**β1-Integrin Deletion From the Lens Activates Cellular Stress Responses Leading to Apoptosis and Fibrosis**

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**PURPOSE.** Previous research showed that the absence of β1-integrin from the mouse lens after embryonic day (E) 13.5 (β1MLR10) leads to the perinatal apoptosis of lens epithelial cells (LECs) resulting in severe microphthalmia. This study focuses on elucidating the molecular connections between β1-integrin deletion and this phenotype.

**METHODS.** RNA sequencing was performed to identify differentially regulated genes (DRGs) in β1MLR10 lenses at E15.5. By using bioinformatics analysis and literature searching, Egfr1 (early growth response 1) was selected for further study. The activation status of certain signaling pathways (focal adhesion kinase [FAK]/Erk, TGF-β, and Akt signaling) was studied via Western blot and immunohistochemistry. Mice lacking both β1-integrin and Egfr1 genes from the lenses were created (β1MLR10/Egfr1f/f) to study their relationship.

**RESULTS.** RNA sequencing identified 120 DRGs that include candidates involved in the cellular stress response, fibrosis, and/or apoptosis. Egfr1 was investigated in detail, as it mediates cellular stress responses in various cell types, and is recognized as an upstream regulator of numerous other β1MLR10 lens DRGs. In β1MLR10 mice, Egfr1 levels are elevated shortly after β1-integrin loss from the lens. Further, pErk1/2 and pAkt are elevated in β1MLR10 LECs, thus providing the potential signaling mechanism that causes Egfr1 upregulation in the mutant. Indeed, deletion of Egfr1 from β1MLR10 lenses partially rescues the microphthalmia phenotype.

**CONCLUSIONS.** β1-integrin regulates the appropriate levels of Erk1/2 and Akt phosphorylation in LECs, whereas its deficiency results in the overexpression of Egfr1, culminating in reduced cell survival. These findings provide insight into the molecular mechanism underlying the microphthalmia observed in β1MLR10 mice.

Keywords: cell stress, lens, development

The ocular lens is transparent tissue composed of two polarized cell types, lens epithelial cells (LECs) and elongated fiber cells, whose basal tips interact with the lens capsule, a thickened basement membrane that completely surrounds the lens. Cell-cell and cell-capsule adhesion and communication are important for lens structural integrity, cellular communication, cell survival, and ultimately, lens transparency. The lens expresses a wide variety of cell adhesion molecules that can regulate lens structure and physiology, although their functional complexity is generally not well understood.

Integrins are heterodimeric transmembrane adhesion molecules that consist of noncovalently associated α and β subunits. They are best known as mediators of bidirectional cell communication with the extracellular matrix and cell surface proteins on neighboring cells. The ocular lens expresses β1-integrins in all cells, and these proteins are proposed to be major regulators of lens cell contact with their basement membrane (the lens capsule) due to their localization at the basal surface of all lens cells. This is consistent with the significant transcript-level expression, in the embryonic day (E)15.5 mouse lens, of several α-integrins that are capable of forming extracellular matrix-binding αβ1 heterodimers. These include the laminin-binding α6- (19 reads per kilobase per million [RPKM]), the fibronectin/vitronectin/osteonectin binding α5- (8 RPKM), the laminin-binding α5- (5.8 RPKM), the collagen-binding α2- (2.3 RPKM), and the fibronectin binding α5-integrin (2.0 RPKM). Of these, αβ6β1 and α3β1 are likely to be the functionally most crucial in the lens, as α6/α5-integrin double-null lenses exhibit abnormalities similar to those arising from β1-integrin deletion from the early lens. However, no one α-integrin is likely responsible for all lens integrin functions, as suggested by the findings that α6-integrin null lenses exhibit only mild defects, whereas α3-integrin and α5-integrin null lenses are morphologically indistinguishable from wild type (WT). Lens integrins also likely regulate growth factor signaling by diverse mechanisms, such as the activation of latent growth factors or direct binding to growth factor receptors in cis, as well as indirectly via integrin-mediated signal transduction.

Conditional deletion of β1-integrin from the lens at different stages of development results in distinct phenotypes. For example, removal of β1-integrin from elongating lens fiber cells (using MLR39-Cre) results in a reduction in F-actin localization at fiber cell membranes and altered gap junctional coupling, leading to defects in lens fiber cell structure. In contrast,
deletion of \(\beta 1\)-integrin earlier, in the lens vesicle (mouse E10.5) using LE-Cre (\(\beta 1\)LE), results in the exit of LECs from the cell cycle, activation of ectopic Erk and bone morphogenetic protein (BMP) signaling, and inappropriate differentiation of the entire lens epithelium into lens fibers.\(^{15,26}\) Furthermore, deletion of \(\beta 1\)-integrin from all lens cells at mouse E12.5 to E13.5 via MLR10-Cre (\(\beta 1\)MLR10) leads to disorganization of the lens epithelium with upregulation of \(\alpha\)-smooth muscle actin (\(\alpha\)SMA) expression by E16.5, and extensive apoptosis of LECs by birth, resulting in the absence of lenses in adults.\(^{26}\) However, the molecular basis of \(\beta 1\)-integrin function in the ocular lens.

**METHODS**

**Animals**

All animal experiments described in this article conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were maintained and bred under specific pathogen-free conditions at the University of Delaware animal facility. Mice lacking \(\beta 1\)-integrin expression in all lens cells via Cre-mediated conditional deletion driven by MLR10-Cre (\(\beta 1\)MLR10) or Le-Cre (\(\beta 1\)LE) were created as previously described.\(^{15,26}\) Egr1 null mice (B6N;129-Egr1\(^{null}\)\(^{+/}\)) were bred to \(\beta 1\)MLR10 mice, to create mice carrying the MLR10-Cre allele and homozygous for both \(\beta 1\)-integrin and Egr1 alleles (\(\beta 1\)MLR10/Egr1\(^{+/}\)). All phenotypic comparisons were done among littermates. No phenotypic differences were found between \(\beta 1\)MLR10 and \(\beta 1\)MLR10/Egr1\(^{+/}\) or \(\beta 1\)MLR10/Egr1\(^{+/}\) mice. All mice carrying \(\beta 1\)MLR10 also carry CP49 mutations due to their genetic background. Noon of the day that a vaginal plug was detected in female mice was considered as E0.5.

**DNA Extraction and Genotyping**

DNA was isolated from tail snips or embryos using the PureGene Tissue and Mouse Tail kit (Gentra Systems, Minneapolis, MN, USA). Mice were genotyped by PCR using primers described in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence of the Primers</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta 1)-integrin</td>
<td>Fwd: 5’-CGG CTC AAA GCA GAG TGT CAG TC-3’&lt;br&gt;Rev: 5’-CCA CAA CTT GCC AAG TTG GCT CT-3’</td>
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<td>MLR10-Cre</td>
<td>Fwd: 5’-GCA TTC CAG CAG GCT GAG TG-3’&lt;br&gt;Rev: 5’-GAG CCC GGA CAC ATG AAG-3’</td>
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<td>Egr1</td>
<td>Common Fwd: 5’-GGG CAC AGG GGA TGG GAA TG-3’&lt;br&gt;Rev for WT: 5’-AAC CGG CCC AGC AAG ACA CC-3’</td>
<td>Recommended by the Jackson Laboratory</td>
</tr>
<tr>
<td>Egr1 (qRT-PCR)</td>
<td>Fwd: 5’-GCA AGT ACC CCA ACC GGC CC-3’&lt;br&gt;Rev: 5’-GCG CGA TCG CAG GAC TCG AC-3’</td>
<td>Designed by A. Terrell</td>
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<tr>
<td>Nab2</td>
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<td>zSMA</td>
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<td>Mt1</td>
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<td>Anxa2</td>
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<td>Ref. 97</td>
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<td>Pla1</td>
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<td>Recommended by the Jackson Laboratory</td>
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<td>Mmp14</td>
<td>RT(^2) qPCR Primer Assay for Mouse Mmp14</td>
<td>Qiagen, PPM03617D</td>
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<td>Tlbs1</td>
<td>Fwd: 5’-CACCCTTCCGGTTATTGAG-3’&lt;br&gt;Rev: 5’-GCACCGCAGACTCCCTTGGAG-3’</td>
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<tr>
<td>Stmn1</td>
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<td>Rpl29</td>
<td>Fwd: 5’-TCCAGTAGACATCGCCTGAG-3’&lt;br&gt;Rev: 5’-TGCAATTCCTTCTCAGGCCTTT-3’</td>
<td>Ref. 100</td>
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</table>

**Table 1.** List of All Primers Used for PCR and qRT-PCR

Fwd, forward; Rev, reverse.
analysis of the resulting data and filtering for significant changes was performed as previously described, except that statistical analysis was done using the pairwise quintile-adjusted conditional maximum likelihood method exact test with a Benjamini Hochberg false discovery rate correction run on the EdgeR BioConductor package (http://bioconductor.org).

RNA Isolation/cDNA Synthesis/Quantitative RT-PCR
RNA was isolated from pooled lenses at different embryonic stages (E13.5, E14.5, E15.5, and E16.5; at least three biological replicates derived from independent tissue pools per stage) using the SV Total RNA Isolation System (Promega, Fitchburg, WI, USA). At E15.5, different RNA samples were used for RNA-seq and RT-PCR validations. The RT2 First Strand Synthesis Kit (Qiagen, Valencia, CA, USA) was used to synthesize cDNA, and this was used in quantitative RT-PCR (qRT-PCR) reactions performed with a QuantiTect SYBR Green PCR Kit (Qiagen) on an Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). See Table 1 for primer sequences used in qRT-PCR. Fold change was calculated via the $2^{\Delta \Delta CT}$ method, and statistical significance was determined by 2-level nested ANOVA.

Bioinformatics Analyses
The filtered differentially regulated gene (DRG) list was analyzed for enriched pathways using PANTHER (Protein Analysis Through Evolutionary Relationships, http://www.pantherdb.org/) and analyzed for enriched expression during normal lens development using iSyTE (Integrated Systems Tool for Eye Gene Discovery, http://bioinformatics.udel.edu/research/isyte/) as previously described. Motif enrichment analysis was performed on the putative regulatory regions surrounding the transcriptional start site (TSS) of the β1MLR10 lens DRGs ($n = 120$). First, we implemented the iRegulon package (https://omictools.com/iRegulon-tool) to identify enrichment of overrepresented transcription factor (TF) binding motifs using the open-access databases for transcription factor binding profiles (Supplementary Table S3). The 10-kb (TSS–5 kb and TSS+5 kb) a priori defined regulatory region (described in Ref. 32) of all 120 DRGs was searched for TF-binding motifs at a significant cutoff of normalized enrichment score (NES) ≥3. Next, Egr1 binding sites similar to the position weight matrix for the Egr1 motif were identified in the region 2.5 kb upstream of the TSS of β1MLR10 lens DRGs ($n = 120$) using an in-house script implemented in the MotifDb R-package (www.bioconductor.org). This Egr1 motif matching was performed with a minimum score match percentage (80%) using the matchPMW function in the "Biostring" package (www.bioconductor.org). The Egr1 logo used in this analysis is shown in Figure 6.

Gross Morphology
Adult mice (age range from 2 to 5 months old) were killed and photographed. Eye tissues were enucleated and photographed using a Zeiss Stemi SV 11 Apo Stereo dissecting microscope (Zeiss, Thornwood, NY, USA) under dark-field illumination. Afterward, lenses were dissected and placed into prewarmed culture Medium 199 (Cellgro; Mediatech, Inc., Manassas, VA, USA) and photographed under bright-field illumination. A minimum of three biological replicates were analyzed for each genotype.

Western Blot (WB)
Protein was extracted from lenses using lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-
Compared with WT, the expression of $b$ at E16.5 was not significant due to the high SD of the data. Compared with WT, the expression of $b$ to E16.5. The elevation in $b$ mRNA at E16.5 was not significant due to the high SD of the data. 

**FIGURE 2.** qRFPqPCR analysis of (A) $\alpha$SMA, (B) Anxa2, (C) Mmp14 (Matrix metalloprotease 14/membrane type 1 matrix metalloproteinase), and (D) Plat mRNA expression in WT and $b$1MLR10 lenses from E13.5 to E16.5. (A) Compared with WT, the expression of $\alpha$SMA was significantly higher in $b$1MLR10 lenses from E14.5 to E16.5. (B) Compared with WT, the expression of Anxa2 was significantly higher in $b$1MLR10 lenses from E13.5 to E15.5. The elevation in $\alpha$SMA mRNA at E16.5 was not significant due to the high SD of the data. (C) Compared with WT, the expression of MMP14 was significantly higher in $b$1MLR10 lenses from E13.5 to E15.5. The elevation in MMP14 mRNA at E16.5 was not statistically significant due to the high SD of the data. (D) Compared with WT, the expression of Plat was significantly higher in $b$1MLR10 lenses from E13.5 to E15.5. The elevation in Plat mRNA at E16.5 was not significant due to the high SD of the data. Error bars represent SD. Statistical significance was determined with nested ANOVA and is given above the error bar in the figure.

Immunofluorescence Staining and Confocal Imaging

All immunofluorescence (IF) analyses were performed as previously described. Briefly, embryonic head tissue was collected, while eyes were collected from newborn or adult mice. Tissues were embedded directly in Optimum Cutting Temperature (Tissue Tek, Torrance, CA, USA) and stored at −80°C until 16-μm-thick sections were obtained with a Leica CM3050 cryostat (Leica Microsystems, Buffalo Grove, IL, USA), and mounted on slides (ColorFrost Plus; Fisher Scientific, Hampton, NH, USA). Slides were fixed with either 1:1 acetone-methanol for 20 minutes at −20°C or 4% paraformaldehyde for 30 minutes at room temperature. Blocking buffer was made in 1X PBS or 1X Tris-buffered saline (TBS), and slides were blocked for 1 hour at room temperature. Sections were then incubated with primary antibody diluted with blocking buffer (see Table 2 for antibodies and dilution rates used) for 1 hour at room temperature, or overnight at 4°C. Sections were then washed in 1X PBS or 1X TBS, and incubated with a solution consisting of Alexa Fluor 488/568 labeled secondary antibody (1:200 dilution; Invitrogen, Grand Island, NY, USA), Draq-5 (1:2000 dilution; Biostatus Limited, Shepshed, Leicestershire, UK), and fluorescein-labeled anti-αSMA (1:200 dilution; Sigma-Aldrich Corp., St. Louis, MO, USA) for 1 hour at room temperature in the dark. Sections were washed in 1X PBS or 1X TBS, then mounted with mounting media (10 mL PBS with 100 mg p-phenylenediamine to 90 mL glycerol; final pH 8.0).

Slides were imaged with a Zeiss LSM 780 confocal microscope (Carl Zeiss, Inc., Gottingen, Germany). For each experiment/comparison, all sections were stained simultaneously and imaged using identical configurations to ensure the validity of staining intensity comparisons. Under some circumstances, images were processed to optimize the brightness and/or contrast for optimal viewing on diverse computer screens. However, in all cases, any such adjustments were applied identically to both control and experimental images. At least six biological replicates were analyzed for each genotype.

Immunohistochemistry

Immunohistochemistry was used to detect pErk1/2 and pAkt levels in the lens as previously described, using the CSA II Biotin-Free Tyratime Signal Amplification System (K150011-2; Dako Laboratories, Carpenteria, CA, USA) and CSA II Rabbit Link (K150180-2; Dako Laboratories), following the manufacturer’s instructions (see Table 2 for the primary antibodies used). At minimum, three biological replicates were analyzed for each genotype.
RESULTS

RNA-Seq Identifies 120 Differentially Expressed Genes in E15.5 β1MLR10 Lenses

Deletion of β1-integrin from the lens after the completion of primary fiber cell elongation (E12.5–E13.5; β1MLR10) causes the lens epithelium to become grossly abnormal by E16.5.26 Thus, RNA-seq was performed a day earlier, at E15.5, on β1MLR10 and WT lenses to identify changes in gene expression that might proximally drive the observed morphological alterations, while minimizing the detection of gene expression changes secondary to the phenotype. Analysis of the gene list confirms that the lens isolations included minimal contamination with nonlens ocular tissues, as keratin 8 mRNA levels are very low in these samples (β1MLR10 lenses, 0.08 RPKM; WT lenses, 0 RPKM), which is expressed robustly in the cornea at this age.19 Although we attempted to completely remove the adherent blood vessels (the tunica vasculosa lentis [TVL]) from these lenses, the RNA-seq suggests that the isolated lenses did likely retain some TVL, as low levels of Pecam mRNA (a blood vessel marker)28 was detected in both WT (1.5 RPKM) and β1MLR10 (2.2 RPKM) lenses. However, this was unlikely to affect the analysis, as these levels were not significantly different (P = 0.18, n = 4). Qualitatively fewer LECs of β1MLR10 lenses had detectable levels of pSmad1/5/8 compared with WT. Blue, DNA; (C–F) red, pSmad3; (I–L) red, pSmad1/5/8; green, αSMA; e, epithelial cell; f, fiber cell. Scale bar: 38 μm.

Figure 3. Analysis of Smad phosphorylation in E16.5 WT and β1MLR10 lenses. (A) Representative WB comparing pSmad3 levels in WT and β1MLR10 lenses. (B) Quantitation of the pSmad3 levels in WT and β1MLR10 lenses showing that pSmad3 levels were significantly lower in β1MLR10 lenses compared with WT (*P = 0.023, n = 4). (C–F) IF staining for pSmad3 (red) and αSMA (green) in WT (C, D) and β1MLR10 (E, F) lenses at E16.5. Qualitatively, pSmad3 staining was reduced in β1MLR10 LECs compared with WT. (G) Representative WB comparing pSmad1/5/8 levels between E16.5 WT and β1MLR10 lenses. (H) WB quantitation showing that pSmad1/5/8 levels in E16.5 WT and β1MLR10 lenses were not significantly different (P = 0.18, n = 4). (I–L) IF staining for pSmad1/5/8 (red) and αSMA (green) in WT (I, J) and β1MLR10 (K, L) lenses at E16.5. Qualitatively fewer LECs of β1MLR10 lenses had detectable levels of pSmad1/5/8 compared with WT. Blue, DNA; (C–F) red, pSmad3; (I–L) red, pSmad1/5/8; green, αSMA; e, epithelial cell; f, fiber cell. Scale bar: 38 μm.
enough levels in either the WT or β1MLR10 lens to plausibly affect cell function (unnormalized RPKM over 2); (2) the difference between WT and β1MLR10 lenses is greater than 2.5-fold to remove genes whose expression is different due to genetic background variations; and (3) exclusion of pseudo-genes or unknown/predicted genes. This filtering resulted in a list of 120 DRGs (76 upregulated and 44 downregulated) that are plausibly involved in the phenotypic changes found in β1MLR10 lenses (Supplementary Table S1). Both the raw and processed RNA-seq data have been deposited in the Gene Expression Omnibus under accession number GSE77188.

**Upregulated DRGs in β1MLR10 Lenses Include Genes Related to Cellular Stress and EMT Responses**

The 120 prioritized candidate genes from the RNA-seq experiment were further analyzed by the bioinformatics tools PANTHER (http://www.pantherdb.org/),30 IPA (Qiagen, Valencia, CA, USA, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/) and DAVID (The Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov). For instance, according to PANTHER, these 120 genes can participate in 33 different pathways, which expectedly included apoptosis and integrin signaling pathways (Fig. 1; Supplementary Table S2). However, none of these tools identified common pathways shared by more than a handful of the 120 genes, and many of the identified “pathways” were represented by only one gene, so these analyses did not give great insight into β1-integrin function in the lens (Fig. 1; Supplementary Table S2; data not shown).

The relative expression of each DRG in the lens compared with a mouse whole embryonic body (WB) reference dataset was determined using iSyTE,31 which has previously been shown to effectively identify lens-enriched genes.35 This analysis revealed that only 12 of the 76 upregulated and 7 of 44 downregulated DRGs exhibited enriched expression in the lens compared with reference (Supplementary Table S1). These findings suggest that β1-integrin is not a major regulator of lens-enriched gene expression after the completion of initial lens morphogenesis. Therefore, PubMed was used to interrogate the scientific literature, and relationships between the genes were manually assessed. This analysis revealed that 37 of the 76 upregulated DRGs are connected to either cellular stress responses, fibrosis or EMT in the scientific literature, whereas only 6 of the 44 downregulated genes had those connections (Table 3). The Fisher’s exact test of independence suggest that the proportion of stress response, fibrotic, and EMT-related genes is significantly different (P = 0.0001) between upregulated and downregulated DRGs.

The time course of induction of four of these fibrotic/EMT marker genes (Table 3), chosen due to their RNA abundance (tissue plasminogen activator [Plat], Mmp14, and Annexin A2 [Anxa2]) and/or importance as a marker of LEC EMT (αSMA), were investigated by qPCR of independent lens RNA samples...
however, Egr1 protein was seen in numerous nuclei of the peripheral epithelium. At E15.5, no Egr1 protein was detected in WT lenses (Fig. 2A), consistent with findings by IF (Figs. 1B, 1E). Anxa2, a phospholipid-binding protein induced in numerous fibrotic conditions, was significantly elevated by E13.5, and remained elevated later (Fig. 2B). MT1-MMP (encoded by MMP14) is a membrane-associated matrix metalloprotease that can activate latent TGF-β. The precise levels of MT1-MMP are critical for normal tissue function, as it is induced in fibrosis. But its absence also results in fibrosis due to defective collagen turnover. Like Anxa2, MMP14 mRNA levels are first elevated at E13.5 in β1MLR10 lenses, and they remain elevated later (Fig. 2C). Platelet-activating factor (PAF) is a pro-fibrotic factor. Its mRNA levels are also first elevated at E13.5 in β1MLR10 lenses, and these levels remain elevated later (Fig. 2D). Unfortunately, attempts to immunolocalize MMP14, Anxa2, and PAF were unsuccessful due to antibody limitations. Thus, it is unknown whether these mRNA differences are reflected at the protein level.

**Phosphorylated Smad Signaling Is Not Elevated in E16.5 β1MLR10 Lenses**

In the lens, EMT is best understood to be mediated by elevated Smad2/5 phosphorylation, driven by TGF-β signaling. However, WB detected a reduction in Smad3 phosphorylation (Figs. 3A, 3B) in β1MLR10 lenses at E16.5, which was also validated by IF analysis comparing phosphorylated Smad3 (pSmad3) levels between WT (Figs. 3C, 3D) and β1MLR10 lenses (Figs. 3E, 3F). Because we recently found that deletion of β1-integrin from the lens vesicle resulted in ectopic activation of Smad1/5/8 phosphorylation in the lens epithelium by E12.5, we also evaluated pSmad1/5/8 levels in β1MLR10 lenses. By WB (Figs. 3G, 3H), pSmad1/5/8 levels were not significantly changed in E16.5 β1MLR10 lenses, and by IF fewer pSmad1/5/8 positive nuclei were detected in β1MLR10 lenses (Figs. 3K, 3L) than WT (Figs. 3I, 3J). The downregulation of pSmad3 and pSmad1/5/8 labeling in E16.5 β1MLR10 lenses appears to contradict our prior observation that nuclear Smad4 levels are elevated in this tissue but may reflect the difference between evaluating levels of an active (pSmad3 or pSmad1/5/8) protein form versus total levels of a protein that interacts with many different Smads (Smad4). Evaluation of both pSmad3 and pSmad1/5/8 distribution in the lens before E16.5 revealed no obvious differences between WT and β1MLR10 lenses (data not shown). Overall, these findings suggest that the mechanism underlying the pathology of β1MLR10 lenses is independent of either TGF-β-driven elevations in Smad2/3 phosphorylation or BMP-driven activation of Smad1/5/8. We then proceeded to investigate other signal transduction cascades that may be responsible for the pathology of the β1MLR10 lens.
β1-Integrin and Cellular Stress in Lens

Figure 6. The putative promoters of genes differentially regulated in β1MLR10 lenses are enriched in Egr1 DNA-binding motifs. (A) Sequence logo for the JASPAR core Egr1 binding site based on the previously reported Egr1 position weight matrix.188 (B) Motif enrichment analysis revealed a statistically significant enrichment of Egr1 DNA-binding motifs in the putative regulatory regions 2.5 kb upstream of TSS of DRGs identified in β1MLR10 lenses. The start and end positions of the promoter region that matches the Egr1 DNA-binding motif (5’ to 3’; position relative to TSS) for each candidate DRG are given. Upregulated DRGs are indicated in red, and downregulated DRGs are indicated in green.
<table>
<thead>
<tr>
<th>Antibodies for IF</th>
<th>Fixation</th>
<th>Blocking Buffer</th>
<th>Dilution Buffer</th>
<th>Incubation Condition and Dilution Rate</th>
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<td>β1-integrin (MAB 1997, Millipore)</td>
<td>1:1 acetone-methanol</td>
<td>2% BSA in PBS</td>
<td>2% BSA in PBS</td>
<td>1 h at room temperature, 1:200</td>
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<td>α-SMA (1A4 F3777, Sigma-Aldrich Corp.)</td>
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<td>2% BSA in PBS</td>
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<td>1 h at room temperature, 1:200</td>
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<td>Aquaporin0 (AB3071, Millipore)</td>
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<td>2% BSA in PBS</td>
<td>2% BSA in PBS</td>
<td>Overnight at 4°C, 1:200</td>
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<td>Cleaved Caspase 3 (9661, Cell Signaling)</td>
<td>4% PFA</td>
<td>2% BSA and 5% goat serum in TBS</td>
<td>2% BSA and 5% goat serum in TBS</td>
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<tr>
<td>cMaf (sc-7866, Santa Cruz)</td>
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<td>2% BSA in PBS</td>
<td>2% BSA in PBS</td>
<td>1 h at room temperature, 1:100</td>
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<td>Prox1, Polyclonal antibody produced in house against homeo-Prospero domain; validated on Prox1 null lenses</td>
<td>1:1 acetone-methanol</td>
<td>2% BSA in PBS</td>
<td>2% BSA in PBS</td>
<td>1 h at room temperature, 1:500</td>
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<td>Egr1 (4153, Cell Signaling); antibody validated on injured Egr1-null lenses (unpublished data)</td>
<td>4% PFA</td>
<td>5% goat serum, and 0.3% Triton X-100 in PBS</td>
<td>2% BSA, and 0.3% Triton X-100 in PBS</td>
<td>Overnight at 4°C, 1:100</td>
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<td>pSmad3 (clone EP823Y, Epitomics)</td>
<td>4% PFA</td>
<td>2% BSA, 10% goat serum and 0.1% Triton X-100 in TBS</td>
<td>2% BSA, 10% goat serum, and 0.1% Triton X-100 in TBS</td>
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<td>pSmad1/5/8 (sc-12353, Santa Cruz Biotechnology)</td>
<td>4% PFA</td>
<td>5% goat serum and 2% BSA in PBS</td>
<td>5% goat serum and 2% BSA in PBS</td>
<td>1 h at room temperature, 1:50</td>
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<td>Nab2 (19601-1-AP, Proteintech)</td>
<td>4% PFA</td>
<td>5% goat serum, 0.3% Triton X-100 in PBS</td>
<td>2% BSA, 0.3% Triton X-100 in PBS</td>
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<tr>
<td>Antibodies for IHC</td>
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<td></td>
</tr>
<tr>
<td>pAkt (9271s, Cell Signaling)</td>
<td>Paraffin section</td>
<td>2% BSA in TBS</td>
<td>2% BSA in TBS</td>
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</tr>
<tr>
<td>pErk (4377s, Cell Signaling)</td>
<td>Paraffin section</td>
<td>2% BSA in TBS</td>
<td>2% BSA in TBS</td>
<td>Overnight at 4°C, 1:50</td>
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<td>Antibodies for WB</td>
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<tr>
<td>p44/p42 MAPK (Erk1/2) (9102, Cell Signaling)</td>
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<td>-</td>
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<tr>
<td>Phospho-p44/p42 MAPK (pErk1/2) (Thr202/Tyr204) (9101, Cell Signaling)</td>
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<tr>
<td>pFAK (Y397) (3283, Cell Signaling)</td>
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<tr>
<td>pSmad3 (Sers423/Ser425) (P8402, Millipore)</td>
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Dashes indicate that these steps are for IF and IHC only, which were not included during WB. PFA, paraformaldehyde.
FIGURE 7. Developmental expression pattern of Nab2 in WT and β1MLR10 lenses. (A) qRT-PCR analysis of Nab2 mRNA in WT and β1MLR10 lenses from E13.5 to E16.5, showing that Nab2 mRNA levels were significantly elevated in β1MLR10 lenses between E13.5 and E15.5, whereas these levels were similar in WT at E16.5. (B–I) IF localization of Nab2 protein (red) in WT and β1MLR10 lenses from E13.5 to E16.5. At E13.5, no Nab2 protein was detected in either WT (B) or β1MLR10 lenses (C). At E14.5, no Nab2 protein was detected in WT lenses (D); however, Nab2-positive nuclei (arrow) were seen in the peripheral β1MLR10 lens epithelium. At E15.5, no Nab2 protein was detected in WT lenses (F); however, Nab2 protein was seen in a few nuclei of the peripheral β1MLR10 lens epithelium (G, arrow). At E16.5, no Nab2 protein was detected in WT lenses (H), whereas Nab2 protein was detected in islands of nuclei in the LECs closest to the transition zone of β1MLR10 lenses (arrow). (B–I) Blue, DNA; red, Nab2; e, epithelial cell; f, fiber cell. Scale bar: 71 μm. Error bars in (A) represent SD. Statistical significance was determined with nested ANOVA and is given above the error bar in the figure.

FIGURE 8. Developmental expression pattern of Egr1 and Nab2 in WT and β1LE lenses. (A–D) Sections from E11.5 eyes stained for Egr1 (red, [A, B]) and Nab2 (red, [C, D]). (A) WT lenses lack Egr1 immunoreactivity at E11.5, whereas (B) Egr1-positive nuclei (arrows) were detected in the lens vesicle of E11.5 β1LE lenses. (C) No Nab2 immunostaining was detected in WT lenses at E11.5. (D) Nab2-positive nuclei (arrows) were found in the lens vesicle of E11.5 β1LE lenses. (E–H) Sections from E13.5 eyes stained for Egr1 (red, [E, F]) and Nab2 (red, G, H). Both WT (E) and β1LE lenses (F) showed no Egr1 immunoreactivity at E13.5. (G) No Nab2 immunostaining was observed in WT lenses at E13.5, whereas (H) many Nab2-positive nuclei (arrows) were found in the abnormally differentiating fiber cells in E13.5 β1LE lenses. (A–H) Blue, DNA; (A, B) and (E, F) red, Egr1; (C, D) and (G, H) red, Nab2; lv, lens vesicle; r, retina; e, lens epithelium; f, lens fibers. Scale bar: 71 μm (A–H).
to the peripheral lens epithelium (Figs. 5G, 5I) in β1MLR10 lenses.

Egr1 Binding Sites Are Present and Enriched in Genes Upregulated in β1MLR10 Lenses

Next, to investigate the molecular connection between upregulation of IER transcription factors and the other genes misregulated in the β1MLR10 lens, we took two approaches: (1) examine if any TF-binding motifs were enriched in the DRGs; and (2) specifically analyze if the Egr1 motif is overrepresented in the putative promoters of the DRGs. The iRegulon package32 was used to identify the enrichment of known TF-binding motifs in the putative regulatory region (10 kb total sequence analyzed from −5kb to +5kb of the TSS of the 120 β1MLR10 lens DRGs. This analysis identified enrichment and overrepresentation of binding sites for Cys2His2 TFs (such as Egr1), leucine zipper TFs (such as the AP1 factors Fos and Junb), and members of the Ets domain family (which encode direct transcriptional effectors of Erk phosphorylation)55 (Table 4) in these putative regulatory regions. Further, iRegulon analysis identified overrepresentation of Egr1 binding sites in 38% (46/120) of the DRGs, including 32 of the upregulated and 14 of the downregulated candidates with a significant (≥3) NES of 5.8 (Table 4).

Egr1 is a multifunctional TF that regulates cellular stress, apoptosis, and fibrosis in different contexts.56–59 As Egr1 is upregulated in β1MLR10 lenses and its binding sites were also overrepresented in the putative regulatory regions of β1MLR10 lens DRGs, we performed a detailed analysis to determine the presence of Egr1 binding motifs in the putative promoters of these genes irrespective of their enrichment. Thus, a 2.5-kb region upstream of the TSS of all 120 DRGs was analyzed for putative Egr1 binding motifs using the JASPAR position weight matrix33 for Egr1, CORE-Egr1-MA0162.1 (Fig. 6A). This analysis revealed that the 5′ flanking sequence of 31 of the 76 upregulated DRGs and 21 of the 44 downregulated DRGs contained consensus Egr1 binding motifs (Fig. 6B). To gain further insight into potential molecular connections between Egr1 and the DRGs, we examined the scientific literature to identify β1MLR10 lens DRGs that may have published connections with this transcriptional regulator. We find 18 of the 76 upregulated DRGs to be either regulated by or coregulated with Egr1 (see Table 3, Supplementary Table S1). In contrast, only 1 of the 44 downregulated DRGs has a previously described connection with Egr1. Among the 19 DRGs with prior evidence connecting them with Egr1, 8 are
validated as direct Egr1 target genes in other cells or tissues (Table 3).

The Established Egr1 Target Gene Nab2 Is Upregulated in β1MLR10 Lenses

Interestingly, Ngfi-A binding protein 2 (Nab2), which exhibits the highest extent of upregulation (14-fold) among these eight known Egr1 direct target genes (Fig. 6B; Table 3), is known to be intricately involved in Egr1 function. Nab2 participates in a negative feedback loop to downregulate Egr1 expression, thus limiting the IER,58–60 while simultaneously stimulating Egr1-mediated transcription at promoters with weak Egr1 binding sites.61,62 Therefore, we investigated the expression pattern of Nab2 in β1MLR10 lenses (Fig. 7). Nab2 transcript levels were significantly upregulated at E13.5, exhibit a further sharp elevation at E14.5, and remain elevated in E15.5 β1MLR10 lenses (Fig. 7A). By immunolocalization, no Nab2 protein was detected in the WT lens at any stage examined (Figs. 7B, 7D, 7F, 7H). Although Nab2 protein was not detected in the β1MLR10 lenses. (A–C) IF of cleaved Caspase 3 (red) in newborn lenses. WT lenses (A) exhibit no cleaved Caspase 3 immunoreactivity under the conditions used. Intense cleaved Caspase 3 immunoreactivity was seen in both the lens fibers and epithelium (arrow) of β1MLR10 lenses (B), whereas little to no cleaved Caspase 3 immunoreactivity was seen in newborn β1MLR10/Egr1−−/− lenses (C). (D–F) TUNEL staining of newborn lenses. WT lenses exhibit no TUNEL-positive nuclei (green) at birth (D), whereas TUNEL staining was detected in both the epithelial cells and fibers of both β1MLR10 (E) and β1MLR10/Egr1−−−−/−−/−−/− lenses (F) (arrows). (G–I) Immunolocalization of cMaf in E16.5 lenses. WT lenses (G) exhibit strong cMaf labeling in the transition zone nuclei in the midst of differentiating into lens fiber cells. cMaf staining is seen in a similar pattern in β1MLR10 lenses, although the number of positive nuclei appears expanded (H). β1MLR10/Egr1−−−−/−−/−−/− lenses (I) appear to exhibit brighter cMaf staining overall, and this staining extends farther into the lens epithelium than either WT or β1MLR10 lenses. (J–L) Immunolocalization of Prox1 in E16.5 lenses. WT lenses (J) exhibit strongest Prox1 labeling in the transition zone nuclei in the midst of differentiating into lens fiber cells, whereas some Prox1 protein is detected in LECs at this age. Prox1 staining is seen in a similar pattern in β1MLR10 lenses, although positive nuclei are brighter in LECs than in control (K). β1MLR10/Egr1−−−−/−−/−−/− lenses (I) stain more intensely for Prox1 immunoreactivity in LECs than either WT or β1MLR10 lenses. (A–L) Blue, DNA; (A–C) red, cleaved Caspase 3; (D–F) green, TUNEL positive; (G–I) red, cMaf; (J–L) red, Prox1; e, epithelial cells; f, fiber cells. (A–F) Scale bar: 142 μm; (G–I) scale bar: 62 μm; (J–L) scale bar: 142 μm.
immunoreactivity in lens tissue, but retain the intense channel alone of the images shown in (arrows). Nab2 immunoreactivity at E16.5. (arrows) were observed in WT E11.5 (Fig. 8A) lenses, numerous Egr1-positive nuclei seen. While occasional Egr1-positive cell nuclei were detected from the lens, as well as at E13.5, when the Nab2 genes from the lens (beta1-integrin loss from the lens. Therefore, we generated mice that lacked both the beta1-integrin and Egr1 genes from the lens (beta1MLR10/Egr1^-/-). As previously reported, adult beta1MLR10 mice lack externally visible eyes, which are severely microphthalmic (Fig. 9A) with little to no identifiable lens tissue on dissection (Fig. 9D), although small islands of cells stain positive for the lens fiber marker, Aquaporin0 (Fig. 9G; n = 6). In contrast, the eyes of adult

**Figure 11.** Molecular phenotype of beta1MLR10/Egr1^-/- lenses. (A–C) Sections from E16.5 eyes stained for beta1-integrin (red) and alpha smooth muscle actin (green). E16.5 WT lenses (A) exhibit beta1-integrin immunoreactivity (red) in both lens epithelium and fiber cells. The intense beta1-integrin staining at the lens periphery is in the tunica vasculosa (arrows). E16.5 beta1MLR10 (B) and beta1MLR10/Egr1^-/- (C) lenses lack beta1-integrin immunoreactivity in lens tissue, but retain the intense beta1-integrin immunoreactivity in the tunica vasculosa (arrows). (D–F) The 25MA (green) channel alone of the images shown in (A–C). No 25MA immunoreactivity was detected in WT lenses (D) at E16.5; however, both beta1MLR10 (E) and beta1MLR10/Egr1^-/- (F) lenses exhibit 25MA immunoreactivity (green, arrows). (G–I) Sections from E16.5 eyes stained for Nab2. (G) WT lenses lack Nab2 immunoreactivity at E16.5. (H) Nab2-positive nuclei are detected in the peripheral epithelium of E16.5 beta1MLR10 lenses. (I) No Nab2 immunostaining was detected in beta1MLR10/Egr1^-/- lenses. (A–C and G–I) Blue: DNA; (A–F) green, 25MA; (A–C) red, beta1-integrin; (G–I) red, Nab2; c, lens epithelium; f, lens fibers. Scale bar: 142 μm (A–F); scale bar: 71 μm (G–I).
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<tr>
<th>Gene ID</th>
<th>Gene Description</th>
<th>Fold Change From WT</th>
<th>FDR, P Value</th>
<th>β1-MLR10 Mean RPKM</th>
<th>WT Mean RPKM</th>
<th>Lens Enrichment</th>
<th>Predicted Egr1 Motif in Promoter</th>
<th>Functional Connection With Egr1</th>
<th>Biological Function</th>
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<td>Ef3j2</td>
<td>Eukaryotic translation initiation factor 3, subunit J2</td>
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<td>5.96</td>
<td>0.01</td>
<td>np</td>
<td>No</td>
<td>nr</td>
<td>Regulates translational control during nutrient depletion⁶¹; selects AUG start codons⁶²</td>
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<tr>
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<td>Metallothionein 1</td>
<td>296</td>
<td>0.00E+00</td>
<td>447.52</td>
<td>1.49</td>
<td>No</td>
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<td>nr</td>
<td>Interacts with heavy metals; stress response⁶³; GPCR ligand; involved in regulating behavior during stress⁶⁵</td>
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<td>Npw</td>
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<td>21.02</td>
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<td>Expression activated by Egr1⁵⁷; Egr1 corepressor but also stimulates Egr1-mediated transcription of promoters with few or weak Egr1 sites⁶⁴,⁶⁵</td>
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<td>Nab2</td>
<td>Ngfi-A binding protein 2</td>
<td>13.76</td>
<td>1.50E-101</td>
<td>7.71</td>
<td>0.55</td>
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<td>Yes</td>
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<td>Transcription factor–negative regulator of Egr1; upregulated by Egr1 as late fibrotic response⁵⁷; promoter has 11 Egr1 sites⁶⁶</td>
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<td>Egr1</td>
<td>Early growth response 1; krox24</td>
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<td>18.07</td>
<td>1.66</td>
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<td>Yes</td>
<td>Egr1 regulates its own promoter⁵⁷,⁶⁸</td>
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<td>Tagln</td>
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<td>Transcription factor that is a key mediator of fibrosis⁵⁷; Egr1 depletion reduces fibrosis⁵⁷; smooth muscle–specific actin-binding protein; EMT marker⁶⁸</td>
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<td>Yes</td>
<td>Egr1 coexpressed in SMA positive cells⁶⁹; Egr1 can bind to acta2 promoter, represses transcription⁷⁰</td>
<td>Transcription factor that is a key mediator of fibrosis⁵⁷; Egr1 depletion reduces fibrosis⁵⁷; smooth muscle actin; EMT marker in lens⁷⁰</td>
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<td>Hmga1-rs1</td>
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<td>6.28</td>
<td>0.77</td>
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<td>Upregulated by BMP signaling, drives EMT via SLUG; coexpressed with βSMA and SM22⁷¹; binds AT-rich DNA in minor groove; regulates chromatin structure⁷²</td>
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<td>Collagen variant; expression restricted to mesenchymal cells⁷³; p53-induced cell cycle arrest gene⁷⁴; negative regulator of cell migration, invasion⁷⁵; transiently upregulated during cellular stress⁷⁶</td>
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<td>Rprm</td>
<td>Reprimo, TP53 dependent G2 arrest mediator candidate</td>
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<td>Collagen variant; expression restricted to mesenchymal cells⁷³; p53-induced cell cycle arrest gene⁷⁴; negative regulator of cell migration, invasion⁷⁵; transiently upregulated during cellular stress⁷⁶</td>
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<tr>
<td>Gene ID</td>
<td>Gene Description</td>
<td>Fold Change From WT</td>
<td>FDR, P Value</td>
<td>β1-MLR10 Mean RPKM</td>
<td>WT Mean RPKM</td>
<td>Lens Enrichment iSyTe at E12.5 1.5 Cutoff</td>
<td>Predicted Egr1 Motif in Promoter</td>
<td>Functional Connection With Egr1</td>
<td>Biological Function</td>
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<td>Tnfrsf12a</td>
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<td>5.55</td>
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<td>Expression activated by Src118, signals via AKT; overexpression in mesenchymal stem cells induces α-SMA expression.119</td>
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<td>Itgb8</td>
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<td>αV integrin binding partner; EMT marker; upregulated during lens EMT22; involved in TGFβ1 activation.120</td>
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<td>Cts5</td>
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<td>1.90E-14</td>
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<td>Yes</td>
<td>No</td>
<td>Coinduced with Egr1 on rabies infection of CNS121</td>
<td>Protease secreted at sites of tissue injury and fibrosis, deletion in muscular dystrophy reduces fibrosis122; upregulated in Smad4 null hearts leading to aneurysm.123</td>
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<td>Klf4</td>
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<td>4.85</td>
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<td>0.43</td>
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<td>Egr1 directly binds to the KLF4 promoter and upregulates its expression.65</td>
<td>Both enhances and inhibits renal fibrosis.124; upregulates αSMA expression and myofibroblast differentiation in cardiac fibrosis, binds directly to the TGFβ1 promoter125; upregulates Pax6 expression in cerebellar granule neuron126; activates or represses SM22a expression depending on context127; KLF4 deletion from lens results in elevation of ALOX12 (see below, Alox12 is repressed here)128</td>
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<td>Fos</td>
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<td>No</td>
<td>cFos and Egr1 are both transcriptional targets of STAT129; Fos and Egr1 are often coinduced130</td>
<td>An early response transcription factor induced in fibrosis.131</td>
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<td>Gene ID</td>
<td>Gene Description</td>
<td>Fold Change From WT</td>
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<td>β1-MIR10 Mean RPKM</td>
<td>WT Mean RPKM</td>
<td>Lens Enrichment byFE at E12.5 1.5 Cutoff</td>
<td>Predicted Egr1 Motif in Promoter</td>
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<td>Chac1</td>
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<td>1.08</td>
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<td>Upregulated in UPR in lens&lt;sup&gt;132&lt;/sup&gt;; induced by ATF4 during UPR; may be proapoptotic; involved in glutathione degradation&lt;sup&gt;135&lt;/sup&gt;</td>
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<td>Pak1</td>
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<td>0.49</td>
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<td>Upregulated by TGFβ signaling in fibrosis&lt;sup&gt;134&lt;/sup&gt;; Key effector of RHO proteins, actin structure; downstream mediator of integrin signaling; regulates FGF23 indirectly&lt;sup&gt;135&lt;/sup&gt;</td>
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<td>7.84</td>
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<td>Cytoskeletal protein induced by TGFβ may be involved with fibrosis&lt;sup&gt;135&lt;/sup&gt;</td>
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<td>Anxa2</td>
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<td>3.81</td>
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<td>6.35</td>
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<td>No</td>
<td>nr</td>
<td>Induced in fibrotic conditions, liver fibrosis marker&lt;sup&gt;94&lt;/sup&gt;; involved in regulation of cellular secretion&lt;sup&gt;139&lt;/sup&gt;</td>
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<td>Mmp2</td>
<td>Matrix metallopeptidase 2</td>
<td>3.55</td>
<td>7.10E-19</td>
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<td>0.72</td>
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<td>Regulates TGFβ-induced matrix contraction in lens&lt;sup&gt;142&lt;/sup&gt;; may regulate lens EMT&lt;sup&gt;145&lt;/sup&gt;</td>
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<td>Feer1g</td>
<td>Fc receptor, IgE, high affinity 1, gamma polypeptide</td>
<td>3.52</td>
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<td>0.67</td>
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<td>nr</td>
<td>Found in immune system, expression activated in CNS by chronic stress&lt;sup&gt;144&lt;/sup&gt;</td>
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<td>Fas</td>
<td>Fas (TNF receptor superfamily member 6)</td>
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<td>1.44</td>
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<td>Involved in apoptosis, regulated by TNF&lt;sup&gt;147&lt;/sup&gt;; can drive lung fibrosis&lt;sup&gt;145&lt;/sup&gt;</td>
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<td>C1qa</td>
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<td>3.70E-09</td>
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<td>1.02</td>
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<td>nr</td>
<td>Initiates complement cascade, involved in neural death on stress&lt;sup&gt;149&lt;/sup&gt;</td>
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**Table 3. Continued**

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<tr>
<th>Gene ID</th>
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<th>FDR, P Value</th>
<th>β1-MIR10 Mean RPKM</th>
<th>WT Mean RPKM</th>
<th>Lens Enrichment by YE at E12.5 1.5 Cutoff</th>
<th>Predicted Egr1 Motif in Promoter</th>
<th>Functional Connection With Egr1</th>
<th>Biological Function</th>
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<td>Plat</td>
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<td>No</td>
<td>nr</td>
<td>Protease; profibrotic factor in vascular injury&lt;sup&gt;150&lt;/sup&gt;; found at the apical tips of lens epithelial and fiber cells in the embryonic lens&lt;sup&gt;151&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fgf15</td>
<td>Fibroblast growth factor 15</td>
<td>3.29</td>
<td>3.80E-02</td>
<td>2.31</td>
<td>0.66</td>
<td>No</td>
<td>Yes</td>
<td>nr</td>
<td>Endocrine FGE requires klothos for its function&lt;sup&gt;152&lt;/sup&gt;; Fgf15 null mice exhibit less liver fibrosis&lt;sup&gt;153&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyr61</td>
<td>Cysteine-rich protein 61 / ccn1</td>
<td>3.27</td>
<td>2.80E-17</td>
<td>3.3</td>
<td>0.99</td>
<td>No</td>
<td>Yes</td>
<td>Egr1 activates cyr61 expression via binding to cyr61 promoter&lt;sup&gt;154&lt;/sup&gt;; Egr1 and cyr61 co-regulated on DNA damage in lens&lt;sup&gt;155&lt;/sup&gt;; MMP12 null mice downregulate expression of both Egr1 and cyr61&lt;sup&gt;145&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Dynlt1b</td>
<td>Dynactin light chain Tctex-type 1B</td>
<td>3.24</td>
<td>3.10E-33</td>
<td>57.1</td>
<td>17.19</td>
<td>np</td>
<td>Yes</td>
<td>nr</td>
<td>Matricellular protein; can induce EMT/fibrosis&lt;sup&gt;156&lt;/sup&gt;; may be a β5 integrin ligand&lt;sup&gt;157&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nes</td>
<td>Nestin</td>
<td>3.18</td>
<td>3.60E-68</td>
<td>59.48</td>
<td>18.25</td>
<td>No</td>
<td>Yes</td>
<td>nr</td>
<td>Molecular motor with role in vesicle transport; binds endoglin and inhibits TGFβ signaling&lt;sup&gt;158&lt;/sup&gt;; involved in neural differentiation&lt;sup&gt;159&lt;/sup&gt;</td>
</tr>
<tr>
<td>S100a13</td>
<td>S100 calcium binding protein A13</td>
<td>3.05</td>
<td>8.90E-05</td>
<td>2.11</td>
<td>0.69</td>
<td>No</td>
<td>No</td>
<td>nr</td>
<td>Neurofilament; promotes vimentin disassembly; can be EMT marker&lt;sup&gt;160,163&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glipr2</td>
<td>GLI pathogenesis-related 2</td>
<td>3.05</td>
<td>1.70E-29</td>
<td>8.35</td>
<td>2.67</td>
<td>No</td>
<td>Yes</td>
<td>nr</td>
<td>Involved in RAGE/inflammation&lt;sup&gt;162&lt;/sup&gt;; Promotes EMT via Erk activation&lt;sup&gt;165&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plagl1</td>
<td>Pleiomorphic adenoma gene-like 1; zac1</td>
<td>3.00</td>
<td>2.40E-18</td>
<td>3.12</td>
<td>1.01</td>
<td>No</td>
<td>Yes</td>
<td>nr</td>
<td>Transcription factor, may regulate TGFβ II expression in retinal development&lt;sup&gt;164&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Gene Description</td>
<td>Fold Change From WT</td>
<td>FDR, P Value</td>
<td>β1-MLR10 Mean RPKM</td>
<td>WT Mean RPKM</td>
<td>Lens Enrichment iSyTe at E12.5 1.5 Cutoff</td>
<td>Predicted Egr1 Motif in Promoter</td>
<td>Functional Connection With Egr1 Biological Function</td>
<td></td>
</tr>
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<td></td>
</tr>
<tr>
<td>Nab1</td>
<td>NgrfA binding protein 1 / EGR binding protein 1</td>
<td>2.99</td>
<td>6.50E-52</td>
<td>4.2</td>
<td>1.38</td>
<td>No</td>
<td>Yes</td>
<td>Corepressor of Egr1, but can also stimulate Egr1 mediated transcription in promoters with few or weak Egr1 sites; Promoter has bioinformatically predicted Egr1 site</td>
<td>Might direct myofibroblast dedifferentiation, transcriptionally regulated by Pax6 during hind brain development</td>
</tr>
<tr>
<td>Emilin1</td>
<td>Elastin microfibril interactor 1</td>
<td>2.88</td>
<td>4.50E-58</td>
<td>6.44</td>
<td>2.18</td>
<td>No</td>
<td>No</td>
<td>nr</td>
<td>Elastin binding glycoprotein, can negatively regulate TGFβ signaling</td>
</tr>
<tr>
<td>Mmp14</td>
<td>Matrix metallopeptidase 14 (membrane-inserted) ; MT1-MMP</td>
<td>2.87</td>
<td>1.30E-54</td>
<td>8.12</td>
<td>2.77</td>
<td>No</td>
<td>Yes</td>
<td>Egr1 directly binds to the MT1-MMP promoter and activates its transcription</td>
<td>Upregulated in kidney fibrosis; may function with αV integrins to activate TGFβ; null mice exhibit fibrosis due to lack of collagen turnover</td>
</tr>
<tr>
<td>Phlda3</td>
<td>Pleckstrin homology-like domain, family A, member 3</td>
<td>2.81</td>
<td>1.00E-17</td>
<td>5.97</td>
<td>2.08</td>
<td>No</td>
<td>Yes</td>
<td>nr</td>
<td>Activated on UPR stress; inhibits Akt activity by competing for membrane binding sites</td>
</tr>
<tr>
<td>Junb</td>
<td>Jun B proto-oncogene</td>
<td>2.8</td>
<td>4.80E-13</td>
<td>5.58</td>
<td>1.27</td>
<td>No</td>
<td>No</td>
<td>Coregulated with Egr1 as IER gene</td>
<td>IER TF involved in fibrosis and other stresses</td>
</tr>
<tr>
<td>Lgals3</td>
<td>Lectin, galactose binding, soluble 3; galectin3</td>
<td>2.57</td>
<td>2.30E-18</td>
<td>6.22</td>
<td>2.37</td>
<td>No</td>
<td>No</td>
<td>nr</td>
<td>Found in lens fibers, binds MP20; carbohydrate binding protein; biomarker of fibrosis in other systems</td>
</tr>
<tr>
<td>Gadd45gip1</td>
<td>Growth arrest and DNA-damage-inducible, gamma interacting protein 1; crifl</td>
<td>−2.51</td>
<td>5.20E-17</td>
<td>1.97</td>
<td>4.81</td>
<td>No</td>
<td>No</td>
<td>nr</td>
<td>Component of mitonobosome, downregulation is associated with mitochondria dysfunction and ROS production</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Gene Description</td>
<td>Fold Change From WT</td>
<td>FDR, P Value</td>
<td>β1-MIR10 Mean RPKM</td>
<td>WT Mean RPKM</td>
<td>Lens Enrichment with E12.5 1.5 Cutoff</td>
<td>Predicted Egr1 Motif in Promoter</td>
<td>Functional Connection With Egr1</td>
<td>Biological Function</td>
</tr>
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</tr>
<tr>
<td>Slc7a11</td>
<td>Solute carrier family 7 (cationic amino acid transporter, y+ system), member 11; Xct</td>
<td>-2.6</td>
<td>6.60E-45</td>
<td>5.46</td>
<td>13.88</td>
<td>Yes</td>
<td>No</td>
<td>nr</td>
<td>Xct deficiency results in autophagy&lt;sup&gt;177&lt;/sup&gt;; drives glutamine release during cellular stress&lt;sup&gt;176&lt;/sup&gt;; can drive resistance to cellular stress&lt;sup&gt;178&lt;/sup&gt;; upregulation of Xct can rescue glutathione deficiency&lt;sup&gt;180&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tbps1</td>
<td>Thrombospondin 1</td>
<td>-2.66</td>
<td>1.5E-23</td>
<td>6.89</td>
<td>17.99</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td>Considered to be profibrotic&lt;sup&gt;182&lt;/sup&gt;; matricellular protein found at sites of active tissue remodeling in adult eye&lt;sup&gt;183&lt;/sup&gt;</td>
</tr>
<tr>
<td>Alox12</td>
<td>Arachidonate 12-lipoxygenase</td>
<td>-2.68</td>
<td>3.8E-65</td>
<td>7.35</td>
<td>19.28</td>
<td>Yes</td>
<td>Yes</td>
<td>nr</td>
<td>Produces 12S-HETE that drives inflammation/oxidative stress&lt;sup&gt;184,185&lt;/sup&gt;; upregulated in KLF4 null lens, KLF4 is activated here, see above&lt;sup&gt;128&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hmga1</td>
<td>High-mobility group AT-hook 1; HMG-I/Y</td>
<td>-3.13</td>
<td>7.9E-31</td>
<td>4.18</td>
<td>12.74</td>
<td>nr</td>
<td>Yes</td>
<td>nr</td>
<td>Bends chromatin, recruits TFs to DNA; can be fibrotic marker/ regulator&lt;sup&gt;115&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nnat</td>
<td>Neuronatin; Peg5</td>
<td>-3.46</td>
<td>1.00E-17</td>
<td>1.06</td>
<td>3.7</td>
<td>No</td>
<td>No</td>
<td>nr</td>
<td>Activated on photoreceptor cell stress&lt;sup&gt;186&lt;/sup&gt;; can regulate calcium signaling&lt;sup&gt;187&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

CNS, central nervous system; FDR, false discovery rate; np, gene not represented in the iSyTE database; nr, none reported.
which the first morphological defects are observed in investigated their molecular phenotype at E16.5, the stage at severe in animals that also lacked the maintenance of lens material in adult cells where it stabilizes cortical F-actin. In LECs, the function localized to the apical tips and lateral membranes of lens fiber deletion of Egr1 from abundant cleaved Caspase 3 (Fig. 10B) staining. Notably, numerous TUNEL-positive nuclei (Fig. 10E, arrows) and lenses. As previously reported, positive nuclei (Fig. 10D) were detected in newborn WT (Fig. 8) was due to alterations in apoptosis. As expected, no levels are reduced in the terminally differentiating lens epithelium and newly elongating cortical fibers, whereas (Figs. 10G–I) and Prox1 (Figs. 10J–L), was affected by Egr1 detection of Egr1 from /mice led to a marked attenuation of cleaved Caspase 3 staining in newborn lenses (Fig. 10C). However, more TUNEL-positive nuclei were detected in /lens fiber cells (Fig. 10F, arrows) than in /lenses (Fig. 10E, arrows). As low-level Caspase 3 cleavage has been associated with lens fiber cell differentiation, we also assessed whether the expression of the Erk responsive fiber cell differentiation regulators, cMaf (Figs. 10G–I) and Prox1 (Figs. 10J–L), was affected by Egr1 deletion from /lenses. Qualitatively, /Egr1–/LECs stain more intensely for cMaf and Prox1 than /LECs, suggesting that the loss of intense cleaved Caspase 3 staining from /Egr1–/lenses is not associated with a loss of the aberrant fiber cell differentiation pathway activation previously reported in /lenses. As the ocular phenotype of adult /mice was less severe in animals that also lacked the Egr1 gene, we investigated their molecular phenotype at E16.5, the stage at which the first morphological defects are observed in /lenses. As we previously reported, WT E16.5 lenses exhibit high levels /integrin immunoreactivity in the lens epithelium and newly elongating cortical fibers, whereas these levels are reduced in the terminally differentiating nuclear fibers (Fig. 11A). In contrast, neither / (Fig. 11B) nor /Egr1–/ (Fig. 11C) lenses exhibit detectable /integrin protein, although, in both cases, other ocular structures, including the TVL (arrows), still stain strongly for /integrin (Figs. 11B, 11C). We then investigated whether the deletion of Egr1 altered the pattern of zSMA upregulation in /lenses, as this is the best characterized fibrotic marker in the lens. As previously reported, WT lenses do not exhibit detectable levels of zSMA protein at E16.5 (Fig. 11A; Fig. 11D is zSMA alone), whereas /lenses exhibit obvious zSMA in LECs (Fig. 11B; Fig. 11E is zSMA alone, arrows). This upregulation of zSMA was similar in /Egr1–/lenses (Fig. 11C; Fig. 11F is zSMA alone, arrows), indicating that Egr1 cannot be the sole driver of zSMA upregulation as a consequence of /integrin deletion in the lens. Notably though, although the expression of Nab2 protein was obviously upregulated in /lenses (Fig. 11H) compared with WT (Fig. 11G), no Nab2 protein was detected in /Egr1–/lenses (Fig. 11I), indicating that Nab2 upregulation is solely under Egr1 control.

**Discussion**

/Integrin is localized to the basal surface of all lens cells and is therefore believed to be a regulator of the interactions between lens cells and the lens capsule. /Integrin is also localized to the apical tips and lateral membranes of lens fiber cells where it stabilizes cortical F-actin. In LECs, the function of /-containing integrins is complex and changes during lens development. Deletion of /integrin from the lens vesicle in /mice leads to defects in lens capsule deposition, as well as in the exit of LECs from the cell cycle, and their differentiation into lens fibers. In contrast, loss of /Integrin after the completion of primary lens morphogenesis (/) leads to a distinct phenotype, wherein LECs initially remain in the cell cycle, but become disorganized, and coexpress the “early” lens fiber cell markers, /crystallin and cMaf, along with the fibrotic marker zSMA, which is followed by their loss via apoptosis. Here we further our understanding of the molecular basis of this phenotype via gene expression profiling by RNA-seq of mutant lenses, and its partial rescue by deletion of an abnormally upregulated transcriptional regulator.

**Deletion of /Integrin After the Completion of Initial Lens Morphogenesis Induces Stress Response and Fibrosis-Related Gene Expression**

Because the morphological abnormalities of /mouse lenses first become apparent at E16.5, we performed RNA-seq expression profiling on lenses a day earlier, at stage E15.5, to identify molecular changes proximally resulting from /integrin deletion, while minimizing the detection of secondary changes. On applying filtering criteria designed to reveal biologically relevant changes in gene expression, 120 DRGs were found. Although several proteins/TFs were found to be altered in /lenses via IF, they were not found to be differentially expressed by RNA-seq. It is likely that either the evaluation of whole lenses masked the subtle changes specifically exhibited by /LECs or the differential protein expression is not controlled at the level of transcription. All the 120 DRGs were prioritized, and analyzed by a variety of bioinformatics tools that use gene ontology databases to identify commonly regulated pathways. However, although “apoptosis,” “integrin signaling,” and several pathways related to Ras/MapK signaling were identified as relevant, the pathways identified by this method (see Fig. 1) were each based on only a few DRGs, leading us to explore more approaches to gain biological insight into the phenotype.

A comprehensive literature search revealed that nearly half of the 76 upregulated DRGs had known connections to stress response and/or fibrotic pathways. This included zSMA, the most commonly used marker of EMT in the lens, which we have previously reported to be upregulated at the protein level in /lenses. Validation of four of these genes by qRT-PCR revealed that their mRNA levels are first elevated shortly after loss of /integrin protein from the /lens, which is 2 to 3 days earlier than the onset of the obvious lens phenotype. Thus, this induction of stress/fibrotic responses in /mice likely proximately results from the loss of /integrin at E12.5 to E13.5. However, although TGF- is the most intensively studied regulator of EMT and zSMA expression in LECs, the levels of its downstream regulator, pSmad3, were significantly downregulated in /lenses (Figs. 3A–F) suggesting that TGFβ/Smad signaling is not driving the /phenotype.

**/Integrin Negatively Regulates Erk1/2 and Akt Signaling in LECs**

FAK is expressed in the rodent lens in a pattern similar to /integrins, and is necessary for fiber cell morphogenesis in zebrafish. Because integrin activation results in the phosphorylation of FAK in other cell types, it was not surprising that pFAK (Tyr997) levels are greatly attenuated in /lenses. The loss of pFAK from /lenses is also consistent with the apoptosis observed in newborn /lenses.
LECs, because pFAK is important for cell survival in other cellular contexts.68–70 Notably, FAK deletion from fibroblasts leads to the upregulation of αSMA expression,49 consistent with the upregulation of αSMA expression found b1MLR10 lenses at E16.5. However, in fibroblasts as well as other cell types, the loss of integrin-mediated FAK phosphorylation often results in a loss of Erk1/2 phosphorylation,49,71–73 whereas Erk1/2 and Akt phosphorylation are upregulated in both b1MLR10 (this study) and b1LE15 LECs. There are other precedents for increased Erk and/or Akt activation on integrin deletion, although the proposed mechanisms are diverse. For instance, deletion of emilin1, an α4β1 and α9β1 ligand, from the skin, leads to a downregulation of the phosphatase, PTEN, leading to sustained Erk and Akt phosphorylation.74 In mesangial cells, α1β1 integrin deletion results in elevated pERK levels due to the ability of this integrin to negatively regulate epidermal growth factor receptor signaling.75 Thus, it appears likely that β1-integrins can limit Erk/Akt pathway activation in a variety of cellular contexts, including the embryonic lens epithelium.

The IER TF, Egr1, Is Upregulated in b1MLR10 Lenses Shortly After β1-Integrin Loss

Interrogation of the b1MLR10 lens DRGs specifically for TFs regulated by pErk/pAKT activation led to identification of the IER TFs Egr1, Fos, and Junb, all of which were upregulated in these mutant lenses. We focused on Egr1 because active Erk can elevate Egr1 levels by phosphorylating the Ets-family TF Elk-1;57,59; Egr1 is the most upregulated, and most highly expressed, of these three IER genes in E15.5 b1MLR10 lenses; Egr1 can both drive,76 and make cells resistant to,77 apoptosis; Egr1 upregulation is first detected coincident with β1-integrin protein loss from both b1MLR10 and b1LE lenses; and the detection of Egr1 protein first in the central lens epithelium, then later in peripheral LECs, correlates with the pattern of LEC loss in the b1MLR10 null lens.

It should be noted that Egr1 mRNA levels remain elevated in the E16.5 b1MLR10 lens, although few Egr1-positive cells were detected at this time by immunolocalization. Although the original reports suggested that Egr1 protein levels are solely controlled at the level of transcription, more recent investigations show that Egr1 protein levels are controlled by microRNA regulation of protein translation78,79 while Egr1 undergoes extensive posttranslational modifications, including phosphorylation, sumoylation, and acetylation, which affect its stability.80–82 Thus, it is not surprising that Egr1 protein and mRNA levels do not always correlate.

This upregulation of IER gene expression is likely to be functionally relevant to the b1MLR10 lens phenotype as an unbiased analysis of the 76 upregulated genes revealed that their promoters were enriched in binding motifs for Ets factors,
which are known direct transcriptional effectors of Erk signaling,53 leucine zipper TFs (like Fos and Junb), and Egr1. This finding suggested that these TFs had the potential to drive the upregulation of many of the DRGs. Further analysis of the DRGs for the presence of Egr1 binding motifs in their putative promoters revealed that 52 of the 120 DRGs had consensus Egr1 binding sites in the 2.5 kb of sequence upstream of their TSS. Notably, eight of these promoters are already known to be directly regulated by Egr1 (Table 3). For example, Egr1 directly binds to and upregulates expression driven by the promoter of the Kruppel-related TF, Klf4, in HepG2 cells.80 Egr1 also directly binds to and upregulates the expression of MMP14, a gene encoding MT1-MMP, a matrix metalloprotease that can activate latent TGF-β.81 Similarly, Egr1 activates the expression of the most upregulated TF in the E15.5 β1MLR10 lens, Nab2, which is best known for its ability to heterodimerize with Egr1, acting as an Egr1 corepressor.85–87 Notably, Nab2 upregulation driven by Egr1 blocks the ability of Egr1 to upregulate its own expression, limiting the IER.57,86 This negative feedback loop likely explains why Nab2 protein upregulation persists in β1-integrin null lens cells longer than Egr1.

However, Nab2-Egr1 interactions also can stimulate gene expression driven by promoters harboring weak Egr1 binding sites.61 This functional complexity may explain the observation that consensus Egr1 binding sites were identified in the promoters of both upregulated (n = 31) and downregulated (n = 21) DRGs. Overall, this analysis suggests that the upregulation of Egr1 may participate in both the positive and negative regulation of β1MLR10 DRGs.

Elevated Egr1 Expression in β1MLR10 LECs Causes a Subset of the β1MLR10 Lens Phenotype

In the eye, Egr1 mediates the connection between image focus on the retina and eye growth, and Egr1 null mice develop myopia associated with elevated growth of the sclera and changes in lens optics.83 In the lens, Egr1 expression is upregulated during cellular stresses such as selenite treatment,89 whereas knockdown of Egr1 in cultured LECs can attenuate selenite-induced cell death.90 Thus, we generated β1MLR10/Egr1−/− mice to evaluate the extent to which the β1MLR10 lens phenotype was a consequence of Egr1 upregulation. Notably, deletion of the Egr1 gene partially rescued the microphthalmia seen in β1MLR10 adults. This partial rescue is likely related to the observed attenuation of Caspase 3 cleavage in perinatal β1MLR10/Egr1−/− lenses, leading to enhanced cell survival of the lens epithelium compared with β1MLR10 lenses. However, it should be noted that β1MLR10/Egr1−/− lenses are still TUNEL positive, particularly within the lens fibers, suggesting that either multiple cell death pathways are active in β1MLR10 lenses, or that Egr1 is able to simultaneously influence prosurvival and proapoptotic pathways in the lens. Alternatively, as β1MLR10 lenses lacking Egr1 appear to upregulate the Erk responsive TFs CMap91 and Proxl,92 Egr1 upregulation in β1-integrin null LECs may limit the ability of these cells to enter the fiber cell differentiation pathway.

Another major feature of β1MLR10 lenses is the upregulation of the “fibrotic” marker, αSMA, in LECs. However, Egr1 expression is also found to be upregulated in the β1LE lens, which does not exhibit upregulation of αSMA,15 and we find that deletion of Egr1 from the β1MLR10 lens did not noticeably affect the upregulation of αSMA. However, Egr1 appears to directly control the expression of Nab2 in the lens. First, although both Egr1 and Nab2 mRNA and protein levels are upregulated in β1MLR10 lenses proximal to the loss of β1-integrin protein, Nab2 upregulation lags behind that of Egr1. Second, Nab2 protein expression is not upregulated in β1MLR10 lenses lacking Egr1, whereas Nab2 is highly upregulated in β1MLR10 LECs. These observations are consistent with the known ability of Egr1 to bind to the Nab2 promoter, activating Nab2 expression, which subsequently downregulates Egr1 expression, limiting the IER.93 The possibility that Egr1 is generally induced in the lens in response to cellular stress, and the role its induction plays in mediating cellular stress responses, is a fruitful topic of future study.

Conclusions

β1-integrin deletion from the lens after the completion of its primary morphogenesis (β1MLR10) leads to activation of Erk/Akt signaling, upregulation of IER genes, as well as numerous markers of cellular stress. As Egr1 expression is also elevated in lenses undergoing stress postnatally,90,92 and deletion of Egr1 from β1MLR10 lenses attenuates part of the phenotype, the role of Egr1 in mediating stress responses in the lens epithelium represents a novel finding worthy of further investigation.

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