AN ABSTRACT OF THE DISSERTATION OF

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Vitamin E was first described in 1922 as an unknown factor required for impregnated rats to carry their offspring to term. In fact, when vitamin E was chemically characterized it was given the name “tocopherol” derived from the Greek: tokos = childbirth; phero = to bear; and –ol, indicating an alcohol. Vitamin E is linked to animal health and wellness, maternal fertility and a human neurodegenerative condition, ataxia with vitamin E deficiency However, *embryonic* vitamin E requirements during development remained unknown. We hypothesized that vitamin E is critical, not only for the mother, but specifically by the embryo for proper development. To separate the embryonic and maternal requirements, we employed an innovative model for the study of vitamin E: the zebrafish. We began by formulating and testing the first fully defined diet sufficient for zebrafish health. We then removed vitamin E from the formula to create our E deficient (E-) diet, which, when fed to adult zebrafish (for >3 months), resulted in E- adults that produced viable, E-

gametes. Deficient embryos initially developed normally; however, by 48 hours post fertilization (hpf), E- embryos developed severe malformations leading to significant mortality. Thus, we demonstrated for the first time an embryonic vitamin E requirement. We provided further insight into the embryonic vitamin E requirement by analyzing the transcriptional changes occurring prior to the observed malformations. The transcriptome revealed a putative mechanism of action for vitamin E in development, in which vitamin E deficiency leads to the dysregulation of key metabolic co-activators. Finally, to understand the trafficking of vitamin E, we identified the zebrafish α-tocopherol transfer protein (TTP). We demonstrated that the zebrafish TTP is homologous to its human counterpart, and its expression is both spatially and temporally regulated during embryonic development. Knocking down the expression of TTP, using morpholinos injected at the one-cell stage, resulted in early and severe malformations in the developing head and tail. Consequently we revealed a definitive role for TTP during development. Taken together the work described here presents a new direction for future research into the role of vitamin E and TTP in post-implantation development.

Vitamin E and the α-Tocopherol Transfer Protein During Zebrafish Embryogenesis

by
Galen W. Miller

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Galen W. Miller, Author

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CHAPTER 1 - INTRODUCTION

The purpose of the research presented herein is exploration of the role of vitamin E in vertebrate development. Vitamin E was first described in 1922 as “substance X”; an unknown factor required for impregnated rats to carry their offspring to term (Evans and Bishop 1922). The name α-tocopherol (from the Greek: tokos = childbirth; phero = to bear; and –ol, indicating an alcohol) was coined in 1936 when “substance X” was first isolated from wheat germ oil (Evans et al. 1936). Over the next 90 years, vitamin E was linked to animal health and wellness (Dierenfeld 1994; Allison and Laven 2000; Berchieri-Ronchi et al. 2011), maternal fertility (Allison and Laven 2000; Jishage et al. 2001; Berchieri-Ronchi et al. 2011) and a human neurodegenerative condition, ataxia with vitamin E deficiency (AVED) (Harding et al. 1985), but its role in fetal development remains unclear. We hypothesize that vitamin E is required, not only for the mother, but specifically for the embryo to ensure proper development. We begin with the conception and description of a novel model to study vitamin E deficiency: the zebrafish (Chapter 2). After establishing and validating the zebrafish model, we query global transcript changes induced by vitamin E deficiency during embryogenesis (Chapter 3). Finally, we characterized and knocked down the α-tocopherol transfer protein (TTP) in embryonic zebrafish, to investigate the role of TTP and vitamin E trafficking during development (Chapter 4).

Vitamin E (α-tocopherol)

α-Tocopherol is one of eight naturally occurring vitamin E isoforms, consisting of the tocopherols and tocotrienols, each synthesized in four distinct isomers (α, β-, γ-, and δ-, **Figure 1**). Natural vitamin E synthesis occurs only in plants (Mene-Saffrane and DellaPenna 2010); correspondingly, the primary dietary source of vitamin E is edible plant oils (Eitenmiller and Lee 2004). After ingestion, vitamin E absorption occurs along with other lipophilic compounds (e.g. dietary fats) by intestinal uptake of lipid micelles. The intestinal cells then pack the lipids into chylomicrons, which are secreted into the blood stream. Chylomicrons circulate through the lymph system and blood stream, accumulating in the liver for further processing and metabolism (**Figure 2**).

Only α-tocopherol can fulfill human dietary vitamin E requirements (Food and Nutrition Board. Institute of Medicine. 2000). The preferential separation of α-tocopherol from the other seven forms occurs in the liver. The hepatic cells actively maintain α-tocopherol levels while the other isoforms are metabolized and excreted. The α-tocopherol transfer protein (TTP) accounts for this specificity, sequestering α-tocopherol and assisting in its secretion from the liver into circulation (blue star, **Figure 2**). The precise mechanism of this transfer is unknown, although it is thought that α-tocopherol is placed into the plasma membrane by TTP allowing non-specific incorporation into nascent

lipoproteins (Qian et al. 2005; Morley et al. 2008). The lipoproteins are secreted by the liver and circulate throughout the body, delivering α-tocopherol to tissues via lipoprotein catabolism. Alternatively, α-tocopherol is easily exchanged between lipid layers (e.g. membrane phospholipids) and lipoproteins, which may contribute to its tissue delivery and uptake. Thus α-tocopherol is the most prevalent in animal tissues.

Functions of α-tocopherol

Vitamin E terminates the lipid peroxidation chain reaction, preventing cellular membrane damage (Traber and Atkinson 2007). Thus, vitamin E is referred to as a lipid-soluble chain-breaking antioxidant (Burton and Ingold 1981; Kamal-Eldin and Appelqvist 1996). In the absence of vitamin E, lipid peroxyl radicals react with nearby polyunsaturated lipids, resulting in the production of a radial (i.e. chain reaction) and the conversion of the original lipid peroxyl radical to a lipid hydroperoxide. In the presence of vitamin E, a lipid peroxyl radical will preferentially react with the chromanol head group of vitamin E (Burton et al. 1983), thereby preventing further lipid peroxide formation and the lipid peroxidation chain reaction. This antioxidant activity is vital in the presence of poly-unsaturated fatty acids (PUFAs). PUFAs are highly susceptible to oxidation, and PUFA oxidation products have been linked to human neurodegenerative and inflammatory diseases (Spiteller 2006; Corsinovi et al. 2011). Recent studies have demonstrated the co-localization

of α-tocopherol and PUFAs (specifically docosahexaenoic acid [DHA]) in cell membranes (Lemaire-Ewing et al. 2010). The co-localization occurs in "non-raft", PUFA-rich lipid domains (Atkinson et al. 2010). This highlights the importance of vitamin E for the protection of specific PUFAs. The antioxidant nature of vitamin E has been demonstrated using in vitro (Seiler et al. 2008; Howard et al. 2011; Sparkenbaugh et al. 2012) and in vivo (Bruno et al. 2006; Hoskins et al. 2012) experiments; as such, the lipid-soluble chain-breaking antioxidant function is considered the primary mode of action for vitamin E (Traber 1997; Traber et al. 2001; Traber 2006; Traber and Atkinson 2007).

Beyond its direct antioxidant function, α-tocopherol has been implicated in the regulation of genes related to lipid uptake and degradation, expression of extracellular proteins, inflammation, cell signaling and maintenance of the cell cycle (Azzi et al. 2004; Zingg and Azzi 2004; Li et al. 2010; Rimbach et al. 2010). In most studies, the noted effects are not directly attributed to α-tocopherol signaling, but instead result from changes in membrane environment that mediate cell signaling (Traber and Atkinson 2007). In addition to its antioxidant function, vitamin E influences membrane fluidity and maintenance (Atkinson et al. 2008; Zhang et al. 2009; Atkinson et al. 2010). A recent study showed that vitamin E deficient myocytes (both in culture and fresh, intact skeletal muscle) were unable to repair their membranes when exposed to oxidative stressors (Howard et al. 2011). This protective effect is

attributed to both the antioxidant and membrane stabilization capacities of vitamin E.

In contrast, a recent study reported that α-tocopherol binds directly to protein kinase Cα (PKCα) (McCary et al. 2012). Previous studies have noted tocopherol-induced modulation of PKCα activity (Traber and Packer 1995; Zingg and Azzi 2004; Betti et al. 2011). PKCα is a serine/threonine kinase that localizes to the plasma membrane, interacts with phosphatidylserine, calcium and diacylglycerol, and is integral in multiple cell signaling-transduction pathways (Spitaler and Cantrell 2004; Gould and Newton 2008). McCary et al. showed that α-tocopherol binds directly to PKCα and works as an antagonist of phosphatidylserine-dependent activity (McCary et al. 2012). This study demonstrates a direct effect of α-tocopherol, suggesting that it may interact more directly than previously thought.

Neuroprotection by vitamin E

Oxidative stress is implicated in many neurological diseases (Dmitriev 2007a, b; Jomova et al. 2010; Galea et al. 2012); accordingly links have been observed between vitamin E and these same diseases (Petersen et al. 2005; Isaac et al. 2008; Nishida et al. 2009; Bostanci et al. 2010; Mangialasche et al. 2010). Oxidative stress is a noted pathology associated with Down's syndrome, specifically in neurons (Busciglio and Yankner 1995), and in the amniotic fluid of pregnant mothers carrying babies with Down's syndrome

(Perrone et al. 2007). Vitamin E supplementation during gestation, using a Down's syndrome mouse model, improved behavior and cognitive abilities, and decreased the associated lipid peroxidation (Shichiri et al. 2011). This benefit was conveyed when mice were supplemented with vitamin E from the point of conception and throughout life (Shichiri et al. 2011). There is a reduced risk of acquiring Alzheimer's disease in individuals with high vitamin E intake (Zandi et al. 2004), and this effect is especially notable in elderly populations (Mangialasche et al. 2010). Consistently, lower levels of vitamin E have been seen in the cerebral-spinal fluid of Alzheimer's patients (Petersen et al. 2005), and depletion of vitamin E inhibits clearing of the amyloid β protein (a component of plaque formation in Alzheimer's disease), resulting in further accumulation of amyloid β and increased plaque formation (Nishida et al. 2009). Increased vitamin E intake is also linked to a lowered incidence of Amyotrophic Lateral Sclerosis (ALS) (Ascherio et al. 2005; Wang et al. 2011).

The most compelling connection between vitamin E and neurological diseases is the aptly named disorder: Ataxia with Vitamin E Deficiency (AVED, OMIM 277460). AVED (originally known as Familial Isolated Vitamin E deficiency [FIVE deficiency]) is an ataxia, which usually presents during childhood. The disease is characterized by α-tocopherol deficiency, progressive peripheral neuropathy with a specific "dying back" of the large caliber axons of the sensory neurons, resulting in ataxia (Harding et al. 1985;

Sokol 1988; Cavalier et al. 1998). The primary manifestations include spinocerebellar ataxia and skeletal myopathy (Sokol et al. 1993).

The α-tocopherol transfer protein

Vitamin E deficiency is rarely the result of dietary deficiency, but instead is caused by mutations of genes responsible for lipoprotein synthesis or assembly, and specifically in the α-tocopherol transfer protein (TTP) (Ouahchi et al. 1995; Cavalier et al. 1998; Morley et al. 2004; Qian et al. 2006; Di Donato et al. 2010). TTP is a hepatic protein responsible for the specific uptake and distribution of α-tocopherol. Initial *in vitro* studies searching for proteins able to bind and transfer vitamin E led to the discovery and characterization of TTP (Catignani 1975). This early work demonstrated TTP's preferential binding of α-tocopherol (Catignani and Bieri 1977; Kuhlenkamp et al. 1993). In the early 1980s, AVED was described as an autosomal recessive disorder, concurrent with low plasma vitamin E levels (Traber et al. 1987; Sokol 1988; Sokol et al. 1988; Ouahchi et al. 1995). In patients with AVED, only vitamin E was affected, while plasma levels of the other lipids remained unchanged. This observation, combined with the recent discovery of a protein able to bind and transfer vitamin E, led researchers to investigate potential mutations in the gene encoding TTP. Soon thereafter the human TTP was characterized (Arita et al. 1995), and as predicted, AVED patients possessed deleterious mutations in both copies of TTP (Ouahchi et

al. 1995; Cavalier et al. 1998; Usuki and Maruyama 2000; Yokota et al. 2000; Morley et al. 2004; Manor and Morley 2007). Clinically, the administration of high oral daily doses of α-tocopherol circumvents the dysfunctional TTP present in AVED patients (Meydani et al. 1998; Schuelke et al. 1999; Di Donato et al. 2010). Unfortunately, while the treatment halts the progression of the symptoms, patients do not regain lost sensation (Eggermont 2006; Gohil and Azzi 2008).

More recently, with *in vitro* studies, many using a fluorescent-labeled vitamin E analog (ω-nitrobenzoxadiazole-α-tocopherol (Morley et al. 2006; Nava et al. 2006)), determined that TTP facilitates the secretion of α-tocopherol from hepatocytes (Arita et al. 1997; Qian et al. 2005; Manor and Morley 2007), putatively for incorporation into lipoproteins. TTP likely binds vitamin E in the endocytic compartments of the hepatocyte (specifically, the late endosomes and lysosomes (Horiguchi et al. 2003; Qian et al. 2005; Qian et al. 2006)) transferring it to the plasma membrane where it is exported via ABC-type transporters (thought to be ABCA1 (Shichiri et al. 2010)) into lipoproteins (Manor and Morley 2007). The α-tocopherol binding pocket of TTP is characterized; the associated sequence and mutations are discussed in detail in Chapter 4.

Vitamin E and TTP during development

Vitamin E was originally discovered for its role in rodent fertility (Evans and Bishop 1922), but its requirement during embryo development is poorly understood (Brigelius-Flohe et al. 2002; Gagne et al. 2009). In humans low maternal vitamin E level is linked to miscarriage, preterm birth, preeclampsia and intrauterine growth restriction (Gagne et al. 2009). Additionally, during normal pregnancies, maternal plasma vitamin E concentrations increase (Horwitt et al. 1972; Brigelius-Flohe et al. 2002; Gagne et al. 2009). This increase is thought to be important as women experiencing abnormal pregnancies (with fetal/maternal complications/risks) present plasma vitamin E concentrations lower than those observed in normal pregnancies at corresponding developmental periods (von Mandach et al. 1994; Brigelius-Flohe et al. 2002; Gagne et al. 2009). Early supplementation with vitamins C and E has a marked clinical benefit when supplied to women at risk of developing preeclampsia (Brigelius-Flohe et al. 2002). In a recent population-based study, E supplementation was linked to a nearly 30% reduction in the risk of preterm birth (Bartfai et al. 2012). However, there are still questions revolving around the appropriate amount of vitamin E required during development, and similarly, the optimal amount to use in prenatal supplements remains unknown.

Fetal resorption and placental failure were noted in TTP knockout mice (Terasawa et al. 2000; Jishage et al. 2001). These outcomes are similar to those observed upon diet-induced vitamin E deficiency (Evans and Bishop 1922). Jauniaux et al. found TTP expression in the yolk sac and placental cells of developing human embryos (Kaempf-Rotzoll et al. 2003; Jauniaux et al. 2004; Muller-Schmehl et al. 2004), explaining the prevalence, albeit at low concentrations (Didenco et al. 2011), of the α-tocopherol form of vitamin E in umbilical cord blood (Acuff et al. 1998). The mouse TTP ortholog is also expressed in uterine/placental tissues (Jishage et al. 2001), specifically at the site of implantation (Kaempf-Rotzoll et al. 2002). In a study involving TTP knockout mice, Jishage et al. showed that embryos, regardless of TTP mutations, developed neural tube defects and failed to come to term if the mother was $TTP^{-/-}$ (Jishage et al. 2001).

Sufficient maternal α-tocopherol is also required for placental formation in mice and sheep, likely coinciding with TTP expression at the implantation site (Jishage et al. 2005; Kasimanickam et al. 2010). Further, a clear association between maternal vitamin E status during gestation and cognitive function of the offspring was experimentally demonstrated. Rats receiving a supranutritional dose of vitamin E produced offspring with compromised synaptic plasticity, while in supplementation in the mouse model of Down's syndrome lessened the severity of the disease (Betti et al. 2011; Shichiri et al.

2011), and may convey a benefit to the developing embryo (Ambrogini et al. 2011).

There are still many questions surrounding the exact role of vitamin E during pregnancy. It is clear that the mother requires α-tocopherol for successful gestation, but there remains a critical gap in the research to date: Does the developing embryo require vitamin E? And, if so, what is the role of vitamin E in the embryo? Previous approaches provide a glimpse into this gap, but fail to determine how much is enough; higher maternal vitamin E status is positively correlated with embryonic head circumference, weight and gestation time (von Mandach et al. 1994; Brigelius-Flohe et al. 2002; Masters et al. 2007; Gagne et al. 2009; Bartfai et al. 2012). Correspondingly, low α-tocopherol in the mother is associated with the development of childhood asthma (Turner et al. 2010). Moreover, higher levels of vitamin E may provide a protective effect from environmental toxicants (Ibrahim and El-Sayed 2012). This void in our understanding exists largely because separation of maternal and embryonic requirements using placental models is nearly impossible. Realizing this, we sought a novel model for embryonic nutrition, one that allows for separation of maternal and embryonic requirements: the zebrafish.

The zebrafish model

The zebrafish is rapidly increasing in popularity as a powerful research tool (Patton and Zon 2001). It is extensively used to model many human-related conditions, including: neurological disorders (Dambly-Chaudiere et al. 2003; Kokel et al. 2010; Rinkwitz et al. 2011; Becker and Rinkwitz 2012; Tal et al. 2012; Tal and Tanguay 2012), addiction (Petzold et al. 2009; Webb et al. 2009), disease treatment/drug development (Shin and Fishman 2002; Lieschke and Currie 2007; Mathew et al. 2007; O'Donnell et al. 2010; Delvecchio et al. 2011; Truong et al. 2011), and gene-environment interactions (Hillwalker et al. 2010). As a vertebrate, signaling and developmental stages are highly conserved across model species and shared with humans (**Table 1**) (Gilbert 2010). The embryonic zebrafish is amenable to high-throughput analyses, providing a screening capacity similar to those of *in vitro* assays (Levin and Tanguay 2011; Truong et al. 2011; Mandrell et al. 2012). Zebrafish embryos develop rapidly and externally from the mother, and are optically transparent, allowing non-invasive whole organism modeling *in vivo* (Kimmel et al. 1995). Most importantly for the work presented herein, zebrafish reproduce by spawning. This separation of mother and embryo, prior to fertilization, coupled with rapid development and optical transparency make the zebrafish an excellent model to answer the 90-year-old question: What is the role of vitamin E during embryogenesis?

We hypothesized that vitamin E is required *by the embryo* for proper development. To investigate this hypothesis we developed the following three aims. 1) Develop and implement a model system to study the embryonic vitamin E requirement. 2) Determine a mechanism of action for vitamin E during development. 3) Determine the role of TTP in early embryogenesis. Upon the completion of these aims, we show that vitamin E is required not only for the mother, but for the developing embryo as well

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Figure 1. Eight naturally occuring forms of Vitamin E

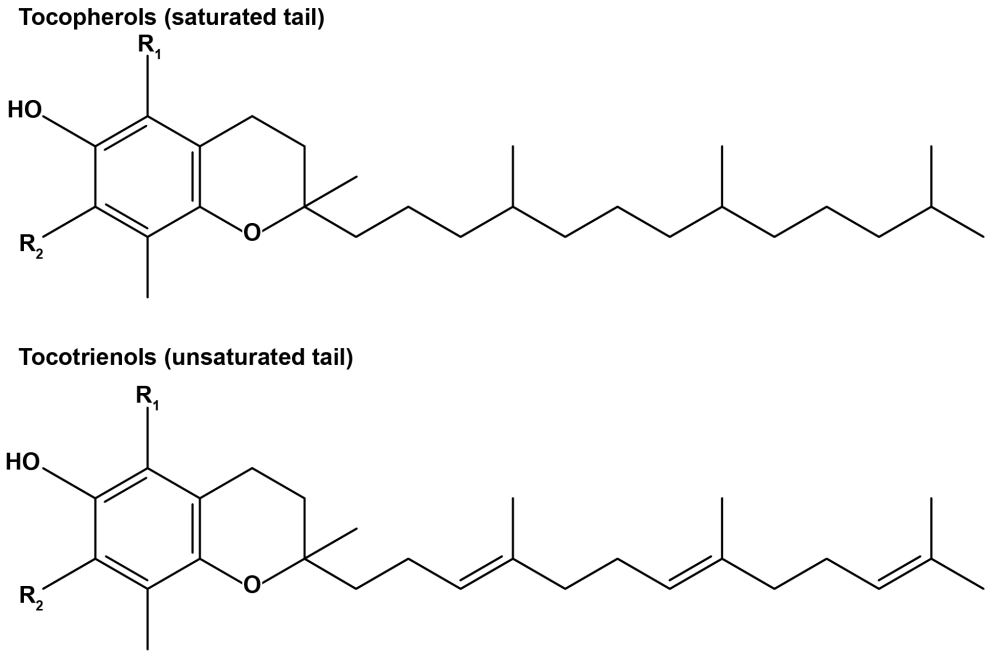

Figure 1- 1. Eight naturally occurring forms of Vitamin E.

Vitamin E naturally occurs with a fully saturated tail (tocopherol, top) and a partially unsaturated tail (tocotrienol, bottom). The distribution of methyl groups on the chromanol head group differentiates the four isomers (α, β, γ and δ).

Figure 1- 2. Vitamin E Uptake.

Ingested vitamin E associates with dietary fats (green oval), which are digested and form micelles in the intestinal lumen. Micelles are taken up by the enterocytes, repackaged into chylomicrons (orange circle) and secreted into circulation (first into the lymphatic system then blood stream). The chylomicrons circulate through the body until reaching the liver. The liver takes up the chylomicron remnants (receptor-mediated uptake), processing the fats for incorporation into nascent lipoproteins (yellow circle). In the liver, α-tocopherol is separated from the other 7 forms of vitamin E by the α-tocopherol transfer protein (TTP, blue star). TTP specifically binds and transfers α-tocopherol into the plasma membrane for incorporation into the nascent lipoproteins, which then circulate through the body delivering α-tocopherol and the lipid cargo. The other vitamin E forms are metabolized and excreted in the bile or urine.

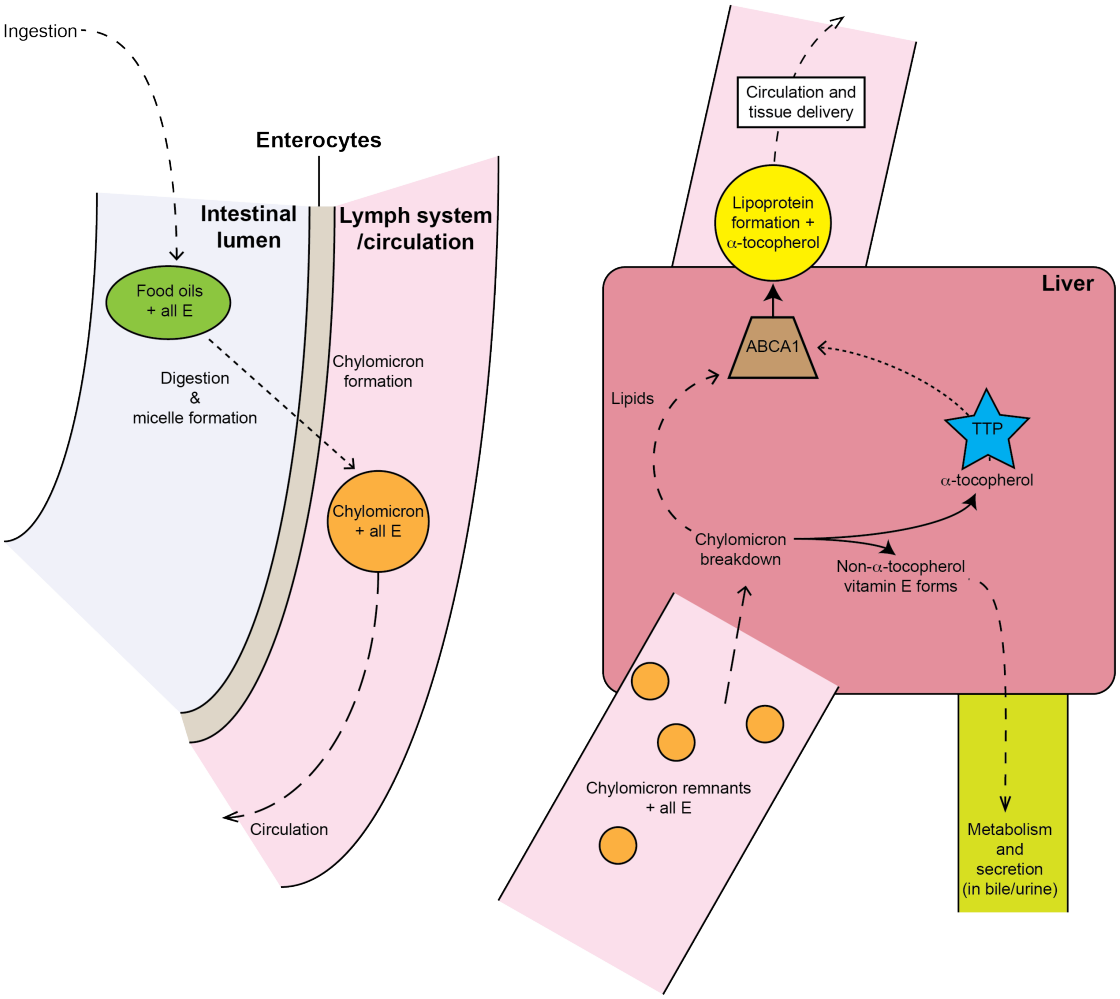
Ingestion
Enterocytes
Intestinal lumen
Lymph system /circulation
Food oils + all E
Digestion & micelle formation
Chylomicron formation
Chylomicron + all E
Circulation
Circulation and tissue delivery
Lipoprotein formation + α-tocopherol
Liver
ABCA1
Lipids
TTP
α-tocopherol
Chylomicron breakdown
Non-α-tocopherol vitamin E forms
Chylomicron remnants + all E
Metabolism and secretion (in bile/urine)

Table 1- 1. Stages of vertebrate embryogenesis.

Developmental Stage	Zebrafish [1]	Rat [2]	Human	Mouse [3]
Blastula/Blastocyst	2-5 hrs	3-5 days	4-6 days [4]	3-5.5 days
Implantation	n/a	6 days	8-10 days [5]	4.5-6 days
Gastrulation	5-9 hrs	8.5 days	13-19 days [6]	7 days
Neural Plate Formation	10 hrs	9.5 days	17-19 days [4]	7.5 days
First Somite	10-11 hrs	9-10 days	19-21 days [4]	7.5-8.75 days
10 Somite Stage	14 hrs	10-11 days	22-23 days [4]	8-9.25 days
Neural Tube Formation	18-19 hrs	9-12 days	22-30 days [4]	8.5-9.75 days
First Pharyngeal Arch	24 hrs	10 days	22-23 days [4]	8.5 days
Organogensisis	48 hrs	5-6 days	21-56 days [7]	8.5-12 days
First Heartbeat	24 hrs	10.2 days	22 days [7]	8.5 days
Birth/Hatching	48-72 hrs	21 days	253 days [7]	19 days

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CHAPTER 2 – ZEBRAFISH (DANIO RERIO) FED VITAMIN E-DEFICIENT DIETS PRODUCE EMBRYOS WITH INCREASED MORPHOLOGIC ABNORMALITIES AND MORTALITY

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Abstract

Vitamin E (α-tocopherol) is required to prevent fetal resorption in rodents. To study α-tocopherol's role in fetal development, a nonplacental model is required. Therefore, the zebrafish, an established developmental model organism, was studied by feeding the fish a defined diet with or without added α-tocopherol. Zebrafish (age, 4-6 weeks) were fed the deficient (E-), sufficient (E+) or lab diet up to 1 years. All groups showed similar growth rates. The exponential rate of α-tocopherol depletion up to ~80 day in E- zebrafish was 0.029±0.006 nmol/g, equivalent to a depletion half-life of 25±5 days. From age ~80 days, the E- fish (5±3 nmol/g) contained ~50 times less α-tocopherol than the E+ or lab diet fish (369±131 or 362±107, respectively; $p<.05$). E-depleted adults demonstrated decreased startle response suggesting neurologic deficits. Expression of selected oxidative stress and apoptosis genes from livers isolated from the zebrafish fed the three diets were evaluated by quantitative polymerase chain reaction and were not found to vary with vitamin E status. When E-depleted adults were spawned, they produced viable embryos with depleted α-tocopherol concentrations. The E- embryos exhibited a higher mortality ($p<.05$) at 24 h post-fertilization and a higher combination of malformations and mortality ($p<.05$) at 120 h post-fertilization than embryos from parents fed E+ or lab diets. This study

documents for the first time that vitamin E is essential for normal zebrafish embryonic development.

Introduction

Vitamin E (α-tocopherol) was discovered in 1922 by Evans and Bishop, who demonstrated that rats fed diets containing rancid fat were unable to carry offspring to term (Evans and Bishop 1922). Both low fetal and maternal α--tocopherol concentrations may be important factors during fetal resorption. Retention of uterine α--tocopherol by the mother appears essential to maintain pregnancy because the α--tocopherol transfer protein (TTP) is increased at the site of implantation (Jishage, Arita et al. 2001; Kaempf-Rotzoll, Igarashi et al. 2002) and is also expressed in the placenta (Kaempf-Rotzoll, Igarashi et al. 2002; Kaempf-Rotzoll, Horiguchi et al. 2003; Muller-Schmehl, Beninde et al. 2004; Rotzoll, Scherling et al. 2008). Both TTP and biomarkers of lipid peroxidation (malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE)) have been detected in the first trimester placenta (Rotzoll, Scherling et al. 2008). Moreover, early failure of pregnancy is associated with lipid peroxidation with resultant damage to the placental syncytiotrophoblast (Hempstock, Jauniaux et al. 2003). Thus, it is likely that α--tocopherol is needed by the mother to protect her from the oxidative stress of the rapidly growing fetus.

TTP is a liver protein in adult rodents and humans. However, Jauniaux et al. (Jauniaux, Cindrova-Davies et al. 2004) showed that not only does the human placenta and uterus express TTP, the human yolk sac expresses TTP. They further suggest that during very early human fetal development, the

human embryo obtains α--tocopherol from the yolk sac. Our hypotheses are dependent upon the observation that TTP is expressed during the first 48 h of zebrafish embryonic development (Usenko, Harper et al. 2008).

The zebrafish is an established vertebrate model organism frequently used in developmental studies (Kimmel 1989; Dodd, Curtis et al. 2000; Wixon 2000; Udvadia and Linney 2003). Embryos undergo maturation from a single cell to an autonomous juvenile fish in 120 hours (5 days) (Kimmel, Ballard et al. 1995). During development, embryos are optically clear, allowing for noninvasive observation of the entire embryo. TTP mRNA is expressed in embryonic zebrafish as early as 24 hours post fertilization (hpf) (Usenko, Harper et al. 2008). Because the zebrafish reproduces by spawning and does not have a placenta, the vitamin E requirements of the embryo can be separated from those of the mother. However, available commercial diets contain many ingredients making the study of a specific nutrient difficult (Westerfield 2000). Many “fish foods” also contain ambiguous ingredients, such as “fish oil” and/or “fish meal”, the sources of which are not immediately apparent. The feeding of live food sources (artemia, paramecium, etc.) to zebrafish present similar difficulties (Peterson and Gustin 2008). This problem has been addressed using zebrafish and other teleost fish in the past with varying levels of success by partially defining laboratory diets (DeKoven,

Nunez et al. 1992; National Research Council (U.S.). Committee on Animal Nutrition. 1993; Markovich, Rizzuto et al. 2007; Siccardi, Garris et al. 2009).

To address these challenges, we hypothesized that development of a defined diet, which is sufficient to sustain zebrafish health, growth, and reproduction, will allow us to study the action of vitamin E during embryogenesis. We further hypothesize based on studies in rodent models of vitamin E deficiency that 1) we can deplete adult zebrafish of α-tocopherol, 2) these adults will remain reproductively active, 3) the embryos that are produced will also be deficient in α-tocopherol, and 4) α-tocopherol deficient embryos will show phenotypic abnormalities as a result of their deficiency.

Materials and Methods

Fish Husbandry

Tropical 5D strain zebrafish (Danio rerio) were housed in the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University. The zebrafish were studied in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Adult zebrafish were kept at standard laboratory conditions of 28°C on a 14 h light/10 h dark photoperiod in fish water (FW) consisting of reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean®, Spectrum Brands, Inc., Atlanta, GA). Zebrafish were fed

experimental diets (described below) or a conventional lab diet comprising of artemia (Inve Aquaculture Inc. Ogden UT) and a combination of commercial flake foods including: Aquatox Flake (77% by weight, Zeigler Brothers Inc., Gardners, PA), Cyclopeez (6% by weight, Argent Laboratories, Redmond, WA), Golden Pearls (8.5% by weight, Artemia International LLC, Fairview, TX), and Hikari Micropellets (8.5% by weight, Hikari, Hayward, CA); hereafter called “lab” diet. Zebrafish were fed twice daily (AM and PM) with an amount of food sufficient for the fish to consume in ~5 minutes. Embryos used for our studies were not fed, but instead received all their nutrition from their yolk sacs. At 120 hpf, embryos were euthanized by an overdose of tricaine. Note that zebrafish younger than 1 month cannot consume standard food because their mouths are too small and instead consume paramecium, artemia and specially created diets (EZ Larval Diet, Zeigler Brothers Inc., Gardners, PA). Thus, we are currently unable to generate adult zebrafish that were spawned from deficient parents and have only consumed the vitamin E deficient diet.

Diet Preparation

Ingredients for the defined diets (**Table 1**) were obtained from Dyets Inc. (Bethlehem, PA) with exceptions, as indicated: wheat gluten (#402100), casein (#400627), egg whites (#401600), cellulose (#401850), vitamin mix (#310064), mineral mix (#210087), tocopherol-stripped soybean oil (#404365),

Stay C (Vitamin C-3, Argent Chemical Laboratories Inc., Redmond, WA), modified food starch (National Starch Food Innovation, Bridgewater, NJ), lecithin (Ultralec without added tocopherol), and Vitamin E (RRR-α-tocopherol). Both the lecithin and the vitamin E were generous gifts from Archer Daniels Midland (Decatur, IL).

Diets were prepared in 100 g batches without added vitamin E (E-) or with (E+, ~500 mg RRR-α-tocopherol/kg diet). Each diet was prepared by mixing ingredients until homogenous, spreading the batter on a large baking sheet and then oven-drying at 212ºF for one hour. The diet was then cooled, crushed into a powder, and stored frozen at -20ºC until fed to the zebrafish. Diets were used within four months of preparation.

α- and γ-Tocopherol Measurements

Tocopherol concentrations of the diets, fish, and embryos were determined by high-pressure liquid chromatography using electro-chemical detection (HPLC-ECD, Shimadzu, Columbia, MD and LC-4C, Bioanalytical Systems, Inc. West Lafayette, IN, respectively), as described previously (Podda, Weber et al. 1996). In brief, the samples were weighed (diet samples ~50 mg, whole fish 5-900 mg, and 5-30 embryos (embryo estimated wet-weight was 1 mg), saponified in alcoholic KOH with 1% ascorbic acid at 70ºC for 30 minutes (young fish, embryos and diets) or up to 1 hour (adult fish). The samples were cooled, extracted with hexane, dried under nitrogen gas,

resuspended in 50:50 ethanol:methanol, and an appropriate aliquot injected into the HPLC-ECD. Tocopherols were quantified by comparison to calibration curves generated from authentic α- and γ-tocopherol standards.

Ascorbic Acid Measurements

Sample collection methods were based on Moreau et al (Moreau and Dabrowski 1998) and Matamoros et al (Matamoros, Loscos et al. 2006). Briefly, fish were euthanized by an overdose of tricaine (MS 222, ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich, St Louis, MO), weighed, and the entire fish homogenized with buffer (5% trichloroacetic acid [TCA, Sigma-Aldrich, St Louis, MO], 0.08% diethylenetriaminepenta-acetic acid [DTPA, Acros Organics, Morris Plains, NJ], 250 mM perchloric acid [PCA, Fisher Scientific, Fair Lawn, NJ], and 0.4 mM dithioerythritol [DTE, Sigma-Aldrich, St Louis, MO]). Samples were then centrifuged, the supernatants frozen and stored at -80°C until analysis. Samples were analyzed by HPLC-ECD, as described previously (Frei, England et al. 1989).

Zebrafish α-Tocopherol Depletion Kinetics

At 4-6 weeks of age, zebrafish were randomly separated into three dietary groups: E-, E+, or lab diets. Zebrafish (n = 3/dietary group) were euthanized by overdose of tricaine at each of the indicated time points.

Zebrafish were weighed and kept frozen until analysis for vitamin concentrations, as described above. This experiment was repeated using three separate generations of fish. α-Tocopherol depletion kinetics were calculated using Microsoft Excel (Microsoft Corporation, Santa Rosa, CA) by fitting a linear regression analysis to the logarithmic-transformed α-tocopherol concentrations measured during the first ~80 days after initiation of the E- diet.

Zebrafish Sensory Testing

To evaluate sensory responses, computer-assisted video monitoring of swimming behavior was assess using modification of the method of Eddins et al (Eddins, Cerutti et al. 2009). Adult zebrafish (n=6) from each diet (E+, E- and Lab; 221 days on diet) were placed in individual 1.75 L tanks containing ~1.5 L FW. Tanks were set in-line on shelves with the broadside facing the camera, separated by dividers to isolate individual fish. Tanks were backed with blank white paper, evenly backlit. Room temperature was controlled at 28° C. Fish were fasted for the duration of the behavior trials and were given 24 h to acclimatize prior to beginning the trials.

Trials were recorded using a Sony HD camcorder (Sony Handycam HDR-SR11) coupled with the Noldus Etho-Vision XT V 7.0 analysis software (Leesburg, VA). The swimming velocity was recorded for 16 minute sessions (separated into 2 minute intervals), and the first 6 minutes were averaged to

obtain the average velocity for the trial, the next 10 minutes were not used for assessment (rest time). Two trials with no stimulus were used to generate "background" swimming information and results from each fish were averaged to generate its own baseline information. Two trials were done using a "single tap" generated by an electro-magnetic solenoid to tap the tank at two minutes; results from each fish were averaged to generate its own single tap information. A final "multiple tap" trial was generated using the solenoids to strike the tanks once every 5 seconds starting at 2 minutes for 90 seconds (18 taps total). Rest time was given between each stimulus trial to allow a return to baseline behavior.

Zebrafish Embryo Handling and Scoring

Fertilized eggs were obtained from natural spawning of adult zebrafish according to methods in The Zebrafish Book (Westerfield 2000). Embryos were collected and staged, as described by Kimmel et al (Kimmel, Ballard et al. 1995). Embryos were treated with a dilute bleach solution (0.0033% = 0.55 ml household bleach diluted to 1 L FW) to clean their chorions, and were then rinsed twice with FW before being placed into 10 cm petri dishes containing methylene blue (0.0002%) to inhibit fungal growth.

To assess morphology of embryos over time, embryos were placed individually in wells of 96-well plates in 150 µl methylene blue (0.0002%) and observed daily using stereomicroscopy, up to 120 hours post-fertilization (hpf).

Scoring was assessed on visible phenotypes (mortality at 24 hpf; mortality at 120 hpf, delayed development, lack of motility, abnormal touch response, spastic movement, and malformations of heart, brain, yolk sac, notochord, body axis, trunk, circulatory system, eye, jaw, somites, snout, otic, fin, pigmentation, or swim bladder).

Quantitative real-time polymerase chain reaction (qPCR)

Fish were euthanized by tricaine overdose, livers removed and placed in RNALater (Qiagen, Valencia, CA) and stored at -20° C until RNA extraction. Total RNA was extracted using an RNeasy kit with DNase I treatment per manufacture's directions (Qiagen). RNA concentrations and purity were determined by UV absorption (NanoDrop ND-1000 UV-Vis Spectrophotometer, Thermo Scientific, Wilmington, DE). cDNA was synthesized following manufacture's directions using Superscript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA). Primers were designed for each target gene using the Primer-BLAST program (Primer3 combined with BLAST, NCBI website) (**Table 2**). Plasmids were cloned from each primer product (TOPO TA cloning kit, Initrogen), sequenced to verify correct product (ABI Prism 3730 Genetic Analyzer, ABI Prism 3730 Data Collection Software v. 3.0, ABI Prism DNA Sequencing Analysis Software v. 5.2, with BigDye Terminator v. 3.1 Cycle Sequencing Kit, Center for Genome Research and Biocomputing core facility, Oregon State

University), and concentrations measured by spectrophotometer. These plasmids were used to generate an absolute copy number standard curve for real-time PCR quantification. Samples were analyzed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) using a DNA Engine Opticon 2 System (Bio Rad, Hercules, CA) with the Opticon Monitor Version 3.0 software for real-time PCR detection. Results were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ß-actin, or ß-2-microglobulin (ß2M) expression. Data are reported as fold-changes relative to values from lab control fish livers. There were no statistically significant differences in the housekeeping genes between the diet groups. The ß2M gene was chosen because its abundance was similar to our genes of interest.

Statistics

GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) and JMP (SAS Institute, Cary NC) were used for statistical analyses. The data was logarithmically transformed, when unequal variances were observed between groups. α-Tocopherol depletion kinetics were calculated using Microsoft Excel (Microsoft Corporation, Santa Rosa, CA) by fitting a linear regression analysis on the log-transformed data to the first ~80 days after initiation of diet feeding. Sensory evaluation was estimated using average velocity of the fish generated by the Noldus EthoVision software. Comparisons between lab, E+, and E- diets were calculated using one-way ANOVA, comparisons between E+ and

E- by Tukey's honestly significant differences, HSD. Relationships between the three diet groups over time were analyzed using two-way ANOVA. Post-hoc tests were carried out using paired comparisons (Tukey's honestly significant differences, HSD). Data are reported as means ± SEM; differences were considered significant at $p<0.05$.

Results

Diet Vitamin E Concentrations

The E- and E+ defined diets were formulated identically with the exception that the E- diet had no added α-tocopherol. The E- diet contained 400-times less α-tocopherol than the E+ diet (p<0.0001, **Figure 1**), which contained ~500 mg/kg. γ-Tocopherol concentrations were also less in the E- than the E+ diets (6.2 ± 1.1 and 10.4 ± 1.0 mg/kg, respectively, p<0.0001). The lab diet contained both α- and γ-tocopherols. The flake food components contained the following α- and γ-tocopherol concentrations, respectively: Aquatox Flake, 440 ± 18 and 16 ± 1 mg/kg; Golden Pearls 305 ± 8 and 10 ± 1; and Hikari Micropellets 283 ± 4 and 5.9 ± 0.1 (n=3 measurements).

Zebrafish Growth on Defined Diets and Tocopherol Concentrations

Zebrafish at ~4-6 weeks of age were divided into three groups: E-, E+ and lab diets; then were fed exclusively the indicated diets and the whole body vitamin E concentrations measured at the indicated time on diet (up to 310 days, **Figure 2**). Although the zebrafish increased weight during the first 3-4 months on diet (time effect p<0.0001), the various dietary treatments (E-, E+ and lab) had no significant effects on body weights over the course of the study (**Figure 2A-C**). The rate of weight increase was 2.3 ± 0.1, 2.1 ± 0.1, and 2.0 ±0.1 g/d for the E-, E+ and lab fish.

Prior to initiating the experimental diets, zebrafish α-tocopherol concentrations were 33.5 ± 2.5 nmol/g. When fed the E+ or lab diets, the α-tocopherol concentrations of zebrafish initially increased over time (**Figure 2E or 2F)**. In contrast, when zebrafish consumed the E- diet, the α-tocopherol concentrations decreased exponentially for the first ~80 days at a rate of -0.029 ± 0.006 nmol/g (n=3 experiments), which yields a half-life of 25 ± 5 days (**Figure 2D**). After 3 half-lives, hypothetically only 12.5% of the starting vitamin E concentration should remain. Consistent with this prediction, between 80 and 300 days of diet consumption, the zebrafish fed the E- diets had α-tocopherol concentrations that were approximately 10% of their starting concentrations and more than ~50-times lower than zebrafish consuming either the E+ or lab diets (**Figure 2E or F, Table 3**, diet effect, p=0.0007, paired comparisons, p<0.05). After this observed initial decrease, the fish maintained consistently low α-tocopherol concentrations (**Figure 2D, Table 3**).

After 80 days, γ-tocopherol concentrations in zebrafish consuming the E- and E+ diets were lower than those in the fish consuming the lab diet; those of the zebrafish consuming the E- diet were less than half of those consuming the other diets (**Figure 2G, H, I, Table 3**, diet effect, p<0.0001, paired comparisons, p<0.05).

Impaired Sensory Perception in Zebrafish Consuming E- Diets

Sensory neuropathy is one of the first symptoms of vitamin E deficiency in humans (Ouahchi, Arita et al. 1995). To assess sensory neuropathy in zebrafish we devised a "startle response" measurement using a percussive "tap" with responses monitored by video recording. In the baseline trial, no significant differences were observed in the average swimming velocity between the diet groups. Application of a single synchronized solenoid tap showed a characteristic startle response in the E+ and Lab diet zebrafish (Eddins, Cerutti et al. 2009). When startled by a single tap the E+ and Lab zebrafish both swam faster, while E- fish had an attenuated response (**Figure 3**, diet effect $p<0.003$, paired comparisons, $p<0.05$). These differences disappeared when the fish were exposed to a higher level of stimulation in the "multiple tap" trial, suggesting that a greater stimulus was needed to elicit a response from the E- fish.

Embryo Tocopherol Concentrations

Fish from each of the diet groups were group-spawned starting at 3-4 months of age (~80 days on diets). Despite differences in α-tocopherol concentrations, all dietary groups of zebrafish routinely produced viable gametes. The embryos were observed up to 120 hours post-fertilization (hpf) without being fed. Normally, zebrafish embryos do not consume food during this period, but receive their nutrition from the yolk sac (Kimmel 1989; Kimmel, Ballard et al. 1995).

Embryo α-tocopherol concentrations reflected those of their parents. For example, adult zebrafish that had consumed the diets for >250 days were spawned and the α- and γ-tocopherol concentrations were measured in representative adult fish and in 48 hpf embryos (**Figure 4**). Adult zebrafish consuming the E+ and lab diets contained nearly 50-times higher α-tocopherol concentrations than did those consuming the E- diet; the embryos from the fish consuming the E+ and lab diets contained nearly 30-times higher α-tocopherol concentrations than embryos in the E- group (diet x life stage interaction, p=0.016; p<0.05 for comparisons, **Figure 4A**).

The adult zebrafish consuming the E+ and lab diets contained nearly 3-times higher γ-tocopherol concentrations than did the adults consuming the E- diet. However, in the E- and lab embryos the γ-tocopherol concentrations were almost double those of the E+ embryos (diet x life stage interaction, p<0.0001, E+ compared with lab p<0.05, **Figure 4B**). Thus, E- embryo γ-tocopherol concentrations were not significantly different from the E- adults' γ-tocopherol concentrations, while the γ-tocopherol concentrations of the embryos from the E+ and lab diet groups were lower than those of their parents (E+ or lab adults, p<0.05). These findings indicate that the adult female zebrafish transfer very little γ-tocopherol to the eggs, even during vitamin E deficiency.

Embryo vitamin E status did not vary significantly from 48 to 120 hpf (Data not shown).

Embryo Morphology and Mortality

Embryos from spawns of each diet group were observed up to 120 hpf for morphological changes. Low levels of abnormalities are inherent in zebrafish embryos and were observed in embryos from all the diet groups. In the E- embryos, there was increased mortality at 24 hpf and at 120 hpf compared with the other diet groups (**Figure 5A**, diet x time interaction, $p<0.0001$), but at 24 hpf, the differences between diet groups did not reach statistical significance for paired comparisons. At 120 hpf, the E- embryos displayed significantly higher levels of mortality compared with the E+ or lab diet embryos (diet effect $p=0.005$, $p<0.05$ paired comparisons).

At 120 hpf, nearly 70% of the E- embryos experienced either malformations or death. These levels were higher than those observed in the E+ or lab diet embryos; E+ had greater malformations than did lab diet embryos (**Figure 5B**, $p<0.05$). Malformations commonly observed included cranial-facial malformations, bent anterior-posterior axis, pericardial edema, swim bladder malformations, and yolk-sac edema (**Figure 6**).

Whole Fish Ascorbic Acid Concentrations

Ascorbic acid concentrations were measured in adult zebrafish after consuming E-, E+ or lab diets for various amounts of time (>181 days). Ascorbic acid concentrations of the fish on the defined diets were nearly double those of the fish consuming the lab diet (diet effect $p<0.0035$, $p<0.05$ paired comparisons, **Figure 7**).

Liver Gene Expression

To assess long-term effects of vitamin E deficiency on mRNA expression levels of liver genes of interest, we used using real-time quantitative polymerase chain reaction (qPCR) to measure transcripts of genes involved in oxidative stress response, lipid metabolism, vitamin E trafficking, and cell death (Johnson, Johnson et al. 2008; Na, Seok et al. 2009; Surh, Kundu et al. 2009; Feng, Liu et al. 2010; Todorcevic, Skugor et al. 2010) (genes are listed in **Table 2**). Expression levels were normalized to ß2M, ß-actin or GAPDH; all three housekeeping genes yielded similar results; there were no statistically significant differences between the diets for each of the housekeeping genes. The data shown are normalized to ß2M and are shown as fold changes over the average of the lab diet control liver mRNA, set to 100. We observed no significant changes in mRNA levels due to chronic vitamin E deficiency in the genes assayed. However, the expression of four genes, which have previously been identified to be important in nerve protection (Seiler, Schneider et al. 2008), were found to be significantly reduced in the zebrafish fed E- or E+ diets relative to those in the control fish (**Figure 8**).

Discussion

Vitamin E deficiency caused developmental abnormalities and death in zebrafish embryos. This finding elucidates for the first time that the embryo, as well as the mother, requires vitamin E. The expression of TTP by the human embryo-yolk sac (Jauniaux, Cindrova-Davies et al. 2004), as well as by the zebrafish embryo-yolk sac (Usenko, Harper et al. 2008), suggested that the embryo itself might require vitamin E. Initially in 1922 vitamin E was discovered because rats fed rancid fat failed to carry their offspring to term (Evans and Bishop 1922). This finding was the basis for the “fetal resorption test” that remains in use today as an assay of biologic activities of various vitamin E forms (Traber 1999). In order to prevent fetal resorption, vitamin E must be administered to the vitamin E deficient rat mother on post-fertilization days 5 to 9 (Leth and Sondergaard 1977; Ames 1979). Interestingly, this is the same critical period where the 12/15-lipoxygenase-dependent pathway appears to mediate implantation (Li, Cheon et al. 2004). Similarly, glutathione peroxidase 4 (GPx4) expression increases at day 7.5 and is the same time at which GPx4-knockout mice embryos are resorbed (Imai, Hirao et al. 2003). Taken together these findings suggest that during days 5 to 9 of rodent embryogenesis, lipid peroxidation can be especially damaging. Importantly, vitamin E is a potent, lipid-soluble antioxidant and thus may be critical for the

embryo to have sufficient amounts to protect against lipid peroxidation (Traber and Atkinson 2007).

Both low fetal and maternal α-tocopherol concentrations may be important factors during fetal resorption. Retention of uterine α-tocopherol by the mother appears essential to maintain pregnancy because TTP is increased at the site of implantation (Jishage, Arita et al. 2001; Kaempf-Rotzoll, Igarashi et al. 2002) and is also expressed in the syncytiotrophoblast of the human placenta (Kaempf-Rotzoll, Igarashi et al. 2002; Kaempf-Rotzoll, Horiguchi et al. 2003; Muller-Schmehl, Beninde et al. 2004; Rotzoll, Scherling et al. 2008). Early failure of pregnancy is associated with lipid peroxidation with resultant damage to the syncytiotrophoblast in the placenta (Hempstock, Jauniaux et al. 2003). Thus, it is likely that α-tocopherol is needed by the mother to protect her from the oxidative stress of the rapidly growing fetus. Herein, we demonstrate that the embryo independently requires vitamin E because the E- zebrafish embryos suffered increased developmental abnormalities, as well as increased mortality (**Figures 5 & 6**). These E- embryos contained significantly lower α-tocopherol concentrations than the embryos in the other diet groups (**Figure 4**),

Importantly, the E- diet depleted the adult zebrafish of α-tocopherol (**Figure 2, Table 3**). The exponential rate of depletion predicted that the zebrafish fed the E- diet would experience nearly 90% depletion of their initial

α-tocopherol concentration by 80 days on diet. The α-tocopherol measurements beyond 100 days showed that further depletion did not occur, or at least was slowed to statistically imperceptible changes. Given that the E- diet had some α-tocopherol and that the zebrafish experienced deficiency symptoms, these data suggest that the dietary α-tocopherol concentration (approximately 2 mg/kg diet) is below the requirement for the zebrafish. Using the estimate that a fish weighing 500 mg eats about 10 mg food (Lawrence 2007), the zebrafish is probably consuming about 50 pmol α-tocopherol, then after 80 days on the E- diet its whole body contains about 2500 pmol α-tocopherol.

Remarkably, like humans (Traber and Kayden 1989; Leonard, Paterson et al. 2005), zebrafish show a preference for α- over γ-tocopherol. This finding is most easily appreciated in the comparison of the vitamin E distribution within the E- diets compared with the E- zebrafish. The E- diets had approximately 5-times more γ- than α-tocopherol (**Figure 1**), while the E- zebrafish contained approximately 2.5-times more α- than γ-tocopherol (**Table 3**). Given that the zebrafish expresses TTP gene (Usenko, Harper et al. 2008), it is not surprising that the zebrafish shows a preference for α-tocopherol. More remarkable is the apparent lack of γ-tocopherol transfer to the embryos (**Figure 4**). Although the E+ and lab adult zebrafish contained γ-tocopherol at 3-times the E- adult zebrafishes' γ-tocopherol concentrations, the E- embryo γ-tocopherol

concentrations were not significantly different from those of either the E+ and lab embryos. These data emphasize that the embryos are preferentially enriched in α-tocopherol and that the E- embryos were limited in γ-tocopherol even when the zebrafish adults were severely limited in both forms of vitamin E. These findings are reminiscent of those in TTP null mice that were fed γ-tocopherol-enriched diets yet did not accumulate γ-tocopherol in tissues or plasma (Traber, Siddens et al. 2005). Thus, vitamin E deficiency does not drive the use of γ-tocopherol in place of α-tocopherol.

This study also demonstrates that the adult zebrafish require vitamin E because we detected abnormal swimming responses to a “tap” test (**Figure 3**). In general, testing of neurologic responses of adult zebrafish has been limited. Eddins et al (Eddins, Cerutti et al. 2009) studied chlorpyrifos neurotoxicity that was induced during early development and then persisted into adulthood. They showed that the neurotoxicity caused an increase in swimming velocity in the affected adult zebrafish (Eddins, Cerutti et al. 2009). In contrast, we found that vitamin E deficiency in zebrafish decreased their responsiveness, as shown by decreased swimming velocity, suggesting diminished sensory perception. It should be noted that muscle degeneration is also associated with vitamin E deficiency, but in the current study the zebrafish were apparently able to swim faster in response to multiple taps, they just did not respond quickly to a single stimulus. This finding suggests that it took greater

stimulus to elicit a response. Given that the vitamin E deficiency symptom in humans is a dying back of the sensory neurons (Food and Nutrition Board and Institute of Medicine 2000), it seems likely that the E- zebrafish also had a sensory deficit, but further studies are needed to evaluate the extent and mechanism of the deficit.

To assess long-term effects of vitamin E deficiency on mRNA expression levels of liver genes of interest, we used using quantitative PCR (qPCR) to measure transcripts of genes involved in oxidative stress response, lipid metabolism, vitamin E trafficking and cell death (Johnson, Johnson et al. 2008; Na, Seok et al. 2009; Surh, Kundu et al. 2009; Feng, Liu et al. 2010; Todorcevic, Skugor et al. 2010). Liver expression of these genes was not dependent upon vitamin E status since the fish from the E- and E+ groups displayed similar responses. However, the lab diet contains various ingredients, such as fish oil, that are not present in the defined diets. These “control” livers had higher expression of GPX4a, PLA2, PLA2gIV, and AIF, suggesting that the more oxidizable lipids had induced expression of these protective enzymes. However, we have not measured lipid oxidation products in these fish, so the mechanisms for the differential expression remain to be investigated. It is also apparent that the embryo is more sensitive than is the adult fish to insufficient vitamin E. We plan further studies to investigate vitamin E-dependent gene regulation in the embryo.

We formulated a diet containing sufficient nutrients for the zebrafish based on previous research in other fresh water teleost fish (DeKoven, Nunez et al. 1992; National Research Council (U.S.). Committee on Animal Nutrition. 1993; Markovich, Rizzuto et al. 2007; Siccardi, Garris et al. 2009). Our diet formulation (**Table 1**) was sufficient for zebrafish nutrition based on growth and body weights (**Figure 2, Table 3**). Animal health, behavior and mortality were observed daily during feeding and no overt visual changes were noted in fish consuming the defined diets. Previously, commercially available diets for zebrafish were insufficient to maintain growth and had to be supplemented with live food (Lawrence 2007). Our diets are, therefore, a major step forward in zebrafish husbandry given that 45-day old zebrafish fed these diets grow as well as those fed the commercial mixture of foods and live food.

In addition, we analyzed whole zebrafish ascorbic acid concentrations, as vitamin C status has been linked to vitamin E status (Bruno, Leonard et al. 2006), and teleost fish lack the L-gulonolactone oxidase gene and thus cannot synthesize ascorbic acid (Dabrowski 1990). Zebrafish ascorbic acid concentrations were higher in the fish fed the defined diets than in the lab controls, showing that the defined diets are more than sufficient in ascorbic acid (**Figure 7**).

In conclusion, our findings document for the first time that vitamin E is required, not only for the mother, but also for the embryo during development.

This zebrafish model has allowed the successful separation of maternal and embryonic nutrition, permitting the study of embryogenesis without the fetal resorption that occurs in mammalian models.

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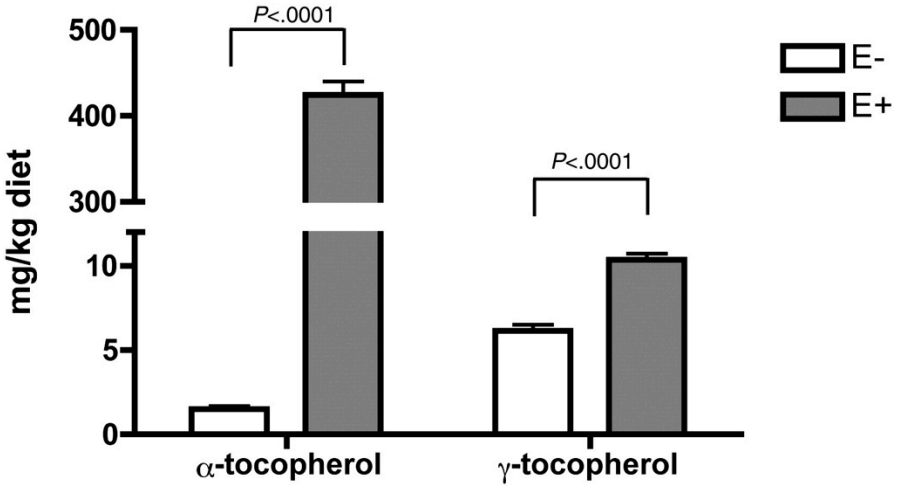
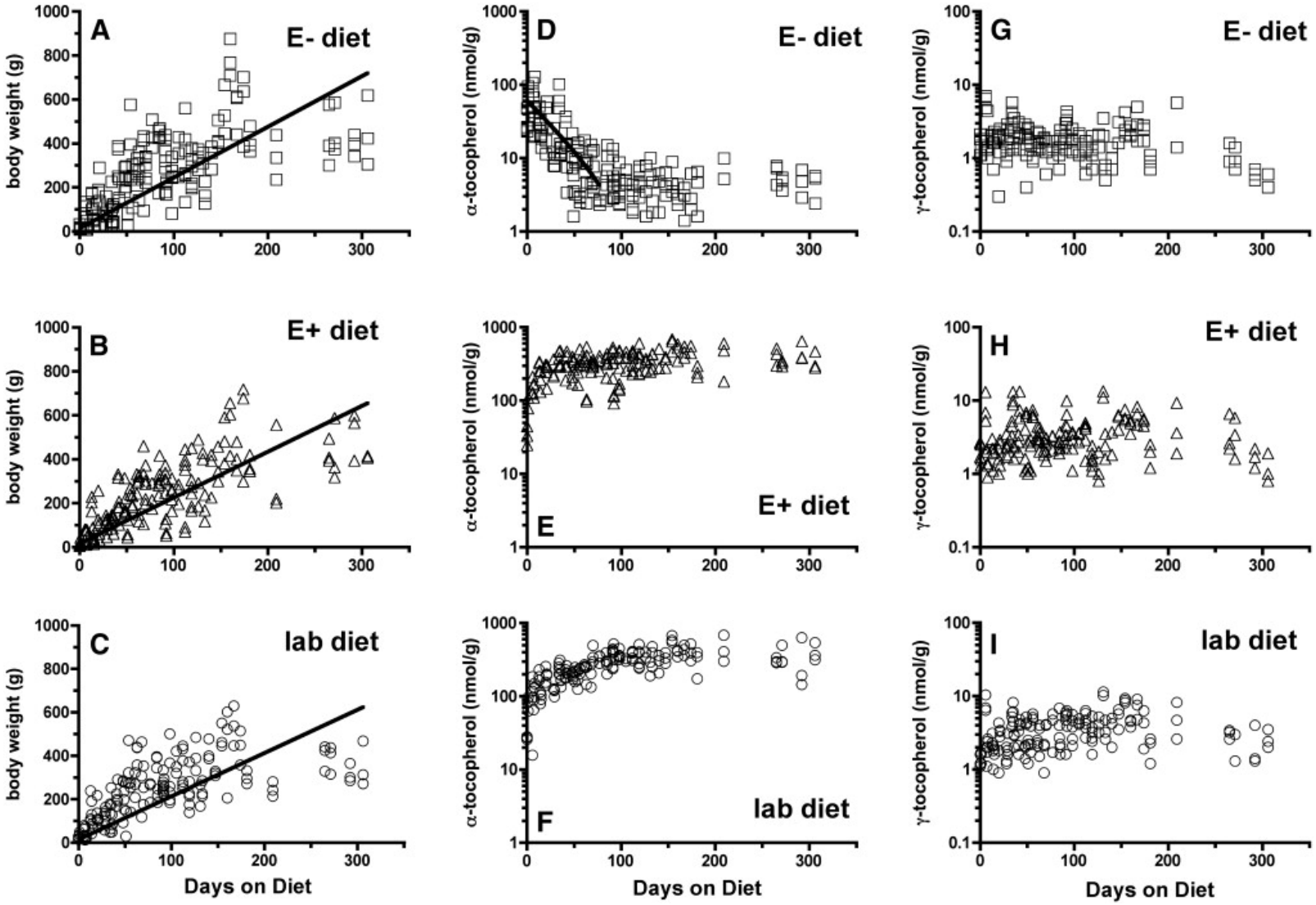


Figure 2- 1. Diet Vitamin E Concentrations.

Measured α- and γ-tocopherol concentrations (means ± SEM, n=3 separate diet batches) of experimental diets.

Figure 2- 2. Vitamin E Depletion Kinetics.

Time course of body weights (panels **A,B,C**) and whole body concentrations of α-(panels **D,E,F**) and γ-tocopherol (panels **G,H,I**) from individual zebrafish fed E- (squares, n=172, panels A,D,G), E+ (triangles, n=177, panels B,E,H) or lab (circles, n=168, panels C,F, I) diets. Results are from three separate generations of fish, samples taken at noted days after initiation of dietary treatments. Body weights (**A-C**) were not significantly different between the diet groups, but increased over time (time effect $p<0.0001$; month 1< month 2< month 3-5 < month 6-10, Tukey paired comparisons, each $p<0.05$). (**D,E,F**) By month 2 (>30 days) and thereafter, the α-tocopherol concentrations of the E- zebrafish were significantly less than those of the other groups [diet x time interaction, p=0.0007; E- for month 2, month 3-5, and month 6-10, different than E+ or lab diet groups (which were not different from each other at each time interval), Tukey paired comparisons, $p<0.05$]. The line indicates the exponential rate of depletion. (**G,H,I**) Within the first month (<30 days) and thereafter, the γ-tocopherol concentrations of the E- zebrafish were significantly less than those of the other groups [diet x time interaction, $p<0.0001$; E- for month 1, month 2, month 3-5, and month 6-10, different than E+ or lab (which were not different from each other at each time interval), Tukey HSD $p<0.05$].


A
E- diet
body weight (g)
D
E- diet
α-tocopherol (nmol/g)
G
E- diet
γ-tocopherol (nmol/g)
B
E+ diet
body weight (g)
E
E+ diet
α-tocopherol (nmol/g)
H
E+ diet
γ-tocopherol (nmol/g)
C
lab diet
body weight (g)
Days on Diet
F
lab diet
α-tocopherol (nmol/g)
Days on Diet
I
lab diet
γ-tocopherol (nmol/g)
Days on Diet

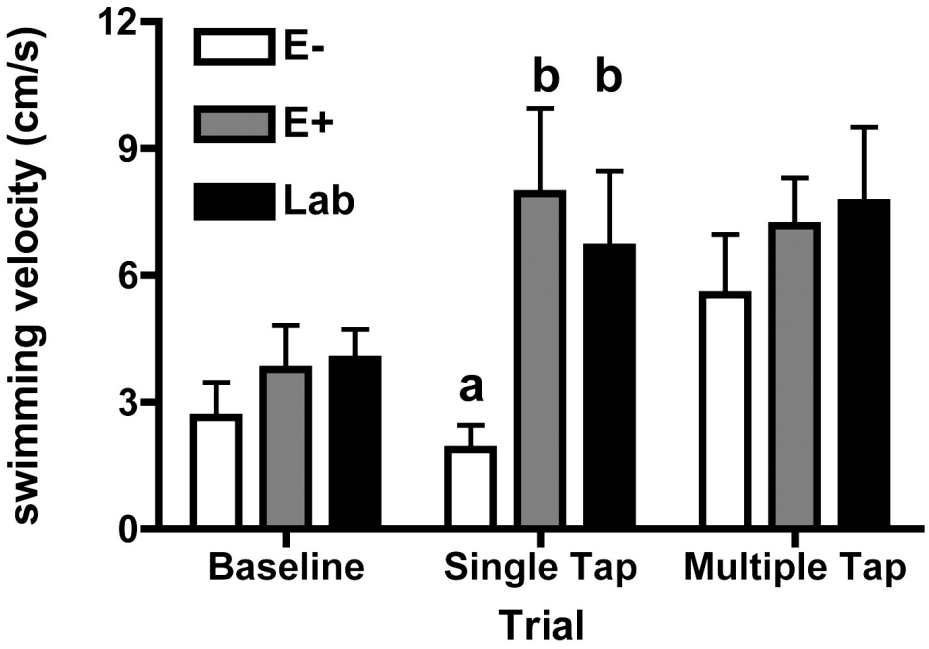


Figure 2- 3. Vitamin E Deficiency Alters Startle Responses.

Adult zebrafish (n=6) from each diet (E+, E- and Lab; 221 days on diet) were placed in individual 1.75 L tanks containing ~1.5 L of FW. In the baseline trials and multi-tap trials, no significant differences were observed in the average swimming velocity between the diet groups. When startled by a single tap the E+ and Lab zebrafish swam faster, while E- fish had an attenuated response (diet effect $p<0.003$, bars not sharing the same letter are significantly different, Tukey HSD $p<0.05$). When exposed to multiple taps, the fish did not show significant differences in swimming velocity.

Figure 2- 4. α- and γ-Tocopherol Concentrations of Embryos and Adults.

Adult zebrafish (n= 12 per diet) were collected for vitamin E analysis between 250 and 300 days of consuming the diets. Zebrafish from this generation were spawned at 270, 278, and 284 days. Embryos were collected for vitamin E analysis at 48 hpf in groups of 15 embryos (E- n=16, E+ n=11, lab n=12 replicates). (**A**) All embryo α-tocopherol concentrations (mean ± SEM: logarithmic scale) were less than those of the adult zebrafish; E- embryo α-tocopherol concentrations were less than those of E- adults; E- embryos compared with E+ or lab embryos had the lowest α-tocopherol concentrations (diet x life stage interaction, p=0.016; bars not sharing the same letter are significantly different, Tukey HSD $p<0.05$). (**B**) Adult E+ and lab zebrafish γ-tocopherol concentrations (mean ± SEM) were greater than the adult E- zebrafish and γ-tocopherol concentrations in all of the embryos; the lab embryo γ-tocopherol concentrations were greater than those of the E+ embryos; E- embryo γ-tocopherol concentrations were not significantly different from the E- adults γ-tocopherol concentrations, while E+ and lab embryos were less than those of the adults (diet x life stage interaction, $p<0.0001$, bars not sharing the same letter are significantly different, Tukey HSD $p<0.05$).

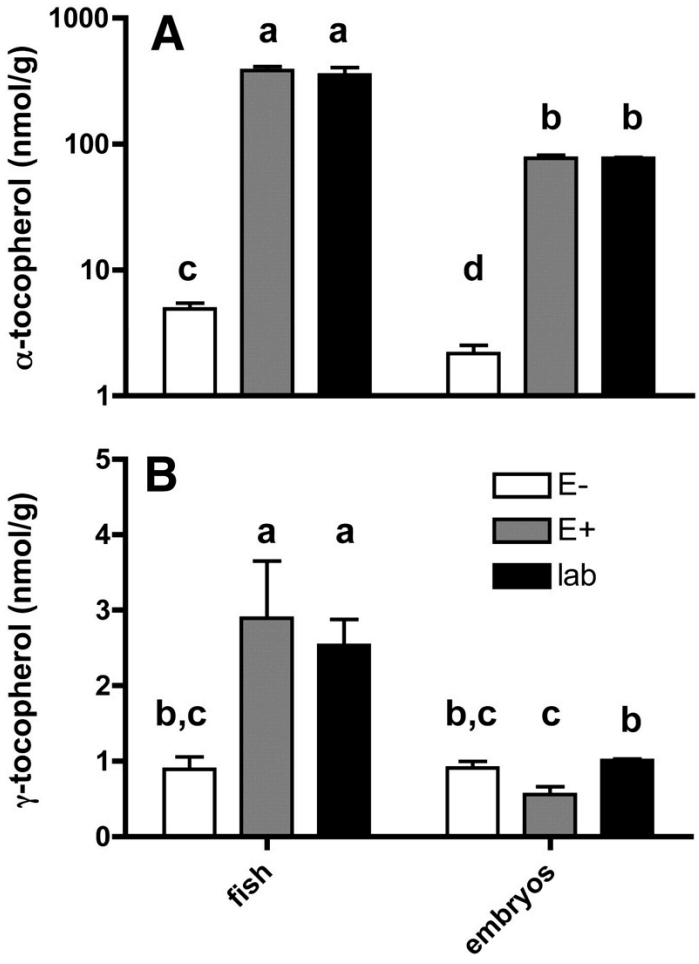
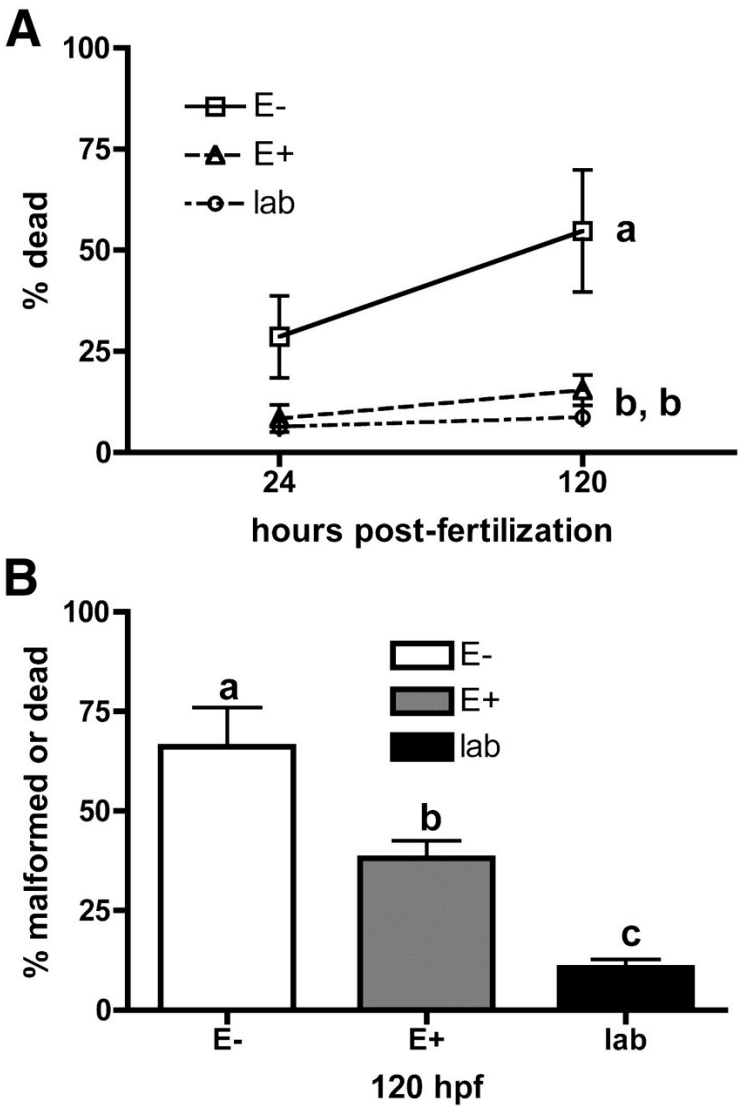
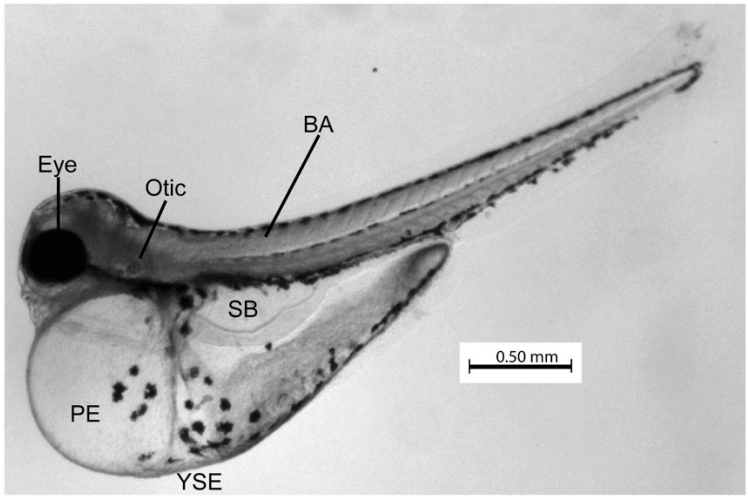

A
B
α-tocopherol (nmol/g)
γ-tocopherol (nmol/g)
1000
100
10
1
5
4
3
2
1
0
a
a
b
b
c
d
b,c
a
a
b,c
c
b
E-
E+
lab
fish
embryos

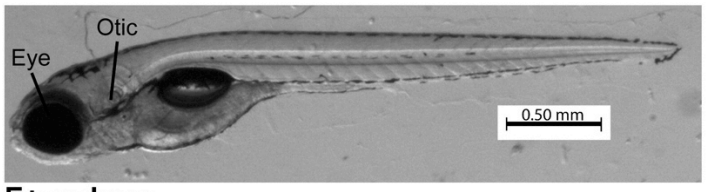
Figure 2- 5. Malformations and Mortality of Zebrafish Embryos.

(**A**) Increased mortality (mean ± SEM) was observed in the E- embryos at 24 hpf and at 120 hpf compared with the other diet groups (diet x time interaction, $p<0.0001$), but at 24 hpf the differences between diet groups did not reach statistical significance. Mortality increased from 24 to 120 hpf in the E- (squares, $p<0.01$) and the E+ embryos (triangles, $p<0.01$), but not in the lab diet embryos (circles). At 120 hpf, the E- embryos displayed significantly higher levels of mortality compared with the E+ and lab diet embryos (diet effect p=0.005; E- (a) > E+ (b) or lab (b), $p<0.05$ paired comparisons). (**B**) Higher levels of both malformations and mortality were observed at 120 hpf in the E- embryos compared with E+ ($p<0.05$, a) or lab diet embryos ($p<0.001$, b); E+ had greater malformations than did lab diet embryos ($p<0.05$, c). Embryos were analyzed in 96-well plates, one embryo per well with 48 to 120 embryos per group per spawn. Results are expressed as percentages affected per total number of embryos (n= 6 spawns per group).

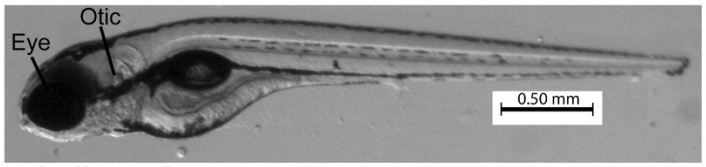

A
100
75
50
25
0
% dead
E-
E+
lab
a
b, b
24
120
hours post-fertilization
B
100
75
50
25
0
% malformed or dead
a
b
c
E-
E+
lab
E-
E+
lab
120 hpf



E- embryo



E+ embryo



Lab diet embryo

Figure 2- 6. Typical Zebrafish Morphology at 120 hpf.

Representative pictures from the three diet groups are shown after 5 days (120 h). The eye and otic vesicle are indicated on all fry; malformations are illustrated on the image of the deficient fish. CF=cranial-facial malformation, BA=bent anterior-posterior axis, PE=pericardial edema, SB=swim bladder malformation, and YSE=yolk-sac edema.

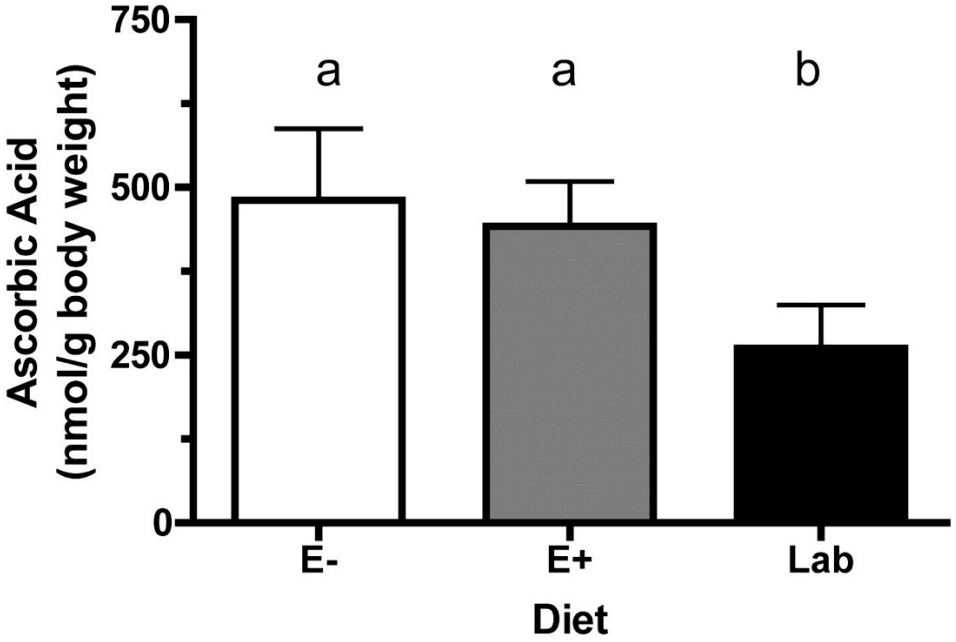


Figure 2- 7. Ascorbic Acid Concentrations in Adult Fish.

Ascorbic acid concentrations (mean ± SEM) were analyzed from two generations of zebrafish (n=7 per diet). (Diet effect $p<0.0035$, bars not sharing the same letter are significantly different $p<0.05$).

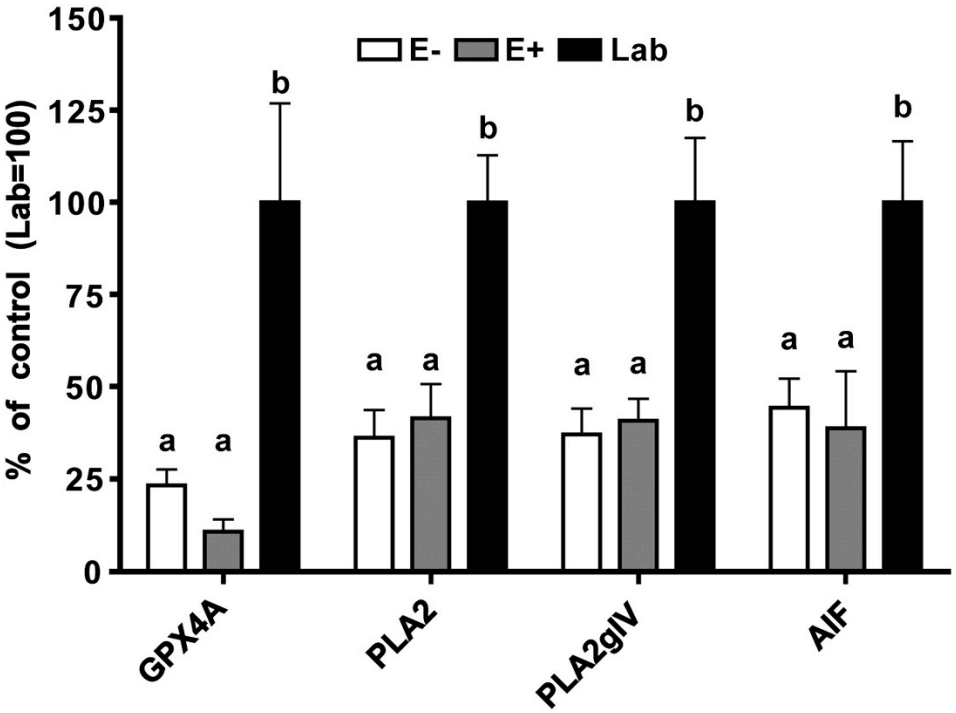


Figure 2- 8. mRNA Expression in Adult Zebrafish Liver.

mRNA expression (mean ± SEM) in adult zebrafish livers from fish from each diet group (n=4 per group, on diets >200 days) was analyzed by qPCR. Genes are defined and primers shown in Table 2. Expression levels were normalized to ß2M and are shown as fold change over the average of the lab diet control liver mRNA, set to 100. (Diet effect for GPX4a p=0.0011, PLA2 p=0.0027, PLA2GIV p=0.0058, AIF p=0.0222; bars not sharing the same letter are significantly different p<0.05).

Table 2- 1. Diet Ingredients.

Ingredient	g/100 g
Wheat Gluten	15
Casein	30.5
Egg Whites	4
Cellulose	3
Vitamin Mix[1]	4
Mineral Mix[2]	4
Starch	26.5
Tocopherol-Stripped Soybean Oil	7
Tocopherol-Stripped Soy Lecithin	5
Stay C (Argent Chemical Laboratories)	1
α-Tocopherol (E+ diets only)	0.05

[1] Containing the following (g/kg vitamin mix): vitamin A (500,000 IU/g), 0.15**;** vitamin D_3 (400,000 usp/ug), 6.2445; vitamin K, 0.025; thiamine, 0.15; riboflavin, 0.25; vitamin B_6, 0.125; pantothenic acid, 0.75; niacin, 1.25; biotin, 0.005; folate, 0.05; vitamin B_{12}, 0.0005; myoinositol, 6.25; PABA, 1; celufil (alpha cellulose), 983.75.

[2] Containing the following (g/kg mineral mix): calcium carbonate, 19.23; calcium phosphate dibasic ($2H_20$), 766.29; citric acid, 5.28; cupric carbonate, 0.36; ferric citrate, 2.99; magnesium oxide, 22.89; manganese carbonate, 5.65; sodium chloride, 28.02; disodium hydrogen phosphate, 11.89; zinc carbonate, 0.97; potassium phosphate dibasic, 74.16; potassium sulfate, 62.26; potassium iodide, 0.01.

Table 2- 2. Primers for genes of interest.

NCBI Gene ID#	Abbreviation[1]	Forward Primer	Reverse Primer
373125	AIF	5'-AAAGTCCGGAAAGAGGGTGT-3'	5'-GCCTGGAGCTCAGCATTAAC-3'
30400	β2M	5'-CCACTCCGAAAGTTCATGTGT-3'	5'-ATCTCCTTTCTCTGGGGTGAA-3'
58100	BAD	5'-CTGAAAGGAGAGCAACTGGG-3'	5'-CCGCATCAGACTCTTTGTGA-3'
57934	β-actin	5'-AAGCAGGAGTACGATGAGTC-3'	5'-TGGAGTCCTCAGATGCATTG-3'
317743	GAPDH	5'-GAATTCTGGGATACACGGAG-3'	5'-AAAGGGGTCACATCTACTCC-3'
326857	GCLc	5'-CTATCTGGAGAACATGGAGG-3'	5'-CATTTTCCTCTGTTGACCGG-3'
352928	GPx4a	5'-CCTTGGAAATGGCATCAAAT-3'	5'-CCACCACACTTGGATCCTG-3'
352929	GPx4b	5'-GAAGTGGATGAAGGAGCAGC-3'	5'-GCCGCAGCATACACACTCTA-3'
79381	GSTp1	5'-TTCAGTCCAACGCCATGC-3'	5'-ATGAGATCTGATCGCCAACC-3'
322732	LOX	5'-GGCACAGCCAAAAAGCTGAGCG-3'	5'-ATCGAGCGTCCTGGGGCAGA-3'
360149	NRF2	5'-CAGACGGAGGAGGAGCGGGA-3'	5'-GGCACTGCTGCAACTCTGGGA-3'
559087	PLA2gIV	5'-ATTTATCCTCGACCCCAACC-3'	5'-ACACCAGCATTGTTTGTCCA-3'
406370	PLA2gVI	5'-AAACTGCAGAGATGAGCCGT-3'	5'-GCCTGGTGTTTCTCCAACAT-3'
325906	TTP	5'-ATGGGAGACGCAAAGAAATG-3'	5'-TGCATGTGAATCCGTTGTTT-3'

[1]**AIF** (pdcd8), apoptosis-inducing factor (programmed cell death 8); **ß2M**, beta-2-microglobulin; **BAD**, BCL2-antagonist of cell death; **ß-actin**, beta-actin; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **GCLc**, glutamate-cysteine ligase, catalytic subunit; **GPx4a**, glutathione peroxidase 4a; **GPx4b**, glutathione peroxidase 4b; **GSTp1**, glutathione S-transferase pi; **LOX**, arachidonate 12-lipoxygenase; **NRF2**, nuclear factor (erythroid-derived 2)-like 2; **PLA2gIV**, phospholipase A2, calcium dependent; **PLA2gVI**, phospholipase A2, calcium independent; **TTP**, α-tocopherol transfer protein.

Table 2- 3. Zebrafish vitamin E concentrations and body weights taken at various times between ~80 to ~300 days consuming experimental diets.

Diet	Observations (N=)	α-Tocopherol (nmol/g)	γ-Tocopherol (nmol/g)	Body Weight (g)
E-	80	5 ± 3[a]	1.8 ± 1.0[a]	379 ± 154
E+	84	369 ± 131[b]	3.6 ± 2.4[b]	334 ± 145
lab	80	362 ± 107[b]	4.2 ± 2.2[c]	334 ± 108

A significant diet effect ($P<0.0001$) was observed for both zebrafish α- and γ-tocopherol concentrations. Data in columns not bearing the same letter are significantly different ($P<0.05$). Time points are indicated in Figure 2.

Table 2- 4. mRNA expression of in adult zebrafish liver from fish fed E-, E+ or lab diets.

Gene Symbol *	Diet E-	E+	Lab
BAD	56 ± 22	57 ± 23	100 ± 29
GCLc	76 ± 42	67 ± 29	100 ± 6
GSTp1	100 ± 48	70 ± 40	100 ± 20
GPX4b	64 ± 19	56 ± 31	100 ± 14
LOX	73 ± 26	77 ± 28	100 ± 42
NRF2	98 ± 42	85 ± 32	100 ± 20
TTP	64 ± 31	42 ± 27	100 ± 38

mRNA expression (mean ± SEM) in adult zebrafish livers from fish from each diet group (n=4 per group) was analyzed by qPCR. Genes are defined and primers shown in Table 2. Expression levels were normalized to ß2M and are shown as fold change over the average of the lab diet control liver mRNA, set to 100. No significant difference was found for a diet effect for the indicated genes.

CHAPTER 3 – THE EFFECT OF PARENTAL DIET ON EMBRYONIC ZEBRAFISH TRANSCRIPTOME

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[1]Linus Pauling Institute, [2]Molecular and Cellular Biology Program, [3]Department of Environmental and Molecular Toxicology, [4]Environmental Health Sciences Center, [5]School of Biological and Population Health Sciences; Oregon State University, Corvallis, OR

Unpublished manuscript

Abstract

Embryonic zebrafish are utilized in diverse biological fields. Although the model has risen in popularity, dietary requirements have not been properly addressed. The typical commercial laboratory diet fed to zebrafish varies within the research community, and even between batches of the same feed. The effects of these differences in adult diets on their progeny have not yet been characterized. Utilizing defined diets we begin to answer two questions: 1) How does the shift from the commercial lab diet, containing many uncontrolled ingredients, to a controlled diet (E+), sufficient for zebrafish health, alter the embryonic zebrafish transcriptome? 2) What effect does removing vitamin E from the parental diet have on the embryonic transcriptome? To answer these questions, we conducted a global gene expression study with embryos produced by adult zebrafish fed a commercial lab diet (Lab), a defined diet with (E+) and without (E-) vitamin E. We sampled embryos from each diet at 36 hours post fertilization (hpf), anchoring the analysis prior to the onset of overt malformations in E- embryos (first observed malformations at 48 hpf), capturing misregulated transcripts preceding the effect. The Lab diet embryos exhibited 946 transcripts significantly (p <0.01) differentially expressed when compared to the E+, and the loss of vitamin E impacted 2656 transcripts. The impact of the Lab diet compared to the E+ diet demonstrates that the parental diet impacts the embryo regardless of overt

morphologic consistency. Functional analysis of the significant transcripts in the E- embryos indicated systemic effects and revealed a putative mechanism by which vitamin E deficiency, and the theorized lipid peroxidation associated with this deficiency, resulted in perturbed energy metabolism, leading to overt malformations and mortality. These results demonstrate that the diet of the adult zebrafish has a direct impact on the transcriptome of the embryos.

Introduction

Zebrafish are increasingly being used as a vertebrate model organism to study human-related conditions, including: neurological disorders (Patton and Zon 2001; Dambly-Chaudiere et al. 2003; Kokel et al. 2010; Rinkwitz et al. 2011; Becker and Rinkwitz 2012), addiction (Petzold et al. 2009; Webb et al. 2009), disease treatment/drug development (Shin and Fishman 2002; Lieschke and Currie 2007; Mathew et al. 2007; Delvecchio et al. 2011; Truong et al. 2011) and gene-environment interactions (Hillwalker et al. 2010). Their developmental stages are well conserved with other vertebrates, including humans (Gilbert 2010). Zebrafish embryos develop rapidly and externally from the mother, and are optically transparent, allowing non-invasive whole organism modeling *in vivo* (Kimmel et al. 1995). Additionally, the embryonic zebrafish is genetically tractable and amenable to rapid-throughput analyses. With the increasing utilization of the embryonic zebrafish model and the lack of a consistent laboratory diet, it is vital to understand the impact of parental diet on their offspring.

The diet of zebrafish in the wild has not been determined (Lawrence 2011), as such, laboratory zebrafish are traditionally fed similarly to aquaria fish. Commercial flake feeds are typically administered in conjunction with various live feeds (e.g. *artemia nauplli*, paramecia, rotifers, etc.). These commercially prepared diets often include unknown or ambiguous ingredients

(e.g. fish oil, or fish meal), which can vary between batches, and may create confounding variables. Our group is not alone in our concern for the lack of consistent zebrafish laboratory diets (Siccardi et al. 2009; Lawrence 2011). We have previously developed a zebrafish diet, which has fully defined components and is sufficient for zebrafish health (Lebold et al. 2011; Kirkwood et al. 2012; Miller et al. 2012). This diet has permitted the controlled removal of key nutrients from the parental diet, allowing the use of zebrafish to query nutrition-related questions.

We have previously demonstrated that, at least in the case of vitamin E, the nutritional status of the adult fish is passed onto the progeny (Miller et al. 2012). The embryos from zebrafish fed a defined diet minus one nutrient, vitamin E (E- diet) were severely malformed and had a heightened incidence of mortality compared to the defined diet (E+ diet). To understand the functional consequences of vitamin E deficiency in the embryos, we used a global transcriptome analysis approach. In this study, total samples were obtained prior to the onset of any overt malformations from embryos of zebrafish fed each of the three diets. The overall goal of this study was application of transcriptome technologies to identify embryonic responses to diverse parental diets in the zebrafish, highlighting the specific role of vitamin E in development.

Materials and Methods

Fish care and husbandry

Tropical 5D (wild-type) zebrafish were reared and maintained at the Sinnhuber Aquatic Research Laboratory at Oregon State University, with protocols approved by the Oregon State University Institutional Animal Care and Use Committee (ACUP # 3897). A schematic of the experimental design is shown in **Figure 1A**. Briefly, juvenile zebrafish (~1 month of age) were divided into 3 groups and exclusively fed one of three designated diets: vitamin E deficient defined diet (E-), sufficient defined control diet (E+) or standard laboratory diet (Lab), as defined previously (Miller et al. 2012). Diets were used as described by our group (Lebold et al. 2011; Kirkwood et al. 2012), with slightly modified ascorbic acid concentrations (350 mg/kg diet, added as Stay-C, Vitamin C-3, Argent Chemical Laboratories Inc., Redmond, WA). Defined diet ingredients by percentage in **Table 1**. Beginning 3 months after diet initiation, adult zebrafish were group-spawned regularly and embryos collected and staged for subsequent experiments (Kimmel et al. 1995).

Embryonic morphology

As depicted in **Figure 1B**, to characterize the morphologic effects of parental diets, embryos (3 hpf) from each of the three diets were placed into individual wells of a 96-well plate containing 100 µl of fish water (reverse osmosis filtered water containing 0.6% Instant Ocean salt solution). Each

embryo was observed daily with a stereomicroscope for visible morphologic effects at 24, 48 and 72 hpf. Vitamin E concentrations were determined as previously described (Miller et al. 2012).

Microarray studies

Four biologically independent sets of 36 hpf embryos (n = 35 per set), collected from adults fed one of the three diets (E-, E+ and Lab) for more than 3 months, were carefully age-matched and collected into RNALater (Life Technologies, Carlsbad, CA). RNA was extracted with Trizol (Life Technologies) as per manufacturer's direction. RNA samples were further purified and characterized to specifications required for microarray analysis, described previously (Truong et al. 2012). Samples from the E- and Lab groups were kept as four independent biological replicates. Samples from the E+ diet were combined into one sample and split into four equal aliquots (technical replicates) to provide a common background comparison for the E- and Lab diet samples.

cDNA synthesis, labeling and array hybridization were performed by the University of Idaho Initiative for Bioinformatics and Evolutionary Studies (IBEST) core facility (Moscow, ID) as described previously (Tal et al. 2012). The NimbleGen Zebrafish 12x135K Array platform (Roche NimbleGen, Madison, WI) was used to hybridize the samples described above. The

University of Idaho core facility normalized raw intensity scores and normalized values were used for subsequent analyses.

Bioinformatics data and pathway analyses

NimbleScan software (Roche NimbleGen) was used to extract raw and quantile normalized data. Gene call files were generated using Robust Multichip Average (RMA) algorithm. Statistical analysis of the normalized intensities was performed using GeneSpring GX 11 (Agilent Technologies, Santa Clara, CA). The E- and Lab diet groups were compared to the E+ diet group using an unpaired t-test with unequal variance. Transcripts were considered significant at $p < 0.01$.

Functional and pathway analyses were performed on the output lists generated using GeneSpring (above). Entrez IDs were updated from the original NimbleGen annotation file (Ensembl version 7) prior to functional and pathway analyses. This updated list was generated by the Pacific Northwest National Laboratory (PNNL), and applied using the Bioinformatics Resource Manager (BRM Version 2.3) (Shah et al. 2007). Multi-Experiment Viewer (MeV) (Saeed et al. 2003) was used for heat map visualization, and analysis. The significant lists were subject to unsupervised bidirectional hierarchal clustering using Euclidean distance metric, and centroid linkage similarity clustering groups with similar expression patterns.

Functional enrichment statistics for the gene clusters were determined using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/, v6.7) (Huang da et al. 2009b, a; Huang da et al. 2009c). DAVID analyses were employed as previously described (Truong et al. 2012). Briefly, gene ontology category terms for significant genes were compared to a background list containing all of the genes probed on the NimbleGen Zebrafish 12x135K Array platform. Functional annotation clustering with medium stringency was used to group similar annotations together into non-redundant functional groups. Functional Gene Ontology (GO) terms were deemed significant with a $p < 0.05$ as determined by DAVID using a modified Fisher's exact test (EASE score) (Huang da et al. 2009b). GO terms with the highest *p*-value from each cluster were compiled and used to describe the functional category of the clustered groups.

Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, Redwood City, CA) was used to further functionally annotate the gene sets. Mammalian Entrez gene identification numbers (obtained using BRM) and fold-change or *p*-values (obtained through GeneSpring analysis) were uploaded into the IPA database. The data sets were subject to IPAs "Core analysis", and the identified biological functions obtained were sorted by their *z*-score (putative activation/repression of biological function), number of

affected molecules and *p*-value. Redundant functional groups were manually removed from the data set.

Statistical analyses

Statistical analysis of microarray results was by unpaired Student's t-test with unequal variance, values were considered significant at $p < 0.01$. These analyses were performed using GeneSpring GX 11 software.

For statistics not related to the microarray: data are presented as means ± SD. Comparisons across groups were by 2-tailed paired Student's t-test or 1-way ANOVA with Tukey's multiple-comparison post-hoc analysis; values of $p < 0.05$ were considered statistically significant. Results were tabulated with Prism 5.0 (Graph Pad, La Jolla, CA, USA).

Results

Embryo morphology and α-tocopherol concentration

We previously reported that E-deficient embryos have high rates of morphologic malformations and mortalities by 120 hpf (Miller et al. 2012). In the present study we characterized the onset of these malformations throughout development to find a morphologic anchor for transriptome studies. Embryos from all diets remained morphologically similar at 24 hpf (**Figure 2A and B**). At 48 hpf, ~50% of the E- embryos showed signs of improper development, which increased to ~75% of the embryos by 72 hpf (**Figure 2A**

and B). Embryos spawned by E+ and Lab diet fish continued to develop normally, showing no significant difference in their morphology up to 72 hpf.

Embryonic α-tocopherol status was confirmed in the three groups at 72 hpf, after the final morphologic observation. Embryos from parents fed E- diet had significantly lower α-tocopherol concentrations than the matched embryos from the E+ and Lab groups (**Figure 2C**).

Global transcriptome changes

To elucidate the mechanism responsible for the developmental malformations noted in the E- embryos, we collected RNA samples for global gene expression analysis at 36 hpf, a time prior to the appearance of any gross, phenotypic malformations. At this time point, embryos spawned from all three groups were morphologically indistinguishable (data not shown). The samples from the E+ group were used as a control for comparison to the Lab and E- diet embryos. Comparison of the E+ and Lab diet embryos identified 946 statistically ($p < 0.01$) different transcriptional changes, while E- compared to E+ diet caused 2656 differentially expressed transcripts. Of these transcript changes only 85 were shared between the Lab and E- diets when compared to the E+ diet (**Figure 3A**).

Lab and E+ diet comparisons

Between the Lab and the E+ control embryos there was, a marked effect on global transcript levels, without an accompanying phenotype (**Figure 2**). Bi-hierarchal clustering of the 946 differentially expressed transcripts between the lab diet and the E+ diet identified 571 transcripts as reduced and 375 as increased in the Lab diet (**Figure 3B**). The reduced expression group contained genes associated with metabolism and processing of carbohydrates and lipids, as well as basic cell cycle processes (**Figure 3B,** bottom). Transcripts with increased expression were categorically centered on nervous system development and function (**Figure 3B,** top). Analysis of the 946 transcripts of interest demonstrated was a decrease in the expression of transcripts linked to cellular growth, apoptosis and movement disorders in the Lab diet group, when compared to the E+ diet group (**Table 2**). There were concomitant increases in genes responsible for transcription, differentiation, and molecular transport (**Table 2**). A list of the 18 significant transcripts with higher then two fold-change is shown in Table 1 of the appendix.

Vitamin E deficiency induced transcriptional changes

The parental fish in the E- and E+ groups were fed identical, fully defined diets, differing only in the vitamin E content. The E-deficient state of the parents (data not shown) was passed on to their progeny (**Figure 2C**). The lack of this single micronutrient in the adult fish resulted in developmental

abnormalities of the offspring by 48 hpf (**Figure 2**). The E- embryos had significant differential expression of 2656 transcripts compared to E+ diet embryos. Of these transcripts, 1661 were decreased and 995 were increased in the E+ diet when compared to the Lab diet embryo transcript levels (**Figure 3C**). The transcripts with elevated expression were implicated in gene expression, amino acid metabolism, organ morphology and nervous system development (cluster #2 and #3, **Figure3C, Table 3**). The transcripts that were repressed represented cell-to-cell signaling and interaction, embryonic and tissue development (cluster #1, **Figure 3C, Table 3**). Notably similar across all three clusters, many differentially expressed transcripts were linked to control of cellular death, organogenesis and cellular growth and proliferation.

The misregulated transcripts also revealed numerous perturbed pathways. When these pathways were clustered together and analyzed in both DAVID and IPA, lipid peroxidation was predicted as a key affected process. Transcripts involved were significantly changed in the E deficient embryos: transcript levels of the amyloid precursor protein (APP) were decreased; apolipoprotein E (ApoE), nuclear receptor subfamily 4, group A, member 3 (NR4A3), cAMP response element-binding (CREB) binding protein (CREBBP), peroxisome proliferation-activated receptor gamma, co-activator 1-alpha (PGC1A) and peroxisome proliferation-activated receptor gamma, co-

activator 1-beta (PGC1B) had increased transcript levels compared to E+ controls. These genes, misregulated by the lack of vitamin E, perturb energy metabolism and mitochondrial function ultimately causing embryonic malformations and mortality. A list of the 323 significant transcripts with higher than two fold-change is shown in Table 2 of the appendix.

Discussion

Hidden" transcriptional differences

The differences in nutrient profiles between our E+ diet and the Lab diet are multifaceted (Lebold et al. 2011; Kirkwood et al. 2012; Miller et al. 2012), but adult zebrafish fed either diet can produce viable, healthy embryos. Interestingly, analysis revealed 946 differentially represented transcripts between embryos from the two diets. Many of these changes were anticipated due to the different dietary compositions. The Lab diet has been formulated to provide more than adequate nutrition for the fish. One such difference is the fatty acid content of the two diets; the E+ diet contains the essential fatty acids, linoleic and alpha-linolenic acid, but not longer chain, n-3 and n-6, fatty acids (Lebold et al. 2011). This forces the adult animals to synthesize all of the required poly-unsaturated fatty acids (PUFAs), thereby affecting the PUFA content of their progeny (Lebold et al., unpublished). Alternatively, the Lab diet provides an excess of fish oil/meal, containing high levels of n-3 PUFAs (e.g. fish oils, DHA, EPA), and may contain oxidized fatty acids as well. Similarly, the differences in lipid and carbohydrate metabolism can be attributed to the nutrient differences between the Lab and E+ diets (i.e. different levels of vitamins, minerals, phytochemicals, toxicants etc.).

The remaining transcriptional differences between the Lab and E+ are less easily explained and therefore of greater interest. There was increased

activity in the Lab embryos of genes associated with tissue development, organogenesis, cellular differentiation, transcription and molecular transport. Pathways involving apoptosis, cell growth, organismal death and neurological disease all had decreased transcript levels compared to the E+ control expression pattern. Although the embryos from the two diets did not present as morphologically different by 72 hpf, at the transcriptional level they were significantly different. These changes are solely due to the different diets fed to the adults spawned to produce the embryonic fish; thereby raising the concern that widespread use of a controlled and well-defined diet has not been properly addressed in this model (Hau and Schapiro 2011; Lawrence 2011). While the optimum nutrient levels are likely not met by either the E+ or the Lab diet, the effect of parental diet on embryonic transcription cannot be denied. More importantly, these concealed embryonic effects may affect all the long-term effects on behavior, fertility and life expectancy.

Vitamin E deficiency

Vitamin E is known as a potent lipid soluble antioxidant (Burton and Ingold 1981; Kamal-Eldin and Appelqvist 1996; Traber and Atkinson 2007), and while this action has been indirectly demonstrated *in vivo* (Traber and Sies 1996; Traber 1997; Traber et al. 2001; Bruno et al. 2006) the precise biological role of vitamin E is still unknown. Through the use of fully defined

diets we have previously demonstrated the requirement of vitamin E for proper zebrafish development (Miller et al. 2012). Similar defined diets were used in the current study, and we sampled the transcriptome prior to onset of E deficient developmental malformations. If left intact, >75% of E- embryos typically displayed gross malformations and mortality by 72 hpf. Remarkably, removing a single micronutrient resulted in a higher number of significantly misregulated transcripts than did the switch from the undefined Lab diet. The transcriptional profile provides a range of possible mechanisms for a vitamin E requirement. Basic developmental functions were chief amongst affected transcripts: organ morphology was differentially regulated, appearing in all significant clusters of E- compared to E+ embryos; transcripts responsible for cell proliferation, differentiation and death were also decreased in the E- embryos. Although these changes preceded the development of a visible phenotype, many of them are indicative of a systemic problem, and as such are likely downstream of the initial E deficient effect.

Our proposed mechanism of action is depicted in **Figure 4**. In theory, vitamin E in embryonic development functions as a lipid soluble antioxidant, halting the lipid oxidation chain reaction. Oxidative stress is produced through endogenous processes and is common throughout development (Jauniaux et al. 2004). Therefore, in the absence of vitamin E, lipid peroxidation would overwhelm the antioxidant capacity of the embryo through a combination of

oxidized PUFAs (Hashimoto et al. 2005; Spiteller 2006; Andreo et al. 2011; Corsinovi et al. 2011), and increased lipid peroxidation by the amyloid precursor protein (APP) (Sung et al. 2004). APP transcripts were decreased in E- embryos, perhaps as an attempt to mitigate the lipid peroxidation resulting from the absence of vitamin E. An additional response to elevated lipid peroxidation was the upregulation of apolipoprotein E (ApoE), which would replace the oxidized lipids and vitamin E (if it were available) in its role as a lipid transporter. ApoE has also been linked with the dysregulation of APP (Cedazo-Minguez et al. 2001; Hoe and Rebeck 2008; Carter 2011). These hypothetical changes in lipid-membranes activate the nuclear receptor subfamily 4, group A, member 3 (NR4A3) (Pearen and Muscat 2010) and decreased APP allowing upregulation of CREB binding protein (CREBBP) transcription factor (Chong et al. 2003; Rouaux et al. 2003), which was increased in our data set. These effects may have culminated in the increase of peroxisome proliferator-activated receptor gamma, coactivator 1-alpha (PGC1A) and -beta (PGC1B). This upregulation could be explained by the increasingly oxidative environment (Kamei et al. 2003; Handschin and Spiegelman 2006; Rhee et al. 2006; Liu and Lin 2011), and the activity of both NR4A3 (Pearen and Muscat 2010) and CREBBP (Puigserver et al. 1999; Herzig et al. 2001). These interactions were demonstrated in our study by the increased transcript levels of ApoE, NR4A, CREBBP, PGC1A and PGC1B in

our E- embryos compared to the E+ control diet embryos. PGC1A and PGC1B are nuclear-receptor cofactors and have global biologic functions, chiefly serving as mediators of energy metabolism (Handschin and Spiegelman 2006; Liu and Lin 2011), theoretically resulting in perturbed energy metabolism in E- embryos. The system-wide importance of these cofactors explains the catastrophic outcome caused by vitamin E deficiency. PGC1A and PGC1B have been implicated in the zebrafish by other work as well; they are necessary for oxytocin production in the zebrafish brain (Blechman et al. 2011), and their expression is co-localized with many known zebrafish nuclear receptors (Bertrand et al. 2007). The identification and description of a likely mechanism of action during development opens new doors into the study of vitamin E. Coupled with the inherent advantages and tractability of the vitamin E deficient zebrafish model, this likely mechanism illuminates a role for vitamin E in development.

Conclusions

The need to standardize zebrafish laboratory diet is recognized within the zebrafish community (DeKoven et al. 1992; Markovich et al. 2007; Siccardi et al. 2009; Lawrence 2011). Current research diets include ambiguous ingredients, content may vary between batches, and they may contain toxins, or toxic levels of some ingredients (e.g. mercury found in *artemia* from the

Great Salt Lake (Peterson and Gustin 2008), and vitellogenin induction due to commercial diets in farmed tilapia (Davis et al. 2009)). Furthermore, these results beg the question of long-term effects on growth, development and behavior due to the parental diet-induced developmental programming alterations. Previous groups have formulated partially defined diets for laboratory fish (DeKoven et al. 1992; Markovich et al. 2007; Siccardi et al. 2009), but the main source of fish nutritional research is found in large-scale aquaculture (National Research Council (U.S.). Committee on Animal Nutrition. 1993), which is focused on adult health. The data presented herein addresses a possible confounding variable in zebrafish research, previously unaccounted for, but one that is readily possible to alleviate.

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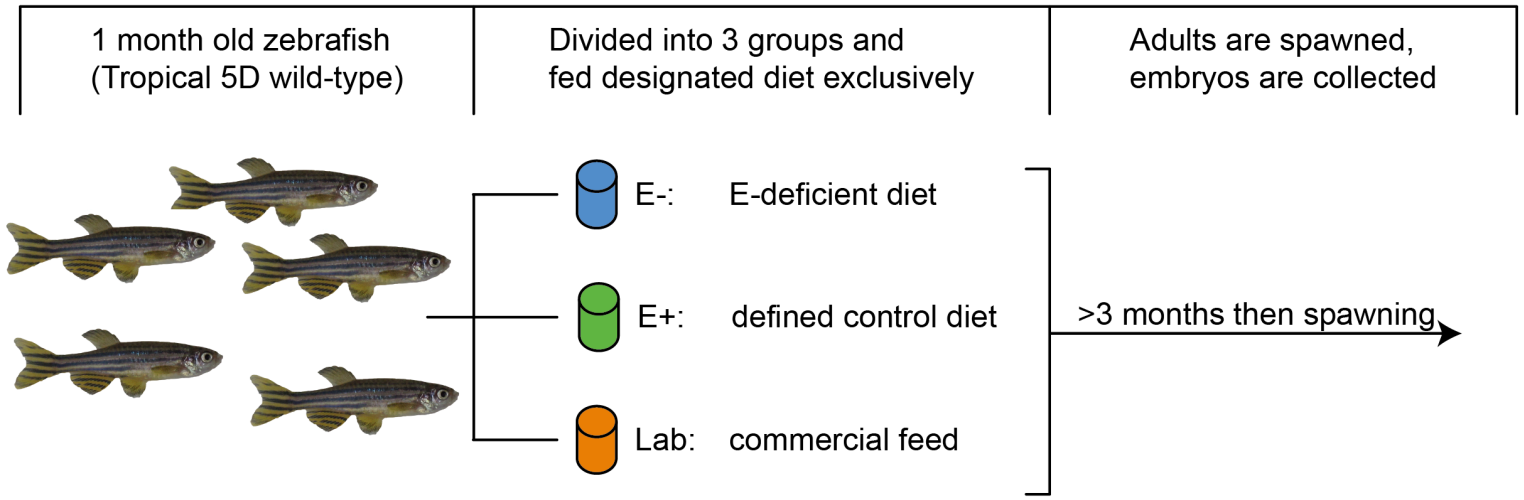
Figure 3- 1. Experimental design and feeding scheme.

A. Schematic of the experimental design and feeding regime. From left to right: 1-month old zebrafish were split into equal groups and exclusively fed one of three diets (E-, E+ or Lab diets). After 3-months of dietary initiation, zebrafish from each group were spawned regularly. **B.** Embryos from each diet were observed until 72 hours post fertilization (hpf). Noted on the timeline are observations at 24, 48 and 72 hpf, samples were collected and euthanized at 72 hpf. *RNA samples were from one clutch only.

Figure 1. Experimental design

A. Experimental set up



B. Embryonic experimental timeline

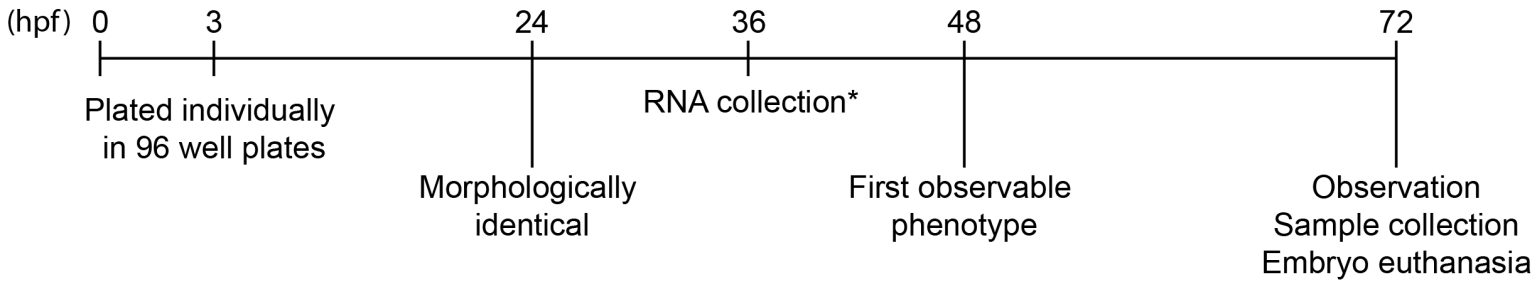
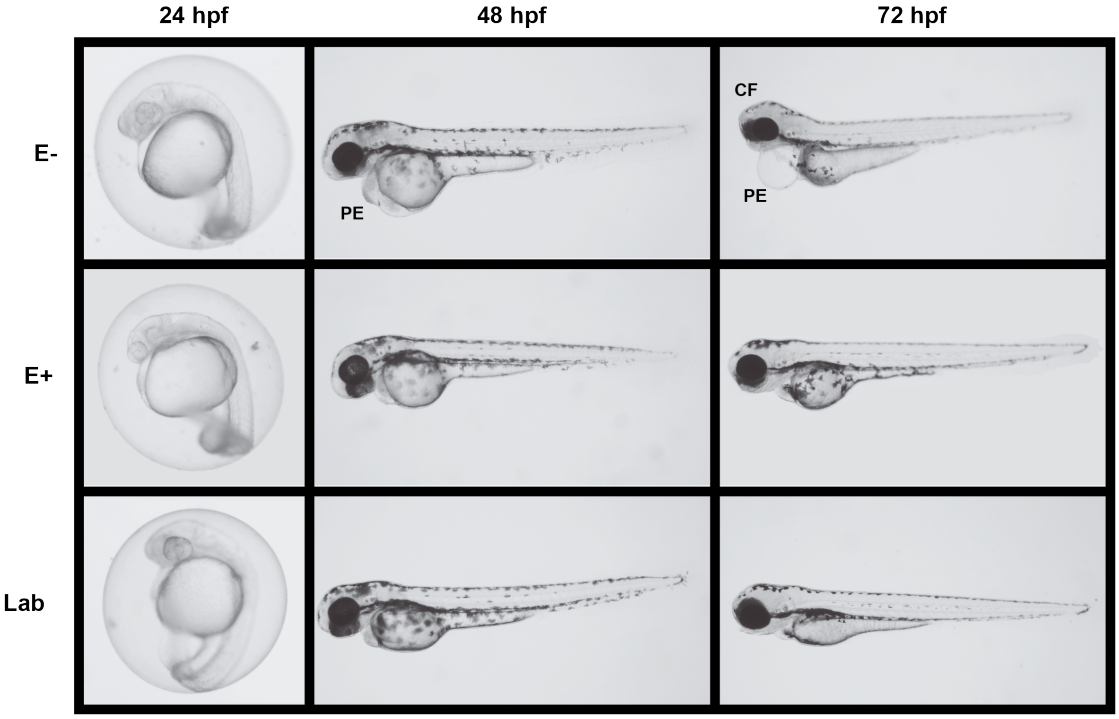


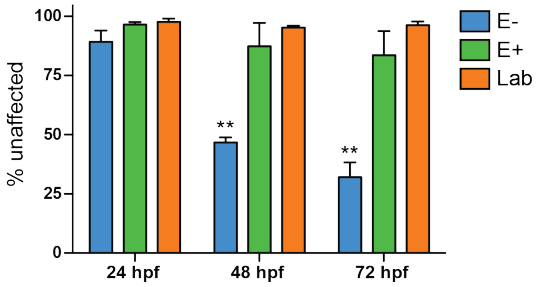
Figure 3- 2. Embryonic development differs with parental diet.

A. Parental diet effects on embryo morphology. Representative images displaying embryo morphology at 3 time points for each diet. At 24 hpf, no adverse effects are noted across groups. At 48 hpf, the E- group begins to display pericardial defects, which are dramatically apparent by 72 hpf. The E+ and the Lab group embryos remain similar throughout. **B.** Embryos from each of the three groups are indistinguishable at 24 hpf, but at 48 hpf, >50% of embryos from the E- group show adverse outward signs of the vitamin E deficiency. At 72 hpf, nearly 70% of the E- embryos display abnormalities or mortalities. Embryos from the E+ and Lab diet groups remain similar throughout the experimental observations. Data are shown as mean ± SD, results shown from 3 separate spawns (E-, n = 358 embryos; E+. n = 396; Lab, n = 296). **C.** Embryos spawned from E- diet-fed fish show deplete α-tocopherol, mirroring their parents. Results shown as mean ± SD, n = 3 pools of embryos for all diet groups. PE = pericardial edema, CF = cranial facial malformation. ** indicates $p < 0.001$ compared to E+ and Lab group (one-way ANOVA, with Tukey's HSD post-test).

A. Representative images of embryonic effects



B. Morphology time course



C. Embryo α-tocopherol concentrations (72 hpf)

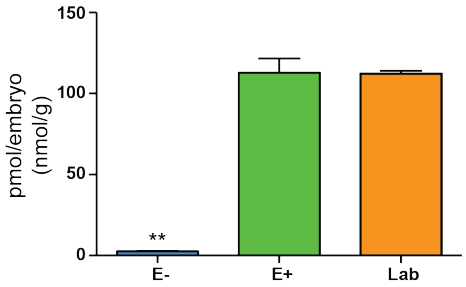


Figure 3- 3. Global effects of parental diet on embryonic transcription.

A. A Venn diagram displaying differentially represented transcripts. Both Lab and E- group were first compared to the E+ defined diet control group; significant transcripts (p<0.01) from each set were then compared as represented in the figure. **B and C.** Transcripts from the Lab (**B**) and E- (**C**) that were significantly different from the E+ controls were group using bi-hierarchical clustering as displayed in the heat map. General annotations for each cluster are noted on the right side the heat maps (annotation generated using IPA and DAVID).

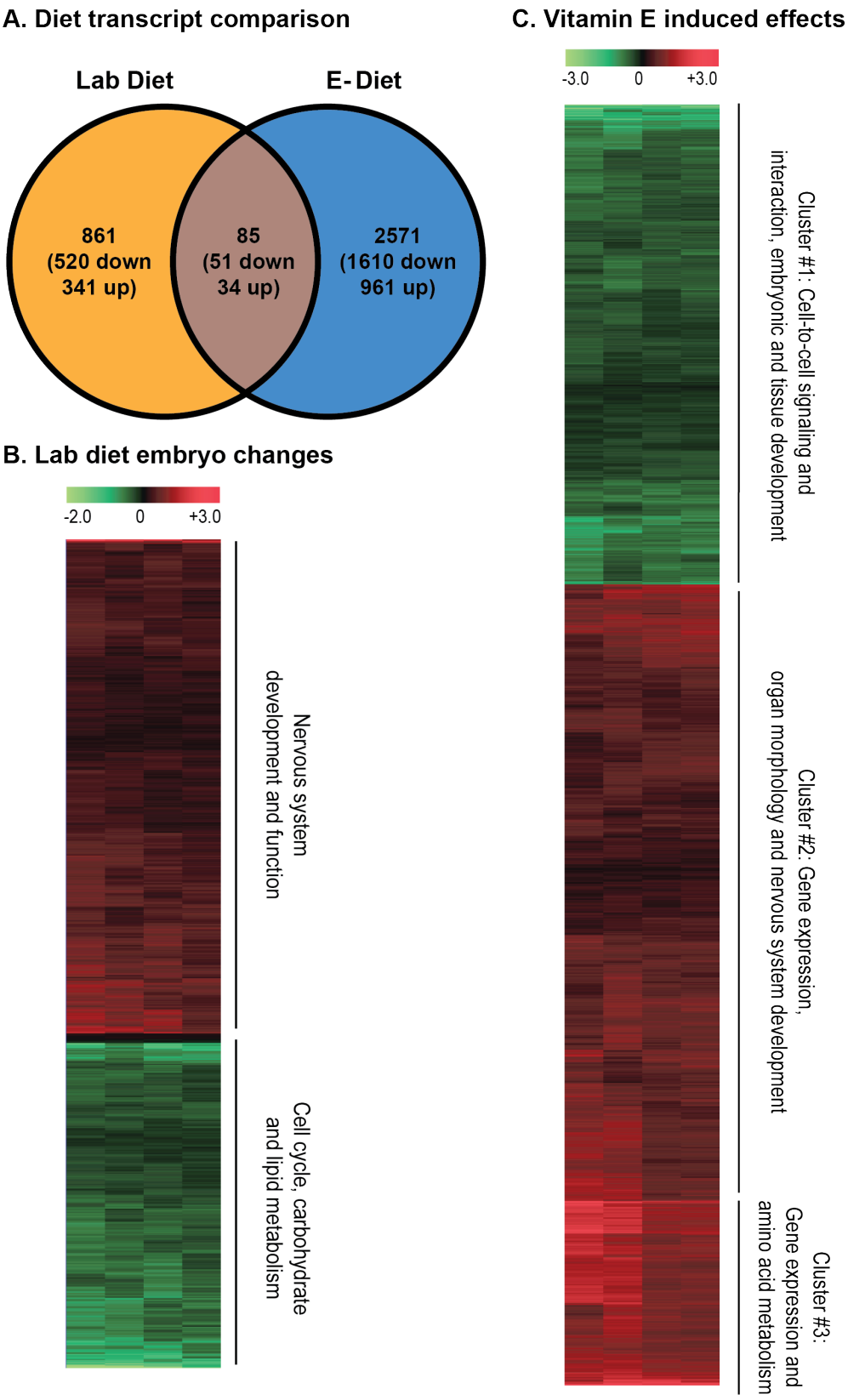

A. Diet transcript comparison
Lab Diet
E- Diet
861
(520 down
341 up)
85
(51 down
34 up)
2571
(1610 down
961 up)
B. Lab diet embryo changes
-2.0
0
+3.0
Nervous system
development and function
Cell cycle, carbohydrate
and lipid metabolism
C. Vitamin E induced effects
-3.0
0
+3.0
Cluster #1: Cell-to-cell signaling and
interaction, embryonic and tissue development
Cluster #2: Gene expression,
organ morphology and nervous system development
Cluster #3:
Gene expression and
amino acid metabolism

Figure 3- 4. Proposed mechanism of action for vitamin E in development.

Vitamin E deficiency results in increased lipid peroxidation, which drives the dysregulation of energy metabolism, reactive oxygen species detoxification and mitochondrial function. Solid arrows indicate direct and dashed arrows are indirect activation, blunted arrows denote repression. Genes colored red are elevated compared to E+ embryos, and green transcripts are decreased. APP, amyloid precursor protein; ApoE, apolipoprotein E; CREBBP, CREB binding protein; NR4A3, nuclear receptor subfamily 4, group A, member 3; PGC1A, peroxisome proliferator-activated receptor gamma, coactivator 1-alpha; PGC1B, peroxisome proliferator-activated receptor gamma, coactivator 1–beta.

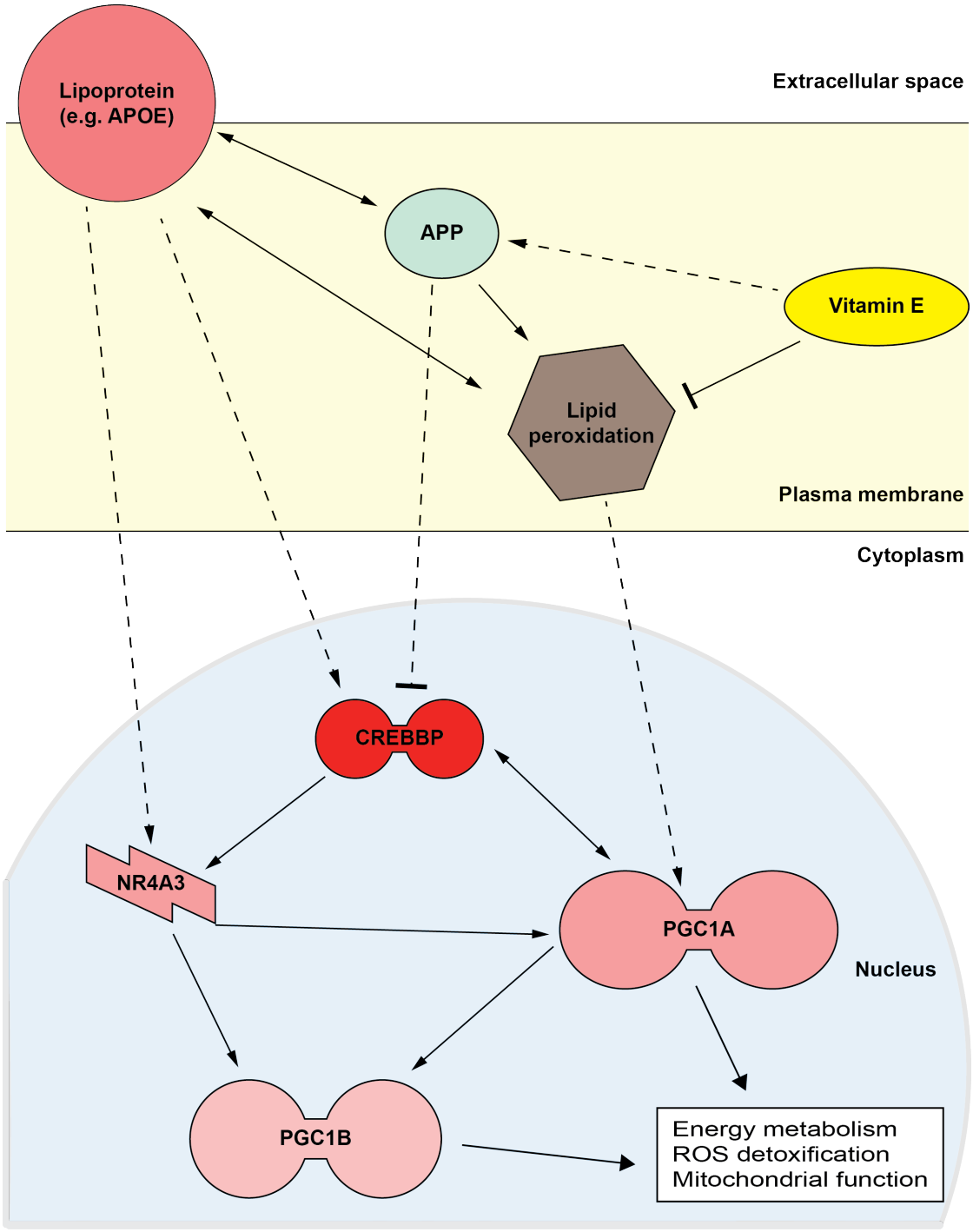

Lipoprotein
(e.g. APOE)
Extracellular space
APP
Vitamin E
Lipid
peroxidation
Plasma membrane
Cytoplasm
CREBBP
NR4A3
PGC1A
Nucleus
PGC1B
Energy metabolism
ROS detoxification
Mitochondrial function

Table 3- 1. Defined diet nutritional profile

Defined nutrient	Amount
Protein	49.5%
Carbohydrate	26.5%
Fat	12%
Fiber	4%
Vitamin and Mineral Mix	8%

Nutrient make-up of defined diets (E- and E+).

Table 3- 2. Lab diet induced transcriptional effects.

Category - Functional annotation	p-value	# Molecules
Tissue Development - Tissue development	3.38E-07	123
Cell Death - Apoptosis	1.51E-02	92
Organismal Development - Organogenesis	1.55E-05	87
Embryonic Development - Development of organ	2.71E-05	85
Cellular Development - Differentiation	1.20E-02	74
Cellular Growth and Proliferation - Growth of cells	3.38E-03	73
Organismal Survival - Organismal death	9.69E-03	62
Gene Expression - Transcription of DNA	4.36E-03	61
Neurological Disease - Movement disorder	1.99E-06	56
Molecular Transport - Transport of molecule	2.53E-04	56

Table 3- 3. Deficient diet cluster annotation.

Category - Functional annotation	p-value	# Molecules
Cluster #1		
Cell Death - Cell death	3.85E-03	142
Cellular Growth and Proliferation - Proliferation of cells	3.17E-03	117
Cell Death - Apoptosis	1.26E-02	107
Organismal Survival - Organismal death	6.00E-04	78
Organ Morphology - Morphology of organ	2.43E-03	69
Molecular Transport - Transport of molecule	3.63E-04	63
Cell Cycle - Cell cycle progression	1.23E-03	53
Cell Death - Cell survival	1.48E-02	52
Cellular Function and Maintenance - Organization of cytoplasm	1.53E-03	50
Cell Death - Cell death of organ	5.48E-03	48
Cluster #2		
Gene Expression - Transcription of DNA	1.19E-24	149
Gene Expression - Activation of DNA endogenous promoter	1.59E-21	111
Tissue Development - Tissue development	9.93E-16	198
Cell Death - Cell death	3.57E-07	196
Cellular Growth and Proliferation - Proliferation of cells	4.36E-06	158
Embryonic Development- Organogenesis	2.66E-13	146

Organismal Development - Development of organ	1.72E-13	145
Cellular Development - Differentiation	3.00E-12	145
Organismal Survival - Organismal death	2.55E-09	115
Organ Morphology - Morphology of organ	2.62E-09	107
Cluster #3		
Cell Death – cell death	2.70E-02	38
Tissue Development – tissue development	5.98E-03	35
Gene Expression - Expression of RNA	7.12E-05	34
Cellular Growth and Proliferation - Proliferation of cells	3.55E-02	31
Cellular Development - Differentiation	5.70E-03	27
Organismal Survival - Organismal death	5.68E-03	23
Cell Cycle - Cell cycle progression	2.47E-04	20
Neurological Disease - Encephalopathy	4.29E-03	20
Cellular Function and Maintenance - Organization of cytoplasm	2.24E-03	17
Skeletal and Muscular Disorders - Neuromuscular disease	8.00E-03	16

CHAPTER 4 – THE α-TOCOPHEROL TRANSFER PROTEIN IS ESSENTIAL FOR VERTEBRATE EMBRYOGENESIS

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Abstract

The hepatic α-tocopherol transfer protein (TTP) is required for vitamin E bioavailability in humans; mutations in the human *TTPA* gene result in the heritable disorder ataxia with vitamin E deficiency (AVED, OMIM #277460). TTP is also expressed in mammalian uterine and placental cells and in the human yolk-sac, underscoring its significance during fetal development. TTP and vitamin E are essential for productive pregnancy in rodents, but their precise physiological role in embryogenesis is unknown. We hypothesize that TTP is required to regulate delivery of α-tocopherol to critical target sites in the developing embryo. We tested to find if TTP is essential for proper vertebrate development, utilizing the zebrafish as a non-placental model. We verify that TTP is expressed in the adult zebrafish and its amino acid sequence is homologous to the human ortholog. We show that embryonic transcription of TTP mRNA increases >7-fold during the first 24 hours. *In situ* hybridization demonstrates that *Ttpa* transcripts are localized in the developing brain, eyes and tail bud at 1 day post fertilization. Inhibiting TTP expression using oligonucleotide morpholinos results in severe malformations of the head and eyes in nearly all injected embryos (88% TRN injected embryos, compared to 5.6% in control MO injected and 1.7% in non-injected controls). We conclude that TTP is essential for early development of the vertebrate central nervous system.

Author Summary

Vitamin E (α-tocopherol) was discovered almost 90 years ago as a plant lipid that restored fertility to diet-restricted rodents. Researchers have since expanded upon the maternal α-tocopherol requirement during pregnancy, but the fetal requirement remains unknown. The critical role of vitamin E in human health has been established, although its relevance to human fertility remains unclear. The α-tocopherol transfer protein (TTP) is responsible for selection of α-tocopherol and facilitates its movement to specific locations within the body. People with heritable TTP mutations are deficient in vitamin E and develop the neurodegenerative disease ataxia with vitamin E deficiency (AVED). In this study we characterize the expression pattern and requirement of TTP during embryonic development of the zebrafish. Our findings demonstrate that TTP has a novel and critical role in brain development during a period corresponding to the first month of human pregnancy.

Blurb

The α-tocopherol transfer protein, which selects and traffics vitamin E (α-tocopherol) within the liver, is required for zebrafish embryonic brain formation.

Introduction

Vitamin E (α-tocopherol) was discovered almost 90 years ago because rats fed an α-tocopherol deficient diet failed to carry their offspring to term; the fetuses were resorbed approximately 9 days into pregnancy (Evans and Bishop 1922). Although the fetal-resorption test is still used to define the international units for vitamin E (Food and Nutrition Board. Institute of Medicine. 2000), the cause of the embryonic failure has never been characterized. Likely the embryonic delivery system for α-tocopherol involves the α-tocopherol transfer protein (TTP) because in the adult liver TTP facilitates α-tocopherol transfer into the plasma. Humans with *TTPA* gene mutations demonstrate a heritable disorder: ataxia with vitamin E deficiency (AVED, OMIM #277460), which manifests in infancy and childhood. TTP, however, is not exclusively a liver protein; it is expressed in human yolk sac (Jauniaux et al. 2004); and has been detected in mammalian placental and uterine cells (Jishage et al. 2001; Kaempf-Rotzoll et al. 2002; Kaempf-Rotzoll et al. 2003). Previously, we utilized the zebrafish model to separate the maternal and embryonic requirements, and characterize the molecular defect of embryonic vitamin E deficiency. We reported that α-tocopherol deficient fish spawn and produce viable eggs, but within days the embryos and larvae display developmental impairment and increased risk of mortality (Miller et al. 2012), establishing a critical embryonic need for α-tocopherol. Zebrafish

nutrients are derived solely from the yolk sac for the initial 4-5 days post fertilization. After demonstrating the embryonic requirement for vitamin E we next queried how it is transferred into the embryo during development. We hypothesized that 1) zebrafish express a protein homologous to the human TTP and 2) TTP is required for early embryonic development. In the present study, we test the hypothesis that adult zebrafish express TTP that is homologous to the human protein. As development is a highly regulated process with specific spatial and temporal control, we evaluate the quantity and location of *Ttpa* during the first day of zebrafish development. To test for embryonic requirement we inhibited translation of TTP using antisense morpholinos (MO) to knockdown protein expression. We conclude that TTP is essential for early brain and axis development.

Results

Zebrafish TTP: identification and mRNA characterization

The zebrafish (NP_956025.2) and human (NP_000361.1) TTP amino acid sequences were compared using Align2 (http://bioinfo.cgrb.oregonstate.edu/), (**Figure 1A)**. The TTP protein sequences are highly conserved between the two species, sharing 64% identical and 85% similar amino acid residues. Even greater conservation (82% identity and 95% similarity) is observed within the ligand binding pockets

of the two orthologs (residues 129-194 of the human proteins and 126-191 of the fish, highlighted in **Figure 1A**). Close inspection of the two sequences revealed that of the 18 residues identified as relevant to human TTP function (identified from AVED patients and *in vitro* studies) (Usuki and Maruyama 2000; Meier et al. 2003; Min et al. 2003; Mariotti et al. 2004; Morley et al. 2004; Min 2007), 15 were identical between the zebrafish and human sequences, 2 were similar, and only one residue (D64) was different (**Table 1)**. This latter unmatched residue, an aspartic acid in the 64th position of the human sequence, has only been reported in one AVED patient, who also harbored an additional point mutation in the TTP coding region (Usuki and Maruyama 2000). The aspartic acid residue has not been otherwise implicated in α-tocopherol binding or TTP function. Thus, it is not likely that this amino acid substitute should alter the activity of the zebrafish ortholog. For additional confirmation of homology we tested for anti-human TTP cross reactivity with western blotting. Adult zebrafish liver homogenate reacted with the anti-human TTP with a single band at 33 kD, the expected size of the zebrafish protein (left lane, **Figure 1B**).

Characterization of the mRNA expression was done by qPCR using a time course of embryonic RNA from 6-24 hpf. Initial expression (6 hpf) increases dramatically beginning ~10 hpf (**Figure 2A**). We chose embryos aged 1-day post fertilization (dpf), prior to development of the liver, to define

the spatial expression pattern of TTP using RNA *in situ* hybridization. TTP mRNA is expressed throughout the developing head, eyes and in the tail bud (**Figure 2**). Prior to 1 dpf, TTP mRNA expression is less spatially restricted and appears throughout the length of the embryo, apparently at greater amounts close to the yolk sac (**Figure 2**).

Disruption of TTP expression

Morpholinos were used to evaluate the requirement for TTP during zebrafish embryogenesis. Our experiments focused on a translational blocking MO (TRN), complementary to a region including the start codon of the mature TTP mRNA (**Figure 3A**). Embryos injected with the TRN showed significant developmental defects along the anterior/posterior axis at 1 dpf, including both cranial and tail malformations ($p<0.0001$ by ANOVA; $p<0.001$ TRN compared to CTR or NON, Tukey's multiple comparison test, **Figure 3C**). These malformations were noted in >88% of injected embryos by 1 dpf, compared with the embryos injected with the control MO (CTR, 5.6%) and non-injected control embryos (NON, 1.7%, **Figure 3B**). It is important to note that these malformations occur in the same regions as the expression of TTP mRNA at 1 dpf (**Figure 2).**

To determine the sequence of the observed malformations, embryos injected with TRN, CTR, and non-injected controls (NON) were followed using time-lapse microscopy from ~6 hpf until ~24 hpf (**Videos M1 and M2**).

Throughout blastula formation, epiboly and gastrulation (6-11 hpf), all embryos appeared to develop normally. At ~12 hpf, the nascent eye of embryos injected with TRN begin to display tissue darkening (**Figure 4**), indicating the initiation of improper head growth. At 1 dpf in the TRN embryos, eye or brain formation was almost completely halted, and a misshapen tail was evident, whereas the CTR embryos developed normally (**Figure 3**). Unfortunately, due to the low level of TTP expression in the developing embryos and interference by the overabundance of vitellogenin-derived yolk-proteins (Link et al. 2006) we were not able to verify TTP knockdown by immunohistochemistry.

To confirm that the TRN specifically knocked down TTP protein expression, we designed two additional, non-overlapping MOs that target the second exon in the TTP pre-mRNA. The exon-exclusion (EXC) MOs are complementary to either end of the second exon (**Figure 3A**). These MOs interfere with the splicing and processing of the pre-mRNA resulting in the deletion of exon two from the mature product (Draper et al. 2001; Morcos 2007). This alteration would result in a truncated protein product, if the aberrant mRNA were translated, due to a reading-frame shift caused by the exon exclusion and resulting in a pre-mature stop codon (**Figure S1**). The efficacy of splice inhibition by the EXC MOs was verified by RT-PCR amplification of a region spanning exon two and size verification by gel electrophoresis (**Figure S2**, primer locations shown as black arrows in **Figure**

S1). The RT-PCR gel shows a complete loss of proper-size TTP mRNA in the EXC MO-treated embryos; instead the products are smaller due to the exclusion of exon two from the final product. Additionally, embryos injected with the EXC MOs present with a significantly lower amount of TTP transcript (**Figure S3**), regardless of mRNA size (primers complimentary with regions not affected by the EXC MOs, orange arrows **Figure S1**). This loss of TTP mRNA is likely due to nonsense-mediated decay of the aberrant transcripts. Importantly, employing the EXC MOs compared with the TRN MO yielded the same phenotype, namely abnormal head and eye formation, and a truncated tail. These results confirm that TTP knockdown using either MO targeting strategy disrupts the normal developmental processes.

Non-specific p53 induction has been observed following injection with some morpholinos (Robu et al. 2007; Gerety and Wilkinson 2011). To confirm that the phenotype observed with TTP knockdown was not a result of off-target p53 induction, co-injections with a p53 knockdown MO were performed. The p53 MO co-injection did not affect the TTP phenotype (data not shown), and was not used in subsequent experiments.

Discussion

This study shows that expression of TTP is essential for early embryonic development in the zebrafish. The high degree of sequence similarity suggests a functional conservation between the human and zebrafish TTP orthologs. This conclusion is further supported by the fact that anti-TTP antibodies recognize a band at the expected size in zebrafish tissues (**Figure 1B**). The cross-reactivity of an anti-human TTP antibody (**Figure 1B**) coupled with the sequence comparisons (**Table 1**) all support that zebrafish TTP is an ortholog of the human protein.

Having established the existence and putative functional conservation of TTP in the zebrafish, we examined its role in development. Expression of TTP mRNA during development is initially low (6 hpf), but increases dramatically by 9-12 hpf and remains elevated thru 24 hpf (**Figure 2A**). Importantly, increased TTP expression precedes formation of the vascular system, and days ahead of liver formation (Kimmel et al. 1995), suggesting a critical role for TTP during development.

The dramatic phenotype, especially the impaired brain formation, due to the loss of TTP has not been noted in vitamin E deficient rodents because their embryos are resorbed prior to neurogenesis or eye formation (Evans and Bishop 1922), or in the case of TTP knockout mouse models, mothers are infertile unless supplemented with high doses of vitamin E (Jishage et al.

2001). The finding in TTP knockdown zebrafish embryos raises the intriguing possibility that low vitamin E status has adverse events in early central nervous system development in other animals, including humans. In TTP knockout mice, Jishage et al. (Jishage et al. 2001) showed that embryos (regardless of TTP mutations) developed neural tube defects and failed to come to term if the mother was TTP-/- (Jishage et al. 2001). While this study addresses the maternal TTP deficiency, the phenotype and link to central nervous system development is similar to our findings in the zebrafish (**Figure 3C**). In support of this notion, previous studies have shown a clear association between maternal vitamin E status during gestation and cognitive function of the offspring (Ambrogini et al. 2011; Betti et al. 2011; Shichiri et al. 2011). The zebrafish model presents an important means to elucidate the fetal requirements for α-tocopherol, independent of the maternal needs. Fetal resorption and placental failure have been noted in TTP knockout mice (Terasawa et al. 2000; Jishage et al. 2001), which are similar to outcomes observed upon diet-induced vitamin E deficiency (Evans and Bishop 1922; Leth and Sondergaard 1977; Ames 1979). The TTP protein is expressed in the placental and uterine cells of mice and humans (Jishage et al. 2001; Kaempf-Rotzoll et al. 2002; Kaempf-Rotzoll et al. 2003; Jauniaux et al. 2004; Muller-Schmehl et al. 2004), and is thought to play an important role in supplying maternal α-tocopherol to the developing fetus to protect against

oxidative stress (Jauniaux et al. 2004). The mammalian studies provide insight into the requirement of TTP for implantation and placental formation, both of which are linked to maternal transfer and need, but fail to determine the TTP requirement of the developing fetus. The mammalian maternal vitamin E requirements occur prior to the developmental stage in which TTP is required in the zebrafish, creating a barrier to the study of TTP in placental models.

TTP specifically traffics α-tocopherol, suggesting that its loss confers an α-tocopherol deficient state in the developing embryo. Our current methods lack the resolution to determine the subcellular localization of α-tocopherol, although we theorize that TTP, which functions as an intracellular transporter of α-tocopherol (Manor and Morley 2007), is required to facilitate delivery of α-tocopherol to critical locations, chiefly within the developing neural tissues. We attempted to determine the distribution of α-tocopherol in early zebrafish development by injecting 1-2 cell stage embryos with the previously characterized fluorescent α-tocopherol analog: ω-nitrobenzoxadiazole-α-tocopherol (NBD-toc) (Nava et al. 2006), but due to technical difficulties could not demonstrate specific transfer and localization.

Morpholino knockdown has been linked to non-specific p53 activation in the zebrafish embryo (Robu et al. 2007; Gerety and Wilkinson 2011). We experienced this first hand with a morpholino targeting the exon1-intron1-2

junction (data not shown). The non-specific activation presents with a phenotype similar to TTP morphant embryos (malformations in the head and tail). These malformations can be mitigated (if not rescued entirely) by co-injection with a MO against p53 (Robu et al. 2007). The p53 MO co-injection alleviated the high occurrence of mortality associated with the exon1-intron1-2 MO, revealing the non-specific p53 activation associated with said MO (data not shown). Co-injection with the p53 MO has recently been called into question, as it may cover specific p53-dependent processes (Bedell et al. 2011), and it has been suggested that MO with phenotypes that are rescued by p53 MO co-injection cannot be reliably studied (Gerety and Wilkinson 2011). As such, we discontinued use of the exon1-intron1-2 targeted MO, and used instead the MO discussed above. All MO were tested for rescue by co-injection. Co-injection with matching concentrations of p53 MO (Robu et al. 2007), failed to rescue the phenotype associated with TTP knockdown, allowing the use of these MO to study TTP function in the developing zebrafish.

We previously demonstrated the requirement of vitamin E during zebrafish development using diet-induced vitamin E deficient embryos (Miller et al. 2012). The malformations associated with TTP knockdown are different from those caused by vitamin E deficiency. Although the α-tocopherol concentration of the E- embryos was >50-fold decreased from the control

embryos, they still possessed detectable amounts of vitamin E. This is likely due to the specific allocation of maternal vitamin E, and its incorporation into the yolk of the developing egg. Loss of TTP, however, precludes the specific trafficking and localization of vitamin E, likely mimicking an absolute deficient state regardless of the ubiquitous yolk sac supply. Furthermore, in our previous studies vitamin E deficiency was imposed by parental diet, while TTP knockdown was preformed using embryos from fish fed commercial lab diets. This difference in parental diets affects not only the nutrient composition but the transcriptional profiles as well (unpublished observation, Chapter 3). Notably, as morphologic outcomes from each study are ultimately due to vitamin E deficiency, they likely involve common mechanisms.

The loss of TTP function results in malformations along the anterior/posterior axis (**Figure 3C**) and early life-stage mortality. We theorize that TTP mediates α-tocopherol transfer to critical sites in the embryo during early vertebrate development and thus, TTP is required for embryogenesis. It is important to note that this requirement for TTP takes place during a time analogous to the first 20 days of human gestation. This window is prior to the detection of most pregnancies, and often before the consumption of prenatal supplements. This early requirement combined with the inadequate consumption of vitamin E (McBurney 2011) could be responsible for early failures in human pregnancy. The role of TTP and vitamin E in post-

implantation development needs to be addressed, as these results highlight the role of TTP and ramifications of its loss.

In summary, we demonstrate that adult zebrafish express TTP, which is homologous to the human protein. As development is a highly regulated process and genes are specifically controlled in both a spatial and temporal fashion, we assayed both the quantity and location of *Ttpa* during the first day of zebrafish development. The function of TTP was determined through inhibition of TTP translation using antisense MOs to knockdown protein expression. We conclude that TTP is essential for early brain and axis development, likely because it delivers α-tocopherol to the developing embryo.

Materials and Methods

Fish husbandry

Wild-type zebrafish (Tropical 5D strain) were kept under standard laboratory conditions at 28.5° C with a 14 h light/10 h dark cycle (Westerfield 2000). Embryos were obtained through natural group spawning; embryos were collected and kept in standard fish water.

Immunoblotting

Adult zebrafish were euthanized by overdose of buffered tricaine, livers were dissected out, frozen in liquid nitrogen and homogenized in RIPA buffer

(150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.) with 1% Protease inhibitor cocktail set III, EDTA-free (Calbiochem, Gibbstown, NJ). The protein concentration was determined using the Bradford assay with the Coomassie Plus reagent per manufacturer's instructions (Pierce Biotechnology, Rockford, IL). Lysates were immunoblotted for endogenous TTP using a rabbit polyclonal CW201P antibody and a secondary HRP-conjugated rabbit antibody in combination with SuperSignal West Dura substrate (Thermo Fisher Scientific, Inc., Rockford, Il) for visualization.

Rabbit anti-human TTP antibody (CW201P)

Recombinant TTP was expressed in bacteria and purified as described previously (Morley, Panagabko et al. 2004). Briefly, GST-TTP fusion protein was isolated from over-expressing bacteria using glutathione affinity chromatography, cleaved with thrombin, re-purified by two ammonium sulfate precipitations and stored at -20°C in 20mM Tris pH 8.0, 150mM NaCl, 50% (v/v) glycerol, 1 mM DTT. For antibody preparation, purified TTP was dialyzed into phosphate-buffered saline. 250 µg at 1mg/ml were used per injection into 2 rabbits (Covance, Denver, PA). The initial protein injection was emulsified in Freund's Complete Adjuvant (FCA), while the 3 boosts, spaced at 3 week intervals, were emulsified in Freund's Incomplete Adjuvant (FIA). The antibody was purified from crude serum using protein G sepharose and stored

at -20°C until use. For Western blotting, antibody was diluted 1:1000 with PBS, 2% bovine serum albumin. TTP reactivity was routinely confirmed as an immunoreactive band near 32 kDa, which is missing from liver extracts prepared from TTP-/- mice.

TTP knockdown by MO injection

Morpholinos (MOs) (GeneTools LLC, Philomath, OR) were designed complementary to the TTP RNA sequence. TRN MO sequence: 5'-TCTCGTCTACTTCTTCGGACTTCAT-3', EXC MO sequences: 5'-AGCTGTGAATTACCAACAATCAAAT-3' and 5'-TGTATGTACCTGCCAATCCGATAGA-3'. A standard zebrafish control MO was used as a control for the injection process (GeneTools LLC). MOs dissolved in UltraPure DNase/RNase-Free distilled water (Invitrogen, Carlsbad, CA), were injected into 1-2 cell-stage embryos at concentrations of 0.06 to 0.07 mM in 3-5 nl injections (0.12 – 0.14 mM total MO in EXC). Phenol red (Sigma Aldrich, St. Louis, MO) was added to verify injection location. To control for spawn quality and embryo handling, a group of non-injected embryos (NON) were collected and observed as well. After injections embryos were placed individually in 96 plates and observed for malformations at 1 dpf by stereomicroscopy.

Time lapse studies.

Embryos (4-7 hpf) into individual wells of a 384-well assay plate, black with 0.9mm clear bottom (Corning Inc., Corning, NY) in ~90 µl of standard fish water and sealed with a MicroAmp Optical Adhesive Film (Life Technologies, Carlsbad, CA). Images were obtained once every 10 min using an ImageXpress Micro Imaging System (Molecular Devices, Inc., Sunnyvale, CA). Images were analyzed and movies created from stacked (time-lapse) images using MetaXpress software, version 3.1.0.93 (Molecular Devices, Inc.).

RNA *in situ* hybridization

Embryos were allowed to develop until the desired stage (Kimmel et al. 1995), euthanized by overdose with buffered tricaine (MS 222, ethyl 3-aminobenzoate methane sulfonate salt; Sigma-Aldrich, St. Louis, MO, USA) and fixed overnight with 4% paraformaldehyde in phosophate buffered saline (PBS) at 4° C, then washed and stored in methanol at -20° C until they were processed. Whole mount *in situ* hybridization was performed using digoxygenin-labeled, antisense RNA probes as in (Thisse and Thisse 2008), using the 2010-updated protocol (zfin.org). Embryos were mounted in glycerol, allowed to clear for >24 h and imaged on glass slides with a Nikon SMZ (800 or 1500) stereomicroscope, using a Nikon CoolPix 4500 camera. The zebrafish *ttpa* transcript was cloned from embryonic cDNA using a pCR4-Blunt TOPO vector with the primers: 5'-TGGACCGCCCGTCGCAGATA-3' and 5'-AGCTGCACCATTCAGTCATGTCCA-3'. The anti-sense probe was

synthesized using a T7 RNA polymerase (Promega, Madison, WI) after enzymatically digested with Pst1 (Promega).

Quantitative real-time PCR

Embryos (n=30) were collected in RNAlater (Invitrogen) at noted time points, RNA extraction and qPCR preformed as described previously (Miller, Labut et al. 2011). Ornithine decarboxylase 1 (odc1) was chosen as a reference gene for normalization (Ho, Dukovcic et al. 2011).

RT-PCR

Embryos (n=30) were collected at 12 hpf and processed as described above. PCR was preformed using primers specifically designed to flank the MO-targeted exons (FOR [UC580] 5'-ATGAAGTCCGAAGAAGTAGAC-3' and REV [UC1441] 5'-GAGCATGAGCAAAACACCAA-3', and arrows in **Figure 3A**) and KOD Hot Start DNA polymerase (EMD Chemicals, San Diego, CA) as per manufacture's direction. Product resolution was achieved using the FlashGel™ System (Lonza Group Ltd, Switzerland).

Statistics

Statistic analyses were preformed using GraphPad Prism software version 5.0d (GraphPad Software, Inc., La Jolla, CA, USA). Relationships between the MO groups were analyzed using one-way analysis of variance on the percentage of viable embryos. Post hoc tests were carried out using paired

comparisons (Tukey's multiple comparison test). Data are reported as means; differences were considered significant at $p<0.05$.

Ethics Statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of Oregon State University (ACUP Number: 3903). All fish were euthanized by tricaine (MS 222, Argent Chemical Laboratories, Inc., Redmond, WA) overdose prior to sampling, and every effort was made to minimize suffering.

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A

```
                10        20        30        40        50        60        70
Danio    MKSEEVDETEELNNLPVDSSRIAPYLSELKEKA-EAELRIRDLDLSKTFLIRFLQARDFDVALALKLLINYHKWRQEC
                 .:: ::  :  . : :. :...: :: . .  : :. .:::.:::.:::::. :: .:: ::.::: ::
Human   MAEARSQPSAGPQLNALPDHSPLLQPGLAALRRRAREAGVPLAPLPLTDSFLLRFLRARDFDLDLAWRLLKNYYKWRAEC
               10        20        30        40        50        60        70        80

        80        90       100       110       120       130       140       150
Danio   PEITADLRPSSVIGLLQNNYHGVLRSRDDAGSRVLIYRIGKWNPKEFTAYEVFRVSLITSELIVQEWETQRNGLKAIFDL
        :::.::::.: :.:::::. .:::::::::: .::.::::::..:.:: :::::.:::::::::::::::: :::::::.::::::
Human   PEISADLHPRSIIGLLKAGYHGVLRSRDPTGSKVLIYRIAHWDPKVFTAYDVFRVSLITSELIVQEVETQRNGIKAIFDL
               90       100       110       120       130       140       150       160

        160       170       180       190       200       210       220       230
Danio   QDWCFAHALQINPSLAKKISSVLTDSFPLKVRGIHLINEPIFFRPVFAMIRPFLPDKIKQRIHMHGCSYARSLCNYFPKA
        . : :.::.::.::.::::..:::::::::::::::::::::..:. ::.::.::: .:::.:::::: .: .:: ..::
Human   EGWQFSHAFQITPSVAKKIAAVLTDSFPLKVRGIHLINEPVIFHAVFSMIKPFLTEKIKERIHMHGNNYKQSLLQHFPD-
              170       180       190       200       210       220       230

        240       250       260       270       280
Danio   VLPPVYGGTGPSVDEVCQEWTEYIMQSEDYLHRLSVDLGGEGGHASQS
        .::  :::   :....:::::..::.:::::  .:
Human   ILPLEYGGEEFSMEDICQEWTNFIMKSEDYLSSISESIQ
     240       250       260       270
```

B

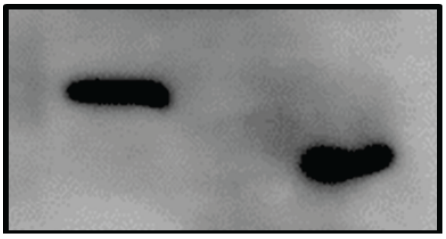


Figure 4- 1. The zebrafish α-tocopherol transfer protein.

A. Alignment of human and zebrafish TTP amino acid sequences is shown. Double dots indicate identical residues and single dots correspond to similar amino acids. Red text signifies α-tocopherol binding pocket. Align2 software at http://bioinfo.cgrb.oregonstate.edu/ was used for sequence comparison. Sequences were obtained from NCBI. **B.** Anti-human TTP antibody cross-reacts with TTP from adult zebrafish liver homogenate. The 33 kD zebrafish protein (left lane) shown with a Ttp-/- mouse sample as a negative control (center lane) and a WT mouse sample with the 32 kD mouse homolog (right lane).

Figure 4- 2. TTP expression is dynamic in the developing zebrafish.

A. Embryonic TTP transcription increases during the first 24 hpf. Bars indicate mean expression change over interval; values are expressed as fold change compared to 6 hpf. Data shown are from four independent collections and mRNA expression analyses. **B-G.** Whole mount *in situ* hybridization of *ttpa* reveals the patterning of mRNA expression. **B.** A lateral view of a whole mount embryo at 12 hpf shows fairly even distribution, however, in **C** a dorsal view of the rostral region with the yolk removed shows specific staining along what may be the developing neural tube. **D.** At 17 hpf expression remains along the length of the embryo, concentrating in the deeper cells, closer to the yolk sac. **E.** A dorsal view of the developing head at 17 hpf, the eyes and neural tube is where the expression appears to be localized (outlined). **F.** By 24 hpf the staining is seen only in the regions of the developing brain, eyes and tail bud. **G.** Dorsal view depicts brain and eye specific patterning. Yolk sacs were manually removed to reduce color interference, and for ease of positioning. fb= forebrain, mb= midbrain, *= midbrain-hindbrain boundary.

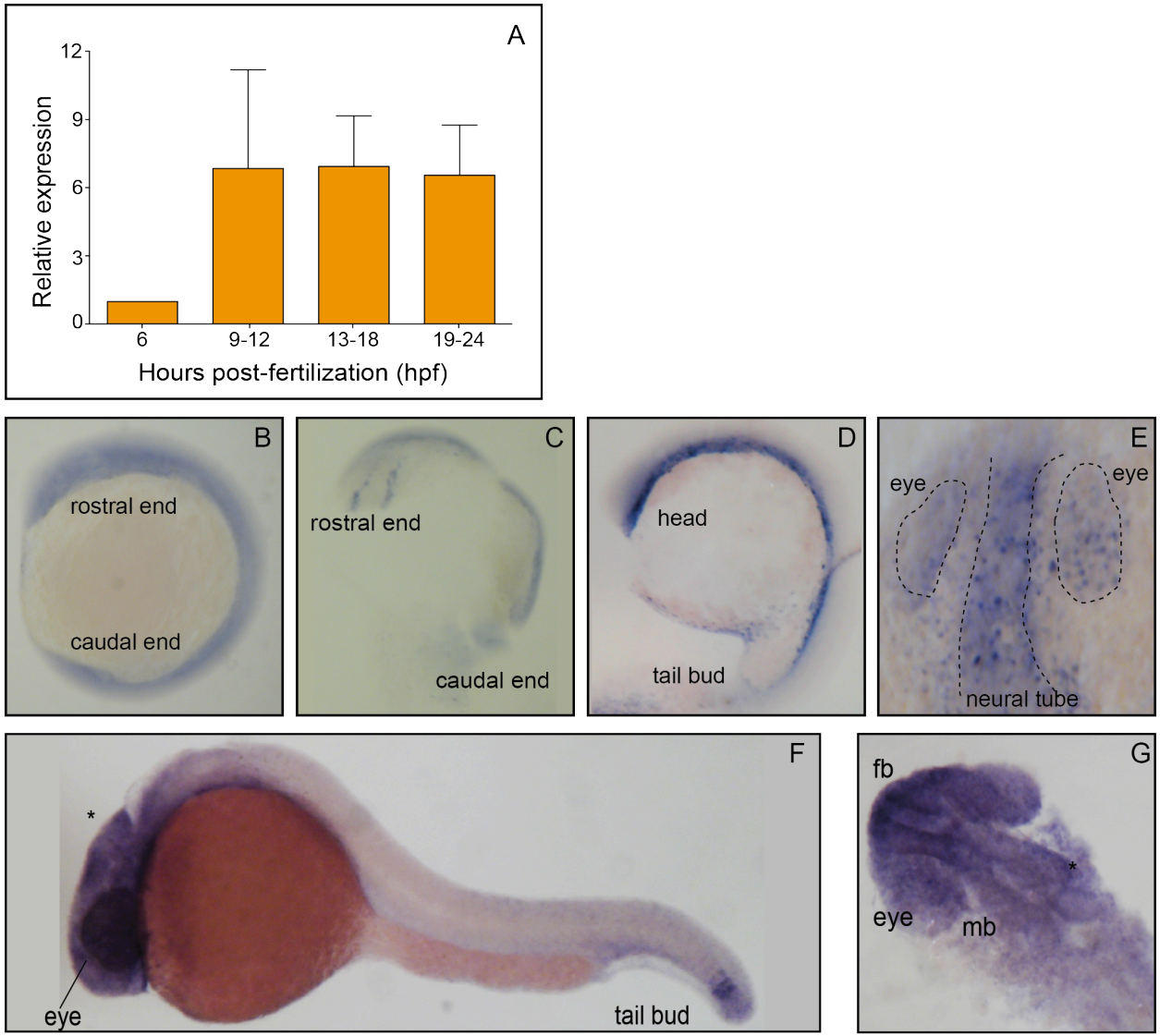
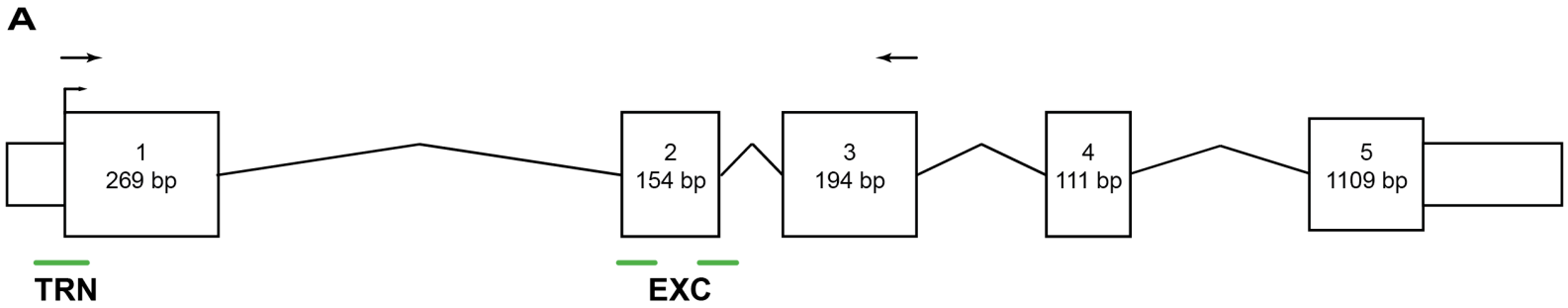
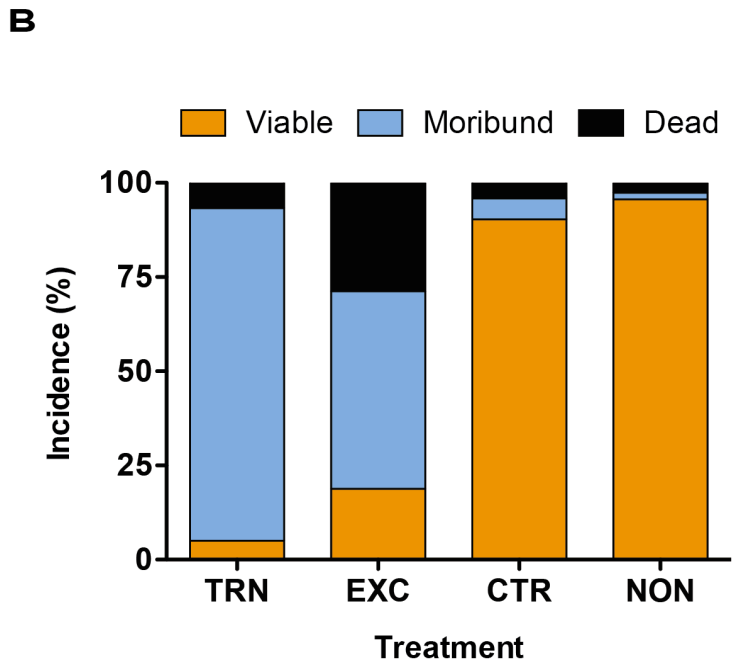

A
12
9
6
3
0
Relative expression
6
9-12
13-18
19-24
Hours post-fertilization (hpf)
B
rostral end
caudal end
C
rostral end
caudal end
D
head
tail bud
E
eye
eye
neural tube
F
*
eye
tail bud
G
fb
*
eye
mb

Figure 4- 3. Morpholino knockdown of TTP causes severe malformations. **A.** MO targeting schematic using a graphic representation of the *ttpa* transcript. The translational blocking morpholino (TRN) is complementary to the translation start-site, while the splice blocking morpholinos (EXC) bind to the intron/exon junctions on each side of the second exon. Arrows mark primers used to verify aberrant mRNA products resulting from the EXC morpholino (**Figure S2**). Numbered boxes represent exons, and spanning lines are introns, smaller unnumbered boxes are untranslated regions. **B.** TTP knockdown leads to high incidence of malformation within the first day of development. Data shown as mean percent incidence from seven (TRN, CTR and NON) or three separate experiments (EXC). **C.** Representative pictures of malformations at 1 dpf due to TTP knockdown. TRN= translational morpholino injected embryo, CTR= standard control injected embryo, concentration and age-matched to the TRN embryo.


A
1
269 bp
2
154 bp
3
194 bp
4
111 bp
5
1109 bp
TRN
EXC


B
Viable
Moribund
Dead
100
75
50
25
0
Incidence (%)
TRN
EXC
CTR
NON
Treatment

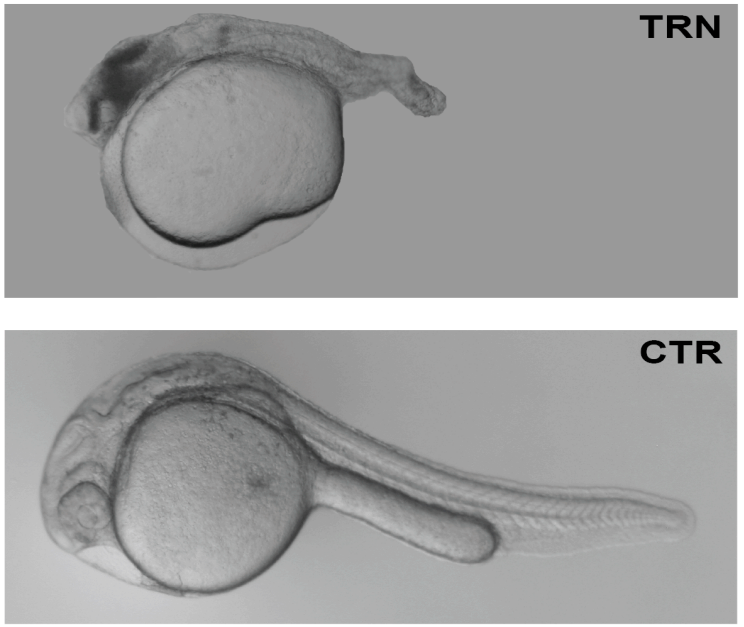

C
TRN
CTR

Figure 4- 4. Early morphant malformations.

Images of embryo development from 6-18 hpf demonstrating early effects of TTP knockdown (right panel) compared to an injected control animal at the same age (left panel). Embryos from each injection type remain constant through 11 hpf. Beginning at 12 hpf, malformations are noticeable in the rostral region of the embryo. These initial malformations occur in the head as soon as the developing eye (marked) becomes distinguishable. The malformations are more pronounced at later stages of development (16 and 18 hpf), while somite formation continues unabated. Images are frames from a time-lapse video (**Videos M1 and M2**).

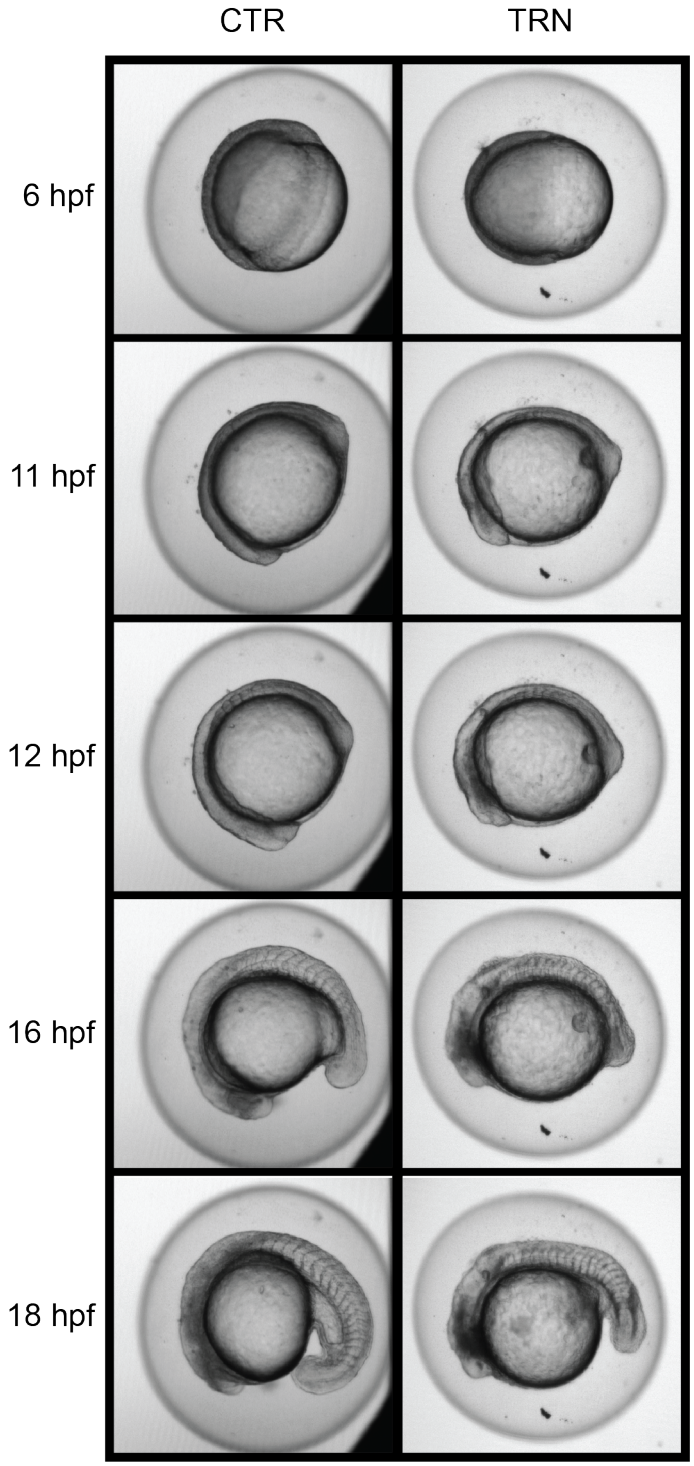

CTR
TRN
6 hpf
11 hpf
12 hpf
16 hpf
18 hpf

Table 4- 1. TTP residues implicated in α-tocopherol binding.

na, information not available.

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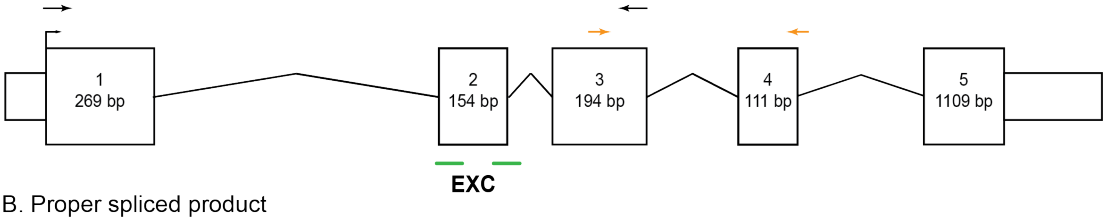
Human residue	Zebrafish residue	Comparison	AVED associated mutations	Ref	α-Tocopherol interaction	Ref
R59	R56	Identical	R59W- early onset	[1]	Decreased binding and transfer	[2]
D64	A61	Dissimilar	D64G- early onset	[1]	na	
H101	H98	Identical	H101Q- late onset	[1]	Similar to wild type	[2]
Y117	Y114	Identical	na		Binding pocket	[3]
A120	G117	Similar	A120T- late onset	[1]	Similar to wild type	[2]
A129	A126	Identical	na		Binding pocket	[4]
F133	F130	Identical	na		Binding pocket	[3,4]
S140	S137	Identical	na		Binding pocket	[3,4]
E141	E137	Identical	E141K- early onset	[1]	Decreased transfer	[2]
I154	L151	Similar	na		Binding pocket	[3,4]
I171	I168	Identical	na		Binding pocket	[3,4]
I179	I176	Identical	na		Binding pocket	[3,4]
V182	V179	Identical	na		Binding pocket	[3,4]
L183	L180	Identical	L183P- NR	[1]	Binding pocket	[3,4]
L189	L186	Identical	na		Binding pocket	[3]
R192	R189	Identical	R192H- late onset	[1]	Similar to wild type	[2]
R221	R118	Identical	R221W- early onset	[1]	Decreased binding and transfer	[2]
G246	G243	Identical	G246R- late onset	[5]	na	

Figure 4S- 1. Putative products of splice variants.

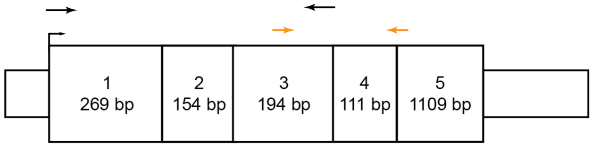
A. TTP transcript is depicted, with EXC morpholinos (green lines), marked. **B.** The proper mature mRNA and associated full-length protein. **C.** A naturally occurring splice-variant (inclusion of intron 1-2), recorded as “non-coding”, if translated, results in a truncated protein product due to a frame shift. **D.** The exclusion of exon 2 from the mature mRNA results in a premature stop codon, and if translated, a truncated peptide product. Sequences of interest are marked: splice-block verification primers (black arrows), qPCR primers (orange arrows) and transcription start site (black right-hand arrow).

Figure S1. Putative products of splice variants

A. TTP transcript schematic

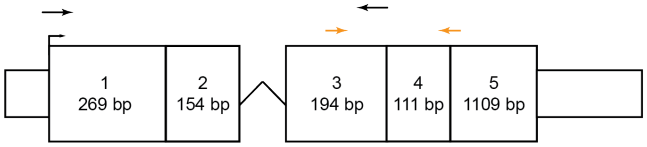


B. Proper spliced product



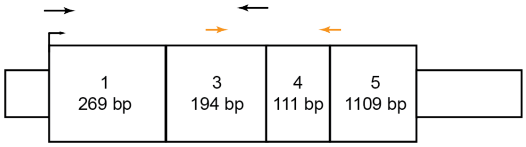
MKSEEVDETEELNNLPVDSSRIAPYLSELKEKAEAELRIRDLDLSKTFLIRFLQARDFDVA
LALKLLINYHKWRQECPEITADLRPSSVIGLLQNNYHGVLRSRDDAGSRVLIYRIGKWNPK
EFTAYEVFRVSLITSELIVQEWETQRNGLKAIFDLQDWCFAHALQINPSLAKKISSVLTDS
FPLKVRGIHLINEPIFFRPVFAMIRPFLPDKIKQRIHMHGCSYARSLCNYFPKAVLPPVYG
GTGPSVDEVCQEWTEYIMQSEDYLHRLSVDLGGEGGHASQS*

C. Native splice variant



MKSEEVDETEELNNLPVDSSRIAPYLSELKEKAEAELRIRDLDLSKTFLIRFLQARDFDVA
LALKLLINYHKWRQECPEITADLRPSSVIGLLQNNYHGVLRSRDDAGSRVLIYRIGRYIHK
Q*

D. Morpholino induced splice variant



MKSEEVDETEELNNLPVDSSRIAPYLSELKEKAEAELRIRDLDLSKTFLIRFLQARDFDVA
LALKVNGTPKSSQPMRFFVSASLRQS*

Figure S2. Splice-blocking confirmation

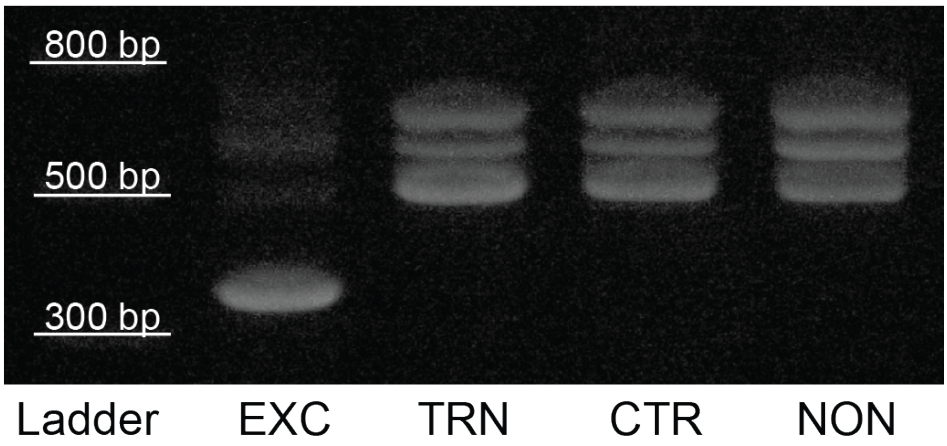


Figure 4S- 2. Splice-blocking confirmation.

PCR products created using primers flanking exon 2 in the TTP mRNA sequence are shown. Products from EXC injected embryos (EXC) display an aberrant transcript when compared to the other TTP knockdown (TRN), or the control groups (CTR and NON). The loss of exon 2 creates a single 346 base pair (bp) product, the proper transcript shows the expected three bands (the result of splice variants) all of which are larger than the EXC induced exon deletion (519-604 bp).

Figure S3. Splice blocking results in decreased TTP mRNA

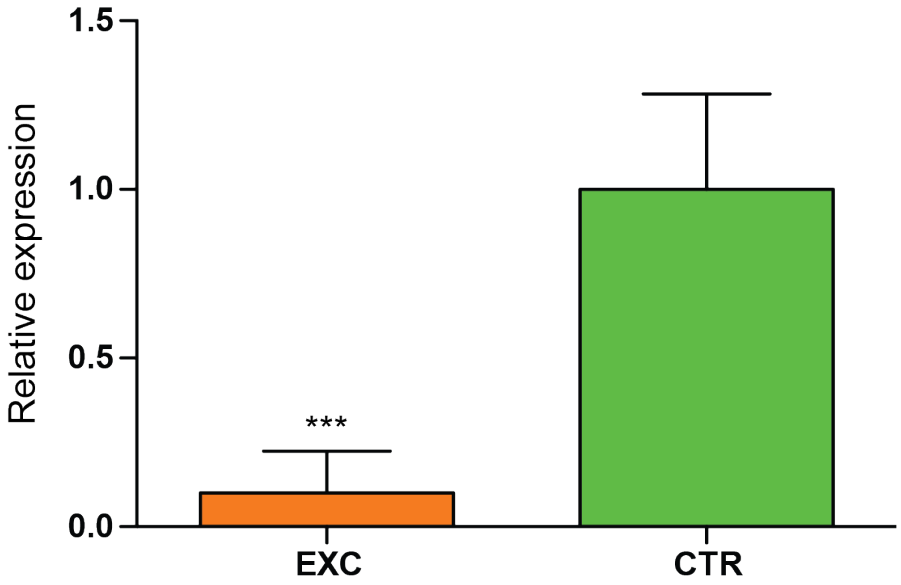


Figure 4S- 3. Splice blocking results in decreased TTP mRNA.

At 12 hpf, prior to overt malformations, TTP transcripts are significantly reduced in EXC embryos compared to the CTR embryos. This ~10-fold reduction in TTP mRNA is likely due to nonsense mediated decay of the aberrant transcript (Gene-tools, personal communication). The qPCR amplicon does not include the excluded exon (primers represented as orange arrows in **Figure S1**), and therefore does not differentiate between proper and aberrant mRNA. Shown as mean ± SD, n = 5, EXC and n = 3 CTR, biological replicates from separate experiments. ***, $p < 0.001$ by Student's t-test.

Video M1. TTP knockdown time-lapse video.

Representative embryo with TTP knockdown from 4-24 hpf (TRN). Loss of TTP causes notable malformations beginning at ~12 hpf. The rostral and caudle parts of the embryo fail to develop, while somitogenesis continues unabated. Arrow appears next to beginning eye-spot at ~12 hpf.

Video M2. Control injected embryo time lapse.

Representative control (CTR) injected embryo from 4-17 hpf. Embryo development proceeds in proper fashion regardless of the injection process, as compared to non-injected, not shown. Arrow appears next to beginning eye-spot at ~12 hpf.

CHAPTER 5 – DISCUSSION

Presented herein is unequivocal evidence that the zebrafish requires both vitamin E and the α-tocopherol transfer protein (TTP). In the 90 years since its discovery, vitamin E has been linked to animal health and wellness (Dierenfeld 1994; Allison and Laven 2000; Berchieri-Ronchi et al. 2011), maternal fertility (Allison and Laven 2000; Jishage et al. 2001; Berchieri-Ronchi et al. 2011) and the human neurodegenerative condition, AVED (Harding et al. 1985). TTP binds and transfers α-tocopherol (Catignani 1975; Catignani and Bieri 1977; Kuhlenkamp et al. 1993), and loss of function mutations in the gene encoding TTP result in drastically decreased plasma α-tocopherol levels and AVED (Traber et al. 1987; Sokol 1988; Sokol et al. 1988; Ouahchi et al. 1995). Previously, both α-tocopherol and TTP were linked with maternal fertility (von Mandach et al. 1994; Brigelius-Flohe et al. 2002; Kaempf-Rotzoll et al. 2003; Jauniaux et al. 2004; Muller-Schmehl et al. 2004; Masters et al. 2007; Gagne et al. 2009; Di Donato et al. 2010; Bartfai et al. 2012). The embryonic requirement, however, remained unknown. Beyond describing the embryonic vitamin E requirement, we propose a central mechanism by which vitamin E affects essential processes during early development. Furthermore, we show a vital role for TTP in early vertebrate development, demonstrating a requirement for the predominantly hepatic protein prior to liver development.

Formulating and implication of a defined diet

Our initial question of embryonic vitamin E requirement was hampered by the lack of a defined zebrafish diet. Furthermore, the zebrafish community lacked a standardized reference diet (Lawrence 2011). This issue has been resolved for most laboratory animals (Hau and Schapiro 2011), but had not yet been addressed in the zebrafish model. To this end, in Chapter 2 we described the formulation and utilization of a fully defined research diet for adult zebrafish. This diet is nutritionally sufficient, palatable to the fish, and allows growth and embryo production comparable to the standard (undefined) laboratory diet fed fish. The importance of parental diet, and the subsequent vertical transfer of nutrients (parent to embryo) are highlighted in Chapter 3. It is important to note that our novel defined diet provides a consistent platform for studying maternal transfer of specific nutrients of interest in a non-placental and external development model.

Our defined diet allows for precise control of micro- and macronutrient content. This is demonstrated in Chapters 2 and 3 with the vitamin E deficient diets. In addition to the work presented here, I have assisted with other defined diet-related zebrafish projects; the creation and implementation of a zinc deficient zebrafish diet with Dr. Emily Ho and her group (unpublished), a metabolomics study with vitamin C and E deficient diets with Dr. Fred Steven's group (Kirkwood et al. 2012), and studies using the vitamin E-deficiency in

relation to poly-unsaturated fatty acid (PUFA) metabolism with Katie Lebold also in the Traber lab group (Lebold et al. 2011) (Lebold masters thesis, in preparation 2012).

Vitamin E depletion and deficiency modeling

The advent of a defined diet allowed the creation of a vitamin E deficient (E-) diet. Zebrafish fed the E- diet maintained growth and health similar to fish the sufficient (E+) diet. Within ~80 days of diet initiation the E- fish had α-tocopherol concentrations ~50-fold lower then the E+ and Lab diet control fish. Despite the marked deficiency, the E- fish continued to gain weight and when spawned produced viable gametes. Moreover, when spawned, the E- adult fish passed their nutrient deficiency along to their progeny, producing E- embryos. E- embryos, although viable, present severe malformations and increased mortality by 120 hpf. These embryos demonstrated, for the first time, that *embryonic* vitamin E is essential for proper vertebrate development (Miller et al. 2012).

Demonstration of an embryonic vitamin E requirement led to the next question: why does the embryo require vitamin E? More precisely: what role, mechanistically, does vitamin E play in embryonic development? E- embryos develop similarly to their E+ and lab diet controls until 48 hours post fertilization (hpf). At 48 hpf ~50% of the embryos show signs of abnormal

development (Chapter 3), and by five days there is a greater then 75% combined morbidity and mortality rate (Chapters 2 and 3). To define the mechanistic role of vitamin E we collected RNA from E- and E+ embryos at 36 hpf, prior to the onset of any overt developmental malformation. Using a whole-genome microarray we found 2656 differentially expressed transcripts in the E- embryos when compared to the E+ control embryos. Separation of these transcripts using a breadth of bioinformatics tools (Chapter 3) revealed a likely mechanism by which vitamin E deficiency results in abnormal growth and failed development. In brief, the postulated accumulation of lipid peroxidation products causes dysregulation in a pathway ultimately causing disruption of energy metabolism, reactive oxygen species detoxification and mitochondrial function. This mechanism works through co-activators responsible for energy homeostasis and metabolism including peroxisome proliferator-activated receptor gamma, coactivator 1-alpha (PGC1A) and peroxisome proliferator-activated receptor gamma, coactivator 1-beta (PGC1B). Through the misregulation of these key nuclear-receptor co-activators, vitamin E deficiency results in embryonic morbidity and death (Chapter 3).

TTP characterization and knockdown

Human vitamin E deficiency is caused almost exclusively by mutations in the TTP gene (AVED, OMIM #277460). Consequently, TTP knockout models are often used to study the effects vitamin E in cell culture and rodent models (Evans and Bishop 1922; Terasawa et al. 2000; Jishage et al. 2001; Lim and Traber 2007; Fujita et al. 2012; Shichiri et al. 2012). Previously zebrafish TTP was identified, *in silico,* as a protein-coding gene in the Ensembl online database (http://www.ensembl.org/Danio_rerio/Info/Index). In Chapter 4, we identified and characterized the zebrafish TTP homolog. Comparing the zebrafish and human amino acid sequences we noted 64% identical and 85% similar residues, with higher conservation in the vitamin E binding region (82% identical and 95% similar). Furthermore, the zebrafish homolog cross-reacted with an anti-human TTP antibody. Having verified the homologous nature of the zebrafish TTP protein, we next studied its role in development. Transcript levels increase at 8 hpf and remain high through the first day post fertilization (dpf). At 1 dpf, using RNA *in situ* hybridization, we showed that TTP mRNA is expressed in the head and eyes, as well as the tail bud. Importantly, this expression occurs prior to the formation of a liver in the zebrafish embryo, even though, in adult animals, TTP is barely detectable in tissues other then the liver (Arita et al. 1995). We then performed transient knockdown using MO antisense technology to inhibit mRNA processing.

Knockdown of TTP resulted in severe malformations of the developing head and tail, coinciding with the mRNA patterning shown with the *in situ* hybridization. These findings suggest a new role for TTP during vertebrate development. We theorize that TTP mediates spatio-temporal α-tocopherol transfer in the embryo during early vertebrate development, and thus, TTP is required for proper embryogenesis.

Conclusions

The culmination of this work is multifaceted. We were successful in the creation and testing of a fully defined zebrafish diet, and in applying it to generate vitamin E deficient adult zebrafish. Using the E deficient model, we demonstrated embryonic vitamin E requirements that are separate from maternal requirements. We then provided a detailed description of the effects of embryonic vitamin E deficiency at the transcript level, and proposed a putative mechanism by which vitamin E deficiency leads to abnormal development. Finally we characterized the zebrafish TTP homolog and established a novel TTP requirement in early vertebrate development. Taken together, this provides both a unique model system and a new path for future research into the role of vitamin E and TTP in early life development.

Human relevance

Vertebrate developmental stages, especially those in early embryogenesis, are highly conserved, and we show the requirement for vitamin E and TTP early in zebrafish development. Deficiency of either adversely affects processes in the zebrafish. The timing of these events is analogous to the first 20-56 days of human gestation. In placental mammals, severe maternal deficiency of vitamin E or TTP impairs fetal implantation into the uterine wall and placental formation. With the zebrafish model, we were able to circumvent the maternal requirement and focused exclusively on the embryonic requirements. Our results demonstrate that vitamin E and TTP are required for proper embryonic development in a time period that is post-implantation in mammals. If the mother has barley sufficient vitamin E (or TTP activity) for successful implantation, but is borderline vitamin E deficient, what are the consequences for the developing embryo? Our studies suggest that vitamin E deficiency has broader effects on embryonic development than previously thought, the mechanisms for which should be investigated further.

It is important to note that effects of vitamin E deficiency begin prior to the detection of most pregnancies, and often before the consumption of prenatal supplements. This early requirement combined with the inadequate dietary consumption of vitamin E (Food and Nutrition Board. Institute of Medicine. 2000; McBurney 2011) could be responsible for early failures in

human pregnancy. The role of TTP and vitamin E in post-implantation development needs to be addressed in the near future, as these results highlight their importance and ramifications of deficiency.

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Bartfai, L., Z. Bartfai, et al. (2012). Rate of preterm birth in pregnant women with vitamin E treatment - a population-based study. J Matern Fetal Neonatal Med: Epub: E-published Mar 26.

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APPENDIX

Table A1. Significantly differentially expressed transcripts ($p < 0.01$) in the Lab diet embryos compared with the E+ control embryos. 18 transcripts with greater then 2-fold difference are shown.

Probe ID	ZF Entrez Gene ID	Gene Symbol	Gene Title	Human Entrez Gene ID	Human Ortholog Gene Symbol	Fold change	
BI880268_R	---	---	---	---	---	2.1	down
CD606624_R	---	---	---	---	---	2.0	up
ENSDART00000073805	---	PCOLCE (2 of 2)	---	5118	---	2.4	down
ENSDART00000079340	100003460	C7 (2 of 3)	---	---	---	2.3	up
ENSDART00000084958	563729	A3KP73_DANRE	---	---	---	2.1	up
ENSDART00000098042	560786	LOC560786	---	---	---	2.1	up
NM_001005973	791531	---	---	---	---	2.3	down
OTTDART00000016891	---	krt1-19d	keratin, type 1, gene 19d	2288	KRT16;KRT17	3.0	down
OTTDART00000017071	---	pkhd1l1	---	2571	---	3.3	up
OTTDART00000019120	---	si:dkey-103e21.5	---	6823	---	2.1	down
TC239167	492320	---	---	---	KRT16	2.8	down
TC244188	100148431	---	---	352909	BC063449 /// AK093458 /// AK097388	2.4	down
TC262743	100004700	cyp24a1l	cytochrome P450, family 24, subfamily A, polypeptide 1, like	1591	CYP24A1	3.5	down
TC264594	---	---	---	28999	KLF15 /// ELK4;KLF15	2.0	up

ZV700S00001856	---	---	---	---	---	3.8	up
ZV700S00003311	30671	hsp70	heat shock cognate 70-kd protein	3303	HSPA8	2.3	up
ZV700S00003502	---	---	---	5090	PBX3	2.0	up
ZV700S00005179	---	---	---	28999	KLF15	2.0	up

Table A2. Significantly differentially expressed transcripts ($p < 0.01$) in the E- diet embryos compared with the E+ control embryos. 323 transcripts with change greater than 2-fold are shown.

Probe ID	ZF Entrez Gene ID	Gene Symbol	Gene Title	Human Entrez Gene ID	Human Ortholog Gene Symbol	Fold change	
AA542593_R	334910	---	---	---	C20orf11	2.2	up
AI384630	336563	wu:fb13g09	wu:fb13g09	---	---	2.2	up
AI478023_R	322111	---	---	---	ITGB1	2.1	up
AI588090	323352	---	---	---	HSPC043 /// BC014881	2.0	up
AI588385	323510	wu:fc01b08	wu:fc01b08	---	---	2.0	up
AI601375	323801	wu:fc10d12	wu:fc10d12	---	KCNJ9 /// KCNJ6 /// KCNJ5	2.2	up
AI601375_R	323801	wu:fc10d12	wu:fc10d12	---	KCNJ9 /// KCNJ6 /// KCNJ5	2.1	up
AI721331_R	751629	setd8a	SET domain containing (lysine methyltransferase) 8a	387893	EBF /// EBF2	2.4	up

AI884093	---	---	---	25900	BC010431	2.3	up
AI957695_R	325673	wu:fd02a07	wu:fd02a07	---	TSN	2.1	up
AL909233	---	---	---	---	---	2.4	up
AL909233_R	---	---	---	---	---	2.2	up
AW115711_R	334542	wu:fj99d11	wu:fj99d11	---	---	2.1	up
AW279740	336030	znf385a	zinc finger protein 385A	---	ZFP385 /// BC029752 /// AK024404	2.1	up
AW279924_R	336072	wu:fj47a01	wu:fj47a01	---	---	3.5	up
AW419597	---	---	---	---	---	2.8	up
AW419597_R	---	---	---	---	---	3.0	up
AW826510	335743	---	---	---	---	2.2	up
BC056563.1	327588	hpx	hemopexin	3263	HPX	2.4	down
BG305949_R	---	---	---	---	---	2.1	up

BG306396_R	---	---	---	---	FLJ12547	2.0	up
BG728391	100000439	LOC100000439	similar to Wnt inhibitory factor 1 precursor (WIF-1)	---	AK027618	2.5	up
BG728718	---	---	---	---	---	2.6	up
BG728977	---	---	---	---	---	2.1	up
BG728977_R	---	---	---	---	---	2.2	up
BG729050	---	---	---	---	---	2.3	up
BG729050_R	---	---	---	---	---	2.3	up
BG729101	---	---	---	---	DDEF1	2.0	up
BG729101_R	---	---	---	---	DDEF1	2.2	up
BG883367	555286	---	---	27324	TNRC9 /// AB018280 /// C20orf100 /// AB018351 /// BC016665	2.0	up
BI430222_R	---	---	---	---	---	2.3	up
BI534083	---	---	---	---	---	2.6	up

BI534083_R	---	---	---	---	---	2.4	up
BI670871	---	---	---	---	---	2.6	up
BI670871_R	---	---	---	---	---	2.4	up
BI671392	---	---	---	---	---	2.1	up
BI671392_R	---	---	---	---	---	2.1	up
BI705687	---	---	---	9969	TRAP240	2.3	up
BI705687_R	---	---	---	9969	TRAP240	2.6	up
BI841461	334379	---	---	---	---	2.5	up
BI879037	---	---	---	---	---	2.0	up
BI879666	---	---	---	---	---	2.1	up
BI879720	---	---	---	---	---	2.2	up
BI879720_R	---	---	---	---	---	2.3	up
BI879864	---	---	---	---	---	2.1	up
BI879864_R	---	---	---	---	---	2.2	up

BI880259	---	---	---	---	---	2.0	up
BI880259_R	---	---	---	---	---	2.0	up
BI880268	---	---	---	---	---	2.0	down
BI880268_R	---	---	---	---	---	2.2	down
BI880511	---	---	---	154881	KCTD7 /// BC042482 /// FLJ11773	2.1	up
BI880511_R	---	---	---	154881	KCTD7 /// BC042482 /// FLJ11773	2.1	up
BI880944	---	---	---	---	---	2.1	up
BI880944_R	---	---	---	---	---	2.0	up
BI885232	567956	zgc:123246	zgc:123246	9170	EDG2	2.3	up
BI885232_R	567956	zgc:123246	zgc:123246	9170	EDG2	2.1	up
BM095178_R	---	---	---	---	---	2.3	up
BM095220	564642	LOC564642	similar to integrator complex subunit 5	80789	KIAA1698 /// AB051485	2.0	down

BM778144_R	334455	zgc:175195	zgc:175195	55218	C14orf114	2.2	up
BQ262458	---	---	---	7007	TECTA	2.4	up
BQ262458_R	---	---	---	7007	TECTA	2.0	up
BQ481028	---	---	---	---	---	2.6	up
CD606127	---	---	---	---	---	2.1	up
CD606127_R	---	---	---	---	---	2.2	up
CD606517_R	---	---	---	---	---	2.5	up
CD606547	---	---	---	---	---	2.0	up
CD606547_R	---	---	---	---	---	2.1	up
CD606560	---	---	---	---	---	2.5	up
ENSDART00000008240	100007926	LOC100007926	hypothetical LOC100007926	5032	---	2.1	down
ENSDART00000008267	---	---	---	---	---	3.5	up
ENSDART00000031510	---	---	---	---	---	2.1	up

ENSDART00000033332	---	---	---	---	---	2.2	up
ENSDART00000044652	100136871	---	---	---	---	2.0	down
ENSDART00000055003	---	---	---	7051	---	2.4	down
ENSDART00000056256	565531	nrxn1a	neurexin 1a	9378	---	2.9	up
ENSDART00000056273	---	---	---	---	---	3.5	up
ENSDART00000056453	793907	igfbp1b	insulin-like growth factor binding protein 1b	3484	---	3.2	up
ENSDART00000057984	---	---	---	---	---	2.8	up
ENSDART00000058297	---	---	---	---	---	2.2	down
ENSDART00000066773	100005862	---	---	---	---	4.3	down
ENSDART00000074715	---	---	---	---	---	3.0	up
ENSDART00000075405	100149351	---	---	---	---	2.2	down
ENSDART00000076831	100008033	---	---	---	---	2.7	down
ENSDART00000077653	---	---	---	---	---	2.1	down
ENSDART00000079373	100002946	---	---	---	---	2.2	up

ENSDART00000080432	---	---	---	---	---	3.6	down
ENSDART00000081020	---	---	---	---	---	2.5	down
ENSDART00000081473	557558	---	---	---	---	2.3	down
ENSDART00000081598	---	---	---	---	---	2.4	down
ENSDART00000084238	---	---	---	3757	---	2.0	up
ENSDART00000092481	560902	---	---	---	---	2.0	up
ENSDART00000097211	---	---	---	---	---	2.7	up
ENSDART00000097914	---	---	---	---	---	2.2	down
ENSDART00000098343	---	---	---	85364	---	2.4	down
ENSDART00000100916	---	---	---	---	---	5.0	down
ENSDART00000100918	100141343	---	---	---	---	3.7	down
ENSDART00000102938	100141330	---	---	---	---	2.7	down
ENSDART00000103562	---	---	---	---	---	4.3	down
ENSDART00000103729	560670	---	---	55803	---	2.2	down

ENSDART00000103852	100148739	---	---	---	---	2.2	down
ENSDART00000103877	---	---	---	---	---	2.5	down
ENSDART00000104361	799220	si:dkey-56d12.4	si:dkey-56d12.4	---	---	2.2	down
ENSDART00000104494	---	---	---	---	---	2.3	down
ENSDART00000106100	558926	---	---	---	---	3.8	down
NM_001030097	555989	---	---	---	---	4.8	up
NM_001080699	791217	---	---	---	---	2.3	up
NM_199605	322453	---	---	---	---	6.0	up
NM_200821	393794	---	---	339488	---	2.0	up
OTTDART00000009023	---	---	---	6087	---	2.5	down
OTTDART00000009197	---	---	---	65059	---	2.3	up
OTTDART00000009477	---	---	---	6457	GATA3	2.1	up
OTTDART00000011885	---	---	---	5080	---	2.5	up
OTTDART00000016888	---	---	---	2297	---	2.3	down

OTTDART00000016891	---	krt1-19d	keratin, type 1, gene 19d	2288	KRT16;KRT17	2.1	down
OTTDART00000017071	---	---	---	2571	---	3.5	up
OTTDART00000019120	---	---	---	6823	---	3.0	down
OTTDART00000019187	---	---	---	2597	---	2.0	down
OTTDART00000020698	100003563	---	---	11075	---	2.1	up
OTTDART00000020706	---	---	---	3471	---	2.0	up
OTTDART00000022817	335497	---	---	---	---	2.7	down
OTTDART00000023042	---	---	---	7833	---	2.2	up
OTTDART00000023625	---	---	---	---	---	2.2	down
OTTDART00000025103	30101	skia	nuclear oncoprotein skia	6497	---	2.3	up
OTTDART00000025386	---	gata2a	GATA-binding protein 2a	323	GATA2	2.1	up
OTTDART00000025910	---	---	---	556	NOLC1	2.0	up
OTTDART00000027394	100148310	---	---	---	TNRC5	2.1	down
OTTDART00000029827	---	---	---	---	---	2.9	down

TC236032	---	---	---	---	---	2.6	up
TC236053	---	---	---	480	AK098076 /// ATP1A4 /// ATP1A3 /// ATP1A2 /// ATP1A1	2.2	down
TC239076_R	553377	prg4	proteoglycan 4	---	PRG4 /// MUC7;PRG4 /// MUC7	2.2	down
TC239773	---	---	---	9080	CLDN9 /// CLDN6 /// BT006989 /// BT007399 /// CLDN3 /// CLDN4 /// CLDN5 /// BT007254;CLDN9 /// CLDN6 /// BT006989 /// BT007399 /// CLDN3 /// CLDN4 /// CLDN5 /// BT007254	3.1	up
TC241153	---	---	---	---	---	2.2	up
TC241778	566086	kif5a	kinesin family member 5A	---	KIF5A	2.1	up
TC242012	334772	---	---	---	AJ272365 /// AK130814 /// CDW92 /// ABCA1	2.9	up
TC242323	---	---	---	---	---	2.1	up

TC242351	571430	wu:fb14a07	wu:fb14a07	---	AK090431 /// AK090476	2.1	up
TC242920	---	---	---	---	---	2.3	up
TC243041	---	---	---	---	---	2.2	up
TC243600	---	---	---	---	---	2.2	up
TC243601	---	---	---	---	---	2.5	up
TC243692	---	---	---	---	---	2.2	up
TC243899	799244	wu:fk30a05	wu:fk30a05	79890	RIN3	2.1	up
TC243942	335127	LOC565024	similar to tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2	---	AK021886	2.1	up
TC243981	553490	LOC553490	hypothetical protein LOC553490	---	BX248299 /// BT006777 /// PSMB5 /// PSMB8	2.1	up
TC244743	---	---	---	---	---	2.4	up
TC244992	---	---	---	---	---	2.1	up

TC245195	---	---	---	---	---	2.2	up
TC245606	327490	wu:fi14b07	wu:fi14b07	---	RUNX1T1	2.1	up
TC246520	394248	nfkbil1	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	7916	NFKBIL1	2.2	up
TC248953	497504	---	---	---	CDC91L1	2.0	down
TC249120	100149027	LOC100149027	similar to GCN1 general control of amino-acid synthesis 1-like 1	10985	STOM /// AL832016 /// SLC7A2 /// SLC7A1	2.4	up
TC249620	324216	---	---	---	BC051883 /// FLJ20321;BC051883 /// FLJ20321	2.1	up
TC250403	---	---	---	---	---	2.1	up
TC250784	323265	---	---	---	ZHX2 /// KIAA0854	2.0	up
TC251353	100002476	---	---	---	CSPG2	2.7	down
TC251388	---	---	---	118	ADD1	2.1	up

TC251572	553410	dpysl5b	dihydropyrimidinase-like 5b	56896	DPYSL5	2.1	up
TC252005	335500	wu:fj17e06	wu:fj17e06	---	ZNF207 /// AK074579 /// MGC34725 /// SLC25A23 /// BC005163 /// DKFZp586G0123 /// AB067483 /// MCSC /// AJ512835 /// MGC2615 /// BC031671	2.4	up
TC252817	562591	---	---	79595	MLLT1 /// MLLT3	2.3	up
TC252823	335859	LOC559001	similar to Fatty acid synthase	2194	FASN	3.4	up
TC252958	---	---	---	---	---	2.2	up
TC253051	---	---	---	---	---	2.1	up
TC253130	---	---	---	---	---	2.5	up
TC253143	---	---	---	---	---	2.5	up
TC253565	---	---	---	---	---	2.2	up
TC253844	---	---	---	---	---	2.5	up

TC254298	559638	wu:fb39e12	wu:fb39e12	---	RBM15	2.3	up
TC254977	---	---	---	9564	BCAR1 /// BC062556	2.0	up
TC255109	---	---	---	---	---	2.1	up
TC255289	---	---	---	---	---	2.1	up
TC255302	336173	wu:fj53f12	wu:fj53f12	---	GPR126 /// AF538954 /// AF539455 /// GPR97 /// GPR64 /// AF539456 /// AY148343 /// AY143365 /// AY143364 /// AY143367 /// AY143366 /// GPR112	2.2	up
TC255376	557285	hcn2	hyperpolarization activated cyclic nucleotide-gated potassium channel 2	610	HCN1 /// HCN2 /// HCN4	2.4	up
TC256037	448893	dopey1	dopey family member 1	---	KIAA1117	2.1	up
TC256052	---	---	---	51621	KLF13	2.7	up
TC256053	---	---	---	---	---	2.1	up
TC256157	---	---	---	---	---	2.5	up

TC256823	---	---	---	---	---	2.4	up
TC256912	335875	wu:fj35f12	wu:fj35f12	---	AF272774 /// F9 /// F10 /// PROC /// F7	2.6	up
TC257030	---	---	---	---	---	2.0	up
TC257144	100150563	LOC100150563	similar to solute carrier family 4, member 4	---	BC030977 /// AF157492 /// SLC4A4 /// AF011390	2.1	up
TC257504	337348	wu:fa56c09	wu:fa56c09	---	BC000377 /// FASTK	2.1	up
TC258033	---	---	---	---	---	2.1	up
TC260604	333936	wu:fi22b07	wu:fi22b07	---	WBSCR18	2.1	up
TC260827	322667	---	---	---	SHARP	2.1	up
TC261476_R	---	---	---	3131	HLF	2.4	up
TC262239	---	---	---	---	---	2.2	up
TC262552	---	---	---	---	---	2.2	up
TC262584	---	---	---	---	---	2.8	up

TC262877	---	---	---	---	---	2.1	up
TC262989	---	---	---	57084	SLC17A6 /// SLC1A2	2.2	up
TC263100	---	---	---	---	---	2.1	up
TC263474	---	---	---	---	---	2.4	up
TC263958	---	---	---	---	---	2.1	up
TC264135	---	---	---	---	---	2.9	up
TC264338	---	---	---	---	---	2.4	up
TC264652	100005144	LOC100005144	similar to fucokinase	197258	FUK	2.2	up
TC264987	---	---	---	---	---	2.5	up
TC265012	335171	wu:fk83g11	wu:fk83g11	---	CDH11 /// AK057922 /// CDH24 /// BX248750	2.2	up
TC265135	386775	gltscr1	glioma tumor suppressor candidate region gene 1	29998	GLTSCR1	2.1	up
TC265146	---	---	---	---	---	2.2	up
TC266222	---	---	---	---	---	3.1	up

TC266514	553726	---	---	---	RFP /// BTN3A3 /// BTN3A1 /// BTN1A1 /// AF327057 /// AL080170 /// RNF36 /// BTN2A2 /// BTN2A1	2.1	up
TC266663	---	---	---	---	---	2.0	up
TC266816	---	---	---	---	---	2.5	up
TC267743	---	---	---	27295	BC001017 /// FGL1 /// BC027870	2.7	up
ZV700S00000006	503776	---	---	1387	EP300 /// CREBBP	2.5	up
ZV700S00000024	335205	---	---	---	COL17A1	2.1	up
ZV700S00000199	---	---	---	---	---	2.4	up
ZV700S00000299	321548	---	---	---	BRD4 /// BRDT /// AY166680 /// BRD2 /// BRD3	2.5	up
ZV700S00000313	553410	dpysl5b	dihydropyrimidinase-like 5b	56896	DPYSL5	2.1	up
ZV700S00000350	---	---	---	27295	BC001017 /// FGL1 /// BC027870	2.2	up

ZV700S00000365	---	---	---	10114	HIPK3 /// AF305239 /// Nbak2 /// HIPK2	2.0	up
ZV700S00000373	335581	atp2b2	ATPase, Ca++ transporting, plasma membrane 2	---	ATP2B1	2.2	up
ZV700S00000398	---	---	---	---	---	2.4	up
ZV700S00000428	334414	LOC559027	hypothetical LOC559027	---	CDC2L5	2.3	up
ZV700S00000459	553231	LOC553231	hypothetical protein LOC553231	440193	---	2.3	up
ZV700S00000545	---	---	---	57724	AK023817 /// AB046852 /// BC003695 /// FLJ11159 /// BC036911 /// AL583915	2.5	up
ZV700S00000566	337295	wu:fk14c11	wu:fk14c11	---	CLDN9 /// CLDN6 /// BT006989 /// BT007399 /// CLDN3 /// CLDN4 /// CLDN5 /// BT007254	2.8	up
ZV700S00000649	---	---	---	---	DDEF1	2.2	up
ZV700S00000661	338102	---	---	---	---	2.0	up

ZV700S00000683	569435	LOC569435	similar to Dmx-like 2	23312	RC3	2.0	up
ZV700S00000723	335055	wu:fa12e08	wu:fa12e08	---	---	2.1	up
ZV700S00000906	---	---	---	---	---	2.2	up
ZV700S00000936	556789	CH211-279M15.2	novel protein similar to vertebrate syntaxin binding protein	134957	---	2.3	up
ZV700S00000975	567956	zgc:123246	zgc:123246	9170	EDG2	2.3	up
ZV700S00001092	566102	LOC566102	hypothetical LOC566102	80314	ITGB1	2.1	up
ZV700S00001133	447931	---	---	---	CLDN9 /// CLDN6 /// BT006989 /// BT007399 /// CLDN3 /// CLDN4 /// CLDN5 /// BT007254	2.4	up
ZV700S00001150	---	---	---	253980	KCTD13 /// MSTP028 /// TNFAIP1 /// BC000567 /// PSK-1 /// AJ245822 /// BC040062	2.6	up
ZV700S00001161	---	---	---	---	---	2.7	up

ZV700S00001198	561503	ncor2	nuclear receptor co-repressor 2	9612	---	2.1	up
ZV700S00001247	---	---	---	2875	GPT	2.0	up
ZV700S00001349	81881	crx	cone-rod homeobox	---	CRX	4.3	up
ZV700S00001350	---	---	---	3131	HLF	2.3	up
ZV700S00001468	---	---	---	3765	KCNJ9 /// KCNJ6 /// KCNJ5	2.1	up
ZV700S00001476	---	---	---	11113	CIT	2.4	up
ZV700S00001533	566432	im:7158925	im:7158925	---	---	2.2	up
ZV700S00001555	560793	mll3a	Myeloid/lymphoid or mixed-lineage leukemia 3a	58508	---	2.1	up
ZV700S00001683	245949	vangl2	vang-like 2 (van gogh, Drosophila)	57216	VANGL2	2.3	up
ZV700S00001771	353179	otx5	orthodenticle homolog 5	---	AF093138	2.3	up
ZV700S00001856	---	---	---	---	---	3.7	up

ZV700S00002000	793907	igfbp1b	insulin-like growth factor binding protein 1b	3484	IGFBP3 /// IGFBP1 /// IGFBP4 /// IGFBP5	7.9	up
ZV700S00002086	---	---	---	153090	DAB2IP /// RASAL2 /// AB067525 /// AB051530	2.3	up
ZV700S00002089	---	---	---	80728	BC013071 /// AB051475	2.5	up
ZV700S00002107	---	---	---	2104	BC064700 /// ESRRG /// ESRRA /// ESRRB	2.6	up
ZV700S00002340	566131	LOC566131	similar to Eukaryotic translation initiation factor 4 gamma 3 (eIF-4-gamma 3) (eIF-4G 3) (eIF4G 3) (eIF-4-gamma II) (eIF4GII)	8672	EIF4G3	2.3	up
ZV700S00002480	324321	wu:fc25e12	wu:fc25e12	---	---	2.1	up
ZV700S00002751	336961	wu:fk35a11	wu:fk35a11	---	WBSCR18	2.1	up
ZV700S00002813	---	---	---	---	C20orf116	2.0	up
ZV700S00002816	100151373	---	---	---	---	2.1	up

ZV700S00002817	100151373	---	---	---	---	2.2	up
ZV700S00002889	553348	---	---	57549	---	2.1	up
ZV700S00002949	323984	LOC559090	similar to PWWP domain-containing protein 2A	---	AK055921	2.1	up
ZV700S00003212	322503	---	---	---	BAT8	2.0	up
ZV700S00003320	570332	zgc:114130	zgc:114130	7538	---	2.1	up
ZV700S00003326	407661	LOC565713 /// ncanl	similar to chondroitin sulfate proteoglycan 3 /// neurocan, like	---	CSPG3	2.2	up
ZV700S00003526	561895	---	---	54910	---	2.1	up
ZV700S00003527	323126	wu:fb82f02	wu:fb82f02	---	AK128062 /// SLC35F2	2.2	up
ZV700S00003533	323301	wu:fb94e12	wu:fb94e12	---	TCBA1 /// SLC31A1 /// AB070452 /// IBRDC1	2.7	up
ZV700S00003539	560688	zgc:152921	zgc:152921	132660	HSPC043 /// BC014881	2.2	up

ZV700S00003555	323843	wu:fc11e07	wu:fc11e07	---	---	2.3	up
ZV700S00003596	321329	---	---	---	PPEF2	2.1	up
ZV700S00003601	---	---	---	10915	TCERG1	2.1	up
ZV700S00003708	406550	zgc:77407	zgc:77407	9931	BC056895	2.3	up
ZV700S00003731	326922	---	---	---	ADSSL1 /// ADSS	2.0	up
ZV700S00003739	---	---	---	---	---	2.1	up
ZV700S00003810	---	---	---	4986	OPRK1 /// OPRM1 /// AY168006 /// AY195733 /// OPRD1	2.5	up
ZV700S00003823	336114	wu:fj49b04	wu:fj49b04	---	---	2.2	up
ZV700S00003829	555837	si:dkey-270i2.3	si:dkey-270i2.3	83992	WNT2	2.0	up
ZV700S00003838	557285	hcn2	hyperpolarization activated cyclic nucleotide-gated potassium channel 2	610	HCN1 /// HCN2 /// HCN4	2.4	up

ZV700S00003901	436917	zgc:92474	zgc:92474	---	LOC147111	2.1	up
ZV700S00003993	321707	si:ch211-57k11.4	si:ch211-57k11.4	---	---	2.6	up
ZV700S00004031	322631	wu:fb68d10	wu:fb68d10	---	---	2.8	up
ZV700S00004089	336180	wu:fj54b06	wu:fj54b06	---	GOPC	2.9	up
ZV700S00004093	---	---	---	120114	FAT3	2.6	up
ZV700S00004158	492287	wu:fa99c11	wu:fa99c11	---	COL5A1 /// COL11A1	2.2	up
ZV700S00004159	492287	wu:fa99c11	wu:fa99c11	---	COL5A1 /// COL11A1	2.1	up
ZV700S00004169	322065	wu:fb48g11	wu:fb48g11	---	---	2.1	up
ZV700S00004186	497482	wu:fb09b10	wu:fb09b10	---	CPEB4	2.1	up
ZV700S00004241	335179	---	---	---	R3HDM	2.3	up
ZV700S00004255	406307	---	---	6234	SARCOSIN	2.6	up

ZV700S00004435	323345	wu:fb95g08	wu:fb95g08	---	AB033061 /// AF268913 /// AK000921 /// AF521670 /// AF521671 /// AK027467 /// AF259792 /// AF253515 /// SMARCF1 /// AF468300 /// ARID1A	2.5	up
ZV700S00004520	326827	---	---	---	SIAT8B	2.6	up
ZV700S00004521	326827	---	---	---	HCFC1	2.5	up
ZV700S00004677	---	---	---	---	---	2.1	up
ZV700S00004829	---	---	---	---	---	2.2	up
ZV700S00004862	553218	zgc:114089	zgc:114089	51599	LSR	2.1	up
ZV700S00004878	406656	zgc:63948	zgc:63948	54602	NDFIP2	2.3	up
ZV700S00004909	100004027	LOC100004027	similar to zinc finger protein 318	---	---	2.1	up
ZV700S00004948	369190	kif1b	Kinesin family member 1B	23095	AB040881	2.0	up
ZV700S00004985	322153	wu:fb51f11	wu:fb51f11	---	---	2.2	up

ZV700S00005108	613141	itih3	inter-alpha (globulin) inhibitor H3	3700	ITIH3	3.1	down
ZV700S00005145	---	---	---	26037	SIPA1L1	2.1	up
ZV700S00005151	337320	---	---	---	FLNB	2.2	up
ZV700S00005154	324488	wu:fc30f10	wu:fc30f10	---	---	2.2	up
ZV700S00005170	564559	zgc:162730	zgc:162730	---	ZFP36	2.6	up
ZV700S00005186	337170	---	---	---	COL12A1	2.2	up
ZV700S00005291	323465	vcanb	versican b	---	BX641036	2.4	up
ZV700S00005485	553277	dido1	death inducer-obliterator 1	---	DIDO1	2.6	up
ZV700S00005510	323979	---	---	---	AJ578034 /// AB086062 /// CHSY1	3.5	down
ZV700S00005528	560260	LOC560260	similar to Protein virilizer homolog	25962	DKFZP434I116	2.1	up

ZV700S00005535	321204	sb:cb319	sb:cb319	---	MYH2 /// MYH1 /// MYH6 /// MYH7 /// MYH4 /// BC007808 /// MYH8 /// AB040945	2.4	up
ZV700S00005575	406391	zgc:65870	zgc:65870	57060	PCBP4	2.3	up
ZV700S00005662	548609	sppl3	signal peptide peptidase 3	121665	SMARCAD1 /// NT5C2	2.1	up
ZV700S00005757	335497	---	---	---	RSHL1	3.0	down
ZV700S00005766	336766	---	---	---	---	2.7	up
ZV700S00005773	336186	wu:fj54e12	wu:fj54e12	---	SV2B	3.6	up
ZV700S00005861	334625	---	---	---	POM121	2.1	up
ZV700S00005883	321197	sb:cb306	sb:cb306	---	PRDM16 /// BX640908 /// BC031019	2.5	up
ZV700S00005970	326754	wu:fe14d06	wu:fe14d06	---	ACSL6	2.3	up
ZV700S00006024	566086	kif5a	kinesin family member 5A	---	KIF5A	2.5	up

ZV700S00006027	336300	---	---	---	IER2	2.0	up
ZV700S00006062	337451	wu:fj67h12	wu:fj67h12	---	FLJ90579 /// AK075060	2.5	up
ZV700S00006100	334772	---	---	---	AJ272365 /// AK130814 /// CDW92 /// ABCA1	2.9	up
ZV700S00006105	334575	---	---	---	GHSR	3.0	up
ZV700S00006116	336058	wu:fj46a10	wu:fj46a10	---	---	2.1	up
ZV700S00006223	567576	LOC567576	similar to spectrin repeat containing, nuclear envelope 1	23345	FLJ23305 /// C6orf97	2.5	up
ZV700S00006392	562131	ints1	integrator complex subunit 1	26173	AL050110 /// BC013367 /// AB037861 /// BC018777 /// AK093809 /// AY358482	2.0	up
ZV700S00006411	321962	---	---	---	---	2.2	up
ZV700S00006431	323264	wu:fb93g02	wu:fb93g02	---	AL834286	2.1	up

ZV700S00006469	406751	srrm1	Serine/arginine repetitive matrix 1	10250	SRRM1 /// TDH /// AK057762 /// AY101187 /// AY101186	2.2	up
ZV700S00006502	555257	wu:fj67h08	wu:fj67h08	---	AP1GBP1	2.1	up
ZV700S00006647	563090	---	---	25942	---	2.4	up
ZV700S00006666	100002476	---	---	---	CSPG2	3.0	down