β1-INTEGRIN MAY REGULATE EGR1

(EARLY GROWTH RESPONSE 1) WITHIN THE LENS

by

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ABBREVIATIONS

αSMA	alpha smooth muscle actin
β1cKO	Mice lacking β1-integrin in the lens
β1MLR10	Mice lacking β1-integrin in the lens
Anxa2	annexin A2
ARVO	Association for Research in Vision and Ophthalmology
B2M	Beta-2 microglobulin
bp	base pairs
BSA	bovine serum albumin
c	capsule
cDNA	complimentary deoxyribonucleic acid
ce	corneal epithelium
cKO	conditional knockout
c-Maf	v-Maf musculoaponeurotic fibrosarcoma oncogene homolog
DNA	deoxyribonucleic acid
dpc	days post coitum
E	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
Egr1	early growth response 1
Egr1-null	Mice lacking Egr1 gene
EMT	epithelial-to-mesenchymal transition
Ep/le/e	lens epithelium or residual epithelial cells (surgical samples)
FDR	false discovery rate
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
fwd	forward
GEO	Gene Expression Omnibus
H&E	hematoxylin and eosin
HDAC1	histone deacetylase 1
HIF-1α	hypoxia inducible factor 1 alpha
IACUC	Institutional Animal Care and Use Committee
IgG	Immunoglobulin G

IOL	intraocular lens
IPA	Ingenuity Pathway Analysis
LF/lf/f	differentiated lens fiber cells
Mmp14	Matrix metallopeptidase 14
Mt1	Metallothionein 1
Nab1	NGFI-A binding protein 1
Nab2	NGFI-A binding protein 2
Nd:YAG	neodymium:yttrium aluminum garnet
OCT	optimum cutting temperature (media)
p21	cyclin-dependent kinase inhibitor 1
p300	histone acetyltransferase (E1A binding protein), 300kDa
p53	transformation related protein 53 (Trp53)
Pax-6	paired-box 6
PBS	phosphate Buffer Saline
PCO	posterior capsular opacification
PCR	polymerase chain reaction
pFAK	phosphorylated Focal Adhesion Kinase
Plat	tissue plasminogen activator (tPA)
Prox1	prospero homeobox 1
qRT-PCR	qualitative reverse transcription polymerase chain reaction
rev	reverse
RNA	ribonucleic acid
RNA-Seq	high-throughput RNA Sequencing
RPKM	reads per kilobase per million
Rpl29	Ribosomal protein L29
S.D.	standard deviation
Sip1 f/f no cre	Sip1 control mice, mix background equivalent to WT
SRE	serum response element
SRF	serum response factor
Stmn1	Stathmin 1 (P18/P19)
TBP	TATA box binding protein
TBS	tris buffered saline
TGF-β	Transforming growth factor beta
Thbs1	Thrombospondin-1
WT	wild type

ABSTRACT

Integrins are heterodimeric transmembrane extracellular matrix receptors that regulate cell-to-cell and cell-to-ECM communication and require an α and a β subunit. The β1-integrin subunit is expressed in both epithelial and fiber cells within the lens, an epithelial tissue important for vision, and also interacts with the largest number of α subunits. Full deletion of β1-integrin from the developing lens causes embryonic lethality at the blastocyst stage. Therefore a conditional knock-out was created, deleting β1-integrin from the lens at embryonic day 12.5 (E12.5). This caused severe disorganization of the lens by E16.5, with the absence of the central epithelium, elongation of the epithelial cells at the transition zone, and highly vacuolated fiber cells. These lens epithelial cells exhibited loss of their normal markers and up-regulation of fiber cell and EMT markers. Analysis of candidate genes based on their known roles in integrin, TGFβ, and MAPK pathways failed to explain the observed phenotype. Hence, I used an unbiased analysis of gene expression changes to identify the likely regulator of the β1-integrin null phenotype.

To do so, I chose to use next generation RNA-sequencing (RNA-Seq), a novel technique for whole genome expression profiling in the lens. Although many researchers in the past have used expression microarrays, it has several limitations that include a reliance on existing knowledge about genome sequence, high background, and limited dynamic range. RNA-Seq, on the other hand, has very low background signal, unambiguous mapping capability, and increased sensitivity. This increased sensitivity allows us to sequence past a large bias towards structural proteins in the

lens. I chose to sequence lenses at E15.5, one day prior to the onset of the β1MLR10 phenotype, in attempt to obtain gene changes that are proximal to the cause of the fibrotic phenotype observed.

With the help of Abby Manthey, I was able to collect wild type C57Bl/6 lenses for RNA-Seq, and compare them to β1MLR10 lens samples. Through this comparison, it was found that over 7,700 genes are expressed in the wild type lens at E15.5. To be able to determine biologically relevant gene changes, we compared two wild type mouse strains: C57Bl/6, an inbred strain, to a mixed background wild type strain. This comparison allowed us to determine that the lens expresses over 7,000 genes over 2 Reads Per Kilobase per Million (RPKM), a measurement of gene transcript level. We also found that only 2%, or 32 genes, of over 2,300 differentially expressed genes between the different strains were above a 2.5 fold change. Therefore any fold change less than this was likely to be due to genetic background strain variability, and not the deletion of β1-integrin from the lens. Therefore, to determine biologically relevant gene changes in the β1MR10 lens, I used the following filtering criteria: mean RPKM ≥ 2 p-value ≤ 0.05, and fold change ≥ 2.5.

Using these filtering criteria, I was able to determine that 90 genes are differentially expressed three days after the deletion of β1-integrin and one day prior to the onset of the phenotype. A large subset of these genes are known to play a role in fibrotic responses and include: *Egr1*, *Nab2*, *αSMA*, *Mmp14*, *Anxa2*, *Plat*, *Mt1*, *Thbs1*, *Stmn1*, and *Rpl29*. *Egr1*, a transcription factor that is directly regulated by integrin signaling, in turn directly regulates *MT1*, *Nab2*, and *αSMA* in other tissues. *Egr1* also has potential to regulate a number of differentially expressed genes within the β1MLR10 lens through EGR1 binding sites (EBS) within their promoter regions. This led me to the conclusion that *Egr1* is likely able to regulate the partial fibrotic phenotype observed in the β1 conditional knock out lens.

Using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis, I was able to validate the differential expression of *Egr1*, *Nab2*, *αSMA*, *Mmp14*, *Anxa2*, *Plat* and *Rpl29* at E15.5 in the β1MLR10 lens. Interestingly, *Egr1*, *Nab2*, *αSMA*, *Mmp14*, *Anxa2*, *Plat* (all genes up-regulated in RNA-Seq) are not only found to be up-regulated at E15.5, but are also up-regulated just one day after the full absence of the β1-integrin protein from the lens. This up-regulation continues to rise through E15.5, and remains high through E16.5. Immunolocalization also showed that *Egr1* is increased at the protein level at E15.5 and E16.5. Altogether, this strongly supports *Egr1*'s role in mediating the partial fibrotic response observed in the β1MLR10 lens.

With *Egr1*'s implication in mediating fibrotic responses in the embryonic lens, it seemed likely that it may be able to play a role in lens diseases such as cataracts and posterior capsular opacification (PCO). Cataract, or the loss of lens transparency, is a major disease of the lens that leads to loss of vision, and is the most common cause of blindness worldwide. Cataracts are commonly treated by extracapsular lens extraction, which involves the surgical removal of the bulk of the lens cells by phacoemulsification, and insertion of a prosthetic lens (or intraocular lens, IOL) into the remaining capsular bag. The remaining lens epithelial cells left on the anterior capsule after surgery proliferate and migrate to accumulate between the capsule and the IOL, where they become fibrotic plaques and cause re-clouding of the lens. This clouding of the lens after cataract surgery is known as posterior capsular opacification (PCO). Studies of PCO models have shown that the fibrotic cells express αSMA, CD44 and other markers of epithelial to mesenchymal transition (EMT). Importantly, EGR1 is able to regulate both *αSMA* and *CD44* in other tissues, and is known to be involved in cataracts induced by selenite in the rat lens. Therefore, I hypothesize that *Egr1* may be an early regulator in lens injury responses such as PCO.

By using a mouse cataract surgery model, I was able to investigate the expression of *Egr1*, *Nab2*, *αSMA* and *CD44* at multiple time points after surgery. Considering *Egr1* is an immediate early response gene, I analyzed samples at 3, 6, 12, 24 and 48 hours post-surgery. Preliminary results using qRT-PCR analysis with one biological replicate seemed promising, with a strong up-regulation of *Egr1* at 3 hours post-surgery. However, subsequent efforts to support this with three biological replicates were unsuccessful. My efforts to detect changes in *Nab2*, *αSMA* and *CD44* expression over this time frame were also unsuccessful using qRT-PCR. However, immunolocalization showed up regulation of EGR1 protein just 3 hours after cataract surgery in the proliferating epithelial cells remaining in the lens capsule.

Overall, *Egr1*is likely to play a major role in the embryonic fibrotic phenotype observed in the β1MLR10 lens; up-regulating pro-fibrotic pathways through *Nab2*, *αSMA*, *Mmp14*, *Anxa2* and *Plat*. It may also play an early role in PCO pathogenesis and other lens diseases.

Chapter 1

INTRODUCTION

1.1. Lens Development

The lens is a transparent, avascular epithelial tissue, which provides us with visual acuity by refracting light to a focused point on the retina (Bassnett et al., 2011; Mathias et al., 2007). Within the eye, the lens is positioned behind the cornea and iris, and in front of the retina (Figure 1.1)(Land, 1988). The development of the lens begins early in embryogenesis, with the optic vesicle instructing the surface ectoderm to form the lens placode. Further signaling between the optic vesicle and the lens placode causes both to invaginate simultaneously, forming the optic cup and the lens pit respectively. The lens pit eventually pinches off to form the lens vesicle, a hollow structure composed of prospective epithelial and fiber cells (Figure 1.2)(Cvekl and Piatigorsky, 1996). At this point, the lens is enclosed by a capsule, a thickened basement membrane composed of structural proteins such as laminin, fibronectin and type IV collagen (Cammarata et al., 1986; Danysh and Duncan, 2009; Yurchenco and Schittny, 1990). This membrane confers structure to the lens and provides a membrane for the lens epithelial and fiber cells to attach. Between E11.5 and E13.5, the cuboidal cells comprising the lens vesicle differentiate into epithelial cells and primary fiber cells depending on their position within the lens.

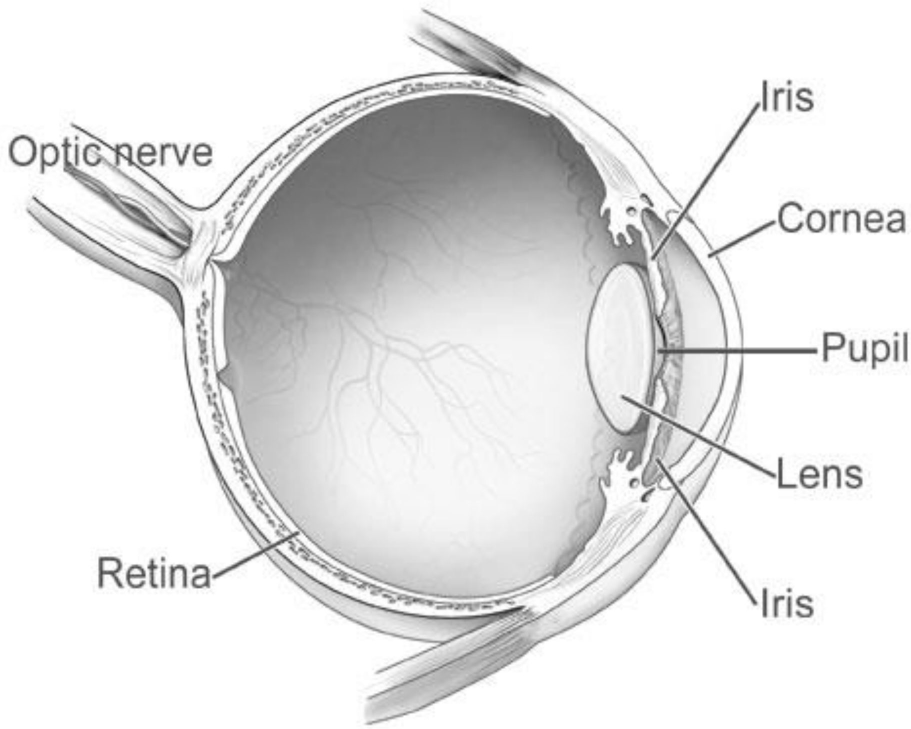

Figure 1.1 Anatomy of the eye. The lens is positioned behind the cornea and iris, and in front of the retina. Light refracts through both the cornea and the lens onto a focused point on the retina which provides visual acuity (National eye institute website: http://www.nei.nih.gov/photo/eyean/images/NEA08_72.jpg).

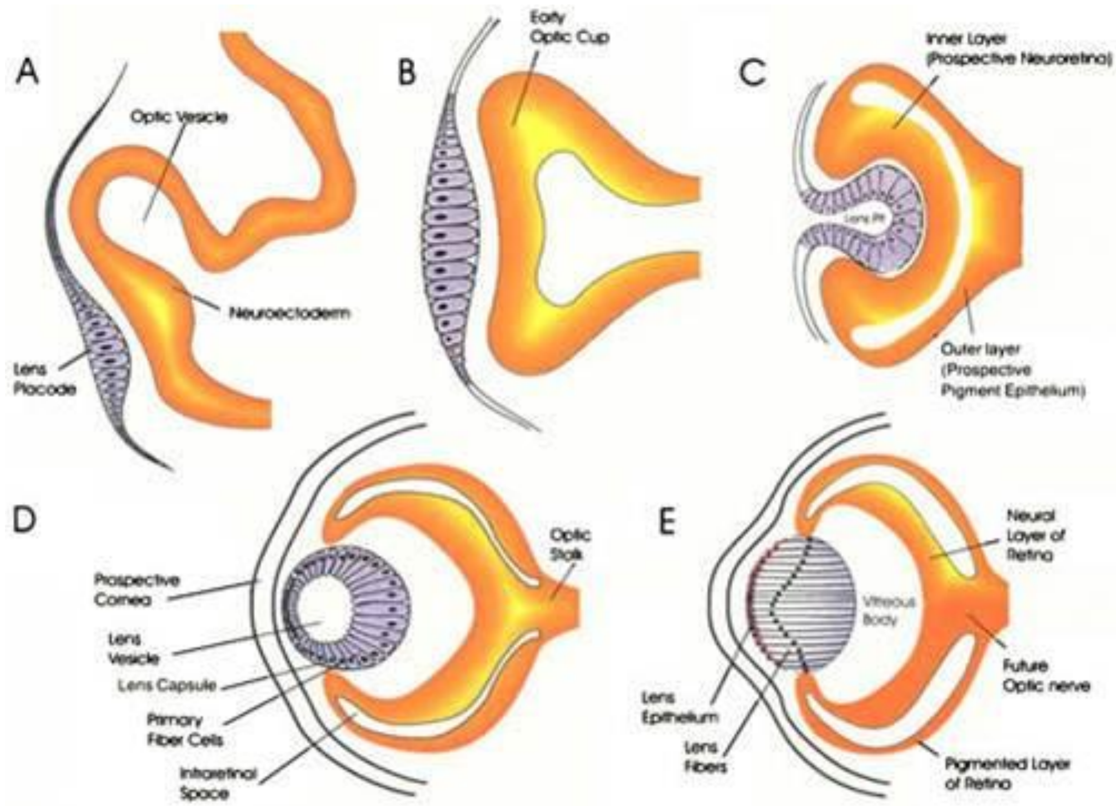

Figure 1.2 Development of the lens. The lens is shown in blue, the retina in orange. Lens development begins at embryonic day 9-9.5 (E9-9.5), with the formation of the lens placode (A) and placode enlargement at E9.5-10 (B). At E10.5 the lens placode invaginates to form the lens pit. At the same time the optic vesicle invaginates to form the optic cup (C). By E11.5, the lens pit has pinched off from the surface epithelium to form the lens vesicle, which is now surrounded by the capsule (D). Around E13.5, the posterior lens vesicle cells elongate to form the primary fiber cells, which make contact with the cuboidal epithelial cells in the anterior portion of the lens (E)(Cvekl and Piatigorsky, 1996).

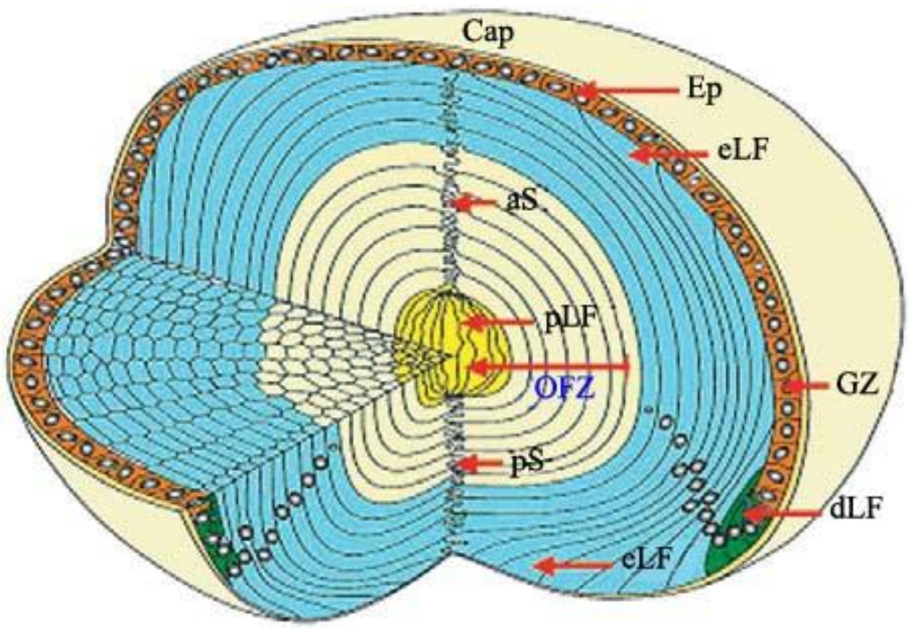

Figure 1.3 Structure of the adult vertebrate lens. The lens is surrounded by the capsule (Cap). The anterior surface of the lens is covered by cuboidal monolayer of epithelial cells (Ep). The primary lens fiber cells (pLF) develop early in embryonic development and make up the nucleus in the adult lens. Secondary fiber cells are formed continuously by the proliferation and differentiation of the epithelial cells at the germinative zone (GZ) at the equatorial region. These secondary fiber cells differentiate (dLF) and elongate (eLF) under the apical surface of the epithelium and along the posterior capsule until their basal surfaces reach the anterior (aS) and posterior (pS) sutures. As lens fiber cells mature, they lose their organelles and nuclei and collect in the organelle free zone (OFZ) Adapted from Bassnett et al., (2011).

1.2 Cell Types in the Lens

Cells in the posterior aspect of the lens vesicle elongate to fill the cavity, differentiating into the primary fiber cells (Bassnett and Mataic, 1997). The anterior cells of the lens vesicle become the lens epithelial cells. These cells continually proliferate at the lens equatorial zone and differentiate into secondary lens fibers which allows for the continued growth of the lens (Cvekl and Piatigorsky, 1996; Ogino and Yasuda, 2000). The continued production of lens fiber cells is important in maintaining the lens and is dependent on the proliferation and migration of stem-like epithelial cells. These cells act to regenerate the lens epithelium as well as provide cells that will migrate towards the transition zone and differentiate into secondary fiber cells (Figure 1.3)(Bassnett et al., 2011; Bhat, 2001; Cvekl and Piatigorsky, 1996). Throughout the growth of the lens, the apical and basal tips of fiber cells migrate towards the center of the lens as the cell lengthens resulting in concentric rings of fiber cells. This process is accompanied by the degradation of their organelles and nuclei, which helps the lens become a transparent tissue (Bassnett and Mataic, 1997; Mathias et al., 2007). Both the lens epithelial and fiber cells are polarized cell types that have their basal membranes attached to the basement membrane, the capsule (Bassnett et al., 1999; Zampighi et al., 2000). This connection to the lens capsule is crucial for maintaining proper cell survival, differentiation, and the general maintenance of the lens; and is maintained by membrane complexes (Bassnett et al., 1999; Coulombre and Coulombre, 1963; Greenburg and Hay, 1982).

As the lens epithelial cells continually proliferate, some of the cells will move towards the equatorial region of the lens, known as the transition zone and differentiate to provide the growing lens with secondary fiber cells (Bassnett and Shi, 2010; Bassnett et al., 2011). For proper fiber cell differentiation, lens epithelial cells must lose their normal gene expression and up regulate fiber cell markers. *Pax6* is a paired-domain and homeodomain containing transcription factor which is necessary for early lens development and maintenance of the lens epithelial cell phenotype (Collinson et al., 2001; Lovicu et al., 2004). It is expressed in the lens progenitor cells before the onset of crystallin expression and serves as an important regulatory factor of crystallins, *c-Maf* and *Prox1* (Cvekl et al., 2004). In the developing and adult lens, *Pax6* is predominantly expressed in the lens epithelium and its expression levels decrease during fiber cell differentiation (Duncan et al., 2000; Li et al., 1994). *Pax6* plays dual roles in lens crystallin expression, in that it activates several genes expressed in adult lens epithelium including αA-, αB-, δ-, and ζ-crystallins (Cvekl and Piatigorsky, 1996; Duncan et al., 2004; GopalSrivastava et al., 1996; Richardson et al., 1995). However, it also acts as a transcriptional repressor of the βB1-crystallin promoter, a member of the β-crystallin family which is required for lens transparency and refraction (Chen et al., 2002; Duncan et al., 1998; Duncan et al., 2004).

c-Maf is a gene that is expressed throughout fiber cell differentiation, and is required for proper fiber formation in the lens (Kawauchi et al., 1999; Reza and Yasuda, 2004). *c-Maf* belongs to the large maf gene family, and is a transcription factor that is able to bind to and regulate genes containing Maf responsive element (MARE) sequences (Kataoka et al., 1994). Importantly, some Mafs, such as L-Maf, are able to recognize and bind to MARE-like sequences such as the αCE2 enhancer sequence in the αA-crystallin promoter (Ogino and Yasuda, 2000). Like, *L-Maf*, *c-Maf* is also able to control crystallin gene expression in the lens, particularly the β- and γ-crystallins (Kawauchi et al., 1999; Reza and Yasuda, 2004).

Prox1, a homeodomain containing factor, is another gene essential for lens fiber cell differentiation (Duncan et al., 2004; Kawauchi et al., 1999; Simirskii et al., 2007; Wigle et al., 1999). In *Prox1* null lenses, γB- and γD-crystallins are down-regulated, but γE- and γF-crystallins are not (Wigle et al., 1999), suggesting that Prox1 specifically regulates γB- and γD-crystallins expression in the lens. Importantly, Prox1 is able to activate βB1-crystallin gene expression, which is a marker for fiber cell differentiation (Chen et al., 2002; Wigle et al., 1999). Overall, *Pax6*, *c-Maf*, *Prox1*, and many crystallins are important and necessary in the lens development and maintenance of transparency and therefore used as markers for the different cell types found in the lens. Their expression, or lack thereof, has been investigated in mouse lenses lacking β1-integrin and will be discussed further in section 1.6.

1.3 EMT

Epithelial to mesenchymal transition, or EMT, is a process in which epithelial cells lose their normal epithelial markers and transition to a more mesenchymal cell phenotype with the up regulation of genes such as α-Smooth Muscle Actin (*αSMA*), Vimentin, N-cadherin, and *TGFβ*, and down regulation of E-cadherin (Figure 1.4)(Hay, 2005). This up-regulation of mesenchymal markers is associated with epithelial cells losing their connection to the basement membrane, losing cell polarity and increasing motility (Greenburg and Hay, 1982). This is a normal process in the development of embryonic tissue, as well as wound healing and cancer metastasis (Hay, 2005; Mendez et al., 2010; Wei et al., 2008).

TGF-β, a growth factor that regulates cell growth, differentiation, apoptosis, and ECM formation, is considered to play a major role in TGF-β activated EMT in response to injury (de Iongh et al., 2005; Massague et al., 2000; Zavadil and Bottinger, 2005). TGF-β can induce the down regulation of epithelial markers and up regulation of mesenchymal markers necessary for the process of EMT (de Iongh et al., 2005). EMT of the lens epithelial cells has been shown to be inducible by TGF-β, and can drive the down regulation of epithelial markers and upregulation of mesenchymal markers necessary for the process of EMT. TGF-β has also been observed to up regulate *αSMA*, a well-known marker of EMT (Mamuya and Duncan, 2012; Saika et al., 2004a). This TGF-β mediated EMT is controlled through the canonical SMAD signaling cascade, which has been one of the most intensely studied pathways (de Iongh et al., 2005; Zavadil and Bottinger, 2005). In the murine lens, TGF-β mediated EMT is morphologically and molecularly similar to anterior subcapsular cataracts (ASC) and posterior capsular opacification (PCO) in human lenses (de Iongh et al., 2005; Lovicu et al., 2004; Wormstone et al., 2009).

Anterior subcapsular cataract (ASC) is a disease characterized by formation of plaques of fibrotic cells underneath the anterior capsule, causing light to scatter through the lens. This can be caused by several conditions including trauma, inflammation and/or irritation of the eye and are often associated with altered levels of *TGF-β* expression or activation (de Iongh et al., 2005; Sasaki et al., 1998; Tang et al., 2003). The cells observed in ASC plaques show accumulations of collagenous ECM which is characteristic of myofibroblast cells (Macky et al., 2001; Novotny and Pau, 1984). Importantly, immunohistochemical studies of ASC samples have shown the presence of αSMA, vimentin, collagens (type I, III and IV), fibronectin, tenascin, laminin and various integrins (Colitz et al., 2000; de Iongh et al., 2005; Hatae et al., 1993; Joo et al., 1999; Marcantonio et al., 2003; Saika et al., 1998; Zhang et al., 2000).

Some features of TGF-β mediated EMT have also been identified in PCO, which is a major complication following cataract surgery. Cataract surgery involves the surgical removal of the bulk of the lens cells by phacoemulsification, and insertion of a prosthetic lens (or intraocular lens, IOL) into the remaining capsular bag (de Iongh et al., 2005). The remaining lens epithelial cells left on the anterior capsule after surgery proliferate and migrate to accumulate between the capsule and the IOL, where they become fibrotic plaques and cause clouding of the lens (Wormstone, 2002). Immunohistochemical studies of *in vivo* and *in vitro* models have shown that the fibrotic cells express αSMA, and aberrant ECM composed of proteoglycans, fibronectin, tenascin and collagens I and III (de Iongh et al., 2005; Ishibashi et al., 1994; Marcantonio et al., 2003; Saika et al., 1995; Wunderlich et al., 2000).

αSMA, a major protein of smooth muscle cells, is expressed during the development of skeletal muscle and cardiac cells. It has been suggested to drive wound contraction during healing (Hay, 2005), although downregulation of its expression did not influence matrix contraction in an *in vitro* model using lens epithelial cells (Wormstone and Wride, 2011). Vimentin, another characteristic marker of EMT, is an intermediate filament protein found in the cytosol (Capetanaki et al., 1989; Mueller et al., 2009). Vimentin is expressed in lens epithelial cells, early differentiating fibers, and its expression is up regulated during injury induced EMT (Capetanaki et al., 1989; Mendez et al., 2010; Zavadil and Bottinger, 2005). In EMT, vimentin is believed to play a role in the formation of the mesenchymal cell shape and motility (Mendez et al., 2010). Considering the embryonic β1MLR10 lens exhibited an increase in αSMA expression, an increase in vimentin would have been expected in a full EMT response. However, this was not observed in the β1MLR10 lens (Riggio, 2011).

Cadherins are transmembrane proteins involved in cell adhesion and signaling. They are known to play integral roles in development and are involved in the maintenance of epithelial cell integrity and morphogenesis (Harris and Tepass, 2010; Pontoriero et al., 2009; van Roy and Berx, 2008). Cadherins have also been found to play a role in development as cells migrate and re-organize, and their expression levels have been found to be altered during EMT (Pontoriero et al., 2009). E-cadherin and N-cadherin are the most studied cadherins in the process of EMT. E-cadherin, a cell-cell adhesion molecule, has been found to be down regulated as cells lose their contacts with the basement membrane during EMT (de Iongh et al., 2005; Hay, 2005). This same down regulation was observed in lenses lacking β1-integrin (Riggio, 2011). N-cadherin on the other hand, is expressed in lens epithelial cells, and its expression increases in differentiated lens fiber cells. Interestingly, this up regulation of N-cadherin was not observed in lenses lacking β1-integrin (Riggio, 2011). This suggests that overall in the embryonic β1MLR10 lens, there is only a partial EMT response being observed.

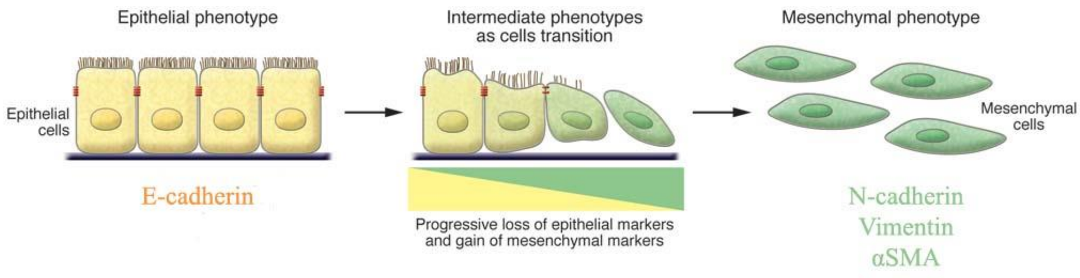

Figure 1.4 Epithelial to mesenchymal transition (EMT). The process of EMT is characterized by the loss of E-cadherin from epithelial cells causing a transition to a migratory mesenchymal phenotype with the upregulation of N-cadherin, Vimentin and *αSMA*. Adapted from Kalluri and Weinberg, (2009).

1.4 Integrins

Like all epithelial cells, the cells of the lens require connection to a basement membrane and this is mediated in part by integrins (Bassnett et al., 1999; Zampighi et al., 2000). Integrins are heterodimeric, transmembrane receptors (Barczyk et al., 2010; Longhurst and Jennings, 1998; Otoole et al., 1994) formed by non-covalent association of an α and a β subunit, for which there are 18 different α subunits and 8 β subunits, making a total of 24 different functional heterodimers (Figure 1.5) (Hynes, 2002; Streuli and Akhtar, 2009). As integrins interact with both the ECM and cytoskeleton, they mediate the transmission of forces from the ECM to the cytoskeleton, providing the cells with structural integrity. They also allow cells to change their behavior in response to changing ECM environments by activating signaling cascades upon ligand binding ("outside-in signaling"). Furthermore, cells have the ability to rapidly and transiently change their integrin function by altering the binding affinity of integrin for its ligand (“inside-out signaling”) (Hynes, 2002; Longhurst and Jennings, 1998; Otoole et al., 1994). This complex cross talk between integrins and their subsequent signal transduction cascades allows integrins to influence many different cellular processes such as survival, proliferation, migration and cellular identity (Barczyk et al., 2010; Longhurst and Jennings, 1998; Otoole et al., 1994).

Once integrins are activated, the subsequent activation of downstream integrin signaling cascades is cell dependent. However, there are many points of crosstalk between integrin and other cell signaling cascades, such as TGF-β and growth factors. This crosstalk can take on many different forms: from ligand binding, to cross talk with the integrin adhesome (Streuli and Akhtar, 2009). The integrin adhesome is comprised of a multi-protein complex that links the cytoplasmic tail of integrins with the actin cytoskeleton (Bhowmick et al., 2001; Kumar et al., 1995; Mitra et al., 2005; Schneiderbauer et al., 2004; Streuli and Akhtar, 2009; Zaidel-Bar et al., 2007). This connection to the actin cytoskeleton aids in the “outside-in signaling”, and “inside-out signaling” (Barczyk et al., 2010; Hynes, 2002).

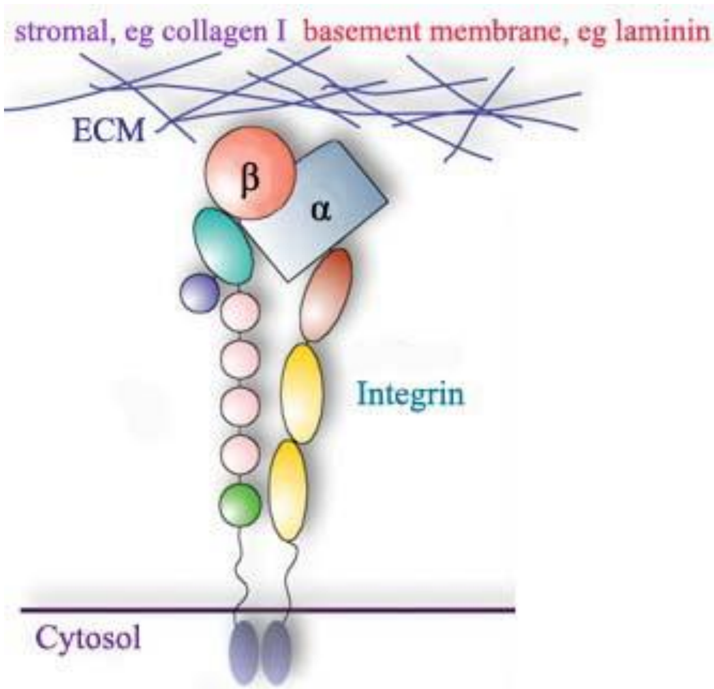

Figure 1.5 Integrin heterodimer structure. Integrins are heterodimers that are composed of an alpha and a beta subunit. There are 18 alpha, 8 beta that comprise a possible 24 functional heterodimers. On the outside of the cell, integrin's bind and interact with the ECM (extracellular matrix) proteins such as collagen I and laminin. Adapted from Streuli and Akhtar, (2009).

In order for integrins to be expressed at the cell surface, the expression of both the α and β subunits are required (Longhurst and Jennings, 1998). There are 18 different α subunits, α1 – 11, αE, D, L, M, v and αX, which are able to associate with 8 different β subunits (Barczyk et al., 2010). The α subunit is comprised of a short cytoplasmic tail, a transmembrane domain and a large extracellular domain, which is formed by the N-terminal region of the protein (Longhurst and Jennings, 1998). In the lens, all of the α subunits have been identified (Walker and Menko, 2009).

The β subunit is similar in shape to the α subunit, containing a large extracellular domain, a transmembrane domain and, a short cytoplasmic tail; with the exception of the β4 subunit which has an extended cytoplasmic domain (Longhurst and Jennings, 1998). As mentioned, there are 8 different β subunits, β1 through β8. In the lens β1, β3, β4, β5, β6 and β8 have been identified in the developing lens (Walker and Menko, 2009)(Fahmy Mamuya, unpublished). β1-integrin is the most common β subunit, and is expressed in both the lens epithelial and fibers cells (Figure 1.6) (Barczyk et al., 2010; Duncan et al., 2000; Humphries et al., 2005; Wederell et al., 2005).

In the lens, the β1-integrin subunit serves as the heterodimeric partner for many α subunits, which creates receptors for various ECM components such as laminins, collagens and fibronectin (Walker and Menko, 2009). The primary receptor of fibronectin is α5β1, which studies in the chick embryo lens showed the highest expression to be in epithelial cells in early lens development, just after lens vesicle closure (Menko and Philip, 1995; Walker and Menko, 2009). Recently, α5β1 has also been found to be important in zebrafish fiber cell morphogenesis in the lens (Hayes et al., 2012). Importantly, $\alpha5\beta1$and $\alpha v\beta3$ are considered to be RGD-binding integrins, which is a binding domain common in many matrix proteins such as fibronectin, vitronectin and tenascin (Hynes, 2002). Unlike $\alpha5\beta1$, however, $\alpha v\beta3$ is found in both the lens epithelial and fiber cells (Walker et al., 2002). In development, $\alpha v\beta3$ mostly binds to tenascin, which is a major component of the chicken lens capsule (Menko and Philip, 1995; Tucker et al., 1997). The receptors $\alpha1\beta1$ and $\alpha2\beta1$ are also expressed in the lens and mostly bind to collagen, specifically collagen I and collagen IV (Walker et al., 2002). In the developing chick lens, $\alpha1\beta1$ was detected only in the lens epithelium (Walker et al., 2002). Whereas in the developing mouse lens, $\alpha2\beta1$ was found to be expressed just prior to and during lens fiber cell differentiation (Wu and Santoro, 1994). Aside from fibronectin, vitronectin, and tenascin, laminin is another major component of the lens basement membrane capsule (Cammarata et al., 1986). The main laminin binding integrins include $\alpha3\beta1$, $\alpha6\beta1$, $\alpha6\beta4$ and $\alpha7\beta1$, and all but $\alpha7\beta1$ are expressed in the developing lens (Hynes, 2002; Walker and Menko, 2009). Without laminin in the lens capsule, the integrity of the capsule cannot be maintained, and without proper connections to laminin, lens development is abnormal (Lee and Gross, 2007; Walker and Menko, 2009). Therefore, it can be postulated that the integrins present in the developing lens could play important roles in maintaining connections, communication, and structural integrity within the lens.

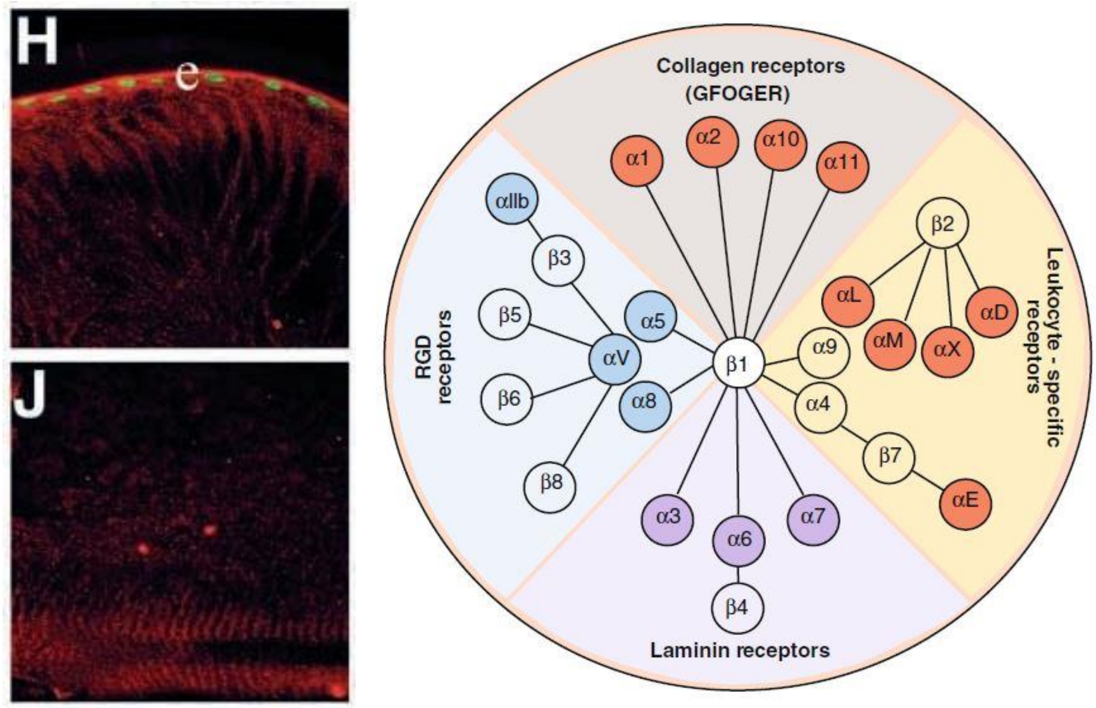

Figure 1.6 β1-integrin expression and integrin subunits. β1-integrin is found in both the epithelial (H) and fiber cells within the lens (J). In vertebrates, the integrin family consists of 18 α, 8 β subunits and 24 functional heterodimers. β1-integrin is used by the widest variety of α subunits and therefore is one of the most common subunits in functional integrin heterodimers. Adapted from Duncan et al., (2000) and Barczyk et al., (2010).

1.5 Integrin Adhesome

One of the most abundant adhesome molecules in the developing lens is FAK (Focal Adhesion Kinase) (Kokkinos et al., 2007). In almost all cells, the integrin cross talk requires the phosphorylation and activation of FAK (Focal Adhesion Kinase) by integrin and SRC (Rous sarcoma oncogene)(Hakuno et al., 2005). However, FAK can be activated by more than just integrins, it can also be activated by growth factors (Greenberg et al., 2006), and lead to signaling processes involving migration, proliferation and cell survival (Mitra et al., 2005; Schaller, 2001)(Figure 1.7).

Most often, the activation of FAK leads to the activation of downstream MAPK (mitogen activated kinase-like protein, map kinase) pathways (Barczyk et al., 2010; Longhurst and Jennings, 1998). The MAPK family of proteins is involved in cell differentiation, cell cycle progression, development and apoptosis and is comprised of three sequentially activated kinases. The activation of MAPKKK leads to the activation of MAPKK, which in turn phosphorylates and activates MAPK, also known as ERK1/2. ERK1/2 can be activated by a number of different cell signaling cascades, and is known to be important in cell proliferation, differentiation, survival and apoptosis (Roux and Blenis, 2004; Shaul and Seger, 2007; Yoon and Seger, 2006). Within the lens, cell proliferation has been found to be dependent on FGF induced ERK activation, and most importantly that FAK is necessary for ERK activation through FGF (Greenberg et al., 2006; Lovicu and McAvoy, 2001). Finally, TGF-β activation of FAK leads to ERK induced αSMA expression (Ding et al., 2008).

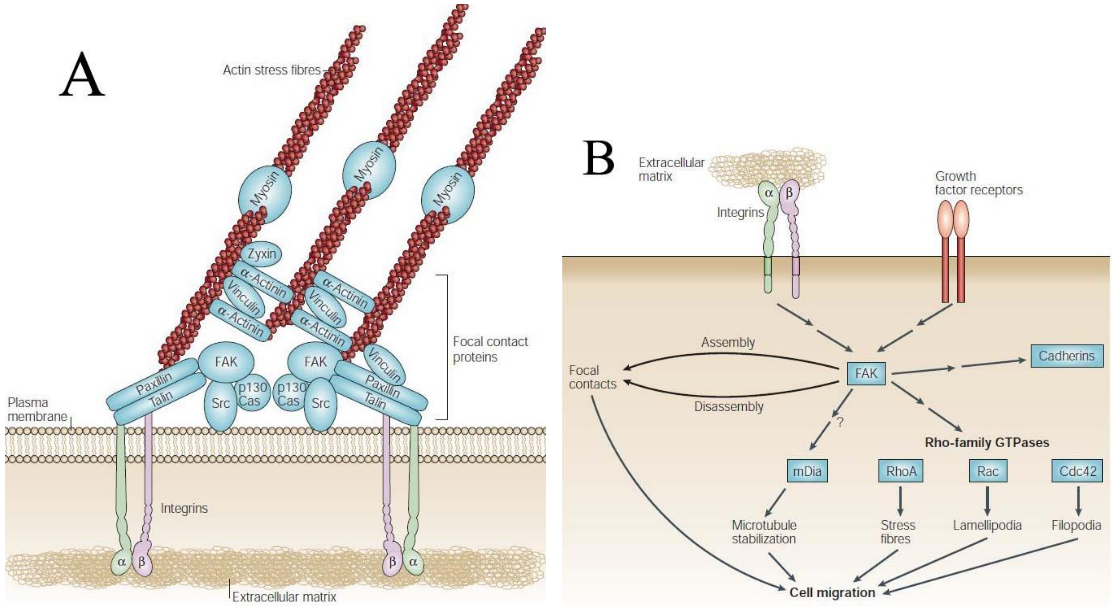

Figure 1.7 Integrin Adhesome and Signaling Pathway Crosstalk. The integrin adhesome (A) is a multi-protein complex that connects the cytoplasmic integrin tail with the cytoskeleton allowing for force transmission as well as “outside-in” and “inside-out” signaling cascades (Barczyk et al., 2010; Mitra et al., 2005). Focal adhesion kinase (FAK), an integral part of integrin/growth factor signaling cascade crosstalk (B), can activate a multitude of different pathways including the MAPK cascade. Adapted from Mitra et al., (2005)

1.6 β1-integrin: Required for Lens Epithelial Cell Maintenance and Survival

Homozygous loss of the β1-integrin subunit leads to lethality just after blastocyst implantation in mice due to its important role in gastrulation (Fassler and Meyer, 1995; Stephens et al., 1995). Therefore, the function of β1-integrin in the lens needed to be studied using a conditional knockout system, utilizing mice homozygous for a loxp flanked β1-integrin allele (Raghavan et al., 2000) and the MLR10 Cre transgene which is first active in the lens at the lens vesicle stage (E10.5) (Zhao et al., 2004). These mice lose β1-integrin subunit protein from the lens shortly after the elongation of the primary lens fiber cells at E12.5 (β1MLR10)(Simirskii et al., 2007).

Simirskii et al., 2007, demonstrated that β1-integrin is essential for maintenance of the lens epithelium. The conditional deletion of β1-integrin at E12.5 led to severe disorganization of the lens cells by E16.5; with the absence of the central epithelium, elongation of the epithelial cells at the transition zone, and highly vacuolated fiber cells. By birth, these mice lack externally visible eyes and the lens epithelial cells exhibit increased apoptosis (Figure 1.8) (Simirskii et al., 2007). The loss of β1-integrin also caused the dysregulation of lens epithelial and fiber cell marker expression in the lens epithelium at E16.5. In the β1-integrin lens, Pax6, a marker for epithelial cells and early differentiating fiber cells, was found to be drastically decreased in the lens epithelium at this time (Figure 1.9)(Simirskii et al., 2007).

Interestingly, β-crystallin, a marker of fiber cells, was found to be up regulated in the lens epithelium at E16.5 (Figure 1.9). c-Maf and Prox1, also markers of fiber cell differentiation and mature fiber cells, were also found to be up regulated in the lens epithelium at E16.5 (Kawauchi et al., 1999; Simirskii et al., 2007; Wigle et al., 1999). Overall this demonstrated that β1-integrin is not only necessary for the maintenance of the lens epithelium, but also preventing lens fiber cell differentiation to ensure lens epithelial cell survival, and lens survival in general (Simirskii et al., 2007).

Surprisingly, α smooth muscle actin (αSMA), a marker for epithelial to mesenchymal transition (EMT), was also found to be up regulated in the lens epithelial cells at E16.5 (Simirskii et al., 2007). This was rather surprising due to the fact that embryonic lens cells lack TGF-β receptors, which in the adult lens are known to initiate the signaling cascade that drives the up regulation of αSMA during EMT (De Iongh et al., 2001). In an effort to determine what other regulatory pathways may be up regulating αSMA, the canonical SMAD pathway was observed. However efforts to observe a dysregulation in the canonical SMAD pathway within the β1MLR10 lens failed to provide sufficient explanation for the observed partial up regulation of pro-fibrotic pathways (Riggio, 2011).

Although target pathway analysis failed to explain the partial up regulation of pro-fibrotic pathways, a direct downstream regulator of the β1-integrin pathway was found to be decreased. Focal adhesion kinase (FAK), an integral protein in the β1-integrin adhesome was significantly decreased at E16.5 (Riggio, 2011). Along with this, a partial EMT response was observed with the down regulation of E-cadherin and up regulation of αSMA in the β1MLR10 lens. Therefore, it seemed that an increase in ERK phosphorylation would be sufficient to explain the partial EMT observed with the loss of β1-integrin. However, a significant decrease in ERK phosphorylation and activation was not observed (Riggio, 2011). Therefore, the loss of FAK activation was found to be insufficient to explain the partial up regulation of fibrotic responses.

This led to the conclusion that a novel pathway or regulatory gene is functioning to up regulate multiple regulatory pathways in the β1MLR10 lens. To be able to investigate the cause of the up regulation of pro-fibrotic responses, I chose to take an unbiased method to determine differentially express genes within the β1-integrin conditional knock-out lens. Two possible methods were considered to determine differential gene expression, microarray and high-throughput – next generation sequencing (RNA-Seq). Specifically, RNA-Seq was used to determine altered gene expression in the β1MLR10 lens. **I will discuss in Chapter 3 how RNA-Seq was used to determine altered gene expression in the β1MLR10 lens.**

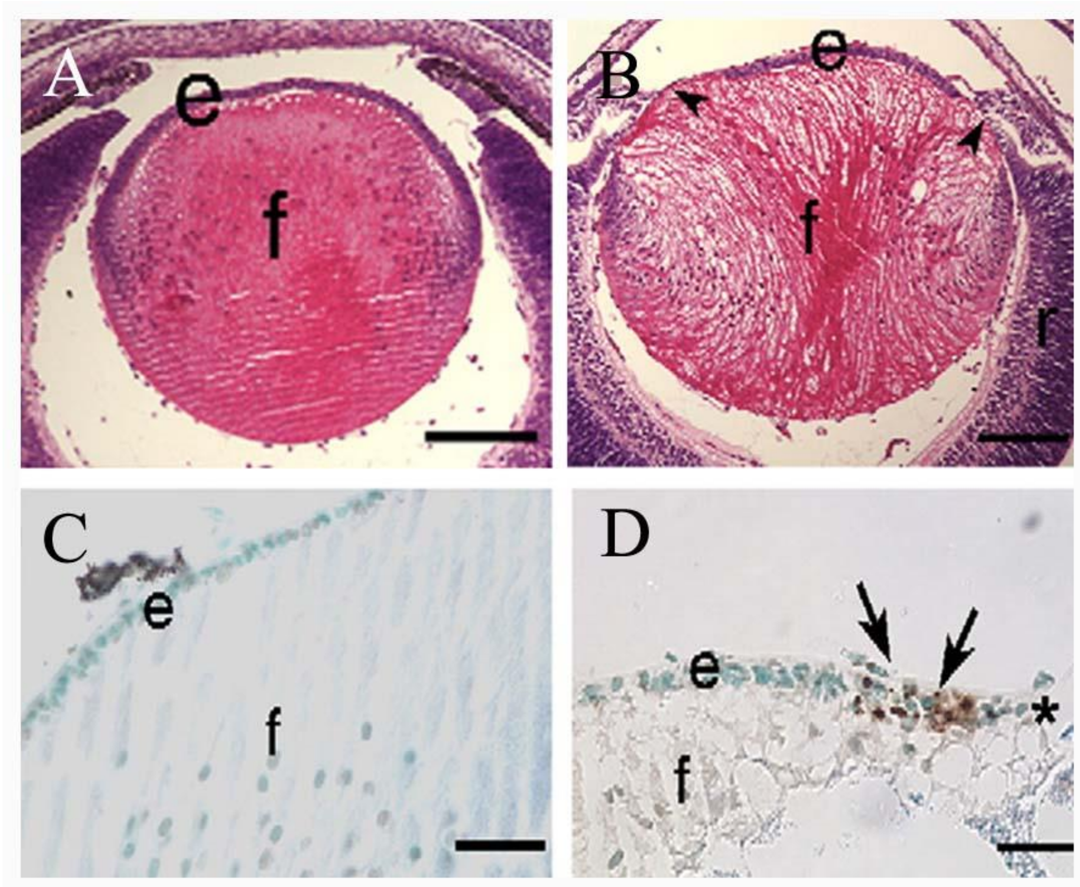

Figure 1.8 β1-integrin required for normal cell morphology and survival. The lens epithelial cells (e) are elongated in the transition zone and are absent in the central epithelial cells of the β1MLR10 lens (B), as compared to the wild type lens (A). The fiber cells (f) exhibit vacuolation. By birth, the lens epithelial cells show an increase in apoptosis as seen in the TUNEL staining of β1MLR10 lens (D) in comparison to the wild type (C) Adapted from Simirskii et al., (2007).

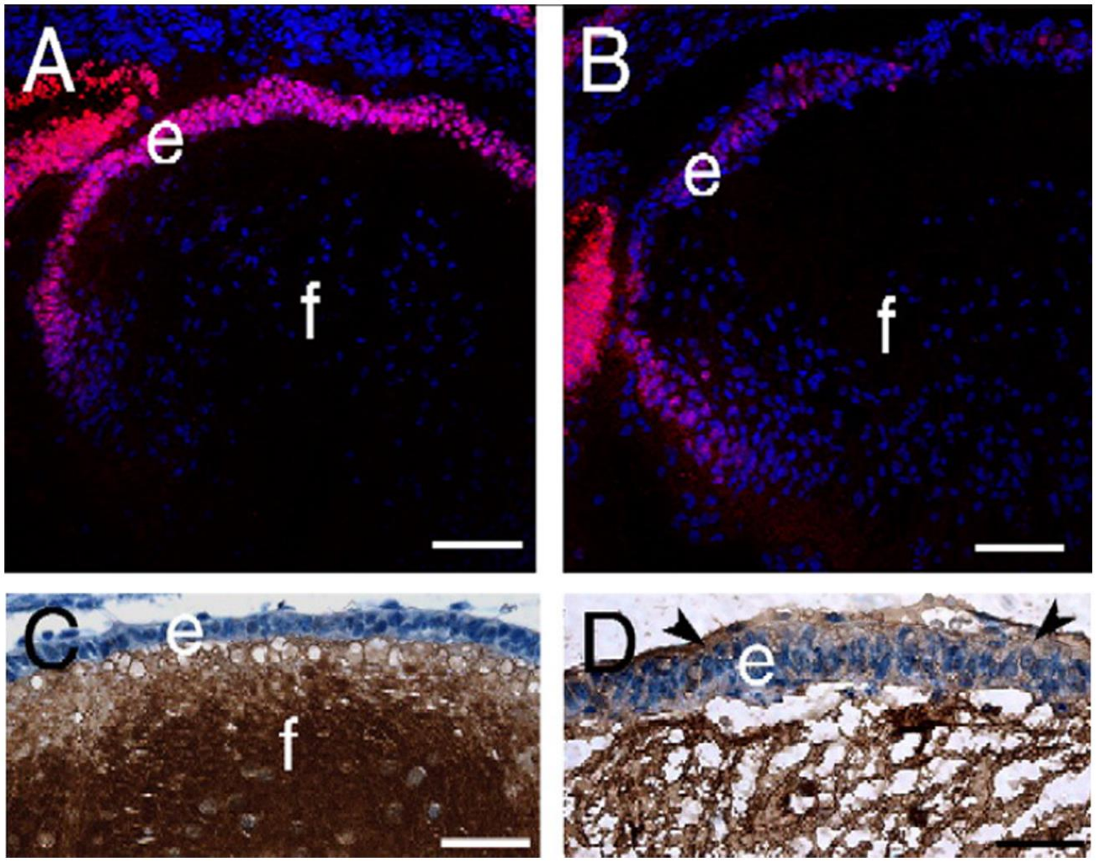

Figure 1.9 β1MLR10 lens epithelial gene dysregulation. Pax6 (red), a transcription factor necessary for normal lens epithelial cell maintenance (wild type, A) is down regulated in epithelium of the β1MLR10 lens (B) at E16.5. β-crystallin (brown), a transcription factor necessary for fiber cell differentiation, is up regulated in the epithelium of the β1MLR10 lens (D, arrows) as compared to the wild type (C) at E16.5 Adapted from Simirskii et al., (2007).

Chapter 2

METHODS AND MATERIALS

2.1 Animals

All animal experiments described here conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Institute for Laboratory Animals Research's Guide for the Care and Use of Laboratory Animals and were approved by the University of Delaware Institutional Animal Care and Use Committee. All mice used in this project were produced in the University of Delaware animal facility in specific pathogen free environment and maintained on a 14/10 light-dark cycle. Wild type mice were C57Bl/6<har> mice (Harlan Sprague Dawley, Indianapolis, Indiana). Conditional knock-out mice were generated by mating mice containing a β1-integrin allele modified to include Lox-P sites surrounding exon 3 (C57Bl/6: 129-*Itgb1*^{tm1Efu}*/J*), obtained from The Jackson Laboratory (Bar Harbor, Maine)(Raghavan et al., 2000). These mice were crossed to MLR10-Cre mice which express Cre-recombinase in all cells within the lens, beginning at the lens vesicle stage (Zhao et al., 2004), as described in (Simirskii et al., 2007), and were obtained from Michael Robinson (Miami University, Oxford, Ohio). Mice were originally genotyped as described in Simirskii et al., 2007; however, the colony is maintained with all mice homozygous for the β1-integrin floxed allele as well as the MLR10-Cre allele, which causes a total loss of β1-integrin in the lens. This line is thus homozygous for both alleles and breeds true in addition to showing an external phenotype, therefore genotyping was not routinely performed. Egr1 null mice, C57Bl/6N;129-*Egr1*^{tm1Jmi}*/J*, were obtained from The Jackson Laboratory (Bar Harbor, Maine). Female Egr1 null mice are sterile (Lee et al., 1996), and were therefore used for cataract surgery experiments. Male Egr1 null mice were maintained homozygous and bred to homozygous β1-integrin floxed mice carrying MLR10 cre. Intercrosses of these two strains generated various heterozygous or homozygous combinations of these alleles, and were used to determine if the loss of Egr1 could rescue the β1-integrin phenotype observed.

2.2 Cataract Surgeries

A modified murine "cataract surgery" or lens fiber cell extraction was performed on anesthetized control (C57Bl/6<har>) or Egr1 null mice as previously described (Desai et al., 2010). Dr. Yan Wang performed the surgery. The pupil was first dilated with Mydriacyl (1% tropicamide ophthalmic solution; Alcon Laboratories, Inc., Sinking Spring, PA) and AK-Dilate (2.5% phenylephrine hydrochloride ophthalmic solution; Akorn, Inc., Lake Forest, IL). After dilation, mice were anesthetized prior to surgery using 15 mg xylazine/75 mg ketamine per kilogram body weight. Balanced salt solution (BSS) was used to rinse the eye to be operated on. Only one eye was used for cataract surgery, the other eye was used as a 0 hour time point. A small scalpel was used to make a 2 – 3 mm incision in the central region of the cornea, going through the anterior lens capsule. BSS was injected into the lens to separate the fiber cell mass from the lens capsule and slight pressure was applied to both sides of the lens to expel the fiber cells, which was then removed with forceps. A single 10-0 nylon suture (Ethicon, Inc., Somerville, NJ) was then used to close the corneal incision. BSS was injected to inflate the anterior chamber to its original size. Finally, erythromycin ophthalmic ointment (E. Fougera & Co., Melville, NY) was applied topically to the eye to minimize infection. The mice were monitored until awakening. For analysis, mice were sacrificed at 0 hours, 3, 6, 12, 24, 48 and five days after surgery, at which time the operated lenses were removed for RNA isolation or embedded in OCT for immunofluorescence.

2.3 DNA Isolation for Mouse Genotyping

PCR amplification was performed on the Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). Mouse tail tissue for DNA isolation was taken according to IACUC regulations, approximately 1 cm of tail tissue was used. The Puregene® Genomic DNA Purification Kit (Gentra Systems, Minneapolis, Minnesota) was used for genomic DNA isolation. The mouse tail samples were placed on ice in microcentrofuge tubes after cutting and stored at -80°C for long term storage. For cell lysis, 600 µl Cell Lysis Solution and 5 µl of 10mg/mL Proteinase K Solution (Invitrogen, Grand Island, New York) were added and the samples were mixed by inverting 25 times. The samples were incubated overnight at 55 °C in a water bath. For protein precipitation, the samples were cooled to room temperature, and 200 µl of Protein Precipitation Solution was added to the cell lysate. The samples were vortexed at high speed for 10 – 20 seconds, and centrifuged at 13,000 – 16,000 x g for 6 minutes to form a precipitated protein pellet. If a pellet did not form, the samples were vortexed again followed by incubation on ice for 5 to 15 minutes and centrifuged again. After centrifugation, the supernatant was transferred to a new clean 1.5 mL centrifuge tube containing 600 µl of 100% isopropanol. The samples were mixed by inverting gently approximately 30 times, and centrifuged at 13,000 – 16,000 x g for 1 minute. The supernatant was then carefully poured off and the tube was drained on a clean absorbent paper. 600 µl of 70% ethanol was then added to the sample and inverted several times to wash the DNA pellet. The sample was centrifuged at 13,000 – 16,000 x g for 1 minute, and the ethanol was carefully poured off. The samples were drained on a clean absorbent paper and allowed to air dry for 15 minutes. Finally, the rehydrate the DNA, 80 – 100 µl of DNA Hydration Solution was added to the samples, and allowed to rehydrated overnight at room temperature, or heated at 65 °C for 1 hour. The samples were gently mixed occasionally to aid in dispersing the DNA. Once the DNA was isolated, the samples were quantitated with the NanoDrop® ND 1000 Spectrophotometer (NanoDrop, Wilmington, Delaware) and stored at 2 – 8 °C until needed.

2.4 Genotyping

Samples were prepared for PCR (polymerase chain reaction) by adding 10 µl of *Taq* Master Mix (Qiagen, Valencia, California), 7 µl Nuclease Free water, 1 µl each of the forward and reverse primers (Table 2.1), and 1 µl of genomic DNA to a 0.2 mL microcentrifuge tube. Samples were evenly mixed by vortexing and spun down. The samples were then placed into a Eppendorf Mastercycler Gradient PCR machine, and the appropriate cycling program was used (refer to Table 2.1). Once the PCR program was completed, 2 µl of 6X Loading Dye (Thermo Fisher Scientific, Waltham, Massachusetts) was added to each sample, and then loaded onto a 2% agarose gel with 2 µl/100 mL agarose of Ethidium Bromide (EtBr)(Thermo Fisher Scientific, Waltham, Massachusetts). The DNA samples were then size separated by electrophoresis, using approximately 150 volts for 30 – 45 minutes. A 100 bp DNA Ladder (Promega, Madison, Wisconsin) was used as a marker for DNA band sizes. Agarose gels were imaged on the Gel Logic 212 Pro Imaging System (Carestream Health, Inc., Rochester, New York).

Table 2.1 Primers used for PCR analysis of mice. β1-integrin and *Egr1* primers recommended by the Jackson Laboratory. MLR10 sequence obtained from Michael Robinson (Miami University of Ohio, Oxford, Ohio) (Zhao et al., 2004).

Allele	**Sequence**	
β1-integrin	5′-CGG CTC AAA GCA GAG TGT CAG TC-3′ 5′-CCA CAA CTT TCC CAG TTA GCT CTC-3'	
MLR10cre	5’-GCA TTC CAG CTG CTG ACG GT-3’ 5’-CAG CCC GGA CCG ACG ATG AAG-3’	
Egr1	5'-GGG CAC AGG GGA TGG GAA TG-3' 5'-AAC CGG CCC AGC AAG ACA CC-3' 5'-CTC GTG CTT TAC GGT ATC GC-3'	Common Wild type Mutant

2.5 Isolation of embryos

Mice were euthanized according to IACUC standards. For embryonic samples, female mice were mated and observed for a vaginal plug, which was considered day 0.5 post coitum (dpc). For specific embryonic time points, pregnant females were euthanized on the appropriate day after a vaginal plug was observed. Using forceps, the skin anterior to the urethra was drawn up to tighten the skin for cutting. Scissors were then used to cut from the urethra, cutting along the ventral midline until at the rib cage, opening the body cavity. Secondary incisions were then made perpendicular to the primary incision on either side of the lower abdomen to open the body cavity more. The uterus with embryos was then removed from the body cavity, and the embryos were removed from their embryonic sacs and placed in 1X PBS for further dissection. If the embryos were to be used for sectioning, the heads were removed and placed in a 1:1 solution of 1X PBS : Tissue-Tek® O.C.T. Compound (optimum cutting temperature)(Sakura® Finetek, Torrance, California) for approximately 1 – 2 minutes. The embryonic head was then embedded in a Tissue-Tek® Cryomold® (Sakura® Finetek, Torrence, California) filled with O.C.T. Compound and immediately placed at -80°C, or on dry ice, to freeze the samples. If the embryos were being used for embryonic lens extractions, then forceps were used to gently remove the eye under a dissection microscope. This was done by gently placing the forceps alongside the developing eye, moving the forceps slowly down towards the optic nerve. Once at the posterior of the eye, the forceps were closed around the optic and used to sever the connection and pull the eye out of the developing socket. The eyes were then placed in a shallow 60mm petri dish containing a small amount of 1X PBS. Once the eyes were collected in the petri dish, fine forceps were then used to further dissect the lenses. This was done by using fine forceps to open the eye through the opening in the posterior of the developing sclera left by the separation of the eye and the optic nerve. The sclera and retina were gently peeled apart, opening the eye to view the posterior chamber and lens. After opening the eye, fine forceps was used to hold the retina and sclera, while another was used to gently separate the lens and vitreous humor from the cornea. After detaching the lens, the retina, sclera and cornea could be discarded. Very gently and carefully, the remaining membranes and ciliary muscles were separated from the lens. Once the lens was entirely clean, it was gently lifted out of the media using both fine forceps. Finally, it was gently placed in a 1.5 mL microcentrifuge tube which was then immediately stored on dry ice to flash freeze the lens.

2.6 Immunofluorescence

Tissue from animal specimens was removed and immediately placed in Optimum Cutting Temperature Media (OCT, Tissue Tek, Torrence California), and placed on dry ice. For long term storage, the specimens were stored at -80°C. The samples were then sectioned 16μm thick using a Cryostat, mounted onto ColorFrost Plus slides (Thermo Fisher Scientific, Waltham, Massachusetts), dried, then stored at -80°C until used. Prior to staining, sections were fixed in fresh cold (4 degree) 3% paraformaldehyde (PFA) or 1:1 acetone/methanol for fifteen minutes, at room temperature or at -20℃ respectively. The slides were then washed in Coplin jars with wash solution (1 X Tris-buffered saline (TBS) and 0.1% Bovine Serum Albumin (BSA)(Sigma Aldrich, St. Louis, Missouri)) for five minutes at room temperature. The slides were transferred into Coplin jars containing blocking buffer (Table 2.2) for one hour at room temperature. One at a time, slides were removed from blocking buffer solution, excess solution was wiped off without letting samples dry and 100 μl of primary antibody (Table 2.2) diluted in blocking buffer was added to the samples. The slides were then incubated in slide incubation chambers at 4°C for one hour, unless otherwise noted. The slides were removed from the incubation chambers, excess antibody dilution was gently removed and the slides were washed in wash solution three times for ten minutes each at room temperature. Once washed, excess wash solution was removed from the slides and the specimens were covered with 100 μl diluted secondary antibody with Draq5 (1:2000)(Bio Status, Leicestershire, UK) in blocking buffer and incubated in slide incubation chambers for 1 hour at room temperature, making sure to keep the slides in the dark. The secondary antibody was then gently removed, and the slides were washed three times for ten minutes each in wash solution. The slides were removed one at a time, excess wash solution was removed and 1 drop of *p*-phenylenediamine antifade media (Johnson and Araujo, 1981) was added along with a cover slip (Thermo Fisher Scientific, Waltham, Massachusetts). The cover slips were then sealed with nail polish, and stored at 4°C until viewing on a Zeiss 780 Confocal Microscope (Carl Zeiss Inc., Göttingen, Germany). Comparisons of staining intensity was performed by staining wild type and β1MLR10 tissue under the same staining conditions and imaging under equal laser power. All staining experiments were performed on at least three independent biological replicates, unless otherwise specified.

Table 2.2 Antibodies used for immunofluorescence. Abbreviation, A:M – acetone:methanol.

Gene	Company	Product number	Fixation Used	Washes/ Solutions	Blocking	Secondary	Dilution
Egr1	R&D Systems	AF2818	3% PFA in 1xPBS	1xTBS	5% Donkey Serum 0.1% Triton 1% BSA	Donkey anti-goat	1:150
Fibronectin	Abcam	AB23750	A:M	1xPBS	1% BSA	anti-rabbit	1:100
Collagen I	Abcam	AB292	A:M	1xPBS	1% BSA	anti-rabbit	1:100
αSMA	Sigma	F3777	A:M or 4% PFA	TBS or PBS	BSA or Donkey Serum	FITC conjugated	1:250
DRAQ5	Biostatus Limited	DR50200	A:M or 4% PFA	TBS or PBS	BSA or Donkey Serum	---	1:2000

2.7 RNA Isolation

RNA for quantitative Reverse Transcription PCR (qRT-PCR) was isolated using the SV Total RNA Isolation System (Promega, Fitchburg, Wisconsin). Prior to starting the protocol, the lab bench, pipettes and pestles were cleaned with Radiacwash™ (Biodex, Shirley, New York), to prevent any foreign nucleic acid contamination. Frozen tissue was then submerged in 175 µl of Lysis Buffer and ground for approximately one minute in a 1.5 mL Eppendorf tube. 350 µl of SV RNA Dilution Buffer was then added to the samples, mixed by inversion, then incubated in a 70°C water bath for three minutes. The samples were then centrifuged for 15 minutes at 13,000 RCF to pellet protein. The supernatant was then transferred to a new 1.5 mL Eppendorf tube and 200 µl of 95% ethanol was added. The samples were then gently mixed, and up to 600 µl was added to the spin column. The samples were then centrifuged at 13,000 RCF for one minute and the flow through was discarded. If there was remaining sample with ethanol, this remaining sample was added and centrifuged. The column was then washed with 600 µl of SV RNA Wash Solution and centrifuged for one minute at 13,000 RCF, discarding the flow-through. To remove genomic DNA, the column was incubated for 15 minutes at room temperature with a DNase solution containing: 40 µl Yellow Core Buffer; 5 µl 0.09M $MnCl_2$; and 5 µl DNase I enzyme. 200 µl of SV DNase Stop Solution was then added to the column, centrifuged for one minute at 13,000 RCF, and the flow through was discarded. Finally, the RNA was eluted from the column with 30 - 70 µl of Nuclease-Free water. The isolated RNA was measured for concentration and quality on a NanoDrop ND 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts) and stored at -80°C until use. RNA concentrations of all samples listed in Table 2.3; number of lenses collected listed in Table 2.4.

Table 2.3 Total RNA yield and quality of lens samples collected for analysis. The RNA yield (ng), calculated based on concentration and amount of RNA originally obtained (70 µl – embryo, 30 µl – PCO), is listed first, the quality of the RNA is listed in parenthesis.

	Total RNA Yield (ng) and Quality (260/280, 260/230)					
	Wild type/Control			**Conditional Knock-out/Experimental**		
Sample time point	**1**	**2**	**3**	**1**	**2**	**3**
RNA-Seq, E15.5	17,255 (1.9, 2.01)	23,471 (1.81, 1.98)	14,007 (1.80, 2.05)	15,295 (1.83, 2.0)	9,275 (1.89, 2.02)	12,173 (1.9, 2.01)
E13.5	3,633	2,789	1,617	3,423	3,346	2,877
E14.5	2,457	3,318	2,401	11,872	4,501	10,325
E15.5	14,427	14,308	13,447	12,320	10,472	11,053
E16.5	23,422	12,341	7,441	32,935	18,564	8,764
PCO Model						
0 hour	600	270	381			
3 hour	399	1,317	255			
6 hour	327	453	630			
12 hour	405	210	372			
24 hour	510	174	207			
48 hour	195	423	660			

Table 2.4 Number of lenses collected for each biological replicate at each time point. These lenses were then used for extracting RNA. The number of wild type E15.5 lenses collected for RNA-Seq is higher than those isolated for the conditional knock out. This is because the wild type was collected first, and it was unclear how many would be needed for quality RNA.

	Number of Lens Samples for RNA extraction					
	Wild type/Control			**Conditional Knock-out/Experimental**		
Sample time point	**1**	**2**	**3**	**1**	**2**	**3**
RNA-Seq, E15.5	70	70	70	29	31	30
E13.5	32	30	30	31	30	29
E14.5	20	22	21	21	22	23
E15.5	22	21	24	20	23	21
E16.5	19	22	20	20	21	21
PCO Model						
0 hour	5	5	5			
3 hour	5	5	5			
6 hour	5	5	5			
12 hour	5	5	5			
24 hour	5	5	5			
48 hour	5	5	5			

2.8 Simultaneous RNA/Protein Purification

2.8.1 RNA Isolation

The SurePrep™ RNA/Protein Kit (BP2806-50)(Thermo Fisher Scientific, Waltham, Massachusetts) was used for extracting RNA and protein from some of the embryonic lens samples extracted by microdissection. The RNA concentration and quality was similar to that produced by the aforementioned SV Total RNA Isolation System (Promega, Fitchburg, Wisconsin). Prior to using the kit, the lab bench, pipettes and pestles were cleaned using Radiacwash™ (Biodex, Shirley, New York) and allowed to dry. It's important to note that no more than 25 mg of tissue can be processed. The centrifuge tube containing lens samples was very briefly centrifuged, then homogenized by adding 600 μl of Lysis Solution and vigorously grinding the sample with a clean pestle for approximately one minute on ice. The samples were then centrifuged at 12,000 RPM (14,000 x g) for two minutes to pellet cell debris. The supernatant was then transferred to another RNase-free microcentrifuge tube, noting the volume of the supernatant/lysate. 70% ethanol was then added to the lysate and the sample was mixed by vortexing for 10 seconds. A spin column was then assembled and 600 μl of the clarified lysate with the ethanol on to the column and centrifuged for one minute at 12,000 RPM, making sure the entire lysate volume passed through the column. The flow-through from this step was retained for the protein purification steps. If used the same day the protein lysate was stored on ice, or at -20 for longer storage. These steps were repeated if there was remaining lysate/ethanol. After the RNA was bound to the column, 400 μl of Nucleic Acid Wash Solution was added to the column and centrifuged for two minutes at 12,000 RPM (discarding the flow through). 100 µl of Enzyme Incubation buffer was added to the column (contains 25 units of RNase-free DNase I) and centrifuged for 30 seconds at 1,500 RPM. The whole column was then incubated for 15 minutes at room temperature, after which 400 µl of Nucleic Acid Wash Solution was added to the column and centrifuged for one minute at 12,000 RPM. This was repeated twice, discarding the flow-through each time. The column was then placed into a fresh microcentrifuge tube, 50 µl of Nucleic Acid Elution Buffer was added to the column and centrifuged for two minutes at 1,500 RPM followed by one minute at 12,000 RPM. The eluent was then measured for RNA concentration and quality on a NanoDrop ND 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts). The column was then retained for the protein purification steps.

2.8.2 Protein Purification

The column from the RNA Isolation steps was retained and assembled with a new collection tube. 500 µl of Protein Column Regeneration Buffer was added to the column and centrifuged at 12,000 RPM for two minutes and the flow-through was discarded. Next, 500 µl of Protein Column Activation and Wash Buffer was added to the column and centrifuged at 12,000 RPM for two minutes, again discarding the flow-through. While the column was being centrifuged, 100 µl of the flow-through from the RNA binding step was transferred to a separate microcentrifuge tube, the volume was then adjusted to 600 µl with molecular grade water. After this, 24 µl of Protein pH Binding Buffer was added to the lysate, mixed well, and the pH was measured to make sure that lysate pH was 3.5 or lower. The entire pH-adjusted protein sample was then applied to the column and centrifuged for two minutes at 8,000 RPM, discarding the flow-through. If the volume of the protein sample exceeded 650 µl, the sample was divided into two spins. 500 µl of Protein Activation and Wash Buffer was then added to the column and centrifuged for two minutes at 8,000 RPM, discarding the flow-through. 9.3 µl of Protein Neutralizer was then added to a microcentrifuge tube for protein collection, and the spin column was transferred to the tube. Finally 100 µl of Protein Elution Buffer was added to the column and centrifuged for two minutes at 8,000 RPM to elute the bound protein. The crude protein eluent was collected and stored at -20° for future use.

2.9 cDNA Synthesis

cDNA was synthesized using the RT^2 First Strand Kit (Qiagen, Valencia, CA). All reagents from this kit, and RNA samples, were briefly (10-15 seconds) centrifuged. A Genomic DNA Elimination Mixture was then prepared by adding 25.0 ng – 5.0 µg of Total RNA, 2.0 µl GE (5X g DNA Elimination Buffer), and nuclease-free water to a final volume of 10 µl. The samples were then mixed gently by pipette, briefly centrifuged, and incubated at 42°C for five minutes. After incubation, the samples were immediately chilled on ice for at least one minute. A RT Cocktail mixture was prepared containing (for one reaction): 4 µl BC3 (5X RT Buffer 3), 1 µl P2 (Primer and External Control Mix), 2 µl RE3 (RT Enzyme Mix 3), and 3 µl water. 10 µl of the RT Cocktail mixture was then added to each sample, gently mixed by pipette, and incubated at 42°C for exactly 15 minutes. The reaction was then immediately stopped by heating the samples at 95°C for five minutes. Finally, 30 – 70 µl of nuclease-free water was added to each sample, depending on the original concentration of the RNA samples. The samples were then analyzed for cDNA concentration and purity using a NanoDrop ND 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts), and stored at -20°C.

2.10 quantitative Reverse-Transcription PCR (qRT-PCR)

Please refer to Table 2 for a comprehensive list of primers used. Quantitative Reverse-Transcription PCR (qRT-PCR) was performed on the Applied Biosystems 7300 Real Time PCR system (Carlsbad, California). Each well contained: 1 μl cDNA (approximately 16 ng/μl final concentration), 12.5 μl of QuantiTect SYBR Green Master Mix (Qiagen, Valencia, California), 1 μl each of forward and reverse primers (Table 2.3) and ddH_2O to 25 μl. The following cycling conditions were used for all experiments: 40-45 cycles of 30 seconds at 95°C, 15 seconds at 58°C and 15 seconds at 68°C. The data which is returned is in the form of a C_T value (threshold value) for each well, which is the cycle number at which fluorescence reached a certain threshold, and depends on the amount of target template in the original sample. In order to determine a fold change for a certain gene, a ratio of expression between the mutant compared to the wild type samples must be calculated. The first step in calculating the relative expression was to calculate the average of the housekeeping gene for each biological replicate, then subtracting the average housekeeping gene C_T value from each experimental C_T value. The housekeeping gene used for all embryonic qRT-PCR samples was TBP (TATA box binding protein), for all post-surgery samples B2M (beta-2 microglobulin) was used. This first value is the ΔC_T value. The next step is to calculate the mean ΔC_T for the control sample, then subtracting the mean ΔC_T(control) from each experimental ΔC_T value, giving you the $\Delta\Delta C_T$. The fold change was then calculated by multiplying the $\Delta\Delta C_T$ by -1 and raising it to the 2nd power ($2^{\wedge -\Delta\Delta CT}$). These individual fold change values were then entered into an excel spread sheet provided by Dr. John McDonald (University of Delaware) to perform a two-level nested analysis of variance to determine the significance of the fold change. Finally, the average fold change value for each gene was determined by taking the average of all the individual fold change values.

Table 2.5 Primer sequences and citations used for qRT-PCR analysis.

Gene	Primer Sequence	Citation
Egr1	Fwd: 5' GCA AGT ACC CCA ACC GGC CC 3' Rev: 5' CGG CGA TCG CAG GAC TCG AC 3'	Designed by A. Terrell
Nab2	Fwd: 5'-GAGGAGGGGTTGCTGGACCG-3' Rev: 5'- GGCTGGAGGCAAAGTCCG-3'	Bhattacharyya et al., 2008
αSMA	Fwd: 5'-CCGAGATCTCACCGACTACCT-3' Rev: 5'-GCACAGCTTCTCCTTGATGTG-3'	Designed by M. Fisher
Mt1	Fwd: 5'-GCTGTCCTCTAAGCGTCACC-3' Rev: 5'-AGGAGCAGCAGCTCTTCTTG-3'	Pankhurst et al., 2012
Anxa2	Fwd: 5’-ACCAACTTCGATGCTGAGAG-3’ Rev: 5’-GCTCCTTTTTGGTCCTTCTC-3’	Huang et al., 2011
Plat	Fwd: 5'-TACAGAGCGACCTGCAGAGA-3' Rev: 5'-AATACAGGGCCTGCTGACAC-3'	Berk et al., 2001
Mmp14	SABioSciences, Catalog number: PPM03617D	
Thbs1	Fwd: 5’-CACCTCTCCGGGTTACTGAG-3’ Rev: 5’-GCAACAGGAACAGGACACCTA-3’	Hayashi et al., 2012
Stmn1	Fwd: 5'-GTTCGACATGGCATCTTCTGAT-3' Rev: 5'-CTCAAAAGCCTGGCCTGAA-3'	Martel et al., 2012
Rpl29	Fwd: 5′-TCCGATGACATCCGTGACTA-3′ Rev: 5′-TGCATCTTCTTCAGGCCTTT-3′	Fowler et al., 2011

2.11 RNA Sequencing

Total RNA was extracted from C57Bl/6<har> (75 lenses per biological replicate), β1-integrin cKO (30 lenses per replicate), and Sip1 floxed/floxed no cre mixed background “wild type” lenses (30 lenses per replicate) at E15.5 and RNA was isolated as described in **2.7 RNA Isolation.** The quantity and quality of the RNA was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California), using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, California). The RNA samples were then given to Sean Blake at Global Biologics (Columbia, Missouri) for library construction and cluster generation. Briefly, mRNA was purified from the total RNA samples using poly-T oligo attached magnetic beads. This was then converted to a library of template molecules for cluster generation and DNA sequencing according to the Illumina® TruSeq™ RNA Sample Preparation Kit v2.

Briefly, the cDNA library was made by using variable heat to denature (65°C), elute (80°C), and fragment the purified mRNA (1 μg total RNA) after being mixed with a solution of divalent cations. The RNA fragments were then copied into first strand cDNA synthesis using DNA polymerase I and RNaseH. cDNA overhangs which resulted from fragmentation are then converted to blunt ends and the 3’ ends are adenylated with a single nucleotide base to prevent the fragments from ligating to each other. This also provides a hybridization target for the adapters. Therefore, using PCR, the purified, ligated cDNA products are enriched to create the cDNA library. Bridge amplification was performed by binding each of the cDNA libraries at both ends to a TruSeq v3 flow cell, to form single strand bridges. These single strand bridges were then amplified by binding single molecules to each strand to form double-stranded bridges. Each bridge was then denatured to form two copies of covalently bound single stranded template. This amplification was then continued to create a “cluster” of identical copies of cDNA on the flow cell. Finally, the reverse strands were then cleaved and washed away, leaving only the forward strands on the flow cell.

These resulting cDNA library clusters were then sent to Brewster Kingham at the University of Delaware, Delaware Biotechnology Institute, Genotyping and Sequencing Center for sequencing on the Illumina® HiSeq 2000 (Illumina® Inc., Madison, Wisconsin). The sequencing kit used was the Sequencing By Synthesis (SBS) Sequencing kit, and sequenced with 50-cycle single-end (50bp) reads. Sequencing By Synthesis (SBS)(Figure 2.1), is an automated process that begins with the incorporation of all four dNTPs, each carrying a base-unique fluorescent dye, onto the flow cell; DNA polymerase than adds to the sequence. Following the base incorporation step, a laser excites the fluorophores, an image of each cluster is taken, and the first base is recorded. Next the chemically blocked 3’-OH group and dye are removed from each dNTP, and the cycle begins again. After each cycle is complete, an image of each cluster is used to determine the sequence using a base-calling algorithm. This converts the raw data into short read sequences which can then be aligned to a reference genome; for this project, *Mus musculus*. The Illumina Pipeline software (version RTA 1.13.48/CASAVA 1.8.2), was used to analyze the images, and translated to generate FASTQ sequence files.

Once sequenced, the RNA-Seq data was initially analyzed by Dr. Shawn Polson at the University of Delaware, Center for Bioinformatics and Computational Biology, and the transcript abundance was determined using the following methods. Overall quality of Illumina HiSeq datasets was assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and CLC Genomics

Workbench (CLC Bio, Aarhus, Denmark). Subsequent data processing was performed using the CLC Genomics Workbench and Genomics Server (version 5.1). Briefly, sequences were trimmed to remove common Illumina adapters and poly-A, and to remove low quality sequence ends (ambiguous base limit: 0; quality limit: 0.01). Following trimming, all sequences shorter than 35 bp were discarded. High quality sequences were aligned to *Mus musculus* reference (Build NCBI-M37.65 ENSEMBL/MGI annotations) using the CLC RNA-Seq reference mapping algorithm (length parameter: 0.9, identity: 0.8). Non-specifically mapping reads were not considered in downstream statistical analysis. FPKM/RPKM values were calculated to rank gene expression. Observed counts were quantile normalized (reviewed in Bolstad et al. 2003) before differential expression (DE) analysis using the beta binomial method of (Baggerly et al., 2003) with FDR (False Discovery Rate) correction for multiple comparisons (Benjamin and Hochberg, 1995) .

The wild type (C57Bl/6<har>) samples were then compared to the β1-integrin MLR10-Cre conditional knock out samples by using the following filtering parameters: Baggerly's p-value ≤ 0.05, weighted proportions fold change ≥ 2.5, RPKM ≥ 1, and mean RPKM difference ≥ 2. Only genes with mean RPKM differences greater than two was used, which corresponds approximately to one mRNA molecule per cell. This rough approximation is based on the estimate that the typical mammalian cell contains 500,000 molecules of mRNA per cell (Bryant and Manning, 1998). However, it is important to note that this is a rough approximation because cells can vary significantly in mRNA content depending on the cell type and other factors (Islam et al., 2011). The fold change threshold of 2.5 or more was chosen based on experimental data comparing relative gene expression changes between lenses of mixed background, Sip1 floxed/floxed no cre "wild type" control mice and C57Bl/6<har> mice, which is an inbred strain typically used for lens research (Manthey, 2013).

Briefly, what was found by Manthey's investigation was that 98.2% of the genes differentially expressed between inbred and random bred embryos exhibited less than a 2.5 fold difference in expression. Sixteen of the remaining 37 genes were either pseudogenes or unknown/predicted sequences (Manthey, 2013). Therefore, the 2.5 fold change was used as a threshold cutoff in the analysis of the β1-integrin cKO samples since any changes below this were likely due to genetic variation in mice with mixed backgrounds and not attributable to the β1-integrin gene deletion. All pseudogenes and unknown/predicted sequences were also removed for this same reason. Importantly, the 21 remaining genes that were altered between the Sip1 f/f no cre controls and the C57Bl/6<har> samples were also accounted for in the comparison to the β1-integrin cKOs. Therefore after filtering by the abovementioned parameters, if any genes remained that were of the 21 genes differentially expressed genes between wild type strains (Table 3.1), they were not considered as biologically relevant in this study.

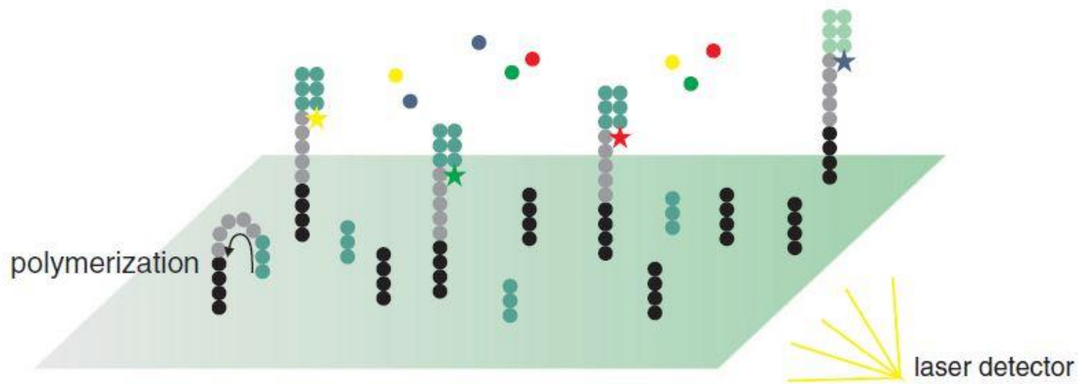

Figure 2.1 Sequencing by synthesis technology utilized by Illumina®. The green background indicates the flow cell used for sequencing. Green and black dots indicate primers utilized for sequencing cDNA fragments. The 'polymerization' depicts how the short fragments are amplified to produce large clusters of one fragment. The blue, dark green, yellow and red dots indicate dNTPs. The blue, dark green, yellow and red stars indicate the fluorescing nucleotides incorporated into the sequence, and detected by the laser. Adapted from Mutz et al., (2013).

Chapter 3

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN LENSES LACKING β1-INTEGRIN

3.1 Global Transcriptome Analysis: Microarray vs RNA-sequencing

Previously, a number of different regulatory pathways able to control the upregulation of αSMA were investigated in the β1MLR10 mice (Riggio, 2011; Simirskii et al., 2007). It was determined that the TGF-β pathway was unlikely to be mediating this phenomenon since the downstream effectors of TGF-β signaling were either unaffected or decreased. FAK activation was found to be nearly eliminated, however, the downstream consequences of this were not identified since pERK1/2 levels were unchanged. Overall, a number of different pathways were investigated, yet none seemed to sufficiently explain the αSMA upregulation, LEC apoptosis or other aspects of the β1MLR10 lens phenotype.

This led to the conclusion that a novel pathway or regulatory gene is functioning to up regulate multiple regulatory pathways in the β1MLR10 lens. To fully understand what was being dysregulated within the β1MLR10 phenotype, I chose to use an unbiased, global transcriptome analysis approach. To do so, I could either use microarrays, or RNA-Sequencing (RNA-Seq) to investigate gene changes. Although a number of researchers have used microarray to study gene changes in the past (Cheng et al., 2005; Clark et al., 2002; Yamada et al., 2003), I chose to use RNA-Seq.

Microarray is a hybridization-based approach which involves incubating fluorescently labeled cDNA with custom-made microarray probes. Specialized microarrays have even been designed, such as arrays with probes spanning exon junctions which can be used to detect and quantify distinct spliced isoforms (Clark et al., 2002). However, these methods have several limitations to them. The first and foremost limitation is that hybridization techniques rely on existing knowledge about genome sequence. They can also have high background levels and can lack specificity due to cross-hybridization (Okoniewski and Miller, 2006; Royce et al., 2007). Importantly, they also have a limited dynamic range of detection due to background and saturation of signals (Wang et al., 2009) and do not provide absolute gene expression levels.

RNA-Seq, on the other hand, is a high-throughput DNA sequencing method that has been used for both mapping (Vera et al., 2008) and quantifying transcriptomes (Wang et al., 2009). It can also be used to determine sequence variations in the transcribed regions of genes (Cloonan et al., 2008; Morin et al., 2008). Another large advantage to RNA-Seq is that it is has very low, if any, background signal. This is because DNA sequences can be unambiguously mapped to unique regions of the genome (Wang et al., 2009). Importantly, RNA-Seq does not have an upper limit for quantification, which correlates with the number of sequences obtained in a certain sample. This provides sufficient sensitivity to be able to quantify low level transcripts. RNA-Seq has also been found to be highly accurate for quantifying expression levels and correlates well with data obtained by quantitative PCR (qPCR) (Nagalakshmi et al., 2008). The results of RNA-Seq have also been found to be highly reproducible, for both technical and biological replicates (Cloonan et al., 2008; Nagalakshmi et al., 2008; Wang et al., 2009). Finally, because RNA-seq provides increased sensitivity, it also allows us to sequence past the bias towards structural proteins in the lens, such as crystallins; allowing us to focus on genes that may be proximal to the cause of the phenotype.

3.2 Global analysis shows a subset of genes that may be responsible for the partial fibrotic β1-integrin phenotype

Since the investigation of candidate genes possibly involved in the β1MLR10 phenotype did not explain the partial fibrotic phenotype observed, I used RNA-Sequencing to compare the transcriptomes of wild type and β1MLR10 lenses. Therefore, sequencing was performed on β1MLR10 and C57Bl/6 wild type lenses at E15.5, just prior to the onset of the morphological phenotype, in an attempt to obtain a subset of genes that are causative, not secondary to the phenotype. What was initially gained from sequencing these lenses at E15.5, was that the mouse lens can be sequenced to a staggering depth of 30 – 40 million transcripts. Overall, greater than 7,000 genes are expressed in the wild type lens. However, the question remained, what are the biologically relevant gene changes within this comparison?

To determine filtering parameters to find biologically relevant gene changes in the β1MLR10 lens, a comparison between wild type lens samples was performed (Manthey, 2013). In this comparison C57Bl/6 lenses, an inbred mouse strain, were compared to a wild type transgenic strain with a mixed background. This analysis showed that the wild type E15.5 lens expresses greater than 7,700 genes at levels over 2 RPKM (Manthey et al., submitted to GEO)(Manthey, 2013) which corresponds approximately to one mRNA molecule per cell. This rough approximation is based on the estimate that the typical mammalian cell contains 500,000 molecules of mRNA per cell (Bryant and Manning, 1998). However, it is important to note that this is a rough approximation because cells can vary significantly in mRNA content depending on the cell type and other factors (Islam et al., 2011). Therefore, only genes with mean RPKM differences greater than two were considered. This analysis also showed that only 2% of the genes differentially expressed between inbred and random bred E15.5 lenses were above a 2.5 fold change (Table 3.1, listed in appendix)(Manthey et al., submitted to GEO)(Manthey, 2013). Therefore, a cut off of 2.5 fold was used to find gene changes that were relevant to the deletion of β1-integrin from the lens.

Importantly, out of the 37 remaining genes out of 90, 16 were either pseudogenes, unknown genes, or predicted genes. For instance, the CP49 gene, which produces one of the beaded filament proteins comprising the lens intermediate filament cytoskeleton, has been linked to familial congenital cataracts in humans. Some inbred mouse strains including FVB/N and 129 (Sandilands et al., 2004; Simirskii et al., 2006) which provide part of the genetic contribution to the random bred strain used in this experiment (Manthey, 2013), also harbor null mutations in this locus, is expressed at 1.2 fold higher levels in C57Bl/6 lenses than the random bred ones. Therefore, any genes expressed greater than 2.5 fold in the strain comparison, or gene changes found to be strain dependent, were not considered biologically relevant to the deletion of β1-integrin in the lens, and were excluded from analysis.

Using these parameters, it was found that only 90 genes were changed more than 2.5 fold, and expressed at above 2 RPKM in the β1MLR10 lens (Table 3.2). Forty nine of these genes were up-regulated and 41 were found to be down-regulated.

Further analysis using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Inc., www.ingenuity.com) showed that a subset of these genes are involved in multiple cellular processes (Figure 3.2). Although useful in determining possible subsets of genes, these subsets were not specific enough to allow us to understand their role in the observed lens phenotype. Notably, manual searches found some of the differentially expressed genes to have been linked directly or indirectly to β1-integrin in the literature while IPA failed to do so, suggesting that the IPA database is incomplete. Through additional manual literature searches, I was able to find one subset of genes that may play a role in the partial fibrotic response observed in the β1MLR10 lens. This subset of genes has the potential to be up-regulated by early response genes, specifically Egr1 (early growth response 1), a Cys2-His2-type zinc-finger transcription factor, which is up-regulated more than 10 fold in the β1MLR10 lens, **discussed further in Chapter 4**.

A subset of ten genes that may play a role in the partial fibrotic response were validated using qRT-PCR (Table 3.3) and include *Egr1*, *Nab2*, *αSMA*, *Mmp14*, *Anxa2*, *Plat*, *Mt1*, *Thbs1*, *Stmn1* and *Rpl29*. This was using three biological replicates that were independently collected from the E15.5 β1MLR10 samples used for RNA-Seq. *Egr1* was found to be up-regulated more than 12 fold at E15.5 via qRT-PCR. Interestingly, Ngfi-A binding protein 2 (*Nab2*), which is activated by and involved in a tightly controlled negative feedback loop with EGR1(Pagel and Deindl, 2011), was up-regulated more than 200 fold via qRT-PCR, whereas, with RNA-Sequencing, it was only up-regulated 14 fold. *Mt1*, was another gene found to have a different fold change. RNA-Sequencing found this gene to be up-regulated close to 300 fold in the β1-cKO, however, using qRT-PCR this gene was only 2 fold up-regulated. Three other genes, *Stmn1*, *Thbs1*, and *Rpl29* were shown to have similar fold changes in both qRT-PCR and RNA-Seq analysis. However, the variability in the biological samples was too great, or the expression levels were too low to be properly accessed using qRT-PCR analysis. Although there were some differences between the RNA-Seq and qRT-PCR gene analysis, these differences could be due to the difference in sensitivity of the analysis systems, as well as the variability in phenotypic progression/timing of the β1MLR10 phenotype.

3.3 Discussion

Through RNA-Sequencing analysis, I was able to show that a small subset of genes in the E15.5 β1MLR10 lens are misregulated at the mRNA level. This misregulation was determined by comparing wild type (C57Bl/6<har>) samples to the β1-integrin MLR10-Cre conditional knock out samples, and determining biologically relevant changes in gene expression by using critical filtering parameters. First, only genes with a *p*-value less than 0.05, for a 95% confidence interval, were used. More importantly, only genes with a 2.5 fold change in normalized RPKM value were reported. This fold change threshold was chosen based on experimental data, analyzed by Abby Manthey, comparing relative gene expression between lenses from mixed background Sip1 floxed/floxed no Cre "wild type" control mice and C57Bl/6<har> mice, an inbred strain typically used for research (Manthey, 2013). This analysis showed that greater than 2,000 genes were differentially expressed between the two strains. Of these, only 2% were found to be greater than 2.5 fold different in expression. Therefore, this 2.5 fold change was used as a threshold cutoff in the analysis of the β1-integrin cKO samples to be able to determine biologically relevant gene changes. Any gene changes below this 2.5 fold change were likely to be due to genetic variation in mice with mixed backgrounds and not the β1-integrin deletion.

Another important parameter with which this data was filtered was to exclude any genes with mean RPKM values less than two for both the wild type and β1-integrin cKO. A RPKM of two relates approximately to one mRNA molecule per cell. This rough approximation is based on the estimate that the typical mammalian cell contains 500,000 molecules of mRNA per cell (Bryant and Manning, 1998). However, it is important to note that this is a rough approximation because cells can vary significantly in mRNA content depending on the cell type and other factors (Islam et al., 2011). Also, wild type E15.5 lens expresses greater than 7,700 genes at levels over 2 RPKM (Manthey et al., submitted to GEO)(Manthey, 2013). Therefore, only genes with mean RPKM differences greater than two were considered, which corresponds approximately to one mRNA molecule per cell. This rough approximation is based on the estimate that the typical mammalian cell contains 500,000 molecules of mRNA per cell (Bryant and Manning, 1998). Also, only genes with a mean RPKM difference greater than two were considered to account for the detectable level of expression in the tissue.

The gene expression changes hypothesized to be biologically relevant using these parameters, included many that have the potential to regulate the pro-fibrotic response observed in the β1MLR10 lens. I was able to validate seven of the pro-fibrotic subset of genes at the mRNA level using via qRT-PCR, that were found to be differentially expressed in the E15.5 β1MLR10 lens. This was using three biological replicates that were independently collected from the E15.5 β1MLR10 samples used for RNA-Seq. Three others genes that I investigated were found to have a similar fold change differences in both qRT-PCR and RNA-Seq, however, the variability between β1MLR10 biological replicate lens samples was too great for there to be a significant *p*-value. In the future, if the number of biological replicates is increased from three to six, this may help to decrease the variability initially observed between the samples.

Notably we found that one of the differentially expressed genes, early growth response 1 (*Egr1*), is a transcription factor with an established role in fibrotic gene expression (Bhattacharyya et al., 2013; Pagel and Deindl, 2011). Egr1 may also be able to regulate many of the differentially regulated genes in the β1MLR10 lens considering a large subset of these genes have predicted Egr1 binding sites (EBS). The potential role of Egr1 in regulating the β1MLR10 phenotype will be further discussed in Chapter 4.

Table 3.1 Truncated table of differentially expressed genes in comparison between Sip1 floxed/floxed no-Cre (AG) and C57Bl/6 wild type strains. This comparison was done comparing Sip1 WT to C57 WT (Sip1WT expression/C57 WT expression), meaning that *Snhg9* is expressed higher in the C57Bl/6 wild type. Only genes changed more than 2 fold shown. The full list of genes is listed in Appendix 1.

Feature ID	Fold Change	FDR p-value correction	WT RPKM Mean	AG RPKM Mean
Snhg9	6.560	0.00002522	1.141	7.789
Ccbe1	5.015	0	0.564	2.977
Eif3j	4.700	0	6.090	19.638
Adamts4	3.686	0.000005195	1.019	3.935
Gm7120	3.640	0	1.823	6.596
Lars2	2.896	9.463E-11	150.924	467.785
Mid1	2.711	0.001724	6.708	18.485
Tdg	2.567	7.948E-14	4.191	16.113
Plxna1	2.419	0.015	7.150	12.987
Pcp4l1	2.315	5.572E-11	2.089	4.963
Tmcc3	2.248	2.329E-08	7.486	9.652
Rab6b	2.119	2.072E-08	2.115	4.747
Zfp365	2.109	0	18.844	41.634
Hipk2	2.098	0.014	6.953	15.085
Rpl3	2.060	0	346.625	605.698
Rpl13	2.042	0.007218	4.342	7.927
Fam103a1	-2.008	3.283E-07	20.573	14.151
Lctl	-2.013	5.959E-09	31.906	16.270
6330403K07Rik	-2.037	2.521E-18	9.950	5.110
Rpa3	-2.038	8.078E-09	8.666	4.680
Gng11	-2.040	1.216E-10	6.281	3.142
Ccdc23	-2.055	1.154E-08	76.365	38.748
Gm7589	-2.067	0.075	16.581	8.972
Emp1	-2.071	8.437E-32	4.456	2.233
Gpx8	-2.082	0.0001141	20.352	9.765
C030030A07Rik	-2.101	5.278E-11	18.057	10.079
Eif2s3y	-2.136	0.0003373	10.132	5.018
Trim13	-2.179	2.415E-29	5.796	3.207
Gm10925	-2.182	1.556E-07	2325.578	1510.424
Rpl39	-2.199	2.783E-09	489.796	328.763
Ceacam10	-2.293	8.356E-07	15.385	7.271
Fam36a	-2.305	0.000001374	45.697	25.535

(Table 3.1, continued)

Phpt1	-2.369	1.291E-11	63.829	46.572
E330034G19Rik	-2.372	1.229E-24	7.095	3.098
Ddx3y	-2.401	1.589E-07	5.633	2.492
Rpl19	-2.432	0.000836	338.370	297.067
Psmc3ip	-2.444	8.233E-13	7.343	3.159
Lmo1	-2.458	1.421E-19	4.651	1.895
Rpl5	-2.581	3.612E-29	228.599	176.678
Cd59a	-2.652	6.013E-14	5.573	2.087
Avp	-2.660	1.556E-08	4.937	1.714
Gm5160	-2.735	0.205	24.926	16.120
Lsm7	-2.739	8.54E-10	70.554	62.081
Gm9234	-2.755	0.284	30.351	19.132
Ccdc117	-2.820	3.51E-19	26.309	9.682
Gm11808	-2.865	0.045	76.110	44.914
Rpl27a-ps1	-2.966	0.072	4.395	1.134
2010002N04Rik	-3.000	7.822E-132	14.428	5.031
Rpl22l1	-3.003	6.19E-14	183.844	71.560
Nme2 (11 93811127..93817573)	-3.268	2.277E-10	174.432	132.824
mt-Atp8	-3.280	7.688E-09	2343.429	1146.269
Ube2l3	-4.167	0.283	12.281	6.733
Scg5	-4.366	3.079E-40	2.714	0.611
Gchfr	-4.381	0.0002886	2.963	0.679
Zfp580	-5.200	0.00222	3.308	1.066
Xlr3b	-5.222	3.623E-12	2.595	0.487
Gm17301	-8.765	0.012	2.621	0.524
4930412F15Rik	-8.857	1.911E-08	24.182	6.202
Rps6-ps2	-19.250	0.058	5.350	3.193
Pcna-ps2	-37.524	0.00001511	3.927	0.249
Gm9781	-167.019	1.06E-109	47.860	1.135
Gm5806	-202.474	1.708E-34	7.665	0.194

Table 3.2 RNA-Seq analysis identified 90 differentially expressed genes in the β1-integrin cKO at E15.5. A value greater than one was used as a cutoff for the un-normalized WT RPKM and β1cKO RPKM means, a value of two was used as a cutoff for the difference between these means. After normalization, the fold change was calculated and reported here; a change greater than 2.5 was used as a cutoff for fold change. In addition, a p-value less than 0.05 was used as a cutoff for significance; the false discovery rate (FDR) p-value correction is listed here.

Gene ID	Gene Name	Fold change	P-value	FDR p-value correction	WT RPKM Means	β1cKO RPKM Means	Mean RPKM Difference
Mt1	metallothionein 1	**279.96**	1.09E-115	2.06E-112	0.86	245.48	244.62
Klk9	kallikrein related-peptidase 9	**183.18**	6.32E-03	6.90E-01	0.01	2.55	2.54
Fgf12	fibroblast growth factor 12	**21.91**	2.02E-09	1.12E-06	0.62	13.66	13.04
Nmb	neuromedin B	**19.74**	3.35E-10	1.97E-07	1.99	39.71	37.72
Hist1h2al	histone cluster 1, H2al	**17.47**	2.77E-08	1.34E-05	0.71	12.4	11.69
Amd2	S-adenosylmethionine decarboxylase 2	**17.14**	1.00E-02	9.90E-01	0.15	2.61	2.46
Nab2	Ngfi-A binding protein 2	**14.25**	7.09E-06	2.02E-03	0.58	8.32	7.74
Egr1	early growth response 1	**10.77**	6.84E-09	3.68E-06	1.72	18.76	17.04
Tagln3	transgelin 3	**10.55**	4.66E-04	8.00E-02	0.51	5.37	4.86
Bend6	BEN domain containing 6	**9.01**	2.81E-03	3.70E-01	0.46	4.17	3.71
Scfd2	Sec1 family domain containing 2	**8.62**	4.79E-06	1.44E-03	1.14	9.94	8.8
Tagln	transgelin	**8.31**	5.75E-05	1.00E-02	0.93	7.83	6.9
Rprm	reprimo, TP53 dependent G2 arrest mediator candidate	**7.1**	8.13E-03	8.50E-01	0.51	3.63	3.12
Atp10d	ATPase, class V, type 10D	**6.33**	1.00E-02	1.00E+00	0.5	3.24	2.74
Acta2	actin, alpha 2, smooth muscle, aorta	**6.07**	3.88E-07	1.40E-04	3.45	21.21	17.76
Zfp949	zinc finger protein 949	**5.46**	1.00E-02	1.00E+00	0.66	3.61	2.95
Tnfrsf12a	tumor necrosis factor receptor superfamily, member 12a	**5.27**	9.95E-03	9.70E-01	0.76	4	3.24
Hebp1	heme binding protein 1	**4.89**	5.46E-07	1.94E-04	3.23	15.94	12.71
Fos	FBJ osteosarcoma oncogene	**4.81**	5.00E-02	1.00E+00	0.53	2.56	2.03
Itgb8	integrin beta 8	**4.78**	1.00E-02	1.00E+00	0.84	4.08	3.24

(Table 3.2, continued)

Hmga1-rs1 (11 1206241 07..1206 25733)	high mobility group AT-hook I, related sequence 1	**4.27**	5.00E-02	1.00E+00	0.61	2.62	2.01
Chac1	ChaC, cation transport regulator-like 1 (E. coli)	**4.22**	3.00E-02	1.00E+00	0.82	3.53	2.71
Crip1	cysteine-rich protein 1 (intestinal)	**4.06**	2.96E-08	1.39E-05	5.53	22.67	17.14
Anxa2	annexin A2	**3.86**	1.37E-06	4.44E-04	4.58	17.86	13.28
Ahnak	AHNAK nucleoprotein (desmoyokin)	**3.72**	4.00E-02	1.00E+00	0.87	3.28	2.41
Zfp580	zinc finger protein 580	**3.55**	3.00E-02	1.00E+00	1.07	3.78	2.71
Fas	Fas (TNF receptor superfamily member 6)	**3.45**	4.00E-02	1.00E+00	1.05	3.64	2.59
Ppp3r1	protein phosphatase 3, regulatory subunit B, alpha isoform (calcineurin B, type I)	**3.44**	4.00E-02	1.00E+00	1.03	3.58	2.55
Plat	plasminogen activator, tissue	**3.33**	6.40E-03	7.00E-01	1.96	6.6	4.64
Nes	nestin	**3.12**	3.33E-11	2.06E-08	13.22	41.5	28.28
Chn2	chimerin (chimaerin) 2	**3.1**	7.49E-03	8.00E-01	2.19	6.87	4.68
Hddc3	HD domain containing 3	**3.09**	1.63E-03	2.40E-01	3.07	9.52	6.45
Ptrf	polymerase I and transcript release factor	**3.03**	6.09E-03	6.70E-01	2.43	7.41	4.98
Mmp14	matrix metallopeptidase 14 (membrane-inserted)	**3.01**	2.00E-02	1.00E+00	1.91	5.8	3.89
Tuft1	tuftelin 1	**2.98**	2.00E-02	1.00E+00	1.76	5.29	3.53
Arhgap4	Rho GTPase activating protein 4	**2.98**	5.00E-02	1.00E+00	1.32	3.96	2.64
Rcan2	regulator of calcineurin 2	**2.94**	3.09E-07	1.14E-04	9.06	26.93	17.87
Glipr2	GLI pathogenesis-related 2	**2.93**	2.00E-02	1.00E+00	1.98	5.85	3.87
Sox4 (13 2904078 7..29045 582)	SRY-box containing gene 4	**2.88**	6.27E-04	1.10E-01	4.22	12.26	8.04
Emilin1	elastin microfibril interfacer 1	**2.87**	4.00E-02	1.00E+00	1.6	4.63	3.03
Gpx8	glutathione peroxidase 8 (putative)	**2.86**	2.67E-07	1.00E-04	9.77	28.2	18.43
Igkc	immunoglobulin kappa constant	**2.83**	5.00E-02	1.00E+00	1.43	4.05	2.62
Phlda3	pleckstrin homology-like domain, family A, member 3	**2.77**	5.00E-02	1.00E+00	1.55	4.33	2.78
Emp1	epithelial membrane protein 1	**2.71**	2.00E-02	1.00E+00	2.23	6.1	3.87
Lgals3	lectin, galactose binding, soluble 3	**2.64**	5.00E-02	1.00E+00	1.66	4.42	2.76

(Table 3.2, continued)

Gadd45gip1	growth arrest and DNA-damage-inducible, gamma interacting protein 1	**2.63**	2.00E-02	1.00E+00	6.74	2.59	4.15
Cd59a	CD59a antigen	**2.56**	4.00E-02	1.00E+00	2.09	5.38	3.29
Nek3	NIMA (never in mitosis gene a)-related expressed kinase 3	**2.52**	5.00E-02	1.00E+00	2	5.06	3.06
Myl9	myosin, light polypeptide 9, regulatory	**2.52**	7.47E-05	2.00E-02	7.95	20.18	12.23
R3hdml	R3H domain containing-like	**-2.51**	1.47E-13	1.21E-10	70.26	28.19	42.07
Pcp4l1	Purkinje cell protein 4-like 1	**-2.51**	5.00E-02	1.00E+00	4.96	2.01	2.95
Hdac1	histone deacetylase 1	**-2.52**	1.51E-06	4.83E-04	29.4	11.73	17.67
Naa10	N(alpha)-acetyltransferase 10, NatA catalytic subunitNalpha acetyltransferase 10	**-2.52**	4.00E-02	1.00E+00	5.15	2.08	3.07
Slc7a11	solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	**-2.57**	3.17E-03	4.00E-01	10.7	4.2	6.5
Alox12	arachidonate 12-lipoxygenase	**-2.6**	6.49E-04	1.10E-01	14.08	5.45	8.63
Hjurp	Holliday junction recognition protein	**-2.64**	2.00E-02	1.00E+00	6.03	2.29	3.74
Rnf26	ring finger protein 26	**-2.65**	1.00E-02	1.00E+00	7.5	2.86	4.64
Parm1	prostate androgen-regulated mucin-like protein 1	**-2.65**	5.00E-02	1.00E+00	4.69	1.79	2.9
Snhg9	small nucleolar RNA host gene (non-protein coding) 9	**-2.68**	1.00E-02	1.00E+00	7.79	2.92	4.87
Thbs1	thrombospondin 1	**-2.74**	6.21E-04	1.10E-01	13.06	4.81	8.25
Dctd	dCMP deaminase	**-2.81**	1.00E-02	1.00E+00	6.46	2.3	4.16
Rpl13	ribosomal protein L13	**-2.84**	6.52E-03	7.10E-01	7.93	2.82	5.11
Tdg	thymine DNA glycosylase	**-2.86**	9.52E-05	2.00E-02	16.11	5.69	10.42
Tagap1	T-cell activation GTPase activating protein 1	**-2.92**	2.00E-02	1.00E+00	6.07	2.09	3.98
Rab6b	RAB6B, member RAS oncogene family	**-2.94**	3.00E-02	1.00E+00	4.75	1.64	3.11
Akr1b8	aldo-keto reductase family 1, member B8	**-2.97**	4.00E-02	1.00E+00	4.1	1.38	2.72
Paqr7	progestin and adipoQ receptor family member VII	**-3**	3.13E-03	4.00E-01	8.67	2.91	5.76
Rplp2-ps1	ribosomal protein, large P2, pseudogene 1	**-3.02**	2.56E-03	3.50E-01	11.1	3.68	7.42
Hgsnat	heparan-alpha-glucosaminide N-acetyltransferase	**-3.03**	2.59E-04	5.00E-02	13.15	4.36	8.79
Pbx4	pre-B-cell leukemia homeobox 4	**-3.04**	3.00E-02	1.00E+00	4.56	1.51	3.05

(Table 3.2, continued)

Ndufs5	NADH dehydrogenase (ubiquinone) Fe-S protein 5	**-3.12**	2.22E-16	2.32E-13	64.8	20.99	43.81
Col6a3	collagen, type VI, alpha 3	**-3.62**	1.24E-03	1.90E-01	8.47	2.36	6.11
Eif3j	eukaryotic translation initiation factor 3, subunit J	**-3.88**	3.48E-07	1.27E-04	19.64	5.07	14.57
Cited4	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	**-3.94**	2.00E-02	1.00E+00	3.87	0.99	2.88
Ahcy	S-adenosylhomocysteine hydrolase	**-4**	1.81E-11	1.18E-08	33.23	8.32	24.91
Rnaset2b	ribonuclease T2B	**-4.05**	1.89E-13	1.51E-10	39.54	9.8	29.74
Olfml3	olfactomedin-like 3	**-4.59**	2.11E-05	5.45E-03	11.98	2.63	9.35
Stmn1	stathmin 1	**-4.69**	0.00E+00	0.00E+00	46.52	9.97	36.55
Rpl29	ribosomal protein L29	**-4.77**	0.00E+00	0.00E+00	97.68	20.6	77.08
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	**-4.79**	0.00E+00	0.00E+00	59.62	12.57	47.05
Ccbe1	collagen and calcium binding EGF domains 1	**-5.31**	3.00E-02	1.00E+00	2.98	0.56	2.42
Rpl35a	ribosomal protein L35A	**-5.47**	1.58E-11	1.04E-08	40.22	7.5	32.72
Nnat	neuronatin	**-5.56**	8.59E-07	2.89E-04	14.05	2.56	11.49
Ccdc85c	coiled-coil domain containing 85C	**-5.58**	7.59E-08	3.21E-05	19.35	3.47	15.88
Ankrd34c	ankyrin repeat domain 34C	**-5.69**	8.64E-05	2.00E-02	8.82	1.56	7.26
Rps4y2	ribosomal protein S4, Y-linked 2	**-5.89**	6.05E-04	1.00E-01	6.63	1.15	5.48
Gm7120	predicted gene 7120	**-7.24**	3.25E-04	6.00E-02	6.6	0.92	5.68
Rps12-ps3	ribosomal protein S12, pseudogene 3	**-7.72**	2.00E-02	1.00E+00	2.73	0.36	2.37
Upp1	uridine phosphorylase 1	**-10.06**	1.50E-08	7.66E-06	14.5	1.46	13.04
Bfsp2	beaded filament structural protein 2, phakinin	**-33.19**	0.00E+00	0.00E+00	1448.84	44.07	1404.77

Diseases and Disorders

Name	p-value	# Molecules
Cardiovascular Disease	1.34E-07 - 7.53E-03	28
Organismal Injury and Abnormalities	3.16E-07 - 6.31E-03	33
Dermatological Diseases and Conditions	1.14E-06 - 1.70E-03	19
Inflammatory Response	1.72E-06 - 7.53E-03	30
Cancer	3.62E-06 - 5.63E-03	86

Molecular and Cellular Functions

Name	p-value	# Molecules
Cellular Growth and Proliferation	4.53E-07 - 7.53E-03	59
Cellular Function and Maintenance	2.20E-05 - 7.53E-03	32
Cell-To-Cell Signaling and Interaction	2.27E-05 - 7.53E-03	24
Cellular Development	2.56E-05 - 7.53E-03	56
Cell Death and Survival	2.86E-05 - 7.53E-03	45

Physiological System Development and Function

Name	p-value	# Molecules
Hematological System Development and Function	1.72E-06 - 7.53E-03	31
Tissue Morphology	1.72E-06 - 7.53E-03	41
Cardiovascular System Development and Function	2.79E-06 - 7.53E-03	31
Skeletal and Muscular System Development and Function	2.56E-05 - 7.53E-03	22
Organismal Development	3.61E-05 - 7.53E-03	28

Figure 3.2 Ingenuity® Pathway Analysis (IPA®) of the 90 differentially expressed genes to determine critical pathways mis-regulated in the E15.5 β1-cKO. IPA® analysis shows multiple cellular processes involving these genes and categorizes them into three main categories: 'Diseases and Disorders', 'Molecular and Cellular Functions', and 'Physiological System Development and Function'. The number of differentially expressed genes involved in each of these processes is identified under '# Molecules'. Although a number of different pathways are described, none of these genes or pathways found by IPA® explained the partial fibrotic response observed in the β1MLR10 lens. Manual searches found some of the differentially expressed genes have been linked directly or indirectly to β1-integrin in literature, while IPA® failed to do so, suggesting that the IPA database may be incomplete.

Table 3.3 Subset of genes, 7 up-regulated and 3 down-regulated in the RNA-Seq, validated with E15.5 biological replicates with qRT-PCR. *Calculated in Microsoft Excel, wild type set equal to 1. **Calculated using nested ANOVA, S.D. – standard deviation, n=3.

Transcript	Fold Change in β1 cKO*	+/- S.D.	Wild Type +/- S.D.	p-value**
Egr1	12.64	14.05/11.36	1.24/0.81	0.000025
Nab2	227.4	289.7/178.5	3.39/0.29	0.0019
αSMA	9.66	10.54/8.86	1.53/0.65	0.0013
Mmp14	4.05	4.61/3.55	1.46/0.68	0.0027
Anxa2	4.7	5.39/4.09	1.7/0.58	0.012
tPA (Plat)	7.38	9.36/5.82	1.53/0.65	0.0032
Mt1	1.93	2.28/1.64	1.17/0.85	0.0012
Stmn1	0.85 (-1.18)	0.92/0.78	1.12/0.88	0.082
Thbs1	0.79 (-1.26)	1.14/0.55	1.93/0.52	0.37
Rpl29	0.014 (-70.7)	0.16/0.001	1.31/0.76	0.06

Chapter 4

EGR1: PROPOSED INVOLVMENT IN THE LENS

4.1 Egr1, Early Growth Response 1

Early growth response 1 (*Egr1*) encodes a Cys2-His2-type zinc-finger transcription factor (Gashler and Sukhatme, 1995; Pagel and Deindl, 2011). It is rapidly and transiently expressed due to many different stimuli such as shear stress, oxygen deprivation, growth factors (GFs) and injury (Cao et al., 1990; Khachigian et al., 1997; Ouellette et al., 1990; Pagel and Deindl, 2011; Yan et al., 1999), allowing it to mediate a multitude of different signaling cascades within the cell. The Egr1 protein is able to do so due to its extensive regulatory regions with functionally independent domains (Figure 4.1). It consists of transactivation as well as repressor domains, along with three DNA-binding zinc-finger domains that recognize GC-rich sequences within their target gene promoters (Bhattacharyya et al., 2011b; Gashler and Sukhatme, 1995; Gashler et al., 1993). The repressor domain is located between the activation domain and the DNA-binding domain and serves as the recognition site for Egr1's nuclear co-repressor NGFI-A binding proteins (NABs); specifically *Nab1* and *Nab2* (Russo et al., 1995; Svaren et al., 1996; Thiel et al., 2000). Nab 2 is able to control EGR1 transcriptional activity by recruiting the inhibitory nucleosomal remodeling and deacetylation (NuRD) complex to the promoter bound *Egr1* (Bhattacharyya et al., 2009; Srinivasan et al., 2006).

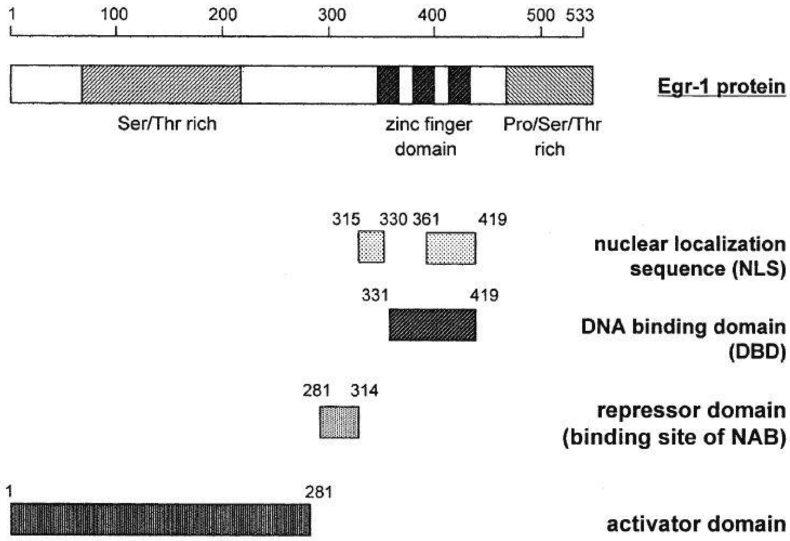

Figure 4.1 Egr1 protein functional domains. Depicting the three Cis2-His2-zinc finger DNA binding domain, allowing it to function as a transcription factor. The nuclear localization sequence is located within and just upstream of the DNA-binding domain. Finally, the repressor domain, where Nab2 can bind, is located between the activator domain and the nuclear localization sequence. Adapted from Pagel et al., (2011).

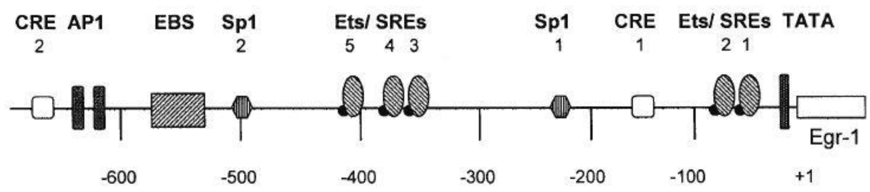

Figure 4.2 *Egr1* promoter. Depicting the TATA-box with two proximal and three distal serum response elements (SREs), with adjacent Etsbinding sites. The promoter also consists of two cAMP response elements (CREs), two Sp1, an AP1 and the EBS (Egr1 binding sequence); where Egr1 is able to bind and regulate itself. Adapted from Pagel et al., (2011).

The *Egr1* gene promoter contains several functional elements that allow for tightly controlled expression, in response to a number of different stimuli (Figure 4.2). At the 3' end of the promoter, and next to the TATA box, there are five serum response elements (SRE), and five Ets family transcription factor-binding sites adjacent to these SREs (Morris et al., 1988; Pagel and Deindl, 2011). There are also two cyclic adenosine monophosphate (cAMP) response elements (CREs), two gene-specific activator protein 1 (Sp1) binding sites, an APETALA1 (AP1) and an Egr binding sequence (EBS) (Cao et al., 1990; Pagel and Deindl, 2011). This EBS sequence is a GC-rich promoter sequence with which early growth response (Egr) genes can bind to within their target gene promoters. Therefore, *Egr1* is its own target, self-regulating by binding to this EBS site to up regulate its own expression levels (Cao et al., 1993).

As highlighted in chapter 3, a number of the differentially regulated genes in the β1MLR10 lens have the potential to be regulated by Egr1. Interestingly, 38 out of

90 differentially expressed β1MLR10 genes were found to have potential *Egr1* binding sites (EBS) by Ensembl (Ensembl Genome Browser, www.ensembl.org) (Table 3.2, gene names highlighted in red) (performed by Jia Ren in Dr. Cathy Wu's lab, University of Delaware). 25 out of 38 genes found to have potential EBS's were up-regulated, and 13 were down-regulated in the β1MLR10 lens. Eight of the genes validated in Chapter 3 (Table 3.3) have known or potential EBS's within their promoter region including *Egr1*, *Nab2*, *αSMA*, *Mmp14*, *Anxa2*, *Plat*, *Stmn1* and *Rpl29* (Table 4.1).

4.2 Nab2 negatively regulates Egr1 expression levels

In normal tissues, *Egr1* expression is maintained at low or undetectable levels. However, *Egr1* expression can rapidly increase due to many different stimuli. Once Egr1 is increased, a number of downstream targets can be activated. One of the major target genes of *Egr1* is *Nab2*, which contains eleven EBS binding domains in its promoter region (Kumbrink et al., 2005). *Nab2* is a delayed early-response gene that lacks DNA binding function. However, Nab2 is able to bind to the *Egr1* inhibitory domain (Pagel and Deindl, 2011) recruiting the nucleosomal remodeling and deacetylation (NuRD) complex, which contains histone deacetylases HDAC1/2 (Bhattacharyya et al., 2013; Pagel and Deindl, 2011; Srinivasan et al., 2006). Therefore, when EGR1 is not repressed at the transcript or post-translational level, it is able to up regulate itself. However, soon after EGR1 up regulates, it initiates the up regulation of *Nab2*, which is then able to post-translationally control Egr1 activity by binding to the proteins repressor domain in a highly controlled negative feedback loop.

4.3 Egr1 stimulation

Egr1 up-regulation can trigger a multitude of different downstream signaling cascades including growth, development, differentiation, apoptosis, inflammation, fibrosis and has been implicated in cancer progression and repression. However, which function is activated is dependent on which stimulus activates Egr1 up-regulation. *Egr1* can be rapidly and transiently expressed due to many different stimuli including shear stress, oxygen deprivation, growth factors and injury. Some of the most studied pathways activating *Egr1* are those occurring after injury, potentially leading to fibrosis. The TGFβ signaling cascade has been found to be a potent activator of *Egr1* expression (Bhattacharyya et al., 2013; Bhattacharyya et al., 2009). Moreover, ectopic expression of Egr1 in fibroblasts can induce collagen gene transcription, suggesting that *Egr1* may be a major mediator of the TGFβ pro-fibrotic response (Chen et al., 2006).

TGFβ activates *Egr1* transcription in a Smad-independent manner, by activating the MEK1/2-Erk signaling cascade which activates Elk-1, allowing it to bind to SRE's in the *Egr1* promoter (Bhattacharyya et al., 2009). Egr1 in turn is able to bind to the *Egr-1* binding sequence on target genes such as *Col1α2* (collagen type 1, α2), and activate collagen transcription with the help of p300 and Smad3 (Chen et al., 2006). Importantly, the TGFβ signaling cascade that leads to fibrosis is able to up-regulate *α-SMA* expression, one of the well-known markers of fibrosis. In pulmonary myofibroblasts, and tubulointerstitial fibroblasts, Egr1 binds to the *αSMA* promoter via SPUR elements, up regulating αSMA expression (Bhattacharyya et al., 2013; Liu et al., 2009b; Nakamura et al., 2002). Therefore, it is possible that Egr1 is able to mediate the up-regulation of the *αSMA* in the β1MLR10 lens.

Egr1 expression is commonly activated by other growth factors such as fibroblast growth factor (FGF) and epidermal growth factor (EGF) also through the MAPK signaling cascade (Biesiada et al., 1996; Damon et al., 1997; Pagel and Deindl, 2011). However, *Egr1* expression can also be induced by EGF (epidermal growth factor) receptor signaling, through a different signaling cascade (Cabodi et al., 2009). Cabodi and colleagues demonstrate that in ECV304 cells, a human cell line, integrin/EGFR stimulation p-regulates *Egr1* through the PI3K/Akt pathway that displaces the negative control exerted by the FoxOI transcription factor on the *Egr1* promoter (Cabodi et al., 2009). In their proposed model, FAK can act as a scaffold that bridges EGFR and integrin-binding components, activating EGFR and the downstream PI3K/Akt (Cabodi et al., 2009; Sieg et al., 2000). They were also able to show that the β1 subunit, not the αV subunit, associates with the EGF receptor on the plasma membrane, which allows for the phosphorylation of the cytoplasmic region of EGFR (Cabodi et al., 2009). It is important to note here that this β1-integrin-dependent EGFR activation, and subsequent Egr1 up-regulation was only observed in adherent ECV304 cells, a human cell line, but not in cells in suspension (Cabodi et al., 2009). This adhesion dependent Egr1 up-regulation was also observed in ovarian carcinoma cell through a similar FAK/SRC mechanism of Egr1 up-regulation (Barbolina et al., 2007). Therefore, *Egr1* has the potential to be mediated by β1-integrin within the embryonic mouse lens. However, the mechanism of Egr1 up-regulation in response to β1-integrin loss in the murine lens is unclear.

4.4 Egr1 has the potential to mediate the β1-integrin cKO embryonic phenotype

Further, Egr1 can upregulate the expression of *Nab2*, a gene is found to be highly upregulated in the β1-integrin cKO mouse lens, which can feedback inhibit *Egr1* levels and function. Importantly, Egr1 can also regulate the expression of a number of other genes up-regulated within the β1MLR10 lens. While αSMA is well known to upregulate expression in response to TGFβ signaling, it can also be directly up-regulated by Egr1 binding to its promoter (Liu et al., 2009b; Nakamura et al., 2002). In a study by Liu and colleagues, they found that TNF-α, was also able to upregulate EGR1 in human pulmonary myofibroblasts, by activating Mek1 and ERK1/2 kinases (Liu et al., 2009b). Egr1 was then found to bind to the SPUR element, a GC-rich element, in the *αSMA* promoter to suppress Smad-mediated transcription of *αSMA* in TGFβ1-activated myofibroblasts (Liu et al., 2009b). This study suggests that TNF-α up-regulated Egr1 is a competitor for TGFβ1 mediated αSMA up-regulation. Therefore, it is possible that *Egr1* may be able to up-regulate *αSMA* in the β1MLR10 embryonic lens.

Matrix metallopeptidase 14, *Mmp14*, also known as MT1-MMP, is a member of a large class of matrix metalloproteinases (MMPs) that function as proteolytic enzymes. These enzymes play an essential role in cellular processes that require coordinated disassembly of the ECM, such as tumor invasion, angiogenesis, fibrosis, EMT, and cellular movement (Chen and Parks, 2009; Galiacy et al., 2011; Gingras and Beliveau, 2010; Sounni et al., 2010). Mmp14 is slightly different than other MMPs in that it is not entirely soluble, however, it does contain an extracellular protease domain, like other MMPs. Importantly, it also contains a transmembrane segment that terminates in a short cytoplasmic domain that allows it to interact closely with intracellular transduction machinery such as β1-integrin (Gingras and Beliveau, 2010; Mori et al., 2013; Sato et al., 1994). *Mmp14* also has the potential to be directly regulated by Egr1(Barbolina et al., 2007). In a study performed in ovarian carcinoma cells, *Egr1* was up-regulated through the adhesion-dependent Integrin/FAK/SRC pathway. This consequently led to the transcriptional activation of *Mmp14*, and Mmp14-catalyzed collagen invasion (Barbolina et al., 2007). Although these results suggest that *Egr1* up-regulation is integrin dependent, it is possible that integrins other than β1 are able to regulate *Egr1* in the lens. However, the direct evidence that Egr1 is able to up-regulate *Mmp14*, another highly up-regulated gene in the β1MLR10 lens, further supports *Egr1*'s role in the β1MLR10 phenotype.

Annexin A2, *Anxa2*, is a member of the annexin superfamily of calcium dependent phospholipid-binding proteins, which is involved in cell migration, angiogenesis, vascular homeostasis and has been implicated in fibrotic diseases (Flood and Hajjar, 2011; Rankin et al., 2012; Zhang et al., 2010). In gastro-intestinal epithelium, *Anxa2* may play a role in the connection between actin cytoskeleton re-arrangement during cell migration. It is able to do so acting as a bridge between actin and β1-integrin, aiding β1-integrin to internalize into endosomes (Rankin et al., 2012). This integrin turn over allows cells to detach and begin migration. In other tissues, mainly the liver, Anxa2 was proposed as a new biomarker of fibrosis (Zhang et al., 2010). Using Ensemble to determine potential Egr1-binding sites, the *Anxa2* gene has at least one potential Egr1-binding site, characterized by a GC-rich region in a target genes promoter (GCG(G/T)GGCG). Considering *Anxa2* was another highly up-regulated gene within the β1MLR10 lens, and evidence to suggest it's involvement in the partial fibrotic phenotype observed, it is possible that Egr1 directly regulates the *Anxa2* gene to up-regulate expression and further the fibrotic response.

Tissue plasminogen activator (*tPA, Plat*) is another gene that is highly up-regulated in the E15.5 β1MLR10 lens and also may be involved in an Egr1 mediated fibrotic response. tPA is a secreted serine protease that catalyzes the conversion of plasminogen to plasmin, and therefore is involved in extracellular matrix remodeling. It also appears to play a role in endothelial cell basement membrane degradation during tumor growth and extravasation (Cottam et al., 1996). Interestingly, the annexin A2 complex binds tPA and also plasminogen on endothelial cell surfaces to aid in ECM remodeling (Dassah et al., 2009; Kwon et al., 2005). In the eye, tPA has been successfully used to clinically treat some retinal disorder and to decrease fibrin formation after cataract surgery (Ghazi et al., 2003; Heiligenhaus et al., 1998).

In the developing mouse, tPA is expressed extensively throughout the head ectoderm, including the lens placode at E9.5 (Collinge et al., 2005). As development of the head and eye progresses, tPA expression decreases in much of the head ectoderm, where as it becomes highly localized to the apical surface of the lens pit at E10.5. After the lens vesicle stage, as the lens fibers elongate to form the early lens, tPA remains detectable and is localized to the apical borders of the lens epithelium and fibers. This expression is maintained until birth, but is usually absent one week after birth (Collinge et al., 2005). Considering *tPA*'s importance in ECM remodeling and it's localization with in the developing lens, it makes *tPA* a possibly important gene in the regulation of lens epithelial cell maintenance. Interesting, at least five Egr1 binding sites (EBS's) has also been found within the *tPA* promoter. This suggests that

Egr1 may be able to mediate *tPA*'s up-regulation in response to cell migration or injury within the embryonic lens.

Overall, a number of genes have been found to be up-regulated within the embryonic lens after the loss of β1-integrin, many of which seem to play some role in fibrotic or cell migratory pathways. Many of the genes discussed, *Nab2*, *αSMA*, *Mmp14*, *Anxa2* and *tPA*, also have the capability to be involved in the partial fibrotic response that has been observed in the β1MLR10 lens. More importantly, all of the genes discussed here have some potential or known connection with *Egr1*. Therefore, *Egr1* may be the mediator of the fibrotic-like response observed when β1-integrin is lost from the embryonic mouse lens.

4.5 Results

Ten genes of the 90 differentially expressed genes within the β1MLR10 lens were chosen for validation due to their possible involvement in the partial fibrotic responses observed in the lens phenotype. I was able to validate *Egr1*, *Nab2*, *αSMA*, *Mmp14*, *Plat*, *Anxa2* at a number of embryonic time points. The mRNA expression of each of these genes was investigated at E13.5, 14.5, 15.5 and 16.5, not only to validate dysregulation of these genes at the time of the RNA-Seq (E15.5), but also to determine their dynamic pattern of expression compared to the onset of β1-integrin loss and the observed β1MLR10 phenotype (Figures 4.3 – 4.9, Tables 4.1 – 4.4).

Egr1 was found to be significantly up-regulated three days prior to the onset of the β1MLR10 phenotype at E16.5, with a fold change of 4.5 (standard deviation (S.D.) +5.61/-3.65, p-value 0.0005) (Figure 4.3). At E14.5, this expression increases to 11.5 fold (S.D. +15.32/-8.62, p-value of 0.005). *Egr1* expression was observed to be the highest at E15.5 with a fold change of 12.6 (S.D. +14.06/-11.36, p-value 0.000025).

Although at E16.5, the expression of *Egr1* decreases from E15.5, it is still significantly high with a fold change of 9.9 (S.D. +11.06/-8.89, p-value 0.00009). Altogether, this shows that *Egr1* expression is significantly upregulated even at E13.5, just one day after the loss of β1-integrin from the lens at E12.5, and continues to increase and remain high through E16.5. Finally, I was also able to validate that Egr1 protein levels were increased in the lens epithelial cells at the transition zone of the E15.5 and E16.5 β1MLR10 lens (Figure 4.4), further supporting *Egr1*'s role in the β1MLR10 phenotype.

Nab2 is also significantly up-regulated at E13.5, although the standard deviation for these samples was large. This was due to one biological outlier in the β1MLR10 samples that had lower expression than the other two biological replicates. This biological replicate's threshold values were between 33.5 to 34.8, whereas the other other two were very close to a threshold of 30. At E13.5, *Nab2* was up-regulated 5.2 fold (S.D. +11.02/-2.45, p-value 0.029)(Figure 4.5). The expression of *Nab2* mRNA then drastically increased by 198 fold at E14.5 (S.D. +309/-126, p-value 0.005). This high expression remained at E15.5, where I observed a fold change of 227 (S.D. +289/-178, p-value 0.002). The expression of *Nab2* at E16.5 was the only time point which was not found to be significantly up-regulated. At E16.5, I observed a fold change of 2.5 (S.D. +123/-0.04, insignificant p-value 0.6). The standard deviation within the E16.5 β1MLR10 samples was too large for the expression to be considered significant. At E16.5, this could be due to phenotypic variability. It could also be due to large sample variability and *Nab2* mRNA expression below the threshold with which the qRT-PCR system could detect; with the threshold of one β1MLR10 biological replicate between 30 to 45. In the RNA-Seq analysis, Nab2

RPKM levels were an average of 0.58 for the wild type, and 8.42 for the β1MLR10 samples. To be able to determine a fold change for *Nab2* using qRT-PCR, some sample values that were undetected by the system were set equal to 45 (the threshold limit of the system).

α-smooth muscle actin (*αSMA*), a common marker of fibrosis in the lens and known to be regulated by Egr1 (Nakamura et al., 2002), was found to be 6 fold up-regulated in the E15.5 β1MLR10 lens by RNA-Seq analysis. Using qRT-PCR to validate, I found *αSMA* not only to be up-regulated at E15.5 but from E14.5 to E16.5. At E13.5 *αSMA* appeared to be up-regulated 2 fold (S.D. +2.9/-1.32 although the p-value (0.06) did not reach significance (Figure 4.6). At E14.5, however, *αSMA* expression in the β1MLR10 lens increased by 8.6 fold compared to WT (S.D. +9.31/-8.0, p-value of 0.0007). At E15.5, this expression increased by 9.6 fold (S.D. +10.5/-8.86, p-value 0.001) via qRT-PCR analysis. By E16.5, coinciding with *αSMA* up-regulation observed at the protein level (Simirskii et al., 2007), it's expression drastically increased by 20 fold (S.D. +22.3/-18.2, p-value 0.000043) in the β1MLR10 lens.

Matrix metallopeptidase 14 (*Mmp14*, MT1-MMP), a protein essential for ECM disassembly and possibly regulated by Egr1 (Chen and Parks, 2009), was also found to be up-regulated 3 days prior to the onset of the phenotype. At E13.5, *Mmp14* was up-regulated 4.3 fold (S.D. +5.7/-3.3, p-value 0.005)(Figure 4.7), but at E14.5 this expression decreased by 2.7 fold (S.D. +3.7/-1.9, p-value 0.02). At E15.5, the expression of *Mmp14* increased by 4.0 fold (S.D. +4.6/-3.5, p-value 0.002), in comparison to a 3 fold expression level as found by RNA-Seq analysis. At E16.5, the expression of *Mmp14* drastically increased by 13.3 fold (S.D. +129/1.4, insignificant p-value 0.25). The large deviation that was seen in the E16.5 samples was due to one β1MLR10 sample with markedly decreased *Mmp14* expression. Again, this is likely due to phenotypic variability that has been observed at E16.5 in the β1MLR10 lens.

Annexin A2 (*Anxa2*), a protein involved in cell migration (Zhang et al., 2010), differentially expressed 3.86 fold at E15.5 via RNA-Seq analysis, was also up-regulated at E13.5 and remained high through E16.5. At E13.5, *Anxa2* was up-regulated 2 fold (S.D. +2.5/-1.7, p-value 0.05)(Figure 4.8), and its expression increased at E14.5 by 8.9 fold (S.D. +11.1/-7.1, p-value 0.008). By E15.5, this expression decreased slightly by 4.7 fold (S.D. +5.4/-4.1), then increased by 15.2 fold at E16.5 (S.D. +202/-1.13, insignificant p-value 0.2). Again, the expression of *Anxa2* at E16.5 was unable to be fully validated due to high expression deviation among the samples, due to phenotypic variability.

Tissue plasminogen activator (*Plat*, *tPA*), a protein involved in ECM remodeling, was found to be differentially regulated 3.3 fold within the E15.5 β1MLR10 lens via qRT-PCR. Interestingly, its expression follows a similar pattern to *Mmp14* and *Anxa2*, increased at E13.5 and remaining high through E16.5. At E13.5, *Plat* was found to be up-regulated 9.1 fold (S.D. +10.8/-7.6, p-value 0.008) (Figure 4.9), and increased at E14.5 by 14.6 fold (S.D. +21.1/-9.9, p-value 0.003). By E15.5, *Plat* expression decreased by 7.4 fold (S.D. +9.4/-5.8, p-value 0.003), and increased by 18.6 fold (S.D. +300/-1.15, insignificant p-value 0.2) at E16.5. Although *Plat* expression was unable to be validated at E16.5, due to high variability among biological replicates, this again is likely due to phenotypic variability at this time point. Therefore, its high expression at E13.5 that continued through E16.5 gives promising evidence that this gene may be involved in the β1MLR10 lens phenotype.

I also attempted to validate *Mt1*, *Thbs1*, *Stmn1* and *Rpl29* at these same embryonic time points. Metallothionein 1, *Mt1*, is a stress response protein that detoxifies heavy metals, regulates zinc/copper homeostasis, and is an important scavenger for reactive oxygen species (ROS) (Barbato et al., 2007; Liu et al., 2009a). RNA-Seq analysis showed *Mt1* to be up-regulated more than 270 fold in the E15.5 β1MLR10 lens. However, qRT-PCR analysis showed only an increase of 2 fold (S.D. +2.3/-1.6, p-value 0.001) (Table 4.1) at E15.5. This 2 fold level of *Mt1* expression remained similar among all embryonic time points investigated. Interestingly, its expression was also found to be increased more than 250 fold in another conditional knockout mouse strain within our lab (Manthey, 2013). Therefore, it is possible that Mt1 is not an important regulated gene in the β1MLR10 lens, but is an important stress response gene within the lens in general.

Thrombospondin-1 (*Thbs1*), a glycoprotein, is known to be involved in activation of latent TGF-β, and is able to bind to various ECM elements (Adams, 2001; Crawford et al., 1998). Lens epithelial cells express Thbs1 in cell culture and at certain stages during lens development (Hiscott et al., 1999; Schmittgraff et al., 1990; Tucker et al., 1997). Importantly, *Thbs1* has also been found to be involved in wound healing processes in other tissues, such as epidermal tissues (DiPietro et al., 1996). In the cornea, Thbs1 expression was altered during the wound-healing process (Hiscott et al., 1999), suggesting that *Thbs1* may play a role in wound healing in the eye in general. A study by Saika et al. (2004b) revealed that in murine post-operative lens cells differentiating to fiber cells, *Thbs1* expression was decreased. Within the E15.5 β1MLR10 lens, a decrease of -2.7 fold was observed, making *Thbs1* a very interesting gene to investigate at other embryonic time points. At E13.5, *Thbs1* was found to be -

1.3 fold decreased (S.D. +0.96/-0.63, p-value 0.0001)(Table 4.2), however, it drastically increased by 4.5 fold up-regulated at E14.5 (S.D. +6.16/-3.26, insignificant p-value 0.19). At E15.5 this expression decreased again by -1.3 fold (S.D. +1.14/-0.55, insignificant p-value 0.37), and decreased even further by -3.9 fold at E16.5 (S.D. +0.37/-0.18, p-value 0.02). Unfortunately, the variation within samples at E14.5 and E15.5 were too great for the fold changes to be significant. This is most likely due to high sample variability. For example at E14.5 the threshold values between wild type biological replicate samples ranged between 26 and 34.

Stathmin 1, *Stmn1*, also known as *P18* and *P19*, is a cytosolic phosphoprotein known to play a role in binding tubulin and controlling microtubule dynamics during cell cycle progression (Belmont and Mitchison, 1996; Luo et al., 1994; Rubin and Atweh, 2004). Importantly, it is also known to play a role in regulating cell-cycle progression, and either the inhibition or overexpression of Stmn1 results in growth suppression and accumulation of cells in the G_2/M phase (Luo et al., 1994; Rubin and Atweh, 2004). Interestingly, it was also found to have six potential Egr1 binding sites within its promoter (Table 3.2), and was found to be -4.7 fold decreased at E15.5 via RNA-Seq analysis. Investigating *Stmn1* expression at E15.5 via qRT-PCR, however, did not result in a large or significant down-regulation; -1.2 fold (S.D. +0.92/-0.78, insignificant p-value 0.08)(Table 4.3). All other embryonic time points showed an up-regulation of *Stmn1* between 2 – 4 fold, however, the variability among biological replicates was too great to be significant. This is most likely due to high sample variability among biological replicates. For example, at E14.5 the threshold values for *Stmn1* expression ranged between 21 and 24.

Ribosomal protein L29, *Rpl29*, is known to play a role in protein synthesis by modulating the rate of protein synthesis (DeLabre et al., 2002; Kirn-Safran et al., 2007). *Rpl29* has recently been implicated in angiogenesis as well, and a loss of *Rpl29* results in a reduction of angiogenesis in cultured endothelial cells (Jones et al., 2013). Its expression in the E15.5 β1MLR10 lens was found to be -4.8 fold decreased, via RNA-Seq analysis. Interestingly, it was also found to have eight potential EBS within its promoter region, making this a possible target for Egr1 control. At E13.5, *Rpl29* expression was found to be -2,492 fold decreased (S.D. +333/-18796, p-value 0.0001)(Table 4.4), and decreased even further by -7,805 fold at E14.5 (S.D. +66.6/-93457, p-value 0.002). This expression drastically changes by -70.7 fold at E15.5 (S.D +5.8/-833, insignificant p-value 0.06), but decreases even further at E16.5 by -9,850 fold (S.D. +3333/-32894, p-value 0.003). Overall, for majority of the embryonic stages, significant p-values were obtained. However, the standard deviation for many of these samples is very large and should be taken into consideration when considering the fold change. For example at E13.5 the fold change was -2,492, however, the standard deviation varied from +333 to -18,796. This range of deviation is too large to assume the fold change is in fact around -2,000.

4.6 Discussion

Early growth response 1 (*Egr1*) is a transcription factor that can be rapidly and transiently expressed due to many different stimuli including shear stress, oxygen deprivation, growth factors and injury (Cao et al., 1990; Khachigian et al., 1997; Ouellette et al., 1990; Pagel and Deindl, 2011; Yan et al., 1999). It is able to up-regulate itself, containing an Egr1 binding site (EBS) within its promoter region (Cao et al., 1990), and is involved in a negative feedback loop with Nab2, Ngfi-A binding protein 2 (Kumbrink et al., 2005). Interestingly, *Egr1* null mice have large eyes and lenses leading to a myopic shift in lens refraction, suggesting *Egr1* may negatively regulate eye and lens growth (Schippert et al., 2007). Notably *Egr1* also has a great potential to be able to control the β1MLR10 lens phenotype. Out of the 90 genes found to be differentially expressed, 38 were found to have potential EBS within their promoter regions. A number of these genes also have the potential to play a role in the partial epithelial to mesenchymal transition (EMT) observed in the β1MLR10 lens. Ten of the 90 genes were chosen for further investigation based on direct connections to *Egr1*, integrins, or possible involvement in fibrosis: *Egr1*, *Nab2*, α smooth muscle actin (*αSMA*), matrix metallopeptidase 14 (*Mmp14*), tissue plasminogen activator (*Plat*, *tPA*), annexin A2 (*Anxa2*), metallothionein 1 (*Mt1*), thrombospondin 1 (*Thbs1*), stathmin 1 (*Stmn1*), and ribosomal protein L29 (*Rpl29*). Not only was the differential expression of these genes validated in most of these cases at E15.5, many of them were also dysregulated at E13.5, one day after β1-integrin expression was lost and three days prior to the onset of the observed morphological anomalies in the β1-integrin cKO lens.

Egr1, was not only up-regulated at E15.5, it was also significantly up-regulated 3 days prior to the onset of the β1MLR10 lens phenotype. This suggests that *Egr1* is up-regulated shortly after the full loss of β1-integrin from the E12.5 lens, and is therefore one of the proximal causes of the β1MLR10 lens fibrotic phenotype. Along with this *Egr1* up-regulation, an up-regulation of *Nab2* was also found. *Nab2* expression is up-regulated 5 fold at E13.5, and jumped to more than 190 fold up-regulated at E14.5 and E15.5. This up-regulation drastically decreased to 2.4 fold at E16.5, coinciding with *Egr1* expression patterns in the embryonic β1MLR10 lens.

This again suggests that *Nab2* may be an early and direct regulator of the β1MLR10 lens phenotype.

I was also able to validate that *Egr1* is not only increased at the mRNA level, but it is increased at the protein level as well. Using immunofluorescent staining, I was able to show that lens epithelial cells at the transition zone of the E15.5 β1MLR10 lens, exhibit strong, nuclear Egr1 staining. This Egr1 nuclear localization remained high within these same cells in the E16.5 β1MLR10 lens. All together, these data suggest an important role for *Egr1* in the β1MLR10, E15.5 day lens.

αSMA, a marker for fibrosis, is able to be directly controlled and up-regulated by Egr1 (Nakamura et al., 2002). In previous work by Simirskii et al., (2007), *αSMA* was found to be up-regulated at the protein level in β1MLR10 lens epithelial cells at E16.5. It was also found to be up-regulated 6 fold at the transcript level at E15.5 using RNA-Seq analysis. Investigating *αSMA* transcript up-regulation using qRT-PCR, it was found to be significantly up-regulated 8.6 fold even at E14.5, increased to 9.7 fold at E15.5, and drastically increased to 20 fold at E16.5; coinciding with the observed up-regulation of αSMA protein at E16.5. This shows that *αSMA* is most-likely secondary to the up-regulation of *Egr1* and *Nab2*, and may be caused by the up-regulation of Egr1 observed at E13.5.

Mmp14, *Anxa2* and *Plat* are all genes that play some role in cellular motility, migration and even fibrosis. *Mmp14* has not only been implicated in fibrosis, but is also up-regulated by Egr1 in other tissues (Barbolina et al., 2007). All of these genes were found to be up-regulated more than 3 fold at E15.5 using RNA-Seq analysis, and this up-regulation of confirmed using qRT-PCR. Importantly, all of these genes were also found to be up-regulated 2 fold or higher at E13.5 using qRT-PCR analysis, and this up-regulation steadily increased through E16.5. Although the E16.5 time point had a large amount of deviation and insignificant p-values, this is most-likely due to high variability seen between biological replicates at E16.5 due to phenotypic variability at this time point. Based on these genes importance in cell migration and fibrosis, it is likely that this up-regulation at E16.5 is correct. However, further biological replicates would need to be collected to prove this.

Mt1, a stress response gene, was found to be more than 250 fold up-regulated using RNA-Seq analysis. However, this same up-regulation was not observed using qRT-PCR in independent biological replicates. Interestingly, *Mt1* was also found to be more than 300 fold up-regulated in another conditional knock-out strain in our lab, therefore suggesting that *Mt1* is a general stress response gene, and may not have a direct connection to the phenotype observed in the β1MLR10 lens. *Thbs1*, *Stmn1* and *Rpl29* were all found to be 3 – 4 fold down-regulated at E15.5 using RNA-Seq analysis. However, *Thbs1* and *Stmn1* down-regulation was not able to be validated using qRT-PCR analysis. This may be due to high sample variability, therefore giving a large standard deviation. *Rpl29*, on the other hand, was able to validate, but had large variation among biological replicates. Its potential role in the β1MLR10 phenotype remains unclear.

Overall, *Egr1* is an excellent candidate to be the proximal cause of the partial fibrotic response observed in the E16.5 β1MLR10 lens. This was supported by the fact that it was found to be up-regulated at the transcript level three days prior to the onset of the phenotype. *Nab2*, its negative regulator, was also highly up-regulated at E13.5 and remained high through E15.5 until *Egr1* expression began to decrease slightly at E16.5. This coincides with evidence that Nab2 in a negative feedback loop with Egr1 expression in other tissues, and is most-likely the same in the lens. Importantly, *αSMA* mRNA up-regulation at E14.5 through E16.5 coincided with previous evidence of protein up-regulation at E16.5 in the β1MLR10 lens epithelial cells. Considering that *αSMA* can be directly regulated by Egr1, this evidence furthers the support for *Egr1*'s importance in the β1MLR10 fibrotic phenotype. Finally, *Mmp14*, *Anxa2* and *Plat* were all found to be similarly up-regulated at E13.5 and remained high through E16.5. Considering all of these genes have been found to play a role in cell migration and fibrosis, and have potential EBS within their promoter regions, it suggests that the *Egr1* up-regulation at E13.5 is able to cause the down-stream up-regulation of these potential target genes. Altogether, these data support the hypothesis that *Egr1* is a likely mediator of the fibrotic phenotype observed in the E16.5 β1MLR10 lens.

Table 4.1 Potential Egr1 binding sites (EBS) in a subset of genes differentially regulated in the E15.5 β1MLR10 lens. 38 total differentially expressed genes in the β1MLR10 lens were found to have potential EBS sites using Ensembl. This table shows eight of these genes, six up-regulated and two down-regulated, that have the potential to mediate the partial fibrotic phenotype observed in the β1MLR10 lens. This analysis was performed by Jia Ren in Dr. Cathy Wu's lab at the University of Delaware

Gene	Number of Sites*	Position**	Sequence
Egr1	2	-191 to -202	GGGCGCGGGCGC
		-201 to 212	GGGCGCGGGCGC
Nab2	4	-34 to -45	CTGCGGGGGCGG
		-41 to -52	CTGCGGGGGCGG
		-47 to -58	CTGCGGGGGCGG
		-581 to -592	CTGCCTGGGGGA
Acta2 (αSMA)	5	-46 to -57	GCAAGTGGGAGG
		-66 to -77	CTGCTTCGGTGC
		-72 to -83	GCAAGTGGGAGG
		-117 to -128	CTGCTTCGGTGC
		-168 to -179	CTGCTTCGGTGC
Mmp14	1	-278 to -289	TAGCCTGGGAGT
Anxa2	13	-585 to -596	AGGCCGGGGCGG
		-528 to -539	AGGCCGGGGCGG
		-587 to -598	AGGCCGGGGCGG
		-593 to -604	AGGCCGGGGCGG
		-597 to -608	AGGCCGGGGCGG
		-603 to -614	AGGCCGGGGCGG
		-583 to 594	AGGCCGGGGCGG
		-586 to 597	AGGCCGGGGCGG
		-627 to -638	AGGCCGGGGCGG
		-229 to -240	GAGTGTGGGGGA
		-501 to -512	CTGCCTTGGCCT
		-85 to -96	TGGCCCAGGAGT
		-61 to -72	TGGCCCAGGAGT
Plat (tPA)	5	-276 to -287	CAGCGGGGGCGC
		-538 to -549	AGGCGGAGGTGA
		-677 to -688	CTGCTGGGGTGA
		-593 to -604	AGGCTTCGGTGG
		-641 to -652	AGGCTTCGGTGG

(Table 4.1, continued)

Stmn1	6	-368 to 379	CAGCGTGGGTGG
		-47 to -58	GGGCGGGGGCGG
		-96 to -107	GGGCGGGGGCGG
		-52 to -63	TAGCTTGGGCGT
		-65 to -76	GCCCGGGGGCGT
		-482 to -493	TTGCCCAGGCTG
Rpl29	8	-501 to -512	GGGCGTGGTGGT
		-435 to -446	GGGCGTGGTGGT
		-486 to -497	GGGCGTGGTGGT
		-453 to -464	GGGCGTGGTGGT
		-374 to -385	GGGCGTGGTGGT
		-472 to -483	GGGCGTGGTGGT
		-421 to -432	GGGCGTGGTGGT
		-502 to -513	GGGCGTGGTGGT

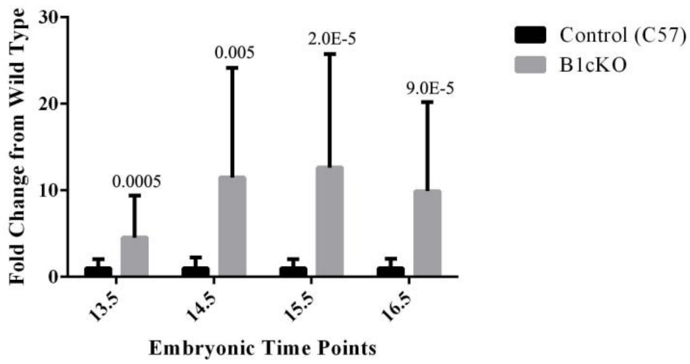

Figure 4.3 *Egr1* mRNA up-regulation using qRT-PCR. Fold change calculated in Microsoft Excel, wild type set equal to one. P-values calculated using nested Anova, sample size of three for both wild type and conditional knock-out (cKO). Bars are standard deviation (S.D.). Fold changes in order of stage E13.5 – E16.5: 4.5 (S.D. +5.6/-3.7), 11.5 (S.D. +15.3/-8.6), 12.6 (S.D. +14.1/-11.4), 9.9 (S.D. +11.1/-8.9).

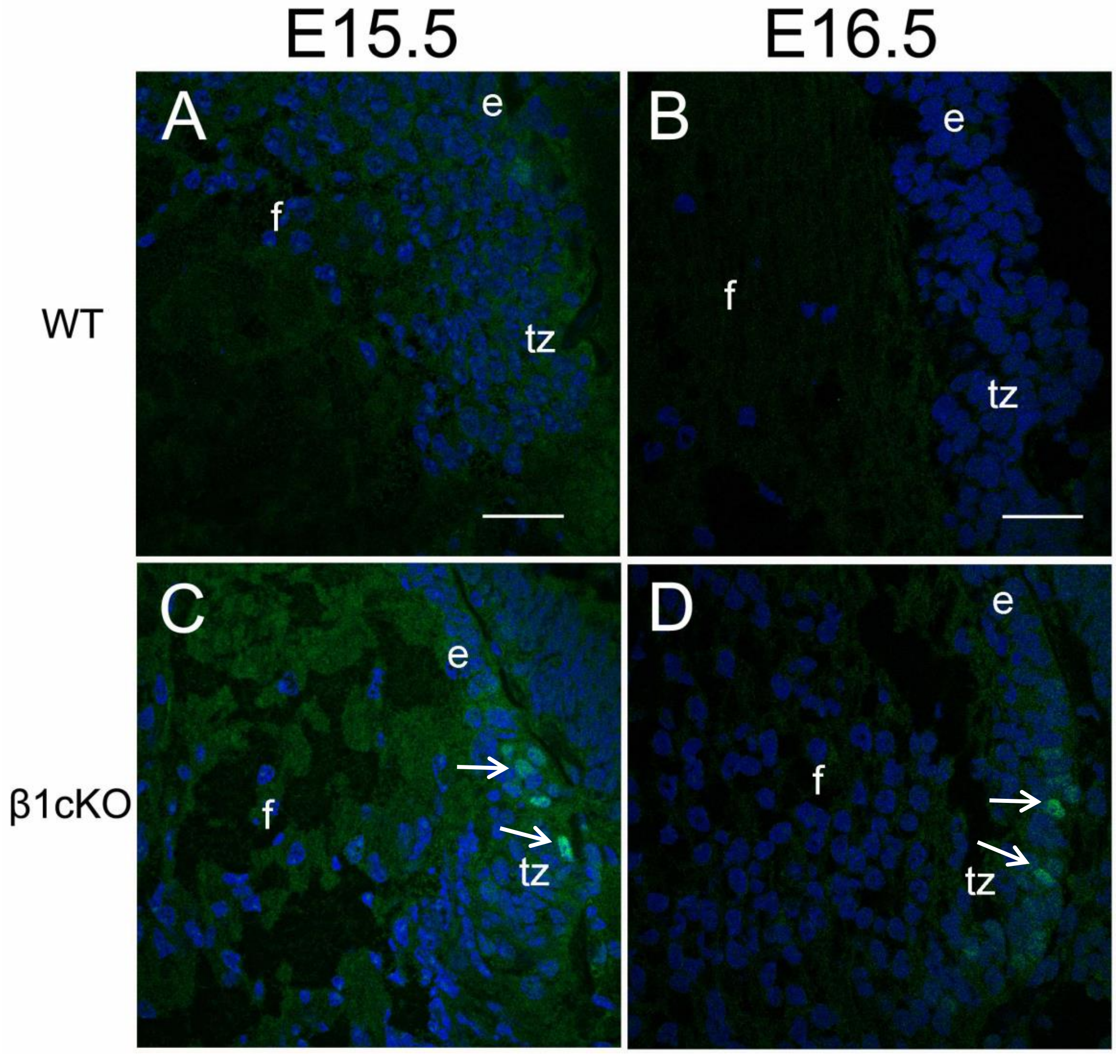

Figure 4.4 Egr1 protein levels are increased at the transition zone (tz) of the E15.5 and E16.5 β1cKO lens. Egr1 (green) localizes to the nucleus (DRAG5: blue) when active (white arrows). Transitioning lens epithelial cells in the E15.5 β1cKO (C) exhibits strong nuclear Egr1 staining. This further validates the Egr1 mRNA up-regulation observed in RNA-Seq observations, but at the protein level. Egr1 protein remains high through E16.5 (D) in the β1cKO lens. Abbreviations: e, lens epithelium; f, lens fiber cells; tz, lens transition zone. Scale Bars = 35 µm.

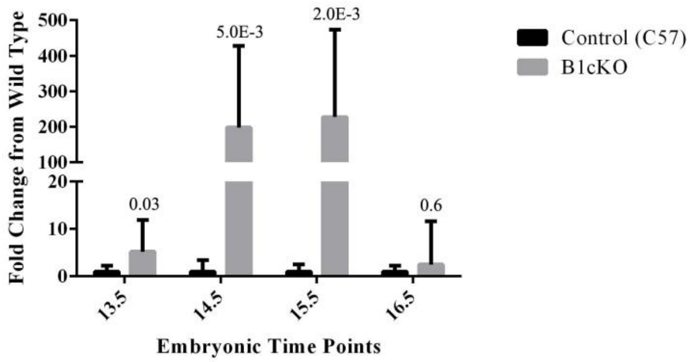

Figure 4.5 *Nab2* mRNA up-regulation using qRT-PCR. Fold change calculated in Microsoft Excel, wild type set equal to one. P-values calculated using nested Anova, sample size of three for both wild type and conditional knock-out (cKO). Bars are standard deviation (S.D.). Fold changes in order of stage E13.5 – E16.5: 5.2 (S.D. +11/-2.5), 198 (S.D. +309/-127), 227 (S.D. +289/-178), 2.5 (S.D. +123/-0.05).

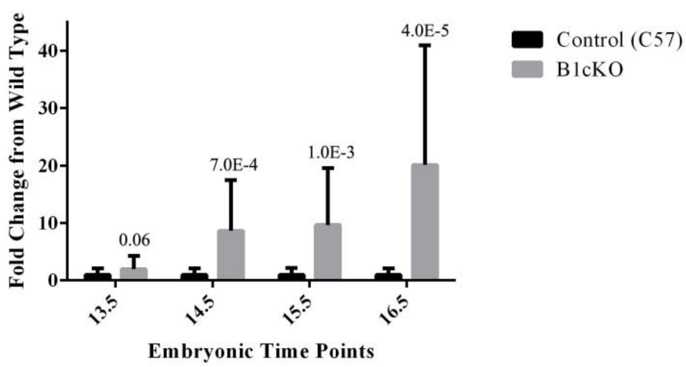

Figure 4.6 *αSMA* mRNA up-regulation using qRT-PCR. Fold change calculated in Microsoft Excel, wild type set equal to one. P-values calculated using nested Anova, sample size of three for both wild type and conditional knock-out (cKO). Bars are standard deviation (S.D.). Fold changes in order of stage E13.5 – E16.5: 2 (S.D. +2.9/-1.3), 8.6 (S.D. +9.3/-8), 9.6 (S.D. +10.5/-8.8), 20.1 (S.D. +22.3/-18.2).

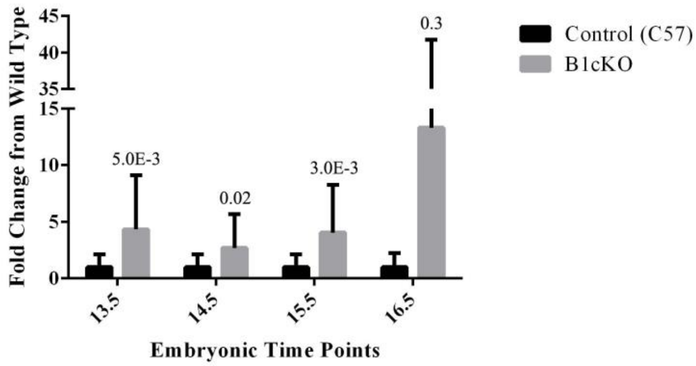

Figure 4.7 *Mmp14* mRNA up-regulation using qRT-PCR. Fold change calculated in Microsoft Excel, wild type set equal to one. P-values calculated using nested Anova, sample size of three for both wild type and conditional knock-out (cKO). Bars are standard deviation (S.D.). Fold changes in order of stage E13.5 – E16.5: 4.3 (S.D. +5.7/-3.3), 2.7 (S.D. +3.7/-2), 4.04 (S.D. +4.6/-3.5), 13.3 (S.D. +129/-1.4).

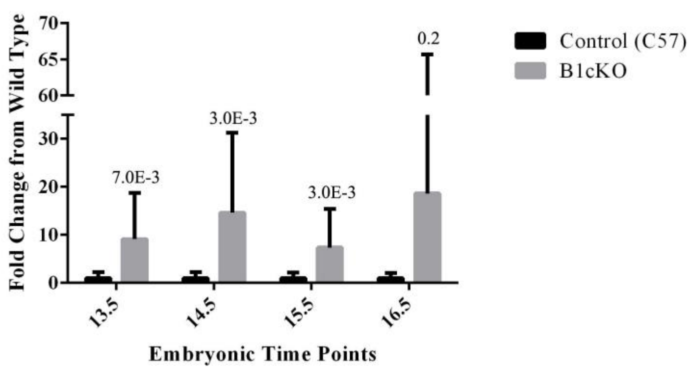

Figure 4.8 *Plat* mRNA up-regulation using qRT-PCR. Fold change calculated in Microsoft Excel, wild type set equal to one. P-values calculated using nested Anova, sample size of three for both wild type and conditional knock-out (cKO). Bars are standard deviation (S.D.). Fold changes in order of stage E13.5 – E16.5: 9.1 (S.D. +10.8/-7.6), 14.6 (S.D. +21.6/-9.9), 7.4 (S.D. +9.4/-5.8), 18.6 (S.D. +300/-1.2).

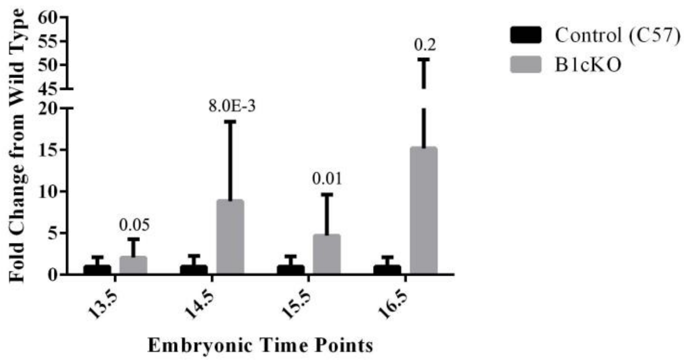

Figure 4.9 *Anxa2* mRNA up-regulation using qRT-PCR. Fold change calculated in Microsoft Excel, wild type set equal to one. P-values calculated using nested Anova, sample size of three for both wild type and conditional knock-out (cKO). Bars are standard deviation (S.D.). Fold changes in order of stage E13.5 – E16.5: 2.1 (S.D. +2.5/-1.7), 8.8 (S.D. +11.1/-7.1), 4.7 (S.D. +5.4/-4.1), 15.2 (S.D. +202/-1.1).

Table 4.2 *Mt1* mRNA expression levels using qRT-PCR analysis. *Calculated in Microsoft Excel, wild type set equal to 1. **Calculated using nested ANOVA, S.D. – standard deviation, n=3.

Embryonic Stage	Fold Change in β1 cKO*	+/- S.D.	Wild Type +/- S.D.	p-value**
13.5	2.63	3.79/1.82	2.44/0.41	0.1
14.5	1.2	1.51/1.07	1.91/0.52	0.57
15.5	1.93	2.27/1.64	1.17/0.85	0.001
16.5	1.56	2.42/1.01	1.35/0.74	0.21

Table 4.3 *Thbs1* mRNA expression levels using qRT-PCR analysis. *Calculated in Microsoft Excel, wild type set equal to 1. **Calculated using nested ANOVA, S.D. – standard deviation, n=3.

Embryonic Stage	Fold Change in β1 cKO*	+/- S.D.	Wild Type +/- S.D.	p-value**
13.5	-1.28	1.04/1.58	1.52/0.66	0.0001
14.5	4.49	6.16/3.26	4.78/0.21	0.19
15.5	-1.26	1.14/1.81	1.93/0.52	0.37
16.5	-3.85	2.7/5.5	1.45/0.68	0.016

Table 4.4 *Stmn1* mRNA expression levels using qRT-PCR analysis. *Calculated in Microsoft Excel, wild type set equal to 1. **Calculated using nested ANOVA, S.D. – standard deviation, n=3.

Embryonic Stage	Fold Change in β1 cKO*	+/- S.D.	Wild Type +/- S.D.	p-value**
13.5	1.6	2.55/1.0	1.36/0.74	0.24
14.5	1.45	1.8/1.18	1.62/0.62	0.33
15.5	-1.18	1.08/1.28	1.13/0.88	0.08
16.5	4.14	56.8/3.3	1.12/0.89	0.46

Table 4.5 *Rpl29* mRNA expression levels using qRT-PCR analysis. *Calculated in Microsoft Excel, wild type set equal to 1. **Calculated using nested ANOVA, S.D. – standard deviation, n=3.

Embryonic Stage	Fold Change in β1 cKO*	+/- S.D.	Wild Type +/- S.D.	p-value**
13.5	-2492	333/18796	1.52/0.66	0.0001
14.5	-7805	66.6/93457	2.31/0.43	0.0023
15.5	-70.69	5.8/833	1.31/0.76	0.06
16.5	-9850	3333/32894	1.62/0.61	0.003

Chapter 5

A POTENTIAL ROLE FOR EGR1 IN POSTERIOR CAPSULAR OPACIFICATION

5.1 Cataracts, Surgery and Outcomes

Cataract, or the loss of lens transparency, is a major disease of the lens that leads to loss of vision, and is the most common cause of blindness worldwide (Asbell et al., 2005). Clinically, there are several different labels for cataracts depending on where the defect manifests. However, the position of a cataract in the lens is quite variable and often depends on the age of onset. All cataracts, regardless of the position of the lens, or the age of onset, are most commonly treated by extracapsular lens extraction. Cataract surgery involves the surgical removal of the bulk of the lens cells by phacoemulsification, and insertion of a prosthetic lens (or intraocular lens, IOL) into the remaining capsular bag (de Iongh et al., 2005). The remaining lens epithelial cells left on the anterior capsule after surgery proliferate and migrate to accumulate between the capsule and the IOL, where they become fibrotic plaques and cause clouding of the lens (Wormstone, 2002). Immunohistochemical studies of *in vivo* and *in vitro* models have shown that the fibrotic cells express αSMA, and aberrant ECM composed of proteoglycans, fibronectin, tenascin and collagens I and III (de Iongh et al., 2005; Ishibashi et al., 1994; Marcantonio et al., 2003; Saika et al., 1995; Wunderlich et al., 2000).

Posterior capsular opacification (PCO) is a major complication following cataract surgery. Essentially, the residual lens cells are interpreting the surgery as an injury to the tissue , which causes the cells to undergo proliferation and migration. This response can cause the cells to enter into the visual axis and cause increase light scatter and decreased visual acuity (Meacock et al., 2003; van Bree et al., 2011). Although PCO can be relatively easily treated by Nd:YAG laser capsulotomy, in some cases, it can lead to a number of other ocular problems including corneal damage, macular edema, glaucoma and retinal detachment (Apple et al., 2011; Billotte and Berdeaux, 2004). Due to these severe side-effects that can occur from laser capsulotomy, emphasis should be placed on preventing PCO from occurring after the first cataract surgery.

PCO can result from two cellular mechanisms that can occur in residual lens epithelial cells remaining in the capsule after cataract surgery: epithelial-to-mesenchymal transition (EMT) and fiber cell differentiation. Both of these processes occur simultaneously with the proliferation and migration of the lens epithelial cells into the visual axis, causing a "secondary cataract", or secondary clouding of the lens. These two very different mechanisms lead to two primary forms of PCO: fibrotic and pearl-type PCO. Fibrotic PCO occurs mostly when the residual cells undergo EMT, causing the cells to become migratory, as well as causing an up-regulation of mesenchymal genes and a down-regulation of epithelial genes (Walker and Menko, 2009). Importantly, TGF-β induces lens cells to undergo the EMT responsible for fibrotic PCO with the deposition of fibronectin/collagen I, lens epithelial cell proliferation and capsular wrinkling (Wormstone et al., 2009).

On the other hand, pearl-type PCO occurs when the cells mostly undergo fiber cell differentiation. This aberrant fiber cell differentiation can lead to two main phenotypes: the formation of Elschnig pearls if the cells migrate into the visual axis, or Sommerring's ring if the cells stay in the periphery of the capsule (Kappelhof et al., 1987; Marcantonio and Vrensen, 1999; Matsushima et al., 2004). Both types of PCO can occur in operated lenses and can lead to multiple lens defects including: decreased visual acuity, capsular wrinkling and inefficient function of accommodating intraocular lenses (IOLs) (Nanavaty et al., 2011; van Bree et al., 2011).

5.2 Time Course of the Mouse Cataract Surgery Model

After fiber cell extraction is performed in the mouse lens, the expression pattern of a number of genes is altered over time (Medvedovic et al., 2006). However, the initial timing of these changes in gene expression is less well understood. Desai et al., (2010) was among the first to look at the immediate effects of fiber cell removal. This study focused on the up-regulation of *CD44*, a cellular receptor for hyaluronan that has been implicated in fibrosis progression. They found that CD44 up-regulates in residual LECs 12 to 24 hours post-surgery, just prior to the up-regulation of αSMA (Figure 5.1)(Desai et al., 2010), however, in the lens, CD44 is abundant on the surface of mouse lens fiber cells, and *CD44* null mice had no deficits in PCO progression so the function of this marker is not certain.

αSMA, a well-known marker for EMT occurring in epithelial cells in response to injury, is up-regulated by the TGFβ pathway (de Iongh et al., 2005; Saika et al., 2004a). In the surgery model used here, (Manthey, 2013), αSMA was up-regulated approximately 24 hours post-surgery, and remained up-regulated at the protein levels 5 days post-surgery (Figure 5.2). Further, E-cadherin, a protein expressed by normal

LECs, is normally down-regulated during EMT in the lens and other tissues (de Iongh et al., 2005) this is apparent by 48 hours after surgery in this model as well (Manthey, 2013).

In this mouse surgery model, the fiber cell differentiation associated with PCO was first detected at five days post-surgery as assayed by the expression of the fiber cell markers Aquaporin 0, cMaf and Prox1in cells not expressing aSMA (Figure 4.4) (Manthey, 2013).Therefore, this allows us to determine a general gene expression pattern in the post-surgery mouse lens. The first changes occurring with CD44 12 to 24 hours post-surgery, followed by αSMA and EMT around 48 hours, and fiber cell marker up-regulation at 5 days post-surgery. This also shows that the murine cataract surgery model is an ideal model for investigating the function of other genes important in the progression and prevention of PCO.

5.3 The Role of Egr1 after Fiber Cell Removal

Notably, *Egr1* is a major player in the development of fibrosis, and has been shown to be up-regulated by a Smad-independent TGFβ pathway, in other tissues. It is also able to directly up-regulate *αSMA* by binding to the EBS in the promoter of the gene (Nakamura et al., 2002). Egr1 has not only been found to be a direct regulator of *αSMA* in other tissues undergoing fibrosis, it has also been found to be a co-regulator of *CD44* expression (Maltzman et al., 1996; Nakamura et al., 2002). In human endothelial cells, Egr1 is a critical transcription factor involved in *CD44* induction by IL-1α (Fitzgerald and O'Neill, 1999). In these cells, IL-1α was able to induce *Egr1*, which in turn bound to an EBS motif at position -301 within the CD44 promoter. Although IL-1α may not be playing a role in *Egr1* up-regulation in the fibrotic lens, as mentioned, many other pathways such as TGFβ are able to regulate *Egr1* expression in fibrosis.

Finally, *Egr1* expression is induced during the formation of cataracts induced by selenite in the rat lens (Nakamura et al., 2002). Therefore, this further supports the hypothesis that *Egr1* is an early and immediate response gene, able to regulate a multitude of genes in response to stress and injury in the lens. Although *Egr1*'s role in these processes within the lens is yet to be determined, it is possible that these same mechanisms of control are operating in the adult lens. Therefore, I hypothesize that *Egr1* functions as an early response gene during PCO, aiding to up-regulate a number of fibrotic pathways. In order to test this, I utilized a previously established murine cataract surgery model that allowed me to investigate the expression pattern of *Egr1* at specific time points after lens fiber cell removal.

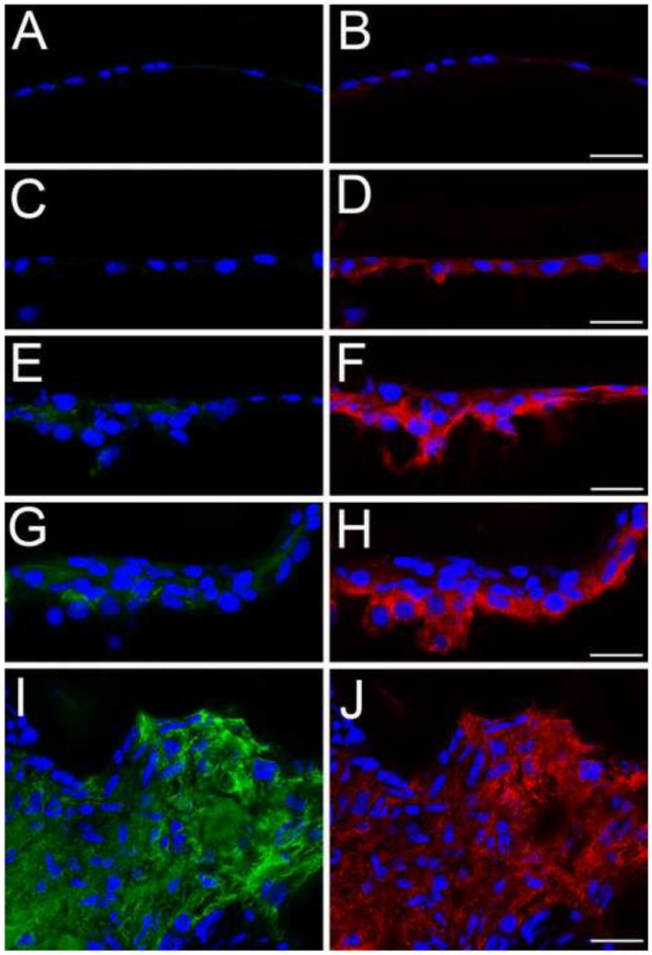

Figure 5.1 CD44 (red) and αSMA (green) protein expression post fiber cell removal. In the murine lens time course, CD44 can be seen up-regulating 12 to 24 hours after removal of fiber cell. The lens epithelium at time 'zero' after surgery shows little to no CD44 (B), and αSMA (A) staining. At twelve hours post-surgery, CD44 (D) can be seen up-regulating, where as there is no αSMA (C) expression. By 24 hours, the level of CD44 (F) protein expression has increased, and αSMA (E) expression has just begun to increase. By 48 hours (G – J) there is robust αSMA (G, I) and CD44 (H, J) expression. Blue staining is representative of cell nuclei, stained by DRAQ5. Scale bar = 24 μm. Adapted from Desai et al., (2010).

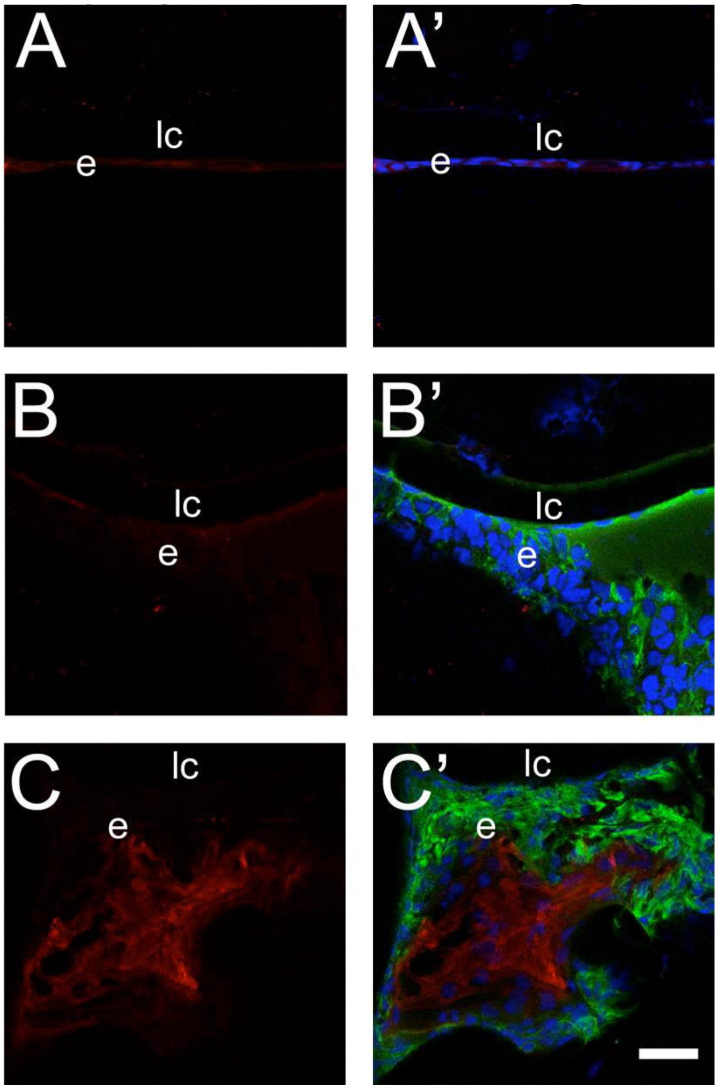

Figure 5.2 Aquaporin 0 expression in a murine PCO model. Aquaporin 0 (red) was not observed in residual lens epithelial cells 0 hours (A) or 48 hours (B) after removal of fiber cells. However, five days post-surgery (C), Aquaporin 0 increased drastically in cells not expressing αSMA (green). Abbreviations: e, residual lens epithelial cells; lc, lens capsule. Prime panels (e.g. A') show Aquaporin 0 expression merged with nuclei (DRAQ5, Blue) and αSMA expression. Scale bar = 35 μm. Adapted from Manthey et al., (2013).

5.4 Egr1: a possible regulator of early gene changes in PCO in wild type lenses

Egr1 is an immediate early response gene which has the ability to up-regulate rapidly in response to injury and stress (Cao et al., 1990; Pagel and Deindl, 2011). It also has the ability to mediate the up-regulation of genes already known to be involved in PCO: *CD44* and *αSMA* (Fitzgerald and O'Neill, 1999; Nakamura et al., 2002). Considering *Egr1* is an immediate early response gene, it was important to investigate early post-surgery time points to determine *Egr1* expression levels. To do so, 0 hour time points were collected as a control to compare to: 3, 6, 12, 24, and 48 hours post-surgery were sampled in wild-type C57Bl/6 lenses. Preliminary results suggested that *Egr1* up-regulation were 127 fold up-regulated 3 hours post-surgery, and gradually decreased to 4 fold up-regulated 48 hours after surgery. However, these preliminary data were found using only one biological replicate.

Although this was very promising evidence of *Egr1*'s involvement in PCO, subsequent trails using three biological replicates were not able to replicate these results. Importantly, *Nab2*, *αSMA* and *CD44* mRNA expression was also investigated alongside *Egr1*. These results did not show the expected up-regulation of *CD44* 12 to 24 hours post-surgery (Table 5.1), nor the expected up-regulation of *αSMA* at 48 hours post-surgery (Table 5.2). *CD44* mRNA expression began at -1.3 fold (S.D. +1021/-2000, insignificant p-value 0.95), and steadily decreased by -7.9 fold at 42 hours post-surgery (S.D. +5.26/-12.5, p-value 0.005)(Table 4.5). *αSMA* expression began at -4.19 fold at 3 hours post-surgery (S.D. +218/-500, insignificant p-value 0.67), and gradually increased by -1.5 fold at 48 hours post-surgery (S.D. +1.3/-1.8, insignificant p-value 0.32)(Table 5.2). Neither *CD44*, nor *αSMA* were able to validate at the expected time point post-surgery in contrast to results obtained by others (Desai et al, Mamuya unpublished). This is most-likely due to poor quality or low quantity of RNA obtained from these post-surgery lens samples. This is evident in the fact that some of the early post-surgery time points yielded 5 ng/μl or less of RNA (Table 2.3).

Nab2 expression post-surgery was similar to that of *CD44* expression, beginning at -1.4 fold 3 hours post-surgery (S.D. +7.68/-16.6, p-value 0.84), and decreasing by -12.7 fold 24 hours post-surgery (S.D. +1.02/-166, p-value 0.09)(Table 5.3). Forty eight hours after surgery *Nab2* expressed was decreased by -3.7 fold (S.D. +3.5/-50, p-value 0.48). *Egr1* on the other hand began at 28.5 fold 3 hours post-surgery (S.D. +1802/-2.2, p-value 0.34), decreased by 2.5 fold 6 hours post-surgery (S.D. +7.6/-1.3, p-value 0.6). By 12 hours post-surgery *Egr1* expression decreased further by -4.5 fold (S.D. +3.5/-5.8, p-value 0.34), and steadily decreased by -5.1 fold at 48 hours post-surgery (S.D. +2.9/-9.1, p-value 0.32)(Table 5.4). Due to the large deviation among biological replicates, more wild-type post-surgery time point samples will need to be collected and analyzed to determine *Egr1* expression in PCO.

Although the mRNA expression analysis using qRT-PCR was inconclusive, preliminary immunofluorescent evidence supports the hypothesis that Egr1 is rapidly expressed after lens injury. Figure 5.3, shows strong nuclear Egr1 immunolocalization in wild type lens epithelial cells 3 hours post-surgery, as compared to 0 hours after surgery.

5.5 Egr1 absence from the lens does not cause a decrease in the fibrotic response 5 days after surgery

Egr1 is known to be direct regulator of *αSMA* and *CD44* (Maltzman et al., 1996; Nakamura et al., 2002), importantly, it’s also known to be a direct regulator of fibronectin in other tissues (Baron et al., 2006). It’s been shown to be an important regulator of fibrosis and is able to be up-regulated by TGF-β signaling (Bhattacharyya et al., 2013; Bhattacharyya et al., 2011b). Therefore, if you are able to remove *Egr1* from the lens, would the fibrosis observed after injury decrease?

To investigate this, *Egr1* null mice were obtained, and the mouse cataract surgery model was used to investigate the fibrotic response to injury five days after surgery. Fibronectin and Collagen I were investigated to determine if the fibrotic PCO, activated by TGF-β signaling, was decreased or absent after cataract surgery (Figure 5.4 and 5.5, respectively). It's important to note that a proper wild type control lens was not used. Due to time constraints a C57Bl/6 four day post-surgery sample was used, instead of a 5 day sample. Although these are preliminary results, a very slight decrease in the Fibronectin, and no decrease in Collagen I was observed in the *Egr1* null five day post-surgery samples.

5.6 Discussion

Posterior capsular opacification (PCO) is a side-effect of cataract surgery which can obscure the light path from the external environment to the retina, resulting in the need for further treatments for the patient to retain vision. The current method for correcting PCO is Nd:YAG laser capsulotomy, which can leave patients with a number of other ocular problems including corneal damage, macular edema, glaucoma and retinal detachment (Billotte and Berdeaux, 2004). To be able to prevent PCO from occurring after cataract surgery, it is important to investigate genes which may be aiding the up-regulation of pro-fibrotic genes such as fibronectin, *CD44* and *αSMA*. *Egr1*, an immediate early response gene that is rapidly and transiently expressed due to many different stimuli including injury, has been known to regulate a number of pro-fibrotic genes in other tissues (Bhattacharyya et al., 2013; Pagel and Deindl, 2011). Importantly, it is has been shown to regulate fibronectin, *CD44* and *αSMA* in other tissues (Fitzgerald and O'Neill, 1999; Nakamura et al., 2002).

To investigate *Egr1* involvement in PCO, two methods were employed. Both of these methods used our mouse cataract surgery model, however, differed in the presence or absence of *Egr1*. The first method was to investigate the normal expression pattern of *Egr1* at both the mRNA and protein level directly after surgery in wild type C57Bl/6 mice. From this investigation, although preliminary, we are able to conclude the *Egr1* is up-regulated at the protein level 3 hours after cataract surgery, and is most-likely highly up-regulated at the transcript level as well. This *Egr1* mRNA expression decreased steadily from 3 to 24 hours post-surgery and remained low through 48 hours. *Nab2*, Egr1's negative regulator, expression was unable to be investigated at the transcript level. This was most likely due to low *Nab2* transcript levels in both the wild type and biological replicate samples. For example, three hours post-surgery, the threshold values for *Nab2* were unable to be detected in one of the biological replicates, and two other technical replicates in other samples. This general low transcript level remained even 48 hour post-surgery, with *Nab2* threshold values ranging between 37 and 45. However, based on results observed in the embryonic lens, it is expected that *Nab2* would drastically increase shortly after *Egr1* expression increased at 3 hours post-surgery to control the amount of Egr1 activity, then decrease as the level of *Egr1* decreased.

The second method employed to investigate *Egr1*'s importance in PCO was to utilize the mouse cataract surgery model on mice lacking *Egr1*. In the absence of *Egr1*, it was expected that the fibrotic response observed after cataract surgery would be decreased or even absent. Preliminary results investigating αSMA, fibronectin and collagen I expression five days post-surgery in the *Egr1* null lens, showed no difference in these markers of fibrosis. This however, does not negate the involvement of *Egr1* in PCO, but rather suggests that other pathways are able to compensate for the loss of *Egr1* from the lens. Overall, the up-regulation of *Egr1* transcript and protein 3 hours post-surgery suggests a very immediate role for *Egr1* within the PCO response. Although *Egr1* may not be the only critical mediator of the PCO response, it could be one of the earliest genes found to be up-regulated after cataract-surgery.

Table 5.1 *CD44* mRNA expression levels using qRT-PCR analysis. *Calculated in Microsoft Excel, wild type set equal to 1 **Calculated using nested ANOVA, S.D. – standard deviation, n=3.

Post-surgery time point	Fold Change in comparison to 0hr*	+/- S.D.	0hr +/- S.D.	p-value**
3 hr	-1.34	1021/2000	1.58/0.63	0.95
6 hr	-1.65	1.2/2.27		0.2
12 hr	-3.42	1.6/7.14		0.09
24 hr	-7.97	5.26/12.5		0.005
48 hr	-4.14	1.75/10		0.09

Table 5.2 *αSMA* mRNA expression levels using qRT-PCR analysis. *Calculated in Microsoft Excel, wild type set equal to 1 **Calculated using nested ANOVA, S.D. – standard deviation, n=3.

Post-surgery time point	Fold Change in comparison to 0hr*	+/- S.D.	0hr +/- S.D.	p-value**
3 hr	-4.19	218/500	1.77/0.56	0.67
6 hr	-2.48	1.2/5.2		0.2
12 hr	-4.55	1.4/16.6		0.15
24 hr	-1.85	1.27/4.3		0.41
48 hr	-1.52	1.3/1.8		0.32

Table 5.3 *Nab2* mRNA expression levels using qRT-PCR analysis. *Calculated in Microsoft Excel, wild type set equal to 1 **Calculated using nested ANOVA, S.D. – standard deviation, n=3.

Post-surgery time point	Fold Change in comparison to 0hr*	+/- S.D.	0hr +/- S.D.	p-value**
3 hr	-1.41	7.68/16.6	8.75/0.11	0.84
6 hr	-5.66	4.03/142		0.5
12 hr	-5.15	3.85/111		0.39
24 hr	-12.72	1.02/166		0.098
48 hr	-3.74	3.9/50		0.48

Table 5.4 *Egr1* mRNA expression levels using qRT-PCR analysis. *Calculated in Microsoft Excel, wild type set equal to 1 **Calculated using nested ANOVA, S.D. – standard deviation, n=3.

Post-surgery time point	Fold Change in comparison to 0hr*	+/- S.D.	0hr +/- S.D.	p-value**
3 hr	28.46	1802/2.2	8.26/0.12	0.34
6 hr	2.47	7.67/1.3		0.6
12 hr	-4.53	3.5/5.8		0.34
24 hr	-4.88	3.3/7.14		0.32
48 hr	-5.07	2.9/9.1		0.32

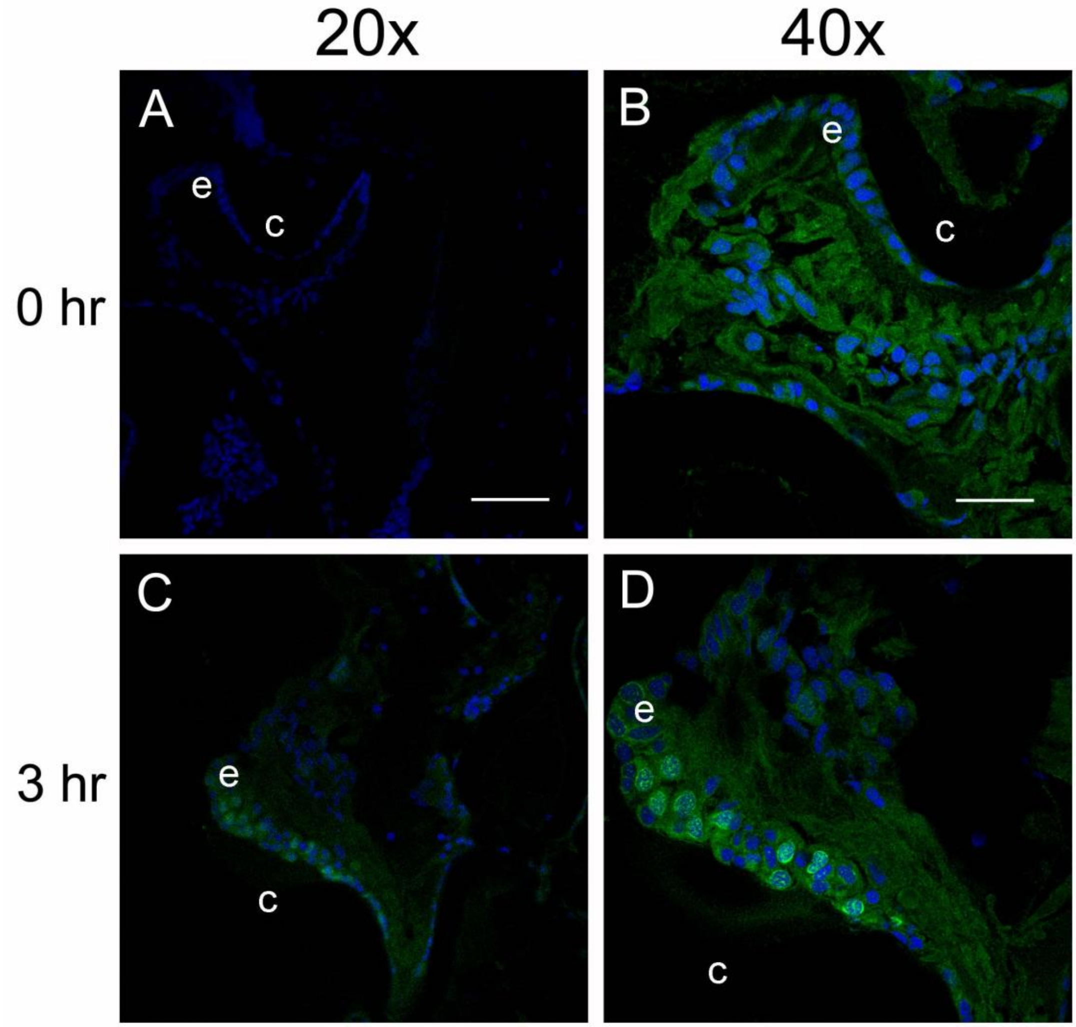

Figure 5.3 Egr1 protein up-regulation 3 hours post cataract surgery. Strong nuclear Egr1 (green) expression can be seen in the 3 hour post-surgery lens epithelial cells (C, D) in comparison to 0 hours post-surgery (A, B). Abbreviations: c – capsule, e – residual lens epithelial cells. Blue stain is Draq5 nuclear staining. Scale bars: A, C – 62 μm; B, D – 32 μm.

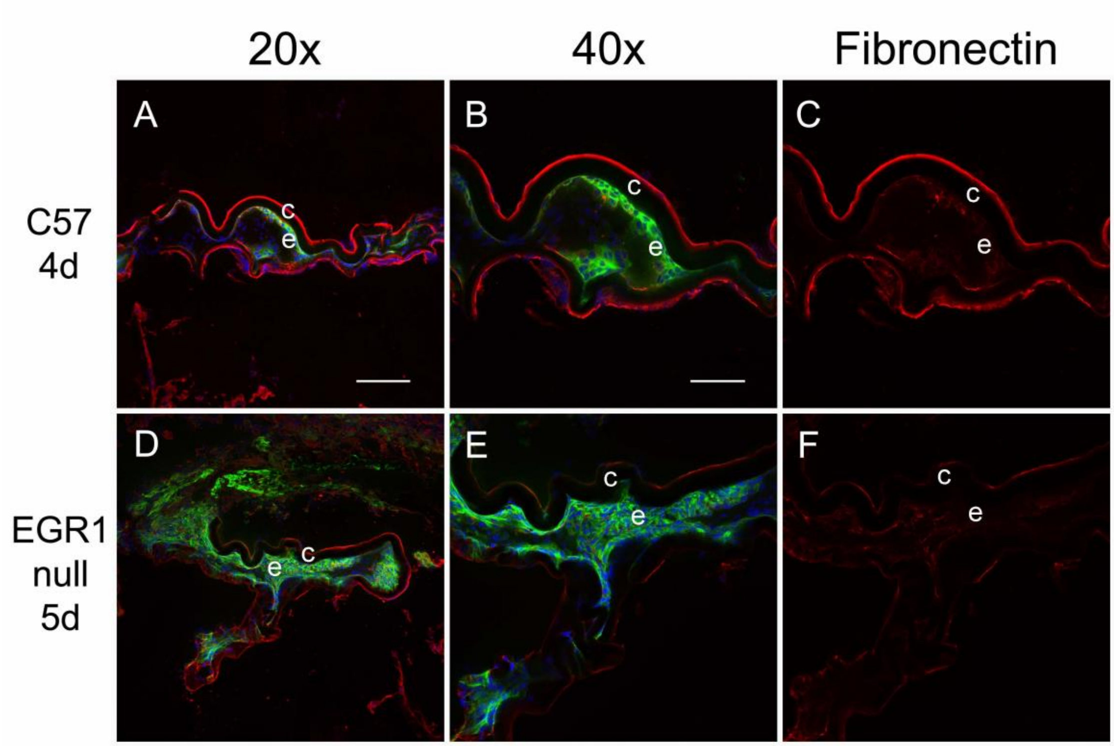

Figure 5.4 Fibronectin expression in Egr1 null 5 day (5d) post-surgery samples. A slight decrease in fibronectin (red) can be observed 5 days post-surgery in lenses lacking Egr1 (F) in comparison to wild type 4 day (4d) post-surgery lenses (C). αSMA (green) expression was not decreased in these samples. Abbreviations: c – capsule, e – residual lens epithelial cells. Scale bars: A, D – 62 μm; B, C, E, F – 35 μm.

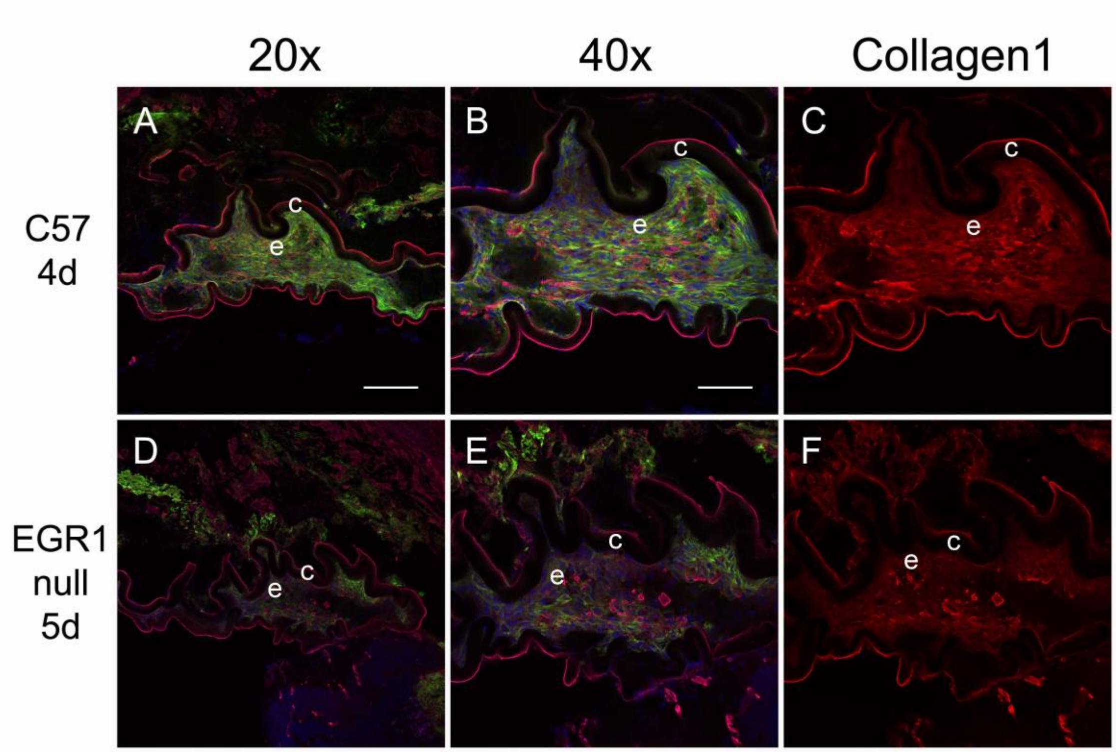

Figure 5.5 Collagen I expression in Egr1 null 5 day (5d) post-surgery samples. No decrease in Collagen I (red) can be observed 5 days post-surgery in lenses lacking Egr1 (F) in comparison to wild type 4 day (4d) post-surgery lenses (C). αSMA (green) expression was slightly decreased in these samples. Abbreviations: c – capsule, e – residual lens epithelial cells. Scale bars: A, D – 62 μm; B, C, E, F – 35 μm.

Chapter 6

CONCLUSIONS AND FUTURE DIRECTIONS

In the absence of β1-integrin from the mouse lens at E12.5, lens epithelial cells lose their normal epithelial markers, and up-regulate some fiber cell markers by E16.5 (Simirskii et al., 2007). Also around this time, αSMA, a marker for epithelial to mesenchymal transition (EMT) was also observed to be up-regulated in the lens epithelial cells (Hay, 2005; Simirskii et al., 2007). Although other markers of EMT, such as vimentin, were not found to be increased in the β1MLR10 lens epithelial cells as expected (Riggio, 2011).

Importantly, one immediate down-stream mediator of the β1-integrin signaling cascade was found to be dys-regulated. Focal adhesion kinase (FAK), a member of the integrin adhesome, was found to be down-regulated in the E16.5 lens (Kokkinos et al., 2007; Riggio, 2011). FAK normally activated the MAPK signaling pathway, however, in the β1MLR10 lens, this pathway expression and activation remained unchanged (Longhurst and Jennings, 1998; Riggio, 2011). Therefore, the decrease in FAK activation was unable to fully explain the partial EMT observed in the E16.5 β1MLR10 lens.

To be able to determine what pathways and genes may be differentially regulated in the β1MLR10 lens, RNA-Sequencing analysis, a novel technique in the lens was used. Wild type and β1MLR10 lens samples were collected and analyzed at E15.5, one day prior to the onset of the phenotype in an attempt to obtain gene changes that are the proximal cause of the E16.5 phenotype. From this analysis, 90 genes were found to be differentially regulated in the E15.5 β1MLR10 lens, using a fold change cut-off of 2.5 or greater. Out of these 90 genes, there were many that were known to play a role in fibrosis. Two genes of particular interest, *Egr1* and *Nab2*, were found to be up-regulated more than 10 fold in the β1MLR10 lens.

Egr1, a transcription factor, is rapidly and transiently expressed due to many different stimuli including shear stress, oxygen deprivation, growth factors and injury (Cao et al., 1990; Khachigian et al., 1997; Ouellette et al., 1990; Pagel and Deindl, 2011; Yan et al., 1999). It is also known to be able to regulate a number of different signaling cascades by binding to target gene Egr1 binding sites (EBS's)(Cao et al., 1990; Pagel and Deindl, 2011). Importantly, *Egr1* is also able to regulate fibrosis, and is known to be an immediate and early response gene in response to injury (Bhattacharyya et al., 2013). *Egr1* is not only able to mediate a multitude of different signaling pathways, it is also able to up-regulate itself, and is involved in a negative feedback loop with Ngfi-A binding protein 2 (*Nab2*) (Pagel and Deindl, 2011). This allows *Egr1* to tightly regulate its own expression in response to a stimulus. Finally, in other tissues, it has been found that *Egr1* is under the control of the integrin/FAK/SRC signaling pathway (Barbolina et al., 2007). Therefore, this makes *Egr1* a likely mediator of the β1MLR10 fibrotic phenotype.

Interestingly, out of the 90 genes found to be differentially expressed in the β1MLR10 lens, 38 of these genes were found to have potential EBS's. Eight of these genes were chosen for further analysis based on their connections with *Egr1*, integrins or involvement in fibrosis. These genes include: *Egr1*, *Nab2*, *αSMA*, *Mmp14, Plat*, *Anxa2*, *Stmn1* and *Rpl29*. Two others were also investigated based on their involvement in stress response and fibrosis, but were not found to have EBS's, including *Mt1* and *Thbs1*. Although some of these genes, *Mt1*, *Thbs1*, *Stmn1* and *Rpl29*, were unable to be validated using qRT-PCR, the others investigated were able to be validated at E15.5.

Egr1 mRNA was found to be highly up-regulated not only at E15.5, but three days prior to the onset of the phenotype at E13.5. This expression level continued to increase, then peak around E15.5. Egr1 protein expression was also found to be up-regulated at E15.5 and E16.5. Coinciding with *Egr1* up-regulation, *Nab2* was found to up-regulated at E13.5. This up-regulation increased drastically to more than 150 fold at E14.5 and E15.5, than decreased to 2 fold at E16.5. This shows that *Nab2* is able to quickly up-regulate to be able to control Egr1 up-regulation in response to injury, in this case the loose of β1-integrin from the early embryonic lens. *αSMA*, a marker for EMT, was found to be up-regulated at E14.5 at the transcript level, and continued to increase steadily to 20 fold at E16.5. Importantly, *αSMA* is not only able to be regulated by TGF-β pathway activation, *Egr1* has been found to directly bind and up-regulate *Nab2* expression in other tissues (Bhattacharyya et al., 2011a; Langmann et al., 2009). The observed *αSMA* mRNA expression coincides with previous evidence of its up-regulation at the protein level at E16.5.

Mmp14, a matrix metalloproteinase, is another gene that is found to be directly regulated by Egr1 in other tissues (Barbolina et al., 2007). *Mmp14* is also known to play an important role in disassembly of the extra cellular matrix (ECM) and fibrosis (Gingras and Beliveau, 2010). Interestingly, *Mmp14* was not only found to be 3 fold up-regulated at E15.5 via RNA-Seq analysis, it was also found to be up-regulated at E13.5 and remained high through E16.5 via qRT-PCR analysis. Other genes implicated in fibrosis, *Plat* and *Anxa2*, were found to have potential EBS's but were not found to be directly regulated by *Egr1* in other tissues. These genes followed a similar pattern as *Mmp14*, up-regulation of expression at E13.5 which remained high through E16.5.

Overall, *Egr1* was found to be highly up-regulated 3 days prior to the onset of the β1MLR10 phenotype. Its known target genes, *Nab2, αSMA* and *Mmp14* were also found to be up-regulated at E13.5 or E14.5 and remained high through E16.5. Altogether, this strongly shows that *Egr1* is an immediate and early response gene that is able to up-regulated *Nab2, αSMA* and other genes involved in fibrosis to drive the partial fibrotic response observed in the β1MLR10 lens at E16.5 (Figure 6.1).

Future directions on this aspect of my thesis will include further validations of *Egr1*'s involvement in the β1MLR10 phenotype. The mechanism with which *Egr1* is able to control the fibrotic phenotype will also need to be elucidated. To do so, *Egr1* null mice have been obtained from Jackson Laboratories, and are currently being bred to our β1-integrin floxed/floxed, MLR10-cre positive mouse strain (β1MLR10). By breeding these two strains together, we hope to be able to rescue the fibrotic phenotype observed in the β1MLR10 lens. To date, Yichen Wang has been able to continue with this aspect of the project to provide compelling preliminary evidence that when both β1-integrin and *Egr1* are lost from the lens, the β1MRL10 phenotype is partially rescued (Figure 6.2).

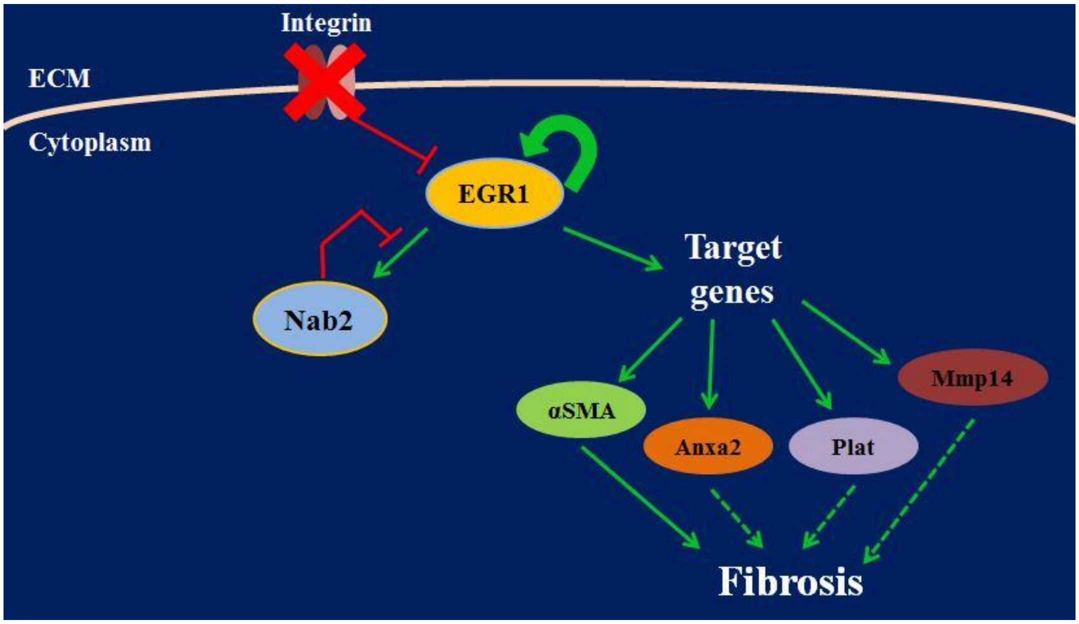

Figure 6.1 Proposed model of the *Egr1* mediated fibrotic response in the lens. In the absence of β1-integrin, *Egr1* is able to up-regulate itself, and concommittingly up-regulates *Nab2* to control its own expression. This up-regulation of Egr1 causes the up-regulation of *αSMA*, *Mmp14*, *Plat* and *Anxa2* which may help to mediate the fibrotic response observed in the E16.5 lens.

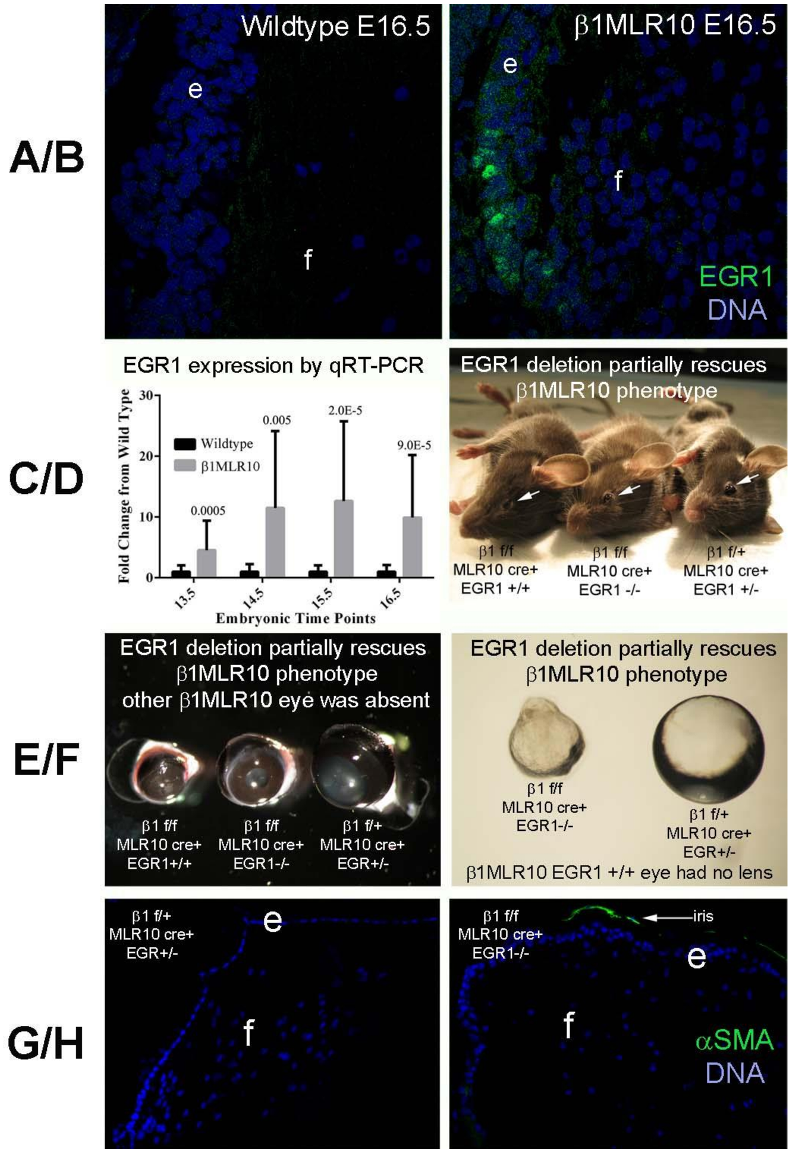

Figure 6.2 β1MLR10 phenotype is partially rescued by the absence of *Egr1*. Egr1 (green), which is suggested to be responsible for the up-regulation of αSMA and fibrotic responses, is shown up-regulated in the β1MLR10 lens at E16.5 (B) in comparison to the wild type (A), and is also shown to be up-regulated 3 days prior to the onset of the phenotype at E16.5 (C). By crossing *Egr1* null mice with B1MLR10 mice, the original β1MLR10 phenotype is able to be partially rescued (E – H). The *Egr1* heterozygous and homozygous null, β1MLR10 mice exhibit increase eye and lens size (E/F), and decreased αSMA expression (G/H) at E16.5.

Egr1 is not only involved in fibrosis, but has also been implicated in selenite cataract development in rats (Nakamura et al., 2002). It is also able to directly up-regulate *αSMA*, as well as *CD44* and fibronectin (Bhattacharyya et al., 2013; Maltzman et al., 1996; Nakamura et al., 2002). This makes investigating *Egr1*'s role in posterior capsular opacification (PCO) particularly interesting, in that it is possibly regulating the EMT/fibrotic response in the process of PCO. To investigate this *Egr1*, *Nab2*, *αSMA*, and *CD44* expression levels were observed in post cataract surgery wild type mouse lenses. The time points observed were 3, 6, 12, 24 and 48 hours post surgery.

Although preliminary results with one biological replicate showed an *Egr1* up-regulation of more than 127 fold 3 hours post surgery, this same expression level was not observed when three biological replicates were used. Using three biological replicates, *Egr1* was found to be up-regulated 28 fold 3 hours post surgery. This expression remained high until 12 hours post-surgery when the expression began to down-regulate. It's important to note that the deviation in all of these samples was too great to be able to obtain a significant p-value. This deviation is most-likely due to the variability of the mouse cataract surgery model itself. Although a very good tool for study PCO in the mouse, the caveat is the number of lens epithelial cells remaining in the capsular bag cannot be controlled. Therefore, some samples may have more or less remaining lens epithelial cells than another.

To overcome the deviation in samples, future work would be to collect more biological replicates to test the expression of these genes on. A sample size of 6 or more may be needed in order to overcome the large amount of deviation observed in the PCO qRT-PCR results.

Although qRT-PCR analysis remains inconclusive, I was able to show an up-regulation of Egr1 protein 3 hours after surgery. Due to time constraints, this was only observed with one biological replicate and would need to be repeated at least two more times.

Finally, to be able to show that *Egr1* is important in the process of PCO, *Egr1* null lenses were subjected to the same cataract surgery model as the wild-type mice. These lenses were then stained for markers of fibrotic PCO using fibronectin and collagen I. Overall, the preliminary results suggest that fibronectin expression may be slightly decreased 5 days post-surgery in the *Egr1* null lens samples. Collagen I, however, remained the same. It is possible that I did not observe a great decrease in fibronectin and collagen I because *Egr1* may not be the only mediator of the fibrotic response in PCO. It is possible that *Egr1* is playing a very early role in the up-regulation of pro-fibrotic genes. It is also possible that by 5 days post-surgery, the absence of *Egr1* is already compensated for by other pro-fibrotic signaling pathways in the lens.

Again due to time contraints, only two bioligical replicates were used, and wild type samples used were only 4 days post-surgery. In the future, this experiment needs to be repeated with 10 - 15 biological replicates and the correct C57Bl/6 5 day post-surgery controls. I suggest 10 – 15 biological replicates for this staining for the same reasons that I would suggest using 6 or more biological replicates for qRT-PCR analysis. The amount of variation in the mouse cataract-surgery model is too great to be able to use only 3 biological replicates. It would also be useful to observe the fibrotic response within the *Egr1* null lenses at earlier time points, such as 24 – 48 hours post-surgery to see if *CD44* and *αSMA* up-regulation are delayed. Altogether, these experiments will need to be repeated several more times to be able to conclude *Egr1*'s early and immediate involvment in the PCO. The preliminary results, however, suggest that *Egr1* is rapidly and transiently expressed post cataract surgey, and may be mediating the fibrotic response within these lenses.

Overall, this project and thesis have shown, that *Egr1* is a novel and most-likely important mediator of fibrosis in the lens.

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Appendix **A**

COMPARISON OF LENSES IN DIFFERENT MOUSE STRAINS

Table A Full list of over 2,000 differentially expressed genes in comparison between Sip1 floxed/floxed no-Cre and C57Bl/6 wild type strains. This comparison was done comparing Sip1 WT to C57 WT (Sip1WT expression/C57 WT expression), meaning that Snhg9 is expressed higher in the C57Bl/6 wild type.

Feature ID	Fold Change	FDR p-value correction	WT RPKM Mean	AG RPKM Mean
Snhg9	6.560	0.00002522	1.141	7.789
Ccbe1	5.015	0	0.564	2.977
Eif3j	4.700	0	6.090	19.638
Adamts4	3.686	0.000005195	1.019	3.935
Gm7120	3.640	0	1.823	6.596
Lars2	2.896	9.463E-11	150.924	467.785
Mid1	2.711	0.001724	6.708	18.485
Tdg	2.567	7.948E-14	4.191	16.113
Plxna1	2.419	0.015	7.150	12.987
Pcp4l1	2.315	5.572E-11	2.089	4.963
Tmcc3	2.248	2.329E-08	7.486	9.652
Rab6b	2.119	2.072E-08	2.115	4.747
Zfp365	2.109	0	18.844	41.634
Hipk2	2.098	0.014	6.953	15.085
Rpl3	2.060	0	346.625	605.698
Rpl13	2.042	0.007218	4.342	7.927
Tssc4	1.997	0.013	5.887	7.890

Tuba1c	1.959	4.145E-08	2.571	6.961
Ankrd52	1.959	0.061	4.649	8.378
Xist	1.957	0.009291	8.743	15.659
Igf1r	1.950	0.00007429	3.798	7.850
Notch3	1.942	0.02	4.162	6.671
Mon1b	1.919	0	2.228	4.421
Parm1	1.888	0.001016	1.813	4.693
Pkd1	1.874	1.577E-07	2.015	4.027
Ints1	1.872	1.318E-11	5.990	11.904
Srcap (7 134655540..134679890)	1.872	0.067	2.544	5.081
Nynrin	1.871	0.225	7.437	10.147
Dhcr24	1.869	0.036	14.881	23.204
Lrp1	1.855	0.038	9.848	19.114
Dcakd	1.819	2.113E-14	35.494	59.519
Trmt61a	1.806	0	2.707	5.124
Gja8	1.798	0.193	28.797	67.695
Fat1	1.795	0.000536	13.758	26.234
Ptbp1	1.787	6.451E-11	29.076	44.609
Trrap	1.785	0.000008243	6.153	11.549
Hspg2	1.783	0.123	33.786	64.747
Plcg1	1.770	0	3.117	5.153
Gcn1l1	1.763	0.00008154	4.696	8.563
Flnb	1.761	0.002639	6.753	12.666
Sec14l5	1.756	1.362E-13	3.007	5.556
Eif2c2	1.756	0.057	3.179	5.987
Cad	1.739	0.00000265	5.068	9.311
Pus1	1.726	0.002283	3.146	5.355
Plxnb2	1.706	1.205E-09	17.374	30.828
Slit3	1.705	0.013	9.755	17.645
Alox12	1.693	0	7.828	14.082
Ubr4	1.693	0.002169	8.256	14.365
Specc1	1.692	2.233E-07	3.252	5.810
Trim32	1.682	0.059	2.923	5.828
Fasn	1.673	0.003011	17.092	29.866
Acvr2b	1.671	0.00001648	3.323	5.804

Fn1	1.670	0.00002004	5.600	10.041
Ap2a1	1.666	0	41.990	64.395
Sirpa	1.649	0	2.912	5.122
Dync1h1	1.647	0.001633	17.316	29.918
Abca2	1.637	2.485E-12	7.096	12.401
Prrc2c	1.630	0.002475	13.981	23.715
Pold1	1.617	0	3.366	5.771
Ndst1	1.613	0.062	3.780	6.506
Tnpo2	1.611	0.014	26.635	31.393
Kif26b	1.608	0.094	2.369	4.403
Mrs2	1.605	0.079	2.957	4.968
Pcnxl3	1.600	6.571E-07	4.252	7.176
Neurl4	1.599	1.023E-07	6.902	11.631
Odz3	1.597	0.009709	11.894	19.658
Copa	1.588	1.318E-11	11.294	18.674
Gatad2b	1.586	0.065	4.712	7.834
Ncor2	1.584	0.000002277	5.612	9.386
Pom121	1.580	0.002548	9.423	14.732
Gm14230	1.573	1.898E-12	4.720	7.682
Crim1	1.562	0.094	168.993	193.182
Wdfy3	1.555	0.018	3.203	5.300
Flna	1.548	0.011	12.433	20.334
Add2	1.541	0.001578	17.657	28.830
Man2b2	1.540	0	6.085	9.922
Ptch1	1.539	0.017	4.103	6.729
Wasf3	1.538	1.528E-11	4.123	6.716
Prpf8	1.537	0.005474	22.396	35.898
Man2a2	1.533	0.0004839	7.110	11.511
Lama5	1.533	0.0009622	6.170	9.942
Ldlr	1.532	0	6.686	10.930
Prrc2b	1.532	0.046	11.092	15.737
Stac2	1.531	0	6.384	10.415
Pdcd11	1.529	0.001225	4.256	6.553
Plk1	1.528	0	7.332	11.888
Ncapd2	1.522	0	8.636	13.884

Il6st	1.522	0.0001199	5.906	9.566
Prmt1	1.520	0	43.639	68.753
Dapk3	1.520	0	11.419	18.460
Cpsf1	1.517	6.6E-13	8.282	13.262
Lama1	1.515	0.004164	18.036	28.427
Carm1	1.507	0	11.776	16.348
Isyna1	1.505	1.869E-08	11.080	17.642
Eif4ebp2	1.505	0.086	6.484	10.311
Ptprf	1.502	0.085	37.550	58.851
Tsta3	1.501	0	12.045	19.179
Sbf1	1.501	1.977E-08	8.066	12.766
Sned1	1.499	0	4.603	7.004
Cdc42bpb	1.493	0.01	6.419	10.005
Arhgef17	1.484	3.769E-10	4.761	7.479
Copg	1.480	5.488E-12	8.550	11.415
Fbxw5	1.479	0	8.021	12.639
Myo9b	1.478	0.0003657	4.066	6.395
Fzd3	1.478	0.045	4.262	6.741
Sin3a	1.477	0.000514	7.647	9.764
Mcm5	1.474	0	7.290	11.468
Rptor	1.473	0.0002374	3.583	5.593
Fads2	1.471	0.0004467	16.408	25.915
Nisch	1.471	0.002239	21.598	33.147
Tmem181b-ps	1.471	0.077	8.203	12.149
Hcfc1	1.470	6.387E-07	10.454	16.118
Reep4	1.469	6.988E-08	4.011	6.123
Tbl3	1.468	0.000003209	4.009	6.261
Sympk	1.466	0	12.008	18.435
Pex19	1.466	0	8.129	12.788
Bub1b	1.466	1.014E-10	5.117	7.958
Nnat	1.466	0.139	9.215	14.049
Btbd2	1.464	3.264E-09	6.306	9.830
Tspan4	1.462	2.114E-08	4.671	7.018
1810055G02Rik	1.460	0.0001014	4.758	7.317
Srebf2	1.457	5.695E-07	19.560	29.620
Dst	1.456	0.006314	8.891	13.635

Ckap5	1.454	1.532E-07	16.262	24.419
Rps6ka2	1.452	0.003498	12.893	19.642
Lamc1	1.452	0.019	20.550	31.303
Arl10	1.452	0.14	4.740	7.254
Bloc1s1	1.451	0.012	24.329	30.040
Kcnq4	1.450	0	3.777	5.837
Tln2	1.450	0.002228	5.890	8.981
Tomm34	1.448	3.372E-10	4.316	6.663
Mamdc4	1.447	0.0005295	2.112	4.784
Spnb1	1.447	0.01	60.220	88.665
Tnks	1.447	0.196	3.851	5.927
Abcc8	1.445	0	8.747	13.396
Celsr2	1.445	0.001356	9.227	13.817
Amotl1	1.443	0.008335	3.913	5.958
Nomo1	1.443	0.014	26.580	39.624
Herc2	1.443	0.052	4.433	6.681
Nup188	1.439	0.000001433	6.145	9.738
Slc25a23	1.439	0.003416	7.966	10.310
Lrrc32	1.437	5.672E-11	4.202	6.401
Llgl1	1.436	1.344E-09	8.530	12.978
Anks1	1.436	5.553E-09	3.685	5.754
Med16	1.436	0.002851	5.367	8.206
3110056O03Rik	1.435	0	4.918	7.485
2610507B11Rik	1.435	0.0002726	13.637	20.048
Igf2r	1.430	0.015	23.694	35.441
Huwe1	1.429	0.002733	7.632	11.474
Olfml3	1.427	0.000008001	7.811	11.978
Plec	1.427	0.011	19.606	29.661
Ubc	1.426	2.473E-09	78.508	117.265
Kirrel	1.426	0.0003203	4.790	7.222
Ehmt1	1.425	0.000002792	3.901	5.961
Txnrd1	1.421	0	12.229	18.254
Eef2	1.420	0.000004746	536.973	782.869
Tubb2c	1.419	0	130.489	188.768
Dhx38	1.419	1.621E-07	5.348	8.078
Tfip11	1.418	0	5.796	8.500

Plxnb1	1.418	0.0002693	4.349	6.524
Kctd10	1.418	0.042	9.361	12.069
Ap1g1	1.418	0.151	5.318	8.249
Ccnd2	1.417	0.007836	65.902	100.820
Acaca	1.417	0.143	4.716	7.086
Kcnj12	1.416	0.005841	14.386	21.573
Thbs1	1.416	0.049	8.784	13.061
Tnrc18	1.414	0.027	6.044	9.011
C230081A13Rik	1.413	0.079	5.509	8.850
Rapgef1	1.411	0	6.846	10.058
Gtf3c1	1.411	0.002353	13.988	20.519
Edc4	1.410	4.047E-12	5.726	8.595
Mapk8ip3	1.409	6.077E-14	7.493	10.676
Dag1	1.409	0.156	15.650	23.069
Cyhr1	1.408	5.726E-11	6.642	9.992
Extl3	1.408	0.000000106	5.292	7.956
Wdr6	1.406	0.00003368	21.775	31.212
Id1	1.402	1.845E-10	12.914	19.196
Mau2	1.402	0.00006383	4.753	7.086
Mtmr9	1.401	0.157	5.865	8.664
Itga3	1.400	5.092E-13	6.151	9.116
Smarca4	1.400	0.000001792	31.373	46.650
Cabin1	1.400	0.000001986	4.659	6.895
1110014N23Rik	1.399	4.121E-14	4.341	6.366
Ccdc85c	1.398	0.189	13.020	19.352
Ncln	1.397	0	10.073	14.926
Ptpru	1.397	2.964E-07	61.234	90.239
Arhgap23	1.395	1.607E-09	8.465	12.512
Cmtm7	1.395	0.002998	7.061	10.259
Lfng	1.393	0	11.506	17.040
Pdgfra	1.393	0.00006581	15.632	22.467
Dnmt1	1.392	0	12.085	17.738
Mki67	1.389	0	9.434	13.495
Thop1	1.389	4.813E-09	9.539	13.952
CAAA01108492.1.1364.1	1.389	0.0002463	5.801	8.389
Grlf1	1.389	0.0002517	7.390	10.847

D2Wsu81e	1.389	0.001251	6.413	9.176
Adam12	1.389	0.111	4.484	6.570
Zfp335	1.388	2.906E-08	5.835	8.254
Tln1	1.388	0.00009355	7.440	10.903
Spnb2	1.387	7.244E-08	65.321	89.211
Map3k4 (17 12420488..12511614)	1.386	8.19E-10	6.178	8.869
Bcr	1.386	9.17E-09	6.640	9.761
Mlec	1.386	2.345E-08	23.514	33.808
Srebf1	1.385	1.249E-07	7.291	10.649
Ncor1	1.385	0.028	12.661	16.772
Uggt1	1.384	0.000004404	5.206	7.585
Hyou1	1.383	0	13.559	19.541
Mtap1s	1.383	9.443E-08	8.939	13.163
Ep300	1.383	0.000005607	4.716	6.840
Jmjd8	1.383	0.0002213	6.421	10.470
Pip5kl1	1.382	4.219E-13	19.498	28.311
Nt5dc2	1.382	9.918E-12	155.246	224.013
Vars	1.381	0	12.895	18.762
Bop1	1.381	0	12.047	17.776
Wdtc1	1.380	0	5.030	7.306
Rgs8	1.380	1.451E-10	13.719	19.761
Rrp12	1.380	0.000008848	5.688	8.357
Kdm5c	1.379	0.013	14.596	20.819
Hip1	1.377	0.00003702	21.895	31.373
Wfs1	1.375	0	5.585	8.158
Mvd	1.374	0.0003257	4.010	6.213
Tomm40l	1.373	0.0006135	6.329	8.562
Fbxw8	1.372	0.131	12.024	16.234
Supt6h	1.371	2.12E-11	15.633	22.071
Sulf1	1.370	0	12.018	17.286
Cand2	1.370	6.927E-13	20.217	28.988
Mgrn1	1.370	0.0001126	17.149	22.672
Atp13a1	1.368	0	9.768	14.125
Hunk	1.368	7.093E-10	5.063	7.361
Tmem132a	1.368	0.001711	34.165	41.909

Scrib	1.367	2.113E-14	9.285	13.252
Xpo5	1.367	0.022	9.239	13.372
Cul7	1.366	0.014	7.710	13.207
2310021P13Rik	1.365	0	8.301	11.970
Scube3	1.365	3.702E-13	11.035	15.777
Zfp523	1.365	2.217E-11	6.717	9.716
Mcm3	1.365	0.000003297	9.913	14.513
Atp9b	1.365	0.0001172	4.761	6.902
Stab1	1.364	1.875E-07	7.099	10.208
Polr2l	1.364	0.027	26.747	32.375
Sec24c	1.364	0.092	12.886	18.093
Stra6	1.363	0	11.088	15.886
Ap3d1	1.363	2.522E-08	14.380	20.551
Slc7a5	1.363	0.084	91.180	117.137
Lrig1	1.361	2.805E-11	6.061	8.700
Lman2	1.359	6.6E-13	14.365	20.488
Polr1a	1.359	0.223	5.869	8.463
Mcm2	1.356	0	20.373	29.016
Pnpla6	1.356	0	12.189	17.279
Th1l	1.356	3.346E-13	12.618	18.184
Ndufs7	1.355	0.0006972	18.494	26.883
Trpm3	1.355	0.094	17.307	24.332
Nacc1	1.353	0	19.986	29.409
Ptpn23	1.353	0	11.149	15.713
Xpo6	1.353	0	6.324	8.913
Mrpl4	1.351	0.00007459	7.572	10.465
Pogk	1.351	0.0007413	6.149	8.686
Upf1	1.350	0	10.569	15.000
Pip5k1c	1.350	0	4.957	7.119
Mast2	1.347	0	8.069	11.330
Flot2	1.347	1.453E-09	19.460	27.368
Uhrf1	1.346	6.1E-12	9.135	13.511
Prox1	1.345	0.009654	69.485	97.627
2510039O18Rik	1.344	0	9.907	14.210
Mtor	1.344	8.626E-11	16.299	22.514
Prrc2a	1.344	2.433E-07	32.420	44.874

Ptpn11	1.344	0.006526	12.083	17.108
Sesn3	1.343	0.201	16.707	23.601
Atp13a2	1.342	1.399E-08	10.707	15.085
R3hdml	1.342	0.00001194	52.371	70.262
Abcb8	1.342	0.0001512	5.109	7.285
Bmp7	1.341	3.409E-07	7.330	10.518
Bysl	1.340	0	11.353	16.007
Leprel1	1.340	5.782E-13	19.136	25.918
Cndp2	1.340	2.911E-10	10.520	15.052
Scap	1.339	2.328E-08	4.679	6.964
Ptprs	1.339	4.437E-08	13.075	18.401
Spnb3	1.338	0.00009849	37.059	51.191
Pacs2	1.337	0	26.066	36.239
Mif	1.337	0.0005925	599.319	848.305
5930434B04Rik	1.334	0.00001741	5.595	7.910
Cic	1.334	0.0008474	7.068	9.964
Ddx39b	1.334	0.005377	33.904	46.440
Pepd	1.333	0	20.274	28.257
Pkdcc	1.332	3.62E-12	7.405	10.504
Mbtps1	1.331	0	11.111	15.630
Tubb5	1.330	0.00001572	218.681	306.723
Supt5h	1.329	0	28.829	39.948
Ogdh	1.329	2.298E-07	16.572	23.303
Myh10	1.329	0.0007998	7.381	10.410
Dvl3	1.328	0.000000566	6.548	9.296
Rpl36	1.327	0.281	332.826	381.762
Ipo4	1.326	8.045E-12	19.864	26.719
Slc2a1	1.326	0.0000191	200.565	225.540
Wdr46	1.325	1.953E-08	9.572	13.515
Ruvbl2	1.325	0.0003495	15.105	21.218
Zbtb17	1.324	1.472E-07	4.971	6.989
Tpra1	1.324	0.0002969	18.268	25.158
Gpr125	1.323	0	5.302	7.484
Spg7	1.323	0.00002379	5.779	7.975
Mapk14	1.323	0.0002323	5.610	7.880
Ascc2	1.322	0	13.244	18.413

Myst3	1.322	0.07	5.273	7.391
Ube3b	1.320	4.121E-14	7.981	11.168
Acly	1.320	0.000007082	19.427	26.664
Slc7a11	1.320	0.005912	7.569	10.704
Adsl	1.319	0	8.732	12.174
Dpp9	1.319	2.799E-12	7.400	10.400
Ccdc97	1.319	1.344E-08	8.901	12.502
Upp1	1.319	0.019	10.384	14.504
Psmb3	1.319	0.141	31.456	42.616
Cdk2ap1	1.319	0.286	25.862	30.761
Yif1a	1.318	0.000001233	10.400	14.454
Rabl5	1.318	0.001454	9.394	13.125
Arhgap39	1.317	4.503E-08	6.845	9.637
Dak	1.317	0.0006035	6.569	8.728
Kdm4b	1.317	0.001475	5.012	7.043
Rnpepl1	1.316	0	12.619	17.749
Por	1.316	3.129E-12	10.613	14.807
Dctn1	1.316	0.00008629	16.077	22.206
Sec16a	1.316	0.017	9.020	12.509
Tbcd	1.316	0.131	6.034	8.218
Fam125b	1.315	2.161E-08	6.929	9.687
Ubr2	1.315	0.002829	5.712	7.950
Slc35a4	1.314	0	14.589	20.308
Actr1b	1.314	0.0009369	12.854	17.840
Fgfr1 (8 26624125..26686186)	1.313	0	9.491	13.417
Grk6	1.313	0	7.625	10.649
Wdr4	1.313	3.686E-12	5.235	7.291
Ogdhl	1.313	0.00004084	5.129	7.171
Obsl1	1.312	0	5.740	7.969
Tbrg4	1.312	9.842E-14	6.928	9.649
Crb2	1.311	0.002934	9.977	13.690
Dcaf15	1.309	1.97E-10	8.707	12.088
Vps33a	1.309	0.0004332	5.902	8.258
Zfp106	1.308	0	14.736	20.046
Arl5a	1.308	0	8.703	12.037

Prmt2	1.308	0.000001943	5.700	7.838
Zdhhc16	1.308	0.000002016	11.420	14.433
Celsr1	1.308	0.077	5.492	7.575
Rhot2	1.307	0.000000236	6.651	10.102
Mfn2	1.307	0.00006132	9.281	12.819
Dpagt1	1.306	0	17.708	24.716
Qtrt1	1.306	0.00135	7.870	10.873
Gas6	1.305	0	20.065	27.412
Zdhhc8	1.305	0	5.498	7.606
Mthfd1	1.304	0	5.875	8.118
Trpc4ap	1.304	6.386E-09	6.192	8.426
Prep	1.304	0.000008065	16.755	23.130
Birc6	1.304	0.063	5.363	7.775
Mfge8	1.303	0	49.082	66.697
Apeh	1.303	0	17.474	24.182
Mybbp1a	1.303	5.782E-13	20.293	27.270
Mllt1	1.303	5.315E-12	9.511	13.241
Adrbk1	1.303	1.205E-09	5.492	7.618
Cgnl1	1.303	0.002949	17.834	24.142
Ptov1	1.302	0	42.120	57.264
Bap1	1.302	0	14.433	19.808
Med24	1.302	0	5.415	7.509
Nphp1	1.302	0.00000155	6.307	8.737
Pcnx	1.302	0.038	6.641	9.116
Cdh4	1.301	0.00008843	9.190	12.679
Acot7	1.301	0.013	8.680	11.977
Gtpbp1	1.300	0.0004907	9.654	13.454
Rbck1	1.299	0	6.024	8.311
Nol6	1.299	0.00013	6.347	8.762
AY036118	1.299	0.239	620.691	849.795
Myh9	1.297	0.000002685	11.240	15.288
41160	1.295	0	28.552	38.181
C2cd2l	1.295	0.000000313	6.366	8.768
Rfc2	1.295	0.003863	16.847	23.137
Fam40a	1.294	0	41.316	55.029
Pde2a	1.294	0.000006262	21.272	28.653

Nup214	1.294	0.00003005	6.206	8.243
Nup205	1.294	0.005233	6.197	8.475
Snrnp200	1.294	0.046	15.648	20.920
Cux1	1.293	1.919E-13	4.616	6.713
Wwp2	1.293	2.017E-09	8.800	12.060
Mafg (11 120486430..120494914)	1.293	2.204E-07	34.325	46.420
Trak1	1.293	0.0001777	13.610	18.627
Dhx8	1.292	0.001555	5.675	7.795
Mavs	1.292	0.013	6.521	8.965
Patz1	1.292	0.047	6.904	8.916
Hyal2	1.291	9.96E-12	8.564	11.736
Gbf1	1.291	0.002398	24.416	32.871
Arhgef12	1.291	0.225	10.184	13.175
Rnf123	1.290	0.00003011	22.238	30.624
Ttll12	1.289	0	6.945	9.514
Fzr1	1.289	1.569E-09	7.545	10.361
Usp11	1.289	9.796E-08	12.477	16.732
Ddx56	1.289	0.00000028	8.616	11.826
Pld3	1.289	3.484E-07	34.630	46.297
Skiv2l	1.288	0.00002703	10.909	14.821
Spns1	1.288	0.003846	6.438	8.779
Elp3	1.287	0	19.253	25.877
Zmiz2	1.287	2.559E-07	6.849	9.371
Lamb1	1.287	0.003089	22.682	29.846
Slc7a2	1.287	0.023	23.454	31.597
Scamp4	1.286	1.67E-12	30.663	40.918
Bckdk	1.285	0	8.211	11.175
Cnot3	1.284	0.0009333	8.892	11.889
Ap2b1	1.284	0.014	13.454	18.388
Ncdn	1.283	0	7.998	10.951
Ift172	1.283	6.831E-10	6.561	8.962
Clasp1	1.283	0.000001618	9.075	12.194
Hras1	1.283	0.001167	11.236	15.282
Itpk1	1.281	0	18.384	24.648
Tmc6	1.281	0.000003141	6.792	9.254

Vav2	1.281	0.00002373	6.371	8.658
Tkt	1.280	0	51.799	68.921
Pik3r2	1.280	0.001411	5.621	7.625
Egln2	1.279	0	8.682	11.850
Ppp1r10	1.279	1.398E-10	9.989	13.394
Pskh1	1.279	3.483E-10	6.188	8.377
Pgd	1.279	5.647E-10	9.164	12.209
Apba3	1.279	0.116	8.277	11.153
Hfe	1.278	1.51E-11	12.087	16.476
Traf7	1.278	1.62E-11	15.944	21.543
Sgta	1.278	6.534E-07	39.382	53.404
Rere	1.278	0.00002978	11.370	15.291
Uck2 (1 169156220..169215258)	1.278	0.000395	11.943	16.119
Aen	1.276	1.098E-12	8.903	11.223
Abcf2	1.276	2.007E-09	18.625	24.480
Ckb	1.275	0.000008681	85.345	113.296
S1pr3	1.274	0	19.127	25.832
2610301G19Rik	1.274	0	11.984	16.215
Eml2	1.273	1.534E-08	19.559	26.172
Dalrd3	1.273	0.001991	12.333	15.377
Itfg3	1.271	4.739E-13	14.114	18.749
Psen1	1.271	3.836E-09	18.574	24.693
Mcm7	1.271	0.00000212	9.851	13.265
Zc3h7b	1.271	0.000766	19.625	25.688
Wsb2	1.270	0	7.578	9.711
Ncstn	1.270	9.897E-07	21.423	28.768
Slc24a4	1.269	0	28.547	37.937
Efemp2	1.269	3.506E-09	8.038	10.853
Rhog	1.269	0.015	6.822	9.149
Plekhg3	1.268	2.445E-10	6.897	9.313
Crtap	1.268	2.466E-07	11.112	13.812
Srprb	1.268	0.0000659	12.295	15.773
Rasl11b	1.268	0.007399	83.728	108.286
Pxdn	1.268	0.092	44.245	59.506
Kdm4a	1.267	0	12.904	17.159

Prr12	1.267	0	8.676	11.416
Cherp	1.266	7.45E-10	16.792	21.205
Igf2bp1	1.266	0.018	8.437	11.280
Aco2	1.265	0	37.873	48.680
Srm	1.265	7.089E-13	27.538	37.041
Bcor	1.265	0.007165	6.546	8.777
Rad54l2	1.265	0.047	6.196	8.221
Men1	1.264	0	7.336	9.870
Ctbp1	1.263	0	27.962	36.849
Mospd3	1.263	0.078	9.046	12.141
Trim28	1.262	0	46.341	61.644
Wdr18	1.262	0	13.568	18.143
Lman1	1.262	0	10.891	14.410
Polr2i	1.262	0.026	12.082	16.238
Fbxo21	1.260	4.723E-09	7.668	10.290
Dennd5a	1.260	0.0004137	7.067	9.298
Hgs	1.259	0	14.549	19.339
Flad1	1.259	0.000001301	28.648	30.891
Pmpca	1.258	0	34.984	41.659
Zyx	1.258	4.121E-14	6.671	8.934
Gna12	1.257	0	21.776	28.850
Rela	1.257	0.02	7.994	10.634
Epb4.1l4a	1.256	3.008E-13	56.284	75.170
Git1	1.256	3.456E-10	5.976	7.983
Ipo13	1.256	2.386E-07	6.096	8.149
Abhd8	1.256	0.000001959	16.976	22.726
Eml4	1.256	0.001072	10.571	13.823
Agpat6	1.256	0.001733	9.483	12.621
BC046331	1.255	5.695E-07	6.763	9.036
Akt1	1.255	0.000001691	38.843	50.347
Ccnd1	1.255	0.001481	41.970	52.648
Cyfip2	1.255	0.108	10.093	13.327
Parp1	1.254	0	28.304	37.086
Trib1	1.254	1.788E-07	11.443	14.953
Lmf2	1.254	2.268E-07	7.110	9.459
Mapk1ip1l	1.254	0.156	15.211	17.630

Dmwd	1.253	0.00001377	25.001	33.210
Grina	1.253	0.00001793	35.295	46.049
Srrm2	1.253	0.0002616	36.519	46.819
Eif3l	1.252	0	69.013	88.621
0610009O20Rik	1.252	0	12.431	16.531
Polr3e	1.252	9.868E-11	6.052	8.088
Mtch1	1.252	1.261E-07	45.946	58.991
Csk	1.252	0.00002303	14.472	19.187
Neo1	1.252	0.001969	9.535	12.471
Scd2	1.252	0.006422	251.561	312.393
Kif13a	1.251	0.021	6.351	8.436
Arhgdia	1.250	0	96.273	128.964
Nr1h2	1.250	2.821E-12	6.701	8.943
Syvn1	1.250	0.0001535	7.210	9.515
Med15	1.249	2.982E-11	7.952	10.564
Gnb2	1.248	4.144E-08	38.127	45.411
Klhl21	1.248	0.0000125	10.788	14.263
Grwd1	1.247	8.189E-10	8.948	11.851
Ddx49	1.247	0.124	10.037	12.848
Zfp445	1.247	0.196	6.626	8.748
Pkm2	1.246	7.576E-08	586.106	755.580
Ttyh3	1.246	8.64E-08	24.169	31.247
Numa1	1.246	0.001432	12.664	16.531
Ctnnd2	1.245	0	27.515	35.511
Rplp0	1.245	2.113E-14	602.265	743.814
Atmin	1.244	0.001925	8.103	10.686
Uhmk1	1.244	0.248	12.550	16.303
Fam134a	1.243	1.876E-12	11.629	15.404
Dhcr7	1.242	1.694E-08	6.586	8.733
Macf1	1.242	0.026	8.300	10.811
Arid1a	1.242	0.1	12.527	16.291
Lonp1	1.241	7.018E-10	15.741	20.606
D19Wsu162e	1.241	0.000001174	7.567	9.995
Sf3a1	1.239	0	12.282	16.009
Top2a	1.239	0	10.545	13.734
Cdca7	1.239	4.531E-12	8.165	10.717

Ubac2	1.239	5.65E-12	13.408	17.661
Brd4	1.239	5.769E-12	7.193	9.262
Phrf1	1.239	8.88E-10	7.140	9.316
Smyd5	1.239	0.000002318	8.425	11.050
Drosha	1.239	0.000009907	17.364	22.480
D17Wsu92e	1.238	6.181E-12	25.402	32.732
Baz1b	1.238	0.0001187	9.515	12.319
Ppm1f	1.238	0.012	17.435	22.785
Fem1a	1.237	0	8.488	11.639
Wars	1.237	8.463E-09	11.328	14.894
Zfyve1	1.236	1.047E-10	8.806	11.567
Tmem214	1.235	0	6.841	8.952
Mcfd2	1.235	0.0002391	24.430	31.482
Eif3d	1.234	0.00002215	69.874	90.731
Jazf1	1.234	0.004604	10.642	14.012
Rcan1	1.233	0	15.403	20.182
Kctd2	1.233	0.008291	6.582	8.599
Ppp6r1	1.232	0	28.940	37.062
Nipal3	1.232	0.00001148	6.703	8.729
Gss	1.232	0.0001086	71.639	82.466
Apbb1	1.232	0.0006587	17.181	22.481
U2af2	1.232	0.000986	74.597	95.800
Gpaa1	1.231	0.000006406	11.562	15.114
Dcaf7	1.231	0.000007776	11.476	14.786
Gdi1	1.231	0.00001033	19.576	25.424
Drg2	1.231	0.00006965	11.923	15.577
Myl12b	1.231	0.003049	17.137	14.795
4632411B12Rik	1.230	0.000007375	7.223	9.422
Usp5	1.230	0.0003773	18.930	24.583
Slc25a1	1.230	0.002202	8.683	11.243
Dpm3	1.230	0.23	12.623	16.318
Odc1	1.229	0.000000869	55.954	74.551
Pofut2	1.229	0.002217	10.886	14.207
Dnm2	1.228	0	11.712	14.628
Rpl6	1.228	0.001874	168.115	183.839
Grik5	1.227	2.113E-14	10.285	13.410

Rnf114	1.227	6.26E-11	13.785	17.871
Cdc20	1.227	0.0000149	8.472	10.941
Tpcn1	1.227	0.001703	10.595	13.747
Cs	1.226	0	34.962	45.376
Asap1	1.226	0	27.626	34.703
Mll5	1.226	3.514E-10	8.382	10.519
Ap2s1	1.226	0.246	41.046	52.458
Gtf2i	1.225	0	37.709	47.467
Atic	1.225	0	10.742	13.697
Hmg20a	1.225	0.00005146	15.817	20.131
Creld2	1.225	0.005752	12.414	16.034
Grn	1.225	0.011	18.712	23.847
Smg5	1.225	0.029	9.457	12.338
Dicer1	1.224	0.0001078	7.625	9.789
Sart3	1.224	0.001527	8.495	11.037
Gstp2	1.224	0.231	46.955	61.128
Itgb5	1.223	0.0000444	38.286	48.070
Sf3b3	1.223	0.000047	28.069	35.250
Col4a2	1.223	0.038	193.042	233.475
Incenp	1.223	0.182	8.314	10.808
Ppp2r4	1.222	0	12.894	16.430
Dbnl	1.222	4.757E-07	147.485	184.721
Zc3h4	1.222	0.002512	6.996	9.047
9430023L20Rik	1.222	0.05	14.145	18.262
Atp6v0e2	1.221	0	28.763	36.807
Mta1	1.221	0	25.946	33.383
Ap2a2	1.221	1.899E-07	12.750	16.639
Adamtsl4	1.221	0.0006254	11.799	15.235
Ints4	1.221	0.031	9.391	12.188
Pygm	1.220	0	96.483	122.569
App	1.220	0	80.339	100.297
Eif3b	1.220	0.000005325	62.405	79.879
9130401M01Rik	1.220	0.004574	8.092	10.442
Cyb5b	1.220	0.025	10.221	13.236
Raf1	1.219	9.842E-14	9.229	11.420
Farp1	1.219	1.364E-11	7.843	10.111

Ap1m1	1.218	0.000002268	13.906	18.000
Stat5b	1.218	0.02	7.911	10.623
Uba1	1.217	1.811E-07	78.241	100.788
Phldb1	1.217	5.587E-07	7.735	9.861
BC057022	1.217	0.004232	8.596	11.120
Cstf1	1.216	2.794E-12	8.549	11.083
Gja3	1.216	0.000002178	305.185	397.490
Arf3	1.216	0.00003087	30.711	38.792
Polr3c	1.216	0.009734	8.761	11.290
Atp6v0a2	1.216	0.03	9.416	12.107
Kat2a	1.215	0	15.900	20.122
Txndc5	1.213	0	21.933	27.889
Ap1b1	1.213	0.003872	9.358	12.059
Tssc1	1.213	0.038	7.471	9.521
Atxn2	1.212	4.432E-10	12.635	16.157
Itga6	1.212	0.015	21.319	27.460
Gck	1.212	0.088	10.860	14.054
Rnf40	1.211	2.833E-13	13.066	16.584
Nup93	1.211	9.092E-11	9.462	12.130
Pacsin2	1.211	7.044E-09	10.547	13.574
Nde1	1.211	0.00001336	7.263	9.362
Ranbp3	1.211	0.028	13.602	17.540
Tmed4	1.210	0	18.973	24.401
Nploc4	1.210	0.001738	11.021	13.984
Rangap1	1.210	0.004236	27.262	34.902
Slc7a6	1.210	0.006878	15.684	19.869
Cryba4	1.209	0	11994.996	11857.705
Gaa	1.209	2.539E-07	16.419	20.833
Pkp4	1.209	0.0001552	11.936	15.114
Bckdha	1.209	0.0008552	12.683	16.403
Gart	1.209	0.0009307	17.817	22.008
Ctxn1	1.209	0.011	29.039	37.379
Lrrc47	1.208	0.099	23.853	31.009
Anapc5	1.207	0	40.319	51.332
Id3	1.207	0.0006518	17.867	22.876
Ddx23	1.207	0.013	11.629	14.624

Kif1a	1.207	0.046	53.738	69.881
Cotl1	1.206	0	377.803	477.194
Rrm1	1.206	0	9.974	12.771
Timp2	1.206	1.251E-12	19.614	24.907
Flii	1.206	2.49E-08	16.179	20.647
Epha2	1.206	0.00002123	14.482	18.201
Cenpt	1.206	0.00002695	7.953	10.166
Nolc1	1.206	0.000139	12.536	16.015
Snrpa	1.206	0.072	36.144	46.359
Sort1	1.206	0.095	9.658	12.157
Tbc1d22a	1.205	1.289E-08	19.482	24.386
Sipa1l3	1.205	2.545E-07	53.240	67.874
Ddb1	1.205	0.046	101.426	129.607
Sdk2	1.205	0.211	9.517	11.826
Pold2	1.204	3.579E-09	15.130	19.315
0610011F06Rik	1.204	0.062	8.494	11.559
Gak	1.203	0.004142	11.451	14.383
Hlcs	1.203	0.005346	11.896	14.872
Eftud2	1.202	1.143E-12	10.508	13.509
Mrps23	1.202	0.000006363	17.033	21.655
Wdr1	1.202	0.00003355	47.581	59.055
Trim35	1.202	0.00004113	15.647	19.727
Csnk1g2	1.202	0.00195	28.396	35.594
Mllt4	1.202	0.003464	11.404	14.270
Abhd14a	1.202	0.226	7.802	9.863
Hdlbp	1.201	0.001988	36.287	45.648
Aip	1.201	0.029	10.598	13.509
Foxk2	1.200	0	12.839	16.047
Sec61a1	1.200	2.113E-14	20.289	25.050
Dnpep	1.200	2.665E-08	8.439	10.738
Cope	1.200	0.277	28.405	35.872
Lrrc59	1.199	0.0004393	27.284	34.201
Ece1	1.199	0.0007746	12.821	16.064
Iars	1.199	0.045	36.095	46.002
Uqcrc1	1.198	0	76.179	94.023
Gba	1.198	2.207E-08	12.139	15.515

Ctsz	1.198	0.0008904	54.003	68.412
Mdh2	1.198	0.002037	57.088	70.862
Gps1	1.198	0.051	51.577	67.236
Surf4	1.197	0	55.024	67.054
Capns1	1.197	0.014	82.986	104.298
Slc25a22	1.197	0.209	8.731	11.083
Cbfa2t2	1.196	0	9.141	11.657
Dnajc5	1.196	4.318E-11	11.292	14.259
Clstn1	1.196	0.000001024	32.140	39.557
Evl	1.196	0.046	17.135	19.719
Fam50a	1.195	0.016	9.244	11.749
Snd1	1.194	0	14.953	18.760
Gprc5c	1.194	0	10.747	13.403
Clk3	1.194	0.000001727	9.800	12.019
Hsp90ab1	1.194	0.000003563	851.563	949.362
Fam168b	1.194	0.085	11.130	13.841
Ilf3	1.193	3.372E-10	12.873	15.924
Hs6st1	1.193	1.279E-08	8.539	10.848
Larp1	1.193	0.081	23.202	28.832
Tubg1	1.193	0.089	17.126	23.082
Ppp2r1a	1.192	0	49.631	61.511
Eif3c	1.192	5.5E-10	103.178	127.497
Pex6	1.192	0.001988	5.280	7.296
Kidins220	1.192	0.013	13.530	16.899
Tmem222	1.192	0.207	7.797	9.825
Fau	1.192	0.228	622.064	582.423
Mip	1.191	0.0002536	3835.280	3621.664
Clpp	1.190	0.194	22.337	28.290
Slc1a4	1.190	0.259	12.838	16.101
Fads1	1.189	0	30.459	37.520
Tprgl	1.189	0.281	11.807	13.910
Dnajc13	1.189	0.285	10.703	13.463
Fsd1	1.188	0.00001173	9.847	12.366
Mest	1.188	0.00003071	68.337	75.279
Gga1	1.187	0.000001562	16.041	20.123
Prmt5	1.187	0.003424	15.761	19.370

Dohh	1.187	0.007876	9.052	11.426
Nup50	1.186	0.00007258	13.393	16.434
Gna11	1.186	0.00009743	9.087	11.477
Hsf1	1.186	0.0003319	12.021	15.174
Etv5	1.186	0.005368	23.182	30.006
Sec14l1	1.185	0.028	23.422	28.610
Polr2a	1.184	6.034E-07	24.751	30.708
Ints3	1.184	0.00001873	21.784	27.061
Atf7ip (6 136467371..136555898)	1.184	0.045	7.915	9.993
Kif1c	1.184	0.049	9.816	11.841
Cpsf3	1.183	1.216E-10	21.057	26.129
Sox13	1.183	0.00009264	11.371	14.134
Peg3	1.183	0.008458	20.642	25.370
Pycrl	1.183	0.048	14.165	17.647
Ubr5	1.183	0.082	17.546	21.536
Clptm1	1.182	0	19.671	24.568
Cdk16	1.182	3.879E-12	20.758	26.018
Nrbp1	1.181	0.000004255	13.359	16.646
Ccnb1	1.181	0.008121	6.112	8.425
Tubgcp2	1.181	0.012	8.631	10.848
P4hb	1.180	0.003203	52.078	62.708
Gbas	1.179	4.39E-12	15.455	19.365
Kars	1.179	0.001534	37.728	47.004
Sec13	1.179	0.012	40.426	49.573
Eif2c1	1.179	0.087	9.681	12.120
Klc2	1.178	2.978E-11	9.630	12.100
Tomm40	1.178	0.00001028	10.090	12.258
Usp19	1.178	0.002182	14.578	18.012
Urod	1.178	0.003744	9.525	11.933
Aldh7a1	1.177	1.528E-11	36.984	45.797
Ip6k1	1.177	1.377E-08	9.866	12.355
Ehd1	1.177	0.000001795	33.403	40.445
Scaf1	1.177	0.001089	11.177	13.934
Atg9a	1.177	0.04	10.003	12.506
Rbm14	1.176	7.639E-09	9.910	12.708

Dpp3	1.176	9.367E-09	17.476	21.792
Prpsap1	1.176	0.000002097	13.481	16.539
Tmem97	1.176	0.0009143	11.504	13.763
Aldh18a1	1.176	0.001868	27.980	34.457
Pi4k2a	1.174	2.384E-10	10.103	12.663
Zfp358	1.174	0.149	8.616	10.677
Csnk1d	1.173	0	14.652	17.862
Tm9sf4	1.173	0	11.746	14.563
Cops7a	1.173	1.255E-08	20.164	24.851
Cyc1	1.173	0.000001637	56.968	69.572
Napa	1.173	0.002469	27.177	33.335
Ehmt2	1.173	0.01	16.239	20.159
Glt25d1	1.173	0.096	11.992	14.959
Nars	1.172	0	44.277	53.892
Gnai2	1.172	1.178E-11	105.839	132.015
Maea	1.171	0	45.383	55.807
Tpp1	1.171	2.721E-09	24.047	29.770
Pak4	1.171	0.0006505	16.783	20.408
Abcf1	1.171	0.0009896	23.643	29.246
Nap1l4	1.170	2.113E-14	475.990	529.669
B230312A22Rik	1.170	4.121E-14	27.311	33.229
Golga2	1.170	1.988E-11	8.652	10.772
Ppp5c	1.170	0.00007689	15.069	18.725
Adc	1.170	0.0007218	11.766	14.544
Ndufv1	1.170	0.002964	19.094	23.642
Cdc37	1.170	0.039	42.397	52.218
Eif2b5	1.169	4.445E-12	19.037	23.159
Ddx24	1.169	1.985E-11	16.865	20.678
Mprip	1.169	0.0005016	11.390	13.939
Man1b1	1.169	0.001158	12.276	14.924
Gstp1	1.168	0.00002945	785.143	870.942
Ptk2	1.167	1.269E-08	10.217	12.562
Actr1a	1.167	2.665E-08	53.249	64.078
E2f4	1.167	0.0009237	12.181	15.186
Man1c1	1.167	0.002356	15.932	19.326
Nubp2	1.166	0.009993	11.052	13.633

Fkbp4	1.166	0.048	63.958	78.175
Tubb2b	1.166	0.178	48.305	59.985
Pa2g4	1.165	0.00003639	55.719	67.525
Uqcrq	1.165	0.235	38.924	47.360
Ywhag	1.164	3.189E-13	46.225	55.958
Tatdn2	1.164	0.0006733	8.780	10.832
Got2	1.164	0.009993	32.413	43.662
Eif4h	1.164	0.025	62.872	76.004
Srsf4	1.164	0.095	15.520	17.844
Ndufs2	1.163	0.007104	43.252	53.041
Idh3g	1.163	0.037	35.703	43.849
Mrps27	1.162	0.002493	10.204	12.817
Cited2	1.162	0.171	14.931	18.390
Ddx41	1.161	0.00001958	10.096	12.452
Tmem63b	1.161	0.028	12.581	16.184
Taf6	1.161	0.105	13.481	16.427
Fem1b	1.161	0.284	16.483	20.031
Iqsec1	1.160	0.036	22.833	27.611
Rnf44	1.160	0.114	16.662	20.410
Srp68	1.159	1.173E-12	20.568	24.864
Psmd3	1.159	2.747E-08	19.967	24.400
Puf60	1.159	0.001053	68.558	81.765
Atp2a2	1.159	0.003135	53.317	64.165
D19Bwg1357e	1.159	0.005371	15.880	19.464
Pvrl2	1.159	0.253	9.160	11.205
Cpxm1	1.158	3.435E-07	35.294	43.246
Ndrg4	1.158	0.005918	9.236	11.374
Adamts18	1.158	0.271	29.447	35.632
Cd99l2	1.157	0.004222	11.395	13.765
Usp22	1.157	0.154	14.813	17.955
Oxa1l	1.156	0.016	11.601	14.237
Rrp1	1.156	0.04	63.821	76.037
Actn1	1.155	0.00001423	22.544	27.487
Arvcf	1.155	0.000488	38.463	46.218
Scnn1b	1.155	0.025	13.665	18.824
Ddost	1.155	0.028	63.377	75.040

Ddx54	1.155	0.21	14.663	17.766
Xpnpep1	1.154	7.007E-09	19.634	23.748
Pgls	1.153	0.000009353	11.520	14.133
Dnajc3	1.153	0.0006331	10.461	12.620
Tpr	1.153	0.0008612	11.342	13.465
Usp10	1.153	0.00217	12.917	16.680
Fam32a	1.153	0.013	11.740	14.425
Atp6v0c	1.153	0.094	43.979	54.373
1500032L24Rik	1.153	0.167	75.449	91.206
Acaa1a	1.152	0.005231	14.692	17.913
Wbp11	1.151	0.001109	15.631	18.817
Ski	1.151	0.021	15.994	19.416
Mapre3	1.151	0.049	17.518	21.012
Mars	1.150	0	24.697	29.857
Blmh	1.150	0.000002801	13.612	16.466
Zfand3	1.150	0.0001779	39.530	47.693
Mrpl43	1.150	0.141	27.624	33.769
Cx3cl1	1.149	2.344E-08	12.067	14.753
Raly	1.149	0.017	27.747	31.993
Rcn3	1.149	0.239	69.628	82.473
Fam193a	1.148	3.002E-08	10.413	12.568
Aars	1.148	0.00003711	11.802	14.168
Parva	1.148	0.057	10.674	12.864
Tecr	1.148	0.091	155.399	188.104
Gldc	1.148	0.234	12.687	15.443
Rhobtb2	1.147	0.003056	20.341	24.432
Sall1	1.147	0.18	19.122	22.774
Fibp	1.147	0.234	11.588	13.959
Dnajb12	1.146	1.665E-07	15.688	18.916
Cd151	1.146	0.002521	19.341	23.867
Snap47	1.146	0.014	24.763	29.473
Vac14	1.146	0.099	12.804	15.651
Ubap2l	1.145	0	23.511	28.073
F2r	1.145	0.000002795	14.172	17.176
Slc38a10	1.145	0.00009975	16.135	19.542
Xrcc1	1.145	0.017	14.665	17.849

Alg2	1.145	0.126	12.558	15.318
Ergic3	1.144	0.047	55.023	65.492
Arhgap1	1.143	0.00004076	10.315	12.585
AI837181	1.143	0.004133	9.689	11.690
Six3os1	1.143	0.005341	12.571	15.639
Anapc2	1.143	0.018	32.823	38.861
Atxn7l3	1.142	0.00000296	9.255	11.284
Drd4	1.142	0.11	10.018	12.212
Psmd4	1.142	0.138	44.713	52.761
2310008H09Rik	1.142	0.14	30.726	33.944
Arfgap2	1.142	0.25	7.938	10.361
Gnpat	1.141	1.714E-12	21.104	25.149
Plagl2	1.141	1.761E-12	22.008	25.890
Trap1	1.141	0.001443	17.091	20.753
Clptm1l	1.140	0.00002492	29.814	35.295
Cttn	1.139	0.0001496	39.976	47.215
Klhdc3	1.139	0.092	18.219	23.481
Ppp2r5d	1.139	0.137	8.076	10.594
Usp4	1.138	1.455E-09	22.271	26.658
Cdk9	1.138	0.007934	11.236	13.441
Ptger1	1.138	0.037	12.484	15.131
Exosc10	1.138	0.157	11.389	13.808
Kcnj11	1.138	0.174	10.157	12.259
Golga4	1.137	0.031	17.462	20.349
Slc25a11	1.136	0.000000112	29.195	34.446
Smpd1	1.136	3.869E-07	17.201	20.788
Jub	1.136	0.00001387	9.936	11.955
Actr8	1.136	0.0001119	15.877	19.079
Lgi2	1.136	0.003662	36.110	43.332
Rpl8	1.136	0.198	608.828	738.524
Klc1	1.135	1.762E-10	36.769	42.279
Gramd1a	1.134	0.00003367	15.950	19.104
Spry1	1.134	0.008458	12.216	14.669
Spna2	1.134	0.141	131.104	155.284
Hadha	1.133	0.0005569	18.313	21.870
Tmem184b	1.132	0.000005213	10.882	13.072

Kctd3	1.132	0.027	19.302	22.764
Grb10	1.132	0.188	13.456	15.791
Tspan14	1.131	0.0009018	46.485	54.360
Tdrd7	1.131	0.001963	339.820	363.676
Jag1	1.131	0.003969	57.573	66.624
Rcc2	1.130	0.000005028	23.073	27.462
Actn4	1.129	0.000000308	23.337	25.409
Yipf3	1.129	0.0006599	15.639	18.862
Tfdp1	1.129	0.003701	19.826	23.637
Cog2	1.129	0.023	13.156	15.361
Thoc5	1.129	0.077	12.647	15.121
Ppm1g	1.129	0.162	35.311	41.864
Tmem147	1.128	0.00008413	37.151	44.478
Scamp3	1.128	0.0001143	27.339	32.603
Rpn2	1.127	2.113E-14	38.866	45.326
Reep2	1.127	0.0001331	27.408	32.614
Mpnd	1.127	0.001073	21.123	25.194
2310044H10Rik	1.127	0.033	14.451	17.366
Ppp2ca	1.127	0.058	13.370	16.014
Timm50	1.127	0.114	10.365	12.378
Mbd3	1.127	0.125	31.051	36.352
Dynll2	1.127	0.13	143.811	171.742
Ctdsp2	1.126	0.000001889	20.382	25.814
Agpat1	1.126	0.01	36.580	41.957
Smo	1.126	0.018	15.728	18.425
2700094K13Rik	1.126	0.085	19.819	23.683
Rai12	1.126	0.176	15.762	18.725
Pum1	1.125	0.001701	13.462	15.955
Eef1a1	1.125	0.084	2275.840	2445.799
Asna1	1.125	0.118	56.452	65.874
Zfp146	1.124	0.0003495	13.505	16.148
H13	1.124	0.01	11.301	13.622
Psat1	1.124	0.012	20.877	24.765
Arpc1a	1.123	0.007274	25.545	29.980
Ctnna1	1.123	0.043	45.956	53.564
Rnf187	1.122	0.00005096	166.864	193.277

Ank2	1.122	0.214	49.177	56.982
Cdh2	1.122	0.273	76.010	72.833
Ddx17	1.121	0.00004671	56.165	64.888
Idh2	1.121	0.005469	68.170	80.056
Dlst	1.120	0.000001679	31.039	36.652
Samd4b	1.120	0.024	10.506	12.535
Pfkl	1.119	0.015	41.226	48.442
Coq2	1.119	0.059	11.043	13.270
Psmd13	1.118	4.697E-09	24.408	28.635
Ptdss1	1.118	0.0001355	14.114	16.712
Pcbp4	1.118	0.009651	51.553	60.778
Pea15a	1.118	0.01	70.787	81.297
Ldb1	1.118	0.01	22.770	26.716
Ubqln4	1.118	0.089	17.902	20.887
Rnf10	1.118	0.19	46.210	52.148
Xpot	1.117	0.136	18.153	21.207
Rae1	1.117	0.25	13.330	15.750
Slc38a1	1.116	0.001635	11.557	13.626
ORF61	1.115	0.000001443	11.646	13.724
Dnlz	1.115	0.063	17.582	20.504
Rbm38	1.115	0.095	20.943	26.296
Dnmbp	1.114	0.162	29.282	33.525
Ctsd	1.113	0.0008502	122.724	140.111
Arsi	1.113	0.01	20.419	23.687
Med25	1.113	0.022	24.547	28.567
Psap	1.112	0.0001427	94.393	107.938
Spire1	1.112	0.0006398	13.609	15.757
Nsun2	1.111	2.319E-11	19.209	22.202
Wnt7a	1.111	0.0003821	27.344	32.103
Ddx19a	1.111	0.001046	19.084	22.940
Ezr	1.111	0.002731	189.513	218.554
Slc35b2	1.111	0.163	14.042	16.534
Fgfrl1	1.111	0.208	69.560	80.649
Wipi1	1.110	0.0004569	16.603	19.165
Rab35	1.110	0.005089	11.032	13.043
Ncbp1	1.110	0.017	18.671	22.456

Stx16	1.109	0.0000264	11.670	13.755
Cxxc1	1.109	0.0003155	13.711	16.121
1810026J23Rik	1.109	0.128	13.429	15.838
Fgfr3	1.108	0.028	35.648	41.431
Ganab	1.107	0.088	34.741	39.472
Atn1	1.107	0.288	17.564	20.037
Map2k2	1.106	0.001205	28.771	33.510
Clu	1.106	0.01	84.038	94.547
Atxn2l	1.106	0.147	20.828	24.397
Brf1	1.105	0.0000339	17.607	20.621
Kpnb1	1.105	0.048	87.954	102.711
Efnb2	1.104	0.0008169	31.176	35.286
Nop2	1.104	0.002397	14.548	17.067
Fkbp8	1.104	0.021	23.316	27.046
Eif4a3	1.104	0.07	30.072	36.806
Mtss1l	1.103	0.0004229	22.721	26.025
Sparc	1.103	0.247	296.648	323.244
Pcbp2	1.102	0.085	78.142	81.107
Bcar3	1.102	0.111	30.530	35.512
Sh3glb2	1.100	0.047	13.461	15.531
Dbn1	1.100	0.05	35.366	40.200
Vps52	1.100	0.233	11.060	13.398
Prpf6	1.099	0.001305	15.209	17.427
Ftsj3	1.099	0.068	14.149	16.256
Cct7	1.099	0.072	128.491	145.326
Chtf8	1.099	0.094	21.387	24.486
Prkag2	1.097	1.183E-08	16.601	19.022
Ctdsp1	1.097	0.038	12.253	14.429
Rgp1	1.097	0.255	9.001	11.155
Gorasp2	1.096	0.246	16.947	19.171
Bcar1	1.095	2.355E-07	26.664	30.898
Htra3	1.095	0.138	95.802	108.003
Bag6	1.095	0.225	38.889	45.446
Gprc5b	1.095	0.257	70.458	75.346
Aldh9a1	1.094	0.001934	14.260	16.722
Tnfaip1	1.094	0.019	13.145	15.184

Rab1b	1.094	0.127	29.914	34.261
Dpf2	1.093	0.000001475	13.848	16.045
Cobra1	1.093	0.23	13.084	15.264
Gpi1	1.092	0.057	46.032	52.150
Psmd2	1.090	5.799E-07	59.102	71.936
Pigt	1.090	0.281	20.161	23.315
Hspa8	1.089	0	589.588	603.285
Ldlrad3	1.089	0.068	15.968	18.481
Gns	1.088	0.0001201	19.892	22.501
Sec31a	1.087	0.005455	38.869	43.564
Mknk2	1.087	0.008373	46.116	51.635
Crygn	1.087	0.245	3354.220	3391.836
Aplp2	1.086	0.19	49.948	57.197
Cap1	1.085	0.228	28.241	32.098
Ergic1	1.081	0.0002223	29.478	33.757
C77080	1.080	0.098	15.698	17.849
Tmbim6	1.079	0.0007409	86.350	95.903
Ran	1.078	0.233	143.570	151.454
Sf3b2	1.077	0.022	59.567	67.637
Pitpnb	1.077	0.18	14.798	17.013
Lta4h	1.076	0.022	18.082	20.617
Gnb2l1	1.075	0.001205	143.465	158.198
Ap3b1	1.075	0.007225	17.908	19.961
Pgm2	1.075	0.069	30.821	35.690
Tgfb2	1.075	0.133	18.506	20.991
Gosr2	1.074	0.000516	22.818	25.421
Mark3	1.073	0.000177	25.397	28.753
Sf1	1.073	0.199	19.327	21.602
Wnt7b	1.073	0.216	73.014	80.159
Ubap2	1.071	0.000004316	27.013	29.833
Gde1	1.071	0.209	18.437	20.894
Dhx32	1.070	0.143	39.008	41.360
Lamp1	1.069	0.0001539	109.921	126.980
Pja1	1.069	0.001125	28.487	32.139
Rnh1	1.069	0.242	35.101	38.998
Vit	1.066	1.937E-12	162.450	186.361

9530068E07Rik	1.066	0.136	43.956	47.834
Atp5a1	1.065	0.136	141.610	162.839
Prkcsh	1.064	0.247	43.146	48.361
Rbm10	1.064	0.275	13.918	16.428
Sfxn1	1.063	0.003272	27.749	30.620
Itm2c	1.063	0.009805	32.504	35.994
Emb	1.063	0.135	16.961	19.403
Amfr	1.062	0.009297	41.717	45.752
Add1	1.061	0.086	30.540	34.064
Palm	1.059	0.267	38.600	43.185
Pigs	1.057	0.248	37.445	42.131
Cdipt	1.056	0.216	17.523	19.534
Atp1a1	1.046	0.000222	47.790	52.822
Cct5	1.045	0.046	135.889	145.534
Snx17	1.040	0.205	23.929	26.200
Eif3f	1.036	0.134	50.009	52.929
Aldh1a1	1.031	0.137	414.180	460.927
Zc3h14	-1.056	0.1	24.798	22.641
Tmed10	-1.062	0.002429	38.922	42.353
Ptpra	-1.063	0.0005737	41.780	39.700
Cdkn1c	-1.069	0.281	688.665	610.140
Gipc1	-1.075	0.03	73.831	71.797
Rbbp4	-1.075	0.066	32.419	28.245
Hexb	-1.082	0.266	25.115	22.732
Ywhab	-1.083	0.041	57.927	55.490
Eif3a	-1.086	0.154	82.662	79.210
Riok3	-1.090	0.091	39.660	37.490
Gpr137b	-1.095	0.004222	106.359	111.618
Calm1	-1.096	8.449E-16	76.925	69.841
Serp1	-1.098	0.034	60.426	56.504
Itgb1	-1.098	0.041	80.868	74.665
Dstn	-1.098	0.202	136.721	126.246
Tm9sf2	-1.102	0.084	25.255	23.025
Cdv3	-1.105	0.00002217	108.759	101.614
Arpc4	-1.106	0.004235	18.945	16.939
Akirin1	-1.106	0.116	37.089	33.861

Rad23b	-1.107	0.000334	37.969	35.535
Bpnt1	-1.108	0.00000597	36.971	34.551
Tsg101	-1.108	0.14	30.704	28.576
Rraga	-1.110	0.012	59.886	56.253
Ptcd3	-1.110	0.076	36.215	32.858
Shc1	-1.111	0.012	57.322	53.534
Adam9	-1.112	0.139	32.266	30.178
Fkbp1a	-1.113	0.000005957	42.692	39.337
Lim2	-1.113	0.004525	1346.183	1139.613
Metap1	-1.114	2.302E-10	94.761	89.897
Cct4	-1.117	0.02	38.205	35.062
Ostc	-1.118	0.002798	56.980	53.540
Nras	-1.119	0.088	32.009	29.494
Tmem59	-1.120	0.0064	95.221	87.491
Cox7a2l	-1.121	0.038	76.112	70.224
Atp5b	-1.122	0.003441	203.261	198.773
Eif3m	-1.122	0.006649	32.619	28.008
Mxra7	-1.122	0.05	45.034	42.034
Tmem176b	-1.123	0.21	40.198	36.993
Cul1	-1.124	0.032	32.192	29.730
Psmd8	-1.125	0.031	66.805	61.785
Dars	-1.127	0.00574	28.014	25.737
Nxn	-1.127	0.015	41.362	38.080
AI848100	-1.127	0.017	27.455	24.981
Cnih	-1.128	2.331E-09	51.434	46.379
Anp32e	-1.129	0.116	49.583	45.442
Ppib	-1.131	0.198	158.943	136.664
Prelid1	-1.132	0.001809	123.073	113.537
Rps9	-1.133	0.269	216.122	202.364
Azin1	-1.134	0.24	29.129	26.748
Stk39	-1.135	5.997E-07	50.593	47.186
Irf2bp2	-1.135	0.075	13.564	10.486
Rer1	-1.136	0.02	40.728	37.370
Rpl4	-1.136	0.123	854.557	754.314
Arf4	-1.137	0.000253	41.741	38.575
Idh1	-1.137	0.00079	43.964	40.412

Mboat1	-1.137	0.002122	44.564	40.219
Ghitm	-1.138	1.672E-23	76.184	71.050
Hnrnpf	-1.138	0.038	110.695	106.321
Atp5c1	-1.138	0.107	59.278	55.311
Uqcrc2	-1.138	0.245	61.793	55.098
Igsf9	-1.139	0.00006139	31.111	29.050
Sar1b	-1.139	0.00221	45.366	41.823
Itm2b	-1.139	0.003404	98.324	89.573
Metap2	-1.140	0.0001109	20.974	18.844
Npc2	-1.140	0.0006518	25.275	22.793
Igfbp7	-1.141	0.056	446.722	394.394
Ddx6	-1.141	0.256	28.649	25.845
Pon2	-1.142	0.0009617	49.397	45.674
Srsf7	-1.142	0.208	37.398	33.994
Hnrnpul2	-1.144	3.401E-11	26.861	24.270
Gltscr2	-1.145	0.006084	40.596	37.074
Gstm1	-1.145	0.222	95.778	91.705
Tcp1	-1.146	0.0001304	109.713	98.519
Dhx9	-1.147	0.003635	44.294	41.200
Gabarapl1	-1.147	0.029	78.843	70.842
Pebp1	-1.147	0.055	202.564	196.191
Psmb1	-1.147	0.088	110.777	102.907
Psma2	-1.147	0.288	27.590	24.928
Atf4	-1.148	0.038	90.660	81.044
Akr1a4	-1.148	0.077	95.706	88.110
Fstl1	-1.149	0.003302	74.003	65.468
Rhob	-1.149	0.004133	34.367	31.073
Klhl9	-1.150	0.001504	26.222	23.931
Marcksl1	-1.150	0.067	146.010	133.574
Nutf2	-1.150	0.228	42.636	46.970
Mff	-1.151	0.0001734	19.352	17.013
mt-Co1	-1.151	0.024	2686.718	2118.899
Eif4ebp1	-1.151	0.049	27.982	25.923
Inpp5a	-1.151	0.141	21.986	19.580
Ddx1	-1.152	0.001497	46.907	42.692
1110004F10Rik	-1.153	0.075	40.782	37.046

Snx4	-1.154	3.736E-08	23.024	20.936
Psmd12	-1.154	0.001213	35.842	32.265
Uqcrfs1	-1.154	0.118	45.420	41.000
Fubp1	-1.155	0.003607	26.644	23.915
Anxa6	-1.155	0.017	32.530	29.286
Actr3	-1.159	1.178E-12	52.895	48.391
Cox5a	-1.159	0.00001189	101.450	87.846
Rab2a	-1.161	0.001583	41.219	36.747
Pgk1	-1.161	0.084	185.161	176.183
Hn1l	-1.163	1.008E-12	48.261	43.443
Eif5	-1.164	1.071E-16	95.091	80.222
Snx6	-1.164	3.863E-10	33.983	30.586
Vdac2	-1.164	2.055E-07	31.408	28.919
Rbbp7	-1.164	0.000003193	35.786	31.714
Snx22	-1.164	0.011	43.550	37.759
Cops5	-1.164	0.068	43.997	38.782
Atp5g3	-1.165	0.0002093	71.847	63.403
Ndn	-1.165	0.286	58.491	52.044
Sod2	-1.166	0.007339	17.638	15.428
Atp1b3	-1.166	0.141	120.055	110.420
Gyg	-1.167	0.058	24.536	22.068
Srsf6	-1.167	0.178	61.921	55.453
Rnf115	-1.168	1.406E-11	26.173	23.452
Yaf2	-1.168	0.072	26.473	24.094
Rpl18	-1.169	0.123	106.368	99.290
Tom1	-1.169	0.194	114.094	106.720
Ssu72	-1.170	0.009415	21.447	19.304
Larp4b	-1.170	0.013	21.552	19.179
Cela1	-1.170	0.097	62.281	55.649
Spcs1	-1.171	0.007972	52.769	46.482
Clta	-1.172	0.002027	48.345	42.645
Mrps33	-1.172	0.189	52.139	41.291
Mrpl11	-1.174	0.141	35.347	31.943
Gm14325	-1.174	0.289	11.500	9.300
Lars	-1.176	9.183E-13	59.441	52.942
Clic1	-1.176	0.275	31.833	28.414

Ensa	-1.177	0.0007238	28.012	24.485
C78339	-1.178	3.564E-07	31.155	27.564
Psmd6	-1.178	0.029	48.534	42.790
Hnrnpd	-1.179	0.085	34.840	28.949
Btf3	-1.179	0.1	209.046	162.882
Arf5	-1.179	0.213	66.242	57.778
Gdi2	-1.180	0.017	183.497	166.977
Aurkaip1	-1.180	0.045	22.519	20.131
Dnaja2	-1.182	0.000006299	34.710	30.362
Nenf	-1.182	0.262	28.879	25.832
Ccar1	-1.183	0.00002883	24.828	21.908
Itfg1	-1.183	0.0001137	26.934	23.574
Psma5	-1.183	0.157	66.089	61.912
Wac	-1.184	0.001118	15.104	12.773
Hmx1	-1.184	0.036	35.799	30.668
Slc20a2	-1.185	0.017	49.719	43.842
5730437N04Rik	-1.185	0.085	45.739	40.934
Nap1l1	-1.186	3.243E-13	621.033	524.631
Ell2	-1.186	9.234E-09	112.158	95.909
H19	-1.186	0.004443	1470.539	1176.224
Epdr1	-1.188	0.0007096	23.577	20.949
Atox1	-1.188	0.143	46.700	41.518
Spg21	-1.189	0.002258	32.434	28.167
Mrps16	-1.189	0.03	16.939	14.891
Apex1	-1.190	0.002502	37.070	31.574
Tmem85	-1.190	0.228	38.372	35.300
Serinc1	-1.191	0.001845	35.247	31.019
Aktip	-1.191	0.025	24.657	20.944
Eef1b2	-1.191	0.245	155.391	135.180
Arpc2	-1.192	1.521E-28	75.516	66.275
C1qbp	-1.192	0.233	51.075	44.687
Med30	-1.193	0.12	22.196	19.536
Tax1bp1	-1.194	0.0003652	19.867	17.352
Ranbp2	-1.195	0.124	14.999	12.912
Srsf1	-1.196	8.831E-33	38.851	34.943
Pafah1b2	-1.196	0.00001011	28.124	24.584

Rps3	-1.196	0.00326	152.719	131.345
Gnl2	-1.196	0.006829	27.290	23.697
Ppdpf	-1.196	0.123	22.524	18.911
Gtpbp4	-1.198	1.969E-22	32.581	28.202
Hnrnpc	-1.198	0.004431	73.563	66.952
Psmb4	-1.198	0.028	125.239	109.218
Tardbp	-1.199	7.552E-11	31.236	27.457
Zc3h15	-1.199	0.011	38.272	33.832
Fn3krp	-1.200	9.436E-26	73.927	64.208
Rab1	-1.201	1.134E-09	31.198	27.287
Dnajb2	-1.201	0.016	35.268	31.122
Oaz1	-1.202	0.000001509	178.932	181.200
Rabggtb	-1.202	0.181	28.810	25.425
Rab14	-1.203	7.658E-08	16.788	14.442
Spcs2	-1.203	0.00278	43.342	38.544
Ptp4a2	-1.204	2.776E-07	25.542	22.340
Ncbp2	-1.204	0.0004155	25.253	22.648
Psma1	-1.204	0.18	34.067	29.431
Atp5e	-1.204	0.283	81.200	69.722
Uba3	-1.206	2.899E-18	27.710	23.895
Fam169a	-1.206	0.001312	17.670	15.073
2810407C02Rik	-1.207	3.926E-08	37.331	31.523
Grifin	-1.207	0.146	356.119	310.861
D4Wsu53e	-1.207	0.18	42.428	36.331
Taf15	-1.207	0.245	38.569	33.400
Hnrnpr	-1.208	0.00007637	39.000	33.725
Ubxn1	-1.208	0.02	123.845	107.415
Kif5b	-1.208	0.072	18.612	15.514
Spryd4	-1.208	0.077	26.030	22.684
Crygb	-1.209	5.466E-46	26959.260	19124.225
Csnk1a1	-1.209	8.684E-09	23.498	20.628
Fcf1	-1.209	0.044	14.903	12.803
Srsf9	-1.209	0.104	30.982	27.049
Tusc3	-1.210	0.0002527	15.177	13.162
Hint1	-1.210	0.01	147.252	127.034
Emg1	-1.211	0.02	29.162	25.471

Psme4	-1.211	0.049	24.784	21.572
Npl	-1.212	1.555E-13	165.794	143.513
Ywhaz	-1.212	2.997E-08	129.985	115.446
Ddx5	-1.213	1.111E-07	107.208	94.635
Pop4	-1.213	0.012	25.214	22.089
Chmp2a	-1.213	0.111	76.489	66.151
Pfdn5	-1.213	0.123	90.548	82.102
Pfn2	-1.214	4.908E-17	20.730	17.878
Cnbp	-1.214	0.003342	104.413	90.029
Ddx3x	-1.215	0.00002138	52.257	45.248
Dpcd	-1.215	0.014	19.358	16.892
Nudt4	-1.215	0.063	17.423	15.118
Mettl9	-1.216	8.161E-07	24.952	21.381
Rpl27a	-1.217	0.137	131.789	102.680
Ndufs6	-1.217	0.207	47.145	41.110
Ubl3	-1.218	3.485E-09	18.153	15.762
Ube2k	-1.218	0.00001544	18.796	15.962
Mrpl54	-1.218	0.025	40.626	35.310
Qk (17 10399335..10512245)	-1.219	0.00292	23.678	20.306
Cyth2	-1.219	0.004397	18.825	16.115
Ccni	-1.220	0.00003075	26.022	23.014
Malat1	-1.220	0.29	766.147	616.503
Atp6v1h	-1.221	0.000008371	16.358	14.197
Pno1	-1.221	0.006671	11.536	5.703
Morf4l2	-1.222	8.161E-34	77.092	64.113
Tmem165	-1.222	3.248E-13	18.534	16.095
Psmb7	-1.222	0.081	136.428	115.530
Hsbp1	-1.222	0.194	99.316	84.257
Cpe	-1.223	6.347E-16	66.682	56.849
Gabarap	-1.223	0.0007837	77.217	65.451
Atp6v1f	-1.223	0.136	64.983	55.268
Tpm2	-1.223	0.157	20.202	17.419
Pdhb	-1.224	0.00008607	38.438	33.078
Vti1b	-1.224	0.00016	25.643	22.115
Mtap7	-1.225	6.93E-21	48.471	41.917
Rps27a	-1.225	0.00748	98.350	81.633

Hnrnpu	-1.226	2.318E-15	79.866	67.808
Fyttd1	-1.226	6.126E-13	15.114	12.718
Mpp6	-1.226	0.0005417	15.035	12.986
Efha1	-1.227	5.689E-09	51.736	44.005
Ogt	-1.228	3.738E-09	17.138	14.493
Nucks1	-1.229	1.855E-27	17.981	14.932
Spon1	-1.229	1.071E-09	25.342	21.655
Khdrbs1	-1.229	2.503E-08	15.202	12.798
Rpl7a	-1.229	0.0001165	73.202	55.587
Oaz2	-1.230	5.683E-09	28.162	23.826
Islr	-1.230	0.001103	14.880	12.828
Cct8	-1.231	0.018	159.121	129.121
Fam96a	-1.231	0.02	24.128	20.781
Lix1	-1.232	2.309E-29	46.709	41.070
Smap2	-1.232	5.823E-11	20.866	17.695
Golph3	-1.232	2.01E-09	22.769	19.313
Glo1	-1.232	0.00007066	72.764	63.043
Bpgm	-1.232	0.0001782	17.508	14.934
4930455C21Rik	-1.232	0.005912	17.402	14.974
Cmas	-1.233	6.765E-13	18.247	15.682
Sri	-1.233	4.939E-09	17.484	14.822
Hmgn1	-1.233	0.0001807	31.001	28.578
Snapin	-1.233	0.00504	17.377	14.989
Thoc7	-1.233	0.007488	47.458	39.769
0610037L13Rik	-1.234	0.019	13.070	11.006
Slc25a4	-1.234	0.029	167.579	137.860
Ppp1r1a	-1.234	0.035	43.980	37.830
Snhg5	-1.234	0.141	16.479	14.019
Rpl28	-1.235	0.047	445.602	424.949
Gstm5	-1.235	0.078	75.929	63.662
Csnk1g3	-1.236	2.794E-18	20.469	17.218
Cd164	-1.236	0.000001479	55.207	46.076
Hnrnpa3	-1.236	0.0001033	25.171	21.038
Brd7	-1.237	0.0003115	19.405	16.253
Eif1ax	-1.238	2.355E-46	28.396	23.594
Mcl1	-1.238	3.72E-24	26.987	22.607

Cops3	-1.239	1.882E-10	21.017	17.041
Lrrc58	-1.239	2.116E-08	17.531	15.289
Pdia3	-1.239	0.044	109.024	91.433
Abi2	-1.240	0.00006577	36.705	31.119
Mki67ip	-1.240	0.001055	15.218	12.947
Rcn2	-1.241	2.292E-16	14.640	12.336
Psip1	-1.241	0.0001127	29.808	24.746
Rpl7	-1.241	0.0005457	333.163	271.744
Rbm24	-1.242	2.472E-93	101.013	85.266
Txndc17	-1.242	0.002276	27.955	23.826
Naca	-1.242	0.014	73.972	60.998
Actr2	-1.243	2.642E-44	21.119	17.624
Tmx1	-1.243	5.231E-11	37.495	31.257
Rnf2	-1.243	0.00000024	17.452	14.886
Ndufa13	-1.243	0.053	90.059	76.543
Mrpl41	-1.243	0.072	35.201	30.586
Rtn4	-1.244	1.281E-23	23.967	19.898
Vps29	-1.244	1.167E-15	18.411	15.909
Syncrip	-1.244	0.00002627	14.648	12.474
Hnrpdl	-1.244	0.0009752	28.942	23.796
Bzw1	-1.245	8.616E-07	82.468	69.544
Rbm45	-1.245	0.003481	21.206	17.873
Eef1e1	-1.245	0.004922	16.393	13.844
Dynll1	-1.245	0.012	66.340	56.526
1810046J19Rik	-1.246	0.149	28.520	22.552
Lyrm2	-1.246	0.173	12.323	10.194
Ube2d2	-1.247	0.00000028	61.729	51.483
AI646023	-1.247	0.045	15.634	13.173
Hist1h1c	-1.247	0.265	40.595	33.822
Tceb2	-1.248	0.085	194.919	184.615
Gtf2f2	-1.248	0.124	14.403	12.133
Cd63	-1.249	0.132	81.347	64.931
Yipf5	-1.250	0.00001377	18.863	15.748
Bfsp2	-1.251	0.038	1649.823	1448.842
Hnrnpk	-1.252	0.0001646	202.700	183.385
Trpc6	-1.252	0.0004471	22.386	18.754

Trappc2l	-1.252	0.001753	17.196	13.304
Psmd10	-1.252	0.004716	12.661	10.599
Atp5g2	-1.252	0.042	214.406	189.718
BC005537	-1.253	1.407E-23	35.336	29.403
Rap1b	-1.253	9.392E-20	31.831	26.589
Dpy30	-1.253	0.0002964	29.182	24.679
Iah1	-1.253	0.005912	14.489	11.710
Hdac2	-1.253	0.006319	18.631	15.332
Rsl1d1	-1.253	0.057	40.516	33.302
Sra1	-1.254	0.045	13.455	11.371
Arl1	-1.255	7.226E-08	25.484	21.331
Mrpl30	-1.255	0.0007347	20.678	17.423
Cfl2	-1.256	8.338E-36	27.421	22.687
Picalm	-1.256	4.204E-35	33.759	27.994
Tra2a	-1.256	0.001142	26.876	22.124
Mrpl34	-1.256	0.03	27.980	23.344
Lsm12	-1.256	0.039	19.625	16.867
Cript	-1.257	0.000002031	16.451	13.730
Myo7b	-1.257	0.18	20.186	16.646
Psmc1	-1.258	7.02E-26	123.943	105.591
Tmem33	-1.258	5.717E-09	11.506	9.495
Ube2r2	-1.258	2.926E-07	20.305	16.689
Srp54c	-1.258	0.058	21.839	18.677
Eif2s1	-1.260	1.22E-19	41.679	34.921
Paip2	-1.261	0.0003555	42.657	34.832
Rps28	-1.261	0.166	315.533	277.148
Cd34	-1.261	0.195	8.567	6.382
Strn3	-1.262	6.85E-11	18.637	15.307
Ufsp2	-1.262	0.0002886	13.341	11.178
Ndufv2	-1.262	0.021	39.642	32.687
Mrpl42	-1.262	0.078	83.282	72.035
Tug1	-1.263	2.64E-09	15.890	12.922
Rbm8a	-1.263	0.155	25.799	22.122
Hmgb1	-1.264	0.059	16.851	12.449
Dmtf1	-1.265	0.0001289	15.746	12.922
Caprin2	-1.265	0.001233	190.885	153.133

Pgrmc1	-1.265	0.006275	40.275	33.183
Pfdn1	-1.265	0.108	55.536	46.509
Hsp90aa1	-1.267	0.00003214	155.534	125.734
Nsa2	-1.267	0.112	14.196	11.811
Zcrb1	-1.267	0.29	13.285	11.035
Mfap4	-1.268	0.0003806	29.896	24.800
Hbxip	-1.268	0.123	22.354	19.900
2900010M23Rik	-1.268	0.211	63.475	52.449
Ube2b	-1.269	0.00004039	17.801	14.163
Calm2	-1.269	0.017	117.688	97.292
Snx5	-1.270	7.492E-10	29.633	24.273
Stxbp6	-1.270	0.00000236	18.114	14.735
Dnajc15	-1.271	0.00000014	40.312	33.626
Rpl13a	-1.271	0.017	270.925	226.597
Myeov2	-1.271	0.169	31.150	25.935
Tmem30a	-1.272	2.258E-12	26.286	21.340
Lnp	-1.272	1.364E-11	10.981	8.937
Nptn	-1.272	2.188E-11	29.809	24.560
Laptm4a	-1.272	0.006737	102.620	84.981
Mrps15	-1.272	0.009279	15.662	12.956
Polr2d	-1.272	0.137	15.133	12.468
Bclaf1	-1.273	1.738E-08	26.884	21.843
Tppp3	-1.274	0.016	33.600	27.943
Rps14	-1.274	0.058	406.091	326.285
Hnrnph2	-1.275	0.00003508	23.021	18.989
2310016M24Rik	-1.275	0.004598	64.596	53.457
Rps21	-1.275	0.062	391.403	325.838
Cul3	-1.276	1.943E-37	75.722	60.885
Ywhaq	-1.276	1.114E-35	67.741	61.505
Efcab1	-1.276	5.14E-28	21.870	18.137
Tmem93	-1.276	0.0003735	20.403	16.846
Ndfip1	-1.276	0.001171	65.556	53.748
Gtf2b	-1.276	0.004518	17.785	14.776
Hnrnph3	-1.276	0.006953	16.076	12.668
Phax	-1.276	0.025	16.875	14.058
Psma6	-1.277	8.024E-45	74.058	59.625

Skp1a	-1.277	6.496E-17	74.543	58.456
Prickle1	-1.277	1.616E-10	15.972	13.098
Hibadh	-1.277	0.000003971	23.269	19.147
Ndufb9	-1.277	0.0001213	89.387	73.812
Dbi	-1.277	0.014	57.249	47.559
Cox5b	-1.277	0.094	188.192	145.164
Jtb	-1.278	6.689E-10	16.628	13.729
Vps35	-1.278	0.00005712	36.819	30.226
Eif1a	-1.279	2.037E-18	19.393	16.063
Ssr3	-1.279	1.931E-15	81.090	65.453
Itpa	-1.279	0.000002313	24.785	22.096
Aldoc	-1.279	0.00002352	18.094	14.908
Rhoa	-1.280	5.091E-39	83.525	73.035
Dek	-1.280	8.44E-18	15.919	13.151
Ndufs3	-1.280	0.000003441	32.357	26.634
Btbd1	-1.281	7.523E-44	41.934	35.934
Sh3glb1	-1.281	0.002342	19.700	15.968
Acadm	-1.281	0.006585	13.753	11.294
Nupr1	-1.281	0.158	87.512	71.467
Rpl15	-1.282	0.014	154.714	148.200
Eif1b	-1.282	0.103	61.125	50.158
Cpeb4	-1.283	1.734E-09	11.650	9.384
Lin7c	-1.283	1.139E-08	13.582	11.102
Larp4	-1.284	3.146E-07	15.966	13.562
Pole4	-1.284	0.0008211	12.200	10.044
Rpl27	-1.284	0.014	69.537	58.506
mt-Cytb	-1.284	0.189	2341.930	1741.568
Ndufa4	-1.285	0.00001581	198.582	159.262
Aimp1	-1.285	0.011	44.485	36.553
Denr	-1.285	0.048	16.345	13.563
Prdx4	-1.286	3.175E-12	13.226	10.851
Impact	-1.286	0.000002229	12.775	10.521
Nudcd2	-1.286	0.0002775	13.386	10.948
Rnf11	-1.287	2.278E-14	17.973	14.507
Gatad1	-1.288	3.213E-11	22.412	18.491
Golga7	-1.288	4.371E-10	30.556	24.956

Mrps14	-1.288	0.0004283	11.645	9.567
Zbtb7b	-1.288	0.004948	35.704	29.025
Txnl1	-1.288	0.006164	27.663	22.437
Plaa	-1.289	1.831E-11	12.258	9.878
Glrx3	-1.290	1.96E-21	80.067	71.534
Adam10	-1.290	7.665E-17	69.964	55.893
Spast	-1.290	1.022E-07	13.309	10.822
Chmp5	-1.290	0.000003054	39.547	32.221
Rexo2	-1.290	0.0005652	86.743	69.179
Mrpl18	-1.290	0.0006658	23.159	19.793
Ube2a	-1.291	0.00005591	11.714	9.514
Swi5	-1.291	0.004438	56.769	45.509
Snrpd3	-1.291	0.246	103.578	84.840
Chmp2b	-1.292	1.057E-07	17.490	14.300
2310036O22Rik	-1.292	0.006031	82.210	66.340
Mrpl51	-1.293	0.000286	12.187	9.735
Pomp	-1.293	0.006147	177.118	142.960
Top2b	-1.294	1.886E-12	30.749	24.773
Tubb6	-1.295	0.000002056	92.610	74.022
Commd6	-1.295	0.0016	17.006	13.881
Fam162a	-1.295	0.003114	58.218	47.700
Rdh14	-1.295	0.00452	11.827	9.541
Bloc1s2	-1.295	0.267	23.675	20.237
Arl8b	-1.297	1.243E-25	21.342	17.298
Cdkn1b	-1.297	9.01E-16	15.536	12.499
Atp6v1d	-1.298	0.0006304	10.769	8.736
Med10	-1.298	0.05	34.073	32.017
St13	-1.299	1.977E-39	85.056	68.428
Vdac3	-1.299	0.001484	66.085	61.703
3110001D03Rik	-1.299	0.003083	31.134	25.459
Srp14	-1.299	0.053	107.040	85.612
Ccdc90b	-1.300	3.63E-14	17.193	12.848
Kdelr2	-1.300	0.00001862	26.977	21.879
Myl6	-1.300	0.031	305.743	284.683
Ola1	-1.301	4.577E-11	15.282	12.345
Hars	-1.302	2.388E-13	28.484	23.222

Txndc12	-1.302	0.00008906	16.927	13.690
Bccip	-1.302	0.0003143	25.946	19.499
Rpsa	-1.302	0.007828	646.844	611.309
Atp5h	-1.302	0.11	100.518	81.548
Ube2n	-1.303	3.838E-16	22.782	17.739
Strap	-1.303	4.099E-15	61.212	49.158
Asah1	-1.303	9.341E-07	12.927	10.468
Ftl1	-1.303	0.00004906	867.384	727.593
Thoc4	-1.304	3.005E-09	16.228	13.553
0610037P05Rik	-1.305	0.001443	11.416	9.153
Gar1	-1.305	0.001478	12.309	9.991
Cdc42	-1.306	3.741E-12	61.944	49.812
41167	-1.306	1.007E-10	61.259	48.975
Ddx50	-1.307	2.343E-10	16.061	13.025
Hnrnph1	-1.307	0.001809	59.707	46.747
Gtf3c6	-1.307	0.007027	33.266	27.039
Psenen	-1.307	0.077	88.671	70.714
Senp6	-1.308	4.001E-11	27.198	21.398
Rps15a	-1.308	0.000006627	54.323	48.602
Pcbd1	-1.309	0.003406	23.191	18.586
Rbm17	-1.309	0.005262	28.152	23.971
Acadl	-1.310	1.25E-09	15.233	12.284
Chchd2	-1.310	0.00000597	64.301	52.360
Nsmce2	-1.310	0.00003895	10.475	8.389
Cacybp	-1.310	0.0000419	59.589	47.820
mt-Nd4	-1.310	0.049	1363.176	1020.515
6720456B07Rik	-1.311	0.0009381	63.241	50.345
AW549877	-1.312	4.05E-14	12.964	10.366
Ndufs4	-1.312	0.001232	36.186	28.951
Rab28	-1.314	1.711E-09	11.158	8.867
Pgcp	-1.314	6.628E-08	18.665	15.036
Upf3a	-1.314	0.002863	15.018	12.005
Sfrs18	-1.314	0.047	9.898	7.757
Rab11a	-1.315	9.599E-22	51.480	40.535
Scfd1	-1.315	1.373E-18	18.505	14.858
Tspan6	-1.315	1.974E-14	20.046	16.160

Mrpl20	-1.315	0.006084	37.787	30.605
Wls	-1.316	1.006E-19	18.850	15.007
Sec22b	-1.316	2.19E-08	13.770	11.047
Naa15	-1.316	0.0000222	31.549	25.038
Rps11	-1.317	0.017	892.802	778.037
Arf6	-1.318	1.35E-10	20.859	16.211
Arpp19	-1.318	0.000004973	14.881	11.813
Anp32b	-1.318	0.08	137.866	110.778
Adi1	-1.319	1.747E-07	19.198	15.352
E030010A14Rik	-1.319	0.004228	10.395	8.286
Immp1l	-1.320	0.054	39.435	32.383
Fxr1	-1.322	2.279E-32	46.123	38.324
1110002B05Rik	-1.322	0.00005243	22.292	17.797
Upf3b	-1.322	0.05	20.348	15.964
Cbfb	-1.323	2.65E-08	11.421	9.123
Sfr1	-1.324	0.006923	98.760	77.297
Stx11	-1.324	0.021	18.364	14.729
Atp6v1g1	-1.324	0.026	86.723	73.218
Dynlt3	-1.325	1.006E-19	27.555	21.639
Prdx3	-1.325	2.612E-08	20.869	16.625
Rps20	-1.325	0.001018	120.076	90.441
Mrps31	-1.325	0.009026	9.677	7.645
Herc4	-1.326	7.121E-14	57.740	45.352
Acbd6	-1.326	6.524E-08	11.630	9.271
Rab6	-1.327	2.647E-31	27.845	21.929
Nes	-1.328	6.525E-07	17.000	13.218
Rpl22	-1.329	2.82E-15	104.287	84.731
Pdcd4	-1.329	1.198E-08	38.155	28.019
Scpep1	-1.329	0.000007249	24.345	19.172
4930583H14Rik	-1.329	0.000027	303.487	239.744
Gadd45g	-1.329	0.242	39.298	31.238
Decr1	-1.330	2.845E-20	10.725	8.549
Atp5f1	-1.330	4.121E-08	39.293	33.077
Fus	-1.330	0.009506	59.642	49.901
Pgap2	-1.330	0.014	25.792	20.024
Rala	-1.332	7.593E-09	34.639	26.787

Cycs	-1.332	0.024	24.748	17.424
Ptbp2	-1.333	8.248E-09	13.958	11.065
BC003331	-1.333	0.00000162	10.207	8.124
Gnl3	-1.334	7.295E-07	20.035	15.873
Polb	-1.335	1.648E-15	19.213	15.154
Fam165b	-1.335	0.046	9.901	7.421
Ift20	-1.336	0.00001111	22.512	17.792
Rpl10	-1.336	0.145	42.346	33.057
Mtpn	-1.337	2.637E-22	68.910	53.965
Ap2m1	-1.337	4.448E-07	47.010	52.222
Esd	-1.337	0.003114	32.810	26.666
Pcp4	-1.337	0.05	111.627	92.143
1110001J03Rik	-1.337	0.077	23.484	18.435
Ppp3ca	-1.339	1.117E-25	15.100	11.695
Sucla2	-1.339	4.248E-23	14.261	11.184
Prdx1	-1.339	0.0001545	85.613	67.959
2810008M24Rik	-1.340	2.465E-18	16.048	12.704
Ppp2r5e	-1.340	9.363E-17	10.616	8.348
Ppid	-1.340	0.000416	13.737	10.663
Mzt1	-1.341	1.179E-15	14.427	11.373
Snrnp27	-1.341	0.07	11.607	9.141
Fam60a	-1.342	0.00003659	4.804	9.821
Srp9	-1.343	0.00006537	38.112	29.348
Anapc13	-1.343	0.118	181.079	141.733
Max	-1.345	0.000007286	29.383	23.023
Mrps21	-1.345	0.005034	43.259	34.748
Shfm1	-1.345	0.118	195.869	150.102
Pnrc2	-1.346	3.116E-07	20.346	15.846
Myef2	-1.347	8.697E-09	14.788	11.256
Cops2	-1.347	1.925E-07	35.950	27.666
Csde1	-1.348	1.641E-108	85.219	65.927
Nit2	-1.348	0.0009896	12.047	9.397
Usmg5	-1.349	0.018	251.516	181.946
Rps4x	-1.350	8.245E-34	373.233	310.625
Atp6ap2	-1.350	7.882E-11	15.375	12.050
Dmrta2	-1.350	9.124E-09	13.620	10.691

Sugt1	-1.350	2.749E-07	25.878	20.222
Eny2	-1.350	0.071	11.251	9.025
Pde4d (13 110744316..110746171)	-1.352	7.696E-12	9.887	7.675
Atpif1	-1.352	0.021	45.089	35.943
Atf5	-1.353	0.033	46.855	36.366
Nhs	-1.354	3.429E-08	17.396	13.308
Fam89a	-1.354	0.000000217	56.849	44.017
Rpl21	-1.354	0.000844	31.450	23.591
Ng23	-1.354	0.005466	64.849	53.030
Hspb3	-1.354	0.025	19.664	15.221
Pla2g16	-1.355	0.0000462	94.496	73.724
Atad1	-1.357	1.092E-15	18.143	14.111
Rbm25	-1.357	5.177E-08	30.674	23.351
Med6	-1.357	0.032	12.222	9.428
Psmc6	-1.358	6.529E-07	41.836	31.781
Rps12	-1.358	0.039	236.509	240.758
Mxd1	-1.359	0.000009285	11.300	8.764
Isca2	-1.360	0.001857	28.839	22.344
Dld	-1.361	1.447E-10	29.016	22.467
Rbm39	-1.361	8.162E-07	28.576	22.053
2310009B15Rik	-1.362	0.001337	8.793	6.478
Uba52	-1.363	1.024E-08	196.260	193.736
Psma3	-1.363	0.00002658	30.531	25.397
Pdcl3	-1.363	0.0001328	8.942	6.921
Ccdc58	-1.363	0.013	10.180	7.839
Cmc1	-1.363	0.081	9.660	6.856
Timm17a	-1.364	0.0004785	35.303	28.304
Wsb1	-1.364	0.001388	15.571	11.954
Gm1673	-1.365	0.0002266	20.696	15.941
Naa50	-1.366	1.761E-66	20.017	15.154
E2f5	-1.366	0.000001513	22.484	17.526
Hcfc1r1	-1.366	0.001552	21.853	17.240
Sec61b	-1.367	0.018	36.203	27.245
Gng5	-1.367	0.062	13.714	9.035
41154	-1.368	1.382E-25	38.481	29.536

Chordc1	-1.368	1.776E-19	29.840	22.989
Set	-1.368	1.443E-13	79.576	66.628
Gpbp1	-1.368	3.86E-09	27.729	20.746
Mocs2	-1.368	0.000416	65.836	49.839
Rab5a	-1.370	6.814E-17	42.411	32.045
Rpl9	-1.370	0.116	98.597	55.767
Ahsa2	-1.371	3.926E-08	15.699	12.081
Frg1	-1.371	0.0001304	22.248	17.154
Snrpg	-1.372	0.146	72.028	60.478
Nop58	-1.373	4.301E-08	27.804	21.121
Hnrnpa2b1	-1.373	0.00227	271.666	194.696
Selk	-1.373	0.03	28.681	22.547
Rpl11	-1.374	0.005166	291.830	213.551
Mtx2	-1.375	1.86E-08	10.404	7.986
Med21	-1.375	0.003523	25.297	19.968
Ogn	-1.377	0.00001756	43.629	35.083
Gm14322	-1.378	0.007016	13.086	10.101
Tmem126a	-1.380	0.000007996	34.073	26.144
Tpt1	-1.380	0.014	690.845	594.222
1110001A16Rik	-1.380	0.126	7.462	5.129
Megf9	-1.381	4.386E-08	38.697	28.974
Gabarapl2	-1.381	0.000002462	14.146	10.641
Hnrnpab	-1.382	1.46E-15	79.619	67.193
Ndel1	-1.382	7.979E-12	19.519	14.826
Sap30	-1.382	0.0000544	10.092	7.572
Mageh1	-1.382	0.041	10.853	8.273
Ufm1 (3 53659133..53667752)	-1.384	3.349E-08	11.684	8.899
l7Rn6	-1.384	0.00001555	9.924	7.627
Ndufb2	-1.385	2.8E-12	7.158	4.921
H3f3b	-1.385	0.0006419	66.029	50.908
Sccpdh	-1.386	2.03E-50	17.441	13.038
Serbp1	-1.386	1.816E-19	79.026	60.348
Nudt2	-1.386	0.005906	19.906	14.974
Lsm3 (6 91466028..91472614)	-1.388	3.187E-07	19.027	14.878
Txn1	-1.388	0.00619	88.989	66.226

Ndufa6	-1.388	0.007826	115.033	86.994
Uchl5	-1.389	0.005146	14.543	11.035
Psmg4	-1.389	0.1	22.659	17.038
0610009B22Rik	-1.390	0.00001157	14.983	11.327
Htatsf1	-1.391	0.000003244	13.732	10.419
Ggct	-1.391	0.000616	25.121	18.567
1810022K09Rik	-1.391	0.011	57.290	32.289
41159	-1.392	2.94E-28	30.091	22.240
2700060E02Rik	-1.393	3.342E-19	62.813	49.250
2300009A05Rik	-1.393	0.005912	14.571	10.922
Gje1	-1.394	2.31E-19	373.450	285.479
Crebzf	-1.394	2.587E-09	8.216	6.183
Sec62	-1.394	0.000001766	21.779	16.214
Bex1	-1.394	0.063	25.935	20.589
Hist1h2bc	-1.395	1.643E-11	70.272	63.446
Igfbp5	-1.396	1.18E-42	80.607	59.251
Rab18	-1.396	4.793E-42	22.149	16.711
Srsf10	-1.396	2.089E-11	14.549	11.263
Bin1	-1.397	3.246E-11	15.649	11.808
Gm561	-1.397	0.006961	22.318	16.638
Ube2d3	-1.398	2.676E-132	93.198	70.547
Ndufb5	-1.398	0.000004973	35.143	26.254
Ppp1r14b	-1.399	0.0005459	136.120	125.952
Cpt1a	-1.399	0.111	13.955	10.423
Snrpb2	-1.401	0.001391	11.367	8.625
Uxt	-1.401	0.008876	8.994	6.737
Birc7	-1.401	0.017	37.337	27.889
Kras	-1.403	8.982E-25	27.790	20.153
Eps15	-1.403	2.351E-16	9.024	6.767
Anp32a	-1.404	0.01	75.943	57.101
Rsl24d1	-1.405	5.381E-07	28.800	23.878
Ppp2r3c	-1.405	0.00002515	9.603	6.923
Ubl5	-1.406	0.006255	25.975	19.322
1500012F01Rik	-1.406	0.013	15.306	11.633
Capza2	-1.408	3.244E-09	13.440	9.950
Vapa	-1.408	0.00004359	54.741	40.162

2700023E23Rik	-1.408	0.049	10.003	7.324
Bbip1	-1.411	0.003478	14.472	10.038
Rnf6	-1.412	1.543E-24	12.376	9.207
Fxyd1	-1.412	0.113	12.086	8.871
Peli1	-1.414	7.498E-20	25.870	19.024
B3gnt5	-1.414	0.0004665	29.317	21.643
Uqcrh	-1.414	0.0008364	99.026	72.126
Ranbp1	-1.415	1.362E-07	72.157	53.463
Ptma	-1.416	0.004157	426.362	347.940
Mrpl32	-1.417	0.025	18.592	13.851
Ube2v1	-1.418	0.111	43.072	45.596
Mnat1	-1.419	2.08E-09	12.107	9.048
Pgam1	-1.420	5.672E-11	192.940	196.553
Slc25a17	-1.421	1.061E-08	17.790	13.244
Vamp7	-1.422	0.000000107	10.040	7.397
Matr3	-1.423	1.452E-27	39.960	29.143
Ing2	-1.423	1.149E-12	16.835	12.399
Pcnp	-1.424	3.749E-13	40.747	29.917
Rpl31	-1.424	0.000007593	520.864	393.458
Fam98b	-1.425	0.000003742	12.917	9.577
Mrpl52	-1.425	0.008376	102.660	75.971
mt-Nd6	-1.426	0.056	1029.845	745.452
Wbp4	-1.427	1.654E-08	9.388	6.956
Tmx3	-1.429	9.77E-12	9.074	6.720
Ccdc53	-1.429	1.653E-07	17.926	13.278
Psme2	-1.429	0.000001077	16.248	11.545
Ndufaf2	-1.429	0.00004517	16.251	11.893
Zmat2	-1.429	0.01	31.423	23.071
Acbd5	-1.430	1.164E-10	10.498	7.771
Mrpl50	-1.430	5.346E-10	21.866	16.047
Sms	-1.433	1.616E-38	12.415	10.120
H47	-1.435	7.913E-08	26.275	19.394
1810063B05Rik	-1.436	0.00009424	14.406	10.452
Cbx3	-1.437	0.001422	44.899	26.088
Scp2	-1.438	3.547E-13	29.533	22.486
Deb1	-1.438	7.023E-07	15.278	11.106

mt-Nd1	-1.438	0.053	1212.644	855.090
Hnrnpa0	-1.439	0.000003906	80.602	59.438
Eif4e	-1.439	0.000007555	82.894	64.940
Ndufc2	-1.439	0.002709	83.756	61.279
Rsrc2	-1.441	5.326E-07	13.199	9.623
Pfdn2	-1.442	0.00574	28.527	22.718
Gkap1	-1.443	3.888E-11	18.188	12.996
1810037I17Rik	-1.444	0.041	24.164	17.723
Ppp1r2	-1.445	4.544E-13	9.245	6.735
Med7	-1.445	0.0002555	16.404	10.102
D1Ertd622e	-1.446	4.397E-16	12.122	9.109
Yme1l1	-1.447	1.374E-20	11.681	8.339
Brix1	-1.447	0.000007285	12.655	9.249
Mrpl46	-1.447	0.003686	12.620	9.066
Smarca5	-1.452	2.489E-19	10.157	7.950
Gm11837	-1.452	2.186E-15	26.216	16.859
Rps19	-1.453	0.00418	57.368	40.786
Zranb2	-1.454	6.005E-21	23.271	16.465
Pcmtd1	-1.454	1.139E-18	8.685	5.861
Tomm20	-1.454	1.728E-17	69.044	57.346
Purb (11 6367608..6369141)	-1.454	1.179E-09	30.555	22.017
Taf7	-1.454	0.000001806	8.998	6.525
Rpl12	-1.455	8.928E-08	181.721	170.676
Txndc9	-1.455	0.000001636	8.097	5.822
Rps24	-1.455	0.005368	754.132	603.466
Atp5g1	-1.455	0.072	17.618	12.661
Scoc	-1.457	4.725E-11	42.992	31.141
Mrfap1	-1.457	0.000001696	214.185	151.481
Paics	-1.458	2.434E-13	137.812	98.816
Atg3	-1.459	1.913E-08	11.299	8.156
Exosc9	-1.459	0.00005394	6.145	4.086
Pcbd2	-1.459	0.0008347	41.778	31.780
mt-Co3	-1.460	6.135E-13	2793.845	1842.444
Mrpl40	-1.460	8.348E-07	12.048	8.602
Bud31	-1.461	0.0001116	28.270	19.969
Cwc15	-1.462	0.006175	66.996	47.803

Mtpap	-1.463	5.898E-14	9.764	7.189
Cfdp1	-1.463	0.022	57.980	41.521
Ctsl	-1.464	0.00001774	43.420	30.782
Ccnl1	-1.466	3.683E-07	9.800	6.998
Mcee	-1.466	0.021	12.972	9.244
Fkbp2	-1.469	0.052	28.914	20.693
Pla2g7	-1.470	0.000007431	219.384	157.778
Rpl35a	-1.470	0.003632	62.477	40.217
mt-Co2	-1.470	0.091	3369.149	2369.574
Morf4l1	-1.472	7.873E-18	147.444	108.967
Rpl29	-1.472	4.807E-11	125.446	97.676
Slc31a1	-1.473	8.405E-08	16.374	12.951
Cetn2	-1.473	0.00001399	23.170	16.629
Ube2g1	-1.474	1.224E-08	7.859	5.616
Prdm16	-1.475	0.0003838	8.792	6.187
Zfp428	-1.475	0.000633	12.965	9.215
Ndufa1	-1.475	0.056	1097.019	743.001
Cxxc5	-1.477	6.883E-68	54.275	37.701
Lhfp	-1.477	0.00006348	10.428	7.433
Krcc1	-1.478	0.000001949	41.049	25.326
Bex2	-1.479	0.00003375	41.629	30.242
Rpl35	-1.479	0.0004422	250.660	189.168
Ccng1	-1.481	1.424E-08	29.938	20.827
Pacsin3	-1.481	3.378E-07	7.895	5.651
mt-Atp6	-1.482	0.0000548	877.776	831.520
Higd1a	-1.482	0.011	32.244	22.883
Ier3ip1	-1.482	0.018	6.131	3.251
Etfa	-1.483	1.011E-11	28.629	20.488
Gtf2e2	-1.483	2.234E-08	20.349	14.449
Dcun1d5	-1.484	1.22E-19	23.331	16.615
Rgs5	-1.484	2.825E-17	9.060	6.449
Ap3s1	-1.484	2.103E-08	31.683	23.597
Ints6	-1.485	3.29E-42	10.150	7.139
Rnf180	-1.486	0.009911	58.297	40.549
Bnip3l	-1.488	4.613E-68	90.685	72.417
Nudt21	-1.488	1.974E-14	38.320	31.944

Rpf2	-1.488	0.000009295	12.221	8.660
Hmox1	-1.489	0.099	47.554	33.258
Timm8a1	-1.490	0.0001552	17.140	13.602
2810001G20Rik	-1.490	0.008344	10.039	7.016
Pisd	-1.491	0.002727	10.427	7.940
Dusp1	-1.493	0.006033	16.905	11.959
Arxes2	-1.494	6.717E-20	15.535	12.948
Eif3e	-1.494	4.044E-15	112.450	81.165
Ttc35	-1.495	8.927E-07	25.430	17.952
1700011J10Rik	-1.497	0.000001194	10.292	7.056
Fam49b	-1.498	4.392E-84	53.820	37.715
Ugp2	-1.499	6.219E-13	15.866	11.208
D630024D03Rik	-1.499	0.00007794	10.990	7.658
2810008D09Rik	-1.499	0.0006789	14.236	9.812
Tmed2	-1.501	1.873E-09	54.894	41.928
Gtf2a1	-1.502	7.227E-21	16.778	11.524
Rps18	-1.502	0.0001128	244.165	236.396
Ndufc1	-1.503	3.657E-10	74.208	50.720
Commd3	-1.505	2.045E-15	16.749	11.778
Hprt	-1.505	2.145E-07	24.787	17.362
Rab39b	-1.506	4.727E-19	8.460	6.360
Trappc6b	-1.506	2.567E-15	31.283	22.639
Eif2s2	-1.506	0.000002182	24.383	15.393
2700029M09Rik	-1.507	0.000646	14.189	9.938
Gas5	-1.507	0.002654	103.261	70.963
S100a11	-1.507	0.023	57.948	43.224
Eif4a2	-1.512	2.651E-17	50.092	35.008
Atp5l	-1.512	0.007156	214.889	172.187
Ufc1	-1.513	0.00001207	37.094	25.696
Mob4	-1.514	3.113E-09	13.743	9.472
Srsf5	-1.516	0.00000628	71.819	51.753
Rps29	-1.517	0.00001977	595.976	482.122
Fundc2	-1.519	4.488E-10	14.806	10.369
Ppp1cb	-1.520	2.259E-21	21.346	14.625
Ermap	-1.520	5.158E-13	13.113	9.002
Haus1	-1.520	0.0005018	11.397	7.784

Hax1	-1.522	0.00004572	43.417	33.429
Clk1	-1.523	0.000002972	11.060	7.540
Basp1	-1.524	8.792E-11	109.789	75.363
Clk4	-1.524	4.846E-08	7.122	4.957
Eif5b	-1.525	2.755E-10	110.905	75.157
2010107E04Rik	-1.525	1.157E-07	201.000	137.885
Sarnp	-1.528	0.001004	50.571	30.815
Rps17	-1.529	0.007139	392.266	306.242
Anxa1	-1.530	0.000184	39.333	26.448
Srsf3	-1.531	3.965E-10	46.334	34.812
Pdcd5	-1.531	0.002771	29.479	20.854
Snhg12	-1.531	0.007417	111.134	76.639
Memo1	-1.536	6.585E-42	108.918	73.784
Zfand5	-1.536	2.822E-12	20.915	14.206
Gm17292	-1.536	5.336E-07	694.988	426.866
Rpl23	-1.538	0.0001961	454.215	308.829
Lims1	-1.539	1.618E-17	10.188	6.917
Gadd45b	-1.539	2.747E-08	23.308	16.008
Thoc1	-1.541	6.976E-14	9.430	6.411
Fkbp3	-1.541	1.876E-12	162.025	100.358
9930023K05Rik	-1.541	0.00007188	10.646	7.273
Bcap29	-1.543	5.38E-14	6.897	4.662
Rheb	-1.547	1.643E-11	8.686	5.902
Npm1	-1.548	0.0001488	152.626	112.819
Polr2k	-1.548	0.0003859	24.617	18.222
Uchl3	-1.551	8.287E-13	13.320	9.041
2410006H16Rik	-1.551	0.00000235	44.845	30.366
Ppia	-1.551	0.009294	107.864	73.982
Cbx1	-1.552	0.000001188	14.768	9.989
Ccdc104	-1.553	0.0005709	26.992	18.262
Rps25	-1.554	0.001012	717.514	548.962
Tm2d1	-1.555	0.00001052	11.617	7.528
Sumo2	-1.558	0.255	20.262	12.511
Zfand6	-1.559	2.188E-13	55.118	36.601
2010011I20Rik	-1.561	0.000002565	7.107	4.803
Snrpe	-1.563	0.0005223	58.005	42.564

Arglu1	-1.564	2.387E-13	63.307	41.384
Casp7	-1.567	3.32E-10	71.007	46.947
2410127L17Rik	-1.569	9.538E-14	15.742	11.892
1810027O10Rik	-1.571	0.00006791	26.280	17.732
Dnaja1	-1.573	5.348E-14	37.032	21.075
Chchd1	-1.575	2.517E-12	64.161	43.010
Fgfbp3	-1.576	9.524E-14	9.150	6.083
Maf	-1.578	1.116E-51	203.535	152.724
Gtf2a2	-1.580	1.097E-10	7.547	5.040
2310003F16Rik	-1.580	0.101	22.821	15.437
Actn2	-1.580	0.125	8.767	5.881
Rpl38	-1.581	7.882E-08	496.752	413.853
Sumo1	-1.583	4.655E-27	43.339	28.364
Rpl30	-1.584	0.0009473	115.723	72.555
Pnrc1	-1.587	8.173E-26	16.029	10.619
Rpp30	-1.589	0.000002527	10.611	6.885
Gapdh	-1.590	6.788E-12	54.597	59.618
Gm15417	-1.591	0.000000781	32.224	22.288
Luc7l3	-1.595	6.267E-09	22.001	14.357
Rps3a	-1.604	9.06E-16	605.021	481.314
Ppp4r2	-1.607	2.765E-32	19.661	14.411
Hsdl2	-1.610	1.43E-17	13.397	8.768
S100a4	-1.610	0.00006691	37.756	24.660
Snhg1	-1.612	0.004145	15.177	9.998
Taf1d	-1.613	1.279E-08	14.746	10.012
Rap1a	-1.614	1.903E-13	15.509	9.637
Btg1	-1.615	2.093E-19	40.011	26.941
5730455O13Rik	-1.616	4.625E-08	12.123	7.861
Pdcd10	-1.617	0.00007578	5.624	3.485
2700089E24Rik	-1.624	3.198E-22	23.670	15.266
1810030N24Rik	-1.629	1.989E-16	12.668	8.155
Dnajc30	-1.630	1.499E-11	7.052	4.889
Rpl18a	-1.635	6.791E-07	385.344	421.921
Cox6c	-1.638	0.0002136	41.300	26.466
Marcks	-1.643	1.733E-29	39.850	25.210
Cetn3	-1.644	0.0001521	54.068	34.474

Ssb	-1.645	0.00002627	60.565	35.903
Ngfr	-1.649	0.016	9.530	6.101
Btf3l4	-1.650	1.269E-09	29.300	17.810
Rpl14	-1.650	8.755E-08	692.278	585.946
B230118H07Rik	-1.652	1.245E-08	15.045	9.845
Hrsp12	-1.653	0.01	7.869	5.014
Ssbp1	-1.659	0.0006568	11.333	7.898
Rpl37a	-1.660	0.000008065	472.520	470.273
Paip1	-1.665	1.38E-28	28.835	17.898
Ogfrl1	-1.666	2.434E-35	6.988	4.382
Hbb-b2	-1.668	0.014	202.003	145.121
Tceb1	-1.669	1.194E-08	50.381	32.086
Tceal8	-1.671	1.046E-13	22.416	13.913
Nol7	-1.676	9.048E-10	33.806	21.014
Sat1	-1.676	3.548E-09	8.016	5.021
Ndufb3	-1.676	0.00351	44.753	28.720
Ddit4	-1.677	1.831E-08	37.210	23.272
Pigp	-1.679	3.981E-37	16.297	10.608
Ift74	-1.679	2.438E-08	20.211	12.675
Sub1	-1.680	1.589E-07	36.110	22.063
Yipf4	-1.683	1.075E-22	12.854	8.009
Hmgn3	-1.691	0.000008903	170.946	103.331
Tmsb4x	-1.692	7.593E-09	140.610	87.100
Cox7c	-1.693	1.024E-12	48.243	21.700
Ptp4a1	-1.698	5.437E-54	6.571	3.696
Rps8	-1.700	0.000006119	418.682	389.884
Rpl36a	-1.703	0.00002919	172.702	132.483
Bnip3	-1.705	9.271E-11	26.279	16.624
Snrpd1	-1.710	0.000187	48.134	29.338
2410004N09Rik	-1.715	0.00001188	14.488	8.728
mt-Nd2	-1.723	0.0006422	930.047	551.176
1190002N15Rik	-1.725	1.975E-07	16.951	10.235
Lsm5	-1.725	0.023	26.603	16.817
D0H4S114	-1.730	1.365E-26	56.994	33.530
Cdca7l	-1.732	1.511E-11	10.452	6.195
Dnase2b	-1.734	0.000002527	28.056	16.905

Rpl36al	-1.734	0.00003077	152.619	126.185
Hmgb3	-1.734	0.0004044	32.567	22.933
Wbp5	-1.736	2.865E-13	55.218	33.120
Dpysl2	-1.739	1.643E-11	32.939	19.317
Rps27l	-1.741	2.527E-14	59.769	37.703
Mrps18c	-1.743	0.0002908	15.151	9.252
Rps2	-1.752	1.236E-14	201.681	111.472
D19Ertd737e	-1.773	0.00001653	20.607	12.456
Hbb-b1	-1.781	0.0009796	409.085	271.890
Rhot1	-1.799	8.024E-48	9.546	5.515
Rwdd1	-1.818	3.355E-16	26.420	17.135
Tbca	-1.820	0.00005747	203.072	134.250
Dnttip2	-1.823	0.000004973	12.794	9.495
Lgals1	-1.848	1.514E-07	38.473	21.943
Hspe1	-1.857	2.591E-08	76.777	51.216
Vbp1	-1.859	8.856E-22	22.033	12.546
Bex4	-1.872	5.047E-10	42.649	26.868
Serf1	-1.876	2.879E-08	27.722	15.598
Atp5j	-1.879	1.903E-07	46.030	24.879
Ccdc72	-1.882	0.000002277	219.917	147.193
Krt10	-1.888	8.906E-12	4.561	2.507
Hmgn2	-1.893	0.0001268	81.437	55.581
mt-Nd3	-1.894	2.397E-07	1133.132	610.352
Pabpn1	-1.900	3.195E-13	23.347	14.798
Uqcrb	-1.907	0.00002095	79.813	49.381
Gm13152	-1.910	3.175E-20	9.635	5.297
BC013529	-1.918	2.832E-35	10.596	5.837
Gadd45a	-1.920	0.00000033	9.329	5.508
Scand1	-1.924	5.88E-23	31.309	18.753
Hspb1	-1.949	0.048	13.626	7.520
Spa17	-1.967	0.00004395	10.245	5.321
Apoo	-1.982	1.109E-22	17.414	9.565
Hspb11	-1.991	6.357E-08	6.529	3.423
Fam103a1	-2.008	3.283E-07	20.573	14.151
Lctl	-2.013	5.959E-09	31.906	16.270
6330403K07Rik	-2.037	2.521E-18	9.950	5.110

Rpa3	-2.038	8.078E-09	8.666	4.680
Gng11	-2.040	1.216E-10	6.281	3.142
Ccdc23	-2.055	1.154E-08	76.365	38.748
Gm7589	-2.067	0.075	16.581	8.972
Emp1	-2.071	8.437E-32	4.456	2.233
Gpx8	-2.082	0.0001141	20.352	9.765
C030030A07Rik	-2.101	5.278E-11	18.057	10.079
Eif2s3y	-2.136	0.0003373	10.132	5.018
Trim13	-2.179	2.415E-29	5.796	3.207
Gm10925	-2.182	1.556E-07	2325.578	1510.424
Rpl39	-2.199	2.783E-09	489.796	328.763
Ceacam10	-2.293	8.356E-07	15.385	7.271
Fam36a	-2.305	0.000001374	45.697	25.535
Phpt1	-2.369	1.291E-11	63.829	46.572
E330034G19Rik	-2.372	1.229E-24	7.095	3.098
Ddx3y	-2.401	1.589E-07	5.633	2.492
Rpl19	-2.432	0.000836	338.370	297.067
Psmc3ip	-2.444	8.233E-13	7.343	3.159
Lmo1	-2.458	1.421E-19	4.651	1.895
Rpl5	-2.581	3.612E-29	228.599	176.678
Cd59a	-2.652	6.013E-14	5.573	2.087
Avp	-2.660	1.556E-08	4.937	1.714
Gm5160	-2.735	0.205	24.926	16.120
Lsm7	-2.739	8.54E-10	70.554	62.081
Gm9234	-2.755	0.284	30.351	19.132
Ccdc117	-2.820	3.51E-19	26.309	9.682
Gm11808	-2.865	0.045	76.110	44.914
Rpl27a-ps1	-2.966	0.072	4.395	1.134
2010002N04Rik	-3.000	7.822E-132	14.428	5.031
Rpl22l1	-3.003	6.19E-14	183.844	71.560
Nme2 (11 93811127..93817573)	-3.268	2.277E-10	174.432	132.824
mt-Atp8	-3.280	7.688E-09	2343.429	1146.269
Ube2l3	-4.167	0.283	12.281	6.733
Scg5	-4.366	3.079E-40	2.714	0.611
Gchfr	-4.381	0.0002886	2.963	0.679

Zfp580	-5.200	0.00222	3.308	1.066
Xlr3b	-5.222	3.623E-12	2.595	0.487
Gm17301	-8.765	0.012	2.621	0.524
4930412F15Rik	-8.857	1.911E-08	24.182	6.202
Rps6-ps2	-19.250	0.058	5.350	3.193
Pcna-ps2	-37.524	0.00001511	3.927	0.249
Gm9781	-167.019	1.06E-109	47.860	1.135
Gm5806	-202.474	1.708E-34	7.665	0.194

Appendix **B**

IACUC LETTER OF APPROVAL

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NOV 28 2012
IACUC

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research and Teaching

(Please complete below using Arial, size 12 Font.)

Title of Protocol: The investigation of developmentally important genes in the mouse and chicken

AUP Number: 1039-2013-0 ← **(4 digits only — if new, leave blank)**

Principal Investigator: Melinda K. Duncan

Common Name: mouse, chicken, rat

Genus Species: Mus musculus, Gallus gallus, Rattus rattus

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: *(Note change of categories from previous form)*

Category	Description
☐ B	Breeding or holding where NO research is conducted
☐ C	Procedure involving momentary or no pain or distress
XX D	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: ____________

Date of Approval: 12/1/12