INVESTIGATING THE TUMOUR SUPPRESSING ROLE OF HACE1 AND ITS NOVEL CONTRIBUTION TO CARDIAC DEVELOPMENT USING THE ZEBRAFISH MODEL

by

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DEDICATION

To Yadi, Zohreh, Bahareh(s), Mehrdad and all my family and friends, whose kind and endless supports never made me feel alone during this journey, even when living thousands of kilometers away. You guys always encouraged me to continue my interests and follow my dreams. THANK YOU ALL!

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ABSTRACT

HACE1 is an E3 ubiquitin-ligase that is epigenetically downregulated in various malignancies. The mechanisms underlying its role in tumourigenesis and in normal vertebrate development have not been well elucidated. We found that loss of *hace1* in zebrafish via morpholino knockdown results in higher expression of gamma H2AX, a marker of double stranded DNA breaks, as well as increased levels of the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2), which was rescued by treatment with NADPH oxidase inhibitors as well as genetic inhibition of the rac1-dependent components of this complex. *hace1* morphants demonstrated an increased incidence of cardiac deformities and increased expression of *rac1*. These cardiac phenotypes appear to be regulated by rac1-dependent NADPH pathway components. Our data reveal a molecular mechanism of HACE1 both in cancer and normal cardiac development, and thus constitutes the first known example of a tumour suppressor that regulates a developmental process via ROS-dependent mechanisms.

LIST OF ABBREVIATIONS USED

5AZ	5-aza-2-deoxycytidine
amhc	Atrial myosin heavy chain
APO	Apocynin
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related
β2AR	β2-adrenergic receptor
bmp4	Bone morphogenetic protein 4
BPM	Beats per minute
BRCA1/2	Breast cancer susceptibility gene 1/2
CCND1	Cyclin D1
CCNE1	Cyclin E1
cdh17	Cadherin 17
CNF1	Cytotoxic necrotizing factor-1
CRISPRs	Clustered, regularly interspaced, short palindromic repeats
CRL	Cullin RING ligase superfamily
Ctrl	Control
Cys876	Cystein-876 residue
DDR	DNA damage response
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
Dpf	Days post-fertilization
DPI	Diphenylene iodonium
DUOX	Dual domain oxidase
E6AP	E6-associated protein
ECM	Extracellular matrix

EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EGFR	Receptor of the epidermal growth factor
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
fli1a	Friend leukemia integration 1a transcription factor
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
Gy	Gray (unit of absorbed dose of irradiation)
H_2O_2	Hydrogen peroxide
HACE1	HECT domain and ankyrin repeat containing E3 ubiquitin-protein ligase 1
HECT	Homologous to the E6-AP carboxyl terminus
HEK293	Human embryonic kidney 293 cells
hpf	Hours post-fertilization
HPV	Human papilloma virus
HR	Homologous recombination
IR	Ionizing radiation
K48	Lysine-48
Kd	Knockdown
KDR	Kinase insert domain receptor
KO	Knockout
krox20	Early growth response 2
LOH	Loss of heterozygosity
myl7	Myosin, light polypeptide 7, regulatory
µg	Microgram
µl	Microliter
mg	Milligram
min	Minutes

ml	Milliliter
MO	Morpholino oligonucleotides
MDM2	Mouse double-minute 2
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NHEJ	Non-homologous end-joining
NOX	NADPH oxidase
NOXA1	NOX activator 1
NOXO1	NOX organizer 1
NRF2	Nuclear factor erythroid 2-related factor 2
nppa	Natriuretic peptide precursor A
PBS	Phosphate buffered saline
PBST	PBS with Tween 20
ProK	Protein kinase
PTU	Phenylthiouracil
qRT-PCR	Quantitative real-time polymerase chain reaction
Rac1	Ras-related C3 botulinum toxin substrate 1
Rb	Retinoblastoma protein
RING	Really interesting new gene
RNA	Ribonucleic acid
SA-β-gal	Senescence-associated β-galactosidase
Ser	Serine residue
siRNA	Short interfering RNA
SOD	Superoxide dismutase
Std	Standard
Tg	Transgenic
TGFβ	Transforming growth factor β
TKI	tyrosine kinase inhibitors
UPEC	Uropathogenic *Escherichia coli*
UPS	Ubiquitin-proteasome system
UV	Ultraviolet

wt	Wild-type
WISH	Whole mount *in situ* hybridization
VEGF	Vascular endothelial growth factor
VHL	Von Hipple-lindau
vmhc	Ventricular myosin heavy chain
γ-H2AX	Phosphorylated H2AX

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CHAPTER 1: INTRODUCTION

1.1 Cancer Pathogenesis

The foundation of cancer has been set in identifying different mutations, which lead to dynamic changes in the genome. These alterations initiate from a multi-step process that leads to progressive transformation of normal cells into highly malignant forms, which ultimately turn into invasive cancers (Hanahan & Weinberg, 2000). Different studies argue that tumour cells are transformed in an evolutionary manner, in which successive genetic changes are inherited and cause conversion of normal cells into cancer cells. These heritable changes can arise as a result of mutations or epigenetic changes such as altered DNA methylation or modification of histones (Fischer et al., 2004; Plass, 2002). In 2000, Hanahan and Weinberg proposed six essential alterations that contribute to malignant growth: sustaining proliferative signaling, evading growth suppressors, resisting cell death or apoptosis, limitless replicative potential, prolonged angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg, 2000). More recently, avoiding immunological destruction and reprogramming cellular metabolism were also suggested as two emerging hallmarks of cancer (Hanahan & Weinberg, 2011). The acquisition of all these hallmarks in cancer cells occurs through two enabling characteristics: the development of genome instability, which refers to alterations in the genome through mutations, and the inflammatory state of malignant lesions that promotes progression of the tumour (Hanahan & Weinberg, 2011).

Cell proliferation is the most fundamental function of the cell. Normal cells typically demonstrate organized proliferative behaviour and are tightly regulated by

growth signals and suppressors as well as other controlling mechanisms, such as cell death regulated by apoptosis-inducing signals and the autophagy machinery (Adams & Cory, 2007; Levine & Kroemer, 2008). On the other hand, enhanced proliferation is one of the important characteristics of cancer cells, allowing them to grow faster and disseminate. This abnormal increase in cell numbers arises due to a deregulated control of cell proliferation and changes in energy metabolism, providing more fuel for cell growth and division. Indeed, one of the main goals of many ongoing research efforts in cancer therapy is to cease the uncontrollable proliferation of tumour cells by reducing their numbers and preventing their accumulation (Andreeff et al., 2000). Otto Warburg proposed glycolysis as the main strategy for energy production in cancer cells, despite the more energy-efficient oxidative phosphorylation commonly employed by normal cells. This irregular behaviour that has been termed "aerobic glycolysis" involves conversion of glucose to lactate even in the presence of normal levels of oxygen, rather than metabolizing it in the mitochondria through oxidative phosphorylation. Although less adenosine triphosphate (ATP) is generated through this mechanism, it results in more glucose uptake by cancer cells to meet their increased energy needs and progression (Warburg, 1956). Thus, through reprogramming glucose metabolism and energy production as well as deregulating growth signals and suppressors, cancer cells sustain chronic proliferation.

In addition to the focus on characteristics of cancer cells themselves, much research has been focused on the interactions of cells and molecules surrounding the tumour cells, which are referred to as the "tumour microenvironment". One of the hallmarks of cancer that is dependent on the microenvironment is the process of

angiogenesis. Angiogenesis is an important factor linked to cancer cell proliferation, since many cancers have the unique ability to generate new blood vessels. This process usually takes place by forming new capillaries from a pre-existing vasculature and is regulated by both pro-angiogenic and anti-angiogenic factors, whose balance is disrupted during tumour development (Tonini et al., 2003). This extensive vascularization is required for tumours to receive their oxygen and nutrient supplies as well as removing metabolic wastes. Unlike transient angiogenesis that is observed during physiological processes such as wound healing or female reproductive cycles, the angiogenic switch is continuously activated during tumour development (Hanahan & Folkman, 1996). Several growth factor pathways regulate the process of angiogenesis, including vascular endothelial growth factor (VEGF). Different groups are developing anti-angiogenic therapies such as anti-VEGF antibodies or small molecule multi-target tyrosine kinase inhibitors (TKIs) for more specific inhibition (Saharinen et al., 2011). A better understanding of all these factors and their control mechanisms will ultimately help researchers develop new drugs and therapies that disrupt angiogenesis. Another microenvironment-dependent hallmark of cancer is the activated invasion and metastasis often referred to as the invasion-metastasis cascade. Just like angiogenesis, tumours may also require lymphatic vessels to help them with their metastatic spread (Saharinen et al., 2011). Metastasis is a multistep process that involves cancer cells which have disengaged from the primary tumour entering into the blood and lymphatic vessels (intravasation), surviving in the circulation, and finally moving to the parenchyma of distant tissues (extravasation), leading to formation of small nodules of cancer cells (micrometastases) (Joyce & Pollard, 2009). One of the essential developmental

regulatory programs involved in metastasis is epithelial-mesenchymal transition (EMT). EMT affects the morphology and behaviour of transformed cells, provides them with stem-like characteristics and allows them to continue their progression and dissemination (Hanahan & Weinberg, 2011). This process is initiated by alteration in cell shapes as well as changes in their attachments to the extracellular matrix (ECM). Here, E-cadherin that plays a crucial role in epithelial cell-to-cell adhesion, is able to suppress this mechanism and its downregulation or mutational inactivation has been frequently observed in human carcinomas as a result of easier cancer cell migration (Berx & van Roy, 2009). Various signals may play different roles in inducing EMT/the invasion-metastasis cascade, but it is important to know that after metastasis, carcinoma cells may also experience a reverse EMT process, termed mesenchymal to epithelial transition and form new tumour colonies in a new location (Hugo et al., 2007).

According to the World Cancer Report published by International Association of Cancer Registries in 2008, the global burden of cancer has more than doubled during the past 30 years, and is estimated to nearly triple by 2030. Thus, besides the ongoing strategies for killing tumours that are often harmful for normal cells too, more effective treatments are forming the future of anti-cancer therapy. During the course of multistep tumourigenesis, each of the previously mentioned hallmarks occurs with distinct mechanisms at various times in different types of tumours, but they all together are essential and allow cancer cells to survive, grow and spread. All these cancer characteristics serve as different frameworks for research to focus more on distinct mechanisms by which cancer cells survive and progress. A better understanding of all

these pathways may provide specific targets for treatment and shed light on more effective therapeutic strategies.

1.2 Oncogenes and tumour suppressors

In general, there are two types of genes responsible for the development and progression of malignant transformation; oncogenes and tumour suppressors, which execute control over a number of cellular functions. In fact, the process is regulated by actions of both oncogenes, which induce downstream signaling pathways, and tumour suppressor genes, which normally function to halt such activation.

Oncogenes are the mutated form of genes called proto-oncogenes. Proto-oncogenes are normal genes that regulate diverse cellular functions including cell division, differentiation and cell death. Transformation of these genes into oncogenes often leads to increased cell division, decreased differentiation and inhibition of apoptosis, which all together contribute to cancer progression (Chial, 2008). The type of mutation that leads to conversion, or activation of a proto-oncogene into an oncogene is generally a gain-of-function, which may occur through point mutation, gene amplification or chromosomal translocation (Lodish et al., 2000). It was in 1911 that Peyton Rous discovered the first tumour-causing virus, called the Rous sarcoma virus (Rous, 1911). This led to the identification of the viral *src* (*v-src*), the first known example of a retroviral oncogene. Later, it was found that the *v-src* caused sarcoma in chickens following transformation of its cellular counterpart, *c-src*, identified as the first proto-oncogene in the vertebrate genome (Martin, 2001). Humans have their own version of this proto-oncogene called *SRC*, whose upregulation has been reported in

different forms of cancer such as colon, breast, lung, pancreas, and liver (Chial, 2008; Dehm & Bonham, 2004). Many proto-oncogenes play critical roles in early embryogenesis and normal growth and their activities are normally turned off when their assigned developmental processes are completed. A number of these genes act as receptors for growth factors such as EGFR, the receptor of the epidermal growth factor (EGF), and Kinase insert domain receptor (KDR), the receptor of VEGF that is involved in angiogenesis. Some others act as downstream signaling proteins such as *HRAS* and *KRAS* as well as cell cycle regulators including cyclin D1 (*CCND1*) and cyclin E1 (*CCNE1*). The activation of some oncogenes can arise due to chromosomal translocations, such as that which happens in the Philadelphia chromosome leading to fusion of BCR and ABL1 proteins from different chromosomes forming the *BCR-ABL* complex, which is associated with chronic myelogenous leukemia (CML) and other forms of leukemia (Chial, 2008; Heisterkamp et al., 1985). Oncogenes also serve as potential therapeutic targets; for instance, inhibiting the tyrosine kinase activity of ABL by imatinib mesylate (also known as Gleevec) has been very successful in CML patients and has been one of the most important breakthroughs in cancer treatment in recent years (Druker, 2002). Therefore, by better understanding the activity of a specific oncogene that plays a critical role in a certain type of cancer, researchers are able to target specific cancer cells more effectively.

Unlike oncogenes, tumour suppressor genes promote cancer when they are inactivated. These are groups of genes that normally act to restrain inappropriate proliferation, regulate levels of apoptosis to keep the number of cells in proper balance and are also involved in DNA repair processes to prevent mutations in cancer-related

genes (Heidi, 2008). The first tumour suppressor gene, *Rb*, was identified in retinoblastoma, which causes a rare eye tumour typically seen in children. Unlike proto-oncogenes, the tumour suppressor genes mostly undergo loss-of-function mutations that are often point mutations or small deletions leading to production of a nonfunctional protein (Heidi, 2008). For most of loss-of-function mutations to yield a tumourigenic phenotype, both functional copies of the tumour susceptibility gene must be mutated. This idea is known as the "two-mutation" or the "two-hit" hypothesis first proposed by geneticist Alfred Knudson in 1971. For instance, in the case of retinoblastoma, the susceptibility gene is inherited from parents as a dominant trait leading to transmission of one defective copy of *Rb*; therefore, retinoblastoma almost always develops following a somatic mutation that leads to the loss of the remaining *Rb* allele that was normal. Knudson also found that, in many cases, retinoblastoma was not associated with a family history of the disease. In fact, based on his statistics, most individuals with one copy of the *Rb*, did not develop retinoblastoma because their mutated cells had already differentiated before they could receive the second hit through a somatic mutation (Cooper, 2000; Knudson, 2001; Knudson, 1971). *Rb* (now called *RB1*) was later found to be involved in many human carcinomas as well. Several other tumour suppressors were identified later in inherited cancers; however, many others were identified following detection of a common mutation or deletion of genes in non-inherited cancers. Another example is the higher risk of cancer in women with germline *BRCA1* or *BRCA2* (breast cancer susceptibility gene 1 and 2) mutations that result in a hereditary predisposition for breast and ovarian cancers (Pruthi et al., 2010). Loss of heterozygosity (LOH) is another mechanism observed in tumour suppressor gene inactivation. In this process, a

heterozygous cell loses the activation of its second copy through LOH, and then becomes homozygous for the mutated gene after receiving a second hit in its remaining functional copy of the tumour suppressor gene (Heidi, 2008). There are generally five classes of proteins recognized as being encoded by tumour suppressors genes. These classes are proteins that promote apoptosis; intracellular proteins able to inhibit progression through a specific stage of the cell cycle such as the p16 cyclin-kinase inhibitor; proteins responsible for checkpoint-controls in the cell cycle; enzymes involved in DNA repair, and finally, receptors for secreted hormones that control cell proliferation and differentiation such as the tumour-derived transforming growth factor β (TGFβ) (Lodish et al., 2000). *TP53*, also known as the guardian of the genome, is another important tumour suppressor gene, whose inactivation has been shown to play a fundamental role in the pathogenesis of multiple cancers. In fact, the p53 protein is either lost or mutated in about half of human cancers (Muller et al., 2011). This protein is a transcription factor that is induced by stress and upon activation can promote cell cycle arrest, apoptosis and senescence to prevent malignant transformation. The p53 protein has two functional domains through which it can bind directly to specific DNA sequences (Levine & Oren, 2009). Normally, the activation of p53 takes place by phosphorylation of this protein following damage of DNA in the genome leading to a cell cycle arrest through its checkpoints. Alternatively, if the DNA damage is not repairable, p53 induces apoptosis through activation of downstream genes such as the pro-apoptotic *Bcl-2* family (Osada & Takahashi, 2002). P53 interacts with several other proteins, but its protein-protein interaction with the mouse double-minute 2 (MDM2) oncoprotein (the human protein is often called HDM2), an E3 ubiquitin ligase, serves as the most important one (Levine &

Oren, 2009). In fact, MDM2 is able to downregulate p53 protein expression (hence its oncogenic activity), while p53 in turn upregulates MDM2 expression through a negative feedback loop (Haupt et al., 1997). The numbers of identified tumour suppressors are growing and the important role of these genes in precise regulation of cell proliferation, growth and death is reported repeatedly. More recently, the HECT domain and ankyrin repeat containing E3 ubiquitin-protein ligase 1 (HACE1) has also been identified as a potent tumour suppressor (Zhang et al., 2007). This protein is the main focus of this thesis, with efforts undertaken to elucidate its tumour suppression mechanisms. In addition, I have identified new roles for this protein in normal development and cardiac embryogenesis, in particular. These findings will be detailed in the next sections of this dissertation

Ultimately, the identification of both oncogenes and tumour suppressors involved in initiation or suppression of tumours, and their correlation with the previously discussed cancer hallmarks, has generated new targets for the development of more specific anticancer drugs discovery.

1.3 Ubiquitin ligases

As previously discussed, tumour development is influenced by a variety of factors, including hyperactivation of oncogenes, downregulation of tumour suppressor genes, and environmental factors. A functional linkage has been proposed between ubiquitylation, a process by which a substrate is labeled with ubiquitin, and degradation by the proteasome (Lipkowitz & Weissman, 2011). Dysregulation of this linkage has been implicated as a causative factor in different cancers and inherited diseases (Pickart,

2001). Ubiquitylation is an ordered cascade, which usually results in formation of a bond between the C terminus of the ubiquitin and ε-amino group of the substrate lysine residue. There are three different enzymes involved in this process: a ubiquitin-activating enzyme (E1), which forms a thioester bond with the carboxyl group of the ubiquitin leading to activation of its C terminus (ATP-dependent); a ubiquitin-conjugating enzyme (E2), which carries the activated ubiquitin molecule; and finally a ubiquitin ligase (E3) that facilitates the transfer of the activated ubiquitin from E2 to the lysine residue of the substrate, which can be in the form of mono- or poly-ubiquitylation (Hershko et al., 1983) (**Figure 1.3.1**). Ubiquitylation controls many critical cellular functions such as cell cycle progression, endocytosis, and apoptosis through selective degradation of master regulatory proteins by proteasomes (Pickart, 2001; Ramakrishna et al., 2011). There are also some additional actions that are regulated by ubiquitylation but do not involve proteolysis. Some examples of these actions include post-replication repair of DNA damage, ribosomal function, regulation of some certain transcription factors, and induction of the inflammatory response (Pickart, 2001).

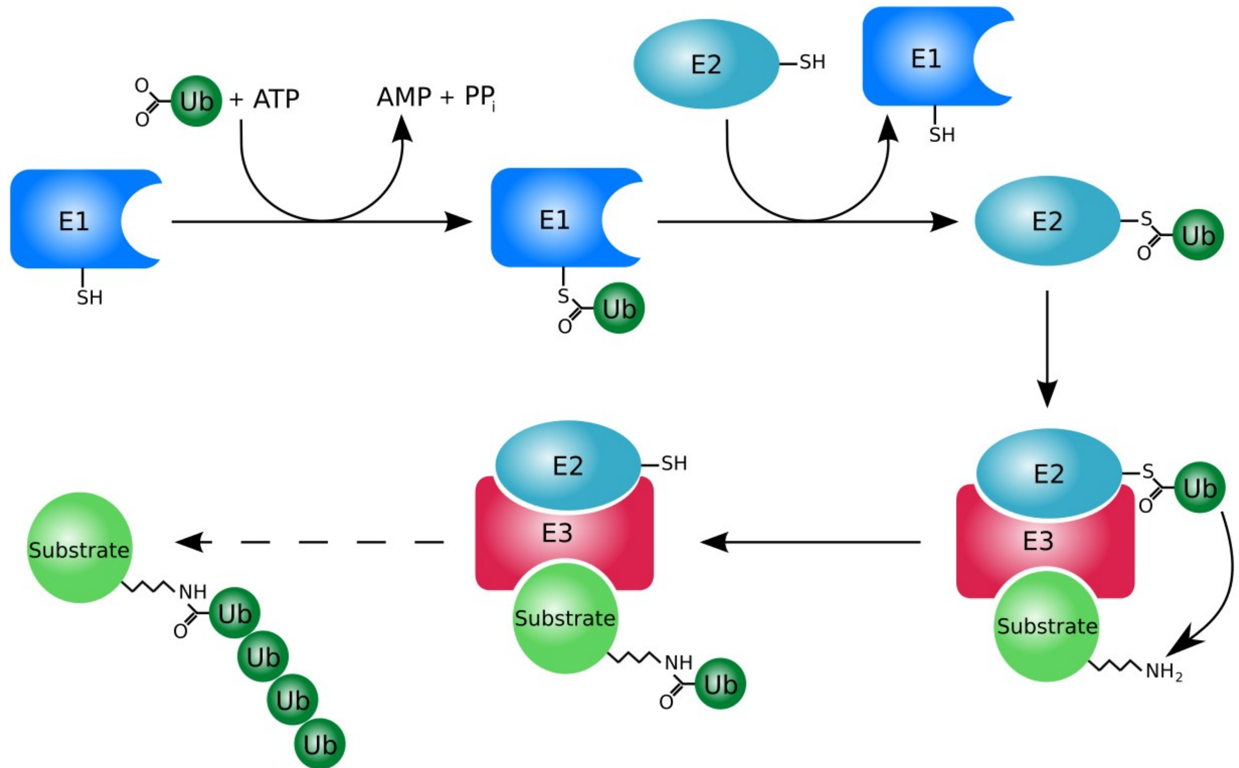


Figure 1.3.1. Schematic diagram of the ubiquitylation system. Ubiquitin is transferred to the targeted protein substrate through a sequence of events starting with the formation of a thioester bond between ubiquitin and an E1 ubiquitin-activating enzyme, which is ATP dependent. The activated ubiquitin is then transferred to an E2 ubiquitin-conjugating enzyme and ultimately, the transfer of ubiquitin to the final substrate is facilitated with the E3 ubiquitin ligase either in the form of mono- or poly-ubiquitylation. (Schematic created by Roger B. Dodd; reproduced with permission)

By contrast, deubiquitylation is a mechanism whereby protein ubiquitylation can be reversed by cleaving ubiquitin from the conjugated substrates with the help of deubiquitylating enzymes (DUBs). Therefore, a balanced action between DUBs and the ubiquitylation system is important to ensure cellular homeostasis (Ramakrishna et al., 2011).

The importance of E3 ubiquitin ligases has been highlighted in many studies based on their various critical roles in regulating normal cellular process and the number of diseases associated with their loss of function. Therefore, E3s are the major group of enzymes that dictate ubiquitylation specificity. There are two major family of E3s: the HECT-type (homologous to E6-AP carboxyl terminus) domain, which have a conserved cysteine residue that is responsible for forming an intermediate thioester bond with the C terminus of the ubiquitin, and the RING (really interesting new gene) finger-type that unlike the direct catalytic role of HECT E3s, are involved in facilitating the transfer of ubiquitin to the appropriate substrates (Rotin & Kumar, 2009). In the human genome, most of the E3s belong to the RING family (~300) and only 28 of them belong to the HECT family (Li et al., 2008). Cullin RING ligase (CRL) superfamily are the largest and the most complex class of RING finger E3s with a great diversity of substrate-receptor subunits including the S phase kinase-associated protein 1 (SKP1), cullin 1 (CUL1), F-box protein (SCF) and CRL2 E3 families (Lipkowitz & Weissman, 2011). Multiple studies have reported different roles for the RING family of E3 ligases such as cell cycle progression, correct regulation of cell growth, angiogenesis and p53 regulation (Metzger et al., 2012). For instance, MDM2 is a RING finger protein and has been shown to function as a major E3 ubiquitin-protein ligase that effectively regulates p53

ubiquitylation (Lee & Gu, 2010). As *MDM2* is itself a p53 target gene, a balanced interaction between the two is critical for normal cellular functions. In addition, MDM2-related protein MDM4 (also known as MDMX) is another key regulator of p53 that interacts by direct inhibition instead of mediating the p53 level by ubiquitylation (Goh et al., 2011). The Von Hipple-lindau (VHL) tumour suppressor also belongs to the RING finger E3 family and makes a complex with cullin-based CRL2 ($CRL2^{VHL}$), which targets the hypoxia-inducible factor- α (HIFα) for proteasomal degradation under normoxic conditions. Downregulation of VHL is associated with tumours of the central nervous system, haemangioblastomas, pheochromocytomas and clear cell kidney cancer (Lipkowitz & Weissman, 2011).

The HECT family of ubiquitin ligases was first identified in human papilloma virus (HPV) E6-associated protein (E6AP) (Huibregtse et al., 1995). Based on the N-terminal architecture of the HECT E3s, they are divided into three different groups: the Nedd4 family, the HERC family and the remainder of the HECTs (Rotin & Kumar, 2009) (**Figure 1.3.2**). The HECT family of ubiquitin ligases plays important roles in various physiological processes and mutations in them have been associated with cancer or other diseases (Rotin & Kumar, 2009). In particular, different studies have reported the important role of HECT family proteins in cancer. *HACE1* is a gene from this group (**Figure 1.3.2**) that maps to human chromosome 6q21. HACE1 was first identified in a Wilms tumour and has been associated with different human cancers, where it functions as a tumour suppressor (Anglesio et al., 2004). In addition, HACE1 was the first documented E3 ligase, in which HECT is linked to ankyrin repeats (Anglesio et al., 2004), and HECTD1 was later added to this group (Rotin & Kumar, 2009). HACE1

ubiquitylates the active form of Rac1, a member of the Rho family (a subfamily of the Ras superfamily) of guanosine triphosphatases (GTPases), leading to proteasomal degradation of this critical protein that localizes to various cellular compartments and regulates multiple processes such as proliferation and reactive oxygen species (ROS) generation (Daugaard et al., 2013) (refer to **sections 1.4.2** and **1.6.2**).

Research is ongoing to better understand these different ubiquitylation pathways and specific inhibitors of the proteasome have been shown to be useful in certain types of cancer therapy. For instance, treatment with bortezomib, a proteasome inhibitor, has shown impressive response rates in multiple myeloma and other hematological malignancies (Crawford et al., 2011).

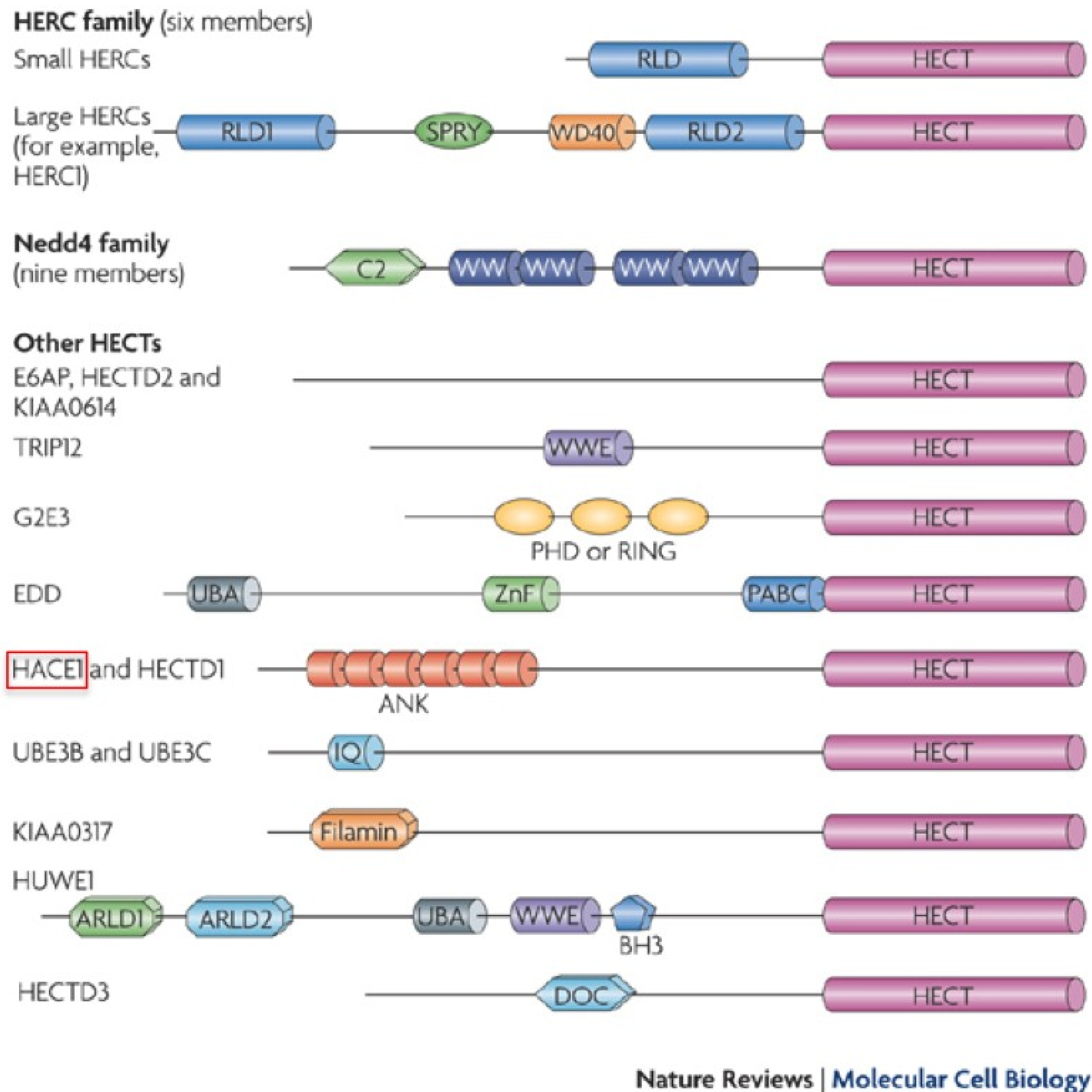


Figure 1.3.2. The mammalian HECT E3 ligases. The HECT E3 ligases are divided into three different groups based on their N-terminal architecture including Nedd4, HERC and the rest of the HECTs. The red box shows the HACE1 tumour suppressor that belongs to the other group of HECTs and contains an ankyrin repeat domain in its structure. (Adapted with permission from Rotin & Kumar, *Nature Reviews Molecular Cell Biology,* 2009; 10, 398-409)

1.4 Reactive oxygen species (ROS)

Reactive oxygen species (ROS), collectively, are oxygen-based reactive molecules and free radicals that play key roles as second messengers in cell signal transduction and cell cycling. Some examples of ROS include superoxide ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), peroxide ($^{\cdot}O_2^{-2}$), hydroxyl radical ($^{\cdot}OH$), hydroxyl ion (OH^-) and nitric oxide (NO) (**Table 1.4.1**). It is the two unpaired electrons in its outer shell that make oxygen susceptible to radical formation. Therefore, the common feature of all these species is that the oxygen is reduced to varying degrees, and as a result, ROS are in a more reactive state than molecular oxygen. Superoxide and hydrogen peroxide are the two most common ROS produced. Superoxide is reduced by dismutation to hydrogen peroxide and molecular oxygen, which can occur spontaneously ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), especially at low pH or be catalyzed through a family of enzymes known as superoxide dismutase (SOD); therefore, once superoxide is generated, the formation of hydrogen peroxide is inevitable (Hancock et al., 2001).

Historically, ROS were believed only to be involved in the host defense mechanisms of phagocytes, especially during microbial invasion, but recent studies have shown that ROS are involved in many biological systems (Hancock et al., 2001). ROS function in normal cells as cell signaling molecules and regulators of apoptosis, however, under stressful conditions, ROS can increase to toxic levels and contribute to various pathologies, including cancer. This elevation in ROS levels may result in significant damages to cell structures, known as "oxidative stress" leading to loss of cell function, and ultimately apoptosis or necrosis (Nordberg & Arnér, 2001). ROS are generated by endogenous (produced by the host organism) and exogenous (coming from outside the

system) sources. Some examples of endogenous sources are mitochondria, peroxisomes, cytochrome P450, and inflammatory cell activation (Klaunig & Kamendulis, 2004). In addition, oxidative enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase generate ROS during normal function (refer to **section 1.4.1**). These reactive species are released primarily into phagosomes for antimicrobial purposes. They are also released into the cytosol, where they can alter the function of proteins and lipids. In addition, ROS can be released into the extracellular environment under pathological conditions, where they may be responsible for tissue injury during unregulated inflammation (Fialkow et al., 2007). Specific proteins are involved in regulation of cellular ROS levels such as proteins involved in the redox (reduction/oxidation) mechanism and their mutations may cause uncontrolled generation of these reactive species. On the other hand, there are also some exogenous sources of ROS such as chlorinated compounds, radiation, xenobiotics, metal ions, barbiturates, phorbol esters and some peroxisome proliferating compounds such as mono-ethylhexyl phthalate (MEHP) (Klaunig et al., 1997). During oxidative stress, superoxide, hydrogen peroxide and other ROS can react rapidly with various biomolecules, including proteins, lipids, carbohydrates and DNA, and can result in their degradation. Therefore, it is important for cells to eliminate these oxidizing species and protect themselves through ROS catabolic enzymes (Lambeth, 2004). Different cellular antioxidant enzymes have been identified as scavengers of these ROS molecules. For instance, hydrogen peroxide can be removed by at least three antioxidant enzyme systems including catalases, glutathione peroxidases, and peroxiredoxins (**Table 1.4.1**). Together with the endogenous enzymes,

exogenous antioxidants such as vitamin C, vitamin E, carotenoids and polyphenols also play an essential role in this defense mechanism (Bouayed & Bohn, 2010).

All in all, high levels of ROS can result in impaired physiological function through cellular damage of DNA, proteins, lipids, and other macromolecules, which can ultimately lead to different diseases including cancers, cardiovascular disease, neurodegenerative disorders and aging (Rowe et al., 2008).

ROS molecule	Main sources	Enzymatic defense systems	Product(s)
Superoxide ($O_2^{\bullet -}$)	'Leakage' of electrons from the electron transport chain	Superoxide dismutase (SOD)	$H_2O_2 + O_2$
		Superoxide reductase (in some bacteria)	H_2O_2
	Activated phagocytes		
	Xanthine oxidase		
	Flavoenzymes		
Hydrogen peroxide (H_2O_2)	From $O_2^{\bullet -}$ via superoxide dismutase (SOD)	Glutathione peroxidase	H_2O + GSSG
	NADPH-oxidase (neutrophils)	Catalases	$H_2O + O_2$
	Glucose oxidase	Peroxiredoxins (Prx)	H_2O
	Xanthine oxidase		
Hydroxyl radical ($^{\bullet}OH$)	From $O_2^{\bullet -}$ and H_2O_2 via transition metals (Fe or Cu)		
Nitric oxide (NO)	Nitric oxide synthases	Glutathione/TrxR	GSNO

Table 1.4.1. Examples of ROS molecules and their metabolism. This table shows the most common intracellular forms of ROS along with their main sources of production and relevant enzymatic antioxidants. (Reproduced with permission from Nordberg & Arner, *Free Radical Biology and Medicine*, 2001; 31(11), 1287-1312.)

1.4.1 The role of NADPH oxidases in ROS production

ROS generation typically happens in mitochondria, peroxisomes, and other cellular elements as a byproduct of oxygen metabolism. The NOX family of NADPH oxidases also generates ROS, not as a byproduct but rather as the primary function of these enzymes, which transport electrons from cytoplasmic NADPH to generate superoxide or hydrogen peroxide with the help of SOD. There are at least seven distinct components of the NADPH oxidase holoenzyme in humans (NOX 1-5, DUOX 1, and DUOX 2), each of which forms unique multiprotein enzyme complexes (Bedard & Krause, 2007) (**Figure 1.4.1.1**). Unique splice variants of NOX2 and NOX4 have also been identified (Anilkumar et al., 2013; Goyal et al., 2005; Harrison et al., 2012). Although Nox enzymes in mammalian organisms have received most attention, they are also widely expressed in zebrafish including Nox1, Nox2, Nox4, Nox5 and a single isoform of Duox (Niethammer et al., 2009) (**Figure 1.4.1.2**). The activation mechanisms differ significantly between different members of this family of oxidases and there are various subunits involved in this process. For instance, NOX2 needs at least five components including the membrane-bound $p22^{phox}$ (required for stabilization of the NOX proteins), cytosolic proteins $p47^{phox}$ and $p67^{phox}$, the modulatory $p40^{phox}$ and the small GTPase, Rac, which all together lead to activation of the complex (**Figure 1.4.1.1**) (Bedard & Krause, 2007). By contrast, NOX1 is less $p22^{phox}$-dependent and becomes activated by forming a complex with NOXO1 (homologous to $p47^{phox}$), NOXA1 (homologous to $p67^{phox}$) and the small GTPase, Rac. Furthermore, NOX3 requires $p22^{phox}$ and NOXO1, as well as NOXA1 in some cases. The activation of NOX4 is usually $p22^{phox}$ dependent and finally, NOX5, DUOX1 and DUOX2 appear to only

require Ca^{2+} for their activation (**Figure 1.4.1.1**). Therefore, there are various subunits in this pathway, and some of which may substitute for one another, such as the two organizer proteins NOXO1 and $p47^{phox}$ that can be combined interchangeably with the two activator proteins leading to different levels of activation (Takeya et al., 2003). The Rac GTPases behave as molecular switches that shift between active and inactive states (depending on the binding of either GDP or GTP to the GTPases), and contain three highly homologous proteins, among which Rac1 is ubiquitously distributed (Bustelo et al., 2007). Another point worth noting is that not only are these Rac proteins important players in the NOX complex, but they are also involved in regulation of many cellular functions that will be discussed in detail in the next section (Heasman & Ridley, 2008) (refer to **section 1.4.2**). NADPH oxidases are the main source of H_2O_2 that transport electrons from cytoplasmic NADPH to generate O_2^-, which is rapidly converted to H_2O_2 in the cell (Bedard & Krause, 2007). More recently a lot of attention has been paid to pharmacological inhibitors of this family of oxidases as potential cancer therapies (Harrison & Selemidis, 2014). Different NOX inhibitors exist with varying degrees of selectivity for different NOX isoforms. Some examples of these inhibitors include diphenyleneiodonium chloride (DPI), apocynin, gp91ds-tat, VAS2870, ML171 and ebselen (Bedard & Krause, 2007; Harrison & Selemidis, 2014). Among these, DPI and apocynin are the two most widely studied inhibitors of NADPH oxidase that are commonly used (Drummond et al., 2011). DPI is a flavoenzyme inhibitor capable of inhibiting all the NADPH oxidase isoforms (Bedard & Krause, 2007; O'Donnell et al., 1993), and apocynin inhibits NADPH oxidase by blocking the association of $p47^{phox}$ and $p67^{phox}$, the components of NOX2 with homologs available in NOX1 isoforms (Stolk et

al., 1994). Although some of these inhibitors have been used for therapeutic purposes, further research is required for identification of inhibitors of such a family of oxidases with different isoforms and subunits that can potentially be used in conjunction with antioxidants.

In general, the family of NADPH oxidases plays an essential role in ROS regulation and various physiological functions. For instance, NOX deficiency may result in immunosuppression, but on the other hand, overactivity of NOX may contribute to different pathologies, particularly cardiovascular diseases and neurodegeneration (Bedard & Krause, 2007). Thus, a proper balance between this complex of enzymes is critical for normal activity of cells and the immune system.

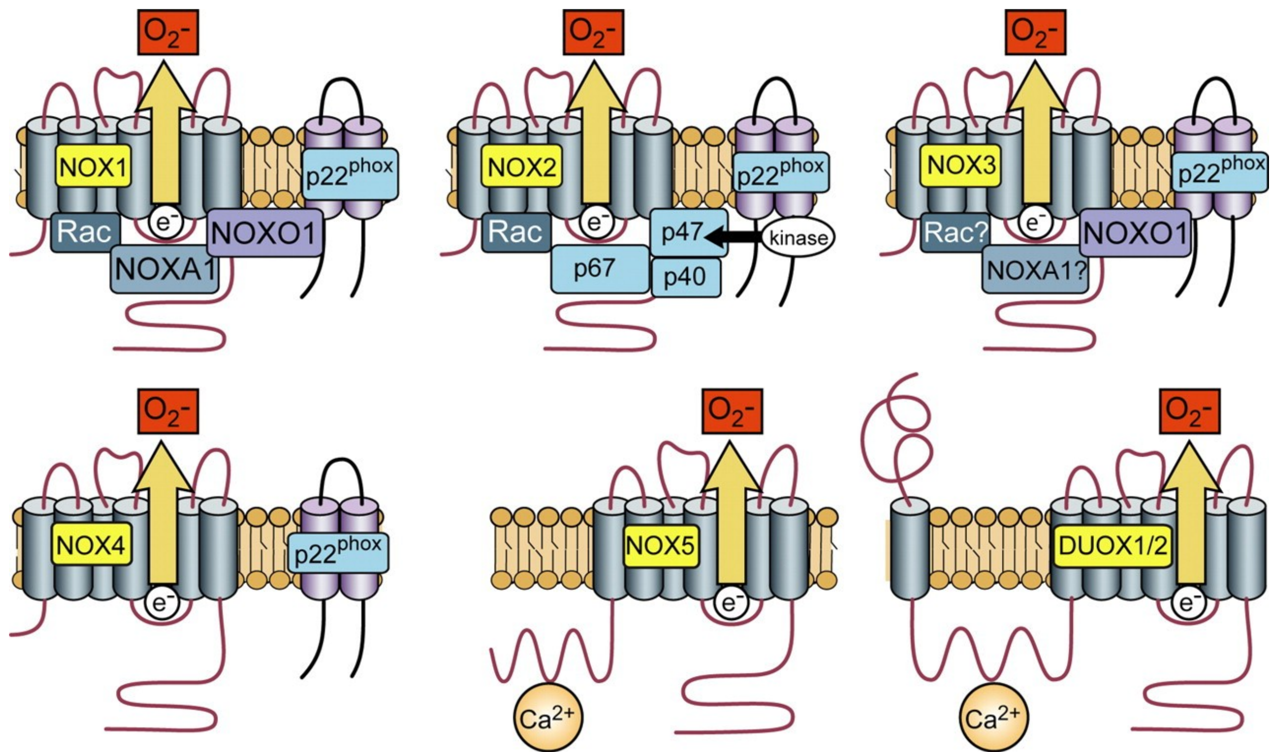


Figure 1.4.1.1. Mammalian NADPH oxidases. Seven distinct NOX enzymes in human cells including: NOX1-5, Duox1 and Duox2 (Dual domain oxidase). (Reproduced with permission from Bedard & Krause, *Physiological Reviews*, 2007; 87(1), 245-313)

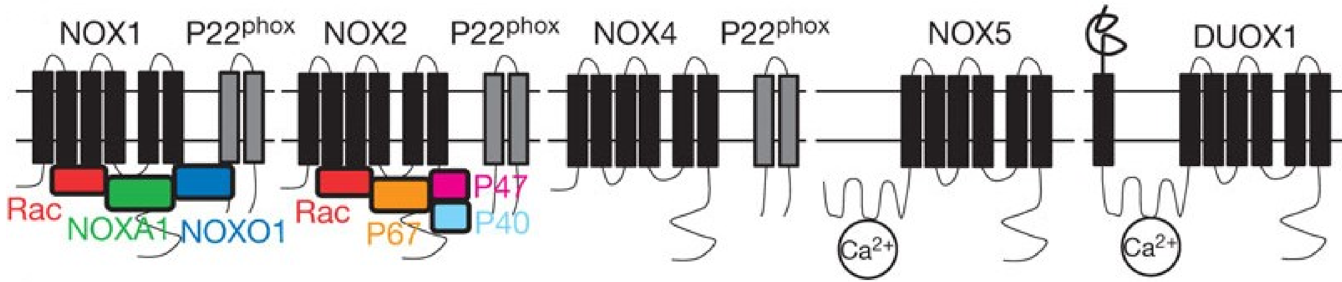


Figure 1.4.1.2. NADPH oxidases in zebrafish. The zebrafish genome encodes Nox1, 2, 4, and 5 and a single isoform of Duox. At the molecular level, Nox1 associates with the membrane subunit $p22^{phox}$, which is necessary for enzymatic activity and it is activated by forming a complex with NoxO1 (homologous to $p47^{phox}$) and NoxA1 (homologous to $p67^{phox}$) and the small GTPase, Rac1. (Reproduced with permission from Niethammer et al., *Nature,* 2009; 459(7249), 996-999)

1.4.2 The role of Rho family GTPase Rac1 in ROS induction, cardiac development and disease

The Rac protein from the Rho-family of GTPases includes three isoforms: Rac1, Rac2, and Rac3, all of which share significant sequence identity. Moreover, Rac1b has also been identified as a Rac1 splice variant that is involved in stimulation of mitochondrial ROS and genomic instability in a model of EMT (Radisky et al., 2005). Among all Rac GTPases, most information has been obtained on the roles of Rac1 and Rac2 in oxidase activation. While Rac2 is only expressed in hematopoietic cells, Rac1 expression is ubiquitous and appears to be the main Rac GTPase for NOX activation in non-hematopoietic cells (Hordijk, 2006). Rac1 GTPase is an essential subunit for the activation of the NOX1 and NOX2 complexes (Hordijk, 2006), and potentially of NOX3 as well (Ueyama et al., 2006). Rac1 undergoes a conformational change of its switch I region after GTP loading, allowing it to bind and interact with effector proteins (Jaffe & Hall, 2005). When in its active GTP-bound conformation state, Rac1 induces cellular ROS generation through its activation of membrane-associated NADPH oxidase complexes (Miyano & Sumimoto, 2012). Importantly, Sundaresan et al. demonstrated a pathway for ROS generation in which Rac1 acts downstream of Ras as expression of a dominant-negative Rac1 mutant inhibited Ras-induced generation of ROS (Sundaresan et al., 1996). In addition, Rac1 is also able to induce the cell cycle regulatory *cyclin D1* gene through activation of nuclear factor-κB (NF-κB), which results in superoxide production (Joyce et al., 1999). These findings are in keeping with studies showing that Rac1 overexpression has been implicated in the progression of multiple cancers (Schnelzer et al., 2000; Wang et al., 2009). For instance, a correlation between Rac1

overexpression and breast cancer has been reported, and its alternative splicing (Rac1b) has been identified to be involved in the development of breast and colon carcinomas (Sahai & Marshall, 2002). More recently, a Rac1 mutation has also been identified in melanoma demonstrating characteristics of gain-of-function mutations (Hodis et al., 2012; Krauthammer et al., 2012).

Rac1 deletion results in embryonic lethality in mammals, suggesting it plays a critical role in early development, while Rac2 deficiency results in hematopoietic cell defects, especially abnormalities in neutrophil function (Roberts et al., 1999; Sugihara et al., 1998). Rac1 is a multifunctional protein that affects vascular development (Tan et al., 2008) as well as cell motility and adhesion (Castillo-Lluva et al., 2013; Parri & Chiarugi, 2010), likely through its association with actin lamellipodia and focal adhesions (Guo et al., 2006). Overexpression of Rac1 has been linked to increased incidence of atrial fibrillation and cardiac hypertrophy (Lu et al., 2006; Reil et al., 2010). Sussman et al. used transgenic mice overexpressing a constitutive active form of human Rac1 in the myocardium and revealed the important role of this protein in development of cardiac hypertrophy using isolated cardiomyocytes from these mice (Sussman et al., 2000). On the other hand, Satoh et al. demonstrated that mice with specific deletion of Rac1 in the heart showed inhibition of myocardial oxidative stress following treatment with angiotensin II (AngII), as a potent inducer of cardiac hypertrophy (Satoh et al., 2006). Several studies have demonstrated the important role of ROS and in particular NADPH oxidases as critical sources of these molecules in different cardiac anomalies (Heymes et al., 2003; Murdoch et al., 2006). For instance, in patients with ischemic or dilated cardiomyopathy, the increased NADPH oxidase-dependent ROS production in failing

myocardium is associated with increased membrane expression and activity of Rac1 (Maack et al., 2003). These studies, all together, show the important roles of the Rac1 GTPase in normal cardiac development through either a direct regulation by this protein or indirectly as a potential outcome of oxidative stress regulated by Rac1. In addition, the essential role of Rac1 signaling for a full DNA damage response has been reported (Huelsenbeck et al., 2012). Therefore, a better understanding of the molecular mechanisms underlying these abnormalities in development and also cancer progression allows for earlier diagnosis and enhanced treatment options in the future.

1.5 DNA damage

Genomic stability is critical for preventing oncogenesis and delivering intact genetic material to subsequent generations. However, DNA damage caused by endogenous or environmental agents is inevitable. Therefore, DNA damage signaling and repair is essential to preserve integrity of the genome and maintain cellular homeostasis (Panier & Boulton, 2014). Some examples of endogenous sources of DNA damage include spontaneous errors during DNA replication and repair, hydrolytic reactions and non-enzymatic methylation, as well as reactive-oxygen compounds (e.g. ROS) generated as byproducts from oxidative respiration or produced as a response to inflammation or infections (Gupta & Lutz, 1999; Jackson & Bartek, 2009). Beyond the classic ROS-generating mechanisms described in **section 1.4**, these molecules can also arise through redox-cycling events involving environmental toxic agents that directly generate or indirectly induce ROS in cells (Valko et al., 2006). ROS-induced DNA damage can result in single- or double-strand breaks, modifications of bases and

deoxyribose as well as DNA cross-linking (Klaunig et al., 2010). The ROS-induced DNA damage response depends on the level of activation of ataxia-telangiectasia mutated (ATM), one of the PI-3 kinase family members, and the tumor suppressor p53, which are both phosphorylated in their active forms following DNA damage (Guo et al., 2010). Other examples of environmental (exogenous) agents that trigger DNA damage include ultraviolet (UV) and ionizing radiation (naturally or during cancer radiotherapy), and of course many other cancer-causing substances such as aflatoxins or chemicals produced by tobacco products (Doll & Peto, 1981; Jackson & Bartek, 2009).

There are many different types of DNA lesions, but in particular DNA double-strand breaks (DSBs) typically resulting from UV radiation are the most harmful ones, because they are difficult to repair and extremely toxic to the cell, such that only one unrepaired DSB is enough to initiate growth arrest and apoptosis (Khanna & Jackson, 2001). In addition, incorrect rejoining of broken DNA DSBs may take place during the process of repair, which can result in loss, amplification, or even translocation of chromosomal material. These changes may result in tumourigenesis through inactivation of tumour suppressors or promoting oncogenic activities of the proto-oncogenes (Khanna & Jackson, 2001; Nikiforova, 2000). In order to detect damage to DNA, signal their presence and initiate repair, cells have evolved a mechanism known as the DNA damage response (DDR) (Jackson & Bartek, 2009). This mechanism involves the action of different functional groups including damage sensors, mediators, signal transducers, and repair, arrest or death effectors that altogether orchestrate the repair of DNA damage as well as keeping the normal cellular physiology (Sancar et al., 2004). There are two complementary mechanisms for the repair of DSBs: homologous recombination (HR)

and non-homologous end-joining (NHEJ) (Khanna & Jackson, 2001). One of the most important proteins in the DDR pathway is the histone, H2AX, which is the main protein component of chromatin. H2AX has been identified as one of the key histones that undergo different post-translational modifications in response to DSBs (Srivastava et al., 2009). After induction of DNA breaks, chromatin is modified by phosphorylation of this histone with the help of PI-3 kinase family members, including ATM, ATM and Rad3-related protein (ATR), and DNA-dependent protein kinase (DNA-PK). Together with phosphorylated H2AX (also referred to as γ-H2AX), these complexes may contribute to the decondensation of chromatin at the site of DNA breaks. Therefore, the status of H2AX phosphorylation determines whether cells can repair the damaged DNA to survive or undergo apoptosis. Ultimately, DDR activation results in phosphorylation or stabilization of p53 and, depending on the extent of DNA damage, type of cells, the stage of cell cycle and the levels of activation of p53 different target genes, this may result in cell cycle arrest, apoptosis or senescence. In fact, mutations in genes regulating these pathways may results in different consequences such as premature aging, neurodegeneration, and increased susceptibility to cancer. Several tumour suppressors are involved in DSB signaling; for instance, mutations in the two DSB mediators, *BRCA1* and *BRCA2*, lead to a major increase in the risk of developing breast and ovarian cancers in women (Blanpain et al., 2011). The most important protein involved in DNA damage signaling and repair is p53, which upon sensing damage, initiates either cell cycle arrest to allow time for repair or apoptosis when the level of damage is excessive (Hanahan & Weinberg, 2000). In fact, p53 DNA damage signaling is lost in most human cancers (Levine, 1997). In addition, many other tumour suppressors are involved in sensing and

repairing DNA damage; therefore, their mutation or loss of function usually results in genome instability and its potential outcomes in development of cancer and other diseases. Thus, our improving understanding of sources of DSBs as well as DNA damage responses provides new avenues for better disease detections and therapies.

1.6 The HACE1 tumour suppressor

HECT domain and Ankyrin repeat Containing E3 ubiquitin-protein ligase 1 (HACE1) is a ubiquitin-protein E3 ligase encoded by the human chromosome 6q21 *HACE1* gene, first described as a potential tumour suppressor in sporadic Wilms tumour (Anglesio et al., 2004). Wilms tumour is the most common pediatric kidney cancer and has been reported as the fourth most common childhood malignancy in North America (Miller et al., 1995). Prior to the first description of the *HACE1* gene, Fernandez et al. reported a translocation with breakpoints at chromosomal regions 6q21 and 15q21, identified in a case of Wilms tumour at the IWK Health Centre in Halifax, Nova Scotia (Fernandez et al., 2001). The 6q21 region had also been reported to be involved in translocations or deletions of other malignancies such as carcinomas of the breast and prostate as well as in leukemias and lymphomas (Hyytinen et al., 2002; Utada et al., 2000; Zhang et al., 2000). Therefore, there was a suggestion that these rearrangements resulted in deletion of a potential tumour suppressor gene located at this common region. In 2004, Anglesio et al. identified the *HACE1* gene at the 6q21 locus encoding a 909 amino acid protein (~103 kDa) with six N-terminal ankyrin repeats and a HECT ubiquitin-protein ligase domain located at the C-terminus of the protein (Anglesio et al., 2004) (**Figure 1.6.1A**). Although the translocation in the mentioned Wilms' case did not

cause direct interruption of the *HACE1* locus, comparing additional Wilms tumour cases with matching adjacent normal kidney of these patients revealed that HACE1 expression at both protein and mRNA levels was downregulated (in fact, was almost undetectable) in more than 75% of Wilms tumours (Anglesio et al., 2004; Zhang et al., 2007). Interestingly, *HACE1* mRNA expression was found to be low in the Wilms tumour cell line (SK-NEP-1), as well as low to undetectable in the neuroblastoma cell line (KCNR) (Anglesio et al., 2004).

HACE1 appears to localize in both the endoplasmic reticulum (ER) and the cytoplasm; however, a significant portion of HACE1 is found in ER, indicating its possible involvement in ER-associated protein degradation (ERAD) (Anglesio et al., 2004). *HACE1* is expressed in multiple normal human tissues. In particular, there is strong expression of *HACE1* in heart, brain and kidney (Anglesio et al., 2004) (**Figure 1.6.1B**). A zebrafish hace1 homologue with 88.9% protein identity to human HACE1 and conserved tissue expression has been identified by the Berman laboratory (Daugaard et al., 2013) (**Fig. 1.6.2A and B**).

Comparing *HACE1* mRNA expression in four different primary human tumours including breast, renal, thyroid and liver with patient-matched normal tissue, Zhang et al. noticed reduced *HACE1* expression in ~50% of tumours studied (Zhang et al., 2007). Genetic inactivation of *Hace1* in mice (*Hace1*$^{-/-}$) resulted in development of multiple spontaneous tumours in various tissues at rates higher than either *Hace1*$^{+/-}$ or *Hace1*$^{+/+}$ littermate cohorts (Zhang et al., 2007). In addition, the loss of *Hace1* made the mice susceptible to different environmental triggers of cancer such as low-dose γ-irradiation and urethane treatment (Zhang et al., 2007). Interestingly, inactivation of *Hace1* together

with the tumour suppressor *Tp53*, significantly increased the incidence of tumours observed in both *Hace1*$^{-/-}$*Tp53*$^{+/-}$ and *Hace1*$^{-/-}$*Tp53*$^{-/-}$ mice (Olivier et al., 2010; Zhang et al., 2007). Loss of *Hace1* together with *Tp53* resulted in a wide variety of tumours, whereas loss of *Tp53* on itself primarily developed thymic lymphomas suggesting that HACE1 cooperates with p53 in cancer formation (Donehower et al., 1992; Zhang et al., 2007). HACE1 is also involved in regulation of cell cycle regulation and cellular proliferation as overexpression of *HACE1* in HEK293 cells significantly reduces cyclin D1 (involved in G1/S transition) expression through degradation, which is rescued by reducing *HACE1* levels using siRNA (Zhang et al., 2007). More recently, we showed that genetic inactivation of *Hace1* in mice or zebrafish, as well as *HACE1* loss in human tumour cell lines enhances cyclin D1 expression through a ROS-dependent mechanism (Daugaard et al., 2013). Therefore, HACE1 downregulation promotes overproliferation and oncogenesis by loss of cell cycle regulation, supporting its role as tumour suppressor.

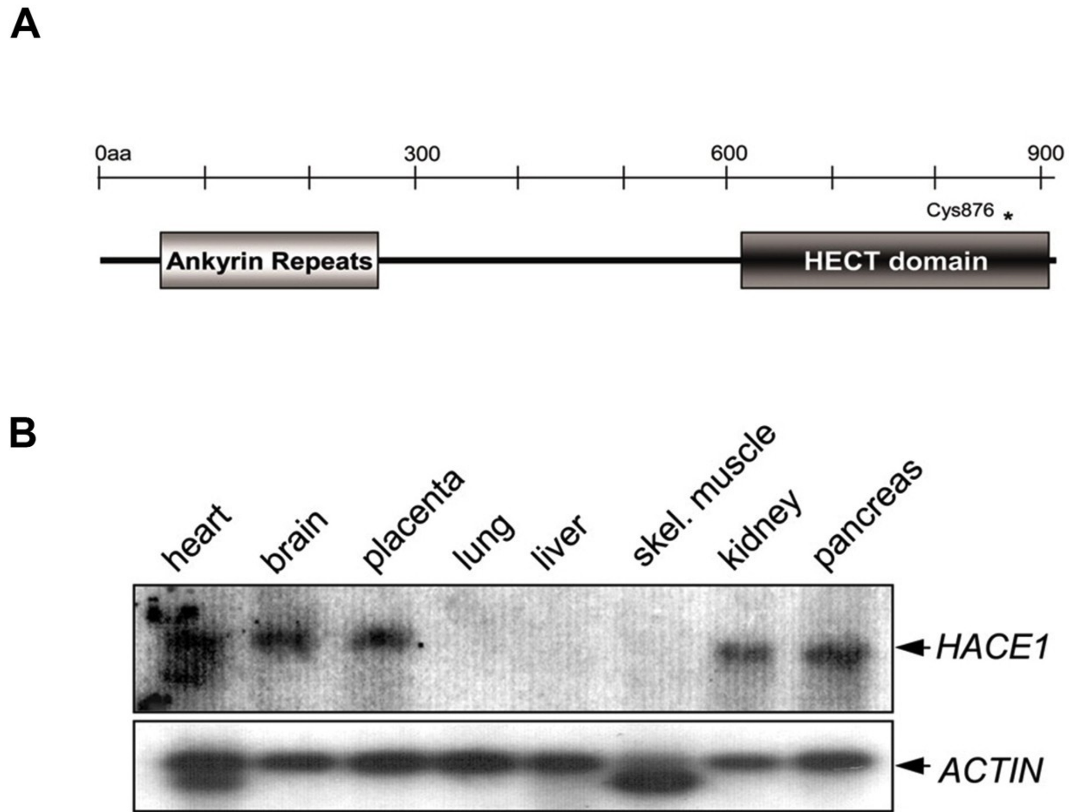


Figure 1.6.1. HACE1 domains and expression in normal human tissues. (**A**) HACE1 has two important domains, including six ankyrin repeats and the HECT domain. *Cys876 on the HECT domain shows the consistent cysteine residue in HACE1 necessary for thioester bond formation with the ubiquitin. (**B**) Northern blotting shows the *HACE1* mRNA expression in a panel of normal human tissues. (Adapted with permission from Anglesio et al., *Human Molecular Genetics*, 2004;13:2061-2074)

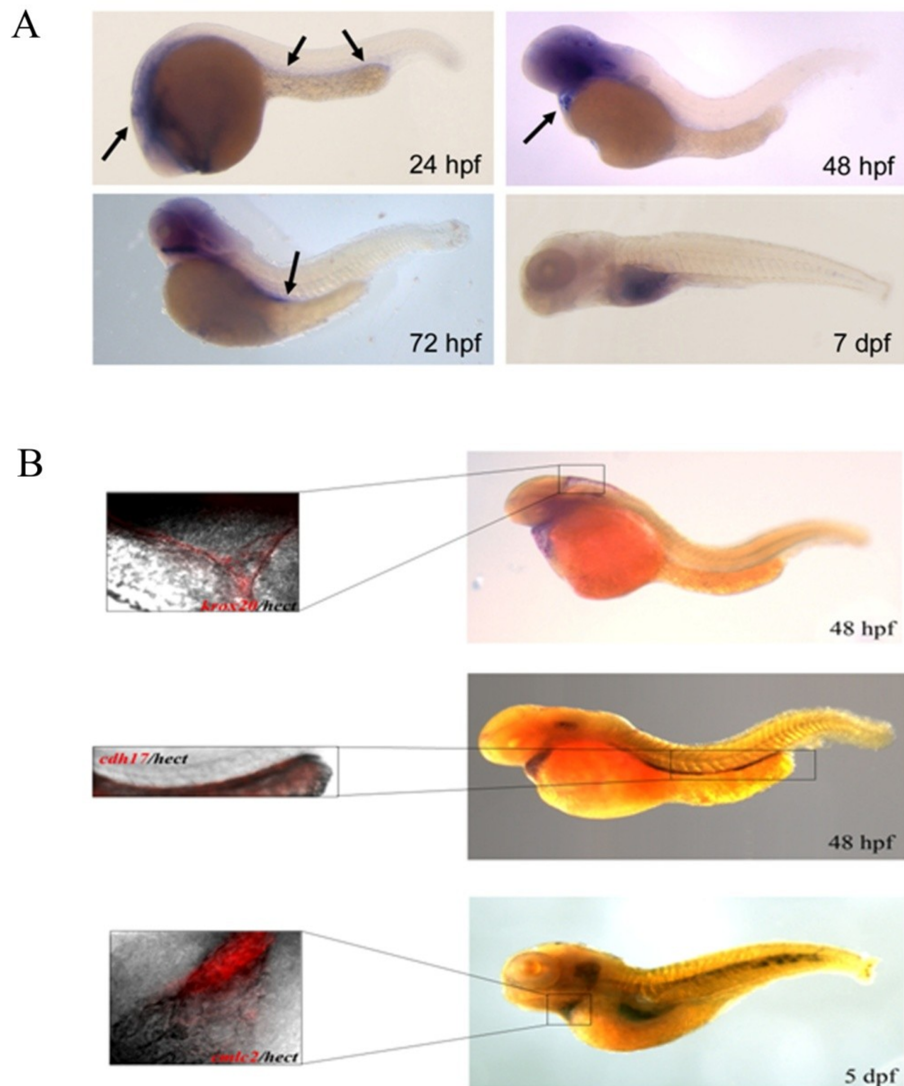


Figure 1.6.2. Zebrafish *hace1* is expressed in the brain, kidney, and heart. (**A**) Whole mount *in situ* hybridization (WISH) of AB zebrafish embryos using a probe to the zebrafish *hace1* HECT domain at 24 hours post-fertilization (hpf) - 7 days post-fertilization (dpf). (**B**) Double WISH of wild type zebrafish embryos at the indicated time points, using Fast Red anti-sense probes to *krox20* (brain), *cdh17* (kidney), and *myl7* (heart), and the zebrafish *hace1* HECT domain (black). (Adapted with permission from Daugaard et al., *Nature Communications*, 2013; 4; WISH experiments and imaging performed by Lindsay McDonald)

1.6.1 Downregulation of HACE1 in different cancers

No evidence of mutation or deletion of HACE1 has been found in sporadic Wilms tumour; however, changes in methylation of two of its upstream CpG islands are associated with its low expression in tumour samples. DNA methylation (hyper/hypo methylation) is the best-known marker for epigenetic regulation, which changes the accessibility of chromatin to transcriptional regulation through modifications of the DNA and nucleosomes (Esteller, 2008). CpG islands are the most common sites of DNA hypermethylation in tumours (Jones & Baylin, 2007). In total, there are three CpG islands associated with *HACE1*: the CpG-88 located in the *HACE1* promoter containing the transcriptional start site, and CpG-29 and CpG-177, which are located upstream of the *HACE1* coding sequence (Anglesio et al., 2004; Zhang et al., 2007). There is no evidence of methylation of CpG-88 and hypermethylation that is usually associated with epigenetic gene silencing is only found at CpG-177, while CpG-29 shows relative hypomethylation, suggesting that the status of methylation at these two CpG sites influences *HACE1* expression (Zhang et al., 2007). In support of this, treatment with the methylation inhibitor, 5-aza-2-deoxycytidine (5AZ), reduced the methylation at the CpG-177 site and resulted in at least 4-fold increase in *HACE1* mRNA re-expression (Anglesio et al., 2004). These findings suggest that the expression of *HACE1* gene is epigenetically downregulated in Wilms tumour (Zhang et al., 2007).

Loss of heterozygosity of chromosome 6q21 has been associated with multiple malignancies including melanoma (Millikin et al., 1991), breast (Noviello et al., 1996), and prostate cancer (Hyytinen et al., 2002). In addition to the epigenetic regulation of the *HACE1* locus associated with loss of HACE1 expression in Wilms tumour (Anglesio et

al., 2004; Zhang et al., 2007), aberrant *HACE1* methylation has also been described in other malignancies such as gastric carcinoma (Sakata et al., 2009), and colorectal cancer (Hibi et al., 2008). *HACE1* genetic variants have also been described in neuroblastoma, where decreased *HACE1* expression is associated with high-risk tumours and poor overall survival (Diskin et al., 2012). Thus, HACE1 is a potent tumour suppressor gene across diverse malignancies.

1.6.2 HACE1 ubiquitin ligase activity and its target for ubiquitylation

E3 ligases are essential components of the ubiquitylation pathway, which in most of the cases lead to proteasomal degradation of a specific substrate (Pickart, 2001). The Cys876 site of the HECT domain is a consistent cysteine residue in HACE1 necessary for thioester bond formation with ubiquitin (**Figure 1.6.1A**). Therefore, in order to test HACE1 ubiquitin ligase activity, Anglesio et al. mutated the Cys876 residue to Serine (Ser) *in vitro* and using a thioester bond formation assay, they demonstrated that substitution at this critical residue completely abolished this bond formation and ubiquitin ligase activity of HACE1 in these mutants. These *in vitro* studies along with immunoprecipitation showing the involvement of HACE1 in the ubiquitylation of proteins *in vivo*, strongly support the role of HACE1 in ubiquitylation and proteasomal degradation of substrates (Anglesio et al., 2004). As mentioned earlier, since it contains ankyrin repeats at the N-terminus, HACE1 is a stereotypical HECT E3 ubiquitin-protein ligase. Ankyrin motifs in general are well known for their role in mediating protein-protein interactions (Bork, 1993). Interestingly, the HACE1 ankyrin repeats have high sequence similarity to those of the cyclin-dependent kinase inhibitors p16INK4A

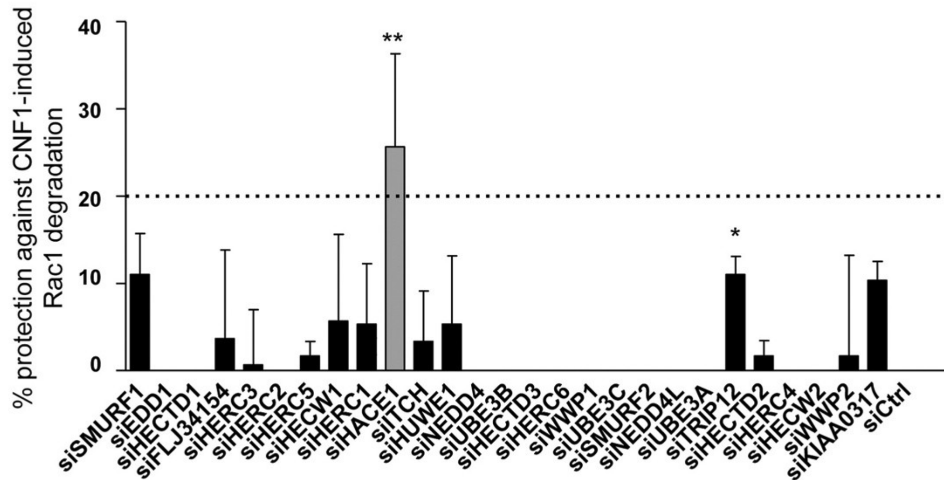
(CDKN2A) and p19INK4D (CDKN2D), which are involved in suppression of the cell cycle by binding to and inhibiting CDK4 and CDK6 (Anglesio et al., 2004).

Recently, a role for HACE1 in the ubiquitylation and subsequent proteasomal degradation of the Rho family small GTPase, Rac1, has been described (Castillo-Lluva et al., 2013; Torrino et al., 2011) (**Figure 1.6.2.2**). Ubiquitin-mediated degradation of activated Rac1 is specifically impaired in different cancer cell lines, suggesting that specific proteins may be involved in its proteasomal degradation (Boyer et al., 2006). Following GTP loading, Rac1 undergoes a conformational change and becomes able to bind and activate target proteins (Jaffe & Hall, 2005). Prior to the identification of HACE1 as an E3 ubiquitin ligase involved in Rac1 degradation, Doye et al. had demonstrated that activation of Rac1 by cytotoxic necrotizing factor-1 (CNF1) leads to targeting of this GTPase to the ubiquitin-proteasome system (UPS) for degradation (Doye et al., 2002). CNF1 is a protein toxin produced by uropathogenic *Escherichia coli* (UPEC) strains responsible for urinary tract infection and found to be one of the Rho GTPase-activating bacterial toxins (Boquet, 2001; Landraud et al., 2000). In order to identify the E3 ligases involved in Rac1 degradation, Torrino et al. transfected cells with a siRNA library targeting 27 known human HECT-domain containing E3 ligases followed by intoxication with CNF1 for induction of ubiquitin-mediated proteasomal degradation of Rac1. Among all the E3 ligases with the HECT domain, HACE1 depletion had the most significant inhibitory effect on Rac1 degradation (**Figure 1.6.2.1A**) (Torrino et al., 2011). In addition, they demonstrated that HACE1 catalyzes the ubiquitylation of the active form of Rac1 to control its cellular level. The two-fold preferential binding of HACE1 to active GTP-bound Rac1 compared to the GDP-bound

form both *in vitro* and *in vivo* showed that, upon activation, Rac1 directly binds to HACE1 for ubiquitylation (Torrino et al., 2011). Furthermore, a significant increase of the GTP over total Rac1 ratio in *HACE1* knocked down cells intoxicated with CNF1 suggests that ubiquitin-mediated regulation of GTP-bound Rac1 by HACE1 is required to achieve the control of Rac1 activity in cells (Torrino et al., 2011) (**Figure 1.6.2.1B**). In keeping with these findings, Castillo et al. demonstrated an enhanced interaction between HACE1 and Rac1 by hepatocyte growth factor (HGF) signaling, a stimulus for cell migration. These results identify HACE1 as an antagonist of cell migration through selective degradation of active Rac1 associated with membrane protrusions (Castillo-Lluva et al., 2013). This regulation of cell migration is independent of HGF signaling and may contribute to the phenotype of invasive cancer cells through enhanced cell migration without requirement of growth factors in HACE1 loss of function (Castillo-Lluva et al., 2013). Rac1 has also been reported to be the target of other E3-ubiquitin ligases such as poly-ubiquitylation by the Ring-domain containing inhibitors of apoptosis proteins (IAPs) regulating cell elongation and migration (Oberoi-khanuja & Rajalingam, 2012).

Therefore, HACE1 is required for ubiquitin-mediated degradation of Rac1 and catalyzes its poly-ubiquitylation at lysine-147 after induction with CNF1, or following activation of HGF signaling (Castillo-Lluva et al., 2013; Torrino et al., 2011). Given the important roles of Rac1 in host defense through regulation of various cellular processes such as apoptosis, phagocytosis, and ROS production, more attention has been directed to the HACE1 tumour suppressor and its role in controlling the cellular level of Rac1. This

key function of HACE1 in degradation of Rac1 is another suppressing activity by HACE1 supporting its protective role in tumourigenesis.



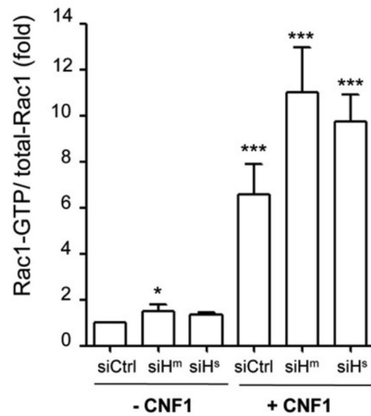


Figure 1.6.2.1. HACE1 controls degradation of Rac1 and regulates its cellular activity. (**A**) Percentage of siRNA-mediated protection against CNF1-induced Rac1 degradation. Cells were transfected with siRNA targeting each of the 27 known HECT-domain containing E3 ubiquitin-ligases and siRNA control (siCtrl). The levels of Rac1 were quantified by anti-Rac1 Enzyme-linked immunosorbent assay (ELISA). (**B**) Quantification of Rac1-GTP normalized to total-Rac1 in siCtrl, siHACE1 mix (siHm) or single (siHs) transfected cells left untreated or intoxicated by CNF1. (Adapted with permission from Torrino et al. *Developmental Cell*, Volume 21, Issue 5, 2011, 959 – 965)

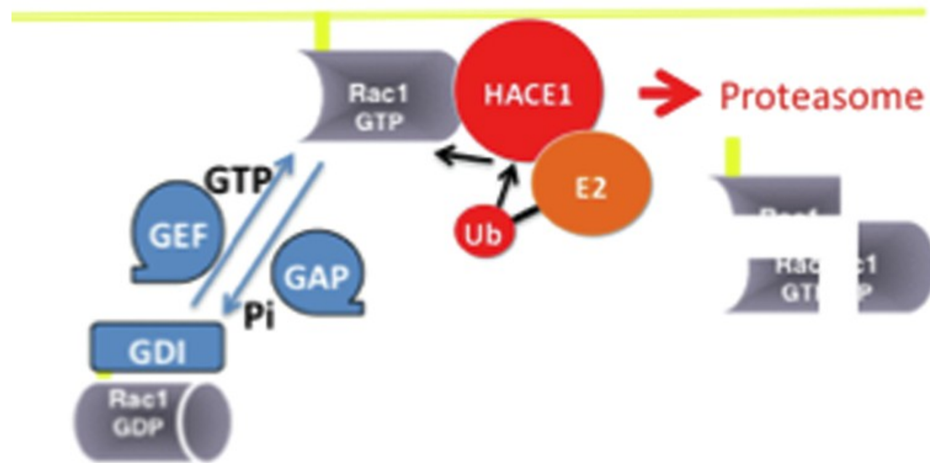


Figure 1.6.2.2. Rac1 ubiquitylation by HACE1. HACE1 binds to the active form of Rac1 (GTP-bound) and to a ubiquitin conjugated to an E2 enzyme. These ubiquitin molecules are then transferred to the Cys876 residue on HACE1 prior to Rac1 conjugation in order to form a lysine-48 (k48) poly-ubiquitylation and proteasomal degradation. (Adapted with permission from Mettouchi & Lemichez. *Small GTPases,* 2012; 3:102 – 106)

1.7 Zebrafish as an ideal model organism for studying human diseases

The zebrafish, *Danio rerio*, a freshwater tropical fish native to the Himalayan region, has emerged as a major model organism for research ranging from developmental biology to cancer genetics and drug discovery. In fact, zebrafish have become one of the most popular model organisms due to several key characteristics. Zebrafish are vertebrates and share high genetic similarity to humans, making them a suitable organism for studying vertebrate gene function (Howe et al., 2013). In support of this contention, comparison with the human reference genome shows that approximately 70% of human genes have at least one obvious zebrafish orthologue (Howe et al., 2013). The small size of zebrafish (~ 1 inch) allows large numbers of these vertebrates to be kept in small spaces under controlled temperature (22-28°C) with a low cost for maintenance. Zebrafish reach sexual maturity at approximately three months of age and each female is able to lay >200 eggs per week with extremely rapid external development of embryos, providing quick and repeated analysis of conserved developmental pathways (Zon, 1999). The transparency of the embryos allows direct visualization of tissue formation and organogenesis (Berman et al., 2003). Furthermore, large scale screens to examine genetic mutations in zebrafish affecting development (Grunwald & Eisen, 2002) as well as reverese genetic technologies such as gene silencing by RNAi or morpholino oligonucleotides (MOs) (Eisen & Smith, 2008) have all been instrumental to expand the ability to manipulate and study this model organism. Morpholinos can block RNA translation transiently for approximately 72 hours, enabling the study of defects and abnormalities resulting from loss of gene function early in development. The widespread popularity of the zebrafish as a model organism has resulted in the emergence of different

resources such as the Zebrafish Model Organism Database (www.zfin.org), the Zebrafish Mutation Project (ZMP) by the Sanger Institute (http://www.sanger.ac.uk/Projects/D_rerio/zmp/), and more recently the Zebrafish Disease Modeling Society (http://www.zdmsociety.org) which all provide useful information and resources for researchers in this filed.

The zebrafish was anticipated to provide important clues to normal embryogenesis and organ development. One key advantage of zebrafish embryos is that they are completely transparent making them ideally suited for studying developmental processes as they occur in real time. Also, transgenic technologies to drive tissue-specific expression of fluorescent proteins as well as different measurement techniques for qualitative mRNA and protein expression such as whole mount *in situ* hybridization (WISH) and immunohistochemistry in an intact whole animal system can be performed to determine where and when particular genes are expressed. Today, researchers around the world are using zebrafish for research in different areas of developmental biology such as neurogenesis, cardiogenesis and hematopoiesis (Delvaeye et al., 2009). In particular, over the last decade, zebrafish has become one of the powerful model systems in the field of cardiovascular research and has been widely used to model human congenital and acquired cardiac diseases (Bakkers, 2011). Forward genetic screens have provided the identification of many novel factors and regulatory mechanisms that play essential roles during cardiogenic specification and differentiation, migration of cardiac progenitor cells, heart tube morphogenesis, and cardiac function (Bakkers, 2011). Furthermore, the zebrafish has become a popular model organism for behavioural studies. Development of computerized behavioural testing methods that allow high-throughput

screening has facilitated using zebrafish to study some behavioural characteristics of certain conditions, such as depression, autism, anxiety, alcoholism, aggression and social behaviour (Miklósi & Andrew, 2006; Sison et al., 2006).

The zebrafish is also an ideal model organism for studying cancer. Early studies in this area started with exposing the zebrafish to water-soluble carcinogens to monitor tumour development (Hawkins et al., 1985). These chemically-induced tumours demonstrated a high degree of similarity to human malignancies with common known hallmarks such as increased rate of proliferation, low degree of cellular differentiation and an overall reproduction of gene signatures involved in the regulation of DNA damage and repair, cell cycle progression and apoptosis (Lam & Gong, 2006). Comparison of the human and zebrafish genome demonstrates high conservation of cell cycle genes, tumour suppressors, and oncogenes involved in tumourigenesis and cancer progression (Amatruda et al., 2002). Gene expression array studies also show comparable signaling pathways involved in tumourigenesis (Feitsma & Cuppen, 2008). These common features set the framework for cancer modeling in zebrafish. Furthermore, based on high similarity of their immune system with humans, several laboratories have developed bacterial and viral disease models with the zebrafish to study immune responses to infection (Sullivan & Kim, 2008). In fact, the zebrafish is an excellent model for studying both innate and adaptive immunity. Since full functionality in the adaptive immune response takes 4 to 6 weeks to develop in zebrafish, it is possible to study innate immune responses exclusively in the first days of development (Cui et al., 2011; Lam et al., 2002).

A part of the present thesis investigates the role of the HACE1 tumour suppressor in normal development of the heart. Thus, the current state of knowledge surrounding the development of the heart in zebrafish will be outlined briefly in the next section.

1.7.1 Zebrafish heart development and morphology

The development of the embryonic zebrafish heart and cardiovascular system, as well as the molecular mechanisms behind this process has been extensively reviewed (Bakkers, 2011; Vogel, 2000). Briefly, heart development begins with the specification of the myocardial and endocardial progenitor cells. These include both atrial and ventricle progenitor cells that are located in the lateral marginal zone at around 5 hours post-fertilization (hpf) (**Figure 1.7.1.1A**). During gastrulation and early somite stages, the cardiac progenitor cells migrate to the mid-line axis and reach their destination at the anterior later plate mesoderm (ALPM), where cardiogenic differentiation is initiated by the expression of cardiac myosins (**Figure 1.7.1.1B**). Cardiogenic differentiation continues during mid- and late-somite stages and occurs simultaneously with formation of a cardiac disc structure with the endocardial cells within the hole at the centre surrounded by ventricular myocytes as well as atrial myocytes at the edge of the disc (**Figure 1.7.1.1C** and **D**). Cardiac morphogenesis transforms the cardiac disc into a cardiac tube and rhythmic contractions of this tube begin at 22-24 hpf, when blood circulation also begins. At 28 hpf, the linear heart tube has formed, with the venous pole located at the anterior left and the arterial pole fixed at the mid-line (**Figure 1.7.1.1E**). The formation of the two chambers of the heart, the atrium and the ventricle, begins at approximately 30 hpf, while the looping of the heart with a clockwise rotation occurs at

33-36 hpf, which continues to form an S-shaped loop by 48 hpf (**Figure 1.7.1.1F** and **G**). At this time, circulation of blood cells within the embryonic vasculature can be clearly distinguished in the trunk and head. By 2 days post-fertilization (dpf), the embryonic zebrafish heart resembles that of other developing vertebrates, with venous blood flowing from the sinus venosus to a single atrium, then to a single ventricle, which pumps blood through the bulbus arteriosus to ventral aorta. By the time the larva is 5 days old, the heart will have its adult shape, with the atrium positioned dorsally to the ventricle.

Finally, besides the rapid development of the heart and vasculature, another key characteristic that makes zebrafish a unique model for studying gene function during cardiovascular development is that unlike avian and mammalian models, zebrafish embryos are not completely dependent on a functional cardiovascular system for their development. In fact, based on the small size of the embryo, oxygen can still enter and reach all tissues by passive diffusion when the blood circulation is not available. Therefore, embryos are able to survive the initial phases of development regardless of severe cardiac abnormalities, which allows for the *in vivo* analysis of these defects in affected embryos (Bakkers, 2011).

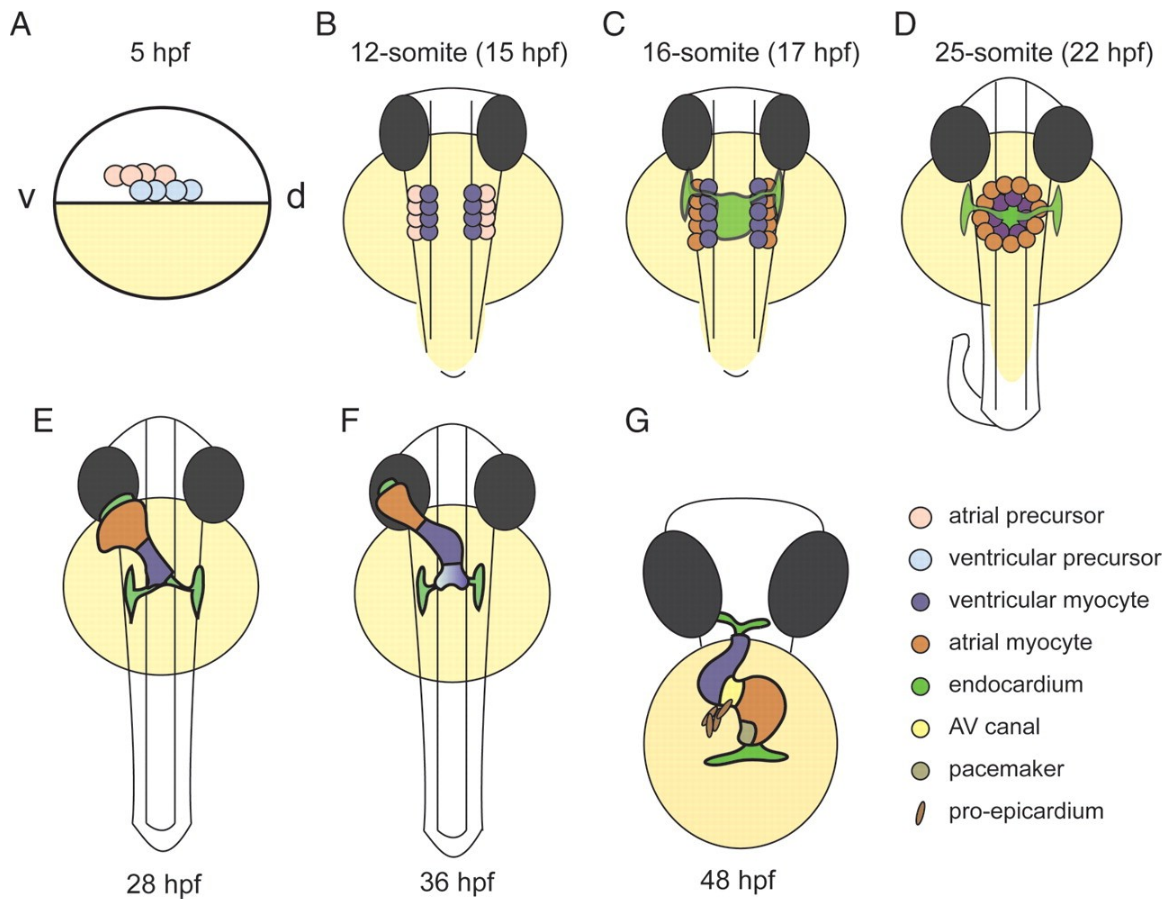


Figure 1.7.1.1. Stages of the embryonic zebrafish cardiac development. Different stages of this process have been explained in the **section 1.7.1**. hpf = hours post-fertilization. (Reproduced with permission from Bakkers, *Cardiovascular Research*, 2011;91:279-288)

1.8 Rationale

The zebrafish is a robust model for studying vertebrate development and modeling human cancers by virtue of conserved genetics and facility for direct evaluation of gene expression through enhanced imaging afforded by transparent embryos. In this project, I will use the zebrafish model system to study the mechanisms underlying the tumour-suppressing activity of the *HACE1* gene as well as its role in normal development of the heart. Downregulation of HACE1 has been reported to be involved in various cancers suggesting it is a potent tumour suppressor; however, the mechanism underlying its mode of action is still unclear. Tumour development is dramatically accelerated by oxidative stress and high levels of DNA damage. I hypothesize that *HACE1* as a tumour suppressor may function to regulate the production of reactive oxygen species (ROS) as well as controlling the levels of DNA damage. The transparency of zebrafish embryos allows direct visualization of these changes and will complement information gained from murine research. Therefore, I will develop *in vivo* assays to monitor and quantify the levels of ROS and DNA damage in zebrafish embryos with/without *hace1* expression.

In humans, *HACE1* is expressed in various tissues including the brain and kidney, with strongest expression in the heart. The high conservation of *HACE1* and its expression in similar tissues has been established in zebrafish. However, the role of HACE1 in normal vertebrate development and the mechanisms by which it contributes to developmental processes have not been well elucidated. Based on the strong *hace1* expression in the heart of zebrafish, I hypothesize that HACE1 contributes to vertebrate cardiac development and may do so via ROS-dependent mechanisms and will investigate

the potential correlation between the tumour-suppressing mechanisms of *hace1* with its novel developmental role using the zebrafish model.

Therefore, this study will exploit the advantages of the zebrafish model to investigate the role of the *HACE1* in regulation of ROS and DNA damage, as well as its contribution to cardiac development. These findings have the potential to impact the development of cancer treatments and a better understanding of congenital heart disease.

CHAPTER 2: MATERIALS AND METHODS

2.1 Zebrafish husbandry

Adult zebrafish [*Danio rerio* (Hamilton, 1822)] were housed in standard zebrafish tank systems (Pentair, Apopka, FL). Zebrafish housing and breeding conditions, as well as developmental staging of larvae, were performed according to Westerfield (Westerfield, 2000). Use of zebrafish in this study was approved by and carried out according to the policies of the Dalhousie University Committee on Laboratory Animals (Protocol 13-131). All zebrafish embryos were maintained in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) in 10 cm petri dishes at 28°C. To prevent pigment formation, the E3 medium was supplemented with N-phenylthiourea (PTU; Sigma) to a final concentration of 0.2 mM.

2.2 Strains of zebrafish used in this study

The translucent *casper* zebrafish mutant with loss of melanocytes and iridophores in both embryogenesis and adulthood (White et al., 2008) was provided by Dr. Leonard Zon's Laboratory (Children's Hospital, Boston, MA). *Tg(myl7::eGFP)* zebrafish were developed by Huang et al. (Huang et al., 2003) and possess green fluorescent protein expression specific to the cardiac myocytes. Both this line and the *Tg(myl7::mCherry)* zebrafish with mCherry-labeled cardiomyocytes were provided by Dr. Ian Scott (Hospital for Sick Children and University of Toronto, ON).

2.3 Generation of the *hace1* morpholino

The *hace1*-HECT (CCCTCGAACTGTTAGACAGAATAAA) and standard control morpholinos (CCTCTTACCTCAGTTACAATTTATA) were purchased from Genetools LLC (Philomath, OR). The *hace1*-HECT splice site resides within the catalytically active HECT domain, and is positioned upstream of the critical cysteine residue required for hace1 ubiquitin ligase function. The knockdown of *hace1* using this morpholino was verified by RT-PCR using the following primers (**Figure 2.3.1 A**): Primer A: Forward-TTGCTGGTCAAATCCTGGGTCTGG; Reverse-AATGCAGTGCGACAAGCAAGCG; Primer B: Forward-AGCCAGGAGGAACTACCATTCAGG; Reverse-ATAACTCCCACAATGCAGTGCGAC. Injection of this morpholino into zebrafish embryos results in aberrant splicing, leading to loss of an exon within the HECT domain, and subsequent loss of hace1 function (**Figure 2.3.1 B**) (Daugaard et al., 2013). The standard control morpholino is a negative control oligo that targets a human beta-globin intron mutation that causes beta-thalassemia and results in little change in phenotype in any known test system except human beta-thalessemic hematopoetic cells. Morpholinos were diluted to a working concentration of 1.6 mM with 0.05% phenol red, and were injected into live zebrafish embryos at the 1-4 cell stage using a high-pressure system PLI-100 from Medical Systems Corp (Greenvale, NY).

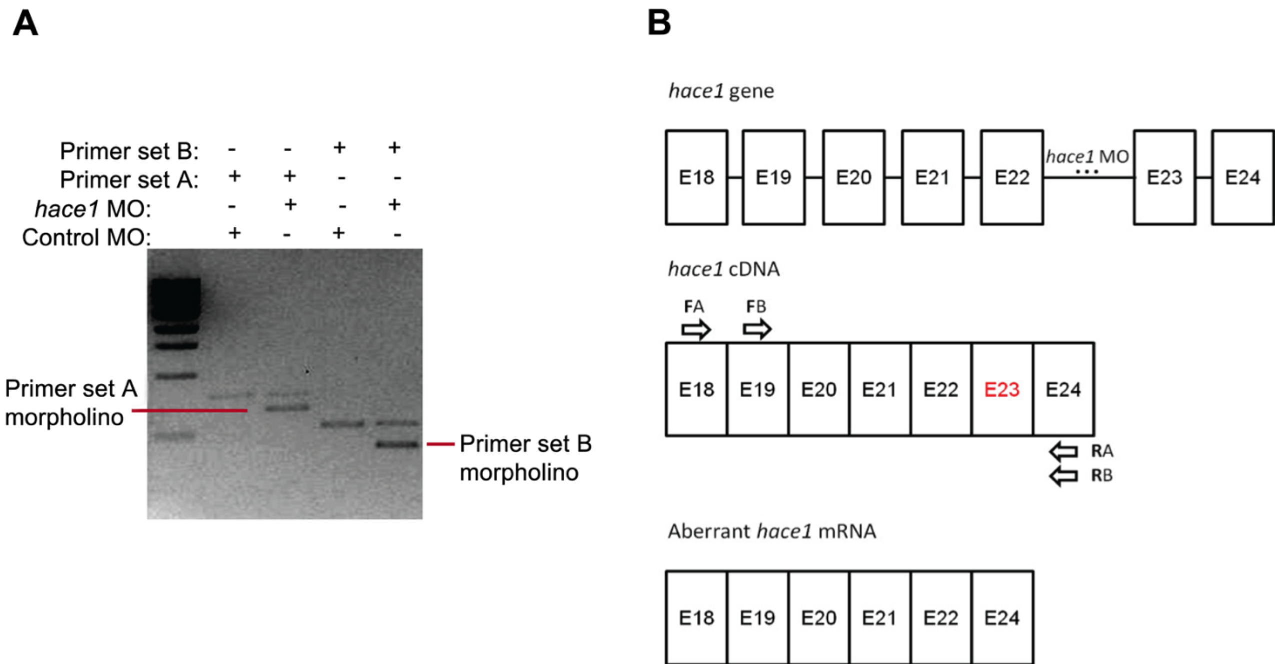


Figure 2.3.1. *hace1* knockdown using splice site morpholino. (**A**) RT-PCR detection of control (Control MO) or *hace1* (*hace1* MO) morpholino constructs in zebrafish 72 hpf using two different primer sets (A and B) as indicated. (**B**) Schematic of the zebrafish *hace1* gene organization showing target site of the *hace1* morpholino construct including binding sites for the primers Forward A (FA), Forward B (FB), Reverse A (RA) and Reverse B (RB) used in (A). Exons are represented by boxes and introns by lines. Dotted lines indicate the region targeted by hace1 morpholino and arrows indicate PCR primer positions. The morpholino inhibited pre-mRNA splicing resulting in E23 deletion. (Adapted with permission from Daugaard et al., *Nature Communications*, 2013;4)

2.4 Imaging of H_2O_2 generation in zebrafish (*in vivo* ROS assay)

H_2O_2 analysis in whole zebrafish larvae using a live cell fluorescein dye was adapted from a previously described ROS staining procedure (Niethammer et al., 2009; Walker et al., 2012) and was outlined in Daugaard et al. (Daugaard et al., 2013). Briefly, 48 hpf morphant embryos were loaded for 45 min to 1 h with 50 μM acetyl-pentafluorobenzenesulfonyl fluorescein (Cayman Chemical) or 5-50 μM 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) in 1% DMSO in E3 embryo medium, and imaged using a 550 nm bandpass filter using a Leica DFC 490 camera connected to Leica Application Suite software (Leica, Wetzlar, Germany) or AxioCam MRm connected to Zeiss AxioZoom.V16 for double fluorescent images (green and red filters) using the Zeiss program Zen 2012 Blue edition (Carl Zeiss, Goettingen, Germany).

2.5 Fluorescence-activated cell sorting (FACS)

Following ROS staining, *Casper* or *Tg(myl7::mCherry)* zebrafish embryos were dissociated to a single cell suspension as described in Covassin et al. (Covassin et al., 2009), and were sorted by a FACS Aria III Cell Sorter (BD Biosciences, San Jose, CA) according to GFP and mCherry fluorescence. Cells were thresholded by forward scatter (FSC) and side scatter (SSC) and interrogated by Coherent® Sapphire™ solid state 488 nm laser with dual band pass filter PE-Texas Red (604 - 627 nm) emission with an LP filter of 610 nm. ROS^+ cells were gated by interrogation for FSC^{LOW} and SSC^{LOW}, and $FITC^{HI}$ (fluorescein isothiocyanate) fluorescence.

2.6 Treatment with antioxidants and NADPH oxidase inhibitors

For all chemical treatments, embryos were incubated beginning immediately after injection in E3 medium with PTU supplemented with or without the following chemicals for 48 h: 50 μM N-acetylcysteine (Sandoz Canada) was used as a general antioxidant precursor to reduce the levels of ROS in morphant embryos; 25 μM of NSC23766 (Santa Cruz Biotechnology) was used to specifically inhibit Rac1 binding and activation. Morphant embryos were also incubated with 200 μM apocynin (Apo), a known NADPH oxidase inhibitor previously described in Daugaard et al. (Daugaard et al., 2013). All embryos were dechorionated at 24 hpf with 10 mg/ml Pronase (Roche Applied Science). To prevent degradation, the chemicals were refreshed and re-applied after dechorionation. Embryos were raised to 48 hpf and heart phenotypes were scored as described in **section 2.9**.

2.7 DNA damage γ-H2AX assay

48 hpf *hace1* or control morphant *casper* zebrafish were stained for phosphorylated histone H2AX (γH2AX) using the rabbit polyclonal antibody to histone H2AX (phospho Ser139) with a 1:250 dilution (GeneTex, San Antonio, TX) and DsRed-conjugated secondary antibody (1:250). Embryos screened for γH2AX foci as a measure for DNA damage using a Zeiss Axio observer Z1 microscope with a Colibri illumination system (Carl Zeiss, Goettingen, Germany). Embryos with more than four γH2AX foci in the tail area were selected and the percentages were reported in a column graph.

2.8 Whole mount *in situ* hybridization (WISH)

Digoxigenin (DIG)- and fluorescein isothiocyanate (FITC)-labelled antisense RNA probes for *nppa*, *amhc*, and *vmhc* were transcribed from linearized cDNA constructs according to manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, IN). WISH assays for zebrafish embryos were conducted as described previously (Dobson et al., 2008). Staining of DIG labeled RNA probes was performed using BCIP/NBT (Vector laboratories, Burlington, ON, Canada). Single WISH images were taken with a DFC 490 camera mounted on a Leica MZ16F microscope (Wetzlar, Germany). For double fluorescence *in situ* experiments staining was first performed with FITC (green) and Fast Red (red), followed by BCIP/NBT, and then imaged with a Zeiss Axio observer Z1 microscope with a Colibri illumination system (all from Carl Zeiss, Goettingen, Germany).

2.9 Sorting larval cardiac phenotypes

Live unanesthetized groups of *Tg(myl7::eGFP)* embryos were sorted for specific cardiac phenotypes at 48 h under a Leica MZ16F stereomicroscope with a fluorescent light source to visualize the green fluorescent protein expressed in the heart to facilitate cardiac visualization. Fish were sorted into three groups: "Normal" (where the larvae had a normal looped heart phenotype with the ventricle oriented to the right side of the fish), "Straight" (where the larvae showed a fully or partially unlooped heart), and "Inverted" (where the larvae had a heart where the ventricle was looped to the left of the fish thus creating a mirror image to the normal heart phenotype). For a diagrammatic

representation of these phenotypes refer to **Figure 3.6.1**. The number of embryos in each phenotypic group was counted and represented as a percentage of the total number of embryos in the groups combined. Images of each group were captured using a Leica DFC 490 camera connected to Leica Application Suite software (Leica, Wetzlar, Germany).

2.10 Measuring larval heart rates

After sorting based on cardiac phenotype, heart rates were measured by direct visualization under the same microscope conditions described above in unanesthetized embryos. At 48 hpf, embryos have sufficiently low sporadic swimming behaviour that hearts can be visualized for extended periods without movement. Heart beats were counted for 15 sec under physiological temperature (28.5-30°C on a temperature controlled stage) and the number of beats multiplied by 4 to obtain a final value of beats per min.

2.11 Phenotypic rescue of morphant embryos using human *HACE1* mRNA

Human HACE1 plasmid was provided by Dr. Poul Sorensen's laboratory at the University of British Columbia, Vancouver, BC. HACE1 mRNA was synthesized from 1 μg of linearized plasmid (pEGFP-C1-HACE1) using the high-yield capped RNA transcription mMESSAGE mMACHINE Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocols. Approximately 100 pg/μl of the resulting mRNA was co-injected with *hace1* morpholino (1.6 mM with 1% phenol red) into embryos at the

1 to 4 cell stage. Embryos were raised to 48 hpf and heart phenotypes were scored as described above.

2.12 Zebrafish protein extraction and Western blot analysis

Zebrafish embryos were manually deyolked at 48 hpf as described by Link et al. (Link et al., 2006). Approximately 50 embryos from each group were homogenized using a 21G needle attached to a 1 ml syringe in 250 µl ice-cold 1x RIPA buffer supplemented with complete protease inhibitor cocktail (Roche, Applied Science, Switzerland). A Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) adapted for use on the NanoDrop 2000 spectrophotometer (Thermo Scientific) was used for protein quantification. Protein extracts were stored at -20°C until use. Protein samples (100 µg of protein per well) were resolved on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes and blocked with 5% skim milk in T-BST for 1 h at room temperature. Membranes were then incubated overnight in 5% skim milk containing one of the following primary antibodies: Anti-HACE1 (1:1000; Sigma-Aldrich SAB2101010 SIGMA). Anti-Rac1 (1:1000; Santa Cruz Biotechnology). Membranes were then washed three times with 1x T-BST and incubated for 1 h at room temperature with 1:2000 anti-rabbit IgG, HRP-linked Antibody (7074;Cell Signaling Technology).

2.13 RNA extraction and Real-Time PCR

Total RNA was extracted from samples of pooled (approximately 30 embryos per sample) 48 hpf *hace1* or standard control morpholino using the Qiagen RNeasy Mini kit (Qiagen) as per the manufacturer's protocol. Total RNA was quantified using a NanoDrop 2000 UV-vis spectrophotometer. Two µg of total RNA from each sample was treated with DNAse I, Amplification Grade (Invitrogen) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) using OligoDT primer as per manufacturer's protocols. Expression of *rac1* and elongation factor 1 a (*ef1a*, used as a housekeeping gene) was determined in cDNA samples using the Brilliant II SYBR® Green Low Rox Master Mix (Agilent). Real-time PCR reactions were carried out using a CFX96 Touch ™ Real-time PCR detection system (BioRad). *rac1* expression levels were normalized to *ef1a* expression using the ΔΔ Cq method and expression in *hace1* MO is expressed relative to expression in standard control morphants (**Figure 3.8.1B**) using CFX Manager software (v 3.1; BioRad). Efficiency of the *rac1* and *ef1a* real-time PCR reactions was determined by the standard curve method (using serial dilutions of cDNA samples) and was confirmed to be between 90%-110%. (*rac1* forward: 5'-CTCCCATCACCTACCCTCAA-3', *rac1* reverse: 5'-TAAGGCAGAGCACTCCAGGT-3', *ef1a* forward: 5'-CCAGCAAATACTACGTCACCAT-3' and *ef1a* reverse: 5'-CAATCAGCACAGCACAATCC-3').

2.14 *rac1*, *nox1*, and *nox2* morpholinos

Splice blocking morpholinos for *rac1* (5'-CCACACACTTTATGGCCTGCATCTG-3'), *nox1* (5'-AGGTAAATAAACGCTCTTACCACGA-3') and *p91^{phox}* (nox2; 5'-

CATAATCCCGATAGCTTACGATAAC-3') were purchased from Genetools LLC (Philomath, OR) and diluted to a working concentration (1.6 mM for *rac1*, and 1mM for *nox1*, *nox2*, and *nox1/nox2* double knockdown) with 0.05% phenol red, and were injected into zebrafish embryos at the 1-4 cell stage using a high pressure system PLI-100 from Medical Systems Corp (Greenvale, NY 11548).

2.15 Statistical Analysis

Fisher's exact test was used to analyze differences in scored heart morphology in RNA rescues as well as NAC/apocynin treatments for *hace1* morphants. ROS-induced fluorescence and Real-time PCR data were analyzed using Student's t-test. Heart rate data were analyzed by two-way ANOVA and Tukey post hoc multiple comparisons test, using GraphPad Prism software (v 6.03; GraphPad Software Inc.).

Notes on chapter 3

Sections 3.1 to 3.3:

Experiments in **sections 3.1 to 3.3** have been done in collaboration with Dr. Sorensen's lab at UBC and parts of the results have been published in Nature Communications as per the following citation:

Daugaard, M., Nitsch, R., Razaghi, B., McDonald, L., Jarrar, A., Torrino, S., Castillo-Lluva, S., Rotblat, B., Li, L., Malliri, A., Lemichez, E., Mettouchi, A., Berman, J. N., Penninger, J. M., & Sorensen, P. H. (2013). Hace1 controls ROS generation of vertebrate Rac1-dependent NADPH oxidase complexes. *Nature Communications*, 4.*

* Proof for copyright and permission from Nature Communication has been attached in **Appendix D**.

Sections 3.4 to 3.9:

Results from **sections 3.4 to 3.9** have been collected and prepared in a manuscript format for submission as following:

Razaghi, B., Steele. S. L., McDonald. L., Lin, W., Daugaard, M., Scott, I. C., Sorensen, P. H. & Berman, J. N. (2014). Hace1 influences zebrafish cardiac development via ROS-dependent mechanisms.

CHAPTER 3: RESULTS

3.1 *hace1* knockdown results in increased levels of ROS

Enhanced generation of reactive oxygen species (ROS) is intimately associated with malignant transformation (Trachootham et al., 2009). We hypothesized that *HACE1* as a tumour suppressor may function to regulate the generation of ROS, such as hydrogen peroxide and free oxygen radicals that result in an oxidative stress condition. Studies from Dr. Sorensen's laboratory demonstrated that knocking down *HACE1* expression in human embryonic kidney (HEK) 293 cells using short interfering RNAs (siRNAs) showed a 4- to 6-fold increase in ROS levels compared with controls. Moreover, organs isolated from the *Hace1* knockout mice (*Hace1*$^{-/-}$) demonstrated dramatic increases in ROS compared with those of littermate controls (Daugaard et al., 2013). To determine whether *Hace1* regulation of cellular ROS was conserved, I used the zebrafish model, which displays 74.7% DNA sequence homology and 88.9% protein identity with humans (McDonald, 2011). I developed an *in vivo* ROS assay to determine if zebrafish could serve as a complementary *in vivo* model, demonstrating a similar inverse trend between *hace1* and ROS levels. I used a *hace1* morpholino previously designed in our laboratory (McDonald, 2011) (**Figure 2.3.1**) to knock down *hace1* expression and assess ROS levels. This morpholino targets a *hace1* splice site located within its catalytically active HECT domain and is positioned upstream of the critical cysteine residue required for *hace1* ubiquitin ligase function (Cys876). Following *hace1* or standard control morpholino injection into *casper* zebrafish embryos (double pigment lacking both melanocytes and iridophores) (White et al., 2008), I incubated the 72 hpf embryos as well

as un-injected group with the H_2O_2-specific probe, pentafluorobenzenesulfonyl fluorescein, which is converted to a fluorescent form upon interaction with H_2O_2 (Soh, 2006). H_2O_2 is a relatively long-lived ROS and is freely diffusible between cells; therefore, it is a suitable signaling molecule for monitoring the ROS levels in the absence of *hace1*. *hace1* morphant embryos showed significant increases in ROS levels compared to control (injected with standard control morpholino) and un-injected embryos (**Figure 3.1.1A**). In order to quantify the ROS elevation, this visual observation was validated by fluorescence-activated cell sorting (FACS) following dissociation of each group of fish. FACS quantification demonstrated that *hace1* knockdown correlated with more intense ROS-related fluorescence (**Figure 3.1.1B**). I repeated the abovementioned ROS assay at 48 h post-injection (hpi). At this earlier stage, I similarly observed increased levels of ROS in the *hace1* morphant group compared to control embryos (**Figure 3.1.2A**). FACS quantification again confirmed a significant increase in ROS levels in 48 hpf *hace1* morphants (**Figure 3.1.2B**). Therefore, these results establish an inverse relationship between *hace1* function and ROS levels in zebrafish, suggesting that *HACE1* is a critical regulator of ROS.

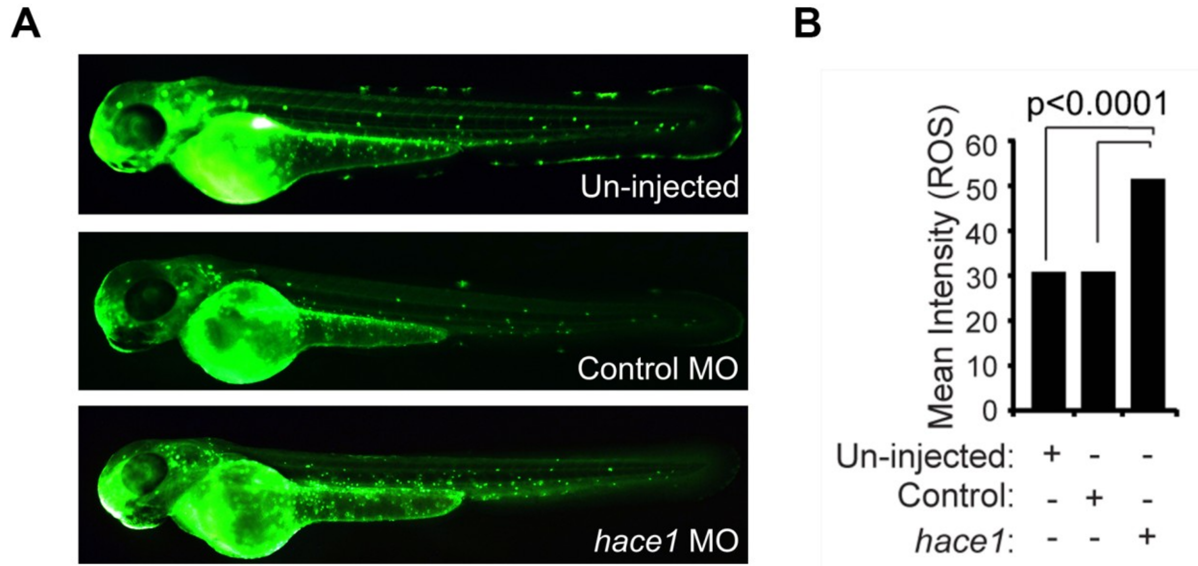


Figure 3.1.1. *hace1* knockdown in zebrafish embryos results in elevated levels of ROS. (A) *Casper* zebrafish *hace1* morphants at 3 dpf were stained for H_2O_2 using pentafluorobenzenesulfonyl fluorescein. **(B)** FACS analysis was used to quantify H_2O_2-induced fluorescence. Results from analysis of 30-40 embryos for each construct and for un-injected embryos were quantified by GraphPad software and displayed in columns as Linear Mean (Intensity) of fluorescence. $p<0.0001$. (see **Appendix A** for representative FACS histograms).

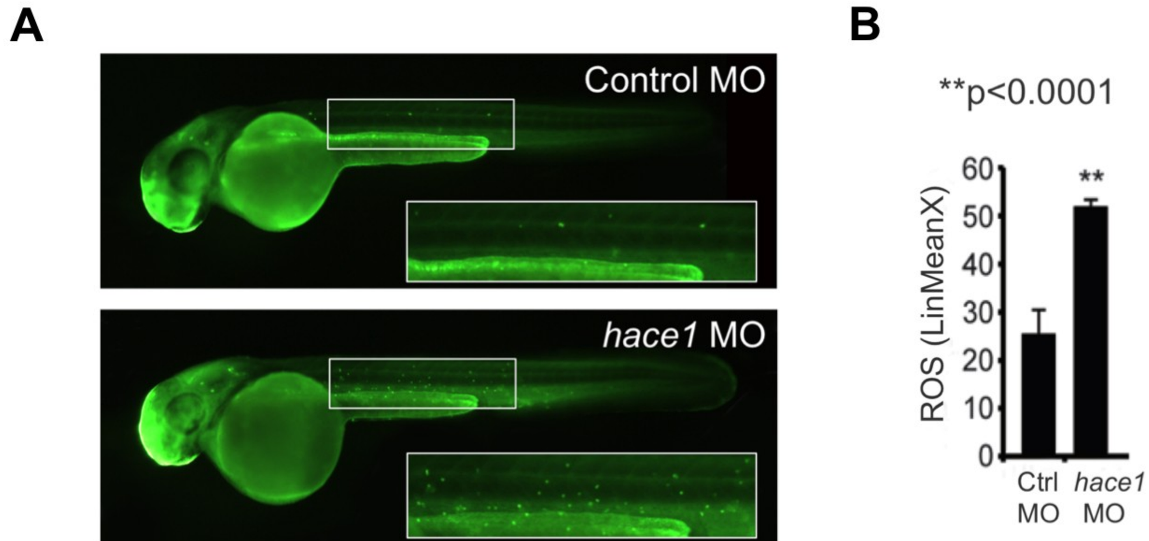


Figure 3.1.2. ***hace1* morphant zebrafish display elevated levels of ROS. (A)** Lateral views of 48 hpf *casper* zebrafish stained for H_2O_2 using pentafluorobenzenesulfonyl fluorescein. Boxes represent a magnified view of the area of the embryo indicated on each panel and demonstrate a higher population of ROS in *hace1* morphant group compared to embryos injected with control morpholino. (**B)** FACS analysis was used to quantify H_2O_2-induced fluorescence in (A). Results were quantified by GraphPad software and displayed in columns as Linear Mean of fluorescence. **p<0.0001

3.2 *hace1* regulates the ROS production via NADPH oxidases

The zebrafish genome encodes Nox1, Nox2, Nox4, Nox5 and a single isoform of Duox (Niethammer et al., 2009). We hypothesized that increased ROS in the absence of *hace1* might result from the hyperactivation of NADPH oxidases. To test for a role of any of the Nox enzyme in generating ROS in the absence of *hace1*, I treated *hace1* morphants and control embryos with diphenyleneiodonium (DPI), a pan-NADPH oxidase inhibitor that has been previously reported to block H_2O_2 production in wounded zebrafish larvae (Niethammer et al., 2009). This treatment efficiently inhibits H_2O_2 production without obvious toxicity. In contrast to untreated *hace1* morphants which exhibit the highest levels of ROS, DPI treated *hace1* morphants demonstrated the lowest levels of H_2O_2 (**Figure 3.2.1A).** These data were also analyzed for ROS-induced fluorescence by FACS, in which elevated levels of ROS in the absence of *hace1* were shown to be completely rescued by DPI and restored to baseline levels (**Figure 3.2.1B** and **C)**. In addition, treatment with the NADPH oxidase inhibitor, apocynin, demonstrated a similar trend and restored the elevated ROS in *hace1* morphants to baseline levels (**Figure 3.2.2).** Treatments with both DPI and apocynin demonstrated that the accumulation of ROS in *hace1* deficiency results from NADPH oxidase complexes. Therefore, HACE1 may play a role in regulation of NADPH oxidases to control the levels of ROS.

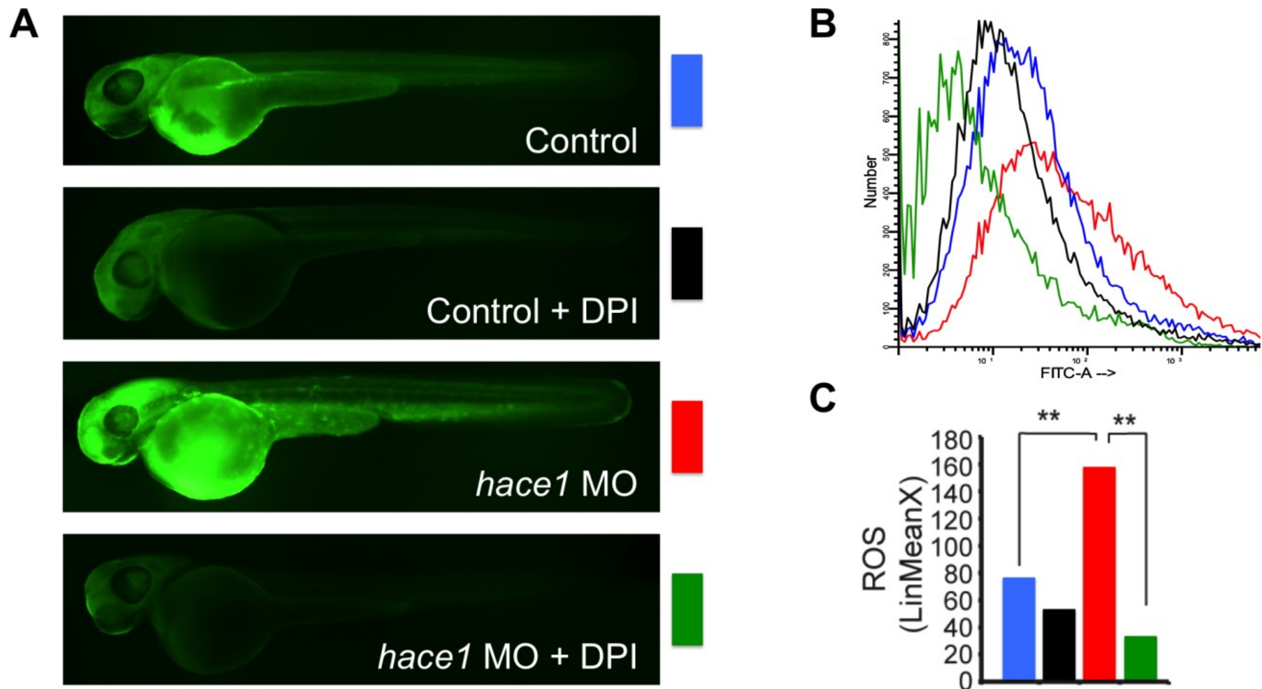


Figure 3.2.1. Elevated ROS in *hace1* morphants is regulated through the NADPH oxidases. (**A**) Representative images of 48 hpf *casper* zebrafish (lateral views) stained for H_2O_2 using pentafluorobenzenesulfonyl fluorescein. Both control and *hace1* morphants were treated +/- 50 μM of the NADPH oxidase inhibitor diphenyleneiodonium (DPI) as indicated, before being analyzed for ROS-induced fluorescence by FACS (colours correspond to the respective treatments). (**B**) & (**C**) FACS analysis was used to quantify H_2O_2-induced fluorescence in (A). **p<0.01

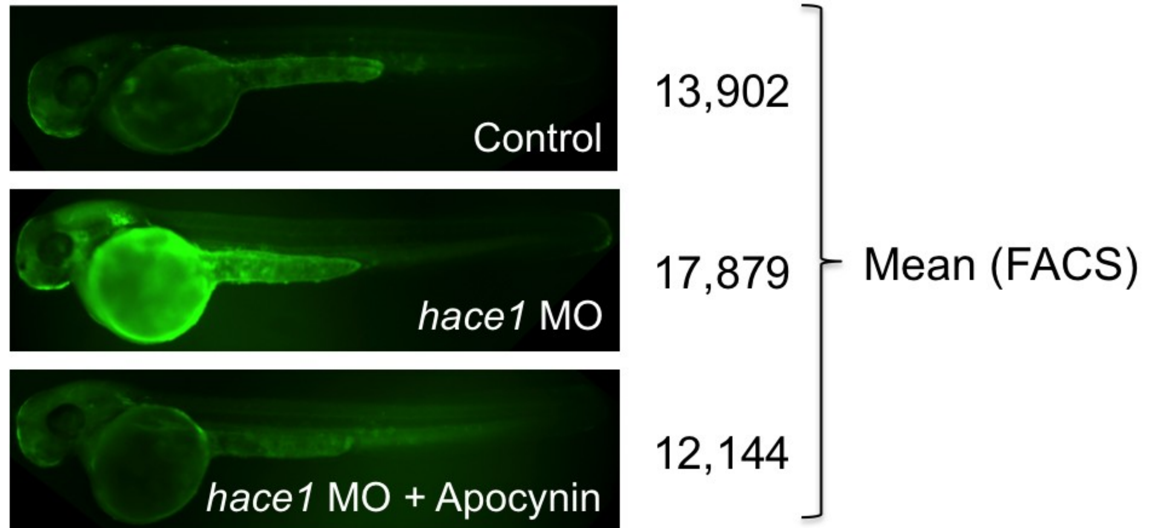


Figure 3.2.2. Elevated ROS in *hace1* morphants originates from NADPH oxidases. Representative images of 48 hpf *casper* zebrafish control and *hace1* morphants (lateral views) stained for ROS using pentafluorobenzenesulfonyl fluorescein after 24 h incubation with 200 μM apocynin as indicated. The numbers on right demonstrate the mean fluorescence intensity after FACS quantification.

As described in the **section 1.4.1**, the NADPH oxidase holoenzyme consists of different subunits and cofactors such as the transmembrane $p22^{phox}$ protein, $p47^{phox}$, $p67^{phox}$, NOXA1 and NOXO1 (Bedard & Krause, 2007). Here, Rac1 GTPase plays a critical role in regulation of this process by binding to NOXA1 within the complex, which is required for activation of Nox1, 2 and 3-containing NADPH oxidase enzymes (Bedard & Krause, 2007). To determine the possible correlation between hace1 and rac1, I used *rac1* morpholino to knock down *rac1* in zebrafish embryos (+/- *hace1* morpholino), as well as using chemical inhibitors of Rac1. Either genetic or chemical inhibition of *rac1* resulted in significant toxicity during embryonic development, and did not allow us to further investigate this hypothesis *in vivo* (please see **Figure 3.8.2** for abnormalities following *rac1* inhibition). *In vitro* studies from Dr. Sorensen's laboratory demonstrated equivalent protein levels of known NADPH oxidase subunits in both *Hace1*$^{-/-}$ and *Hace1*$^{+/+}$ MEFs, with the exception of Rac1, which was markedly elevated in *Hace1*$^{-/-}$ MEFs (Daugaard et al., 2013). Indeed, Rac1 knockdown using independent siRNAs significantly reduced ROS in *Hace1*$^{-/-}$ MEFs (Daugaard et al., 2013). This suggests that the elevated ROS in Hace1-deficient cells originates from Rac1-dependent NADPH oxidases. In keeping with these data, morpholino inhibition of *hace1* and *nox1* or *nox2*, the only NADPH oxidase components that are rac1-dependent in zebrafish, resulted in reduced ROS levels in these embryos (please see **Figure 3.9.1**). Taken together, these results suggest that HACE1 regulation of ROS is highly conserved. Therefore, HACE1 negatively regulates NADPH oxidases in ROS production by controlling the Rac1-dependent components of this complex.

3.3 *hace1* protects against DNA damage

Excessive production of ROS, leading to oxidative stress, enhances cell proliferation, DNA damage, and acquisition of genetic mutations that contribute to tumour progression (Trachootham et al., 2009). We hypothesized that based on its role in ROS regulation, HACE1, as a tumour suppressor, may also protect against DNA damage. To determine a possible role of *hace1* in mediating the resistance to DNA damage in zebrafish, I studied the survival rate of different groups of embryos following irradiation. In order to induce DNA damage and enhance ROS production, I irradiated the 24 hpf *hace1*morphants and control embryos with 16 Gy of radiation for 3.5 min (Berghmans et al., 2005). Interestingly, at 24 h post irradiation, the *hace1* morphant embryos demonstrated the lowest survival rate compared with embryos injected with the control morpholino (**Figure 3.3.1A**). To further study the role of *hace1* in protecting against DNA damage, I looked at the levels of histone H2AX phosphorylation (γH2AX), an established readout of the genotoxic stress response (Ciccia & Elledge, 2010). *hace1* morphants showed a striking increase in H2AX phosphorylation under basal conditions (no radiation) compared with controls (**Figure 3.3.1B** and **C**). These results demonstrate the role of *hace1* tumour suppressor in regulation of DNA damage susceptibility.

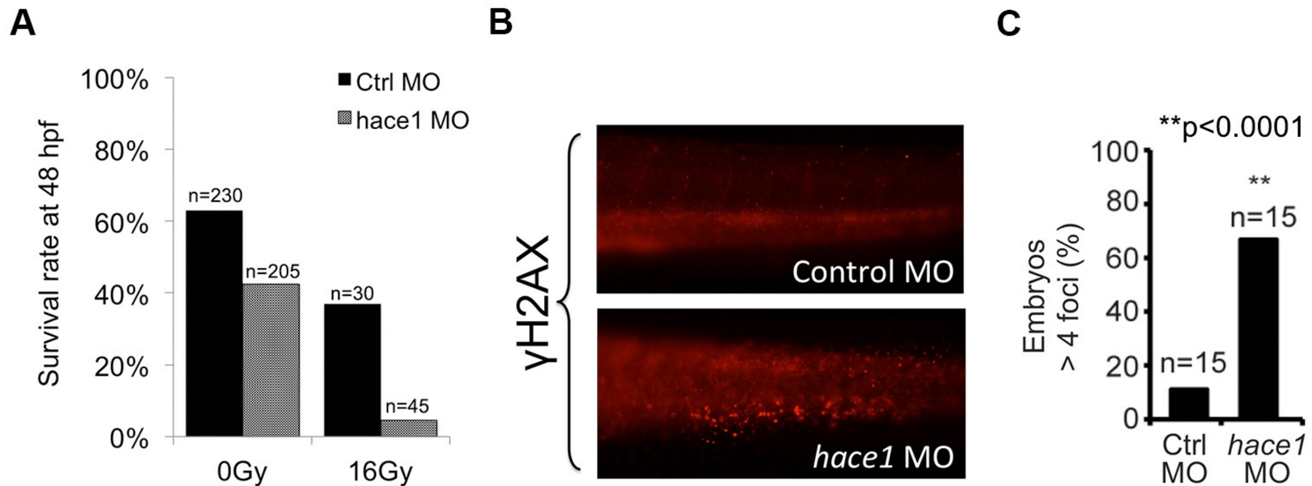


Figure 3.3.1. Increased phosphorylation of H2AX following *hace1* knockdown. (**A**) Survival rate of *hace1* morphant embryos and controls at 48 hpf, which were exposed to 16 Gy radiation for 3.5 min at 24 hpf compared to the survival rate of these groups without radiation (0 Gy) shown on left (collected from a separate experiment). (**B**) Representative images of 48 hpf *casper* zebrafish control and *hace1* morphants (lateral views) stained for phosphorylated histone H2AX (γH2AX). (**C**) The column graph displays % embryos (n=15) with more than 4 γH2AX foci per tail. **p<0.0001

3.4 *hace1* knockdown results in perturbed cardiac development

In 2004, Anglesio et al. demonstrated the high expression of the human *HACE1* mRNA in multiple tissues including brain and kidney, with the strongest expression in the heart (Anglesio et al., 2004). We also observed strong expression in the hearts of zebrafish embryos using double *in situ* hybridization with probes to the *hace1* HECT domain and also to the cardiac-specific *myosin, light polypeptide 7, regulatory* (*myl7*) (Daugaard et al., 2013) (**Figure 1.6.2**). Double fluorescence whole mount *in situ* hybridization (double WISH) similarly demonstrated co-localization of *hace1* and *myl7* expression in the embryonic zebrafish heart at 48 hpf (McDonald, 2011). Injection of a *hace1* morpholino into AB embryos revealed abnormal cardiac development with evidence of a tubular-appearing heart by WISH (**Figure 3.4.1A**). These morphant embryos demonstrated a significant increase in the number of abnormal hearts with a perturbed development compared to controls. Transgenic zebrafish lines that exhibit fluorescent labeling of different organs allow direct visualization of organogenesis. I employed the *myl7::eGFP* transgenic line (provided by Dr. Ian C. Scott, Hospital for Sick Children and University of Toronto), which expresses green fluorescent protein (GFP) under the *myosin, light polypeptide 7, regulatory* promoter exclusively in the myocardium, to more easily visualize the heart morphology of these embryos. *hace1* knockdown embryos at 48 hpf resulted in a linear cardiac structure and abnormal heart tube assembly rather than forming an S-shaped structure as a result of proper cardiac looping (**Figure 3.4.1B**).

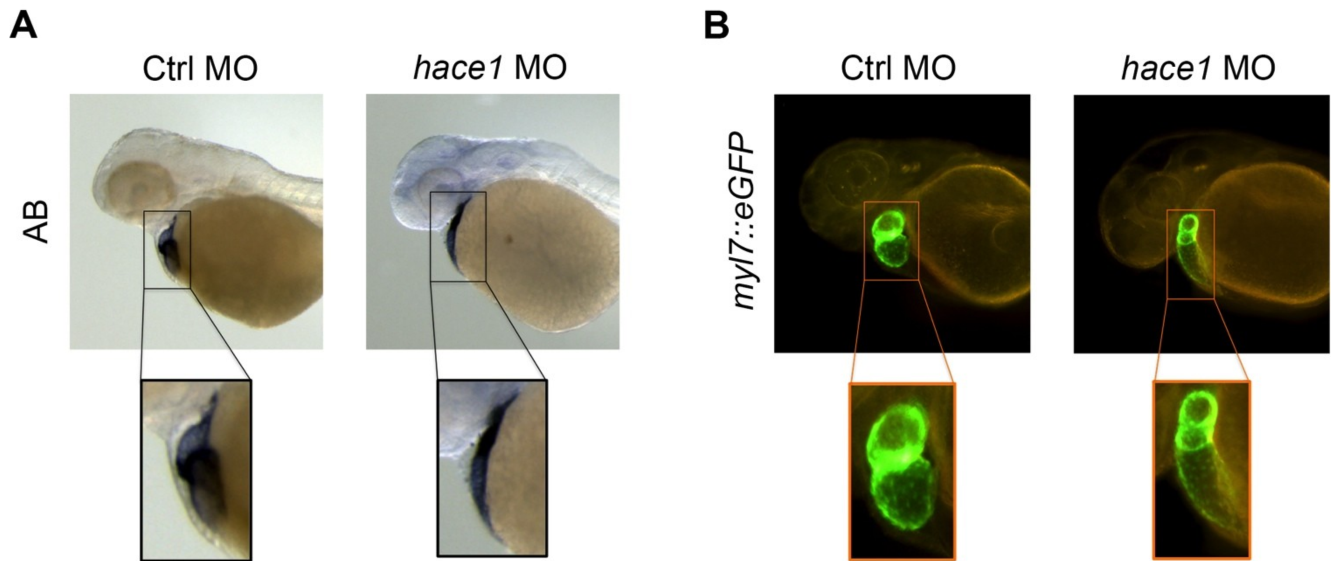


Figure 3.4.1. *hace1* knockdown results in a cardiac looping defect in zebrafish embryos. (**A**) WISH of AB control and *hace1* morphant embryos at 48 hpf using the *myl7* probe with magnified views show in the boxes as indicated; lateral views (WISH experiments performed by Lindsay McDonald). (**B**) Fluorescence imaging of *Tg*(*myl7::eGFP)* morphant embryos at 48 hpf; lateral views. The cardiac phenotype was observed in approximately 50% of each clutch of *hace1* morphant embryos (n=90-130). p=0.0007

3.5 *In situ* hybridization of *hace1* morphants using heart specific markers demonstrates abnormal patterns of expression

To better delineate the defects in morphogenesis of the cardiac chambers in the absence of *hace1*, I performed WISH at 48 hpf using molecular markers for *atrial myosin heavy chain* (*amhc*) and *ventricular myosin heavy chain* (*vmhc*). The simple architecture of the heart at this developmental stage facilitates the analysis of differences between normal and aberrant phenotypes. When heart looping is completed by 48 hpf, the *amhc*-expressing atrium and the *vmhc*-expressing ventricle become morphologically distinct (Berdougo et al., 2003). I confirmed by WISH for both *amhc* and *vmhc*, the previously observed abnormal structures in the heart (**Figure 3.5.1**). The most significant changes in *hace1* morphants were observed in ventricular rather than atrial structures (**Figure 3.5.1A** and **B**). The ventricles of the *hace1* morphant fish appeared longer and thinner than in controls (**Figure 3.5.1B**). Natriuretic peptide precursor A (*nppa*) marks fish myocardium and is normally absent at the atrioventricular (AV) junction (Takeuchi et al., 2011). Using a probe for *nppa*, I also found that the formation of the AV boundary was lost or disrupted in *hace1* morphants versus standard control morphant embryos (**Figure 3.5.1C**).

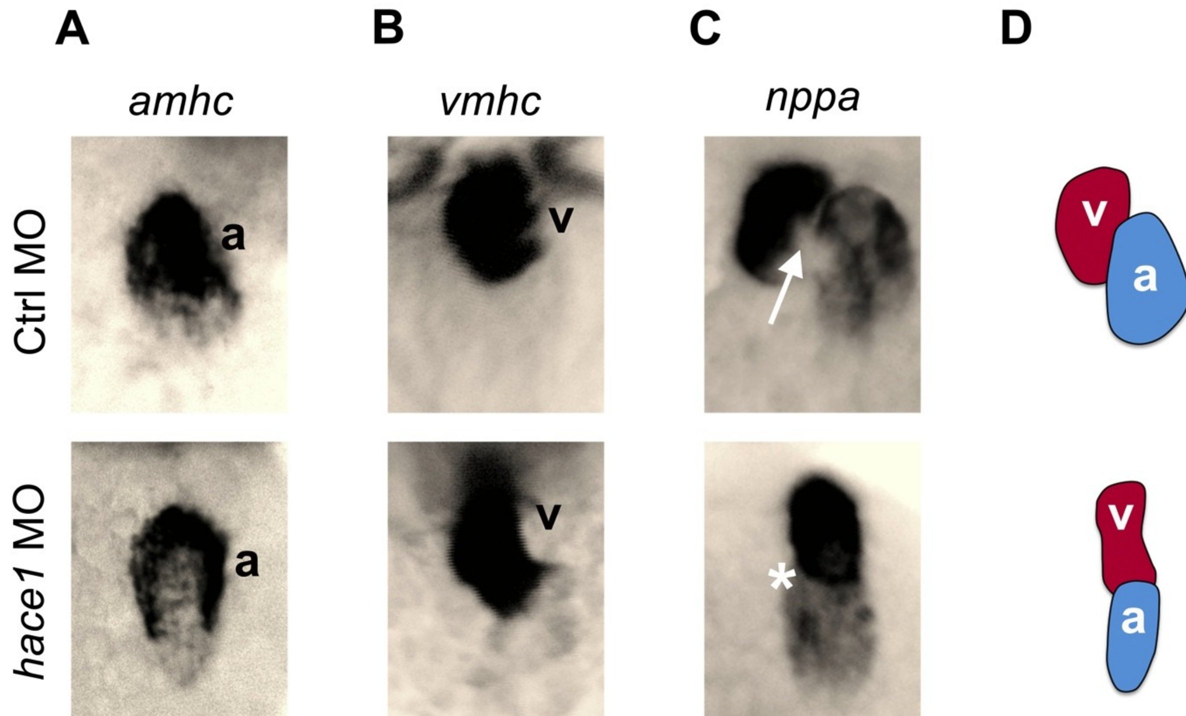


Figure 3.5.1. *hace1* knockdown results in more significant ventricular than atrial defects. Ventral views from WISH of *hace1* morphant and control embryos using *amhc* (**A**), *vmhc* (**B**) and *nppa* (**C**) probes. (**D**) Schematic diagram of cardiac structures in control or *hace1* morphants. *nppa* is expressed in both the atrium and ventricle, distinguishing the atrioventricular (AV) junction following WISH, as indicated by the white arrow in the control embryos in (C). By contrast, the AV junction is not clearly delineated in *hace1* morphants with the straight heart phenotype (as indicated by *).

3.6 *hace1* knockdown results in cardiac looping defects and bradycardia in zebrafish embryos

Tg(myl7::eGFP) 48 hpf *hace1* morphants demonstrated a looping defect resulting in two distinct cardiac phenotypes, including straight tubular hearts and "inverted" hearts, in which the atrium and ventricle are on opposite sides of their normal orientation with the ventricle positioned to the left of the atrium (**Figure 3.6.1A** and **B**). In addition to these structural abnormalities, *hace1* loss resulted in bradycardia that was most pronounced in morphant embryos with the straight heart phenotype (**Figure 3.6.2**; note that heart rate data for the inverted phenotype was not included in this figure due to the small group size compared to the other two major phenotypes). Surprisingly, heart rates in structurally normal appearing hearts of *hace1* morphants were significantly lower than in control morphant embryos, suggesting that the observed bradycardia in the absence of *hace1* is independent of defects in cardiac structure (**Figure 3.6.2**). Zebrafish and human HACE1 share 74.7% DNA homology and 88.9% protein identity (Daugaard et al., 2013). To show that the effects of the morpholino are specific, I performed a rescue experiment, using human *HACE1* mRNA. Co-injection of *hace1* morpholino with human *HACE1* mRNA successfully rescued both the structural cardiac phenotypes as well as the bradycardia, confirming the specificity of *hace1* loss as the etiology of these anomalies (**Figures 3.6.1C** and **3.6.2**).

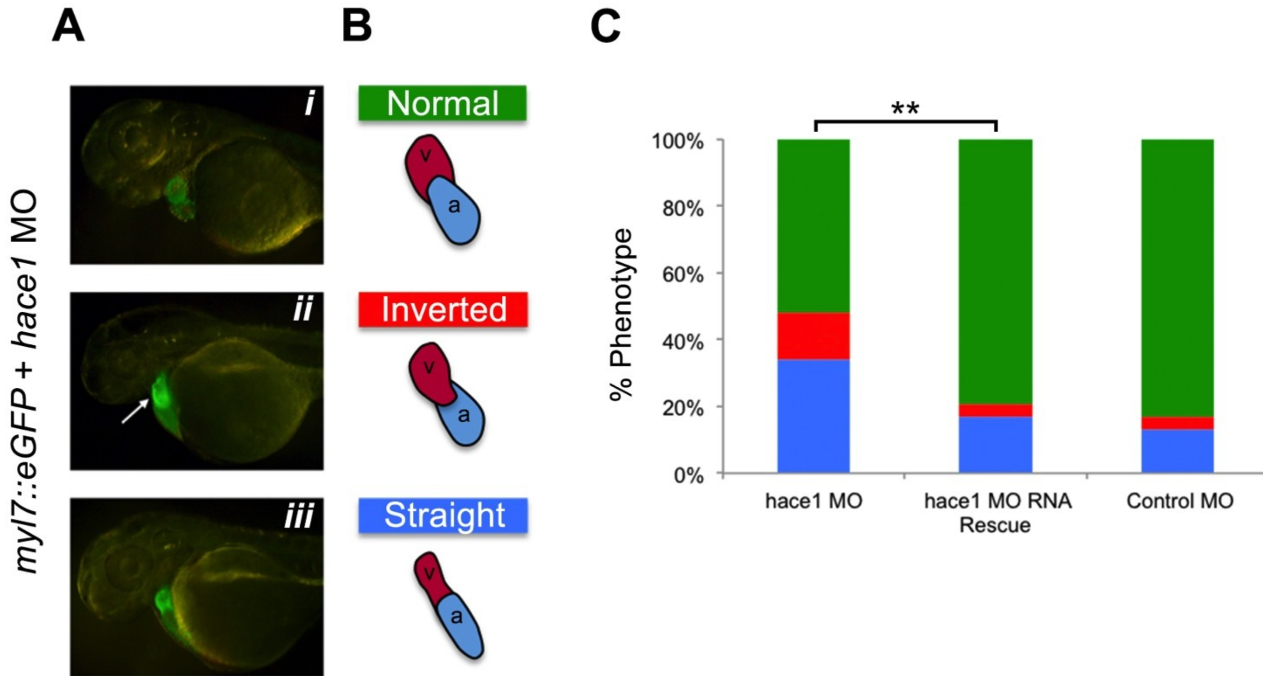


Figure 3.6.1. ***hace1*** **morphant embryos display specific cardiac phenotypes that can be rescued by human** ***HACE1*** **mRNA.** (**A**) The looping defect that results from *hace1* knockdown results in one of two abnormal cardiac phenotypes -either misalignment of the atrium and the ventricle (the ventricle is located on the left side (white arrow), "inverted") in (***ii***) or a tubular heart ("straight') in (***iii***). (**B**) Diagrammatic representation of each phenotype is included adjacent to a fluorescent image of a *Tg(myl7::eGFP) hace1* morphant embryo with the associated phenotype. (**C**) Bar graphs showing the percent of each of the representative anatomic phenotypes shown in panel (A) and (B) (colours correspond to the respective phenotype and n=90-130 embryos for each group; lateral views at 48 hpf; **p<0.0001).

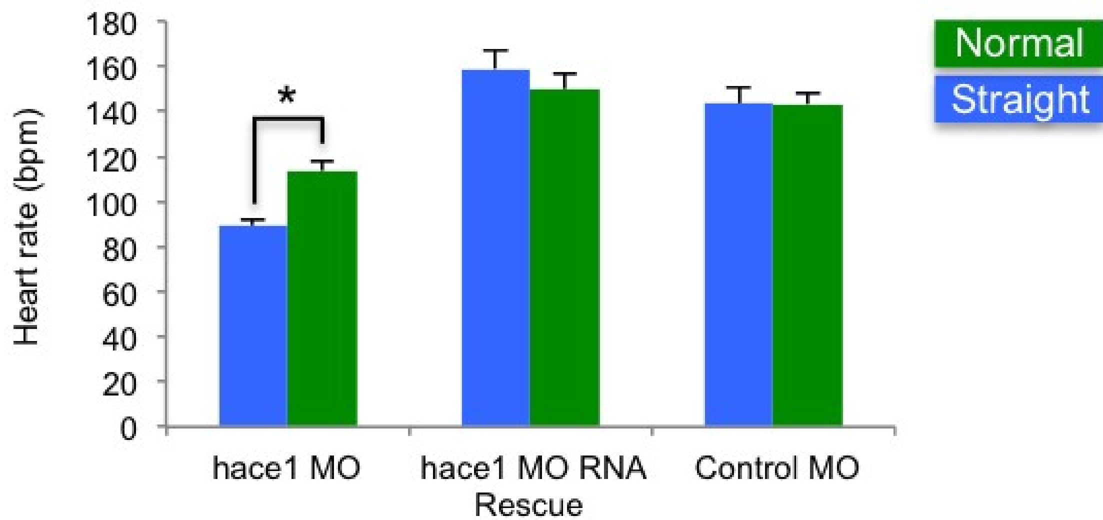


Figure 3.6.2. ***hace1*** **knockdown results in bradycardia that can be rescued by human** ***HACE1*** **mRNA.** Bar graphs showing the heart rate in *hace1* and control morpholino-injected embryos under the conditions listed on top right (n=30-40 embryos for each group; * denotes a significant difference between the two groups of *hace1* MO, which also differ significantly with all other groups included in this figure at *$p<0.05$).

3.7 Cardiomyocytes demonstrate elevated levels of ROS in *hace1* morphant zebrafish

Oxidative stress has been reported to play a crucial role in various types of cardiovascular diseases, and antioxidant therapy may prove beneficial for treatment of these problems (Dhalla et al., 2000). For instance, treatment of mice *in vivo* with mitoTEMPO, a mitochondria-targeted antioxidant, demonstrated therapeutic benefits for hypertension (Dikalova et al., 2010). In **section 3.1**, I demonstrated elevated levels of ROS in the whole zebrafish embryo following *hace1* knockdown. Here, to investigate if ROS is increased specifically in cardiac cells of *hace1* morphants, I monitored the production of H_2O_2 in the heart using FACS to count the number of cells positive for the cell-permeable ROS probe, 2′, 7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is cleaved and trapped inside living cells, and become oxidized by ROS to yield 2′, 7′-dichlorofluorescein (DCF) and emit strong fluorescence; therefore, it is useful for detecting H_2O_2 and other ROS as a general marker of intracellular oxidative stress (Soh, 2006). To monitor ROS specifically within the heart, DCFH-DA was used in combination with *Tg(myl7::mCherry)* embryos (provided by Dr. Ian C. Scott, Hospital for Sick Children and University of Toronto), which expresses mCherry under the *myl7* promoter exclusively in the myocardium. Both *Tg*(*myl7::mCherry*) and AB embryos injected with control morpholino demonstrated structurally normal hearts as well as low levels of ROS in the whole organism and in the heart at 48 hpf (**Figure 3.7.1A** and **C**). In contrast, *hace1* morphants exhibited a cardiac looping phenotype and increased accumulation of ROS throughout the entire embryo and in particular, a significant increase of ROS within cardiac structures (**Figure 3.7.1B** and **D**). I next sorted the

myl7::mCherry cardiac myocytes from both control and *hace1* morphants and quantified the number of H_2O_2-induced fluorescent cells in each population by FACS. An increase was also found in the number of ROS positive cardiac cells amongst *hace1* deficient cells (**Figure 3.7.2**), which is in keeping with my previous findings that *hace1* loss leads to increased ROS.

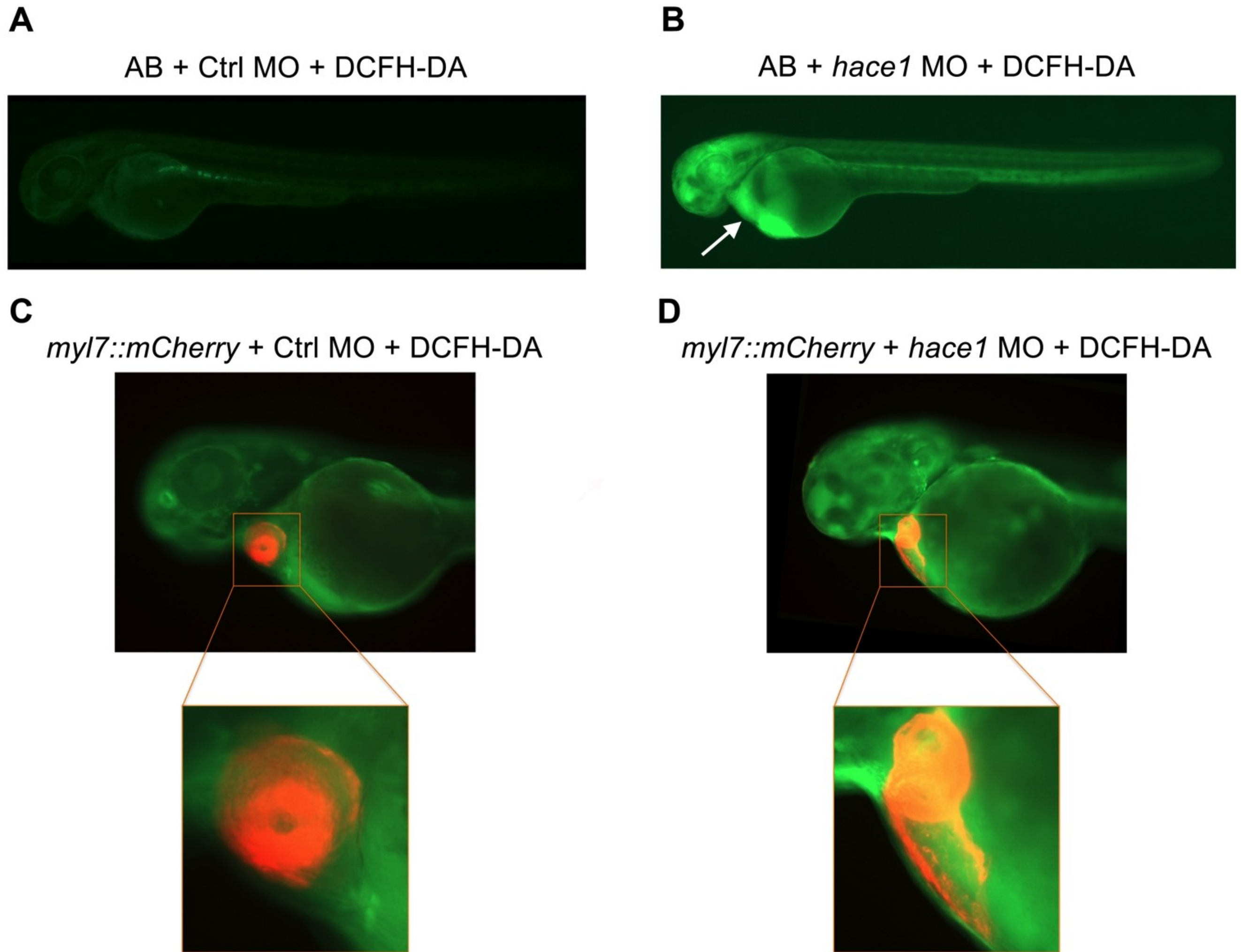


Figure 3.7.1. ROS levels are elevated throughout the embryo and in particular in the heart following *hace1* knockdown. H_2O_2 is labeled by green fluorescence with 2',7'-dichlorofluorescein diacetate (DCFH-DA). (**A**) AB embryos injected with control morpholino demonstrate baseline levels of H_2O_2 in the whole organism. (**B**) AB embryo following *hace1* knockdown exhibits elevated levels of ROS throughout the embryo, in particular in cardiac structures (indicated by the white arrow). (**C**) *Tg(myl7::mCherry)* embryo injected with control morpholino demonstrates a normal two chamber heart (zoom of heart underneath) in red with baseline levels of H_2O_2. (**D**) *Tg(myl7::mCherry)*

embryo injected with *hace1* morpholino demonstrates a cardiac looping phenotype. Boxes contain magnified images of the heart, demonstrating the “straight” phenotype and accumulation of ROS in the heart area. (Lateral views of embryos at 48 hpf)

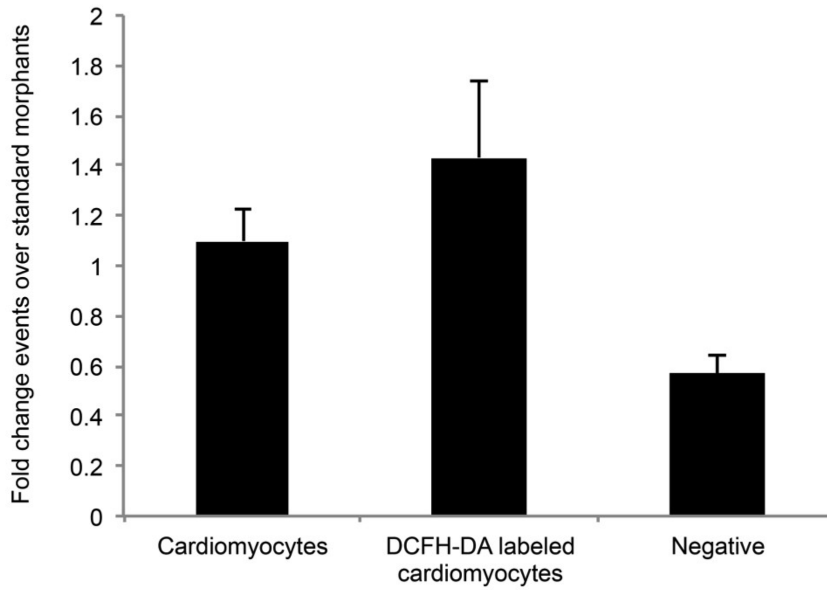


Figure 3.7.2. Elevated levels of ROS positive cardiomyocytes in the hearts of *hace1* morphant embryos. FACS analysis of DCFH-DA stained *Tg (myl7::mCherry) hace1* and control morphants. Cardiomyocytes labeled with DCFH-DA positive cells were sorted as number of events out of 200,000 for both groups. Values are plotted as fold change in positive events in *hace1* deficient cells compared to controls (n = 2, +SEM).

3.8 *hace1* knockdown results in *rac1* overexpression in the whole embryo and specifically in the heart

Rac1 has been identified to play a critical role in the cardiovascular system. Studies have demonstrated different roles of Rac1, including an essential role in proliferation and migration of vascular smooth muscle cell (VSMC) as well as a role in cardiomyocyte hypertrophy (Hordijk, 2006; Lezoualc'h et al., 2008; Sawada et al., 2010). To investigate the levels of *rac1* in zebrafish, I performed WISH for *rac1* at 48 hpf, which showed higher expression of this gene in the *hace1* morphant embryos with specific expression detected in the heart of the morphant embryos but not in control embryos (**Figure 3.8.1A** and **B**). Quantitative RT-PCR analysis similarly revealed the higher expression of *rac1* in *hace1* morphants (**Figure 3.8.1C**). Based on previous studies and our own recent findings showing Rac1 as the only NADPH oxidase subunit overexpressed in the absence of Hace1 (Daugaard et al., 2013), I first knocked down *rac1* in zebrafish embryos to see if this would rescue the previously observed elevated ROS and the subsequent cardiac phenotypes. I co-injected a *rac1* splice-site morpholino along with the *hace1* morpholino at the 1-2 cell stage and monitored the survival and cardiac development as well as the levels of H_2O_2 in heart and the whole embryo at 48 hpf. Somewhat surprisingly, I found that the double knockdown resulted in overall abnormal embryonic development, with the morphant embryos displaying more severe cardiac phenotypes with pericardial edema and accumulation of blood around the heart (**Figure 3.8.2B**). In addition to the genetic inhibition of *rac1*, I also treated *hace1* morphants with NSC23766, a Rac1-GTPase small molecule inhibitor that has been identified to have specific inhibitory effects on Rac1 activity both *in vitro* and *in vivo* (Gao et al., 2004), as

well as EHT 1864, another Rac inhibitor with high affinity of binding to Rac1 (Shutes et al., 2007). Like *rac1*/*hace1* double morphants, treatment with NSC23766 or EHT 1864, demonstrated an exacerbated cardiac phenotype compared to untreated *hace1* morphants. These results are consistent with findings from previous studies indicating the essential role of Rac1 in regulation of cardiovascular development and postnatal endothelium function, in which specific endothelial Rac1 deletion resulted in lack of lamellipodia formation leading to reduced motility, hypertension and impaired angiogenesis (Fiedler, 2008; Tan et al., 2008).

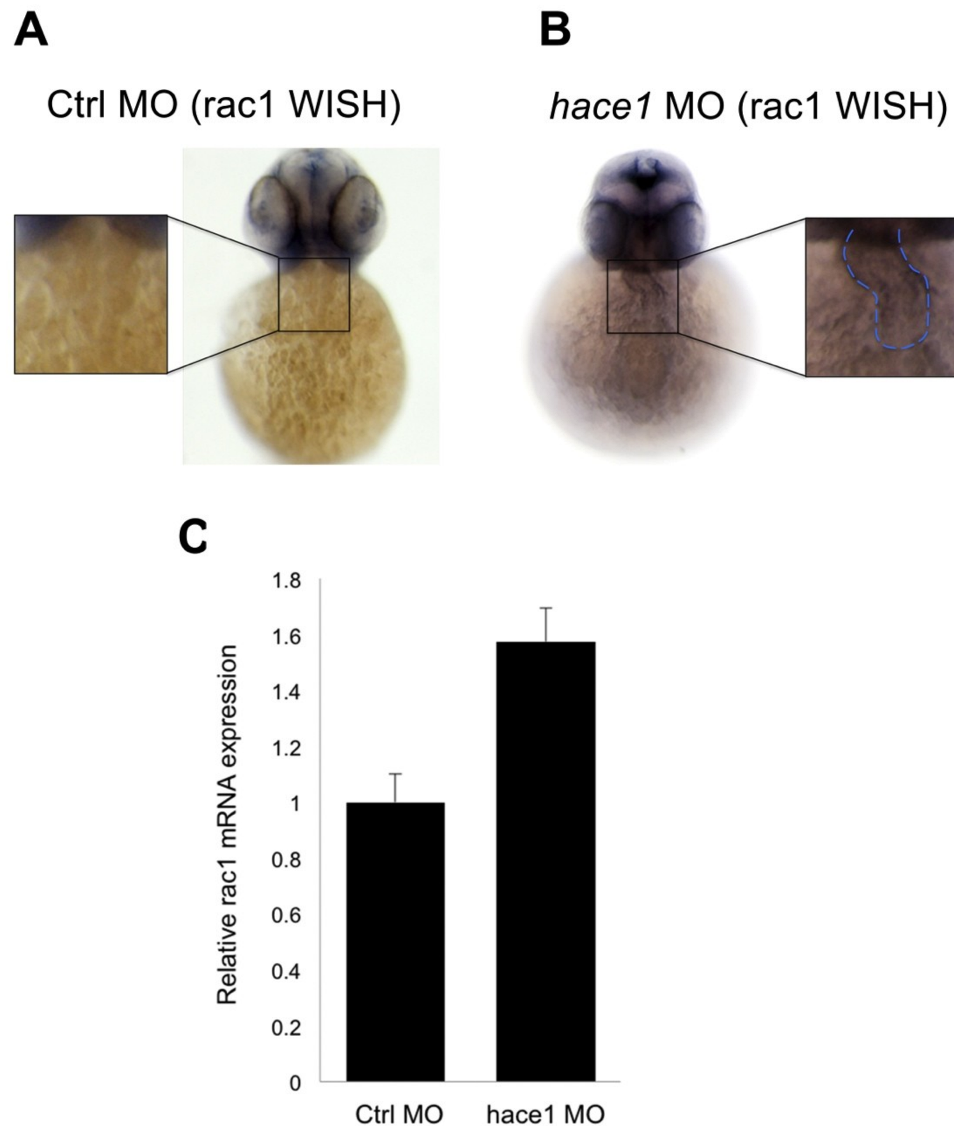
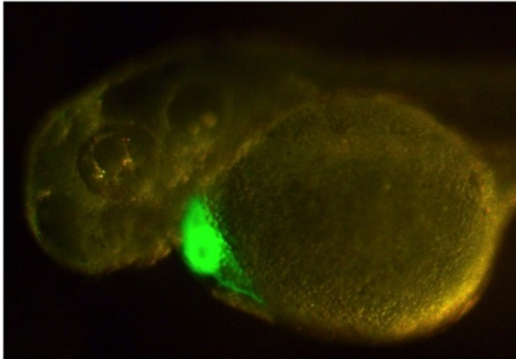


Figure 3.8.1. *In situ* hybridization and quantitative PCR demonstrate increased expression of *rac1* in the absence of *hace1*. Ventral views of *rac1* WISH in 48 hpf *casper* control (**A**) and *hace1* morphant (**B**) embryos. *hace1* morphant embryos demonstrate higher expression of *rac1* in the whole embryos and particularly in the heart (see the blue dotted line showing the magnified region of the heart in the box on the right). (**C**) Real-time PCR analysis of *rac1* expression in whole 48 hpf *hace1* morphants relative to expression in control morphants. All data were normalized to expression of *ef1a* (n = 5-6, +SEM).



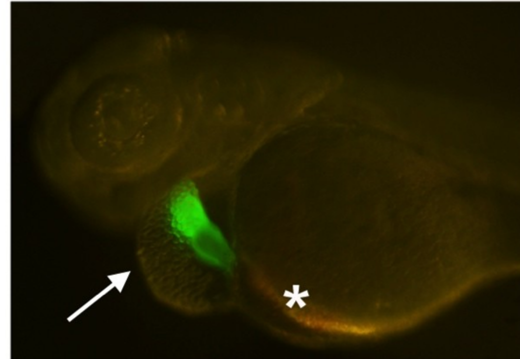


Figure 3.8.2. ***rac1*** **morpholino knockdown results in a more severe cardiac phenotype.** (**A**) *Tg(myl7::eGFP)* embryo injected with *hace1* morpholino showing the straight cardiac phenotype. (**B**) Co-injection of *hace1* morpholino along with a low concentration of *rac1* splice-site blocking morpholino (0.2 mM) in the *Tg(myl7::eGFP)* embryos causes a more severe cardiac phenotype with pericardial edema (white arrow) and apparent venous congestion (*); lateral views at 48 hpf.

3.9 *nox1* and *nox2* knockdown or treatment with NADPH oxidase inhibitors rescue the cardiac abnormalities and elevated ROS

Rac1 has been found to be essential for activation of NOX1, 2 and 3-containing NADPH oxidases (Hordijk, 2006). Given that the zebrafish genome encodes *nox1*, *2*, *4* and *5* (Niethammer et al., 2009), I examined the possible role of the rac1-dependent nox enzymes in augmenting ROS levels and cardiac abnormalities in *hace1* morphants. Again, I used the *Tg*(*myl7::mCherry)* embryos stained with DCFH-DA at 48 hpf to concurrently track overall embryonic development, the levels of H_2O_2 generated and most importantly, cardiac structural changes. To investigate the potential role of *nox1* and *nox2*, I injected their corresponding splice site morpholinos alone, together or in combination with *hace1* morpholino to compare phenotypes. Both *nox1* and *nox2* knockdown as well as *nox1/nox2* double knockdown reduced the overall ROS generation in the embryos and resulted in normal cardiac development in each group (**Figure 3.9.1C**, **D** and **E**). Interestingly, combined knockdown of *nox1*, *nox2* and *hace1* demonstrated the most significant reduction in the levels of ROS and a normal cardiac phenotype was maintained in these embryos (**Figure 3.9.1F**). The significant reduction in ROS levels and normal cardiac development observed in this combination knockdown reveals a direct correlation between *hace1* and Rac1-dependent NADPH oxidases. These results also support previous findings implicating Rac1 in cardiovascular development as well as serving as the direct substrate of Hace1 in controlling cardiomyocyte levels of ROS.

Given the evidence for a role of ROS in mediating the cardiac phenotypes observed, I wanted to examine potential therapeutic features of known general or specific anti-

oxidants. I applied two different treatments and monitored the effect of these drugs on the zebrafish embryonic phenotypes in the absence of *hace1*. I first treated *hace1* morphants with *N*-acetylcysteine (NAC), a well-known general ROS scavenger. NAC has been widely used to treat various diseases such as cancer, human immunodeficiency virus (HIV) infections, paracetamol (acetaminophen) poisoning and, particularly, cardiovascular diseases (Zafarullah et al., 2003). Embryos were incubated in 50 μM NAC following morpholino injection until 48 hpf. Compared to untreated morphants, NAC-treated *hace1* morphants demonstrated a 40% reduction in the number of embryos with a cardiac phenotype, supporting the hypothesis that ROS levels contribute to the perturbed cardiac development (**Figure 3.9.2**). I next examined the effects of NADPH oxidase inhibitor, apocynin, in *hace1*-deficient embryos. Apocynin is reported to inhibit Rac1-mediated actin cytoskeleton formation during cell migration (Klees et al., 2006). Similarly, treatment with apocynin significantly rescued the aberrant cardiac phenotype in the *hace1* morphant group (**Figure 3.9.3**), demonstrating the critical role of NADPH oxidase-derived ROS accumulation in previously described cardiac abnormalities.

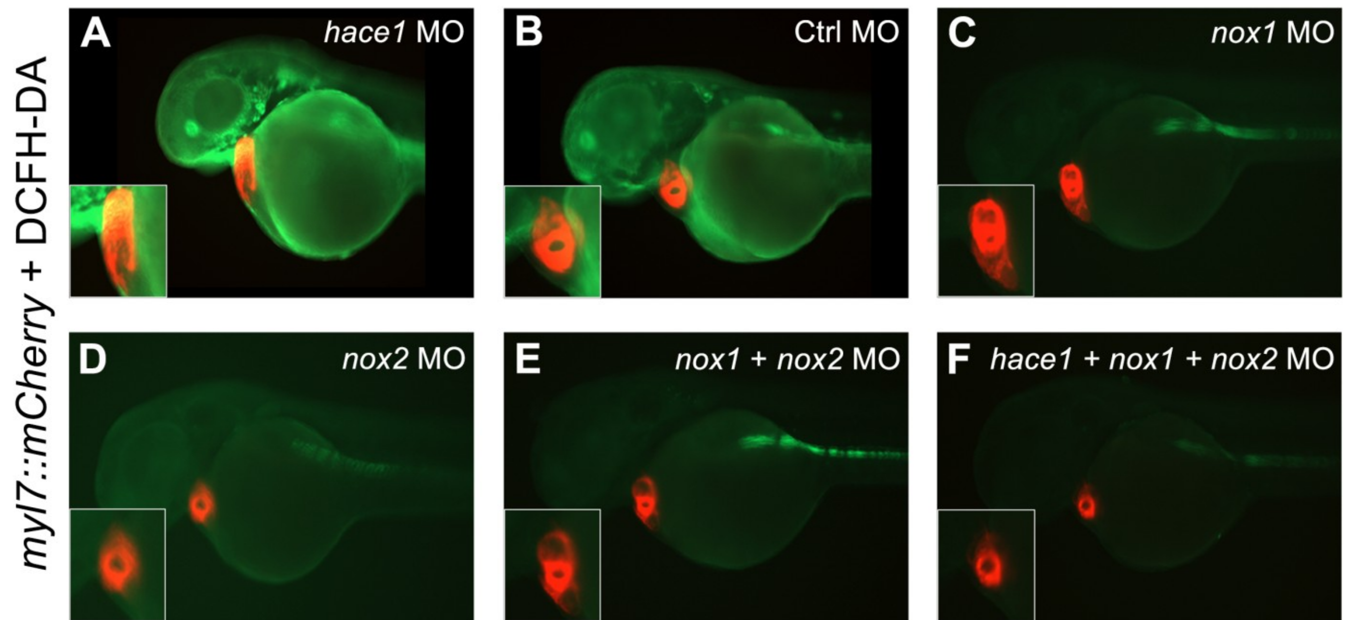


Figure 3.9.1. Knockdown with *nox1* and *nox2* morpholinos rescues the cardiac phenotype in *hace1* morphant embryos by decreasing ROS levels. Lateral views of *Tg*(*myl7::mCherry)* embryos stained with DCFH-DA (5 μM) at 48 hpf following morpholino knockdown of *hace1* (**A**), injection of control morpholino (**B**), morpholino knockdown of *nox1* (**C**), *nox2* (**D**), *nox1/nox2* (**E**) and knockdown combination of *hace1*/*nox1*/*nox2* (**F**). Co-injection of *hace1* MO + *nox1/nox2* MOs rescues the abnormal cardiac phenotypes demonstrating the lowest levels of ROS and normal cardiac structure in (F).

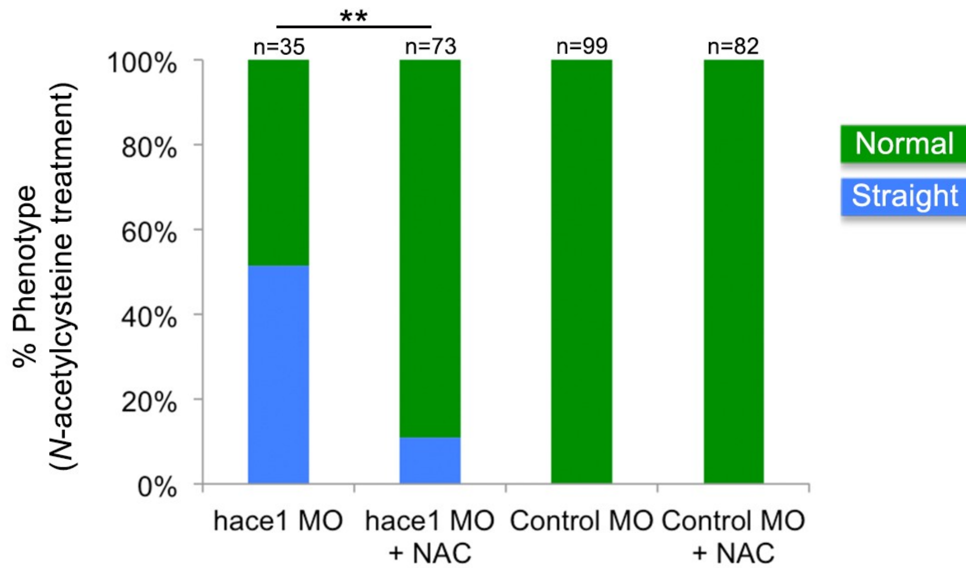


Figure 3.9.2. Treatment with the ROS scavenger, *N*-acetylcysteine, restores the normal cardiac structure. Quantification of abnormal cardiac phenotypes in *hace1* morphants following treatment with *N*-acetylcysteine (NAC). The number on top of each bar represents the total number of embryos in the group. **p<0.0001

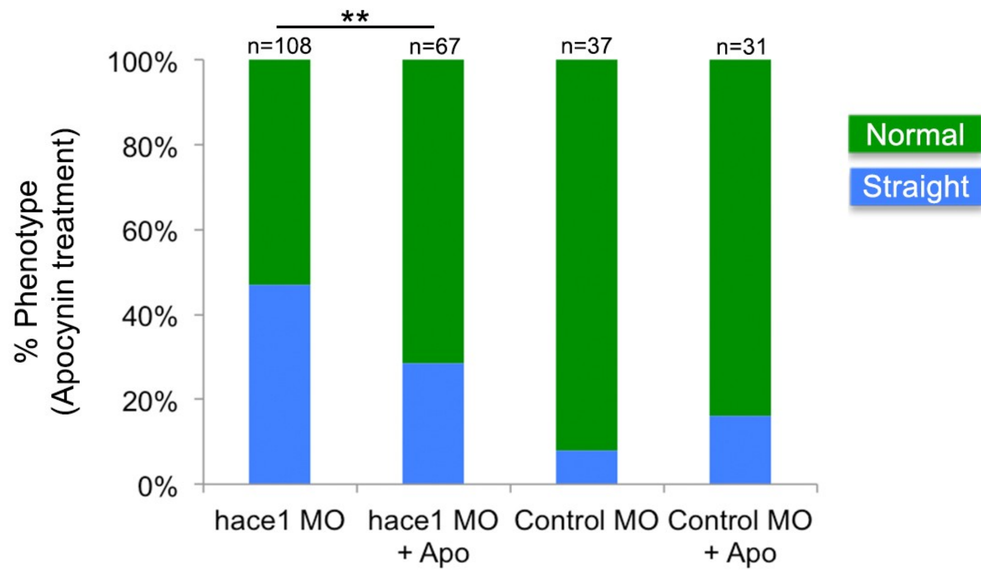


Figure 3.9.3. Treatment with apocynin, an inhibitor of NADPH oxidases, rescues the cardiac phenotype. Quantification of abnormal cardiac phenotypes in *hace1* morphants following treatment with apocynin (Apo). The number on top of each bar represents the total number of embryos in the group. **p=0.0098

CHAPTER 4: DISCUSSION

4.1 *hace1* protects against the generation of ROS and DNA damage via influencing Rac1-dependent NADPH oxidase complexes

Generation of ROS is an inevitable consequence of normal metabolism of oxygen, therefore, the balance between ROS generation and the activity of both enzymatic and non-enzymatic antioxidant pathways that may function as scavengers or reducers of ROS is essential (Shah & Channon, 2004). During zebrafish development, innate immune cells emerge as early as 15 hpf and become competent to respond to different stimuli from around 22 hpf (Bennett, 2001; Feng et al., 2010). Therefore, in early days of zebrafish embryonic development, when only innate immunity is present, ROS can be an indicator of the intensity of the innate immune response as well as other sources involved in production of these reactive molecules. High degree of genetic conservation and ease of observing *in vivo* readouts justifies use of the zebrafish for studying *HACE1* in oncogenesis and putative functional mechanisms, such as ROS. In this study, I showed that loss of *hace1* in zebrafish results in elevated levels of H_2O_2, a stable form of ROS, as well as increased phosphorylation of histone H2AX, a marker of DNA damage. The reciprocal relationship between *hace1* function and ROS levels in zebrafish suggests that *HACE1* is involved in regulation of ROS. In keeping with these results, Rotblat et al. recently demonstrated that HACE1 mediates resistance to oxidative stress (Rotblat et al., 2014). They challenged *Hace1* wild-type (WT) and knockout (KO) mouse embryonic fibroblasts (MEFs) with H_2O_2 or arsenite to induce acute oxidative stress and then measured the ability of these cells to survive in this condition. Cell death analysis

showed that *Hace1* KO MEFs were significantly more sensitive to both forms of oxidative stress compared with WT MEFs, suggesting the role of HACE1 in mediating the oxidative stress response (Rotblat et al., 2014). In particular, Rotblat et al. demonstrated that HACE1 is essential for activation of the nuclear factor erythroid 2-related factor 2 (NRF2), one of the master regulators of the cellular antioxidative stress response. The authors showed that HACE1 depletion results in reduced activity and deregulation of NRF2 in neurodegenerative disorders such as Huntington disease, where oxidative stress is an important player besides its key role in cancer (Rotblat et al., 2014). Therefore, taken together, these results demonstrate that *HACE1* is a critical regulator of ROS and mediates resistance to oxidative stress.

In addition to the mitochondrial electron transport chain, a second major source of cellular ROS is the NADPH oxidase complex (Trachootham et al., 2009). I showed that the increased levels of H_2O_2 in *hace1* morphant embryos were reduced using inhibitors of NADPH oxidase, as well as through the genetic inhibition of the rac1-dependent components of this complex. In support of this contention, staining with MitoSoxRed, which specifically measures mitochondrial ROS, was largely unchanged between *Hace1*$^{+/+}$ and *Hace1*$^{-/-}$ cells (Daugaard et al., 2013). In addition, the recently reported role of HACE1 in ubiquitylation of the Rac1 GTPase (Castillo-Lluva et al., 2013; Torrino et al., 2011), as well as the important role of Rac1 in activation of the NADPH oxidase enzymes suggest a correlation between HACE1 and Rac1-dependent NADPH oxidase complexes for ROS regulation. Rac1 has been reported to be involved in many cellular activities, including cell motility (Doanes et al., 1998), stress signaling (Naumann et al., 1999), proliferation (Debidda et al., 2005), protein translation (Chou & Blenis, 1996) and,

most importantly, in generation of ROS (Cheng et al., 2006; Ueyama et al., 2006). During ROS generation *HACE1* plays an important role as a direct regulator of the active (GTP-bound) form of Rac1 (Daugaard et al., 2013). Despite the previously reported role of HACE1 in proteasomal degradation of Rac1, I was not able to measure the rac1 protein levels in *hace1* morphant embryos to validate this process in zebrafish (refer to **section 4.4**). However, I showed an overexpression in *rac1* RNA levels with *in situ* hybridization and RT-PCR, which might be due to a possible interaction of *hace1* with other genes in the regulation of the expression of *rac1*. Ultimately, to date, HACE1 is the only tumour suppressor that has been reported to directly control ROS generation by regulating the activity of Rac1-dependent NADPH oxidase complexes through ubiquitylation (Daugaard et al., 2013). However, Rac1 is not the only known target for ubiquitylation by HACE1. More recently, Lachance et al. reported that β2-adrenergic receptor (β2AR), a prototypical G protein-coupled receptor, in conjunction with HACE1 triggers ubiquitylation of Rab11a (a Rab GTPase) leading to its activation and regulation of β2AR recycling (Lachance et al., 2014). Therefore, it will be interesting to further investigate the potential correlation between HACE1 and other Ras-related proteins.

Zhang et al. demonstrated that low-dose ionizing radiation (IR) or urethane treatment, both of which induce DNA damage through ROS generation, dramatically enhances tumour formation in *Hace1*$^{-/-}$ mice (Zhang et al., 2007), suggesting that these cells are highly sensitive to DNA damaging agents. However, the basis of this hypersensitivity remains unknown. I demonstrated that loss of *hace1* in zebrafish results in higher expression of γH2AX, a marker of double stranded DNA breaks. However, I cannot rule out the potential role of other phosphorylated proteins involved in ROS-

induced DNA damage susceptibility. In keeping with these results, parallel studies at Dr. Sorensen's laboratory demonstrated higher phosphorylation of histone H2AX in *Hace1*$^{-/-}$ versus *Hace1*$^{+/+}$ MEFs following low-dose IR, as well as increased phosphorylation of ATM and p53 (Ser-15) following exposure of *Hace1*$^{-/-}$ MEFs to low concentrations of exogenous H_2O_2, which were all reversed by transient wild-type Hace1 re-expression or treatment with the ML171 NADPH oxidase inhibitor (Daugaard et al., 2013). In addition, the H_2O_2-induced p53 phosphorylation in *Hace1*$^{-/-}$ MEFs was blocked following Nox1 knockdown. Therefore, chronic elevated Rac1-dependent NADPH oxidase-mediated ROS might lead to increased DNA damage in Hace1-deficient cells. Furthermore, the Sorensen lab showed that the previously reported cyclin D1 regulation by HACE1 (Zhang et al., 2007) is also mediated by Rac1-dependent NADPH oxidases, as induced cyclin D1 levels in *Hace1*$^{-/-}$ was significantly reduced by ML171, apocynin, or Rac1 siRNA knockdown (Daugaard et al., 2013). Therefore, Hace1 also controls cyclin D1 expression and cell cycle progression through its ability to block activity of Rac1-dependent NADPH oxidase complexes. Collectively, these findings demonstrate that HACE1 deficiency leads to chronic elevated levels of ROS, which is associated with ROS-induced DNA damage and cyclin D1 expression. Therefore, these findings provide insight into the underlying tumour suppressing mechanism of HACE1 whereby it targets complex-bound Rac1 to regulate NADPH oxidase, ROS production, cyclin D1 expression and DNA damage susceptibility (**Figure 4.1.1A** and **B**).

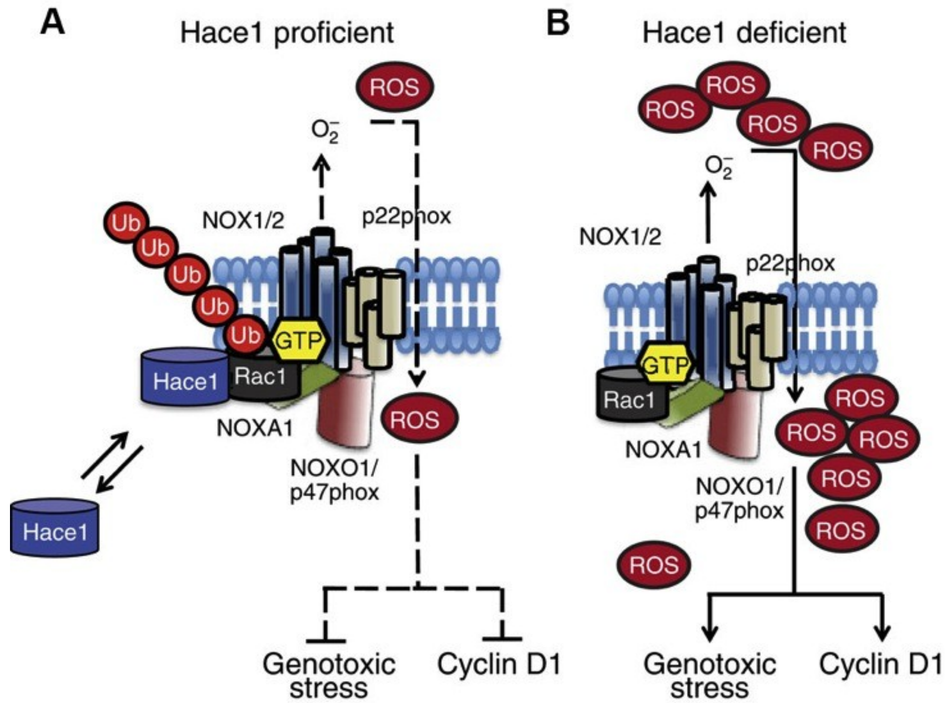


Figure 4.1.1. Hace1 controls Rac1-dependent NADPH oxidases. (**A**) Hace1 targets complex-bound Rac1 to regulate NADPH oxidase, ROS production, cyclin D1 expression and DNA damage susceptibility. (**B**) Loss of Hace1 leads to hyperactive NADPH oxidase activity, increased ROS generation, high cyclin D1 expression and ROS-induced DNA damage. (Reproduced with permission from Dauggard et al. *Nature Communications*, 2013;4)

4.2 *hace1* regulates ROS production in a manner necessary for normal heart development

Zebrafish cardiac development undergoes a stereotypical process during the first several days of life that governs the proper orientation of the two chambers, the disruption of which can potentially mirror cardiac defects seen in humans due to defects early in development. A beating heart and a functional circulatory system form within 26 hpf; however, the two-chambered heart does not fully develop until 48 hpf, making this time frame a crucial point for studying heart development (Malone et al., 2007). Based on the strong *hace1* expression in the heart, the purpose of the current study was to investigate the role of this tumour suppressor in normal vertebrate cardiac development. *hace1* knockdown embryos at 48 hpf exhibited a linear cardiac structure along with an abnormal tube assembly rather than forming an S-shaped structure as a result of proper cardiac looping. In addition to this dominant abnormal phenotype, another phenotypic group that we termed "inverted" was also identified. In these embryos, the ventricle is malpositioned to the left of the atrium, demonstrating a completely reversed orientation and patterning of the heart. This phenotype might arise due to defects in the initial assignments of the embryonic left-right (L-R) axis (Glickman & Yelon, 2002) and the genes involved in this process such as bone morphogenic protein 4 (*bmp4*), *nodal*, or *lefty2* (Smith et al., 2011). I also found evidence of increased numbers of cardiomyocytes in the zebrafish heart in which ROS levels are highly induced following *hace1* knockdown. This observation is in keeping with the previous finding by Buggisch et al., demonstrating that treatment with low levels of H_2O_2 stimulated proliferation of cardiomyocytes derived from embryonic stem cells and neonatal mice, as well as

highlighting the role of NADPH oxidase in cardiovascular differentiation by showing reverse outcomes following treatment with DPI and apocynin (Buggisch et al., 2007). Remarkably, in the current study, co-injection of human *HACE1* mRNA, treatment with NAC as a general antioxidant, or treatment with apocynin as a NADPH oxidase-specific inhibitor rescued the abnormal cardiac structure, suggesting that *hace1* may regulate ROS production in a manner necessary for normal heart development in these fish.

4.3 Implications in the pathogenesis of human cancers and congenital heart defects

Elevated levels of ROS and downregulation of ROS scavengers promote tumorigenesis and cancer progression (Waris & Ahsan, 2006) and HACE1 appears to serve as a gatekeeper of ROS levels through the NADPH pathway (Daugaard et al., 2013). The findings from this project along with ongoing research suggest that HACE1 protects against detrimental effects of elevated ROS levels by downregulation of Rac1. HACE1 ubiquitylates Rac1, which in turn controls cellular ROS levels (Castillo-Lluva et al., 2013; Daugaard et al., 2013; Torrino et al., 2011) as well as important processes such as cell motility, adhesion, and the cell cycle progression, all of which are closely tied to cancer development (Ellenbroek & Collard, 2007). Overexpression of Rac1 is associated with metastasis and poor clinical outcome (Schnelzer et al., 2000; Wang et al., 2009), while inhibition of Rac1 significantly reduces cell migration in various cancer cell types (Liu et al., 2008; Toyama et al., 2010).

Cell migration is intimately linked to cancer metastasis as well as normal development. Based on the findings in the current study, the cardiac abnormalities in zebrafish, with *hace1* temporarily silenced by morpholino, appear to be regulated by a ROS-dependent mechanism linked to rac1. Rac1 has been identified as an important source of ROS in the cardiovascular system and there has been recent interest in the use of Rac1 inhibitors for different cardiovascular diseases (Adam & Laufs, 2014). I demonstrated that *rac1* knockdown in a *hace1* deficient condition results in a more severe cardiac phenotype (see **Figure 3.8.2B**) following either genetic or chemical inhibition. Although this co-knockdown may have been capable of reducing the increased ROS due to elevated rac1 level in the absence of hace1 (as demonstrated in *Hace1*$^{-/-}$ MEFs in Daugaard et al), global knockdown of the *rac1 in vivo* resulted in more severe abnormalities in the hearts of *hace1* morphant zebrafish embryos. However, I showed that morpholino inhibition of *hace1* and rac1-dependent NADPH oxidases in zebrafish (*nox1*/*nox2*) resulted in reduced ROS levels and a normal cardiac development was maintained in these embryos (**Figure 3.9.1C**, **D, E** and **F**) suggesting that preventing ROS overexpression by inhibiting the mentioned rac1-dependent components restores the normal cardiac development in the absence of *hace1*. Therefore, given our findings and the importance of Rac1 in cardiovascular development and being a critical regulator of ROS production in this system, caution should be exercised in knocking down or employing inhibitors of Rac1.

The findings in the current study may inform human cardiac development, of which many similarities have been found to zebrafish cardiac embryogenesis. For example, the zebrafish heart also develops functional β-adrenergic and M2 muscarinic

receptors early in larval development, which are sensitive to the same pharmacological stimulation as the human cardiac receptors (Steele et al., 2011). The data in the current study reveal a link between HACE1, ROS and normal cardiac development for the first time. These new findings may have implications in the pathogenesis of human congenital heart disease and may provide an explanation to the higher frequency of congenital heart defects in children with certain malignancies like Wilms tumour, in which HACE1 was initially identified (Anglesio et al., 2004; Fernandez et al., 2001). Ultimately, if HACE1 is similarly found to influence human cardiac embryogenesis, germline expression levels could serve as a new biomarker for screening, with elevated ROS levels being a potentially modifiable factor in utero.

4.4 Limitations

Despite many advantages to using zebrafish in various areas of research such as developmental biology and cancer, there remain some limitations to this model system. The presence of the yolk sac at early stages of embryonic development may lead to technical difficulties in interpreting different assays due to the intense absorbance of dyes and levels of autofluorescence emitted by yolk. For instance, we had hypothesized that ROS-induced DNA damage in the absence of hace1 might cause premature senescence in cardiomyocytes contributing to demonstrated cardiac phenotypes, but high absorbance of senescence-associated β-galactosidase (SA-β-gal) staining by the yolk sac hindered the ability to evaluate this phenotype on account of the close anatomic proximity of the yolk and cardiac structures. Knowing that the majority of total protein content in early embryos is in the yolk, removing the yolk prior to performing protein extraction may

reduce the signal intensity facilitating accurate quantification of protein levels by Western blotting. In addition, there remain relatively few antibodies specifically available for zebrafish compared to other model systems such as mice. Antibodies raised in other species often show low cross-reactivity with the corresponding zebrafish antigen. For instance, we were not able to detect the levels of DNA damage in *hace1* morphant embryos using a polyclonal anti-γH2AX antibody from rabbit or evaluate the levels of zebrafish rac1.

Morpholinos are extremely valuable in evaluating defects and abnormalities resulting from loss of gene function early in development. However, due to the transient nature of morpholinos and the absence of adaptive immunity during the early stages of embryonic development, examining the crosstalk between innate and adaptive immune responses to different stimuli such as oxidative stress is limited using this approach. More permanent long term *hace1* knockout fish are needed for these types of studies. Permanent gene knockouts have historically been an important limitation in the zebrafish model due to challenges in initiating homologous recombination in this species compared with mice (Lieschke & Currie, 2007). More recently, zinc finger nucleases (ZFNs) have been employed for gene inactivation in zebrafish. ZFNs create double-stranded DNA breaks, enabling error-prone non-homologous end joining and introduction of an insertion or deletion mutation at the target site (Foley et al., 2009). Customized ZFNs targeting zebrafish *hace1* were generated in collaboration with the Context-dependent assembly (CoDA) consortium (Sander et al., 2011) and used to generate *hace1* knockout zebrafish. However, following multiple approaches commonly used for screening mutations, including high-resolution melting analysis (HRMA), no obvious mutation was

identified in embryos injected with *hace1* customized ZFN encoding RNAs (see **Future directions**).

4.5 Future directions

In my thesis work, I demonstrated that zebrafish *hace1* expression negatively regulates NADPH oxidase-dependent ROS generation and that this function is required for normal cardiac development. I found that rac1 and NADPH oxidase-dependent ROS generation are both elevated in the absence of hace1; however, while reducing the expression of rac1-dependent NADPH oxidase isoforms rescued the phenotype, targeting rac1 directly actually worsened the phenotype. Considering that the elevated ROS is rescued by siRNA knockdown of Rac1 in cell lines, further studies on cardiac development in zebrafish performing a tissue-specific knockdown is indicated. Tissue-specific knockdown has been reported to be feasible in zebrafish using RNA polymerase II promoter-driven microRNA-based shRNA knockdowns (Dong et al., 2009). Therefore, a heart-specific knockdown of rac1 (+/- *hace1* knockdown) rather than ubiquitous loss of function may provide additional evidence on the role of hace1 in normal cardiac development. I also demonstrated that there was an increased number of cardiomyocytes labeled with ROS in *hace1* morphant embryos. Future studies can more specifically analyze cardiac myocyte hyperplasia or hypertrophy in the absence of hace1 using confocal microscopy of transgenic fish with GFP-labeled hearts, which may also provide additional insights into the contributions of hace1 to normal cardiac development. WISH studies can be undertaken for specific genes known to be involved in proper left-right patterning of the heart such as bone morphogenic protein 4 (*bmp4*),

nodal, or *lefty2* (Smith et al., 2011) to determine potential pathways affected by *hace1* loss of function in zebrafish. Moreover, based on high expression of *hace1* in brain and kidney (see **Figure 1.6.2**; *early growth response 2* (*krox20*; brain) and *cadherin 17* (*cdh17*; kidney)), future work is required on the role of this tumour suppressor in the development of these other organs.

A transgenic zebrafish line that was developed in our lab (McDonald, 2011), harbouring a dominant-negative (DN) mutation for human *HACE1* (C876S), showed early signs of tumours starting at 11 months, but interestingly did not have a cardiac phenotype. Due to the transient nature of morpholinos and the incomplete phenotype of DN construct, permanent gene knockout is required to properly investigate the role of hace1 in cancer progression and normal development. As mentioned above, an attempt using ZFNs was unsuccessful. Transcription activator-like effector nucleases (TALENs) have been widely used in zebrafish laboratories for gene-targeted mutagenesis with fewer off-target effects than corresponding ZFNs (Clark et al., 2011). Similar to ZFNs, TALENs rely on nucleases engineered to target a specific sequence and induce DSBs in target sites (Huang et al., 2012). However, dramatic progress in this field has been made more recently by using the clustered regularly, interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system for genome editing. This system has been found to have high efficiency in the zebrafish that is much easier and more cost-efficient to generate than ZFNs or TALENs (Li et al., 2013). CRISPR/Cas is a bacterial adaptive immune system against foreign nucleic acids that uses short RNA molecules to direct the degradation of target sequences (Li et al., 2013). The CRISPR system has been adapted to employ customized guide RNAs (gRNAs) that direct site-specific DNA cleavage by

the Cas9 endonuclease (Hwang et al., 2013). With Dr. Sergey Prykhozhij's help (postdoctoral fellow in Berman laboratory), we have generated specific gRNAs targeting *hace1* and co-injected these constructs together with Cas9 mRNA into one-cell-stage zebrafish embryos. Using the T7 endonuclease I assay (developed by Dr. Keith Joung's laboratory), we found that our targeting was successful, which was very encouraging (see **Appendix B**). At the time of this writing, these fish are about two months old and by the time they reach the age of breeding (> 3 months), further screening for mutations will be done by sequencing. Founders will be crossed to AB, *myl7::eGFP* and *myl7::mCherry* fish with the *Casper* background. The *hace1*$^{-/-}$ embryos will be evaluated for ROS levels, cardiac development, gene expression +/- irradiation, perturbations in cell cycle (BrdU, phosphohistone H3), senescence levels (SA-β-gal assay), and developmental phenotypes by WISH. A proportion of these fish will be crossed to a *p53*$^{-/-}$ line that is lacking expression of these key tumour suppressor gene to potentially accelerate the development of tumours as was observed in *Hace1*$^{-/-}$ mice (Zhang et al., 2007). These fish can then be examined histologically. The precise mechanism by which hace1 operates to impact heart development remains unknown. Recently, RNA-seq technology has offered a more accurate and comprehensive approach to the sequencing of transcripts compared to microarray (Zhao et al., 2014). Based on the well-established cardiac phonotypes in the absence of hace1 in zebrafish, RNA derived from cardiac tissue can be extracted from *hace1* mutant fish expressing GFP/mCherry in cardiomyocytes isolated using FACS. These heart-specific RNAs can be used for RNA-seq analysis to detect transcripts alterations in the heart and identify other potential genes involved in these cardiac defects and pathways that link hace1 function to heart development.

Recently, approximately 8% of Wilms tumour patients have reported to have cardiac abnormalities (personal communication, Dr. Conrad Fernandez, Renal Committee, Children's Oncology Group). We hypothesize the link between these developmental defects and Wilms tumour may be HACE1 and the regulation of ROS and the cytoskeleton by Rac1; however, further characterization of germline and somatic mutations of HACE1 is required. Wilms tumour patient tissues can be screened for germline HACE1 mutations by sequencing or alterations in methylation by methylation-specific MLPA (multiplex ligation-dependent probe amplification), as well as elucidating additional genes involved using RNA-seq/microarray analysis. These data will be correlated with patient echocardiograms to determine the frequency of congenital cardiac abnormalities. Moreover, using the *hace1* knockout zebrafish line, hace1/rac1 localization and cytoskeletal structure can be investigated by immunocytostaining. These future steps may further illuminate HACE1 and Rac1 as a link between congenital heart defects and cancer.

4.6 Conclusions

In summary, taking advantage of the translucency of zebrafish embryos and high conservation of hace1 in this model organism, this work has shed light on critical tumour suppressing mechanisms of HACE1 as well as its novel role in normal development of the heart. By employing unique tools available in the zebrafish, such as the *Tg(myl7::eGFP)* and *Tg(myl7::mCherry)* transgenic lines; *hace1*, *rac1* and *nox* morpholinos; and the facility with which ROS studies can be visualized *in vivo* in real time, the present study extends our understanding of these interactions. Developing an

in vivo ROS assay, I showed that *hace1* knockdown in zebrafish results in a significant accumulation of ROS. The inverse relationship between *hace1* function and ROS levels suggests that *hace1* is a critical regulator of these reactive molecules. The increased levels of ROS in *hace1* morphant embryos were restored with NADPH oxidase inhibitors as well as morpholino knockdown of the rac1-dependent components of this complex. These findings suggest a correlation between HACE1 and Rac1-dependent NADPH oxidase complexes for ROS regulation. Moreover, I showed that *hace1* deficient embryos are hypersensitive to radiation and DNA damage as low-dose IR resulted in a dramatic increase in mortality of these embryos and a γH2AX DNA damage assay revealed a significant increase in the levels of genotoxic stress in the absence of *hace1*. These data suggest that HACE1 is involved in regulation of ROS and DNA damage susceptibility.

The specific role HACE1 plays in normal development remains uncertain. The zebrafish is well-established as a robust model organism for studying vertebrate development. Based on the highly conserved strong *hace1* expression in the heart of zebrafish, I studied the contribution of this gene to normal cardiac development. I found that knockdown of *hace1* in the zebrafish results in abnormal heart structure and bradycardia. These cardiac abnormalities were rescued using human *HACE1* mRNA confirming the specificity of *hace1* loss as the etiology of these anomalies. Given the previously demonstrated elevated levels of ROS in the absence of hace1, I was interested to see how the loss of hace1 impacts cardiac development and whether elevated ROS levels represent an underlying mechanism. I found that *hace1* zebrafish morphants have characteristic defects in cardiac embryogenesis associated with elevated ROS levels that

can be effectively ameliorated with anti-oxidant compounds. Interestingly, *hace1* morphant embryos demonstrated an increased expression of *rac1*. Inhibition of rac1-dependent components of NADPH oxidases reduced ROS levels and restored normal cardiac structure suggesting that these cardiac phenotypes are regulated by rac1-dependent NADPH pathway components. Ultimately, these findings link HACE1 and ROS production to vertebrate cardiac development for the first time.

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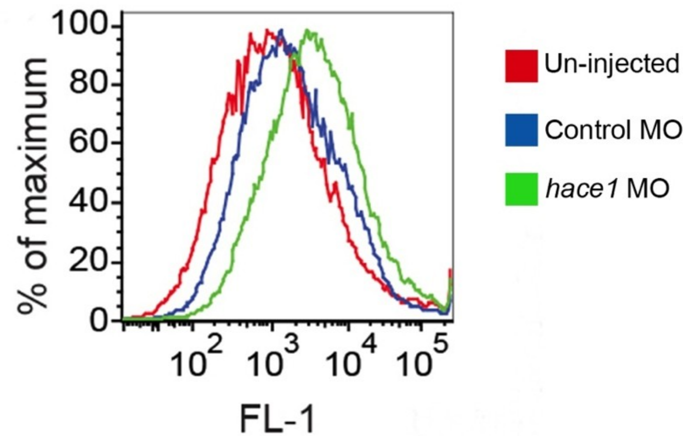
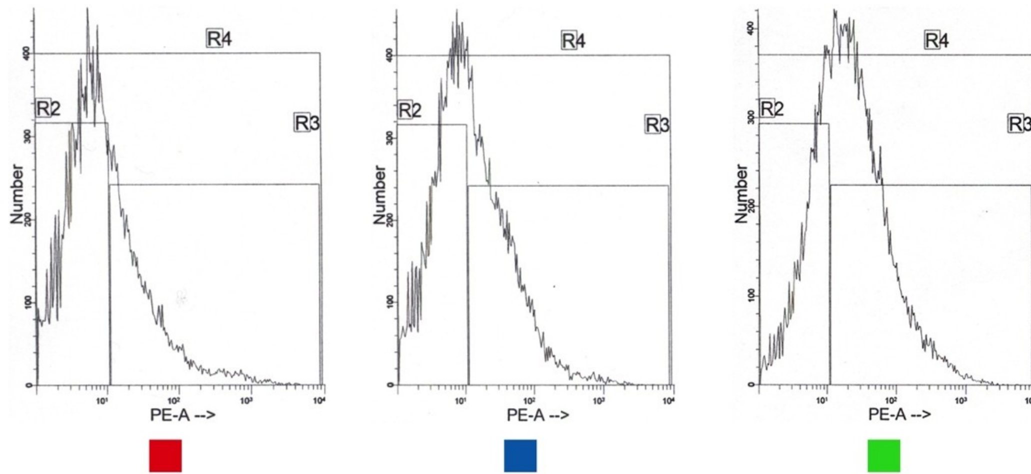
Appendix A. FACS histogram plots of 72 hpf zebrafish embryos.

A

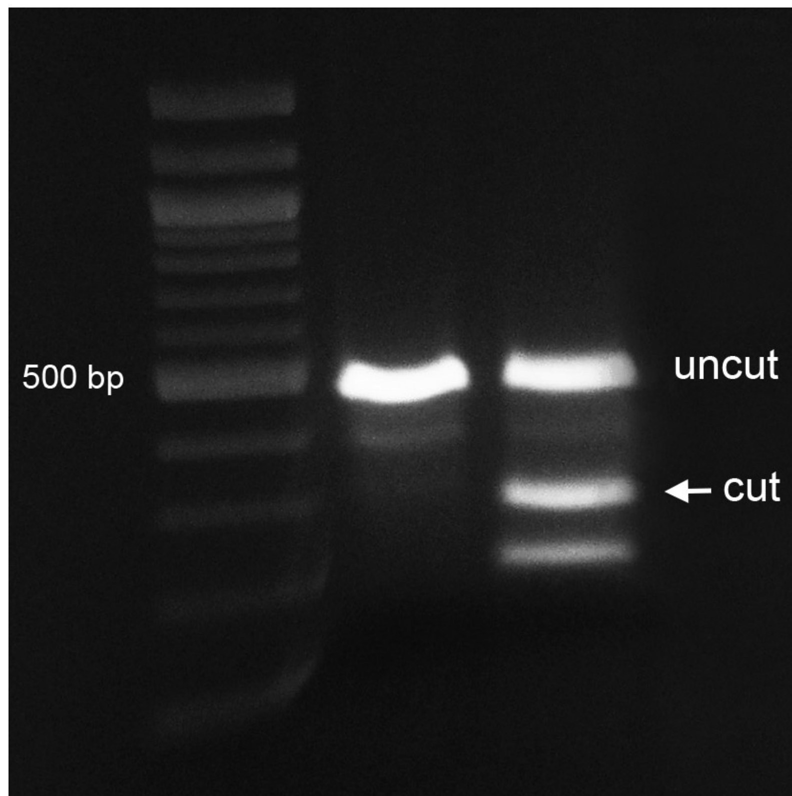


B



Appendix A. Zebrafish embryos knocked down for *hace1* display higher than normal ROS levels. FACS quantification (described earlier) was performed on 72 hpf embryos in un-injected, control or *hace1* morphant groups. Colours correspond to the respective treatments demonstrating the maximum population of each group based on their intensity of fluorescence as multilayer in (**A)** and as individual histograms in (**B)**.

Appendix B. T7 endonuclease I assay.



Appendix B. T7 endonuclease I assay. Genomic PCR digested with T7 endonuclease I. The cut shows mismatch at the target site. T7-hace1-CRISPR_sense: TAATACGACTCACTATAGGATATCGCATATGATGGAAGTTTTAGAGCTAGAA ATAGC. Primers used were: hace1_CRISPR_for GGCGGAATGAGTCGTGAAC and hace1_CRISPR_rev TGAATGTCAAGACAGGAATGCT.

Appendix C. Notice of Protocol Approval for the Hace1 Project

NOTICE OF PROTOCOL APPROVAL
UNIVERSITY COMMITTEE ON LABORATORY ANIMALS

Protocol Number: 13-131

Investigator: Jason Berman

Expiry Date: Dec 1, 2014

Category/Level: B

Title of Study: Using Zebrafish to Study the role of HACE 1 in Normal Development and Cancer

Species: fish

Leslie Lord
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IMPORTANT FUNDING INFORMATION:

In compliance with granting agency and Dalhousie University policy, the Office of Research Services in not permitted to release funding instalments into research accounts until documentation of all necessary approvals are submitted to Research Services (ie. Human ethics, animal ethics, biohazard and radiation permits).

*** To ensure the research funds related to this animal protocol are released, please fill out the information below, sign and Fax the entire sheet to the Research Office.

- Dalhousie University Research Office   FAX 494-1595
- IWK Research Office   FAX 470-6767

Appendix D. Permission from Nature Communications

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Title: Hace1 controls ROS generation of vertebrate Rac1-dependent NADPH oxidase complexes

Author: Mads Daugaard, Roberto Nitsch, Babak Razaghi, Lindsay McDonald, Ameer Jarrar, Stéphanie Torrino et al.

Publication: Nature Communications

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