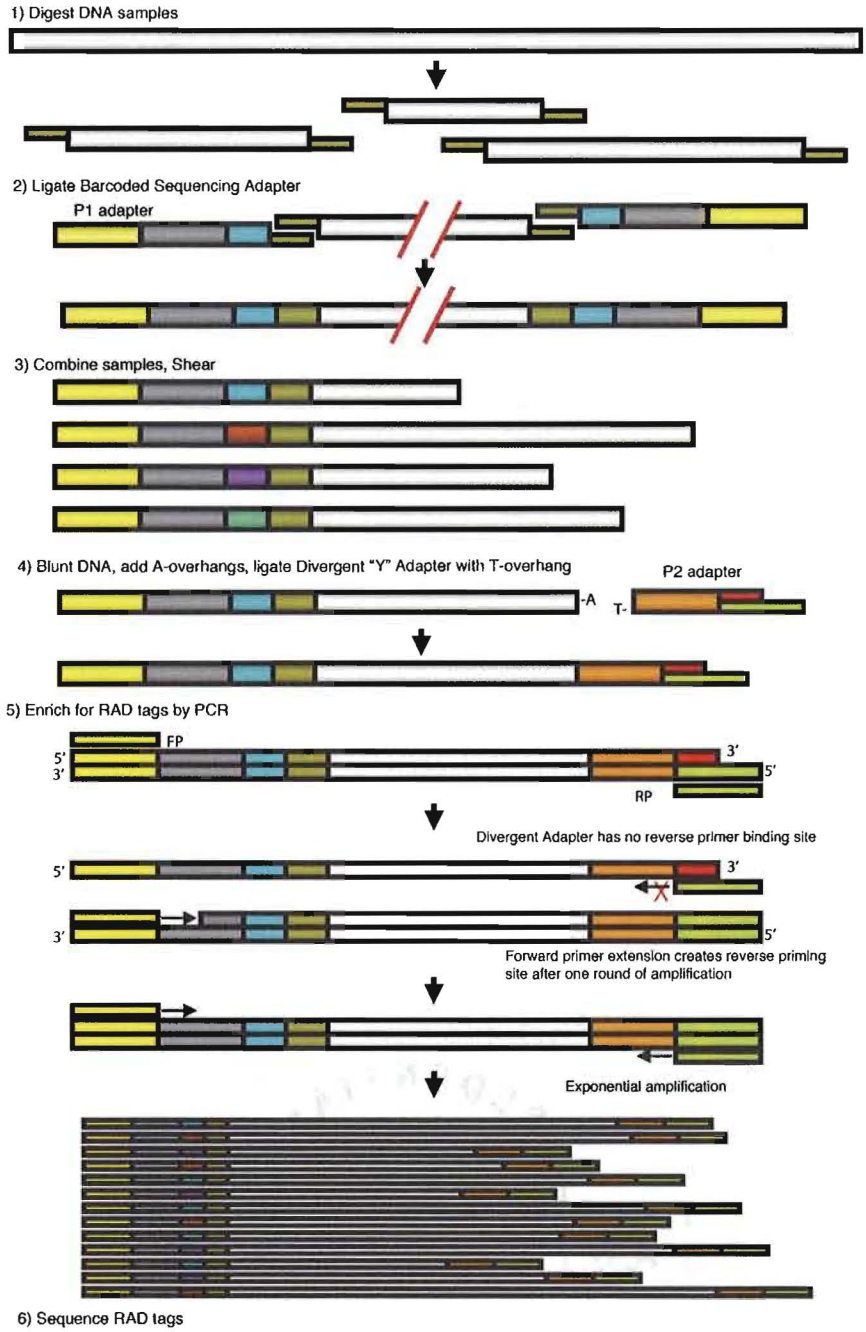

Figure 1. RAD marker generation. Genomic DNA is digested with a restriction enzyme and an adapter (P1) is ligated to the fragments. The P1 adapter contains a P1 amplification primer, a Solexa sequencing primer, and a barcode. Adapted fragments are sheared and ligated to a second adapter (P2). P2 adapter is a divergent "Y" adapter; containing a site for amplification that only binds the P2 reverse amplification primer after the complementary strand is synthesized by the P1 primer. Therefore, only fragments ligated to P1 at the restriction cut site (RAD markers) will be amplify by PCR.

and then ligated to a uniquely barcoded adapter that allows one to distinguish and track RAD markers from individuals by the first few nucleotides in each read of the sequencing run. Additionally, this allows us to pool all the samples once the barcoded adapters have been ligated, which in turn enables the remainder of the protocol to be carried out with only a single sample. Therefore, multiple samples can be handled at once with ease, allowing simultaneous sequencing of RAD markers from every individual in a genetic cross. This pooling method eliminates problems that may arise from mishandling when working with 10s or 100s of samples or from differences in shearing and amplification efficiencies between the many reactions had the RAD markers been treated individually.

I first confirmed to feasibility of barcoded RAD sequencing by cloning a small subset of RAD markers that had been amplified. After sequencing these tags using conventional sequencing methods, I confirmed the correct sequence and orientations of the appropriate adapters on all of the RAD tags (Fig. 2). With the small amounts of Solexa sequencing data that I have gotten back, I have begun to map the natural phenotypic differences between two populations of stickleback at a know locus. Mapping the locus that causes the reduction of bony lateral plates in multiple freshwater populations has been achieved by several techniques with varying degrees of precision (Colosimo et al. 2005; Colosimo et al. 2004; Cresko et al. 2004; Miller et al. 2007b). I hope to increase the resolution of this mapping with my new RAD marker sequencing technique (Fig. 3).

In addition, by using unique barcodes for each individual, I will be able to map a second natural phenotypic variant (which possesses a decreased pelvic structure) that

B

5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC
GATCTCCCGTGCAGGGTCTGGAGGAGCGGCCGCAGAGACGGGTGAGGCTAACAGCAG
CTGCTGTGCTTATAAACACTCATGATGCACTTATAATACTCGTGATGCGCTTATAAACACTCATGATGCACT
TATAATACTTGTGATGCGCTTATAATGCACGTGATGCACTCATAATCCTGGTGATGCACTCATAACCCTCGT
GATGCCCTTATAATGCTCGTGATGCGCTCATAACCCACGTGATGCGCTTATACTCAGGCATCACTCGATTC
CTCCGTCGTATGCCGTCTTCTGCTTG-3'

Figure 2. RAD markers confirmed to be appropriate size and sequence orientation. A) Amplified RAD markers were TOPO cloned and colonies were grown overnight. Individual clones were screened using PCR to check that all the markers were the correct size. B) Approximately 100 clones were individually full-length sequenced. Roughly 95% of the RAD markers had the correct orientation of adapters and primer sequences flanking genomic DNA from the appropriate organism. Above is an example of a sequence read from a cloned RAD marker (blue: forward P1 amplification primer site; red: Solexa sequencing primer; purple: barcode; black [bold]: genomic DNA that would be sequenced by Solexa; black: genomic DNA; green: reverse P2 amplification primer site).

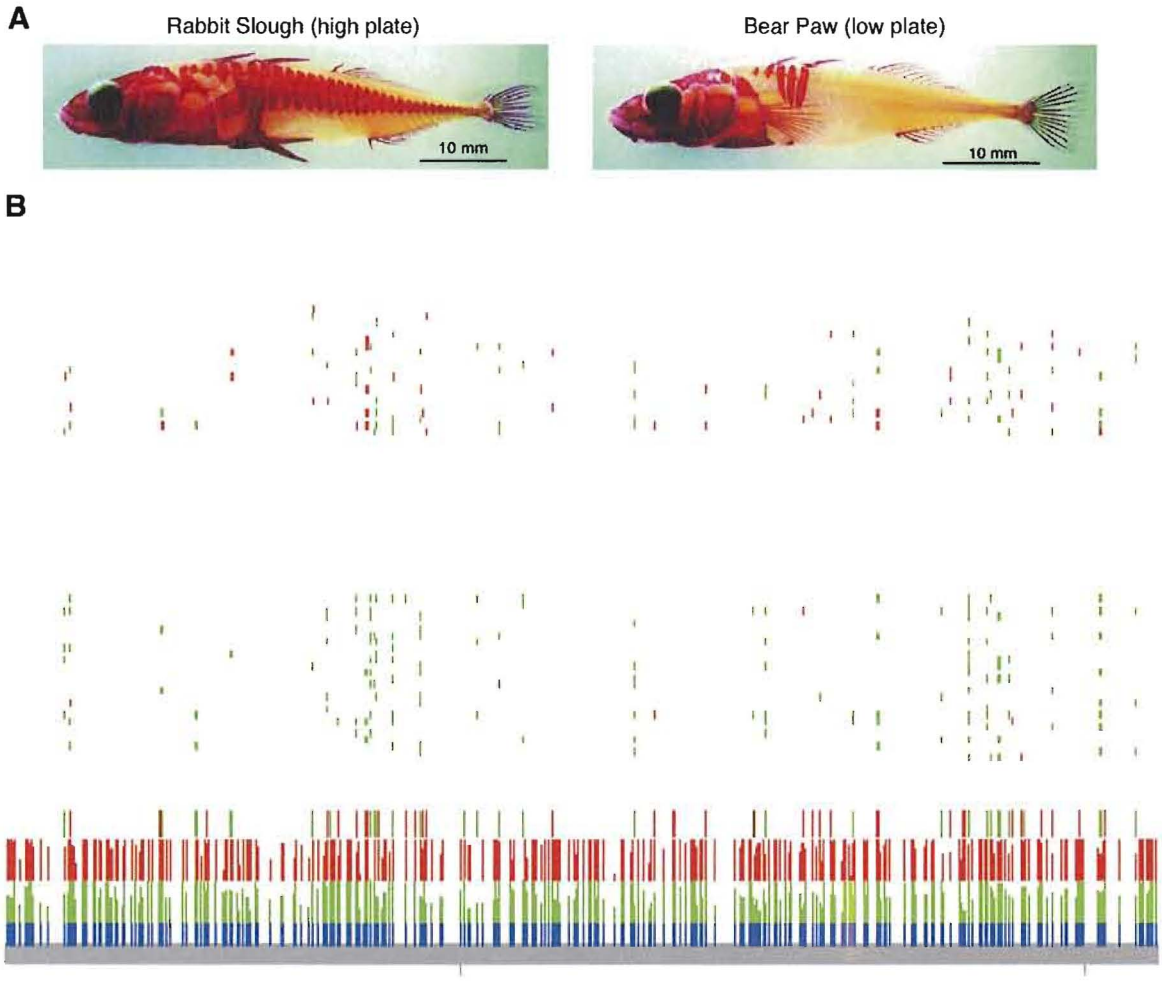

Figure 3. Genetic mapping with RAD marker sequencing. RAD marker sequencing can be used to genetically map phenotypic variation. A) Native saltwater Rabbit Slough stickleback have full lateral plate armor while there is an absence of this phenotype in the derived, freshwater Bear Paw stickleback. B) Crosses were performed as described for bulk segregant mapping and RAD markers were generated and sequenced. Blue tick marks are predicted RAD marker locations over a 2 megabase region previously mapped to the lateral plate locus. Green tick marks are RAD markers derived from Bear Paw parents and red ticks are from Rabbit Slough parents. The top cloud of mapped RAD markers is tags from the pooled F_2 progeny phenotyped as "high lateral plate". The lower cloud of mapped RAD markers is tags from pooled F_2 progeny phenotyped as "low lateral plate". There is an absence of Rabbit Slough markers in the pooled "low lateral plate" progeny in the exact location that has previously been genetically mapped for this phenotype. Therefore, high-throughput RAD marker sequencing is a promising means of genetic mapping.

segregates independently in the F_2 generation of the dihybrid cross that was used to map the lateral plate phenotype. Therefore, using barcoded adapters I will be able to track RAD markers that follow this phenotype differentially from markers associated with wild-type F_2 individuals.

Bulk segregant mapping in other organisms

While tracking every F_2 individual can be a powerful technique for mapping, as shown above, I will also use a bulk segregant method to map mutations in zebrafish and *N. crassa* in parallel with the stickleback sequencing. F_2 pools of either mutant or wild-type phenotypes were generated using the same crossing approach as described with the stickleback. When the sequence data is obtained, I can look for genomic regions that are enriched in the RAD tags of the mutant pool, compared to the wild-type pool, as was done with the stickleback mapping. These regions should be genetically linked to the original mutation. Therefore, I can then go to the original mutant and analyze that region to find the exact genomic alteration. This bulk segregant mapping technique should be very useful because of its ease of set-up and analysis.

Discussion

Understanding how developmental and physiological processes are based upon an organism's genetic code is a critical area of research. It is only beginning to be understood just how powerful genomic research can be for our comprehension of all forms biological study. It is because of this that there has been a great increase in new

methods and techniques to elucidate the power of DNA. The ability to obtain the DNA sequences of organisms was the first critical technology that had to be established. Now there are various methods and the rate and accuracy of sequencing is increasing exponentially.

As the knowledge of organisms' DNA sequences has increased, so has the ability to analyze this information. By applying computer science to genomics the field of bioinformatics has come to the forefront of genetic research. Techniques that utilized established genetic manipulation, like bulk segregant analysis, has also helped researchers develop methods to use DNA sequences as a markers to map new genes (Doerge 2002). These methods have dramatically increased the ease determining which specific gene is involved in a developmental, physiological or disease process. Complex phenotypes, such as ageing, can also be analyzed with methods like quantitative trait loci mapping, to determine many genes that affect the phenotype in degrees (Lai et al. 2007; Mackay 2001).

I have applied all of these areas of research together to devise a new technique for genomic research. RAD marker analysis using microarrays has been proven to be an effective method of mapping genomic alteration in various species. However, the technique suffers from the fact that species-specific arrays must be made for each organism of interest. Also, for every comparison that wants to be made, a new array hybridization must be performed. Therefore, this technique is both time consuming and cost-prohibitive. I have adapted the positive aspects of this RAD approach with new, high-throughput Solexa sequencing. Using this method, researchers can perform the

equivalent of nearly 1,000 RAD array experiments, all in parallel. The ability to sequence heterogeneous DNA samples with Solexa sequencing allows the researcher to multiplex the experiments so that many individual mapping projects can be performed in parallel. Obviously, this saves researchers both time and money.

Methods

Isolation of RAD markers for Solexa sequencing

Genomic DNA (0.1-1 μg) was digested for 15 min at 37° C in a 50 μL reaction with 20 units (U) of EcoRI or SbfI (New England Biolabs [NEB]). Samples were heat inactivated for 20 min at 65° C. 2.5 μL of 100 nM P1 Adapter, a modified Solexa adapter, (for EcoRI digestion, top: 5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxx-3′ [x=barcode], bottom: 5′-Phos-AATTxxxxxAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT-3′, for SbfI digestion, top: 5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTxxxxTGCA-3′, bottom: 5′-Phos-xxxxAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT-3′) was added to the sample along with 1 μL of 100mM rATP (Promega), 1 μL 10x EcoRI buffer, 0.5 μL (1000 U) T4 DNA Ligase (high concentration, NEB), 5 μL H_2O and incubated at room temperature (RT) for 20 min. Samples were again heat

inactivated for 20 min at 65° C, pooled, when appropriate, and randomly sheared (Bioruptor or Branson sonicator 450) to an average size of 400 bp. Samples were then run out on a 1x agarose (Sigma), 0.5x TBE gel and DNA 300 bp to 700 bp was isolated using a MinElute Gel Extraction Kit (Qiagen). A Quick Blunting Kit (NEB) was used to polish the ends of the DNA. Samples were then purified using a Quick Spin column (Qiagen) and 15 U of Klenow exo$^-$ (NEB) was used to add adenine (Fermentas) overhangs on the 3' end of the DNA at 37° C. After another purification, modified Solexa adapters were ligated to the DNA fragments at RT. Samples were again purified and eluted in 50 μL. 10 μL of this product was used in a PCR amplification with 50 μL Phusion Master Mix (NEB), 2 μL of 10 μM Amplification primer mix, and 38 μL H_2O. Phusion PCR settings followed product guidelines (NEB) for a total of 18 cycles. Samples were gel purified as needed and diluted to 10 nM with all other samples. Solexa protocols were followed for sequencing.

Sequence Analysis

We processed the sequence reads to optimize read number and reduce artifactual data. First, sequences were trimmed to 24 nucleotides to avoid higher error rates at the end of the reads while containing enough information to map most reads unambiguously to the zebrafish reference genome. Sequences were then filtered to eliminate low-quality reads. Any sequences with ambiguous 'N' nucleotides were eliminated, and any that contained more than two nucleotides with a quality score less than 20 (as determined by Illumina) or any nucleotides with a quality score less than 13 were eliminated. Quality

scores provide an estimate of error - a score of 13 implies 1 in 20 are wrongly called while 20 implies 1 in 100 are wrongly called (10^1.3 and 10^2.0 respectively).

Next, the sequence reads were grouped by barcode. Polymorphic RAD tags in the parental strains were identified by the presence of multiple reads in one strain and none in the other. Polymorphic markers were mapped to the reference genome by first identifying perfect matches to the RAD marker Individual F_2 progeny's markers were then classified as non-polymorphic, or polymorphic and maternal or paternal. Chromosomal blocks are then determined to be heterozygous in inheritance or homozygous for maternal or paternal genetic material, using the assumption that only at most a few crossing over events occurred in the F_1 generation. Chromosomal blocks containing biased representation within the mutant and wild type F_2 populations were then determined.

Crosses and DNA isolation

The stickleback fish used were from lines originally derived from wild samples collected in the Matanuska-Susitna Borough of Alaska. A freshwater derived individual lacking lateral plates and pelvic structure (Bear Paw Lake; 320.00.003) was crossed to a native saltwater individual possessing both phenotypes (Rabbit Slough; 280.00.009). A full-sib cross between two F_1 individuals produced the F_2 individuals that were used for the mapping analysis. The F_2 individuals were pooled as follows: 60 by full lateral plate phenotype and 31 low lateral plate embryos. For the stickleback mapping, all F_2 individuals were uniquely barcoded. By using the barcodes we also tracked the individuals with differing pelvic structures. Crosses and rearing were performed as

described in Cresko et al. (2004). Genomic DNA was isolated from pectoral fin clips from the original parental fish or entire five-day-old F_2 embryos using DNeasy Tissue Kit (Qiagen). Phenotypic analyses were performed as in Cresko et al. (2004).

The zebrafish mutant was isolated in an AB background and out-crossed to a WIK individual. Heterozygous F_1 individuals were in-crossed to produce F_2 embryos, which were pooled into two phenotypic groups: wild-type and mutant. Each parent and F_2 pool was given a unique barcode.

To isolate *N. crassa* mutants that are defective in DNA methylation, we mutagenized strain N2977 (*a his-3::bar^m; ::hph^m; ; Δinl am^{132}*), which contains methylated, and therefore, silent copies of basta and hygromycin resistance genes (*bar^m*and *hyg^m*, respectively). Following mutagenesis, we selected mutant strains that grew in the presence of both drugs. Subsequent Southern analysis revealed that the selected strains displayed global defects in DNA methylation patterns. The original mutant isolates were backcrossed to strain N3311 (*A sad-1 his-3::bar^m; ::hph^m; ; Δinl am^{132}*) to isolate homokaryons and to confirm that the mutant phenotype was caused by a mutation in a single gene. To generate recombinant progeny, mutants were crossed to the polymorphic Mauriceville wild type strain (strain N32). For cross AX6, mutant strain zx143-18 was crossed to strain N32. 33 wild type progeny were obtained for RAD genotyping. Mutant progeny were not obtained from this cross. This is probably a result of meiotic silencing of unpaired DNA (MSUD) (see Shiu, P.K. et al, 2001). For cross AX7, mutant strain u.v. 43-7 was crossed to strain N32. 28 mutant progeny and 24 wild type progeny were isolated. For RAD genotyping, RAD tags were isolated from parental

strains N32, zx143-18, and u.v. 43-7 and pools of DNA created from mutant or wild type progeny of cross AX6 and AX7. With respect to DNA methylation, strain u.v. 43-7 displayed a complete loss of DNA methylation at all regions tested and strain zx143-18 displayed an ~50% reduction in DNA methylation at all regions tested (data not shown).

Bridge to Chapter IV

Chapter III discussed a new technique I developed to facilitate parallel, high-throughput genetic mapping. This technique was used to map natural and induced phenotypic variation in various organisms. This newly developed method should be a useful tool for genomic research. Chapter IV will summarize the findings of Chapters II and III, as well as give further details about the impact of these results.

CHAPTER IV

CONCLUSION

Hypoxic Gene Regulation

Cell must adapt to low oxygen levels or die. Activation of Hsps is critical to cell survival during hypoxia, as well as the oxidation stress of reoxygenation. For decades it has been known that Hsf regulates Hsps at a transcriptional level, but my results demonstrate an unexpected regulatory link between the oxygen sensing and heat shock pathways. I have shown that *Hsf* is transcriptionally up-regulated during hypoxia and that this up-regulation is due to direct activation by HIF-1. I show the exact HIF-1 response elements in an *Hsf* intron that act as a binding site for the transcription factor. I also show that this increase in Hsf transcripts is necessary for full *Hsp* induction during hypoxia and reoxygenation. The HIF-1-dependent increase in Hsps has a functional impact, as reduced production of Hsps decreases viability of adult flies exposed to hypoxia and reoxygenation. Thus, HIF-1 control of *Hsf* transcriptional levels is a regulatory mechanism for sensitizing heat shock pathway activity in order to maximize production of protective Hsps. This cross-regulation represents a mechanism by which the low oxygen response pathway has assimilated complex new functions by regulating the heat shock pathway's

key transcriptional activator. Additionally, the junction of the hypoxia and heat shock stress pathways offers a possible therapeutic target for lessening cellular damage due to hypoxia and tumor growth.

High-throughput genetic mapping

All phenotypic traits, as well as many disease states are based upon genetic variation. I have described a new method that I developed to identify small, yet important, changes within entire genomes. This technique quickly and accurately maps genetic changes due to natural phenotypic variation or produced by genetic screens utilizing the abundant number of SNPs throughout a genome. I employed the massively parallel, high-throughput technology of Solexa sequencing and RAD markers, which are short tags of DNA adjacent to the restriction sites. These RAD markers generate a genome-wide signature of fragments for any restriction enzyme. I have used this technique to begin identifying polymorphisms between two lines of inbred zebrafish. When more sequencing data is available, I can analyze the differences in the restriction fragment profiles between F_2 individuals to genetically map regions associated with the natural variation in the lateral plate and pelvic structures of stickleback. I will also be able to use bulk segregant analysis of RAD tags from pooled F_2 populations to identify a mutated genetic region that caused novel phenotypes in zebrafish and *Neurospora*. This method of using SNPs as high-density genetic markers to genotype phenotypic variation should be a useful tool for many researchers because it does not require prior sequence knowledge and can be performed in parallel with many other samples.

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