The Functions of E2F3a in Mouse Development

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Abstract

The $E2f3$ gene is thought to be important for promoting cellular proliferation and normal growth. It is known that deficiency of $E2f3$ combined with deficiency of the other activating E2Fs causes compounded effects on cellular proliferation, but studies with $E2f3$ deficiency in development have been limited because the majority of $E2f3$ deficient mice do not survive past birth. The $E2f3$ gene locus encodes for two protein products, $E2f3a$ and $E2f3b$. Based on their expression patterns during the cell cycle and their ability to interact with other proteins, it was hypothesized that $E2f3a$ is an activator and $E2f3b$ is a repressor of transcription. Disruption of the $E2f3$ locus has been targeted in the mouse, but these models failed to discriminate between $E2f3a$ and $E2f3b$ since both products were simultaneously deleted. The research presented here utilizes an $E2f3a$ knockout to study the functions of $E2f3a$ individually, and also to investigate the relationships of the functions that $E2f3a$ has with the other activating E2Fs. We find that $E2f3a$ loss does not negatively affect mouse development and growth, but when combined with $E2f1$ loss, it results in developmental defects in the adrenal gland, lung, bone, fat tissue, testicles, and pancreas.

Introduction

The Retinoblastoma Protein Family and its interaction with the E2F Family (E2F) of Transcription Factors has been long documented and researched. The Retinoblastoma Protein Family, which includes the three pocket proteins (Rb family), Retinoblastoma (Rb), p107, and p130, regulate the effects of the E2F’s by selectively binding to them. Rb is a tumor suppressor, which is mutated in many types of human cancer (1). The pocket proteins are themselves regulated by cyclin and cyclin dependent kinases (CdKs), which
phosphorylate the pocket proteins and cause them to lose their ability to bind to the E2Fs. The E2Fs have been proven to be important regulators of cellular growth and the cell cycle (see Figure 1). E2F family members either positively or negatively affect the expression of genes important for the entry of cells into S-phase of the cell cycle. The common feature of the E2F family members is a highly conserved DNA binding domain, which allows these transcription factors to bind to specific E2F consensus DNA binding elements on target promoters.

Eight E2F genes have been discovered in mammals. The E2F family can be broken down into groups based on their hypothesized function and their binding partners (Figure 2 shows a depiction of the E2F Family Protein Sequences). The E2Fs are thought to function primarily either as activators (E2F1, E2F2, E2F3a) or repressors (E2F3b and E2Fs 4-8). E2Fs 1-6 all share a pocket protein binding domain and a DP heterodimerization domain, used to bind with their dimerization partner, DP, to allow them to bind to DNA.

E2Fs 1, 2, and 3a are thought to be transcriptional activators which are important for promoting cellular growth. These E2Fs bind only to Rb (2), which is thought to inhibit their function. These E2Fs are also regulated at multiple levels, including by transcription, protein degradation, post-translational modifications, and by miRNAs. E2F1, E2F2, and E2F3a expression oscillates during the cell cycle with peak expression during the G1/S transition (2). E2F3b and E2Fs 4-8 are thought to be repressors that prevent growth, and also cell cycle reentry in quiescent cells (3, 4). E2F4 can bind to all three Rb-related proteins and E2F5 can bind to p107 and p130. E2F6 is believed to repress transcription through binding to the polycomb protein groups (5). E2F7 lacks a
pocket binding domain and a DP heterodimerization-binding domain. Evidence supports
that E2F7 is a repressor, but its mechanism of regulation and binding is unknown. E2F8
was recently discovered and it shares some similarities in function to E2F7 (6).

Although the E2Fs are thought to act primarily as either activators or repressors, it
has been found that their functions seem to have more complexity. It has been suggested
that the individual E2Fs have some specificity in function within their subgroups and that
that E2Fs have different roles in different cell tissues. For example, E2F1 and E2F3 have
been found to be important for promoting adipogenesis, or fat tissue development, while
E2F4 has been found to be important for inhibiting adipogenesis (7). E2F1 and E2F2
deficiency has been found to cause diabetes in mice, showing that E2F1 and E2F2 are
important for normal pancreas function. E2F4 has been suggested to be important for
promoting liver development (8). Evidence suggests that when expressed at high levels
E2F1 and possibly the other activating E2Fs can induce apoptosis, or programmed cell
death, although E2F1 has been observed to be the most effective at inducing it (9).

Evidence suggests E2Fs 1-3 are very important for cellular growth, but E2F3 is
the most critical of the three. E2f3 deficiency in mice causes partial embryonic lethality
(10), which is unique among the activator E2Fs. In vitro experiments using Mouse
Embryonic Fibroblasts (MEFs), a type of cell, deficient in E2F3 showed severely
decreased cellular proliferation. MEFs deficient in either E2F1 or E2F2 showed almost
no defect in cellular proliferation, however, combining deficiency in E2F1 or E2F2 with
E2F3 deficiency was found to compound the decrease in proliferation seen in E2F3
deficient MEFs. MEFs deficient in all three showed almost no proliferation (11). These
results suggest that a loss of one of the activators is compensated by the function of the
other ones, and loss of more activators causes increased effects on cellular growth. These results suggest that these genes may have functions in common, although it is not known to what extent.

The activator E2Fs’ importance for promoting cellular growth is also why they are believed to be linked to cancer. As stated previously, Rb has been found mutated in a startling percentage of human tumors. Its inhibitory function with the activator E2Fs is believed to be key to its role in cancer and also tumor suppression (reviewed in 12). It is believed that losing normal regulation of the activating E2Fs causes their unchecked action and uncontrolled growth in cells (13). There is some evidence the activator E2Fs may have a causative role in initiating some cancers (14). The relationship between the E2Fs and cancer seems to be complicated, as E2Fs 1 and 3 also appear to function as tumor suppressors in some circumstances, as well, (15) possibly through their ability to induce apoptosis.

The E2f3 DNA locus was found to encode for two distinct proteins, E2F3a and E2F3b (16). The protein sequences for these two are identical except for at their N-termini, which are encoded by exon 1a for E2F3a and exon 1b for E2F3b (Figure 3a shows a cartoon of the DNA sequence). The expression of E2F3a and E2F3b are driven by two distinct promoters. E2F3a was thought to be an activator since its expression pattern resembled that of the other activator E2Fs and has the ability to transactivate target promoters. E2F3b was expressed equally throughout the cell cycle and can complex with Rb in quiescent cells, resembling the expression pattern and activity of other repressor E2Fs in expression pattern; leading to the hypothesis that it functions as a transcriptional repressor. The different expression patterns led to the question of which
functions of E2F3 could be attributed to each protein isoform, and whether the critical function of E2F3 was due to a single isoform or a combination of both.

Knockout Experiments are commonly used to investigate the role of the Rb/E2F pathway in development. Experiments using knockouts investigate gene function by observing the effects caused by deficiency of a particular gene. Knockouts are commonly used to study the Rb/E2F. \textit{E2f1\textsuperscript{-/-}} mice are found to undergo testicular atrophy with aging, decreased T-Cell apoptosis, and increased thymic proliferation as a result of a decrease in apoptosis (17, 18). \textit{E2f2\textsuperscript{-/-}} mice show increased thymic proliferation and autoimmunity defects (19, 20). As stated previously, \textit{E2f3\textsuperscript{-/-}} mice show a partially penetrant embryonic lethality and heart defects. \textit{Rb\textsuperscript{-/-}} mice are found to be unable to develop past embryonic day 14.5 (21), although \textit{E2f3} loss is able to rescue these mice and allow a significant number to survive to embryonic day 17.5 (22).

In order to individually investigate the functions of E2F3a and E2F3b, an \textit{E2f3a} deficient mouse was created. Mice were chosen as a model organism for these experiments because E2F’s are conserved in mammals and also because of the physiological similarities between mice and humans. The \textit{E2f3a} knockout allows E2F3a to be studied separate from E2F3b, and thus we expect that the analysis of these mice may provide new insights to the functions of E2F3a and E2F3b and how these contribute to the overall E2F transcriptional program.

\textbf{Materials and Methods}

\textbf{Generation of the \textit{E2f3a} Knockout}

The \textit{E2f3a} KO was created prior to my involvement with the project using the Cre Lox-P system. Lox-P sites were inserted upstream and downstream of the exon 1a to
prevent the $E2f3b$ gene from being affected. Embryonic Stem (ES) cells with incubated with the vector to produce chimeric mice. These ES cells were used to create mice that were then bred with Cre positive mice to excise desired gene exon and create $E2f3a^{+/−}$ mice. Mice with the desired gene were verified by using Southern and Western analysis. These mice were further crossed to produce $E2f3a^{−/−}$.

**DNA isolation and PCR**

Tissue samples were incubated with Proteinase-K overnight, and then mixed with isopropanol. DNA was then precipitated in 70 % ethanol, centrifuged, and then dissolved in TE buffer. We performed Polymerase Chain Reactions using standardized protocols to determine the genotype of mice in the experiment.

**Southerns and Westerns**

We used Southerns and Westerns in some cases to prove whether a gene was present or being expressed to produce protein. The Southerns verified that genomic DNA was present. Westerns were used to verify protein expression. Shih-Yin Tsai mainly conducted them both, although I assisted occasionally.

**Breeding Methods.**

In order to attain a good mix of $E2f1^{−/−} E2f3a^{−/−}$ mice and acceptable controls ($E2f1^{+/+} E2f3^{+/+}, E2f1^{+/−} E2f3^{+/+}, E2f1^{+/+} E2f3^{−/−}$, and $E2f1^{+/+} E2f3^{−/−}$ for wild type and both single knockouts) breeding usually paired a heterozygous male with two or three heterozygous females. This allowed for a one sixteenth chance of each pup being $E2f1^{−/−} E2f3a^{−/−}$ or $E2f1^{+/+} E2f3a^{+/+}$.
Autopsies

Freshly dissected organs were washed in water or PBS, and then fixed in formalin for preservation. In some autopsies, the blood was centrifuged and the decante removed to filter out the serum for future testing.

Histology

Organ samples were fixed in paraffin, and cut onto microscope slides for analysis. Tissue samples were investigated using Hematoxylin-Eosin to observe the structure and cell types of tissue. Other stains were used depending on the test. The Ki 67 test used a mouse anti-human antibody from BD BioSciences Pharmigen. The Caspase anti-body was a rat anti-goat antibody from Cell Signalling.

Tunnel Assay

TUNNEL Assays were performed using the BIO-RAD test kit. Slides were deparafinized and then the procedure was followed as is given in the kit. Slides were counterstained in Hematoxylin.

Quantification

I counted of rates of Cellular Expression by comparing the number of positive cells to all normal cells. These were analyzed to find averages and standard deviations. Charts and Tables were created in Microsoft Excel, and error bars values were taken from the standard deviation within the genotype. Chi-squared tests were performed from standard statistical method and a critical value of \( P<.05 \) was considered significant. I performed T-test using the standard formula to generate a t-value, and I used a t-chart to get a p-value from the t-value.

Generation of the \( E2f3a^{E2F1} \) Knock-in Gene
The \( E2f3^{E2f1} \) knock-in gene was created prior to my involvement with the project by Rene Opavska using the Cre Lox-P system. An \( E2f1 \) cDNA locus was inserted after the 1a exon, with a start before and a stop codon after it. This produced a gene that expressed E2F1 under E2F3a’s regulation without affecting the expression of E2F3b. Embryonic Stem (ES) cells with incubated with the vector to produce chimeric mice. These ES cells were used to create mice that were \( E2f3a^{+/E2f1} \). Mice with the desired gene were verified by using Southern and Western analysis. These mice were further crossed to produce \( E2f3a^{E2f1/E2f1} \).

**Results**

**Generation of \( E2f3a \) Knockout**

In order to gain an improved knowledge of the specific functions of E2F3a, \( E2f3a \) deficient mice were created in the Leone lab by Rene Opavsky. This allowed the study of E2F3a function separate from E2F3b. \( E2f3a^{+/−} \) mice were crossed and the pups’ genotypes were analyzed at birth (Table 1). Unlike deletion of both \( E2f3a \) and \( E2f3b \), which leads to embryonic lethality, \( E2f3a^{−/−} \) mice were viable and later found to be fertile. There were no apparent anatomical or histological abnormalities found in \( E2f3a^{−/−} \) mice. From this, it is clear that E2F3a does not alone cause the critical functionality of E2F3. This leads to the hypothesis that either E2F3b alone or a combination of E2F3a and E2F3b comprise the totality of E2F3 function.

The activator E2Fs seem to have linked function because they have similar expression patterns. As stated earlier, evidence from *in vitro* studies showed that deficiency in E2F3 caused a cellular growth decrease. E2F1 and E2F2 deficient cells did not have an impact on growth, although combing either E2F1 or E2F2 deficiency with
E2F3 deficiency compounds the growth deficiency further from the effect of E2F3 deficiency. Cells deficient for all three showed nearly no growth. This suggests that E2F3 has a critical role in promoting normal growth and E2F1 and E2F2 also act to promote growth but their role is not as critical. Losing one may cause the others to compensate for its loss, and combining deficiencies compound the effects of their loss. It was not known whether this effect could be attributed to E2F3a or E2F3b.

To explore the overlapping functions between E2F activators, we crossed $E2f3a^{-/-}$ mice with either $E2f1^{-/-}$ or $E2f2^{-/-}$ mice to generate double mutant mice of $E2f1^{-/-} E2f3a^{-/-}$ or $E2f2^{-/-} E2f3a^{-/-}$. We successfully generated double mutant mice of $E2f1^{-/-} E2f3a^{-/-}$ or $E2f2^{-/-} E2f3a^{-/-}$. First, we analyzed $E2f2^{-/-} E2f3a^{-/-}$ mutant mice. $E2f2^{-/-} E2f3a^{-/-}$ mutant mice were indistinguishable from wild type littermates. Histopathological analysis did not identify any defect in $E2f2^{-/-} E2f3a^{-/-}$ mutant mice. Next, we analyzed $E2f1^{-/-} E2f3a^{-/-}$ mutant mice. $E2f1^{-/-} E2f3a^{-/-}$ mutant mice were generated at the expected Mendelian rates at birth, but showed a reduced lifespan after that, as is shown in Table 2. The average lifespan of mice in a laboratory is about two and a half years, but the majority of $E2f1^{-/-} E2f3a^{-/-}$ mice die within twenty-one days after birth. The P-test in Table 2 shows that by twenty-one days past birth there was a significant difference between the expected and actual genotype ranges. No $E2f1^{-/-} E2f3a^{-/-}$ mice survived past ten months of age (Figure 4). It was also found that although $E2f1^{-/-} E2f3a^{-/-}$ mice exhibited normal size at birth, soon after $E2f1^{-/-} E2f3a^{-/-}$ mice show severe postnatal growth retardation. As is shown in Figure 5, by six months old the $E2f1^{-/-} E2f3a^{-/-}$ mice are about a third the size of their littermates, and this size discrepancy continued throughout their aging process. This shows that E2F3 and E2F1 are important for promoting cellular growth in development.
E2f1-/- E2f3a-/- mice exhibited other phenotypes as well. The E2F’s have been shown in the past to have specific functions in different cell tissues, and it was found that the E2f1-/- E2f3a-/- mice had several tissue specific defects that were not observed in mice deficient for one of these genes. The E2f1-/- E2f3a-/- mice seemed to show an enlarged head, which is a physical characteristic of dwarves. It was found that E2f1-/- E2f3a-/- males and females were infertile. E2f1 deficient male mice undergo atrophy in their testicles and reduced fertility later in life and the loss of E2f3a seemed to compound this and cause it from young age. Younger E2f1-/- E2f3a-/- mice behaved much like their control littermates, but after reaching adulthood, they seemed listless much of the time and eventually became sickly looking. The E2f1-/- E2f3a-/- mice were much thinner than their control counterparts, showing much less adipose tissue (Figure 7). Generally, two types of fat exist: white fat and brown fat. White fat tissue serves the biological function of insulation and storage of energy. In mice it is located in the front of the abdomen. Brown fat is used to generate heat. In mice it is located in their upper back. As stated previously, other work had already suggested that E2F1, E2F2, and E2F4 have some role in controlling fat tissue development (22). E2F1 and E2F3 deficient mice have reduced adipogenesis and E2F4 deficient mice have increased adipogenesis. In our results, mice deficient for either E2f1 or E2f3a had some reduction in brown fat levels and E2f1-/- E2f3a-/- mice had a similar reduction in brown fat. In regards to white fat, or white adipose tissue, E2f3a-/- mice had a small reduction in white fat levels and E2f1-/- mice showed the same level as wild type littermates. E2f1-/- E2f3a-/- mice, however, were found to have greatly reduced levels of white adipose tissue. We tested to see if E2f1-/- E2f3a-/- mice of young and old ages were absorbing less food by collecting feces samples
after feeding the mice food with a known fat concentration. The feces was collected and sent to the Department of Pathology and Laboratory Medicine at the University of Cincinnatti. It was found out of all the $E2f1^{-/-} E2f3a^{-/-}$ mice tested, young and old had no significant difference in the percentage of fat digested in their food relative to controls (data not shown). This means that the reduced fat in these mice is due to a defect with developing fat tissue and not to a defect in absorbing/digesting them. These results showed that E2F3a performs the E2F3 function of promoting fat tissue development, and that combined loss of $E2f3a$ and $E2f1$ can further decrease fat tissue growth.

**$E2f3a^{-/-} E2f1^{-/-}$ Organ Phenotypes**

In order to determine whether the reduced size of $E2f1^{-/-} E2f3a^{-/-}$ mice was due to an overall reduction in organ size, we weighed several of the organs of twenty-one day-old $E2f1^{-/-} E2f3a^{-/-}$ mice and compared the normalized body weight percentages to control littermates (Figure 7). It was found that the weight percentage of the pancreas, thymus, salivary gland, and brown fat and white fat in $E2f1^{-/-} E2f3a^{-/-}$ mice were significantly lower than in controls. Confirming what was seen visually, the brains of $E2f1^{-/-} E2f3a^{-/-}$ mice represented a much higher percentage of body weight than in the controls. These results showed that there were clearly organ-specific effects of E2F1 and E2F3a loss and that these organs would have to be investigated individually.

The development of the skeletal structure in $E2f1^{-/-} E2f3a^{-/-}$ mice was investigated by staining for cartilage and bone individually. This test showed that the bone structure of these $E2f1^{-/-} E2f3a^{-/-}$ mice seemed to develop normally from a broad perspective (data not shown). Bone samples were viewed microscopically with HE to give a better view of the cellular development of the structure. As can be seen in Figure 8, the growth plate,
where most of the growth of long bones emanates from, showed a clear lack of organization in $E2f1^{-/-} E2f3a^{-/-}$ animals. Therefore, E2F1 and E2F3a are seen to be important for the bone development, without these genes, they are unable to maintain the normal structure and normal growth.

Some organs showed a phenotype on an organ and cellular level, while others did not. The testicles of $E2f1^{-/-} E2f3a^{-/-}$ mice were smaller and had a less organized structure compared to controls. The twenty-one day old male $E2f1^{-/-} E2f3a^{-/-}$ mouse shown in Figure 8 had Leydig cells in the testicles and also a thin layer of Sertoli cells surrounding the semiferous tubules, but no spermatocytes could be seen. This is unlike the controls, which have an organized structure and pre-differentiated spermatocytes at this stage. This observation explains why the male $E2f1^{-/-} E2f3a^{-/-}$ mice are infertile. They lack the stem cells, spermatocytes, and sperm to reproduce. Female $E2f1^{-/-} E2f3a^{-/-}$ mice sexual organs also appear to be less disorganized but the phenotype is not as clear in comparison (data not shown).

The lungs of $E2f1^{-/-} E2f3a^{-/-}$ have thinner walls than their controls, both in the thicker bronchioles and in the alveoli, and also have increased space in their alveoli (Figure 8). Thinner walls in the alveoli could potentially affect oxygen consumption of an animal or make their walls more susceptible to damage. Either of these defects could cause harm to the animals and potentially contribute to the shortened life of $E2f1^{-/-} E2f3a^{-/-}$ mice.

The $E2f1^{-/-} E2f3a^{-/-}$ mice were found to have a clear defect in the development of their adrenal gland (Figure 8). The adrenal gland is an endocrine organ with two main divisions: the adrenal cortex and the adrenal medulla, which come from different cell
lineages. The inner portion, the adrenal medulla, produces the hormones epinephrine and norepinephrine, which help to regulate metabolism and initiate a fight or flight response. The outer portion, the adrenal cortex, has three layers: the zona reticularis, zona fasiculata, and zona glomerulosa, from inside to outside. These layers produce different hormones. HE stains of $E2f1^{-/-} E2f3a^{-/-}$ mice adrenal glands showed that the adrenal medulla seemed to be relatively healthy, but that the adrenal cortex showed a lack of organization. The layers of the cortex were different sizes when compared to control, and the cortices of $E2f1^{-/-} E2f3a^{-/-}$ mice glands were missing one of the layers of the adrenal cortex. Oil red stains, which stain fats and fatty acids in tissue samples, were used to differentiate between the layers and determine the missing layer (Figure 8) because the adrenal gland produces several different steroid hormones. It was determined to be the outermost layer, or zona reticularis. The zona reticularis produces adrogen hormones that act to control development of the sexual organs, especially in males. If this layer is in fact missing in $E2f1^{-/-} E2f3a^{-/-}$ mice adrenal glands, it could help explain the disorder of the $E2f1^{-/-} E2f3a^{-/-}$ male mice testicles.

The pancreas of $E2f1^{-/-} E2f3a^{-/-}$ mice appeared to be normal at an early age, but with time a phenotype was observed (Figure 9). By early adulthood, which is at three months old for mice, some cells with enlarged nuclei were apparent in the exocrine portion of the pancreas. The pancreas has two portions of tissue that have two different functions, exocrine and endocrine. The endocrine tissue produces hormones insulin and glucagons that regulate blood sugar levels and help to regulate metabolism. The exocrine tissue produces enzymes, which are secreted into the small intestine and digest fats, carbohydrates, and proteins. By six months old, the pancreas of $E2f1^{-/-} E2f3a^{-/-}$ mice
atrophied to be a fraction of the size of the controls and even smaller than they were at younger ages. Many of the cells of the exocrine tissue show the enlarged nuclei and the structure of the pancreas appears to be withering away. The endocrine tissue seemed to be for the most part intact in its original structure, although there was much less endocrine tissue in the older $E2f1^{-/-} E2f3a^{-/-}$ mice pancreas than in their controls.

The functions of the endocrine and exocrine pancreatic tissues were investigated at different ages by using antibodies to check for insulin and glucagons in the endocrine tissue and amylase, a carbohydrate-digesting enzyme. These tests showed that these enzyme and hormones are being produced even after the pancreas has degenerated (data not shown). The effectiveness of endocrine tissue to regulate blood sugar and metabolism was investigated by testing serum from autopsied mice. These did not show a significant difference (data not shown), so even the pancreases of even the older $E2f1^{-/-} E2f3a^{-/-}$ mice seem to be able to regulate blood sugar with some effectiveness. The feces absorption test, described previously, showed that there was no significant decrease in fat absorption in $E2f1^{-/-} E2f3a^{-/-}$ mice of any age tested. The pancreas produces lipase, an important enzyme that breaks down fats in the small intestine. Without pancreatic lipase, the body could not digest fats as effectively, so it is believed that the pancreas maintains some effectiveness at producing digestive enzymes even after the pancreas has undergone significant atrophy. In spite of this, due to the extreme decay of the pancreases exocrine tissue in $E2f1^{-/-} E2f3a^{-/-}$ mice, it is believed that their pancreases likely lose effectiveness at producing their digestive enzymes. This likely contributes to the $E2f1^{-/-} E2f3a^{-/-}$ mice wasting away and the death of $E2f1^{-/-} E2f3a^{-/-}$ mice later in life. It is believed that the $E2f1^{-/-} E2f3a^{-/-}$ mice death at younger ages, prior to the pancreas defect, could be due to
the defect in their lungs, adrenal gland, or some other defect caused by the deficiency of these genes.

It was believed that the rapid decay of the pancreases of these mice could be due to apoptosis, or programmed cell death. Apoptosis levels were measured using either Caspase-3 antibody or Tunnel Assay stainings at different age points to see if there was an increase in apoptosis in the $E2f1^{-/-} E2f3a^{-/-}$ mice. The apoptosis level was counted and found to increase as the animal ages (data not shown). There is very little apoptosis at twenty-one days old, but by six months the rate of apoptosis in the $E2f1^{-/-} E2f3a^{-/-}$ mice is far above that of the control mice. It is believed that this apoptosis is the cause of the decay of the pancreas in the $E2f1^{-/-} E2f3a^{-/-}$ mice. E2F1 is proven to be an important regulator for apoptosis. It can act to induce apoptosis when expressed in large levels. That the deficiency of $E2f1$ and $E2f3a$ caused this increase in apoptosis in the pancreas is unexpected. The fact that this decay was only found in the pancreas suggests some role for E2F3a and E2F1 in the pancreas exocrine tissue.

**Proliferation Assays**

In order to ascertain if the reduced size of the $E2f3a$ and $E2f1$ deficient animals was due to a reduced cellular growth, Ki 67 antibodies were used to investigate the rates of cellular proliferation in different organs. Ki 67 is a nuclear protein that is commonly used as a marker for proliferating cells. As is shown in Figures 10 and 11, the organs of $E2f1^{+/-} E2f3a^{+/-}$ twenty-one day old mice that were tested had reduced levels proliferation in comparison to both mice deficient for one gene and wild-type controls. This was seen in organs that had a similar body weight percentage compared to the control and in those that had a lower body weight percentage relative to the control. These differences in
proliferation show that loss of E2F3a and E2F1 cause a reduction in growth in cells throughout different organs *in vivo*, and not just in cells *in vitro*, and helps to explain the reduced size of the animals.

**E2f1 Knock-in to E2f3a Experiment**

The results described above indicate that E2F1 and E2F3a deficiency causes defects that were not seen in mice solely deficient for one of these genes. This suggests that these genes likely play a synergistic role in promoting growth and had similar functions. These results also show that some of the relationship between E2F1 and E2F3 function that was found in cellular studies is due to E2F3a. These genes have similar gene sequences and similar expression patterns during the cell cycle, with their expression peaking during S-phase. Their functions have been found to be similar also, but it was not known how much specificity in function these genes really had, or to what extent these genes function was the same *in vivo*. In order to investigate this, a novel knock-in gene was created by Renee Opavska that allowed the replacement of exon 1a of the *E2f3a* gene locus with an entire *E2f1* gene cDNA (Figure 12). This allowed the creation of an *E2f1* gene that was under the regulation and expression of the *E2f3a* locus. In essence, E2F3a was replaced with E2F1 in living animals.

**E2f3a*E2f1/E2f1** *E2f1* */* Mice Phenotypes

*E2f3a*<sup>E2f1/E2f1</sup> *E2f1* */* mutant mice were generated and crossed with *E2f1* */* mice to generate mice that had a deficiency in *E2f3a* and *E2f1*, but had expression of *E2f1* under *E2f3a*’s natural regulation (Figure 13). Both *E2f3a*<sup>E2f1/E2f1</sup>, and *E2f1* */* *E2f3a*<sup>E2f1/E2f1</sup> mice appeared to develop normally with body sizes nearly the same as controls (Figure 14). They also did not seem to show the shortened life span that *E2f1* */* *E2f3a* */* mice
experience (data not shown). Mice with the $E2f3a^{E2f1}$ gene were found to have a reduced fertility compared to control mice, but were able to produce offspring unlike $E2f1^{-/-}$ $E2f3a^{-/-}$ mice.

When we investigated the $E2f1^{-/-}E2f3a^{E2f1/E2f1}$ mice for organ defects, it was found that $E2f1^{-/-}E2f3a^{E2f1/E2f1}$ mice did not experience the organs defects observed previously in $E2f1^{-/-}E2f3a^{-/-}$ mice, such as the lung walls, adrenal gland layers, testicle disorganization, bone growth, and pancreas decay (Figure 15). Ki 67 assays were performed to investigate the proliferation of the $E2f1^{-/-}E2f3a^{E2f1/E2f1}$ tissue, and it was found that $E2f1^{-/-}E2f3a^{E2f1/E2f1}$ had much higher levels of cellular proliferation than $E2f1^{-/-}E2f3a^{-/-}$ but seemed to have somewhat less proliferation than wild type controls (Figure 16).

For the most part, these $E2f1^{-/-}E2f3a^{E2f1/E2f1}$ mice seemed to develop similarly to mice deficient solely for $E2f1$ or $E2f3a$. They showed that the $E2f1$ gene could perform the functions of $E2f3a$ when under the regulation that $E2f3a$ usually experiences, suggesting that E2F1 and E2F3a have similar functions. This was an interesting and unforeseen observation, but unexpectedly there was a new discovery. Older $E2f3a^{E2f1}$ mice began to show an expected phenotype (see below).

**Long-term Analysis of $E2f3a^{E2f1}$ Gene**

It was discovered that mice with the $E2f3a^{E2f1}$ gene were developing hepatocellular carcinoma. This is a malignancy that forms in hepatocytes, the cell type that forms the majority of the liver and produces several important enzymes. Dr. Shan Naidu, a DVM pathologist in the Leone lab, analyzed the liver samples. All livers observed with a greater number of E2f1 alleles than natural ($E2f1^{+/+}E2f3a^{E2f1/E2f1}$,
$E2f1^{+/+}\ E2f3a^{+/E2f1},\ E2f1^{+/+}\ E2f3a^{E2f1/E2f1}$ and of at least twelve months of age were diagnosed with hyperplasia, adenoma, or carcinoma in their liver (Figure 18 and Table 3). Hyperplasia is new growth in tissue caused by an increase in the number of cells. It often does not negatively affect neighboring tissue and does not necessarily indicate that a tissue will become cancerous. Adenoma is a collection of growths that originates in tissue that is glandular in origin. Although usually benign, adenoma growths can negatively affect neighboring tissue by compressing them and can develop to become malignant over time. Carcinoma is a malignant tumor of epithelial tissue. The diseased livers formed “off color” spots throughout the organ and grew in size. In some cases the growth became larger than the largest lobe of the liver (Figure 17). As shown in Table 3, the two $E2f1^{+/+}\ E2f3^{E2f1/E2f1}$ mice we tested did not develop these abnormal growths. All mice autopsied with three $E2f1$ gene copies (this includes $E2f1^{+/+}\ E2f3a^{E2f1/E2f1}$ and $E2f1^{+/+}\ E2f3a^{+/E2f1}$ mice) developed unusual liver growths. Among these mice, there were four incidents out of ten that had developed hepatocellular adenoma and four that had developed hepatocellular carcinoma. We have not tested any mice with four $E2f1$ gene copies because the incidents of hepatocellular carcinoma were unexpected in these older mice. It is likely that these mice would have similar results to the mice with three or $E2f1$ gene copies.

These results suggest a number of things. Previous studies have suggested that E2F1 can be oncogenic in certain circumstances (23, 24, and 25) and these results seem to support that claim. It seems that too much E2F1 protein is causing the tumors to form. $E2f1^{+/+}\ E2f3a^{+/+}$ and $E2f1^{-/-}\ E2f3a^{E2f1/E2f1}$ mice did not develop the liver growths. Mice with more than the natural number of $E2f1$ gene copies, either by $E2f1^{+/+}\ E2f3a^{E2f1/E2f1}$ or
$E2f1^{+/+} \ E2f3a^{+/E2f1}$ genotypes developed hyperplasia, which are likely to go on to develop into adenoma and carcinoma. This suggests that there is a critical level of E2F1 protein that causes growth in these mouse livers. Having more E2F1 protein makes these growths more likely and also more likely to be malignant.

**Discussion**

It has been known for some time that E2F3 has two isoforms, E2F3a and E2F3b, and since their discovery it was desired to know how much of these proteins functions is individual or in common between the two of them. These genes have the same gene locus except E2F3b lacks the initial exon, or N-terminus, which is found in E2F1 and E2F2 and believed to encode for the cyclin A binding site in E2F3a (26, 27). This binding site has been hypothesized as a key part of the activating E2Fs’ regulation; interestingly E2F3a has it and E2F3b lacks it, suggesting that this domain may provide E2F3a with some specific functions. The $E2f3a$ knockout was generated to allow new experiment to allow the functions of E2F3a to be evaluated separate from that of E2F3b. Mice deficient for E2F3a were generated at their expected rate and developed normally. This shows that E2F3a does not solely perform the critical function of E2F3, which makes it important for normal development and growth. Possibly E2F3b or a combination of E2F3a and E2F3b causes E2F3’s critical function in embryonic development. This could be tested by generating an $E2f3b$ knockout, and observing whether mice deficient in this isoform exhibit the shortened lifespan characteristic of $E2f3$ deficient mice.

Earlier *in vitro* experiments showed that $E2f3$ was unique among the $E2fs$ in being important for promoting cellular growth. Cells that were $E2f3$ deficient had severely
decreased cellular growth. Cells with $E2f1$ or $E2f2$ deficiency cells showed no decrease in cellular growth, but combining $E2f1$ or $E2f2$ deficiency with $E2f3$ deficiency compounded the effect $E2f3$ loss on cellular growth. It was hypothesized, that this effect might have occurred because the activator E2Fs have some redundancy in function and may be able to compensate for the loss of one. Previous studies of E2F3 in mouse development had limits because the majority of $E2f3$ deficient mice died before birth, making $E2f3$ deficiency difficult to examine over further developmental points. The $E2F3a$ deficient mice did not exhibit this embryonic lethality. In order to investigate whether relationships were observed in mouse development between $E2f3a$ separate from $E2f3b$ and the other activators, the $E2f3a$ deficient mice were combined with $E2f1^{−/−}$ and $E2f2^{−/−}$ mice to create double knockout mice deficient with both. It was found that $E2f2^{−/−}$ $E2f3a^{−/−}$ mice did not seem to exhibit any clear phenotypes. The $E2f1^{−/−} E2f3a^{−/−}$ mice, on the other hand, were found to have significant phenotypes, suggesting a functional link between E2F1 and E2F3a in development.

The $E2f1^{−/−} E2f3a^{−/−}$ mice were born at expected Mendelian rate, but the mice experienced a decreased survival rate, with less than half of $E2f1^{−/−} E2f3a^{−/−}$ mice surviving to one month of age. The $E2f1^{−/−} E2f3a^{−/−}$ mice were found to be significantly runted. They were found to be of normal size at birth but by adulthood these mice were only about 40% the weight of their littermates. These results show that E2F3a and E2F1 are necessary for normal organism growth, similar to the results seen with $in vitro$ experiments that showed that E2F3 and E2F1 have some redundant function in promoting cellular growth.
When we investigated $E2f1^{-/-} E2f3a^{-/-}$ mice closely we found that they had several defects on an organ and tissue level that were not seen in $E2f3a^{-/-}$ or $E2f1^{-/-}$ mice. Defects were found in the adrenal gland, lungs, bone growth plate, testicles, and pancreas. Fat tissue was reduced in $E2f1^{-/-} E2f3a^{-/-}$ mice in comparison to mice deficient in one gene and wild-type controls. E2F1 and E2F3 were found previously to be important for promoting fat growth. These results show that E2F3a contributed to E2F3’s function in promoting adipogenesis. It is likely that some of these defects, most notably the lung, adrenal gland, or pancreas, contribute to the shortened lifespan of $E2f1^{-/-} E2f3a^{-/-}$ mice.

As stated before, the E2Fs seem to have organ specific functions in growth, differentiation, and development. The organs with defects are likely some of the organs where the E2F1 and E2F3a have an increased importance. Loss of one of the genes did not seriously affect them, possibly causing the other to compensate for it. The loss of both, however, caused a defect in several tissues.

The knock-in $E2f1$ into $E2f3a$ gene locus study was performed to give an idea of how much of these genes’ functions is individual and specific. E2F1 and E2F3a have similar components to their structure, with a DP hybridization domain, an Rb binding domain, a DNA binding domain, and a N-terminus where they are believed to be regulated by cyclins. They have different promoters but have similar expression patterns.

The $E2f1$ knock-in experiment attempted to find if E2F1 could perform the functions of E2F3a. It was found that $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ mice did not experience the effects caused by $E2f3a$ deficiency and natural $E2f1$ deficiency. $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ mice had normal growth and lacked the organ defects described earlier in $E2f1^{-/-} E2f3a^{-/-}$ mice. This shows that E2F1 can act as E2F3a in these mice and prevent defects, causing these
mice to be like $E2f1^{-/-}$ mice. To some extent, the E2F1 seems to be able to perform the functions of E2F3a, meaning that there is some redundancy in their functions in promoting normal growth and development.

The $E2f1$ knock-in gene was found to have an unforeseen effect in older mice of inducing liver growths and in some cases hepatocellular carcinoma. Evidence suggests E2F1 can cause tumorigenesis when overexpressed in liver and other epithelial tissues (24, 25, and 26). The occurrence of hepatocellular carcinoma in mice with more $E2f1$ alleles than natural suggests that there is a critical amount of E2F1. Thus, there is a critical amount of E2F1 in animals, where too little or too much may adversely affect the health of the animal. Above this critical level of expression, E2F1 promotes unhealthy growths in the liver that can develop into tumors. The results seen from the $E2f3a^{E2f1}$ experiment suggest that E2F1 can replace E2F3a in mice in the short term, but with prolonged time negative effects are experienced.
References


The E2F Family

Figure 1: E2Fs and the Cell Cycle. Cells grow by progressing through the cell cycle. First cells grow (G1), then synthesize DNA to create duplicate chromosomes (S), then grow again (G2), and then undergo mitosis (M), creating two daughter cells. Quiescent cells stay in G0. The E2Fs have been found to be important for the Cell Cycle, specifically for reentry of quiescent cells from G0 and the G1/S transition. The activators seem to promote growth and the repressors seem to inhibit it.

Figure 2: The E2F Family of Transcription Factors. The E2F family are transcription factors, or genes that influence others genes’ expression. The activator E2Fs (1-3a) have been shown to positively affect transcription of E2F targets, while the repressors negatively affect it. The common component of the E2Fs is their DNA Binding Domain (DBD), which is boxed in red. E2Fs 1-4 have an Rb binding domain where they can bind to the pocket proteins. E2Fs 1-6 all have the LZ section, where they dimerize with DP. The NTD section is the N-terminal domain, where it is believed that E2F1-3a may be regulated.
Generation of the \textit{E2f3a} Gene

![Diagram of the E2f3a Knockout](image)

Figure 3: Creation of the \textit{E2f3a} Knockout. The \textit{E2f3a} Knockout was created by inserting lox-P sites upstream and downstream of the 1a exon of the \textit{E2f3a} DNA gene locus (a). The 1a exon was later deleted by the introduction of CRE, eliminating the \textit{E2f3a} protein but not the \textit{E2f3b}. Southernns (b), Westerns ©, and PCRs (d) show that the \textit{E2f3a} gene was ablated in the Knockout mice.

Analysis of embryos derived from 5\textsuperscript{th} generation FVB \textit{E2f3a}\textsuperscript{+/-} and \textit{E2f3}\textsuperscript{+/-} crosses

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<th>\textit{E2f3a}\textsuperscript{+/-}</th>
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P-value for \textit{E2f3a} Generation = .600, P-value for \textit{E2f3} Generation = 1*10^-5

Table 1: Mendelian Rate of \textit{E2F3} and \textit{E2F3a} Generation. \textit{E2F3a}\textsuperscript{+/-} mice were crossed and \textit{E2F3}\textsuperscript{+/-} mice were crossed to analyze the occurrence of the genotypes. P-tests were performed afterward and gave a significant value for the \textit{E2F3} breeding (1*10^-5), but a non-significant value for the \textit{E2F3a} breeding (.6). A P-value of .05 was used to test for significance.
Phenotypes of $E2f1^{-/-} E2f3a^{-/-}$ Mice

### Analysis of embryos derived from 5th generation FVB $E2f3a^{+/-} E2f1^{+/-}$ crosses

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Table 2: Mendelian Rates of $E2f1^{+/-} E2f3a^{+/-}$ crosses: The Mendelian rates were found at embryonic day 13.5, birth, and 21 days old. The death of the $E2f1^{-/-} E2f3a^{-/-}$ mice created a significant difference by 21 Days (P-value = .001). A P-value of .5 was used as a test for significance.

Figure 4: $E2f1^{-/-} E2f3a^{-/-}$ Survival Chart. The $E2f1^{-/-} E2f3a^{-/-}$ mice experienced a reduced life span that was not seen in single deficient of control littermates. The average lifespan of mice is about two and a half years but the mean life of the $E2f1^{-/-} E2f3a^{-/-}$ mice was about twenty days. The control group includes any $E2f3a$ and $E2f1$ genotype besides $E2f1^{-/-} E2f3a^{-/-}$. 

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**Figure 4**
Figure 5: E2f1−/− E2f3a−/− Mice Postnatal Growth Retardation. The E2f1−/− E2f3a−/− mice were about normal size at birth but afterwards experience growth retardation. The mice weighed for this graph were taken from male and female mice at set age points. The data is not all taken from the same mice at different age points. The 1 month old E2f1−/− E2f3a−/− mice weighs about 4 grams, while its littermate weighs about 10 grams. The control group includes E2f1+/+ E2f3a+/+, E2f1+/+ E2f3a−/−, E2f1−/+ E2f3a+/+, and E2f1−/+ E2f3a−/− mice.

Figure 6: 21 Day Organ Body Weight Percentage. The organ weight of 21 days old E2f1−/− E2f3a−/− mice were compared to control littermates and it was found that several organs had different sizes (normalized to body weight) to their controls. The control group includes E2f1+/+ E2f3a+/+, E2f1+/+ E2f3a−/−, E2f1−/+ E2f3a+/+, and E2f1−/+ E2f3a−/− mice.

Figure 7: Fat Levels of 21 Day E2f1−/− E2f3a−/− Mice. E2f3a−/− and E2f1−/− and singly deficient mice showed reduced fat levels, confirming the results of prior experiments which showed reduced fat levels. The data here was taken by weighing white adipose tissue and brown adipose tissue after autopsy and then taking a relative weight by body mass. Controls include E2f1+/+ E2f3a+/+, E2f1+/+ E2f3a−/−, E2f1−/+ E2f3a+/+, and E2f1−/+ E2f3a−/− mice. Only male mice were used in the data shown, although females showed similar trends. The yellow arrows in the picture to the right indicate white adipose tissue in a E2f1−/− E2f3a−/− mouse and a control.
Figure 8: Organ Defects of E2f1−/− E2f3a−/− Mice (continued on next page). E2f1−/− E2f3a−/− mice had clear phenotypes in several organs that were not seen in single deficient mice. The bone growth plate was disorganization. The testicles showed a lack of structure in the seminiferous tubules and lack of spermatocytes. The lungs had thin lung walls and larger alveoli. The adrenal gland was disorganized and seemed to be missing the zona reticularis. The control group includes E2f1+/+, E2f3a+/+, E2f1+/− E2f3a+/+, and E2f1+/− E2f3a+/− mice.
| Adrenal Gland | Control |  |  |
|--------------|---------|  |  |
| HE           | 4x      | 40x |  |
| Oil Red      |  |  |  |

<table>
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- HE: Hematoxylin and Eosin staining.
- Oil Red: Oil Red staining.
Figure 9: Pancreas Atrophy Progression in $E2f1^{-/-} E2f3a^{-/-}$ Mice. Pancreases of young mice appeared normal. By three months, some exocrine cells in $E2f1^{-/-} E2f3a^{-/-}$ mice pancreases showed enlarged nuclei. At six months the $E2f1^{-/-} E2f3a^{-/-}$ mice pancreases had atrophied to have nearly no exocrine tissue left. The control group includes $E2f1^{+/+} E2f3a^{+/+}$, $E2f1^{+/+} E2f3a^{+/-}$, $E2f1^{+/-} E2f3a^{+/-}$, and $E2f1^{+/-} E2f3a^{++}$ mice.
Figure 11: E2f1−/− E2f3a−/− Tissue Proliferation Assay. Proliferation Assays showed reduced cellular growth in several tissues of twenty-one day old E2f1−/− E2f3a−/− mice, explaining their reduced size. Pictures were taken of tissue sections of these mice and the amount of cells counted varied depending on the size of the organ. The adrenal gland did not have a large enough area to count more than about four hundred cells. Larger organs were counted with at least 1000 cells per animal. At least four animals were used for each organ, and I calculated standard deviations by comparing rates among different mice of the same genotype. The control group includes E2f1+/+, E2f3a+/+, E2f1+/− E2f3a+/+, E2f1−/+ E2f3a+/+, and E2f1+/− E2f3a+/− mice.

Figure 10: E2f1−/− E2f3a−/− Tissue Ki 67 Stainings. Ki 67 Assays were performed on tissues E2f1−/− E2f3a−/− mice. A general reduction in proliferation was observed. The liver and adrenal gland used histochemical antibodies and the pancreas staining used fluorescent antibodies. The control group includes E2f1+/+, E2f3a+/+, E2f1−/+ E2f3a−/−, E2f1+/− E2f3a+/+, and E2f1+/− E2f3a+/− mice. The brown cells in the liver and the adrenal gland and the red cells in the pancreas are Ki 67 positive and proliferating.
Generation of $E2f3a^{E2f1}$ Mice

Figure 12: $E2f1$ Knock-in to $E2f3a$. The Genomic Locus of $E2f3$ (a) has the 1a and 1b exons, which compose the initial exons of $E2f3a$ and $E2f3b$, respectively. The vector has an $E2f1$ cDNA inserted into the 1a exon, with a start codon before the $E2F1$ cDNA and a stop codon afterwards.

Figure 13: The Western shows that mice deficient in natural $E2f1$ but with an $E2f3a^{E2f1}$ allele produce the $E2F1$ protein. The anti-$E2F1$ antibody allowed $E2F1$ protein existence to be tested. The anti-tubulin antibody was used as a control to ensure that the Western worked.
Phenotypes of $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ Mice

Figure 14: Body Weight of 21 Day $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ mice. $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ mice do not experience the growth retardation seen in $E2f1^{-/-} E2f3a^{-/-}$ mice. I found the weights were found by averaging several mice of the same genotype together, and I calculated the standard deviations from the weight values of individual mice. The control group includes $E2f1^{+/-} E2f3a^{+/-}$, $E2f1^{-/-} E2f3a^{+/-}$, and $E2f1^{+/-} E2f3a^{E2f1/E2f1}$ mice. The $E2f1^{-/-} E2f3a^{-/-}$ mice shown come from a different group but their controls were of similar size to the controls shown.
Figure 15: Phenotypes of $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ mice. $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ did not show any of the organ defects seen in $E2f1^{-/-} E2f3a^{/-}$ mice.
Figure 16: $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ Mice Tissue Ki 67 Proliferation Assay. $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ do not show the reduced proliferation seen in different tissues in $E2f1^{-/-} E2f3a^{-/-}$ mice. I performed a T-Test to compare the $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ tissues’ and $E2f1^{-/-} E2f3a^{-/-}$ tissues’ proliferation rates to their controls. The data shows that the $E2f1^{-/-} E2f3a^{-/-}$ tissues have a more significant difference from their controls than $E2f1^{-/-} E2f3a^{E2f1/E2f1}$ do from their controls, although not enough to pass the significance test ($P<.05$) for all but one type of tissue. The control group includes $E2f1^{+/+} E2f3a^{+/+}$ and $E2f1^{+/+} E2f3a^{E2f1/E2f1}$ mice.
Long Term Analysis of \(E2f3a^{E2f1}\) Gene

Figure 17: \(E2f3a^{E2f1}\) Mice Develop Hepatocellular Carcinoma. The mouse on the left has developed a hepatocellular carcinoma. Hepatocellular carcinoma is cancer of hepatocytes, an epithelial cell in the liver which comprises the majority of the liver’s mass. The liver with malignant tumor shown in center.

Figure 18: The Liver Tumors formed by a progression from Hyperplasia, to Adenoma, and finally to Carcinoma. The following 10x microscope pictures shows examples of these cell conditions. Hyperplasia (left) is an increase in the number of cells in an organ, but usually does not affect neighboring cells. Adenoma (center) is a benign increase of cells which compresses nearby tissue and deforms their shape. In this case, the growing tissue in the center is compressing the neighboring cells, altering their shape from the normal tubular to a spindle shape. Carcinoma is a cancer of epithelial cells, it is characterized by intrusion of malignant cells into normal cellular tissue and disruption of normal cellular structure (right).

Table 3: Liver Dissection Observations. Record of observation of dissected \(E2f3a^{E2f1}\) mice livers. These samples were autopsied at varying ages past 12 months and the incidence of hyperplasia, adenoma, and carcinoma were recorded. 2 \(E2f1\) hard copies includes \(E2f1^{+/-}\) \(E2f3a^{E2f1/E2f1}\) mice. 3 \(E2f1\) hard copies includes \(E2f1^{+/-}\) \(E2f3a^{E2f1/E2f1}\) and \(E2f1^{+/+}\) \(E2f3a^{+/E2f1}\) mice.