Exploring the Early Effects of AP Endonuclease 1 Knockdown in Zebrafish Embryogenesis

A thesis presented by
Benjamin Snow

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Abstract of Thesis

Submitted in partial fulfillment to the requirements for the degree of Master of Science in Biology in the Graduate School of Science of Northeastern University, May, 2012
ABSTRACT

AP Endonuclease 1 (Apex1) is a major player in the base excision repair (BER) pathway, which accounts for the majority of oxidative DNA damage repair in eukaryotic cells. Loss of Apex1 in developing vertebrate embryos, whether by artificial knockdown or genetic knockout, is lethal and halts development near the onset of gastrulation. To date, no one has successfully cultured Apex1 -/- cells from any source, suggesting its vital role for Apex1. Our lab has recently discovered a regulatory relationship between Apex1 and the cAMP response element binding (Creb) complex in zebrafish and mice. Partial knockdown of Apex1 using morpholino oligonucleotides (MO) in zebrafish embryos leads to a diminution of transcript and protein of the Creb complex genes by 24 hours post-fertilization (hpf), as well as transcripts of some genes controlled by Creb. However, the means by which Apex1 controls the Creb complex, and thereby embryonic development, remain a mystery.

Because loss of Apex1 protein following MO knockdown begins at the 256 cell stage (2.7 hpf) and progresses to 75% epiboly (8 hpf), when 54% of the protein is lost, we asked what other transcript changes were detected at early times that might account for the phenotype seen at 24 hpf. By the dome stage (4.3 hpf), the transcript for hist2h2l, the histone H2B ortholog, is upregulated 6-fold in the Apex1 hypomorphant relative to the control, while the transcripts for the other core histones remain unchanged. Transcripts for three ribosomal genes are also slightly diminished. By the shield stage (6 hpf), transcript of hist2h2l is even further increased to 26-fold in the hypomorphant relative to the control, while those of the other core histones still remain constant. Furthermore, monoubiquitination of Lys\textsuperscript{120} on H2B, a modification associated with transcriptional upregulation and DNA double strand breaks, increases 7-fold in the hypomorphant. Transcripts for hsp70, fos, crtc1 and crem are also significantly increased,
although changes in hist2h2l far outstrip these other mRNAs. Despite the fact that hsp70 transcript is upregulated 29-fold in the Apex1 hypomorphant relative to the control at the shield stage, Hsp70 protein cannot be detected until later in development. Creb1 protein is also reduced by almost 70% relative to the control at this stage. By 75% epiboly, the transcripts of most of these genes have reached control levels, but Creb1 protein is still reduced by almost 70%, and the NAD+/NADH ratio has risen significantly, another sign of cell stress. We therefore conclude that in Apex1 hypomorphants, early disturbance of chromatin architecture leads to abnormal zygotic genome activation, and that the early disturbance of developmental programs is responsible for lethality.
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LIST OF ABBREVIATIONS

Apex1  Apurinic/apyrimidinic endonuclease 1
AP site  Apurinic/apyrimidinic site
BER  Base excision repair
Cbp  Creb binding protein
Creb1  cAMP response element binding protein 1
Crem  cAMP response element modulator
Crtc1, 3  Creb-regulated transcription coactivator 1/3
dpf  Days post-fertilization
dRP  Deoxyribose-phosphate
ExoIII  Exonuclease III
Fen1  Flap endonuclease 1
Fos  v-fos FBJ murine osteosarcoma viral oncogene homolog
H2afx  H2A histone family, member X
H2BK120ub1  Monoubiquityl-histone H2B Lys^{120}
H3f3b.1  H3 histone, family 3B.1
H3K4me3  Trimethyl-Histone H3 Lys^{4}
Hdac1  Histone deacetylase 1
Hist1h4l  Histone 1, H4, like
Hist2h2l  Histone 2, H2, like
Hmgb3a  High-mobility group box 3a
hpf  Hours post-fertilization
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<td>Mesoderm posterior A</td>
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<td>MutL homolog 1</td>
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<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MO</td>
<td>Morpholino oligonucleotide</td>
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<td>Mpg</td>
<td>N-methylpurine-DNA glycosylase</td>
</tr>
<tr>
<td>MTS</td>
<td>Mitochondrial targeting sequence</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
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<td>8-oxoguanine DNA glycosylase 1</td>
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<td>DNA polymerase β</td>
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<tr>
<td>qrt-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
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<td>Replication protein A1</td>
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<td>TBST</td>
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<td>Tdg</td>
<td>Thymine DNA glycosylase</td>
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<td>TS-MO</td>
<td>Translation start site morpholino oligonucleotide</td>
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<td>Xrcc1</td>
<td>X-ray cross complementing protein 1</td>
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<td>ZGA</td>
<td>Zygotic genome activation</td>
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INTRODUCTION

DNA Damage and Repair Systems

Significance of DNA Damage Repair

The evolution of DNA from RNA as the information-carrying molecule of all life increased the stability of genetic information, and ultimately permitted the development of single cells and later, multicellular life (1). DNA is not immune from damage, however, so methods to prevent insult to DNA and to repair it when it does occur have evolved. Biologists have identified several systems by which genetic damage is mended: among them are nucleotide excision repair (NER), mismatch repair (MMR), non-homologous end joining (NHEJ), homologous recombination (HR), and base excision repair (BER). Each of these systems recognizes specific types of damage, although they share several steps in common, and some enzymes participate in multiple pathways.

The importance of these systems cannot be overstated because all life is routinely bombarded by harmful energies and chemicals. Ultraviolet light can dimerize adjacent pyrimidines to form bulky cyclobutane dimers, and damage to the bases by ionizing radiation can even destabilize phosphodiester bonds. DNA is not safe even from the cell itself. Reactive oxygen species produced by mitochondria during oxidative phosphorylation or by oxidase enzymes can pollute the cytosolic nucleotide pool, so oxidized nucleotides become incorporated into DNA during replication (2). The nitrogenous base uracil, usually present only in RNA, can be incorporated into DNA either by accident of a replicative DNA polymerase or by spontaneous deamination of cytosine (3). Unrepaired damage inhibits transcription and replication, and can be lethal (4, 5).
Nucleotide Excision Repair

NER is the key mechanism for the replacement of UV-damaged nucleotides, and deficiencies in any of the several genes associated with this pathway lead to diseases in which the patient is sensitive to UV light (6). The NER pathway is activated by recognition of pyrimidine-dimers or 6-4 photoproducts by a complex formed by the enzymes xeroderma pigmentosum complementation group A (Xpa)\(^1\) and replication protein A1 (Rpa1). This complex recruits TFIIH, a multi-subunit helicase that unwinds the lesion and several nucleotides on either side. Two nucleases, xeroderma pigmentosum, complementation groups G and F (Xpf and Xpg), cleave the strand with the damaged bases on the 3’ and 5’ sides, respectively, releasing a single-stranded fragment of 24-32 nucleotides. The newly-created gap allows DNA polymerase δ or ε to synthesize a new strand using the undamaged strand as a template, and the gaps are sealed by a DNA ligase (7).

Mismatch Repair

The highly-conserved MMR pathway repairs errors introduced to the genome during normal semiconservative DNA replication that were not repaired by the replicative polymerases’ proofreading function (8). The exception is U:A, U:G, or T:G mispairs, which are repaired by BER (9). In E. coli, the rate of misincorporation is one in every \(10^4\) to \(10^5\) bases, although the polymerase proofreading lowers the error rate to only one in every \(10^7\) bases (10). This rate is estimated to be the same in mammals (11). Because of efficient repair, the mutation rate in E. coli is much lower, at approximately one in every \(10^9\) to \(10^{10}\) bases (12). In MMR, improper base pairing forms a lesion in the double-stranded DNA which is recognized by the MutSα.

\(^1\) For the sake of simplicity, this thesis will follow zebrafish (Danio rerio) nomenclature for gene and protein names.
heterodimer (formed by mutS homologs 2 and 6), or the MutSβ heterodimer (formed by mutS homologs 2 and 3). The former demonstrates more activity when there is only a single mismatch, whereas the latter is preferentially activated when there are several consecutive errors. The enzyme mutL homolog 1 (Mlh1) then heterodimerizes with postmeiotic segregation increased 2 (Pms2) to cleave the daughter strand at one side of the mismatch, and exonuclease 1 (Exo1) cleaves at the other end. Interaction with the Mlh1-Pms2 heterodimer is required for Exo1 to cleave 3’ to the error, while interaction with either of the MutS heterodimers and Rpa1 assist in 5’ cleavage. DNA polymerase δ fills in the gap, and DNA ligase 1 seals the nick (8).

Non-homologous End Joining and Homologous Recombination

Double strand breaks induced by ionizing radiation or errors in recombination are repaired by either NHEJ or HR. NHEJ utilizes the Ku heterodimer (formed by Ku70 and Ku80) to tether the two strands so DNA ligase 4 (Lig4) can ligate the ends. If the ends have been modified by the break, such as by formation of 5’ hydroxyls and 3’ phosphates rather than the other way around, the ends must be repaired before Lig4 can seal the break. Polynucleotide kinase 3’-phosphatase, aprataxin, and some nucleases have been shown to modify broken ends for NHEJ. DNA polymerases λ or μ can fill in gaps left behind by degradation of one of the strands by nucleases or the damage-causing event itself. HR is a much more complicated process, which involves the coordination of several proteins to twist the DNA in such a manner that the original chromosomes are reformed (13).

Base Excision Repair

The final repair mechanism, BER, repairs small, non-bulky lesions formed by both
exogenous and endogenous agents. It also repairs uracil residues inserted during replication, or formed by deamination of cytosine, as well as T:G mispairs. It is a versatile pathway, recognizing several modifications to the nitrogenous bases, and proceeds in the stepwise manner to excise the damaged nucleotide and then replace it with the correct one. One of many DNA glycosylases recognizes the damaged base and breaks the N-glycosyl bond that bonds it to the deoxyribose backbone. This removal leaves behind a toxic apurinic/apyrimidinic (AP) site. Glycosylases include 8-oxoguanine DNA glycosylase (Ogg1), which removes 8-oxoguanine; uracil-DNA glycosylase (Udg), methyl-CpG-binding domain protein 4 (Mbd4), and thymine-DNA glycosylase (Tdg), which all remove uracil depending on how it was introduced to the DNA; and N-methylpurine-DNA glycosylase (Mpg), which removes hypoxanthine, the product of spontaneously deaminated adenine. Some glycosylases, such as Ogg1, also have inherent lyase activity, so they cut the backbone 3’ to the AP site in addition to removing the damaged base (2).

Following generation of the AP site, AP endonuclease 1 (Apex1) cleaves the phosphodiester bond 5’ to the lesion. In the case of monofunctional glycosylases, cleavage of the AP site leaves behind a single strand break sandwiched between a 5’ deoxyribose phosphate (dRP) and a 3’ hydroxyl group. When the glycosylase also nicks the backbone, Apex1 leaves a single nucleotide gap (2, 14, 15). Poly(ADP-ribose) polymerase 1 (Parp1) then binds to the strand break, which stimulates the enzyme to attach branching chains of poly(ADP-ribose) to itself and nearby histones, thereby unwinding the nucleosomes. This chromatin rearrangement is necessary for recruitment of downstream enzymes to the damaged site (16–20).

BER can then undergo one of two subpathways: short patch or long patch repair. In short patch repair, the interaction of DNA polymerase β (Polb) with Apex1, automodified Parp1, and the scaffolding protein X-Ray cross complementing protein 1 (Xrcc1) fills in the gap by adding a
single nucleotide. Polb lyase activity removes the 5’ dRP, so that DNA ligase IIIα (Lig3) can then seal the nick. In long patch repair, the polymerase continues synthesis for an extra two to ten nucleotides, which results in the displacement of the original strand by the new strand (21). To remove the displaced strand, flap endonuclease 1 (Fen1) cleaves the displaced DNA before the ligase seals the nick (22). In early stage zebrafish embryos, Polb protein is lacking, so aphidicolin-sensitive polymerases fulfill the base insertion step (23).

While the reasons for the cell to choose which pathway to follow are unclear, evidence suggests that the structure of the strand break plays the determining role. If the lyase activity of Polb can remove the 5’ dRP site left behind by Apex1, then BER will proceed by short patch. However, if the glycosylase possessed lyase activity, then the 3’ aldehydic end blocks Polb, so strand displacement is required to permit the modification’s elimination by Fen1 (24, 25). Another hypothesis concerns the availability of energy in the form of ATP. Under normal conditions, Lig3 is readily able to seal the newly-introduced nucleotide into the chromosome. When energy levels are low, however, long patch repair can work in conjunction with other enzymes to create the ATP needed for the completion of BER (26).

BER is the eukaryotic cell’s most frequently used DNA damage repair pathway (2). This system heals injuries resulting from exposure to natural mutagens such as oxygen radicals and alkylating agents, and from normal metabolic processes and spontaneous chemical reactions, like base deamination. Given the significance of this pathway, it follows that loss of activity of many of the BER enzymes can be catastrophic for the organism. Impaired BER leads to mutagenesis, tumorigenesis, or death (for review, see 21). Other diseases, such as Alzheimer’s, Parkinson’s, and Graft-versus-Host are also associated with improper BER due to decreased cell survival after damage repair failure (28, 29). Conversely, various DNA damage repair genes are popular targets
for chemotherapy. Many drugs, such as cisplatin and oxaliplatin, attack tumor cells by inducing DNA damage, although the cells are still able to repair some of the insult and survive. In order to make such chemotherapeutic drugs more effective, BER inhibitors can weaken the tumor cell’s ability to reverse the damage, which thereby induces cell death (30–34).

**AP Endonuclease 1**

*Identification of Apex1*

Human Apex1 was first cloned in 1991 by Bruce Demple, Tory Herman, and Davis Chen at Harvard University, and independently by Craig Robson and Ian Hickson at the University of Oxford, who identified it as the ortholog of bovine AP endonuclease and *E. coli* exonuclease III (ExoIII) (35, 36). Further investigations revealed several eukaryotic orthologs, underlining the antiquity and importance of this gene: apurinic endonuclease-redox protein in *A. thaliana*, AP endonuclease II in *S. cerevisiae*, exo-3 in *C. elegans*, and recombination repair protein I in *D. melanogaster* (37). Robson, Hickson, and colleagues later mapped the 2.6 kb human gene to chromosome 14q11.2, and confirmed a structure of four introns and five exons, with the start codon in the second, and the bulk of cross-species homology in the fifth (38).

*Apex1 Structure and Endonuclease Function*

A nearly complete crystal structure of the human enzyme lacking the first thirty-five amino acids was first resolved in 1997 at a resolution of 2.20 Å. Because this truncation had previously been characterized with the same endonuclease activity as the full-length enzyme, it was suitable for analysis. The crystal structure displayed the common architecture of nucleases, with two similar domains each built with six α-helices flanking a six-stranded β-sheet. Human
Apex1 overlaps neatly with its *E. coli* homolog ExoIII, apart from the human’s 61 residue N-terminus and slightly shorter helix $\alpha_8$. Comparison with bovine DNase I revealed three helical loops unique to AP endonucleases that specify AP site recognition and binding (39).

The exact mechanism of Apex1 incision into the DNA backbone is not clear, but crystal structures, sequence alignments, and site-directed mutagenesis have informed an hypothesis. Helical residues in domain 1 interact with phosphates on the strand opposite the AP site, and the minor groove is extended by Gly$^{127}$, Tyr$^{128}$, and Met$^{270}$, while the major groove is opened by Arg$^{177}$. As the structure of the loop in which this residue resides is not found in other endonucleases, this particular interaction seems to be specific to Apex1’s orthologs and their AP site binding capability (40).

For a tetrahydrofuran substrate, a single step mechanism coordinated by Glu$^{96}$, Tyr$^{171}$, Asp$^{283}$, and His$^{309}$ then catalyzes the hydrolysis reaction. Glu$^{96}$ binds a divalent metal cation (probably Mg$^{2+}$ *in vivo*, but Sm$^{2+}$ or Pb$^{2+}$ in crystal structures) which helps to expose the negatively-charged phosphate to nucleophilic attack. His$^{309}$, stabilized by a hydrogen bond with Asp$^{283}$ and guided by Tyr$^{171}$, generates a nucleophilic attack with a water molecule to break the phosphodiester bond (41–43). These residues are conserved amongst all known Apex1 homologs; much of the interspecies divergence lies in the disordered N-terminus (39). Mutations of residues close to or at the active site result in a significant loss of activity. For example, the loss of an hydroxyl moiety in the Y171F mutation results in a 25,000-fold decrease of endonuclease activity (42). A natural variant identified by genomic sequencing, D283G, results in an enzyme with only 10% the repair capacity of the wild type, and may predispose the individual for neurodegenerative disease (44).
Non-Endonuclease Functions of Apex1

There is much discussion in the literature regarding a redox function for Apex1, first identified in 1992 by Steven Xanthoudakis and Tom Curran, and now principally supported by Mark Kelley (45, 46). Xanthoudakis and Curran identified an approximately 37 kDa protein that could reduce the heterodimerizing transcription factors Fos and Jun in vitro, thereby increasing their affinity for DNA. The researchers first named this protein Ref-1, but they soon identified it as Apex1 and not a novel gene after all. The residues that confer redox activity were pinpointed to a domain distinct from the repair domain. Furthermore, it appeared that deletion of either function did not affect the other in vitro (45, 47, 48). Several more transcription factors that Apex1 regulates have been identified by the same methodology. These include Creb1, NFkB, p53, Hif-1α, and Egr1 (47, 49–52). Chemical inhibition of this redox activity thereby affects gene expression and cell proliferation, turning it into another potential chemotherapeutic target (for review, see 50).

Of the seven cysteine residues in the human sequence, five are conserved amongst all vertebrates; Cys$^{65}$ and Cys$^{138}$ evolved in a therian ancestor. Non-therian vertebrates, for example zebrafish, do not display the redox modulation, suggesting that one of these two residues is the one responsible for the redox activity. Georgiadis, et al. co-transfected Skov-3X ovarian cancer cells with a plasmid containing the coding sequence for luciferase driven by a promoter containing the NFkB consensus sequence, plus one of seven constitutively-active plasmids expressing either an Apex1 cysteine-to-alanine mutation or the wild type. Overexpression of five of the seven mutant plasmids luminesced equally as well as wild type, but the C65A and C93A constructs luminesced by only about half. As Cys$^{65}$ is the non-conserved residue, the authors attributed redox function to it. To support this conclusion, they also mutated the corresponding
zebrafish residue, a threonine, to cysteine and measured its in vitro redox function on AP-1 DNA binding, which they determined to be equal to the wild type human (54).

There are three main gaps in the understanding of Apex1 as a redox factor. Firstly, careful kinetics of the redox activity have never been performed, and the assay used to identify redox activity could not be duplicated in the Strauss laboratory (P. Strauss, personal communication). Kelley’s binding assay protocol itself is also not particularly precise, because his group used nuclear extracts rather than purified proteins as their source of transcription factors, so the reactions may have contained other unknown molecules that affected DNA binding (54). Secondly, in all crystal structures resolved to date, Cys65 is buried deep within the protein. Extensive remodeling would be required to expose it so that it can reduce other proteins. Evidence for such an event is lacking, and no mechanism for refolding has been proposed (39, 54, 55). And thirdly, the redox function is a therian (i.e. evolutionarily recent) adaptation. The zebrafish, frog, and platypus homologs lack the redox-active cysteine residue (54). That this function is absent in most metazoans, and for that matter most vertebrates, suggests that other mechanisms exist to modulate transcription factor binding, so Apex1’s activity in that regard may be redundant. This is supported by the observation that mice homozygous for C64A (the equivalent residue to human Cys65) and cells cultured from these embryos are identical to the wild type, even though disruption of the apex1 gene is embryonic lethal in both mouse and zebrafish (23, 56–58).

Apex1 is also reported to possess RNase activity in addition to its normal DNA cleavage function, and in fact the enzyme uses the same active site to catalyze both classes of reactions. Barnes, et al. in Chow Lee’s lab at the University of Northern British Columbia identified Apex1 as one of two RNases that cleave in vivo the mRNA of the oncogenic transcription factor c-myc.
RNAi-mediated knockdown of Apex1 maintains steady-state transcript levels of c-myc, and endonuclease-incompetent mutations E96A or H309N do not cleave the RNA in vitro (59). They further characterized this RNase activity to be cation-independent, whereas the DNase activity is Mg$^{2+}$-dependent, and for Asp$^{283}$ to be involved only in RNA binding and not cleavage as it is with an AP site substrate (60). The specific biological contexts and kinetics of this activity remain unclear.

**Apex2**

An ancient paralog of apex1 also exists in eukaryotes, the 518 amino acid, and 59.1 kDa apex2, mapped to human chromosome Xp11.21. Apex2 is distinct from Apex1 by virtue of three features: a mitochondrial targeting sequence (MTS) N-terminal to the nuclease domain, a PCNA binding motif, and a topoisomerase III homologous domain on the C-terminal side (61). Northern blot analysis of mouse tissue detected expression throughout the animal with the exception of the stomach and salivary glands (62). The first studies of this gene suggested that Apex2 is redundant to Apex1 in nuclear BER, but serves as the primary endonuclease for mitochondrial BER, since Apex1 lacks a clear MTS (61). However, recent studies on Apex1 have identified a putative unique MTS within residues 289-318, which, while normally buried within the three-dimensional structure of the protein, may be exposed during oxidative stress, permitting translocation from the nucleus to the mitochondria (63, 64). Therefore, Apex2 may simply serve as backup when Apex1 is knocked out or mutated (65). Studies from the Strauss lab indicate that Apex2 is also highly expressed in very early stage zebrafish embryos, although the significance of this observation is unknown at this time (65).
Base Excision Repair and Embryonic Development

BER in Early Development

The importance of BER in everyday cell survival is well appreciated. However, the system in the context of embryonic development, specifically among vertebrates, is still not well characterized. Nevertheless, it stands to reason that it is an essential system, since knockout or knockdown of several BER genes is embryonic lethal.

The Strauss lab has begun to describe embryonic BER using the zebrafish model. Many of the elements for BER are maternally deposited into eggs, but Polb is a notable exception. For at least the first three days post-fertilization, another unidentified aphidicolin-sensitive polymerase executes the gap-filling role. Embryonic zebrafish extracts are able to repair a DNA oligomer containing uracil, although less efficiently than adult extracts. Surprisingly, depletion of the zebrafish Apex1 homolog via morpholino oligonucleotide (MO) microinjection does not abolish BER activity. *In vitro* assays of Apex1 knockdown extracts at three hours post-fertilization suggest another Mg$^{2+}$-dependent endonuclease can compensate for Apex1, possibly Apex2, which is highly expressed in early embryos (65).

Mouse BER Knockouts

The mouse model has been the primary tool for investigating homozygous knockouts of BER genes, and it has provided some fascinating insights. Knockout *fen1* mice die around embryonic day E3.5, when the blastocyst hollows and the inner cell mass is formed. When cultured in media containing bromodeoxyuridine, knockout cells display no sign of incorporating it into their DNA, whereas the controls do, suggesting that *fen1* knockouts lose the ability to enter the S phase of the cell cycle just prior to gastrulation. Although there is no evidence that
these embryos die from apoptosis, cells do apoptose when exposed to ionizing radiation, as expected from DNA damage repair-deficient cells (24). Although Xrcc1 is only known to be a scaffolding protein that assembles the final enzymes required for BER, its loss is also lethal to mice at E6.5 to 7.5. Knockouts display increased incidence of DNA strand breaks, and the endoderm does not form the proper flattened morphology (66). Knockouts of lig3 halt developmental programs by E8.5, and die with excessive apoptosis within the next 24 hours (67).

Knockout parp1 mice are viable and display no obvious phenotype besides enhanced sensitivity to DNA damaging agents, which is surprising considering Parp1’s known involvement in various pathways (68). Knockout of parp1 does not completely abrogate levels of poly(ADP-ribose) in nuclear extracts, which is explained by the redundant activity of the paralog Parp2 (69). Knockout of parp2 displays a similar phenotype to parp1 -/-, but double knockouts die by E7.5. Similarly, knockout of poly(ADP-ribose) glycohydrolase (parg), which reverses the activity of Parp1/2, is lethal at E3.5, and cultured cells are extremely sensitive to DNA damage (70). Interestingly, the genotype parp1 +/- parp2 -/- is embryonic lethal only for females, which die at E9.5. X chromosome instabilities are detectable at E8.5, suggesting a role for Parp1 in X chromosome maintenance (71).

Knockout of polb is also not embryonic lethal, but pups die shortly after birth due to respiratory failure, and also display retarded growth and abnormal brain development, as evidenced by increased neuronal cell death in knockout embryos compared to wild type (72). Cells cultured from knockout embryos display increased sensitivity towards DNA damaging agents (73). Similar results have been found in zebrafish. While MO knockdown is not lethal, the fish are more sensitive to methylmethane sulfonate and fail to inflate their swim bladder, an organ which derives from the same embryonic tissues as does the tetrapod lung (23).
Interestingly, until recently no glycosylase has been demonstrated to possess an embryonic lethal phenotype, suggesting either tolerance of absence or a compensatory mechanism which downstream genes of BER lack (74–78). Cortázar, *et al.* have provided evidence of one exception: thymine DNA glycosylase (Tdg), a monofunctional glycosylase which targets the thymine of T/G mismatches that arise from spontaneous deamination of 5-methylcytosine. Knockout mouse embryos died by E11.5 from internal hemorrhaging and necrosis. Through chromatin immunoprecipitation experiments, they determined that Tdg is normally enriched on CpG islands in gene promoters, likely to reverse DNA methylation. In *tdg* knockout cells, these promoters are instead enriched for transcriptional repressive histone markers (H3K27me3 or H3K9me3) with a concomitant decrease in the stimulatory marker H3K4me2. The authors also observed this enrichment and embryonic lethality when wild type Tdg was replaced with the catalytically inactive protein bearing the N151A mutation. The authors therefore concluded that Tdg is required to help modify chromatin in such a way that the right genes are expressed at the right times during embryogenesis, and that the genes remain silent in the absence of Tdg (79).

*Knockout and Knockdown of Apex1*

Knockout of *apex1* is unique among the BER genes. Homozygous null mice die at some point between late E4 and E6.5 (57). Apex1 MO knockdown in zebrafish is also lethal at the mid-blastula transition, which roughly corresponds to the same stage. Partial knockdowns display severe heart and brain abnormalities by 1 day post-fertilization (dpf), and die by 7 dpf, when a fully-functional heart is required (23, 58). But unlike all of the above genes, no *apex1* knockout cell cultures have been developed to date. High levels of *apex2* message in young
zebrafish embryos relative to adult fish suggests that embryos may use Apex2 to compensate for
the loss of Apex1, but this is clearly insufficient at later stages in both zebrafish and mice. MO
and RNAi-mediated knockdown in zebrafish and human cancer cell lines, respectively, can be
rescued only by providing exogenous endonuclease-competent apex1 mRNA; the redox function
is irrelevant. Zebrafish, which naturally lack the requisite cysteine in any case, can be rescued by
wild type human apex1 mRNA, but fail to be rescued by the catalytically-inactive Y171F
mutation (23, 58). Human cancer cells can be rescued with endonuclease-competent S. cerevisiae
apn1, which also lacks the redox function of therian Apex1 homologs (80). As mentioned
previously, the normal development of redox-incompetent C64A mice further supports the
contention that viability is dependent on the endonuclease activity (56).

**Zebrafish as a Model Organism**

*Benefits of the Zebrafish Model*

The Strauss lab employs the zebrafish (*Danio rerio*) as a model to explore the role of
BER in vertebrate embryonic development. Like all models, zebrafish are small, easy to care for,
and share common genes and pathways with humans. The embryos are transparent, which allow
researchers to carefully monitor organogenesis in real time. While mating behavior in the wild
seems to be timed with the monsoon season of southern Asia from where the fish originate, they
are capable of breeding year-round, and a single female can lay dozens of eggs every week (81).
The first maps of the zebrafish genome were published in 1998, and the latest annotation of the
fully sequenced genome was released in November, 2010 (82–84). This species is a particularly
powerful genetic tool because gene overexpression and knockdown studies are very simple to
perform. An *in vitro* transcribed capped RNA of a particular gene can be microinjected into a
zygote to overexpress the gene. To knock down expression, morpholino oligonucleotides can be microinjected.

James Summerton first developed MOs in 1980 as an antisense therapy for genetic diseases. MOs are nucleic acid analogs, with the bases bound to morpholine rings rather than to deoxyribose, and oligomerized with phosphorodiamidate instead of phosphate. MOs function similarly to siRNA and shRNA in principle, in that they are designed to be complementary to a small sequence of the RNA to be blocked. Unlike these RNA antisense methods, though, MOs do not activate the cell’s RNAi machinery, and so do not promote the degradation of the target transcript. Rather, translation of the proper protein is inhibited by either blocking the progression of the ribosome down the mRNA (translation start or TS-MO), or by sterically blocking spliceosome binding to the pre-mRNA, so introns are retained and the reading frame shifts (splice site or SS-MO). Knockdowns can be rescued by co-injection with the RNA of the MO target gene so long as the sequence to which the MO binds is absent or modified (85).

**Zebrafish Embryogenesis**

Embryogenesis is a complex process that guides an initial single cell to grow, divide, and differentiate into the dozens of tissue types that make up the mature, autonomous organism. Zebrafish embryogenesis is typically completed at 3 dpf, and can be divided into seven stages. Upon fertilization (stage 1), the cytoplasm of the zygote segregates to one end to form the blastodisc, whereas maternally-deposited nutrients remain in the yolk on the opposite end. During cleavage (stage 2), which lasts from second through seventh cell divisions, the blastodisc divides at regular intervals, typically every 15 minutes when the embryo is incubated at 28.5°C. The cells continue to divide rapidly and synchronously during the first half of the third stage, the
blastula. After the tenth cell division, a period known as the mid-blastula transition (MBT), the cell cycles lengthen and asynchronize, and gastrulation begins (86).

The gastrula period (stage 4) is characterized by epiboly, the flattening of cells into sheets that envelop the yolk, a process which is typically completed in five hours. The germ layers also begin to differentiate during this stage. The somites and later other rudimentary organs form during segmentation (stage 5). By the time the sixth stage begins at 24 hpf, the embryo has a clearly defined head and tail, and the eyes, brain, heart, kidneys, and other organs are partially functional. This period is called the pharyngula, so named because of the development of the pharyngeal arches, which will later grow into the mature fish’s jaws and gills. The head and tail straighten out, and pigmentation is produced, which darkens the eyes and the body. The final stage, from 48 to 72 hpf, is the hatching period. As most organs have largely matured, morphogenesis noticeably slows. At some point during this period, the embryo breaks out of the chorion and will later inflate its swim bladder so that it can move freely through the water. Full development and sexual maturity are typically reached within three months (86).

Mid-Blastula Transition and Zygotic Genome Activation

The first ten cell divisions are synchronous, and during this time, the embryo relies almost exclusively on maternally deposited RNAs and proteins. The embryo does not actively transcribe its own genes until the cell cycles lengthen and diverge at the MBT which occurs around 3.5 hpf (86). Two recently-published papers provide the first high-resolution descriptions of the transcriptomics of zebrafish zygotic genome activation (ZGA). The first, by Håvard Aanes and Cecilia Winata, et al., employed RNA-seq to examine differential transcription in unfertilized eggs and embryos spanning the first 5.3 hpf. The authors defined three
“superclusters” of transcripts. Maternal transcripts are present at high levels in the unfertilized egg, and steadily degrade either immediately after fertilization, or following the MBT. The Pre-MBT supercluster genes are not detectable until after fertilization, and then levels of transcript either increase or decrease at the MBT. The MBT supercluster represents genes that are either not actively transcribed until the MBT or shortly thereafter, or are present even in the unfertilized eggs but are then upregulated at the MBT. The authors then used quantitative real time polymerase chain reaction (qrt-PCR) to confirm the RNA-seq data, and found that for the pre-MBT supercluster, they could only confirm their results if they examined polyadenylated transcripts; PCR using cDNA derived from total RNA displayed no significant differences between stages. The authors concluded that this supercluster represents maternally-deposited transcripts that are not polyadenylated until later in development, and so delayed polyadenylation is a means of regulating the timing of gene expression. Furthermore, treatment of embryos with the polyadenylation inhibitor cordycepin significantly retarded epiboly, and the embryos died by 10 hpf, demonstrating the significance of this process (87).

Leif Lindeman and Ingrid Andersen, et al., followed up with a study examining trimethylation of lysine residues on histone H3 in zebrafish before and after the MBT. Chromatin immunoprecipitation followed by microarray analysis demonstrated that H3K4me3, which is generally associated with enhanced transcription, increased between the 256-cell stage and the MBT, and then stabilized through 50% epiboly. Levels of H3K9me3 and H3K27me3, which are both repressive markers for transcription, increased between the MBT and 50% epiboly. Several promoters were simultaneously enriched for both the stimulatory and repressive markers at different developmental stages. Further analysis incorporating Aanes and Winata’s mRNA-seq data revealed that the presence of these markers correlated with transcription of the enriched...
genes; genes enriched for just H3K4me3 were actively transcribed at the MBT and later, and those enriched for the repressive markers (with or without concurrent H3K4me3) were not actively transcribed (88). Together, these two studies demonstrate that the ZGA is not a simple matter of timely recruiting of RNA polymerase 2 to gene promoters. Rather, the ZGA is characterized by temporally-regulated epigenetic alterations and post-transcriptional modifications that permit the rapid and dynamic gene expression required for progression through embryogenesis.

Apex1 Studies in Zebrafish

Zebrafish Apex1

A genome duplication event approximately 350 million years ago has left teleosts with two copies of many genes, some of which have lost or altered function during evolution (83). Zebrafish possess duplicates of almost one-third of their genome (87). It came as no surprise, then, when the Strauss lab identified two genes for *apex1* in zebrafish, one with introns and one without. The full-length gene consists of four introns and five exons, the first of which is not translated, just like the human ortholog. The second gene only has exons and may be the product of a retrotransposon. Northern blot analysis revealed two RNA transcripts in adult fish: one measuring 2.1 kb and a shorter one of 1.3 kb that lacks the full 3’ UTR. This second transcript may arise from the smaller gene, or may be an alternatively spliced transcript of the full-length gene. In either case, only one protein species was identified, a 35 kDa molecule displaying 78% homology (64% identity) with the human protein. The transcript and protein are both ubiquitously expressed through early embryos, but become concentrated in the head and heart by 48 hpf (58).
Consequences of Apex1 Knockdown in Zebrafish Embryos

TS-MO knockdown of Apex1 was observed to be lethal to embryos at the MBT, which roughly corresponds to the same stage at which apex1 -/- mice die. Microinjection with a lower concentration of the TS-MO resulted in larvae with severe brain and heart abnormalities detectable by 1 dpf. Death followed by 7 dpf when a functional heart is required for survival. These deformations were also observed in larvae microinjected with a combination of three SS-MOs, none of which affected the fish individually. The full TS-MO knockdowns could be rescued by co-injection with the RNA for human apex1, only so long as it was endonuclease-competent. Co-injection with the RNA encoding the Y171F mutation, which essentially possesses no endonuclease activity, failed to rescue. The deaths that resulted from the loss of Apex1 were not apoptotic, which is a concern with MO usage. Hypomorphants at 4 hpf did not stain for acridine orange to a greater extent than the controls (58). Furthermore, the full and partial knockdown phenotypes were also observed in p53^{M214K/M214K} fish, which cannot apoptose via p53 (23).

While investigating the role of Polb in zebrafish embryogenesis, a previous graduate student in the lab discovered that partial knockdown of Apex1 resulted in failure of Polb transcript and protein to appear. Since cyclic-AMP response element binding protein 1 (Creb1) is known to regulate Polb expression, another lab member then looked at the expression of Creb1 and its partners to see if they were also affected by the loss of Apex1 (89, 90). As predicted, transcript and protein of Creb1, Creb binding protein (Cbp), cAMP response element modulator (Crem), and Creb-regulated transcription coactivators 1 and 3 (Crtc1 and Crtc3) were all diminished by 24 hpf in Apex1 hypomorphants. Transcript and protein of Polb, Creb1, and its
partners were restored by co-injection with \textit{creb1} mRNA (23). Since the Creb complex is known to regulate brain and heart development, these findings suggested that the Apex1 hypomorphant phenotype may be the result of altered Creb1 expression. In fact, four neural markers with the Creb1 consensus sequence in their promoters are heavily downregulated at 24 hpf following Apex1 knockdown (\textit{D. Pei, unpublished data}).

**How does Apex1 regulate zebrafish early embryonic development? – Studies in this thesis**

The question remains of how Apex1 regulates Creb1 and its partners to determine the fate of the embryo, and moreover why the gene’s endonuclease activity is vital for rescue. To answer this, I proposed an investigation to identify the earliest changes to zebrafish embryos after Apex1 knockdown. Our lab has documented genetic and morphological alterations at the pharyngula stage and later, but we are still unclear about changes that happen earlier. Uncovering a sequence of events at the beginning of embryogenesis after partial MO knockdown of Apex1 will provide clues as to the mechanisms by which Apex1 regulates development. My project is broken down into four primary questions:

1. After microinjection of the Apex1 MO, when do protein levels of Apex1 drop, and how quickly is maternal protein depleted?
2. When does expression of Creb1 and its partners change relative to the loss of Apex1?
3. What other genes change expression following Apex1 knockdown?
4. Are there any biochemical or morphological changes than accompany early Apex1 knockdown?

Zebrafish embryos rely on maternally-deposited RNA and protein until the MBT, at which point they become genetically autonomous. Since unfertilized zebrafish eggs are loaded
with Apex1 transcript and protein, I predict that protein levels will not decrease following MO microinjection until at least the MBT, since the embryo will not be able to translate newly transcribed mRNA (58). At this point, DNA damage and other cell stressors will accumulate, which will in turn retard the expression of Creb1 and, not long after, genes that it regulates.
METHODS

Zebrafish husbandry and breeding

Outbred zebrafish were purchased from Aquatic Tropicals (Plant City, FL) and kept at standard laboratory conditions of 28-29°C on a 13 hour light, 11 hour dark cycle. To breed, adult fish were placed in a small tank the night prior to mating, with a removable plastic barrier separating the males and females. Just before the beginning of the light cycle the following morning, the tanks were set at a slight incline to create a depth gradient (81), the barrier was removed, and two green florets were added to the tank. Fish were allowed to mate for at least 30 minutes before collection of fertilized embryos to be used for experiments.

Morpholino oligonucleotide and microinjection

TS-MO of zebrafish apex1 mRNA was synthesized by GeneTools (Philomath, OR) and has been described previously (23, 58, 65). The sequence is 5’-GTT CTT CTT GGC TCT TTT GGG CAT G-3’. Embryos no older than the 4 cell stage were injected with 0.23 mM TS-MO directly into the yolks to ensure equal distribution amongst all daughter cells. A 25% phenol red solution (in water) served as a tracking dye. Controls were injected with the phenol red solution without additions. Injected embryos were then raised in a 29°C incubator until they reached the desired developmental stage for further experiments.

RNA isolation, cDNA synthesis, and qrt-PCR

Total RNA was extracted from embryos at the desired developmental stages by homogenizing the embryos in 1 mL TRI reagent (Sigma Aldrich, St. Louis, MO or Life Technologies, Carlsbad, CA) and then following the manufacturer’s instructions. After pellets
were resuspended in RNase-free water (Life Technologies, Carlsbad, CA), the concentration and purity were measured with a NanoDrop 1000 (ThermoScientific, Waltham, MA). To ensure that there was no degradation, 2 µL of the extracted samples was resolved on a 1% agarose gel; two sharp, distinct bands without any smearing, which represent 28S and 18S rRNA, signified a successful extraction. For each sample, 1 µg RNA was then reverse transcribed into cDNA using either Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN) or Tetro cDNA Synthesis Kit (Bioline, Taunton, MA), with random hexamer or oligo-(dT)$_{18}$ primers as required for the particular experiment.

Oligonucleotide primers for qrt-PCR were designed using Primer3 software (available online at [http://frodo.wi.mit.edu/](http://frodo.wi.mit.edu/)) (see Table 1) (91). The instrument used for qrt-PCR was the ABI PRISM 7000 Sequence Detection thermal cycler (Life Technologies, Carlsbad, CA). The reaction solution consisted of 1 µL cDNA (diluted 1/10 in 10 mM Tris-Cl, pH 8.5), 0.5 µL each forward and reverse primers, 12.5 µL RT$^2$ SYBR® Green qPCR Mastermix (SABiosciences, Frederick, MD), and 10.5 µL nuclease-free water. The PCR followed the standard profile of an initial 95°C denaturation for 10 minutes, followed by 45 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 1 minute, followed by a dissociation step that ranged 65°C-95°C to ensure that only a single product was amplified. Each sample was assayed in triplicate, and fold change was quantified using the $\Delta\Delta$CT method, with beta-actin as a housekeeping control. The results of three or more independent experiments were averaged to determine overall fold change.
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| beta actin | NM_131031 | F’ 5-CAACAGAGAGAAGATGACAGAGATCA-3  
                     |               | R’ 5-GTCAACCACCTCAACGAGTTGACCATAC-3               |
| apex1    | NM_213421 | F’ 5-CGAAGCAGAGAAGATGACAGCTCAGATCA-3  
                     |               | R’ 5-GTCAACCACCTCAACGAGTTGACCATAC-3             |
| creb1    | NM_200909 | F’ 5-AGGAGCAGAGAAGATGACAGCTCAGATCA-3  
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| crem     | XM_677932.5 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
                     |               | R’ 5-GTCAACCACCTCAACGAGTTGACCATAC-3           |
| cbp      | NM_001089455.2 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
                     |               | R’ 5-GTCAACCACCTCAACGAGTTGACCATAC-3          |
| crtc1    | XM_003199425.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
                     |               | R’ 5-GTCAACCACCTCAACGAGTTGACCATAC-3         |
| crtc3    | XM_693452.5 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| ogg1     | NM_001123308.2 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| parp1    | NM_001044942.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| hsp70    | NM_00117518.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| hist2h2l | NM_200117.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| fos      | NM_205569.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| jun      | NM_199987.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| hmgb3a   | NM_001122836.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| mespa    | NM_131551.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| h2afx    | NM_201073.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| h3f3b.1  | NM_001017599.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| hist1h4l | NM_001105706.2 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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| hdac1    | NM_173236.1 | F’ 5-CAACAGAGAGAAGATGACAGCTCAGATCA-3  
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### Protein extraction and quantification, and Western blotting

Embryos were homogenized in a solution of 10 mM Tris-Cl (pH 7.8) and 200 mM KCl supplemented with one tablet of cOmplete Mini, EDTA-free, protease inhibitor (Roche Applied Science, Indianapolis, IN) in a volume of 2 µL per embryo. This inhibitor cocktail consists of the following proteases: aprotinin, bestatin, calpain inhibitors I and II, chymostatin, E-64, leupeptin, α2-macroglobulin, pefabloc SC, pepstatin, phenylmethyl-sulfonyl fluoride, tosyllysine chloromethyl ketone hydrochloride, and two trypsin inhibitors (chicken egg white and soybean). An equal volume of a solution of 10 mM Tris-Cl (pH 7.8), 200 mM KCl, 2.5 mM EDTA, 2.5 mM DTT, 0.25% NP-40, and 50% glycerol was added to the homogenate. After one hour incubation on a slowly rotating shaker in 4°C, the solutions were centrifuged to pellet insoluble material, and then the supernatants were collected. Concentrations of the protein extractions were quantified with a Bradford Assay (92). For Apex1, Creb1, and Hsp70 immunoblotting, 50 µg protein extract were resolved on a pre-cast Precise 4-20% Tris-HEPES SDS polyacrylamide gel (Thermo Scientific, Rockford, IL), and then transferred onto a PVDF membrane.

A different method was used to ensure extraction of histones (93). Equal numbers of Apex1 hypomorphant and control embryos were dechorionated by incubation for 5 minutes in 2 mg/mL pronase (CalBiochem, San Diego, CA) in E2 medium (15 mM NaCl, 0.5 mM KCl, 2.7 mM CaCl2, 1 mM MgSO4, 0.7 mM NaHCO3, 0.15 mM KH2PO4, 0.05 mM Na2HPO4) warmed to 37°C. The pronase was carefully removed and embryos washed with E2 medium. The embryos
were then transferred to a microfuge tube with 1 mL ½ Ginzburg Fish Ringer’s solution without calcium (55 mM NaCl, 1.8 mM KCl, 1.25 mM NaHCO₃). Agitation for 5 minutes dissolved the yolk and dissociated the cells. Cells were pelleted with a brief centrifugation at 300 xg for 30 seconds, and then dissolved in 2 µL/embryo 4x NuPAGE LDS sample buffer (Life Sciences, Carlsbad, CA), and 0.1 M dithiotreitol. After samples were heated at 95°C for 5 minutes, equal volumes of Apex1 hypomorphant and control were resolved on a 4-20% SDS polyacrylamide gel. Resolved proteins were then transferred onto a PVDF membrane as above.

For all Western blots, the membrane was then blocked in 5% dry milk dissolved in Tris-buffered saline with 0.1% Tween-20 (TBST) either overnight at 4°C for Apex1, or for 1 hour at room temperature for Creb1, Hsp70, or H2BK120ub1. To probe for Apex1, the membrane was incubated in a solution of 1% milk in TBST with a primary antibody directed against zebrafish Apex1 residues 140-155 (custom prepared for the Strauss lab by Sigma-Genosys, The Woodlands, TX (58)) at a dilution of 1:2000 for one hour at room temperature. For Creb1 (AbCam, Cambridge, MA), Hsp70, or H2BK120ub1 (Cell Signaling Technologies, Danvers, MA), the primary antibodies were prepared in a solution of 5% BSA in TBST at a concentration of 1:1000, and then incubated overnight at 4°C. An antibody directed against human beta-tubulin (AbCam, Cambridge, MA) at a dilution of 1:10,000 in 1% milk served as a loading control.

Following incubation with the primary antibody, membranes were washed with five volumes of TBST at room temperature with mild agitation, twice for five minutes each and three times for fifteen minutes each. The membrane was then incubated with anti-rabbit IgG-HRP secondary antibody (AbCam, Cambridge, MA) at a dilution of 1:5000 in either 1% milk or 1% BSA in TBST for one hour at room temperature, followed by another five washes with TBST as above. In order to visualize the blot, the membrane was soaked in Western Lightning
chemiluminescence reagent (Perkin Elmer, Waltham, MA), and then briefly exposed to X-ray film for development. Bands were quantified using Image J software (available online http://rsbweb.nih.gov/ij/).

**NAD+/NADH Quantification**

Concentrations of NAD+ and NADH in embryos were quantified using the BioVision colorimetric kit per manufacturer’s instructions (Milpitas, CA). Equal numbers of Apex1 hypomorphant and control embryos were homogenized in 400 µL extraction buffer. The supernatant was then passed through a 10 kDa spin filter (Pall Life Sciences, Ann Arbor, MI) to remove enzymes which might consume NAD. To detect total NAD, 10 µL extract were added to a 96-well plate in duplicates, and then the volume was brought up to 50 µL with extraction buffer. To measure NADH, 50 µL extract which had been heated at 60°C for 30 minutes to decompose NAD+ were added to the plate in duplicates. One hundred microliters NAD cycling buffer and 2 µL NAD cycling enzyme were then added to each well. Following 5 minute incubation at room temperature to convert all NAD+ to NADH, 10 µL NADH developing solution were added to each well. OD$_{450}$ was measured after color was allowed to develop for 1 hour at room temperature. The difference between total NAD and NADH yielded the concentration of NAD+.

**Statistical analyses**

GraphPad Prism was used to conduct paired two-tailed t tests for qrt-PCR, Western blots, and NAD quantification. In all cases, $p < 0.05$ was considered significant. All experiments were repeated at least three times independently of one another.
RESULTS

**Loss of Apex1 protein following MO knockdown is detectable after the MBT**

MO knockdown of a gene does not eliminate protein already present in the embryo, it only prevents the synthesis of new proteins. Amount of protein in the embryo will decrease over time as extant protein is turned over. To determine the rate at which Apex1 protein was lost following TS-MO microinjection, and to identify the relevant embryonic stages to examine for further experiments, control and hypomorphant embryos were immunoblotted for Apex1 at the dome, shield, and 75% epiboly stages. While these stages typically correspond to 4.3, 6, and 8 hpf, respectively, embryos were only harvested when the morphology matched the desired stage. Apex1 was lost in a linear fashion, beginning at around the dome stage (Fig. 1). By 75% epiboly, the hypomorphant only had 46% as much Apex1 protein as did the control.

To confirm that there was no loss of Apex1 prior to the MBT, the line was extended back to the time point when the amount of Apex1 in the hypomorphant reached 100% relative to the control, which was calculated to be 2.7 hpf, corresponding to the 256-cell stage. Western blot for Apex1 of embryos at this stage verified that prediction: loss of Apex1 was not detectable at this stage (Figure 1).

**Expression of Creb1 transcript and protein are enhanced following the MBT**

Prior studies from this laboratory showed that knockdown of Apex1 results in a loss of Creb1 transcript and protein at the pharyngula stage (24 hpf). We wanted to determine whether and how Creb1 loss might follow loss of Apex1 protein. To calculate normal Creb1 expression and identify the stages at which it might fail to be properly expressed following Apex1
knockdown, RNA and protein levels of non-injected embryos were examined by qrt-PCR and Western blotting, respectively. Transcript and protein were both present in recently-fertilized embryos, and expression of both increased following the MBT. There was reproducible loss (~30-40%) in both transcript and protein between the dome and 50% epiboly stages, but expression recovered by the shield stage and continued to increase through 75% epiboly. By this stage, embryos possessed four times as much creb1 transcript as they did at 1 hpf, and seven times as much protein (Figure 2A, C).

Because mRNA maturation plays an important role in zebrafish embryonic gene expression, polyadenylation of transcript over time was also observed with qrt-PCR on cDNA derived from oligo-(dT)18 primers (87). As displayed in Figure 2B, polyadenylated message increased between fertilization and 50% epiboly, but at a relative magnitude lower than the total message. Polyadenylated message then decreased between the shield stage and 75% epiboly, although it remained higher than 1 hpf embryos.

**Creb1 protein is lost in Apex1 hypomorphants concurrently with Apex1**

After determining that Apex1 protein is not lost in the hypomorphant until after the MBT, and that expression of Creb1 is normally upregulated at the MBT, I then asked how Creb1 expression is altered in the Apex1 hypomorphant. As depicted in Figure 3, Western blotting revealed that like Apex1, there was no change in Creb1 protein in hypomorphants at the dome stage. However, protein levels dropped significantly over the next two hours. At the shield stage, Apex1 hypomorphants had only 37% Creb1 protein relative to controls, and this decrease failed to recover through the 75% epiboly stage.
Knockdown of Apex1 does not affect embryonic morphology

Following microinjection of TS-MO to partially knock down Apex1, embryos were observed over time to identify any prominent morphological changes. At these early stages, no changes could be detected. Hypomorphants and controls progressed through the cleavage and blastula stages at the same rates, began gastrulation at the same time, and displayed no differences in the rate of epiboly (Figure 4).

Knockdown of Apex1 alters the transcriptional profile of the embryo

The Strauss lab has previously demonstrated that Apex1 knockdown leads to a loss of Creb family transcripts and proteins, which includes Cbp, Crem, Crtc1 and Crtc3 by 24 hpf (23). As explained above, the loss of Creb1 protein in Apex1 hypomorphants occurs early in embryogenesis, between the dome and shield stages. In order to determine whether Apex1 knockdown leads to other transcriptional changes between the dome stage and 75% epiboly, I performed qrt-PCR on total RNA extracts to examine transcript levels of Creb family genes (creb1, crem, cbp, crtc1, and crtc3), as well as three genes involved in BER (apex1, ogg1, and parp1). I also included seven genes that were identified by the Affymetrix GeneChip Zebrafish Genome Array as altered after Apex1 knockdown at the shield stage. The Strauss lab performed this microarray in collaboration with the MIT Center for Environmental Health Studies in 2008. Three of these genes were determined to be downregulated at the shield stage after Apex1 knockdown: the chromatin remodeler and transcription factor hmgb3a, the mesodermal transcription factor mespa, and the stress response protein gpx4b. The other four were upregulated according to the microarray: the stress response chaperone hsp70, the zebrafish ortholog of histone H2B hist2h2l, and the transcription factors fos and jun.
At the dome stage, qrt-PCR revealed only two significant changes in the Apex1 hypomorphants: a 2-fold increase of \textit{crtc1}, and a sixfold increase of \textit{hist2h2l}. Message of \textit{hsp70} was also increased 5-fold, but statistical significance could not be confirmed (Figure 5A).

At the shield stage, \textit{crtc1} message remained enhanced in the hypomorphant, up 3-fold from the control. Message for \textit{crem} and \textit{fos} were also upregulated, at 2.5- and 4-fold, respectively. Transcript of \textit{jun} was up about 4-fold on average, although statistical significance could not be confirmed. Message of \textit{hsp70} and \textit{hist2h2l} were particularly striking. Transcript for \textit{hsp70} was increased 29-fold in the Apex1 hypomorphant, and \textit{hist2h2l} up more than 26-fold (Figure 5B, C).

The qrt-PCR results for 75\% epiboly were very different than those from the earlier stages. At this stage, there was no significant difference between Apex1 hypomorphants and controls for \textit{crtc1}, \textit{crem}, \textit{hist2h2l}, \textit{fos}, or \textit{jun}. While the latter three were on average higher in the hypomorphants, the changes were not statistically significant. Message for \textit{hsp70} was about 3-fold higher in the hypomorphant, while messages for \textit{ogg1}, \textit{hmgb3a}, and \textit{mespa} were increased two-fold or less (Figure 5D).

No change to \textit{creb1} transcript at any stage was observed even though the protein levels decreased at the shield and 75\% epiboly stages, nor to its partners \textit{cbp} and \textit{crtc3}. Likewise, none of the three BER genes were affected by partial Apex1 knockdown, with the exception of a very minor increase of \textit{ogg1} at 75\% epiboly.

\textbf{Apex1 knockdown does not affect polyadenylation of pre-mRNA}

Partial Apex1 knockdown results in a loss of Creb1 protein by the shield stage, but not \textit{creb1} transcript. To investigate the possibility of a post-transcriptional effect, qrt-PCR for \textit{creb1},
cbp, and mespa using cDNA derived from oligo-(dT)$_{18}$ primers was performed. However, there was no observed change to either creb1 or mespa polyadenylated transcript at the dome stage, and a statistically significant but very minor increase for cbp (Figure 6A). At the shield stage, cbp again was up on average in the hypomorphant, but it was not statistically significant. Similarly, polyadenylated transcript of creb1 and mespa were down on average, but also not statistically significant (Figure 6B). There was no noticeable change to any of these three genes at 75% epiboly (Figure 6C).

**Zebrafish embryos do not express Hsp70 protein at early stages**

Transcript of hsp70 increased significantly following partial knockdown of Apex1. To investigate changes in protein expression of Hsp70, an antibody against human Hsp70 was obtained. To test the antibody’s suitability for zebrafish protein, 4 and 24 hpf embryos were heat shocked for one hour at 37°C, and then protein extracts were immunoblotted for Hsp70. As displayed in Figure 7, no protein was detectable in the 5 hpf control embryos (lane 8), and one hour of heat shock induced only minor expression (lane 7). At 25 hpf (lanes 9 and 10), some protein was present in the control, and it increased substantially after heat shock.

To determine expression of Hsp70 protein following partial Apex1 knockdown, hypomorphant and control protein extracts were immunoblotted, but no protein was detected in either sample at any of the three stages examined (Figure 7 lanes 1-6).

**Minor deregulation of ribosomal genes follows Apex1 knockdown**

The reduction of Creb1 protein levels in hypomorphants without parallel loss of creb1 transcript (total or polyadenylated) suggests that Apex1 regulation of Creb1 may be at the
translational level, and that diminished Creb1 protein may be caused by means similar to the one that represses Hsp70 translation. In addition, reports that Apex1 may be capable of cleaving RNA require us to pay close attention to the status of ribosomes after Apex1 knockdown (59, 94). Our microarray identified three genes for ribosomal proteins that were downregulated at the shield stage following Apex1 knockdown: rpl21, rpl35, and rps15. Diminished expression of these genes could affect ribosomal assembly and thereby explain the reduced Creb1 protein. The expression of these genes was examined by qrt-PCR. On average, all three genes were reduced at the dome stage (Figure 8A). However, fold changes varied between the replicate experiments, so statistical significance could not be confirmed. At the shield stage and 75% epiboly, only transcript of rpl35 was still reduced in the hypomorphant, but the change was still not significant. There was no change to transcripts of rpl21 or rps15 at these stages (Figure 8B, C).

Transcription of the other core histones and two histone deacetylases are not affected by partial Apex1 knockdown

The staggering increase of hist2h2l raised the question of whether expressions of the other core histones were also affected by partial Apex1 knockdown. To investigate this possibility, primers for the other three core histones (h2afx/H2A, h3f3b.1/H3, and hist1h4l/H4) and for two histone deacetylases (hdac1 and sirt1) were designed and used for qrt-PCR. Partial Apex1 knockdown did not affect the expression of any of these genes between the dome and 75% epiboly stages, however (Figure 9).

Apex1 knockdown alters NAD+/NADH ratio by 75% epiboly

Partial knockdown of Apex1 causes increased oxidative stress in the embryo by the
pharyngula stage, as evidenced by increased levels of 8-oxodG, AP sites, and thioredoxin (Pei, et al., unpublished data). Another indication of oxidative stress is an increased ratio of NAD+ to NADH; increased oxidized NAD+ at the expense of reduced NADH signifies oxidative stress, DNA damage response, and/or impaired metabolism (95–99). Additionally, a recent report in the literature suggests that the NAD+/NADH ratio regulates the binding of transcription factors to $h2b$ loci in human cells (95, 100, 101). Therefore it seemed likely that Apex1 knockdown might perturb the natural NAD+/NADH ratio in favor of the oxidized form, and that this alteration would be detectable at early stages.

When concentrations of NAD+ and NADH in Apex1 hypomorphant and control embryos were measured at the dome, shield, and 75% epiboly stages, the results were the opposite of what was expected. The NAD+/NADH ratio was not different between hypomorphant and control at the dome and shield stages, but was significantly higher in the hypomorphant at 75% epiboly (Figure 10). Therefore, increase in NAD+ at the expense of NADH did not precede the increase of $hist2h2l$ message, but rather followed it.

**Apex1 knockdown enhances monoubiquitination of histone H2B at Lys$^{120}$**

Partial knockdown of Apex1 results in a substantial increase of message for the zebrafish H2B ortholog, $hist2h2l$, but without any change to the messages of the other core histones (Figures 5, 9). This change is only relevant to the cell if there is also a change in translation of the H2B protein or its modifications. The best characterized modification to H2B is monoubiquitination at Lys$^{120}$, catalyzed by the E3 ligase ring finger protein 20 (Rnf20) (102). This modification is generally associated with increased transcription of the loci on which it is enriched (103–107). Since upregulated transcription of several genes, including $hist2h2l$, is
observed following Apex1 knockdown, monoubiquitination of H2B was investigated in hypomorphants and controls. At the high and sphere stages (3 and 4 hpf, respectively), very little H2BK120ub1 was detected in either embryo. However, a 7-fold increase in the hypomorphant was observed at the shield stage, concurrent with the nearly 27-fold increase of the message (Figure 11).
**FIGURES**

**Figure 1:** Loss of Apex1 following TS-MO knockdown is detectable after MBT. Quantification of Western blot for Apex1 following microinjection of 0.23 mM Apex1 TS-MO. Each point represents the relative amount of Apex1 protein in the hypomorphant compared to the control in each individual experiment. Below is a representative blot for each time point.
**Figure 2**: Dynamic expression of Creb1 during normal early zebrafish embryogenesis. Data were generated from three independent experiments, error bars represent SEM, and * indicates p < 0.05 as determined by paired two-tailed t test. See Table 2 for details.

**A.** qrt-PCR quantification of creb1 message derived from total RNA during the first 8 hpf of zebrafish embryogenesis, relative to 1 hpf (2-4 cells). β-actin was used as a housekeeping control.

**B.** Polyadenylated creb1 message (mature message) during the first 8 hpf relative to 1hpf, as quantified by qrt-PCR. As above, β-actin served as a housekeeping control.

**C.** Quantification of Creb1 protein. Equal amounts of protein were resolved on a 4-20% SDS polyacrylamide gel and then transferred to a PVDF membrane for Western blot. β-tubulin was used as a loading control. Below is a representative Western blot.
Figure 3: Creb1 protein decreases following Apex1 knockdown, as determined by Western blot. Representative blot is show below. Data presented are the average ± SEM of three independent experiments. * represents p < 0.05 as determine by paired two-tailed t-test. See Table 2 for details.
Table 2: Paired two-tailed t test for Creb1 expression as determine by qrt-PCR and Western blot. See Figures 2 and 3.

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<th>p value</th>
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**Figure 4:** Apex1 knockdown does not affect general morphology or gastrulation through 75% epiboly. Hypomorphant and control embryos were photographed at the same time points. 31X magnification.
**Figure 5:** Following Apex1 knockdown, transcription of several genes is altered at the dome stage (A), shield stage (B, C), and 75% epiboly (D). Data presented are the average ± SEM of three independent experiments (four for dome). * indicates p < 0.05, and ** indicates p < 0.01 as determined by paired two-tailed t test. See Tables 3, 4, and 5 for all t and p values.
Figure 6: Polyadenylation of select transcripts is not altered following Apex1 knockdown. (A) dome stage, (B) shield stage, (C) 75% epiboly. Data were generated from three independent experiments, error bars represent SEM, and * indicates p < 0.05 as determined by paired two-tailed t test. See Tables 3, 4, and 5 for all t and p values.
Figure 7: Translation of Hsp70 following heat shock or Apex1 knockdown. No protein was detected before 75% epiboly except after a one hour heat shock at 37°C (lanes 1-8). Hsp70 is nominally expressed in pharyngula-stage embryos, and is considerably upregulated following a one hour heat shock (lanes 9-10).
**Figure 8:** Apex1 knockdown may affect ribosomal production. Message for three ribosomal genes were measured with qrt-PCR for three stages: (A) dome, (B) shield, (C) 75% epiboly. Data were generated from three independent experiments; error bars represent SEM. See Tables 3, 4, and 5 for all t and p values.
**Figure 9:** Apex1 knockdown does not affect transcription of other core histones or two major histone deacetylases. (A) dome stage, (B) shield stage, (C) 75% epiboly. Data were generated from three independent experiments, error bars represent SEM, and * indicates $p < 0.05$ as determined by paired two-tailed t test. See Tables 3, 4, and 5 for all t and p values.
Figure 10: Apex1 knockdown significantly increases the NAD+/NADH ratio at 75% epiboly. Data were generated from three independent experiments, and error bars represent SEM. Below are details of the t test.

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Figure 11: H2BK120ub1 is significantly upregulated after the MBT in Apex1 hypomorphants. Data were generated from three independent experiments, error bar represents SEM, and * indicates $p < 0.001$ as determined by paired two-tailed t test. Below are a representative Western blot and details for the t test.

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<th>Embryonic stage</th>
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Table 3. Results of paired two-tailed t tests for qrt-PCR at dome stage. Refers to Figures 5, 6, 8, and 9.

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Table 4. Results of paired two-tailed t tests for qrt-PCR at shield stage. Refers to Figures 5, 6, 8, and 9.

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Table 5. Results of paired two-tailed t tests for qrt-PCR at 75% epiboly. Refers to Figures 5, 6, 8, and 9.

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DISCUSSION

Overview

Cells are continuously exposed to chemicals and energies that are harmful to DNA. The damage that these agents cause must be quickly and accurately repaired in order to ensure cell survival (4, 5, 108). Ancient life evolved several response systems to DNA damage which are highly conserved amongst all extant organisms (6–8, 13, 109). Among these is BER which identifies, excises, and replaces small non-bulky lesions. Loss of any one of many genes involved in this pathway is detrimental to the eukaryotic cell, and in many cases is also lethal to developing vertebrate embryos (24, 66, 67, 70–72, 79). AP endonuclease 1, which removes the highly toxic AP site generated by the glycosylase that initially recognizes the lesion, is of particular interest to our lab. Not only is Apex1 knockout an embryonic lethal in mice and zebrafish, but to date no viable knockout cell cultures have been made, suggesting that Apex1 loss cannot be tolerated (23, 56–58). More specifically, it is the endonuclease function of Apex1 that is vital and not the recently evolved redox function, because knockdown of Apex1 by RNAi or MO can be rescued by endonuclease-competent but redox-incompetent Apex1 (56, 58, 80).

The zebrafish model is a particularly powerful tool for investigating early embryogenesis, and our lab has successfully used it to uncover the consequences of Apex1 loss. Brain and heart development abnormalities follow partial knockdown of Apex1, detectable as early as 24 hpf, which may be the result of deregulated Creb1, an important transcription factor that regulates approximately one quarter of the mammalian genome (18, 19, 21, Pei et al, unpublished data). The goal of my project, therefore, was to identify the earliest detectable genetic, biochemical, and morphological changes to the zebrafish embryo following MO knockdown of Apex1 in order to elucidate the causes of knockout lethality. Specifically, I wanted to determine if Creb1
deregulation is one of the first responses. I observed the following:

1. Maternal Apex1 protein is cleared from the embryo after the MBT, to be replaced by zygotic Apex1 afterwards;
2. Expression of Creb1 is upregulated following the MBT on both the transcriptional and translational levels, but the pattern of message maturation is not consistent with total transcription;
3. Following partial Apex1 knockdown, Creb1 protein fails to be upregulated at the MBT; however, knockdown affects neither total transcription nor polyadenylation of the message;
4. The overall morphology of the Apex1 hypomorphant is not different from control embryos during the first 8 hpf;
5. Several transcripts are upregulated in the Apex1 hypomorphant as early as the dome stage, including *hsp70* and *hist2h2l*, although they approach control levels by 75% epiboly;
6. Loss of Apex1 does not affect polyadenylation of select transcripts;
7. Although *hsp70* message is significantly upregulated early following Apex1 knockdown, no protein is detectable;
8. Transcripts of three ribosomal genes experience delayed expression following Apex1 knockdown;
9. Transcription of the core histones besides *hist2h2l* as well as two well-known histone deacetylases is not affected by Apex1 knockdown;
10. The ratio of oxidized NAD+ to reduced NADH increases in the hypomorphant by 75% epiboly, but not earlier; and
11. Knockdown of Apex1 leads to a substantial increase in histone H2B monoubiquitination on Lys\textsuperscript{120} by the shield stage, but not earlier.

**Partial Apex1 knockdown results in a loss of the protein following the MBT**

Full knockdown of Apex1 is not lethal until the MBT, which is the point in embryogenesis when the embryo starts to clear maternal gene products and instead rely on its own genome (58, 86). Therefore, it stands to reason that there is a shift of reliance on maternal Apex1 to zygotic Apex1 at the MBT, and that the MO prevents accumulation of zygotic Apex1 even as the maternal protein is cleared. To test this hypothesis, 0.23 mM Apex1 TS-MO was microinjected into embryos, and then protein extracts from three stages past the MBT were immunoblotted for Apex1: dome (4.3 hpf), shield (6 hpf), and 75% epiboly (8 hpf). An insignificant amount of protein was lost at the dome stage, but the decrease proceeded in a linear fashion, resulting in a loss of nearly half of the protein by 75% epiboly. The line was then extended back to the point when Apex1 in the hypomorphant reached 100% relative to the control, which was determined to be 2.7 hpf, corresponding to the 256-cell stage. As predicted, there was no difference in Apex1 protein between hypomorphants and controls at this pre-MBT stage.

Extracts from pre-MBT full Apex1 knockdown embryos perform BER *in vitro*, which has been explained by the abundance of Apex2, a relative of Apex1 that mostly appears to be redundant in function (62, 65). The results above suggest an alternative explanation. It is possible that even though the dosage of TS-MO is high enough to completely abrogate translation of new Apex1 protein, there is enough maternally deposited protein to maintain BER early in development. Consistent with transcriptomic data, the MBT marks a distinct shift from maternal
to zygotic protein, as evidenced by the decaying amount of Apex1 in the partial hypomorphant after the MBT (Figure 1) (86–88, 110).

*Creb1 is dynamically expressed through early embryogenesis*

Gene expression during embryogenesis must be tightly regulated to ensure the proper deployment of developmental programs. Perturbation of developmentally important genes can have substantial effects on the maturation of the organism. Reduced expression of Creb1 and Creb-controlled neurodevelopmental genes following Apex1 knockdown raises the hypothesis that loss of Apex1 is lethal through Creb1 deregulation. To better understand the natural context for Creb1 expression, embryos at several early stages were examined for RNA and protein expression of the gene. As displayed in Figures 2A and C, recently fertilized embryos possess both RNA and protein for Creb1, and the levels of both increase following the MBT. They then suffer a small loss as gastrulation begins, which may represent clearance of maternal transcript and protein without concomitant expression of zygotic Creb1, but quickly recover as gastrulation proceeds.

A recent investigation of zebrafish embryological transcriptomics by RNA-seq revealed a subset of genes that are upregulated following fertilization, but qrt-PCR of total RNA extracts could not confirm the results. When the authors reversed transcribed the RNA using oligo-(dT)$_{18}$ primers so they could quantify just mature, polyadenylated message, the qrt-PCR results matched the RNA-seq data (87). The authors concluded that zebrafish embryos regulate maturation of RNA in order to temporally control gene expression before activation of their own genome at the MBT. To determine if *creb1* maturation is regulated separately from its transcription, qrt-PCR of polyadenylated message was also performed. Interestingly, my results did not mirror those of the
total transcript (compare Figures 2A and B). While there was a significant upregulation of polyadenylated message following the MBT, it peaked soon after, and then began to decline, but by 75% epiboly was still higher than at 1 hpf. This suggests that only a portion of creb1 transcript at any given stage is fully matured. The early upregulation of transcription and mRNA maturation may serve to permit rapid translation of protein at later stages.

To our knowledge, this is one of the first determinations of Creb1 expression during vertebrate embryogenesis. One previous study examined the presence of the protein in pre-implantation mouse embryos. The authors did not quantify expression of the transcript or protein, but did demonstrate that both the phosphorylated and non-phosphorylated forms of Creb1 localized to the nucleus in two waves: once at the two-cell stage, and then once again at the eight-cell stage (111). Although implantation into the uterine wall in placental mammals corresponds to the same embryonic stage as the zebrafish MBT, the zygotic genome is actually activated by the two-cell stage for mice, which is much earlier than for teleosts (112). Therefore for mice, nuclear localization of phospho-Creb1 may represent the beginning of Creb-controlled gene expression. It remains to be seen if zebrafish Creb1 is present in the nucleus prior to the MBT, and if and when phosphorylation of Creb1 changes during embryogenesis.

Loss of Apex1 results in a rapid depletion of Creb1

As mentioned previously, our lab has demonstrated a regulatory relationship between Apex1 and Creb1, and MO knockdown of Apex1 results in substantially reduced expression of Creb1 and its partners by the pharyngula stage (23). The question remains if loss of Creb1 is an early consequence of Apex1 or if it is the result of prior events and does not occur until a later stage. To answer this question, the same extracts used to quantify Apex1 protein in
hypomorphants were immunoblotted for Creb1. No loss was detected at the dome stage, which was expected because there was almost no loss of Apex1 protein at that stage. However, Apex1 hypomorphants displayed a nearly 70% reduction of Creb1 at the shield and 75% epiboly stages, when hypomorphants possessed 66% and 46% Apex1 relative to controls, respectively (Figures 1, 3).

These results are best explained when considered along with the normal expression of Creb1 as depicted in Figure 2. Normally, protein production increases immediately following the MBT. The decrease at 50% epiboly may represent removal of maternal product without simultaneous production of zygotic Creb1, but the embryo quickly upregulates expression again before the shield stage. The Apex1 hypomorphant displays the same amount of Creb1 as the control at the dome stage, so the brief initial production still occurs. This is expected considering there is almost no loss of Apex1 yet. But by the shield stage, when the hypomorphant has begun to lose Apex1, the second upregulation of Creb1 is vastly diminished, so it possesses only approximately one-third of the Creb1 as the control. This loss of production is not recoverable (23). Deregulation of Creb1 protein, then, appears to be a very early effect of Apex1 knockdown, which lends further support to the hypothesis of Creb1 dependence on Apex1.

**Morphology of early embryos is not altered following Apex1 knockdown**

Partial knockdown of Apex1 causes brain and heart abnormalities that are detectable in pharyngula-stage embryos (58). The brain derives from the ectoderm whereas the heart and notochord, which secretes some of the signals that induce neurogenesis, derive from the mesoderm. In zebrafish, the germ layers begin to differentiate after the shield stage (113). Following the observation that Creb1 protein is markedly decreased in hypomorphants also at the
shield stage, I then asked if there are any detectable morphological changes to early embryos. However, hypomorphant and control embryos appeared identical through 75% epiboly (Figure 4). These observations may just be a weakness of the method; the germ layers are not visually distinguishable from each other, so changes may simply not be visible with light microscopy. In situ hybridization or immunohistochemical staining of germ layer markers may be necessary in order to visualize morphological changes in Apex1 hypomorphants prior to segmentation.

**Apex1 knockdown results in an altered transcriptional profile**

The proteomic and morphological changes in pharyngula-stage Apex1 hypomorphants are correlated with transcriptomic changes (23). To determine if there are also early transcriptomic changes, especially to creb1 and its partners, qrt-PCR of fifteen genes was performed for the three stages. These genes included the Creb family (creb1, crem, cbp, crtc1, and crtc3), three BER genes (apex1, ogg1, and parp1), as well as seven genes identified by a microarray as altered after Apex1 knockdown: hsp70, hist2h2l, fos, and jun which were upregulated, and gpx4b, hmgb3a, and mespa which were downregulated.

Since there was no change to Apex1 or Creb1 protein levels at the dome stage, no RNA fold changes were predicted. However, transcripts for crtc1 and hist2h2l were significantly increased in the hypomorphant, as was message for hsp70, although that change was not statistically significant. These transcripts remained increased at the shield stage, and were joined by crem, fos, and hsp70. Message for jun was also increased on average, but the results were not statistically significant. By 75% epiboly, all of these messages returned to control levels except for hsp70, which remained increased threefold. A very small but statistically significant increase was also observed at this stage for ogg1, hmgb3a, and mespa (Figure 5).
Overall, I could not confirm all of the results of the microarray. While *hsp70*, *hist2h2l*, *fos*, and maybe *jun* transcripts were increased in the Apex1 hypomorphant at the shield stage, *hmgb3a*, *mespa*, and *gpx4b* were not changed. Message of *apex1* also did not change, which was expected since the MO does not promote degradation of the target transcript, and there is no evidence that Apex1 positively regulates its own transcription (114).

The observed changes fluctuated between each stage. The results for *hsp70* and *hist2h2l* were the most dramatic. Transcript levels were up even at the dome stage, which is when Apex1 protein has only just started to decrease in the hypomorphants. These messages spiked at the shield stage, but fell back to near-control levels by 75% epiboly. These results suggest that either the Apex1 hypomorphant’s *hsp70* and *hist2h2l* message decays between the shield stage and 75% epiboly, or transcription is enhanced in the control and meets the levels of the hypomorphant by 75% epiboly.

Perhaps the most surprising result is that *creb1* transcript did not change at all in the hypomorphants during these stages, even though the protein levels were greatly reduced. Loss of Creb1 protein then precedes the loss of *creb1* message evident at pharyngula. This is reasonable because Creb1 regulates its own transcription by virtue of the Creb consensus sequence in its own promoter (115). So at this early stage, Apex1 regulation of Creb1 must be at the post-transcriptional level.

*Apex1 does not regulate polyadenylation of creb1 transcript*

Apex1 may post-transcriptionally regulate *creb1* by affecting maturation of the message. The time course of *creb1* RNA as determined by qrt-PCR (Figure 2B) demonstrated that there is an increase in polyadenylated *creb1* message following the MBT. The loss of Creb1 protein in
Apex1 hypomorphants, then, may be the result of impeded polyadenylation during mRNA maturation. To test this hypothesis, I reverse transcribed Apex1 hypomorphant and control RNA using oligo-(dT)\textsubscript{18} primers rather than the random hexamer primers used in the previous section, and then examined polyadenylated mRNA fold changes of \textit{creb1}, \textit{cbp}, and \textit{mespa} with qrt-PCR.

The results did not strongly support the hypothesis. Mature message for \textit{creb1} and \textit{mespa} remained unchanged at the dome stage in hypomorphants, although there was a very minor but statistically significant increase for \textit{cbp}. Similarly at the shield stage, though the fold changes for \textit{creb1} and \textit{mespa} in the hypomorphant were on average lower than the control, and higher for \textit{cbp}, they were not enough to be considered statistically significant. By 75\% epiboly, \textit{creb1} was decreased on average relative to the control, but not significantly so.

Due to the variation between replicate experiments, I cannot say for certain that the failure of Creb1 protein to rise at the shield stage is due to inhibited polyadenylation of \textit{creb1} message. Polyadenylation of transcripts of two other genes, one in the Creb family (\textit{cbp}) and one predicted by the microarray to decrease (\textit{mespa}), also did not appear to change following Apex1 knockdown. Apex1 therefore does not seem to play a role in mRNA maturation of these genes.

\textit{Hsp70 is not expressed in early embryos even after enhanced transcription}

One of the first responders to Apex1 knockdown was \textit{hsp70}, which experienced a 6-fold increase relative to the control at the dome stage, and increased to a 29-fold difference at the shield stage (Figure 5A, C). This suggests that the hypomorphants were encountering stress that threatened cellular stability, as Hsp70 is a chaperone that repairs protein misfolding and prevents protein aggregation due to heat, heavy metal exposure, reactive oxygen species, and other
stressors (116–119). In addition, Hsp70 is required for proper lens development in zebrafish (117, 120). Therefore, deregulation could have significant effects for young embryos.

Protein extracts were immunoblotted for Hsp70 to determine if enhanced translation correlated with the observed enhanced transcription. However, no protein at all was detectable at any stage in either the hypomorphants or the controls (see Figure 7, lanes 1-6). The absence of protein in the controls is consistent with a previous report that levels of Hsp70 protein are very low prior to hatching (121).

Extracts from 30% epiboly and pharyngula stage embryos, which had either been raised at the standard incubation temperature of 29°C or heat-shocked for 1 hour at 37°C, were also analyzed to ensure that the anti-human antibody could recognize the zebrafish protein. Both pharyngula samples reacted with the antibody, and the heat-shocked sample predictably displayed a substantial increase relative to the control (Figure 7, lanes 9 and 10). No protein was detectable in the 30% epiboly control, and very little was detected for the heat-shocked sample (Figure 7, lanes 7 and 8). The amount of heat-shocked induced Hsp70 at 30% epiboly was far less than the protein displayed by the control pharyngula extracts.

The lack of Hsp70 protein in early embryos is reminiscent of normal expression of Polb in zebrafish embryos; whereas the unfertilized egg is loaded with maternal polb message and transcription increases after fertilization, no protein is detectable until 13 hpf (23, 65). Some unidentified factors are likely inhibiting the translation of these actively transcribed genes. In mammalian cells, PI3K-mTORC1 signaling negatively regulates Hsp70 translation even when there is ample message, so a similar system be at work in zebrafish (122).
Transcription of select ribosomal genes is delayed in Apex1 hypomorphants

The above results suggest another explanation for the reduced Creb1 protein in hypomorphants: repression of translation. The system(s) that prevents early translation of Polb and/or Hsp70 may be regulated by Apex1, and therefore may become deregulated following loss of Apex1. Another look at our microarray revealed that three genes that code for ribosomal proteins are downregulated in hypomorphants: rpl21, rpl35, and rps15. If these genes are not being properly expressed, then ribosomes may not be properly assembled, and global translation may thereby be inhibited. Additionally, one report in the literature posits that Apex1, regulated by nucleophosmin 1 (Npm1), is required for rRNA quality control; Apex1 silencing by RNAi increases the levels of oxidative lesions in rRNA, which in turn curtails translation (94).

Analysis of transcription of these genes by qrt-PCR did not strongly support this hypothesis, however. At the dome stage, transcription of all three genes was on average lower in the hypomorphants than the controls, but variation between replicate experiments was too high to confirm statistical significance. At the shield and 75% epiboly stages, transcripts for rpl21 and rps15 were not different from that for the controls, and rpl35 was slightly decreased, although again statistical significance could not be confirmed (Figure 8).

If these results reflect a relevant phenomenon, then Apex1 may be required for the ZGA. Presumably, one of the embryo’s major requirements for genetic autonomy is the assembly of translational machinery. Microarray and sequencing data of genes upregulated at the MBT support this contention (88). The results in Figure 8 suggest that rather than abrogate transcription of these ribosomal genes, Apex1 knockdown just delays their expression, as evidenced by the rise of rpl21 and rps15 message to control levels by the shield stage. This delay
could hinder ribosomal assembly, so hypomorphants miss the critical second upregulation of Creb1 translation that normally occurs between 50% epiboly and shield (Figure 2C).

Much stronger evidence for this hypothesis is required. Ribosomes are intricate structures that consist of several RNA complexes and over seventy distinct proteins (123). The levels of both message and proteins of ribosomal genes in hypomorphants need to be examined. Perhaps more importantly, the actual process of ribosomal assembly in hypomorphants and controls at different stages needs to be investigated (124). Even if there is a difference between hypomorphants and controls in the expression of ribosomal genes, there will only be any actual physiological relevance if altered expression affects the building of these organelles.

**Apex1 knockdown does not affect the transcription of the other core histones or two histone deacetylases**

The other transcript that experienced a substantial increase very early after Apex1 knockdown was hist2h2l, which encodes histone H2B. The staggering early increase of hist2h2l expression suggests that knockdown of Apex1 may affect chromatin structure quite early after the MBT. If the expression of all core histones increases following Apex1 knockdown, then improper formation of nucleosomes may affect expression of multiple other genes (125). The transcripts of the other core histones were examined by qrt-PCR. Surprisingly, no difference between hypomorphants and controls was observed for genes encoding H2A, H3, or H4 between dome and 75% epiboly stages (Figure 8). This experiment may not have been entirely representative of histone transcripts, though. In mammals, each of the four core histones is encoded by multiple distinct open reading frames (126). ZFIN identifies five genes each for H2A and H3, and thus far only one each for H2B and H4 (127). The paralogs have divergent
sequences due to codon wobbling, so the primers designed for PCR only examined one single gene. It is possible that the single genes examined (h2afx for H2A and h3f3b.1 for H3) were not altered by Apex1 knockdown, whereas one or more of the others were. It is also possible that the transcription for each locus changed only slightly, but the cumulative effect was significant.

Two well-characterized histone deacetylases, hdac1 and sirt1, were also included in this study. MO knockdown of Hdac1 in zebrafish has been demonstrated to abrogate expression of genes involved in neurogenesis, whereas MO knockdown of Sirt1 in zebrafish hinders proper formation of blood vessels (128, 129). However, neither of these genes was affected by Apex1 knockdown, except for a very minor but statistically significant increase of hdac1 at the dome stage, which was lost by the shield stage.

**Apex1 knockdown increases NAD+/NADH ratio at 75% epiboly**

The ratio of oxidized NAD+ to reduced NADH in Apex1 hypomorphant and control embryos was examined for two reasons. Firstly, Yan Luo at the Institute of Molecular and Cell Biology in Singapore has reported that increasing the amount of NAD+ relative to NADH enhances transcription of h2b genes in human cells (95, 100, 101). Secondly, the ratio may have implications for the redox status of the embryo, as well as its energy production capabilities and DNA damage response (96–98, 130). An alteration of the NAD+/NADH ratio early in development shortly following Apex1 knockdown could then explain the increase in hist2h2l transcription without the concomitant increase of the other core histones, and also hint at biochemical changes that the hypomorphant encounters.

As displayed in Figure 10, however, there was no difference in this ratio between hypomorphants and controls at the dome and shield stages, when the hypomorphants displayed
the most substantial increase in hist2h2l message. The ratio did significantly increase in hypomorphants relative to controls at 75% epiboly, which may suggest an increase in cell stressors or incapacitated metabolism, but this is a later consequence of Apex1 knockdown. The altered NAD+/NADH ratio followed hist2h2l upregulation, so it cannot be considered a causal agent in the early changes following Apex1 knockdown.

Apex1 knockdown results in a substantial increase of monoubiquitination of histone H2B at Lys^{120}

Apex1 hypomorphant and control protein extracts before, during, and after the MBT were immunoblotted for the primary modification to histone H2B, monoubiquitination at Lys^{120}, in order to determine if there was any physiological relevance to increased hist2h2l message. This modification is generally associated with enhanced transcription of associated loci, and is required for trimethylation of histone H3 at Lys^{4} and Lys^{79}, which also stimulates transcription (103–107). Recent evidence also implicates these modifications in the repair of double strand DNA breaks by HR or NHEJ, independently of their function as transcriptional enhancers, possibly by opening chromatin to grant access to repair enzymes (131, 132).

Very little H2BK120ub1 was detectable in either hypomorphant or control embryos at the high or sphere stages (3 and 4 hpf, respectively), while shield stage hypomorphants displayed a sevenfold increase of this modification (Figure 11). Sphere occurs immediately prior to dome, so presumably the hypomorphants at this stage were beginning to stimulate transcription of hist2h2l. However, it was not until the hypomorphant possessed more than twenty times as much hist2h2l message as the control that the monoubiquitination also increased, so this modification
is not the earliest responder. It does, however, appear when loss of Apex1 protein is detectable, which suggests that Apex1 may negatively regulate H2BK120ub1.

The increase in this modification may explain the increased transcription of select genes at the shield stage (Figures 5B, C). H2BK120ub1 stimulates transcription by signaling for H3K4me3, which allows the rapid dissociation and reassembly of the nucleosome as RNA polymerase 2 proceeds along the gene (106). Western blotting for H2BK120ub1 and qrt-PCR for fos, jun, crtc1, crem, hsp70, and hist2h2l at time points between the dome and shield stages will confirm if the modification precedes transcriptional upregulation of these genes, thereby driving their expression. Chromatin immunoprecipitation can also be performed to determine if these loci are enriched for this histone modification. The enhanced modification may also signal the accumulation of DNA damage in the hypomorphant, perhaps in the form of double strand breaks and not BER-mediated lesions or AP sites. As replication of lesions or AP sites can generate strand breaks, these types of damage may also be accumulating in the hypomorphant during the rapid cell division preceding gastrulation (133, 134).

Significance and Model

Endonuclease-competent Apex1 is vital for the survival of eukaryotic cells. Genetic knockout, artificial knockdown, and chemical inhibition are disastrous and often lethal for the cell or developing vertebrate embryo (23, 30, 56–58). I have identified several changes in early zebrafish embryos following MO knockdown of Apex1. Shortly after the MBT, which marks the beginning of gastrulation and activation of the zygotic genome, hypomorphants experience a significant increase in transcription of Creb-family member crtc1 and the histone hist2h2l. Transcripts for ribosomal genes also may fail to be upregulated. Several changes occur over the
next two hours as the embryo progresses into the shield stage, when the loss of Apex1 is first detectable: transcription of several more genes increases, with *hsp70* and *hist2h2l* experiencing the most substantial changes; Creb1 protein fails to be upregulated; and monoubiquitination of H2B at Lys\(^{120}\) increases sevenfold. By the time the embryo reaches 75% epiboly, most of the perturbed transcripts have recovered, but more than half of the Apex1 and Creb1 proteins have been lost, and the NAD+/NADH ratio has also risen significantly compared to controls.

These data support the hypothesis that loss of Creb1 is an early result of Apex1 knockdown, and that decreased Creb1 contributes to embryonic lethality. However the means by which Apex1 regulates Creb1 are still unclear. Total *creb1* message does not change during the stages examined, nor does polyadenylated message, so the regulation is probably at the translational level. The decreased transcription of three ribosomal genes prior to Creb1 loss may support this hypothesis, although the data are not particularly strong because statistical significance could not be confirmed.

The most surprising results are the differences observed at the dome stage: the increase in *hist2h2l* and *crtc1* messages, and the moderate decreases in ribosomal gene messages. These changes precede any detectable loss of Apex1 following MO knockdown. One explanation is that transcription of these genes is very sensitive to even minor perturbations to Apex1, and that Western blotting is not sensitive enough to detect such a small loss. Alternatively, while the amount of total Apex1 is not significantly different between hypomorphants and controls at this stage, maternal protein may be marked for clearance from the embryo, and these post-translational modifications inhibit the normal function of Apex1. The control embryos may be able to replace maternal with zygotic protein instantly, whereas the hypomorphants are slowed
down, and this inhibition then results in the observed transcriptional changes. Proper subcellular localization of Apex1 may be disrupted in the hypomorphant.

My results explain why the endonuclease function rather than the redox function of Apex1 is required for survival. Firstly, zebrafish lack the redox-sensitive cysteine residue, so such a reaction is irrelevant to this model (54). And secondly, I present indirect evidence of accumulation of cell stressors which, when taken together with previous studies, suggests that hypomorphants may suffer debilitating genetic damage. Hypomorphants display a substantial increase at the shield stage of H2BK120ub1, which is associated with DNA double strand breaks, as well as an increased NAD+/NADH ratio at 75% epiboly, which results from ROS that also cause oxidative DNA damage (130). The upregulation of hsp70 despite the absence of translation also suggests the accumulation of cell stressors. Our lab has previously shown that pharyngula-stage hypomorphants display increased incidence of 8-oxodG lesions and AP sites relative to controls, and that this damage can be traced back to elevated ROS during late gastrulation (Pei, et al., unpublished data). Direct evidence of ROS, 8-oxodG, AP sites, and/or DNA strand breaks at earlier stages is still required.

Based on the data that I have gathered along with evidence in the literature, I propose the following model to explain embryonic lethality following Apex1 knockdown in zebrafish (Figure 12). A hallmark of the ZGA is global chromatin remodeling by way of histone modifications. Opening up chromatin permits the transcription of zygotic genes so that the embryo may achieve genetic autonomy (110). One recent study identified changes of methylation patterns on histone H3 at the ZGA, which are followed by the initiation of transcription of genes involved in gene expression and metabolism (88). One of the two histone demethylase enzymes, Lysine-specific demethylase 1 (Lsd1) creates H₂O₂ as a byproduct of the
reaction, which damages the surrounding DNA. This damage must be repaired by BER, or the locus will not be properly transcribed (135). Therefore, I predict that the histone modifications at the ZGA necessarily result in oxidative damage to DNA which must be repaired by BER. With Apex1 expression inhibited by the MO, AP sites will accumulate rapidly, and the embryo will soon die because it cannot transcribe or replicate its genome.

Partial Apex1 knockdown results in a less potent scenario. Since the hypomorphants still possess control levels of Apex1 at the MBT, most of the damage will be reparable. But as the protein is cleared, BER becomes less efficient, so DNA damage accumulates, presumably at sites of highly active transcription, such as genes involved in regulating gene expression and metabolism. These genes can be thought of as primary responders to the ZGA, since they are required for progress through the MBT and for attaining genetic autonomy. Ribosomal genes may be included in this group. Following the deregulation of these genes, expression of genes involved in the initiation of gastrulation and differentiation may begin to suffer. These secondary responders, which include transcription factors to activate genes for differentiation, patterning and morphogenesis, may still be transcribed properly, but synthesis of the final product will be affected because of the absence of the primary responders. Finally, specific developmental programs will not be appropriately followed, which will result in organ abnormalities, specifically in the heart and brain.

Testing this model will require careful experiments to identify not only the presence of DNA damage in young embryos, but also the rate of damage accumulation and localization of damage throughout the genome. Furthermore, high-throughput transcriptomic and proteomic analyses of hypomorphants and controls at several stages before and after the MBT will serve to identify a wide variety of the genes that experience altered expression. These experiments will
also need to be replicated in a mammalian model to determine if my proposal is widely applicable or teleost-specific.

The primary impediment to studying the in vivo effects of complete loss of Apex1 is the inability to culture cells from homozygous null organisms. To test my model in the context of individual cells, RNAi knockdown of Apex1 followed by the same genetic analyses as for embryos should be performed. Active transcription during the cell cycle is expected to bring about substantial genetic damage and rapid suppression of cell growth and replication. As a consequence, even those cells that do not die quickly should be unable to be passaged into a second culture.

**Figure 12**: Model of effects of partial Apex1 knockdown in zebrafish embryogenesis. Lsd1-mediated histone demethylation generates oxidative stress that will damage actively-transcribed genes at the MBT. The failure to express genes required for embryonic autonomy will ultimately repress expression of genes required for differentiation of the germ layers and later for specific tissue types. This will result in the heart and brain deformities observed in pharyngula-stage Apex1 hypomorphants.
**Conclusion**

Apex1 is unique among BER genes in that it is the only one that is absolutely required for cell survival. While knockouts of *lig3*, *xrccl*, *fen1*, and *tdg* are embryonic lethal, viable cell cultures can still be made from the null embryos (24, 66, 67, 79). Such is not the case with *apex1* (57). My project represents one of the first investigations into the immediate effects of Apex1 loss in the context of a developing vertebrate embryo. These results contribute to a better understanding of vertebrate embryonic development and can be generalized to all metazoan cells. They can also be applied to drug development. BER inhibitors are being investigated to enhance the potency of DNA damaging chemotherapeutic agents (30–34). Identification of the first responders to Apex1 knockdown could provide leads for new drug targets, as well.

The Strauss lab has recently discovered a potential positive regulation of the important transcription factor Creb1 by Apex1, and proposed that lethality is the result of the loss of Creb1. Through a series of genetic experiments, I determined that while Creb1 protein does significantly decrease in the hypomorphant concurrently with Apex1, it is not the first detectable change. Furthermore, the diminution is at the protein level, and not the level of the transcript. I conclude that embryonic lethality of Apex1 knockdown does not result from just a change in what genetic information is being read, as previously assumed, but also how the information is read. Transcriptomic and proteomic changes in the hypomorphants at the ZGA are later accompanied by histone modifications that are known to precede chromatin rearrangement. These alterations drive an altered genetic profile which leads to developmental abnormalities. Apex1, an integral participant of BER, is essential for proper genetic expression and maintenance of the epigenome.
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