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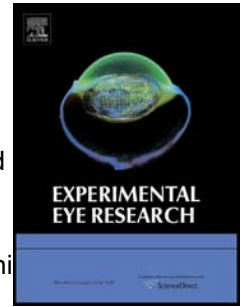
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Integrin linked kinase (ILK) is required for lens epithelial cell survival, proliferation and differentiation

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1 **ABSTRACT**

2 While the role of growth factors in lens development has been investigated extensively, the
3 role of extracellular matrix signalling is less well understood. The developing lens expresses
4 predominantly laminin-binding integrins (such as $\alpha3\beta1$, $\alpha6\beta1$), which are cooperatively
5 required in the lens epithelium during development. We investigated the role of ILK, a
6 downstream mediator of integrin signalling in mice conditionally null for *Ilk*. Mutant lenses
7 showed epithelial thinning at E17.5 with reduced proliferation and epithelial cell number and
8 aberrant fibre differentiation. There was complete loss of the central epithelium from
9 postnatal day (P) 2 due to cell death followed by fibre cell degeneration and death by P10 as
10 well as rupture of the lens capsule between P10 and P21. At E17.5 there was significant
11 inhibition (~50%) of epithelial cell cycle progression, as shown by BrdU incorporation,
12 cyclin D1/D2 and phospho-histone H3 immunostaining. The epithelial marker, E-cadherin,
13 was decreased progressively from E17.5 to P2, in the central epithelium, but there was no
14 significant change in Pax6 expression. Analyses of ERK and Akt phosphorylation indicated
15 marked depression of MAPK and PI3K-Akt signalling, which correlated with decreased
16 phosphorylation of FRS2 α and Shp2, indicating altered activation of FGF receptors. At later
17 postnatal stages there was reduced or delayed expression of fibre markers (β -crystallin, c-
18 Maf, and p57^{kip2}) in fibres. Loss of *Ilk* also affected deposition of extracellular matrix, with
19 marked retention of collagen IV within differentiating fibre cells. By quantitative RT-PCR
20 array there was significantly decreased expression of 19 genes associated with focal
21 adhesions, actin filament stability and MAPK and PI3K/Akt signaling. Overall, these data
22 indicate that ILK is required for complete activation of signalling cascades downstream of
23 the FGF receptor in lens epithelium and fibre cells during development and thus is involved
24 in epithelial proliferation, survival and subsequent fibre differentiation.

1 INTRODUCTION

2 The vertebrate lens comprises two cell types (epithelial and fibre cells) that arise from head
3 ectoderm during embryonic development (Gunhaga, 2011; Lovicu et al., 2011; Martinez and
4 de Iongh, 2010). Growth and differentiation of the lens involves mitoses of a population of
5 pre-equatorial epithelial cells, whose progeny migrate posteriorly and differentiate into
6 secondary fibre cells that form concentric layers of fibre cells around the embryonic nuclear
7 lens fibre cells (Lovicu et al., 2011; Martinez and de Iongh, 2010). The regulation of these
8 processes by various growth factors and cell-cell signalling, particularly fibroblast growth
9 factors (FGFs), bone morphogenetic proteins (BMPs), Wnts and Notch has been extensively
10 reviewed (Lovicu and McAvoy, 2005; Lovicu et al., 2011; Martinez and de Iongh, 2010).

11 In addition to growth factor signalling, the association and interaction of lens cells with the
12 extracellular matrix (ECM) is of critical importance during lens development. From the
13 earliest stages of lens formation, the vesicle is surrounded by a basement membrane that
14 continues to thicken and form the characteristic lens capsule. Cell interactions with the ECM
15 are mediated by integrins, an extensive family of glycosylated, heterodimeric,
16 transmembrane adhesion and signalling molecules (Humphries et al., 2006). Binding of
17 integrins to their matrix ligands is required for cell attachment to the ECM and can result in
18 activation of various signalling cascades that control cell adhesion, proliferation,
19 morphogenesis, differentiation, and survival. It is becoming increasingly apparent that
20 signalling via growth factor receptors, particularly receptor tyrosine kinases, requires
21 functional adhesion complexes and the cooperation of integrins and their adaptor protein
22 complexes (Alam et al., 2007; Cabodi et al., 2010 ; Streuli and Akhtar, 2009).

1 The lens expresses a range of integrin receptors, particularly the $\beta 1$ family, including $\alpha 6\beta 1$,
2 $\alpha 3\beta 1$, and to a lesser extent $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 5\beta 1$ in patterns that are regulated during lens
3 development (Walker and Menko, 2009; Wederell and de Iongh, 2006). To date, only $\alpha 3$
4 and $\alpha 6$ integrins have been shown to have functional, albeit redundant, roles in lens
5 development. Individual knockouts of $\alpha 3$ and $\alpha 6$ genes showed no gross effects on
6 embryonic lens development but compound $\alpha 3/\alpha 6$ null mice show a disrupted anterior
7 epithelium/capsule with extrusion of the fibre cells into the overlying cornea (De Arcangelis
8 et al., 1999; Wederell and de Iongh, 2006). Consistent with this, conditional deletion of the
9 $\beta 1$ integrin gene resulted in epithelial apoptosis and abnormal fibre cell differentiation
10 (Samuelsson et al., 2007; Simirskii et al., 2007).

11 Integrin-linked kinase (ILK) is a cytoplasmic protein that is commonly localised to focal
12 adhesions. It comprises five ankyrin repeat domains at the N-terminus, a kinase domain at
13 the C-terminus and a central a pleckstrin homology domain. The C-terminus of ILK binds
14 the cytoplasmic tails of $\beta 1$ - and $\beta 3$ -integrins and by its association with various actin-
15 binding and actin regulatory proteins, such as PINCH, parvin, paxillin and kindlin has been
16 shown to regulate the actin cytoskeleton (Bottcher et al., 2009; Legate and Fassler, 2009;
17 Legate et al., 2009; Moser et al., 2009). While recent *in vivo* and molecular structural studies
18 suggest that ILK is a pseudokinase (Fukuda *et al.*, 2009; Lange *et al.*, 2009), various *in vitro*
19 studies have shown that it can regulate the activation of cell signalling molecules such as
20 protein kinase B (PKB/AKT) to promote cell proliferation, survival and migration (Hannigan
21 et al., 2007; Hannigan et al., 2011; McDonald et al., 2008).

22 The lens has been shown to express ILK in the epithelium and early differentiating fibre cells
23 (Cammass et al., 2012; de Iongh et al., 2005; Weaver et al., 2007) and previous conditional

1 knockout studies using nestin-Cre and LeCre lines, suggest that it is also required for lens
2 development (Cammass et al., 2012; Samuelsson et al., 2007). In this study, we analysed the
3 role of ILK in the developing epithelium and fibre cells, using the MLR10 Cre line. The
4 data indicate that ILK is required for complete activation of FGF receptors in lens epithelial
5 cells and during early fibre cell differentiation and regulate lens epithelial cell survival,
6 proliferation and subsequent differentiation predominantly via the phosphatidylinositol 3-
7 kinase (PI3K) pathway. Loss of ILK also results in aberrant focal adhesion gene expression,
8 altered matrix protein trafficking in lens cells.
9

1 MATERIALS AND METHODS

2 All experimental procedures on animals conformed to the ARVO Statement for the Use of
3 Animals in Ophthalmic and Vision Research and were approved by the Animal Ethics
4 Committee of the University of Melbourne.

5 Generation of conditional mutants

6 The lens specific MLR10 Cre transgenic mice on an FVB background have been described
7 previously (Cain et al., 2008; Martinez et al., 2009; Zhao et al., 2004). The floxed *Ilk* mouse
8 (C57Bl6/129Sv), in which LoxP sites have been inserted in introns between exons 4 and 5
9 and exons 12 and 13, has also been described previously (Terpstra et al., 2003). Cre-
10 mediated recombination at the floxed *Ilk* locus results in deletion of the kinase domain and
11 generates a null allele. MLR10 Cre mice were bred with *Ilk*^{lox} mice and genomic DNA from
12 resultant progeny were screened by PCR, using specific primers (Table 1) for the desired
13 genotypes (*Ilk*^{lox/lox}/MLR10Cre⁺). Homozygosity for the Cre transgene and lack of the *Bfsp2*
14 mutation was confirmed after back-crosses with *Ilk*^{lox/lox} mice. Mutant mice were maintained
15 by inbreeding as a separate line, ILK10 (*Ilk*^{lox/lox}/MLR10Cre^{+/+}). In some experiments,
16 ILK10 mice that were hemizygous for the Cre transgene (*Ilk*^{lox/lox}/MLR10Cre^{+/-}) were used.
17 Wild-type (Wt) controls were age-matched tissues from MLR10, *Ilk*^{lox/lox}, or FVB/N mice.

18 Histology and Immunofluorescence

19 Ocular postnatal and embryonic tissues from Wt and mutant mice were fixed in 10% neutral
20 buffered formalin and embedded in paraffin. Paraffin sections (5 µm) were rehydrated and
21 stained with haematoxylin and eosin, periodic acid Schiff (PAS), or further prepared for
22 immunofluorescence or immunohistochemistry. Immunofluorescence with heat-mediated
23 sodium citrate antigen retrieval was performed as described previously (Cain et al., 2008;
24 Martinez et al., 2009). The antibodies used were mouse anti-E-cadherin (1:200; #610182,

1 BD Transduction, Franklin Lakes, NJ), mouse anti-ILK (#611802, BD Biosciences, San
2 Jose, CA), rabbit anti-Pax6 (1:300, #PRB278P, Covance, Princeton, NJ), mouse anti- β -
3 crystallin (Clone 3H9), rabbit anti-phospho-histone H3 (1:200, 07-424, Millipore, Billerica,
4 MA), rabbit anti-p57^{kip2} (1:200; #4058-500, Abcam, Cambridge, UK), rabbit monoclonal
5 anti-cyclin D1 (1:1, #21699 (SP4), Abcam), rabbit anti-collagen IV (1:500, ab19808,
6 Abcam) and rabbit anti-phospho-Y⁵⁴² Shp2 (1:100; #3751, Cell Signaling Technology).

7 Horse-radish peroxidase (HRP) immunohistochemistry for p44/42 extracellular signal
8 regulated kinase (ERK), phospho-p44/42 ERK (T²⁰²/Y²⁰⁴), Akt and phospho-Akt (S⁴⁷³) was
9 carried out using the CSA II Biotin-free Tyramide Signal Amplification System (Dako
10 Cytomation Inc, Carpinteria, CA), according to the manufacturer's instructions. Antibodies
11 used were rabbit anti-ERK (1:200, #9102, Cell Signaling Technology, Boston, MA), rabbit
12 anti-phospho ERK (1:200, #4377 Cell Signaling Technology), rabbit anti-Akt (1:200, #9272,
13 Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-phospho-Akt (1:200, #4060, Cell
14 Signaling Technology).

15 Immunofluorescence for Frs2 α was conducted on frozen sections of formaldehyde-fixed
16 tissues. E17.5 embryo heads were fixed in 4% paraformaldehyde in phosphate buffered
17 saline (PBS) for 1 h, rinsed extensively in PBS and cryo-protected by infiltration in 10%,
18 20% and 30% sucrose in PBS. Tissues were embedded in Tissue-Tek Optimal Cutting
19 Temperature (OCT) compound and sectioned at 12-14 μ m. Following incubation with
20 blocking solution (5% goat serum, 0.1% bovine serum albumin (BSA) in PBS), sections
21 were incubated overnight at 4°C with anti-phospho-Y⁴³⁶ FRS2 α (1:100, AF5126, R&D
22 Systems, Minneapolis, MN) diluted in blocking solution. Antibody binding was detected
23 with anti-rabbit IgG secondary antibody, conjugated with Alexa-488 (Invitrogen).

1 Immunoblotting

2 Western blotting was used to quantify the activation (phosphorylation) status of Akt and Erk,
3 and expression of ILK in Wt and ILK10 lenses. Embryonic lenses (E17.5) from Wt and
4 ILK10 mice were dissected in cold, sterile PBS to remove surrounding tissues. Due to the
5 difficulty in obtaining cleanly dissected lenses from ILK10 mice that were homozygous for
6 Cre ($Ilk^{lox/lox}/MLR10Cre^{+/+}$), lenses were obtained from mice that were hemizygous for the
7 Cre transgene ($Ilk^{lox/lox}/MLR10Cre^{+/-}$). Lenses from individual litters were pooled, snap
8 frozen in liquid nitrogen and stored at -80°C . Protein was extracted from the pooled samples
9 in lysis buffer [2.5mM EDTA, 25mM Tris-HCl (pH 7.5), 0.375M NaCl, 1% IGEPAL,
10 1.5mM sodium orthovanadate and a protease inhibitor cocktail (Roche)] as previously
11 described (Newitt et al., 2010). Protein concentration was determined using a Pierce BCA
12 Protein Assay Kit (Thermo Scientific, Rockford, USA). For each sample, 50 μg of total
13 protein was diluted in water and mixed with an equal volume of 2x Laemmli sample buffer
14 (BioRad, Gladesville, NSW, Australia) containing 5% β -mercaptoethanol to a final volume
15 of 20 μl . Samples were electrophoresed through 10% precast polyacrylamide gels (BioRad)
16 and transferred to nitrocellulose membrane. Membranes were blocked with 0.5% BSA in
17 Tris-buffered saline (TBS) pH8.0 for 1 hour at room temperature prior to incubation at 4°C
18 overnight with the same ILK, ERK and Akt antibodies as described above and mouse anti-
19 GAPDH, 1:1000 (G8795, Sigma-Aldrich, Castle Hill, NSW, Australia). Blots were washed
20 3x5 minutes in TBST with 0.5% BSA followed by incubation with secondary antibodies
21 (IRDye 680LT goat anti-rabbit IgG; #926-68021; Li-Cor Biosciences) and IRDye 800CW
22 goat anti-mouse IgG, 1:10000 (#926-32210; Li-Cor Biosciences) diluted 1:10000 in
23 TBST/BSA for 2 hours at room temperature. Blots were then washed 3x10 minutes in TBST
24 and once in TBS prior to visualisation with a Li-Cor Odyssey Infra-Red Imaging System (Li-
25 Cor Biosciences) according to the manufacturer's specifications. The densities of the

1 scanned bands were quantified using Image-J and data analysed by ANOVA and Student's *t*-
2 test.

3 **Proliferation and TUNEL Assays and Morphometry**

4 Bromodeoxyuridine (BrdU) labelling to quantify cell proliferation and terminal
5 deoxynucleotidyl transferase dUTP Nick End Labelling (TUNEL) to identify apoptotic cells
6 in lenses was carried out as described previously (Cain et al., 2008; Martinez et al., 2009).

7 The percentage of BrdU⁺ cells was quantified in at least three sections of lens epithelium
8 from three to five E17.5 embryos, by counting cells from one equator to the other.

9 Measurements of central epithelial thickness were made from high magnification images
10 (40x objective) of haematoxylin and eosin stained mid-sagittal sections, using the
11 measurement function in Axiovision software (Carl Zeiss AG, Oberkochen, Germany). Ten
12 measurements of the anterior epithelium (between the margins of the irises) from the basal
13 lamina to the apical membrane were made on a minimum of three slides from four animals in
14 each group. Data expressed as mean thickness (\pm SEM) were analysed by ANOVA and
15 Student's *t*-test.

16 Measurements of nuclear density in the transitional zone of E15.5 lenses were conducted by
17 delineating a 50 μ m by 50 μ m square region bounded by the equator and lateral lens capsule.
18 In Wt lenses this encompasses the transitional zone fibres undergoing early elongation but
19 does not include nuclei in the bow zone. The number of nuclear profiles was counted in at
20 least two sections from a total of four animals, using Axiovision software (Carl Zeiss).

21 All data are expressed as mean (\pm SEM) and were analysed statistically by ANOVA and
22 Student's *t*-test.

1 **PCR Array profiling**

2 Mouse Focal Adhesion Pathway RT² Profiler PCR Arrays (SuperArray Bioscience
3 Corporation, Frederick, MD) were used to interrogate the expression of 84 extracellular
4 matrix and focal adhesion genes, including a panel of five housekeeping genes to normalise
5 PCR array data, genomic DNA control primers, reverse transcription controls and positive
6 PCR controls.

7 Embryonic lenses were dissected from at least two litters of Wt and ILK10
8 (*Ilk^{lox/lox}/MLR10Cre^{+/-}*) embryos at E17.5, taking care to remove surrounding optic cup tissue
9 and as much of the adherent tunical vasculosa lentis as possible. Total RNA was isolated
10 from four Wt and four ILK10 pooled samples, each containing 8-9 individual lenses, using a
11 Qiagen RNeasy Mini Kit (Qiagen Pty Ltd.) with on-column DNase-digestion. RNA integrity
12 and concentration was quantified using a NanoDrop spectrophotometer (ThermoFisher
13 Scientific, Wilmington DE). For each array, 400 ng RNA from each sample was reverse
14 transcribed using the RT² First Strand kit (SuperArray/Qiagen) and resultant cDNA was
15 amplified by real-time PCR using RT² Real-Time SYBR Green PCR master mix
16 (SuperArray/Qiagen) as follows: (a) denaturation for 10 min at 95°C, (b) 40 cycles of two-
17 step PCR including denaturation for 15 sec at 95°C, and (c) annealing for 1 min at 60°C. The
18 cycling program was immediately followed by a melt curve analysis to confirm generation of
19 a single amplicon. Four independent pooled samples of lens RNA were reverse transcribed
20 and used for real-time PCR analyses on independent arrays. Average Ct values for each gene
21 were analysed by the $\Delta\Delta C_T$ method using a Microsoft Excel analysis template
22 (SuperArray/Qiagen). Relative expression values were determined between Wt and ILK10
23 samples by normalizing differences in ΔC_T values ($\Delta\Delta C_T$) to the average of 5 housekeeping
24 genes. Fold change of gene expression in samples was calculated as $2^{-\Delta\Delta C_T}$.

1 RESULTS

2 Loss of *Ilk* in the lens causes microphthalmia

3 The *Ilk* conditional null mice were generated by breeding mice with *Ilk^{lox}* allele with mice
4 carrying the MLR10 Cre transgene, which is expressed from E10.5 in the lens vesicle and
5 becomes expressed in both epithelial and fibre cell lineages. Previous studies indicate that
6 effective deletion of a floxed target gene with the MLR10 transgene occurs from E12.5 to
7 E13.5 (Cain et al., 2008; Martinez et al., 2009; Zhao et al., 2008). Mutant mice derived from
8 MLR10 crosses (*ILK10, Ilk^{Lox/Lox}/MLR10-Cre^{+/+}*) had a mild microphthalmia (Fig. 1B) that
9 was evident from eye opening. In dissected eyes from weanling mice, the ILK10 eyes
10 showed a smaller lens that did not protrude through the iris (Fig 1D) as seen in Wt (Fig 1C)
11 mice. Similar to previous studies (Cain et al., 2008; Martinez et al., 2009) it was found that
12 the mutant phenotype was more severe when the mice were homozygous for the MLR10 Cre
13 transgene. Unless specifically stated all mice analysed were homozygous for the MLR10 Cre
14 transgene.

15 To confirm deletion of *Ilk*, we examined ILK expression in mutant lenses by
16 immunofluorescence. In Wt lenses (Fig. 2A, C, E), reactivity for ILK was found in the
17 epithelium and primary fibres at E13.5 (Fig. 2A) and E15.5 (Fig. 2C) but was strongest and
18 most reliably detected in the epithelium at E17.5 (Fig. 2E). By contrast, ILK10 lenses
19 showed complete loss of ILK reactivity in the lens from E13.5 onwards (Fig. 2B, D, F). This
20 pattern of gene deletion and loss of protein expression with MLR10 Cre mice is consistent
21 with previous studies (Cain et al., 2008; Martinez et al., 2009)

1 **Loss of *Ilk* disrupts epithelial cell survival and fibre cell elongation**

2 To characterise the phenotype, we conducted histological studies of embryonic and postnatal
3 eyes. Examination of neonatal (P2) eyes revealed gross disturbances of lens development in
4 ILK10 lenses (Fig. 3). At E15.5 the ILK10 lenses (Fig. 3B) were similar to Wt lenses (Fig.
5 3A), but close examination of the elongating cortical fibres suggested that these have not
6 elongated as extensively as Wt fibre cells. By E17.5, this defect in fibre cell elongation was
7 more evident (Fig. 3D, K) as the fibre cells just below the equator in ILK10 lenses were
8 abnormally arranged (Fig. 3K) compared to the Wt lenses (Fig. 3J). This may be due to
9 altered cell packing, an altered modiolus [arrangement of apical membranes (Zampighi et al.,
10 2000)] or altered nuclear positioning or all three and suggest there is decreased fibre cell
11 migration along the capsule. Analyses of nuclear density in the transitional zone below the
12 equator revealed a statistically significant ($p=0.0001$) increase in the numbers of nuclei in the
13 ILK10 lenses (29.9 ± 0.9 ; $n=4$) compared to the Wt lenses (21.2 ± 0.3 ; $n=4$), confirming the
14 abnormal arrangement of the fibre cells below the equator.

15 In addition, the epithelial layer of ILK10 lenses (Fig. 3D, K, M) at this stage appeared to be
16 much thinner than the Wt (Fig. 3C, J, L) and this was confirmed by quantitative analysis (see
17 Figure 6G). Similarly, counts of epithelial cells showed there was a significant reduction
18 ($p<0.001$) in the total number of epithelial cells at E17.5 (ILK10, 132 ± 4 , $n=5$; Wt, 170 ± 2 ;
19 $n=3$) but not ($p=0.81$) at E15.5 (ILK10, 132 ± 5 , $n=3$; Wt, 134 ± 6). By P2, the ILK10
20 lenses (Fig. 3F) showed a much flatter profile than the round Wt lenses (Fig. 3E) with a
21 deficient anterior epithelial layer. In many lenses at this stage, the equatorial, germinative
22 zone epithelium appeared to still be present, but the anterior epithelial cells were completely
23 absent (compare Fig. 3O and 3N).

1 Complete loss of the central anterior epithelium occurred variably in postnatal mice between
2 P2 and P10. By P21, the mutant lenses were grossly abnormal, with evidence of fibre cell
3 vacuolation and rupture of the fibres through the anterior lens capsule (Fig. 3H). The
4 anterior epithelium was largely absent with only a few remnant epithelial cells evident in the
5 germinative zone (Fig. 3H). In adult ILK10 mice of three to six months of age, the lens was
6 absent (not shown).

7 To examine the epithelial phenotype in mutant lenses we examined expression of E-
8 cadherin. As shown previously (Cain et al., 2008; Martinez et al., 2009), E-cadherin in Wt
9 lenses is strongly localised to epithelial cell membranes at all ages and is rapidly down-
10 regulated as cells undergo fibre differentiation (Fig. 4A, C, E). In ILK10 lenses, there was
11 reduced intensity of E-cadherin staining in the anterior epithelium at E17.5 and P2, but
12 intense staining of the equatorial cells remained (Fig. 4B, D). By P10, all epithelial cells and
13 thus reactivity for E-cadherin in the anterior lens was absent, but germinative zone cells were
14 still intensely stained (Fig. 4F). We also examined expression of Pax6 and the localisation
15 pattern was consistent with the pattern of epithelial cell loss from P2 onwards, with no
16 changes in localisation or intensity prior to P2 (not shown).

17 To examine fibre cell differentiation in E17.5 mutant lenses we examined expression of β -
18 crystallin, p57^{Kip2} and c-Maf. In Wt lenses, β -crystallin expression is initiated as fibre cells
19 start to elongate (Fig. 5A), whereas p57^{Kip2} is detected in the nuclei of cells that have exited
20 the cell cycle and initiated differentiation below the lens equator (Fig. 5C). β -crystallin
21 expression decreases in the more mature central fibres, presumably as the fiber cells become
22 increasingly compacted and dehydrated and β -crystallin protein undergoes post-translational
23 modifications. However, in the ILK10 lenses at E17.5 this loss of reactivity in central fibres

1 does not occur, suggesting reduced fibre cell differentiation (compaction) or reduced post-
2 translational modification of β -crystallins. In Wt lenses, c-Maf is most intensely detected in
3 elongating fibre cells (Fig. 5E). In ILK10 lenses at E17.5, expression of β -crystallin was
4 delayed in cortical fibres (Fig. 5B) and reactivity was detected in the central mature fibres.
5 This is consistent with the indication above that these fibre cells are differentiating less
6 quickly than in the Wt and thus accumulation and compaction of crystallins occurs less
7 rapidly, leading to increased availability to antibody binding. In ILK10 lenses, p57^{Kip2}
8 expression was markedly decreased (Fig. 5D). A similar reduction was observed in p21^{Kip1}
9 staining (not shown). There appeared to be no difference in expression of c-Maf (Fig. 5F).
10 Similar patterns of delayed β -crystallin expression in early differentiating fibres were
11 observed at later post-natal stages (not shown).

12 ***Ilk* is required for epithelial cell cycle progression and survival.** The earliest detectable
13 phenotype in the ILK10 lenses was a decrease in central epithelial thickness from E15.5 to
14 E17.5 and subsequent loss of the anterior epithelial cells by P2. To determine if the loss of
15 the anterior epithelium in ILK10 lenses was due to altered cell proliferation or cell death, we
16 examined markers of the cell cycle (BrdU, phospho-histone H3 (PH3) and cyclin D1) and
17 carried out TUNEL assays. In Wt lenses, intensely stained BrdU⁺ nuclei were detected
18 throughout the anterior epithelium and germinative zone (Fig. 6A). By contrast, very few
19 BrdU⁺ nuclei were detected in the thinned epithelium of ILK10 lenses, and the staining
20 detected was markedly fainter than seen in Wt lenses (Fig. 4B). Quantification of central
21 epithelial thickness showed a significant decrease in the epithelium of ILK10 lenses between
22 E15.5 and E17.5 (Fig. 6G). Similarly, quantification of BrdU⁺ cells in these lenses showed
23 that at E17.5 there is significant decrease in the percentage of proliferating cells in ILK10
24 lenses. This occurred against a background of a rapid increase in the percentage of

1 proliferating cells in the Wt epithelium (Fig. 6H). Consistent with the decrease in BrdU
2 labelling, we also detected decreased reactivity for cyclin D1 (Fig. 6D) and PH3 (Fig. 6F) in
3 ILK10 lenses compared to Wt (Fig. 6C, E). A similar reduction in staining was observed for
4 cyclin D2 (not shown).

5 To determine whether the epithelial phenotype in ILK10 lenses was due to cell death we
6 performed TUNEL assays on E17.5 to P10 lenses. TUNEL staining revealed very little to no
7 evidence for cell death in Wt lenses (Fig. 7A-C). In E17.5 ILK10 lenses, no TUNEL⁺ nuclei
8 were detected in the epithelium (Fig. 7D). However, by P2, apoptotic nuclei were detected
9 in the central anterior epithelium (Fig. 7E) and at P10 numerous TUNEL⁺ nuclei were
10 detected in the fibre cells (Fig. 7F), with some lenses showing large regions with TUNEL⁺
11 nuclei (Fig. 7F, inset).

12 ***Ilk* modulates ERK and Akt activity**

13 Previous studies have indicated that the activity of ERK and Akt signalling pathways are
14 required for epithelial cell proliferation (Iyengar et al., 2006; Iyengar et al., 2009) and fibre
15 cell differentiation (Lovicu and McAvoy, 2001; Wang et al., 2010; Wang et al., 2009). In
16 addition, past studies indicated that Akt may be a direct substrate for ILK (Hannigan et al.,
17 2005; Kimura et al., 2010; Persad et al., 2001; Troussard et al., 2003). To determine whether
18 these pathways were affected in ILK10 lenses we carried out immunohistochemistry and
19 western blotting for total and phosphorylated forms of these proteins. By
20 immunohistochemistry, distinct granular reactivity for phospho-Akt was detected in the
21 cytoplasm of epithelial cells (Fig. 8A) and differentiating cortical fibre cells (Fig. 8J) of Wt
22 lenses at E17.5. By contrast, intense reactivity for total Akt was detected uniformly in the
23 epithelium and weaker reactivity was detected uniformly throughout the fibre mass (Fig. 8E).
24 In ILK10 lenses, the reactivity for total Akt appeared unchanged (Fig. 8F), but the reactivity

1 for phospho-Akt was greatly reduced in both the epithelium (Fig. 8B) and in the
2 differentiating cortical fibres (Fig. 8K). Reactivity for phospho-ERK was weakly detected in
3 the anterior epithelial cells of Wt lenses as granular puncta in the cytoplasm (Fig. 8C) and
4 intense staining was detected in the cortical fibres (Fig. 8L). Total ERK reactivity was
5 detected relatively uniformly in the lens epithelium and fibre cells (Fig. 8G). However, in
6 ILK10 lenses, there appeared to be enhanced staining for total ERK in the epithelium but
7 decreased reactivity for phospho-ERK in both epithelium (Fig. 8 D) and cortical fibre cells
8 (Fig. 8M).

9 To quantify changes in Akt and ERK, lenses from E17.5 Wt and ILK10 (*Ilk^{lox/lox}/Cre^{+/-}*)
10 embryos were dissected and extracted for immunoblotting (Fig. 9A). In these lenses the
11 level of ILK expression was approximately 45% that of Wt lenses (Fig. 9B). The level of Akt
12 phosphorylation was significantly decreased ($p=0.001$, Student's *t*-test) by ~62% (Fig. 9C).
13 While in some preparations there appeared to be decreased levels of phosphorylated ERK
14 (lane 4, Fig. 9A), quantification of eight independent extracts indicated that this trend was
15 not significantly different ($p=0.11$, Student's *t*-test) in *Ilk^{lox/lox}/Cre^{+/-}* mice compared to Wt
16 (Fig. 9D).

17 **FGFR activation is reduced in ILK10 lenses**

18 One of the major activators of Akt and MAPK activation in the lens is signalling via FGF
19 receptors (Lovicu and McAvoy, 2001; Lovicu et al., 2011; Zhao et al., 2008). Moreover, ILK
20 has been shown to act as a scaffold protein, linking integrins and receptor tyrosine kinases
21 via the PINCH proteins (Hehlgans et al., 2007) into active signalling complexes. To
22 examine whether loss of ILK may have impacted on signalling directly downstream of
23 FGFR, we examined for the presence of active, phosphorylated FGF receptor substrate 2 α
24 (FRS2 α) and active phosphorylated tyrosine phosphatase (Shp2) in Wt and ILK10 lenses.

1 FRS2 α is a membrane-tethered docking/scaffolding protein that is essential for FGFR
2 signalling via the ERK and PI3K-Akt pathways and becomes phosphorylated on various
3 tyrosine residues when FGFRs are activated by FGF ligands (Gotoh, 2008). Shp2 is also
4 recruited to active FGFRs by binding to Frs2 α and together they provide docking sites for the
5 adaptor protein Grb2 to activate the MAPK or Akt pathways (Gotoh, 2008). In Wt lenses
6 there was intense, specific reactivity for phospho-Y⁴³⁶ FRS2 α in the equatorial regions of the
7 lens, where cells proliferate and differentiate into lens fibre cells (Fig. 10A). Staining was
8 also present on the basolateral surfaces of lens epithelial cells (Fig. 10A, C) and in the
9 developing iridial epithelium (Fig. 10A). Similarly, phospho-Shp2 was strongly detected in
10 the equatorial region of Wt lenses (Fig. 10E). However, in ILK10 lenses, reactivities for
11 phospho-Y⁴³⁶ FRS2 α and phospho-Y⁵⁴² Shp2 were greatly decreased (Fig. 10B, D, F),
12 indicating decreased activation of FGF receptor signalling in these lenses.

13 ***Ilk* modulates extracellular matrix and focal adhesions**

14 Previous studies indicated that loss of *Ilk* results in aberrant matrix assembly in various
15 tissues, (Gagne et al., 2010; Gkretsi et al., 2008; Guo and Wu, 2002; Mills et al., 2006;
16 Niewmierzycka et al., 2005), including the lens (Cammass et al., 2012; Samuelsson et al.,
17 2007). Consistent with this we found abnormal localisation of collagen IV in ILK10 lenses
18 at E17.5, with abnormal retention of collagen reactivity within the fibre cells and
19 irregularities in the lens capsule (Fig. S1). Failure of the lens capsule is evident in P10
20 lenses when there is commonly rupture of equatorial lens cells into the posterior chamber of
21 the aqueous (Fig. S1) and fibres into the vitreous (not shown). PAS staining, which labels
22 polysaccharides, revealed that the lens capsule of ILK10 mutants was grossly deficient in
23 anterior and equatorial lens regions, but was still detectable, albeit thinned, in the posterior
24 regions (Fig. S1). To further examine changes in gene expression associated with the

1 extracellular matrix and focal adhesions we employed quantitative RT-PCR using a
2 commercial PCR array on *Ilk*^{lox/lox}/MLR10Cre⁺ lenses. The analysis at E17.5 showed
3 significant decreases in 17 genes and increases in 3 genes (Table 2, S1). As expected there
4 was a large and significant decrease in *Ilk* expression. The other differentially expressed
5 genes were associated with focal adhesions (*Itga2, Itga9, Itga5, Actn2*), the actin
6 cytoskeleton (*Actn2, Pak1, Pak4, Crk, Crkl, Arhgap5, Dock1, Dst*) and protein trafficking
7 (*Cav1, Dst*) as well as the PI3K/Akt (*Pten, Akt2, Akt3*) and MAPK/ERK pathways (*Hras1,*
8 *Rap1a*).

9

1 DISCUSSION

2 Previous studies indicate that, in addition to the critical role of growth factors, signals from
3 the ECM, mediated by integrins, are essential for lens development. Double null mutations
4 of the laminin-binding integrins, $\alpha 3$ and $\alpha 6$ integrin subunits result in a deficient lens
5 epithelium during lens formation (De Arcangelis et al., 1999; Wederell and de Iongh, 2006).
6 The intracellular signalling mediators for integrin signalling, such as focal adhesion kinase
7 (FAK)(Kokkinos et al., 2007) and ILK (Cammass et al., 2012; de Iongh et al., 2005; Weaver
8 et al., 2007) are expressed in the lens and there are indications that these molecules have
9 altered activity in some cataracts (Menko and Andley; Weaver et al., 2008). Moreover,
10 recent studies using the nestin-Cre and Le-Cre lines have demonstrated a requirement of ILK
11 for cell survival and matrix deposition during lens development (Cammass et al., 2012;
12 Samuelsson et al., 2007). In this study we have similarly deleted *Ilk* from the developing
13 lens using the MLR10-Cre line and show that ILK affects ERK and Akt pathway activation
14 as well as FGF receptor activation required for lens epithelial cell proliferation and survival.
15 Consistent with previous studies (Cammass et al., 2012; de Iongh et al., 2005; Weaver et al.,
16 2007), we detected ILK expression predominantly in lens epithelial and early differentiating
17 fibre cells. In ILK10 lenses there is complete loss of ILK staining from E13.5, which is a day
18 earlier than documented with the *Nestin*-Cre and two days after that obtained with Le-Cre
19 (Cammass et al., 2012). The subsequent phenotype of the ILK10 lenses is similar to that
20 observed in the Nestin-Cre/ILK^{fl/fl} mice, with a progressive epithelial deficiency, attenuated
21 fibre elongation and disruption of the lens capsule. Consistent with the earlier gene deletion,
22 these defects appear slightly earlier in the ILK10 lenses (E17.5) than in Nestin-Cre/ILK^{fl/fl}
23 lenses (E18.5).

1 ***ILK has a role in epithelial cell proliferation and survival***

2 The epithelial phenotype in ILK10 lenses was characterised by decreased central epithelial
3 thickness, epithelial cell number and proliferation at E17.5 with subsequent loss of the
4 central epithelial cells by P2. Cells in the germinative zone were less affected than central
5 anterior lens epithelial cells and some of these cells showed evidence of cell cycle activity
6 albeit greatly reduced. A similar sparing of equatorial compared to central epithelial cells
7 was seen in lenses with a conditional null mutation of $\beta 1$ integrin (Simirskii et al., 2007) and
8 in Nestin-Cre/ILK^{fl/fl} mice (Cammass et al., 2012).

9 In contrast to findings by Cammass et al. (2012), ILK10 lenses showed decreased levels of
10 cell cycle entry as shown by reduced BrdU incorporation, and immunostaining for cyclin D1
11 and phospho-histone H3 at E17.5. One possible explanation for the difference in the
12 proliferation assay results between these two studies is that the loss of protein in Nestin-
13 Cre/ILK^{fl/fl} lenses occurs a day later and thus the effects on proliferation may have occurred
14 later than the analyses conducted at E14.5-17.5. Our data suggest that during the peak
15 growth period of the foetal lens, there is decreased epithelial cell generation and that this
16 contributes to the epithelial reduction and altered fibre differentiation. Intriguingly the
17 number of cells expressing the CDKI, p57Kip2, at the equator was also reduced in the ILK10
18 lenses compared to the Wt, suggesting that the number of differentiating cells that exited the
19 cell cycle was also reduced. Similar studies of ILK function in the developing cerebellum
20 (Mills et al., 2006) have also shown that loss of *Ilk* results in decreased precursor cell
21 proliferation.

22 Our data suggest that, in addition to the reduced cell cycle activity, cell death (TUNEL) is
23 also a contributor to the loss of epithelial cells from P2 and fibre cell death from P10 in *Ilk*-
24 deficient lenses. By contrast, a previous study (Cammass et al., 2012) did not demonstrate

1 TUNEL positivity or caspase reactivity and suggested that loss of epithelial cells is
2 independent of the classical intrinsic apoptotic pathway as there is no amelioration of this
3 loss in mice that lack the central effectors (*Bak*, *Bax*) in this pathway. However, loss of *Bax*
4 in this study was reliant on the same Cre activity required to delete *Ilk* and thus residual *Bax*
5 protein may have been sufficient to induce apoptosis. Moreover, studies of *Bax/Bak* double
6 null mice indicate that organs regulated by apoptosis such as kidney, heart, lungs and liver
7 were unaffected (Lindsten et al., 2000), suggesting there may be other effectors of the *Bcl2*
8 family in this pathway. Indeed, a recent study has suggested that the closely related BOK
9 protein may function in a BAX/BAK-like manner (Ke et al., 2013). It remains to be
10 determined if combinations of *Bax/Bak/Bok* function to regulate apoptosis in the lens and
11 whether other cell death processes such as autophagy or necroptosis contribute to cell loss in
12 *Ilk*-deficient lenses.

13 ***ILK has a role in fibre cell elongation and differentiation***

14 Loss of ILK also affected fibre cell differentiation. In ILK10 lenses at E15.5 there was
15 evidence of mild defects in fibre cell elongation that became increasingly noticeable from
16 E17.5 onwards. As a result, many ILK10 lenses appeared elongated in the anterior-posterior
17 axis as shorter fibre cells became added posteriorly in the lens without extending completely
18 in the apical direction. The fibre cells in ILK10 lenses at E17.5 were abnormally arranged in
19 the transitional zone just below the equator and showed altered orientation with respect to the
20 posterior capsule, suggesting altered migratory behaviour. At later stages the fibre mass
21 became increasingly disorganised and eventually fibre cells underwent apoptosis. A similar
22 pattern of altered fibre cell elongation is seen in transgenic mice that express a C3-
23 exoenzyme that inactivates Rho GTPase (Maddala et al., 2004). This suggests that ILK
24 plays a role in regulating fibre cell elongation via disruption of actin filaments at focal

1 adhesions. Consistent with this, the PCR array data indicated disrupted expression of various
2 focal adhesion and cytoskeletal components and regulators (see below).

3 An alternative scenario is that loss of *Ilk* in the epithelium has had an indirect effect on the
4 fibres, as there is known to be exchange of signals between the epithelium and fibre cells via
5 connexins and also cell to cell signalling (Martinez and de Iongh, 2010; Mathias et al.,
6 2010). This may particularly apply to the cell death observed in the fibres postnatally as this
7 occurs after the defect in the epithelium. However, the abnormal differentiation of early
8 fibres at E17.5 coincides with the epithelial defect and thus a more parsimonious explanation
9 is that this is a direct effect of loss of *Ilk* in these fibre cells.

10 The finding of delayed β -crystallin and reduced p57^{Kip2} expression in the ILK10 fibres is
11 consistent with the notion that the effects of ILK on the fibres are via signalling pathways
12 (MAPK and PI3-Akt) that regulate fibre differentiation (see below). However, the changes
13 do not seem to be via major changes in the expression of c-Maf. While the Maf genes are
14 important regulators of crystallin gene expression, regulation is likely to be combinatorial
15 (Ring et al., 2000). Indeed α A-crystallin expression is regulated by combined actions of
16 Pax6, c-Maf and CREB (Yang et al., 2006). As this study has shown no differences in Pax6
17 or c-Maf expression in the ILK10 lenses, it remains to be determined whether the delay in β -
18 crystallin expression is due to a decrease in CREB or deficiency of another transcription
19 factor.

20 ***ILK has dual functions as integrin signal mediator and scaffold protein***

21 ILK appears to mediate its effects on lens cell survival, proliferation and differentiation by
22 regulating the activity of growth factor signalling pathways (MAPK and PI3K-Akt),
23 particularly the PI3K-Akt pathway, as this was markedly depressed in ILK10 lenses. Similar

1 decreases in Akt phosphorylation on Ser473 have been reported in various tissues with
2 conditional null mutation of *Ilk*, including cardiac muscle (White et al., 2006), T cells (Liu et
3 al., 2005) and Schwann cells (Pereira et al., 2009). Numerous *in vitro* studies have shown
4 that ILK either directly or indirectly regulates Akt phosphorylation (Hannigan et al., 2005;
5 Kimura et al., 2010; Maydan et al., 2010; Persad et al., 2001; Troussard et al., 2003). Indeed
6 certain cancer cells appear to be preferentially dependent on *Ilk* for phosphorylation of Akt
7 and cell survival compared to normal cells (Troussard et al., 2006).

8 By contrast, the role of ILK in regulating ERK phosphorylation is less clear and the results
9 of the present study suggest this is decreased in lenses that have a more severe phenotype
10 with total loss of ILK but not in mice with only reduced ILK expression. There is some
11 evidence to suggest that ILK may indirectly affect ERK phosphorylation via glycogen
12 synthase kinase 3 β (GSK3 β) (Naska et al., 2006), but direct association of ILK with ERK
13 has not been demonstrated. Alternatively, as ILK is known to bind the PINCH adaptor
14 protein, which, can interact with receptor tyrosine kinases in the membrane via Nck2,
15 (Hehlgans et al., 2007; Wu, 2005) it is possible that loss of ILK scaffolding function results
16 in disrupted growth factor signalling (Figure 13). Previous studies (Lovicu and McAvoy,
17 2001; Lovicu et al., 2011; Zhao et al., 2008) have indicated that FGFR signalling is the major
18 contributor to ERK-MAPK and PI3K-Akt activations in the lens. Activation of FGF
19 receptors leads to phosphorylation of specific FRS2 α tyrosine residues that function as
20 binding sites for Shp2 and Grb2 and activation of Ras-ERK and PI3K-Akt pathways (Gotoh,
21 2008). Both adaptor proteins showed reduced levels of phosphorylation in ILK10 lenses,
22 suggesting loss of ILK abrogated FGFR activation in these lenses. Loss of Shp2
23 phosphorylation may also have had an impact on adherens junction re-assembly as it has
24 been shown to bind to β -catenin and, in endothelial (Timmerman et al., 2012) and corneal

1 epithelial (Ng et al., 2013) cells, it is required during injury-mediated recovery of cadherin-
2 based adhesion junctions. Consistent with this we documented progressive loss of E-
3 cadherin reactivity in the lens epithelium. It remains to be determined whether activation of
4 other receptor tyrosine kinases (PDGFR, IGFR EGFR) that are known to activate MAPK or
5 Akt in the lens (Lovicu et al., 2011) are similarly affected by a loss of *Ilk*. Overall these data
6 suggest that ILK in the lens may function as a scaffold protein and promotes optimal
7 activation of FGFRs in focal adhesions, resulting in efficient activation of Ras-ERK and the
8 PI3K-Akt pathways (Figure 13). This is consistent with models that show ILK functions in
9 mediating interactions with other scaffold proteins such as parvins in focal adhesions
10 (Fukuda et al., 2009; Wickstrom et al., 2010)

11 Further support for ILK functioning as a scaffold protein is the delay between the deletion of
12 *Ilk* (~E13.5) and the effects on lens epithelial phenotype (~E17.5). It is plausible that
13 another integrin signalling pathway partially compensates for *Ilk* loss in the early embryonic
14 lens but not at later stages. For instance, FAK is expressed and active in the developing lens
15 from E12.5 to postnatal stages. However, during late foetal (E18.5) and early postnatal
16 stages its expression and activity in the anterior epithelium decreases and it becomes
17 restricted to the germinative zone cells (Kokkinos et al., 2007). Notably these cells are
18 spared in perinatal ILK10 mice. While a preliminary report indicates that loss of FAK affects
19 lens development (Schlieve and Beggs, 2007), further studies are required to determine the
20 role of FAK in the lens epithelium and whether FAK can compensate for ILK loss. Indeed in
21 cardiomyocytes that lack ILK, there is loss of FAK activation suggesting that ILK is required
22 for FAK recruitment to focal adhesions in these cells (White et al., 2006).

23 Our PCR array data also suggest there is disruption of focal adhesions as there were
24 significant changes in several genes associated with focal adhesions (*Itga2*, *Itga9*, *Itga5*,

1 *Actn2*), the actin cytoskeleton (*Actn2*, *Pak1*, *Pak4*, *Crk*, *Crkl*, *Arhgap5*, *Dock1*, *Dst*) and
2 protein trafficking (*Cav1*, *Dst*) as well as the PI3K/Akt (*Pten*, *Akt2*, *Akt3*) and MAPK/ERK
3 pathways (*Hras1*, *Rap1a*). Of the most dysregulated cytoskeletal and focal adhesion genes,
4 actinin-2 (*Act2*) binding to β 1 integrins has been implicated in the maturation of the focal
5 adhesion and it is involved in the transmission of force between the integrins and the actin
6 cytoskeleton (Roca-Cusachs et al., 2013). *Pak1* and *Pak4* are members of the
7 serine/threonine p21-activating kinase family, which are critical effectors that link Rho
8 GTPases to cytoskeleton reorganization and in growth factor signaling networks that control
9 proliferation, cell polarity, invasion and actin cytoskeleton organization.

10 In summary, this study has shown that integrin linked kinase is required for lens epithelial
11 cell survival, proliferation and differentiation and that it potentially acts as a key scaffolding
12 protein to permit optimal FGFR activation as well as a kinase to facilitate Akt
13 phosphorylation and complete activation of the MAPK and PI3K-Akt pathways.

14

1 FIGURE LEGENDS**2 Figure 1. Ocular phenotype of ILK10 mutant mice**

3 Comparison of adult Wt (A), and ILK10 (B) mice shows ILK10 mice have mild
4 microphthalmia. Dissected Wt eye (C) show a spherical lens protruding through the iris into
5 the anterior chamber of the iris whereas the ILK10 lens (D) is smaller and does not protrude
6 through the iris (D). Scale bars: A, B, 5mm; C-D, 1mm.

7 Figure 2. Loss of ILK protein in lenses of ILK10 mutants

8 Immunofluorescence for ILK protein in sections of Wt (A, C, E) and ILK10 (B, D, F) and
9 lenses at E13.5 (A,B), E15.5 (C, D) and E17.5 (E,F). In Wt lenses at E13.5 (A) and E15.5
10 (C) strong ILK reactivity is detectable in the epithelium (arrowheads), with weaker staining
11 in the primary fibre cells (lf). By E17.5 (E), staining is mainly limited to the epithelium with
12 background staining detected in the fibre cells. No detectable staining for ILK (*) is
13 detectable in the epithelium or the fibres of ILK10 lenses (B, D, F). Scale bar: A, B, 50 µm;
14 C-F, 100 µm.

15 Figure 3. Disrupted lens differentiation in ILK10 mutants

16 Histological sections of Wt (A,C,E,G, J, L, N) and ILK10 (B, D, F, H, K, M, O) lenses at
17 E15.5 (A, B), E17.5 (C, D, J, K, L, M), P2 (E, F, N, O) and P21 (G, H). At E15.5, ILK10
18 (B) lenses were similar to Wt (A), with an apparently normal epithelium and normal fibre
19 elongation. Extent of fibre elongation in the transitional zones of these lenses is indicated by
20 curved lines. By E17.5, ILK10 lenses (D) have a thinned epithelium and abnormal fibre
21 elongation compared to Wt (C). The fibre cells (K) show attenuated fibre elongation in the
22 transitional zone compared to Wt (J) and the epithelium shows decreased central epithelial
23 thickness (arrows) in the ILK10 mutant (M) compared to Wt (L). At P2, many ILK10

1 mutants (**F, O**) lack anterior epithelial cells (arrowheads) but appear to retain germinative
 2 zone epithelium (arrows) similar to Wt (**E, N**). By P21, ILK10 lenses (**H**) are grossly
 3 disrupted with extensive fibre cell vacuolation (*) and rupture of the lens through the capsule
 4 (arrow). Scale bar: **A, B**, 100 μm ; **C, D**, 150 μm ; **E, G, F, H**, 200 μm ; **J, K**, 40 μm ; **L, M**,
 5 **N, O**, 50 μm .

6 **Figure 4. Loss of epithelial cells in ILK10 mutants**

7 Immunofluorescent localisation of E-cadherin in Wt (**A, C, E**) and ILK10 mutant (**B, D, F**)
 8 lenses at E17.5 (**A, B**), P2 (**C, D**) and P21 (**E, F**). At E17.5, staining intensity for E-cadherin
 9 is reduced in the anterior epithelium (arrowheads) of ILK10 lenses (**B**) compared to Wt
 10 lenses (**A**) but is maintained in the germinative zone. The insets in A and B show higher
 11 magnification views to highlight loss of E-cadherin staining in anterior epithelial cells
 12 compared to pre-equatorial region (arrows). In contrast to the uniform staining for E-
 13 cadherin in Wt lenses, ILK10 lenses show reduced lateral membrane staining but retain
 14 intense puncta apically. In P2 lenses (**D**) the loss of E-cadherin staining is more noticeable
 15 with distinct patches of cells showing greatly reduced E-cadherin staining (arrowheads).
 16 However the germinative zone epithelium shows intense labelling of epithelial cells
 17 (arrows). By P10, the anterior epithelium of ILK10 lenses (**F**) is largely absent but the
 18 germinative zone epithelium is still present and intensely labelled with E-cadherin (arrows).
 19 Hoechst labelling indicates very few cell nuclei (arrowheads) in this region (dotted line).
 20 Scale bar 100 μm .

21 **Figure 5. Abnormal fibre differentiation in ILK10 mutants**

22 Immunofluorescent localisation of β -crystallin (**A, B**), p57^{Kip2} (**C, D**) and c-Maf (**E, F**) in
 23 Wt (**A, C, E**) and ILK10 mutant (**B, D, F**) lenses at E17.5. **A, B**. In ILK10 lenses initiation
 24 of β -crystallin expression (arrows) in the transitional zone is delayed compared to Wt. **C, D**.

1 Expression of the CDKI, p57^{Kip2}, in cells below the equator (dashed line) is markedly
 2 reduced in the ILK10 mutant fibre cells (**D**, arrow) compared to Wt (**C**, arrow). **E, F**.
 3 Expression of c-maf does not appear to be changed in ILK10 lenses compared to Wt. Scale
 4 bars: **A-B**, 100 μm ; **C, D**, 70 μm ; **E, F**, 50 μm .

5 **Figure 6. Disrupted cell cycle in epithelium of ILK10 lenses**

6 Analysis of cell cycle markers shows decreased BrdU incorporation (**A, B**) and cyclin D1 (**C**,
 7 **D**) and phospho-histone-H3 (**E, F**) staining in ILK10 (**B, D, F**) lens epithelial cells
 8 (arrowheads) compared to Wt (**A, C, E**). **G**. Quantification of central epithelial thickness
 9 (yellow bar, **A, B**) and BrdU incorporation (**H**) show that the ILK10 epithelium becomes
 10 significantly (*, $p < 0.05$; Student's t-test) thinned and has a significantly reduced percentage
 11 of cells in S phase at E17.5 but not at E15.5. Scale bars: **A, B**, 50 μm ; **C-F**, 100 μm .

12 **Figure 7. Increased apoptosis in ILK10 lenses**

13 TUNEL reaction (green) and DNA (blue) in Wt (**A-C**) and ILK10 (**D-F**) lenses at E17.5 (**A**,
 14 **D**), P2 (**B, E**) and P10 (**C, D**). Wt lenses at E17.5 (**A**), P2 (**B**) and P10 (**C**) do not show any
 15 TUNEL⁺ nuclei. In E17.5 ILK10 lenses, TUNEL⁺ nuclei are not detected in the epithelium
 16 (**C**, inset) but are evident at P2, particularly in the central epithelium (**E**, arrows). In P10
 17 ILK10 lenses, apoptotic nuclei are more evident in the fibre mass (**F**, arrows). In some
 18 lenses there were distinct foci of TUNEL⁺ fibre cells (**F**, inset). Scale bar: A-F, 150 μm .

19 **Figure 8. Altered Akt and ERK reactivity in ILK10 lenses**

20 Immunoperoxidase staining for phospho-Akt (**A, B, J, K**), total Akt (**E, F**), phospho-ERK
 21 (**C, D, L, M**) and total ERK (**G, H**) in Wt (**A, E, J, C, G, L**) and ILK10 (**B, F, K, D, H, M**)
 22 lenses at E17.5. In ILK10 lenses there is dramatically reduced punctate reactivity for
 23 phospho-Akt in epithelial cells (**B**, arrowheads) and in differentiating fibre cells (**K**, arrows)

1 compared to Wt (**A, J**), but there is no apparent change in total Akt staining in the lens
2 epithelium or fibre mass of ILK10 (**E**) compared to Wt (**F**). Reactivity for phospho-ERK is
3 only weakly detectable in the Wt epithelium (**C**, arrowheads) and is strongly up-regulated in
4 differentiating fibre cells (**L**, arrows). In ILK10 lenses, the reactivity for pERK is greatly
5 reduced in the epithelium (**D**) and fibre cells (**M**), despite an apparent increased reactivity for
6 total ERK in the epithelium (**H**) compared to Wt (**G**). Scale bar: A-H, 50 μm ; J-M, 75 μm .

7 **Figure 9. Reduced Akt activity in ILK10 lenses**

8 Western blots of two separate Wt and ILK10(Cre^{+/-}) E17.5 lens extracts from , probed for
9 ILK, phosphorylated Akt and ERK, total Akt and ERK and GAPDH (**A**). Blots for phospho-
10 proteins were stripped and re-probed for total protein. Densitometric analyses indicate
11 significant decreases in ILK (**B**) and phospho-Akt (**C**) but not combined phospho-Erk1/2 (**D**)
12 expression in ILK10 lenses compared to Wt. Analyses were conducted on the number of
13 samples indicated from at least two independent gels.

14

15 **Figure 10. Reduced phospho-FRS2 α and phospho-Shp2 in ILK10 lenses**

16 Immunofluorescent localization of phospho-FRS2 α (red) and DNA (blue) and phospho-Shp2
17 (red) in Wt (**A, C, E**) and ILK10 (**B, D, F**) E17.5 lenses. **A-D**. In frozen sections, reactivity
18 for FRS2 α was detected strongly in differentiating lens fibre cells of the transitional zone
19 (arrowheads) and in the ciliary body (large arrow) of Wt eyes (**A**); weaker reactivity was
20 detected in the epithelial cells (small arrows, **A, C**). In ILK10 lenses (**B, D**), reactivity for
21 phospho-FRS2 α was greatly reduced in the transitional zone (*) and only weakly detectable
22 in the anterior epithelial cells (small arrow, **B**). Reactivity in the ciliary body was similar to
23 Wt eyes (large arrow, **B**). In paraffin sections, reactivity for phospho-Shp2 is strongly

1 detected in the elongating fibres and weakly detected in the epithelium of the Wt lens (**E**) but
2 virtually absent in the ILK10 lens (**F**). Scale bar, A, B, 50 μm ; C, D, 65 μm ; E, F 100 μm .

3 **Figure 11. Proposed model of ILK function in lens cells**

4 Ilk is known to associate with the β -subunit of integrins and via PINCH and Nck link to
5 receptor tyrosine kinases such as FGF receptors. ILK has also been show to phosphorylate
6 Akt. In this study, loss of *Ilk* results in altered phosphorylation of Akt, Frs2 α , Shp2 and to a
7 lesser extent ERK. The data obtained in this study (in red) support a model whereby ILK
8 directly affects Akt phosphorylation and indirectly regulates the efficient phosphorylation of
9 FGF receptors to affect lens cell survival, proliferation and differentiation, via its function as
10 a scaffold protein.

11

12 **Supplementary Figure S1. Abnormal ECM deposition in ILK10 lenses**

13 Immunofluorescent localization of collagen IV (**A-D**) and PAS staining (**E, F**) in Wt (**A, C,**
14 **E**) and ILK10 (**B, D, F**) lenses. **A, C.** In Wt lenses, collagen IV is normally detected in lens
15 epithelial cells (arrows), the lens capsule, the tunica vasculosa lentis (double arrows, **C**) and
16 in the vitreous (*,**C**). **B, D.** In mutant lenses, increased reactivity is seen in epithelial and
17 fibre cell cytoplasm (arrowheads) but decreased staining is found in the lens capsule. **Insets.**
18 Higher magnification of equatorial lens regions in **C, D** show decreased reactivity for
19 collagen IV in the lens capsule of the mutant (**C**) compared to Wt (**D**). **E.** PAS staining
20 shows the typical thick anterior lens capsule (double arrowhead) and slightly thinner
21 posterior capsule (open arrowhead) in the Wt lens. **F.** In ILK10 lenses, PAS reactivity is
22 greatly reduced in the thinned anterior capsule (double arrowhead), absent in the equatorial
23 regions where lens cells have ruptured into the posterior chamber of the aqueous (*) and only

- 1 slightly decreased in the posterior capsule. Scale bar: A, B, 200 μm ; C, D, 50 μm ; E, F, 100
- 2 μm ; insets, 10 μm .
- 3

ACCEPTED MANUSCRIPT

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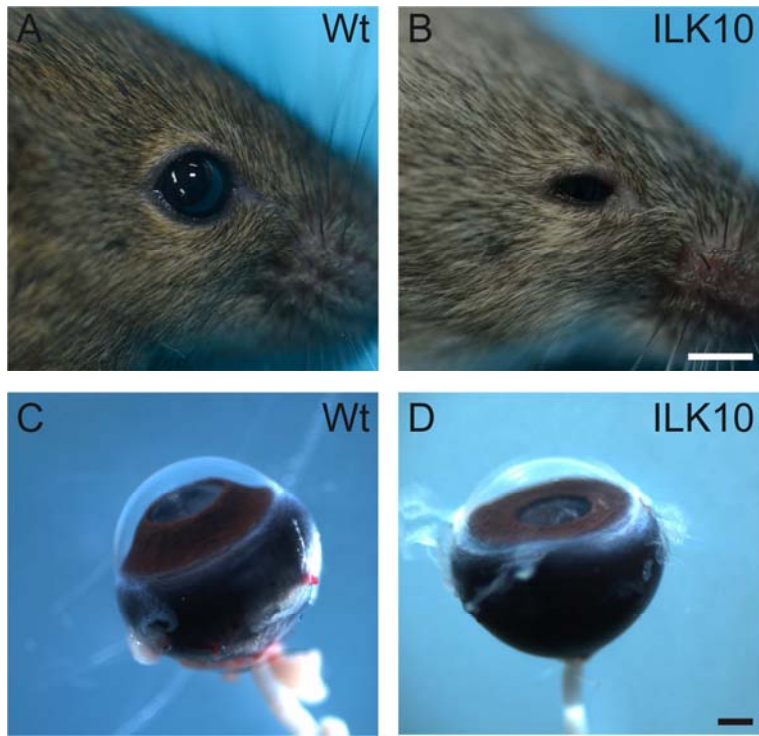
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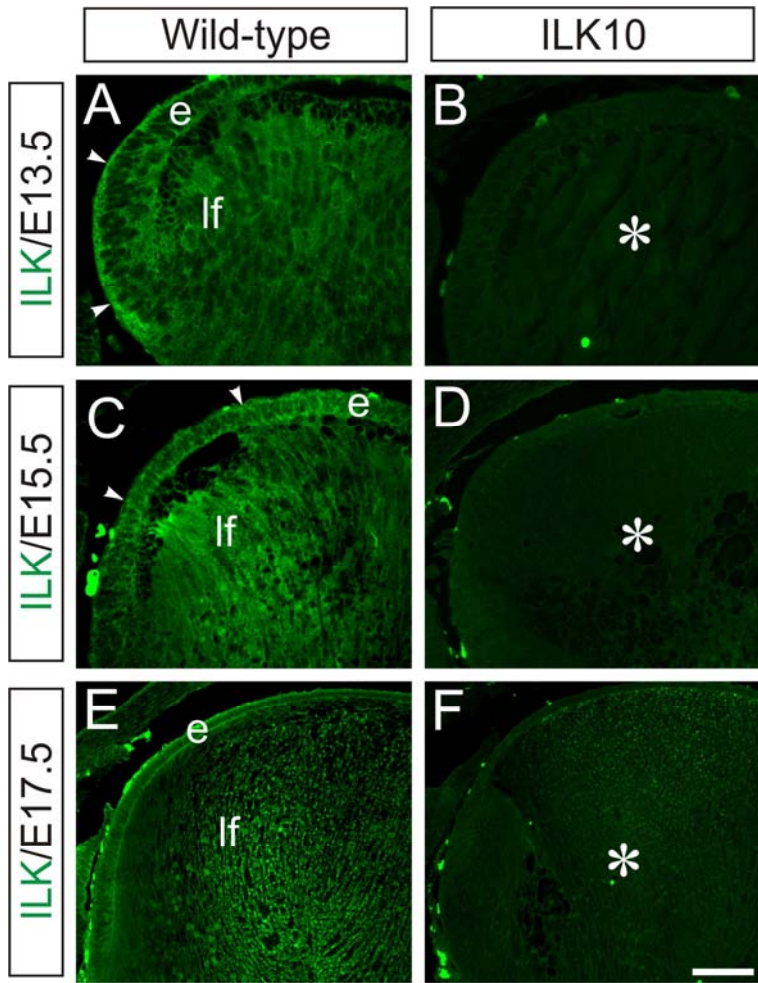
Table1. Genotyping primers

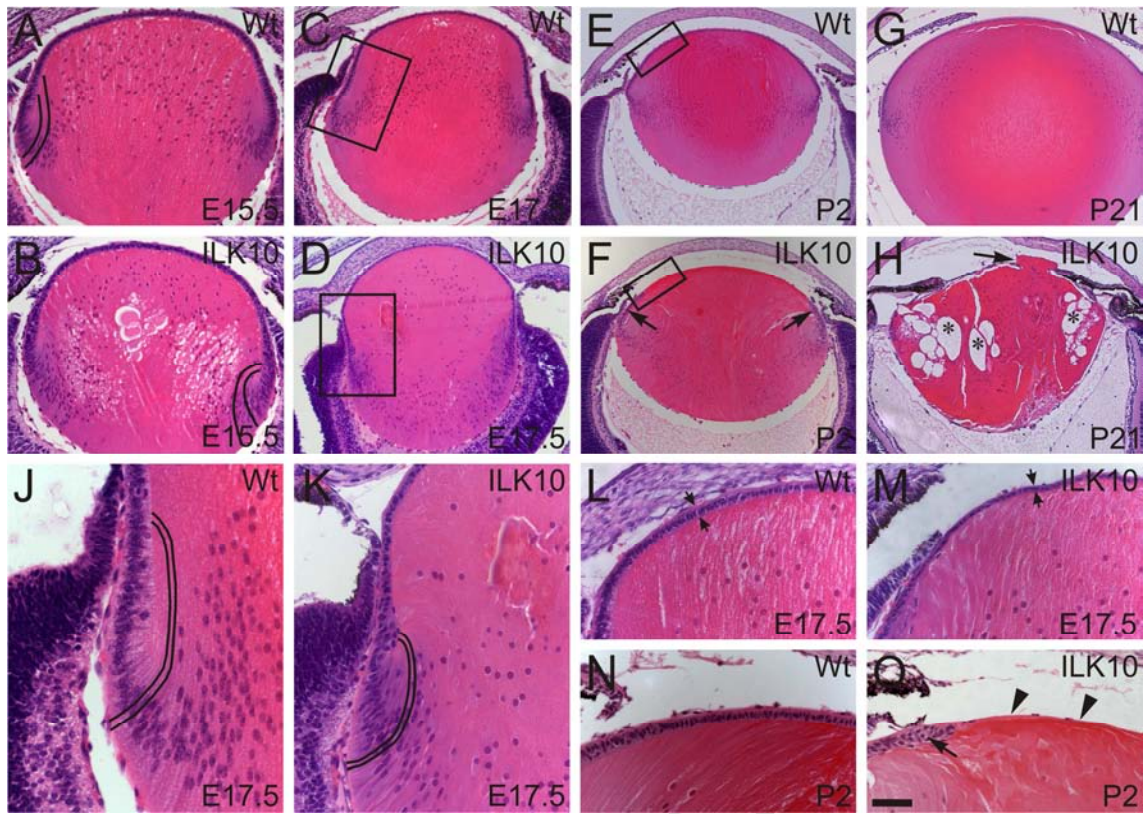
Name	Sequence (5'-3')	Ta	Amplimer Size
<i>Floxed Allele</i>			
ILK-Lox1	TTCTGTGGGAAGCTGGTGACA	61°C	450 bp (LoxP)
ILK-Lox2	GTGCCACCTGCAAATTACAA	61°C	280 bp (Wt)
<i>Cre Transgene</i>			
PR4	GCATTCCAGCTGCTGACGGT	61°C	
Cre-AS	CAGCCCGGACCGACGATGAAG	61°C	577 bp (Cre)
<i>CP49 (Bfsp2)</i>			
Exon2 FwA (C57)	CAGTCATGTGGTTCTGGAAGC	60°C	203 bp (Wt)
Exon2 FwB (129)	AAGTTTCACCCACATTCTCCAGC	60°C	335 bp (Mt)
Exon 2 Rev	CCGTGGGGAGTCTGG	60°C	

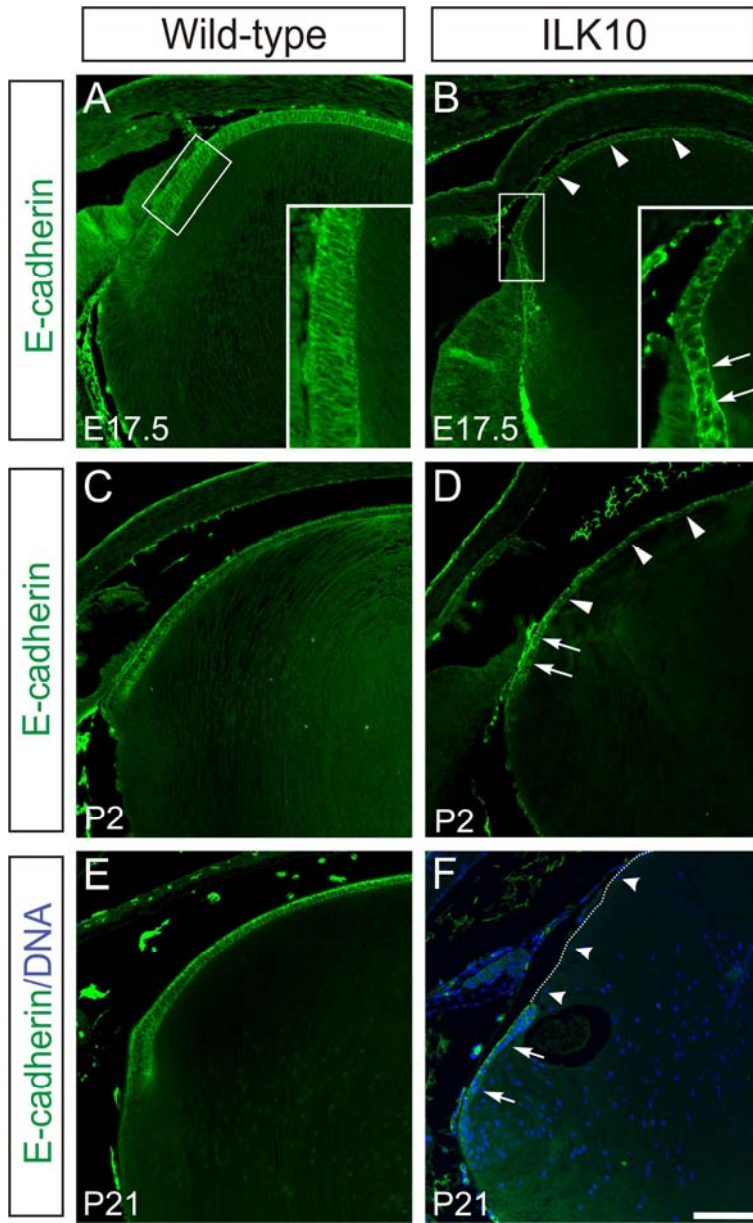
Table 2. QPCR Array Analysis on E17.5 mRNA from Ilk10 and Wt lenses

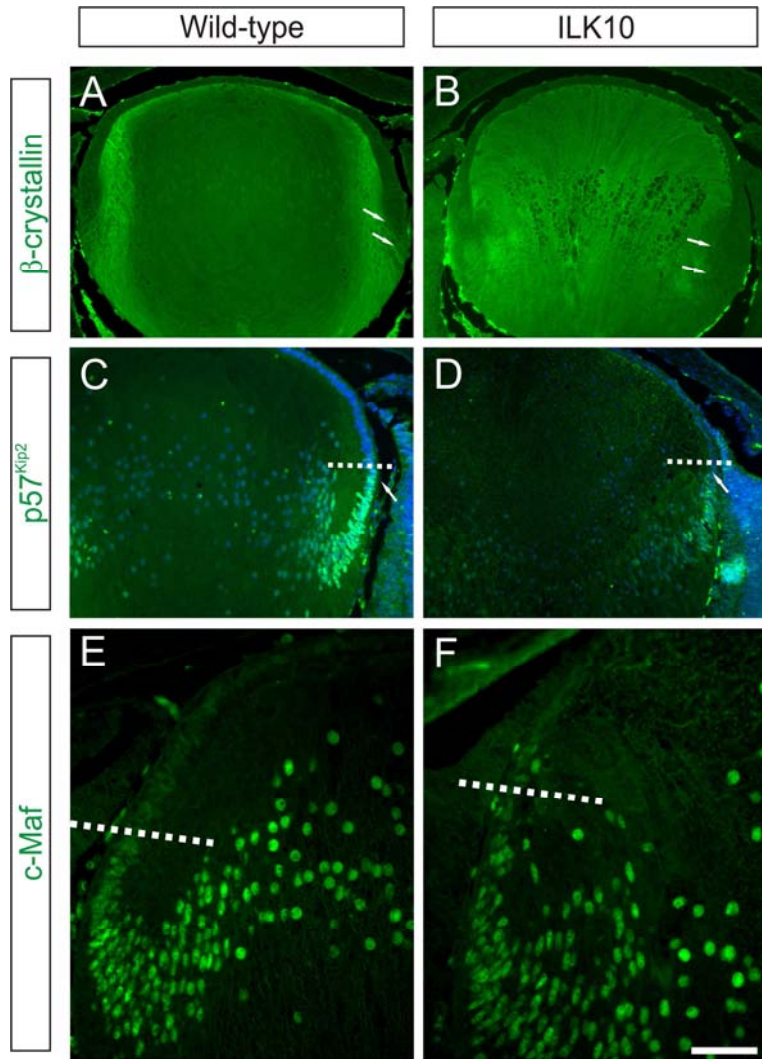
Symbol	$2^{\Delta Ct}$		Ratio	Fold Change	t-test
	ILK10	Wt	ILK10 /Wt	ILK10 /Wt	p value
Ilk	2.8E-02	1.7E-01	0.17	-6.05	0.0003
Actn2	7.1E-02	2.9E-01	0.24	-4.10	0.0012
Pten	1.1E-01	2.2E-01	0.49	-2.04	0.0026
Pak4	3.4E-02	6.7E-02	0.50	-1.98	0.0086
Itga2	3.0E-02	5.8E-02	0.52	-1.94	0.0099
Itga9	6.3E-03	1.1E-02	0.56	-1.80	0.0167
Akt2	1.8E-01	3.0E-01	0.60	-1.67	0.0112
Akt3	7.2E-02	1.1E-01	0.65	-1.54	0.0202
Dst	6.1E-02	8.8E-02	0.70	-1.44	0.0044
Crkl	6.0E-02	8.3E-02	0.73	-1.38	0.0394
Ptk2	1.8E-02	2.4E-02	0.73	-1.37	0.0055
Crk	1.2E-01	1.6E-01	0.74	-1.35	0.0015
Ctnnb1	7.4E-01	1.0E+00	0.74	-1.35	0.0286
Arhgap5	6.6E-02	8.7E-02	0.75	-1.33	0.0264
Dock1	3.2E-02	4.3E-02	0.76	-1.32	0.0137
Hras1	1.6E-01	2.1E-01	0.77	-1.30	0.0455
Rap1a	1.2E-01	1.5E-01	0.82	-1.22	0.0389
Itga5	1.3E-02	7.8E-03	1.62	1.62	0.0221
Pak1	5.3E-03	3.2E-03	1.65	1.65	0.0254
Cav1	3.2E-02	1.7E-02	1.92	1.92	0.0305

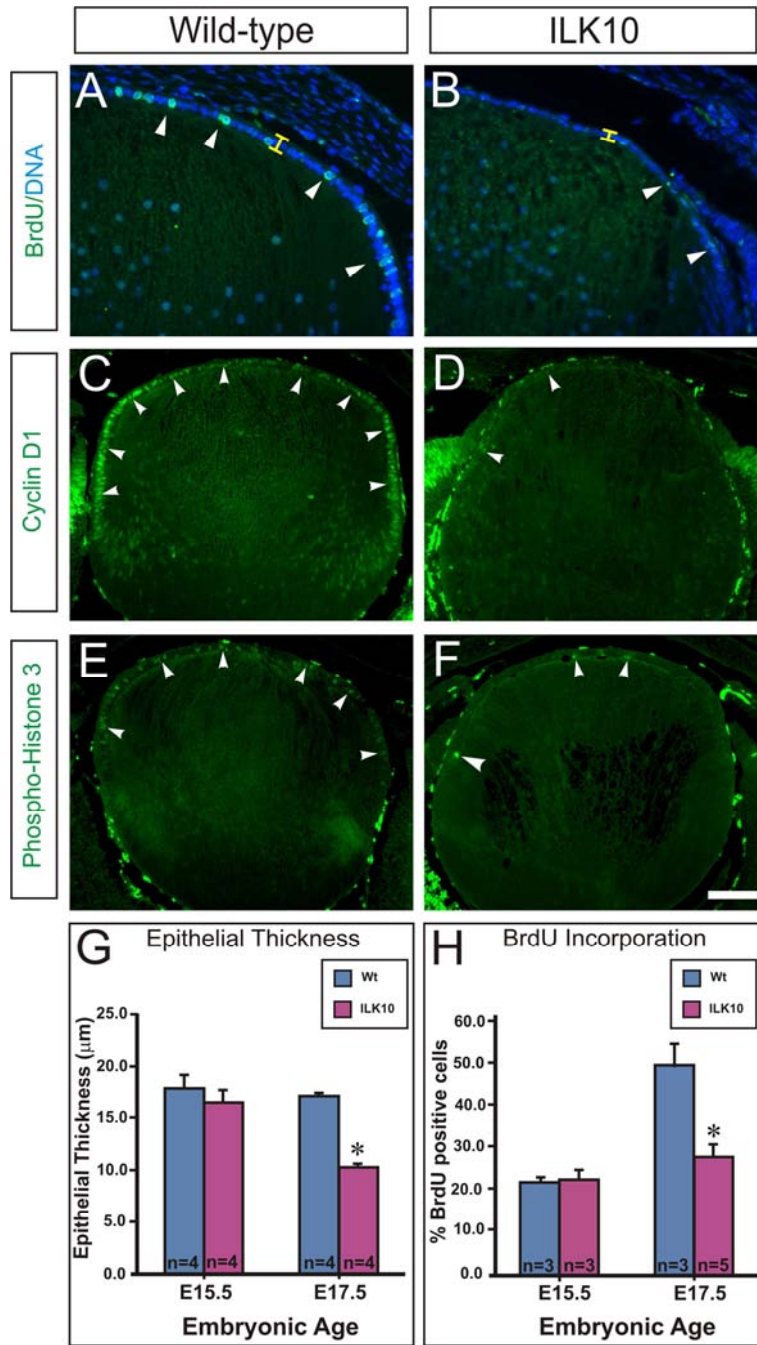


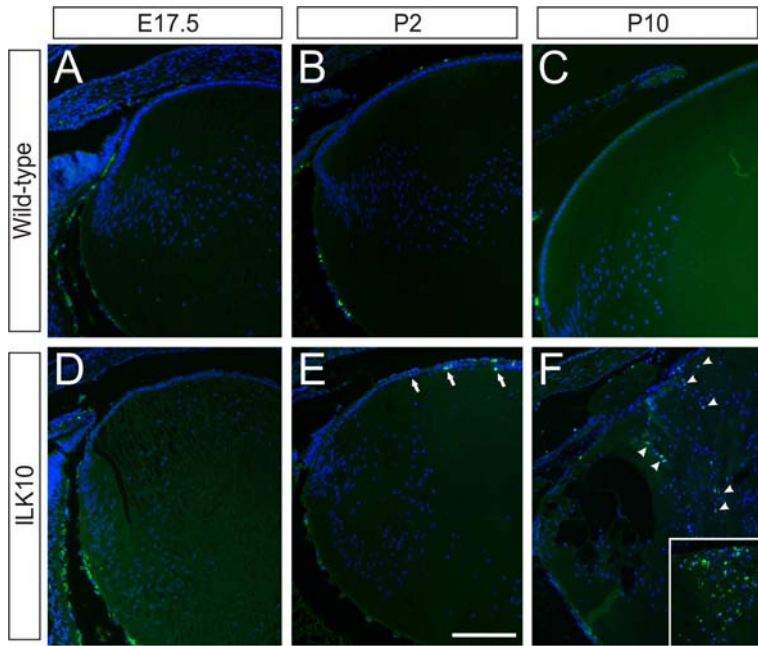


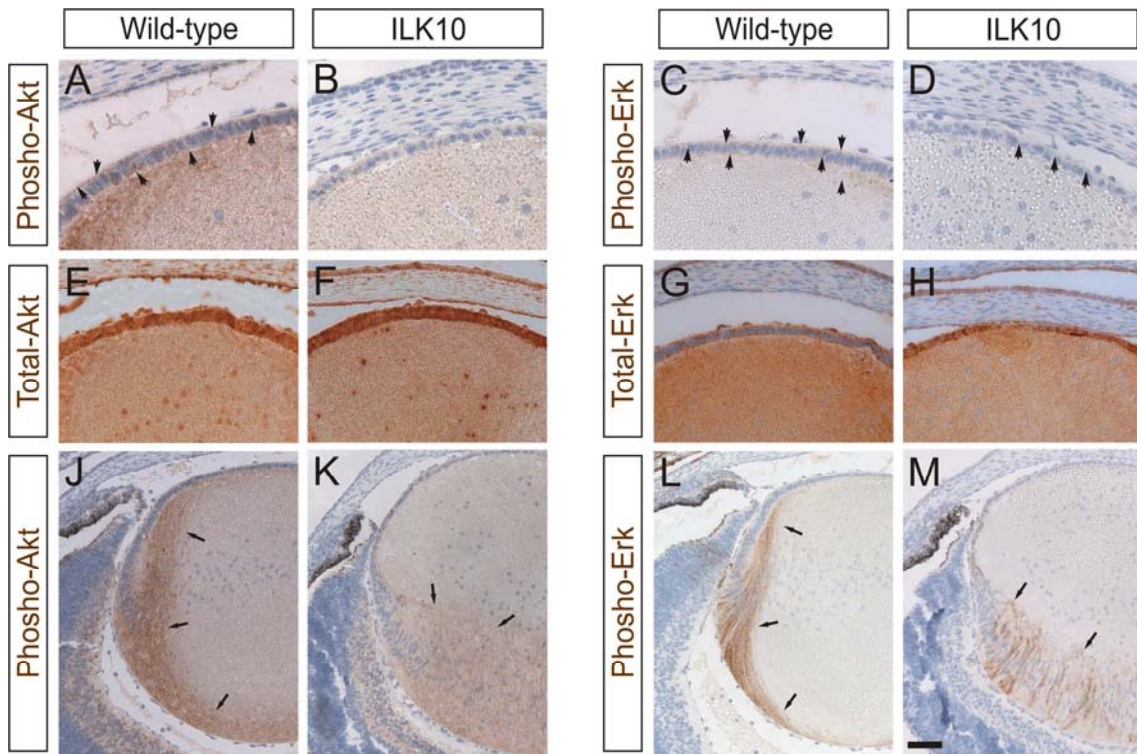


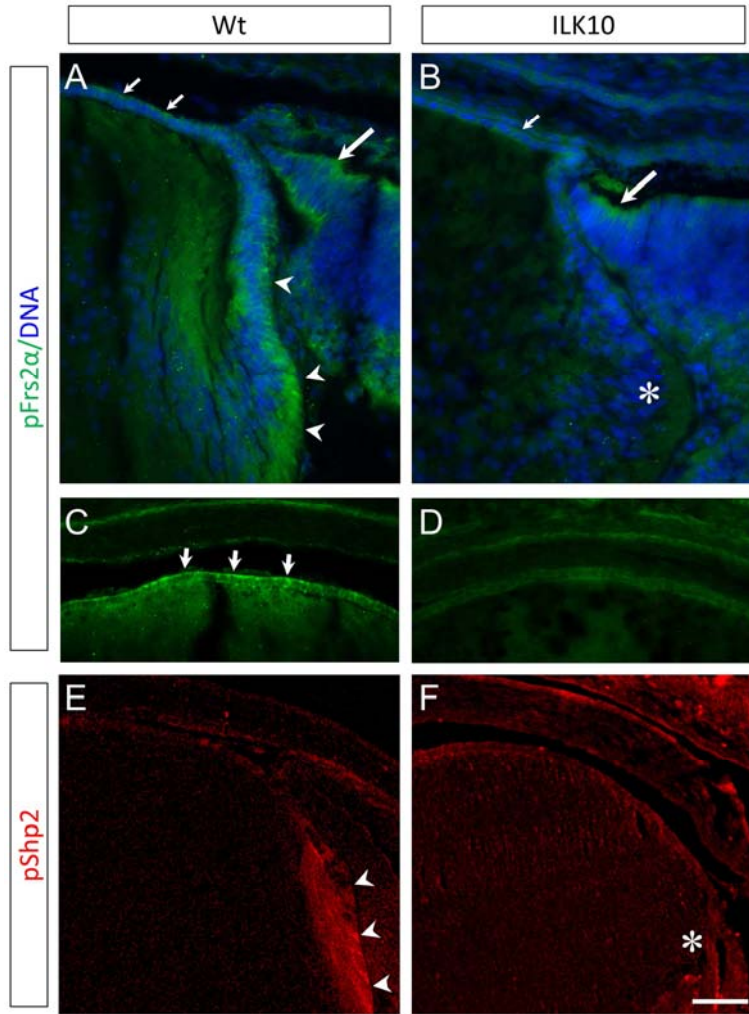


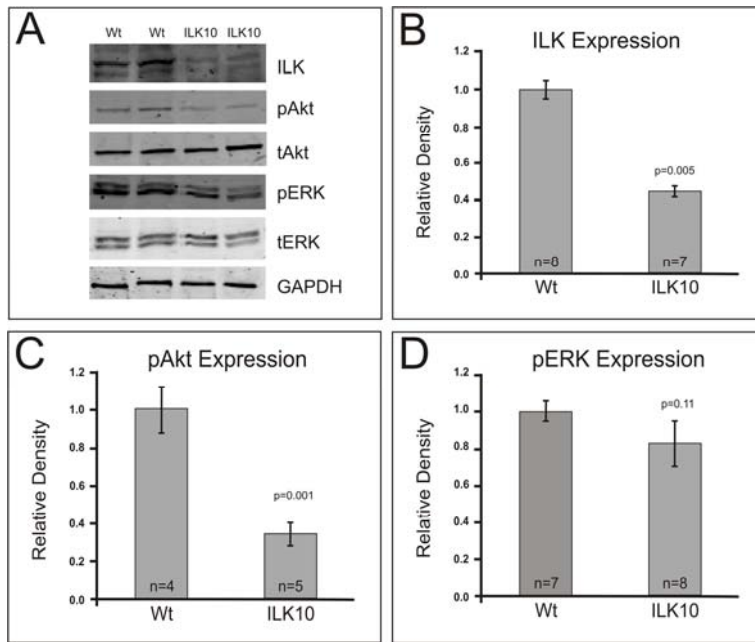


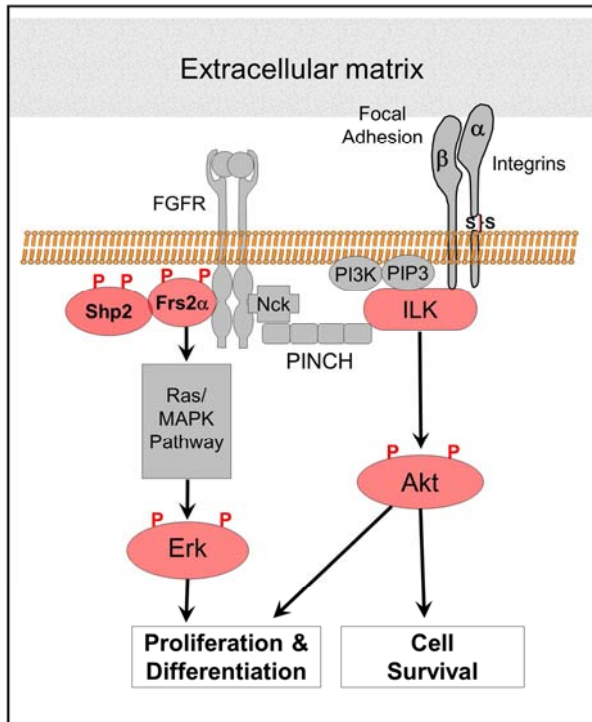






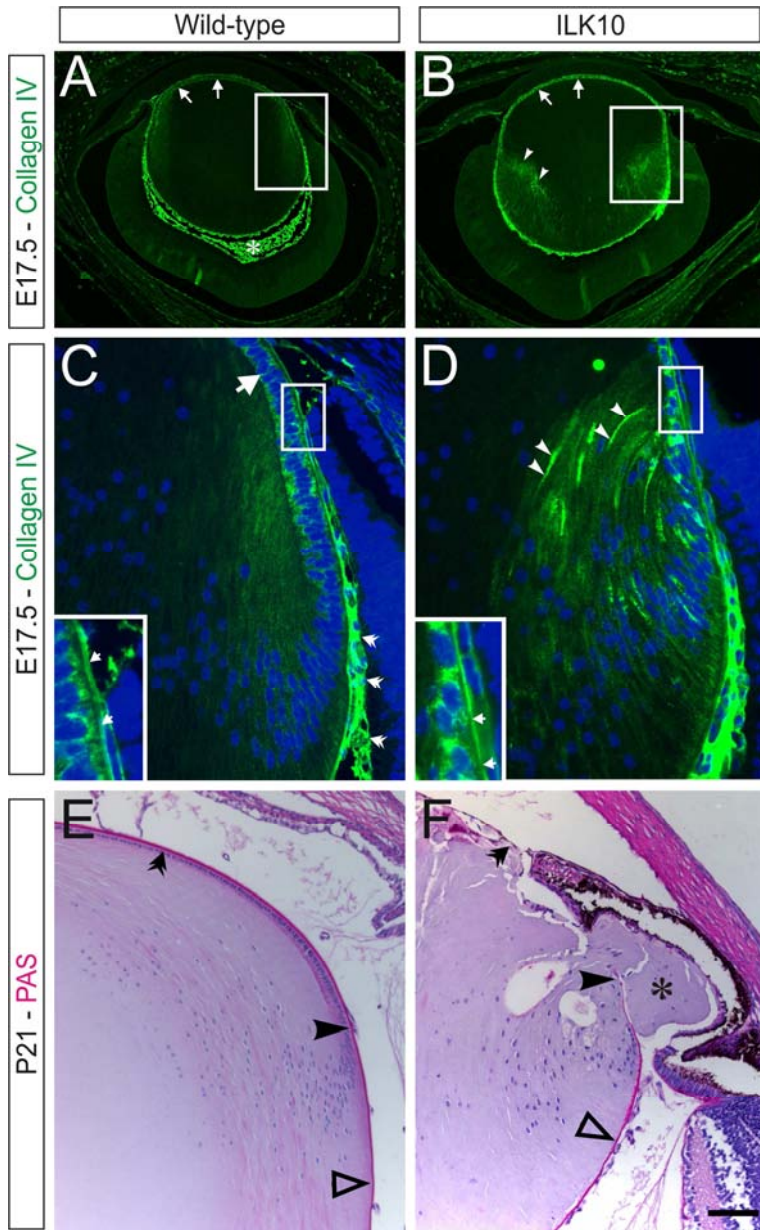






Research Highlights – . EXER13-496; Teo et al.

- We studied the role of *Ilk* in lens differentiation using conditional gene deletion
- *Ilk* loss results in reduced proliferation and fibre differentiation, followed by cell death
- MAPK and Akt activation was reduced in *Ilk* mutant lenses
- FGF receptor activation was decreased and ECM deposition was compromised
- *Ilk* is required for optimal activation of the PI3K and MAPK pathways, activated by FGF



Gene Symbol	ILK10 (n=4)		Wt (n=4)		AVG ΔC_t		$2^{\Delta C_t}$		Ratio	t-test	Fold Change
	AVG Ct	SD	AVG Ct	SD	ILK10 (n=4)	WT (n=4)	ILK10	Wt	ILK10 /WT	p value	ILK10 /Wt

Actn1	22.8	0.7	23.2	0.3	3.26	3.17	1.0E-01	111E-01	0.94	0.53727	0.53727
Actn2	23.3	0.3	21.8	0.3	3.81	1.78	7.1E-02	2.9E-01	0.24	0.0012	-4.10
Actn4	23.3	0.3	24.0	0.5	3.75	4.00	7.4E-02	6.2E-02	1.19	0.4948	1.19
Akt1	22.1	0.5	22.0	0.6	2.63	2.01	1.6E-01	2.5E-01	0.65	0.1195	-1.53
Akt2	22.0	0.8	21.7	0.3	2.46	1.72	1.8E-01	3.0E-01	0.60	0.0112	-1.67
Akt3	23.3	0.9	23.2	0.3	3.79	3.17	7.2E-02	1.1E-01	0.65	0.0202	-1.54
Arhgap5	23.5	0.7	23.5	0.2	3.93	3.52	6.6E-02	8.7E-02	0.75	0.0264	-1.33
Bcar1	22.9	1.5	22.5	0.2	3.39	2.54	9.6E-02	1.7E-01	0.56	0.2574	-1.80
Capn2	22.1	0.8	23.0	1.1	2.55	2.96	1.7E-01	1.3E-01	1.33	0.6197	1.33
Cav1	24.5	0.2	25.9	0.3	4.96	5.90	3.2E-02	1.7E-02	1.92	0.0305	1.92
Cav2	25.6	0.5	26.7	0.5	6.11	6.74	1.4E-02	9.4E-03	1.54	0.0750	1.54
Cav3	31.9	1.3	32.6	1.7	12.41	12.64	1.8E-04	1.6E-04	1.17	0.9671	1.17
Cdc42	20.0	0.7	20.2	0.3	0.48	0.19	7.1E-01	8.7E-01	0.82	0.1328	-1.22
Crk	22.6	0.7	22.7	0.1	3.10	2.66	1.2E-01	1.6E-01	0.74	0.0015	-1.35
Crkl	23.6	0.9	23.6	0.3	4.05	3.59	6.0E-02	8.3E-02	0.73	0.0394	-1.38
Ctnnb1	19.9	0.8	20.0	0.2	0.43	0.00	7.4E-01	1.0E+00	0.74	0.0286	-1.35
Diap1	26.1	1.0	26.7	0.4	6.61	6.67	1.0E-02	9.8E-03	1.04	0.9056	1.04
Dock1	24.5	0.6	24.6	0.2	4.95	4.55	3.2E-02	4.3E-02	0.76	0.0137	-1.32
Dst	23.6	0.7	23.5	0.1	4.04	3.51	6.1E-02	8.8E-02	0.70	0.0044	-1.44
Flna	23.4	1.8	23.5	0.1	3.90	3.55	6.7E-02	8.5E-02	0.78	0.7650	-1.27
Flnb	24.7	0.7	24.9	0.2	5.14	4.91	2.8E-02	3.3E-02	0.85	0.4536	-1.18
Fyn	25.1	0.8	25.6	0.5	5.61	5.57	2.0E-02	2.1E-02	0.97	0.7394	-1.03
Grb2	23.7	0.8	23.8	0.3	4.13	3.84	5.7E-02	7.0E-02	0.81	0.1644	-1.23
Gsk3b	25.6	0.7	26.2	0.3	6.12	6.16	1.4E-02	1.4E-02	1.02	0.9610	1.02
Hras1	22.1	0.6	22.2	0.2	2.62	2.24	1.6E-01	2.1E-01	0.77	0.0455	-1.30
Ilk	24.7	1.2	22.6	0.3	5.18	2.58	2.8E-02	1.7E-01	0.17	0.0003	-6.05

-1.06

Gene Symbol	ILK10 (n=4)		Wt (n=4)		AVG ΔC_t		$2^{\Delta C_t}$		Ratio	t-test	Fold Change
	AVG Ct	SD	AVG Ct	SD	ILK10 (n=4)	WT (n=4)	ILK10	Wt	ILK10 /WT	p value	ILK10 /Wt
Itga1	26.5	0.8	27.5	0.4	7.03	7.45	7.7E-03	5.7E-03	1.34	0.1637	1.34
Itga11	30.0	1.1	31.0	1.2	10.48	11.03	7.0E-04	4.8E-04	1.46	0.5966	1.46
Itga2	24.6	0.7	24.1	0.4	5.06	4.11	3.0E-02	5.8E-02	0.52	0.0099	-1.94
Itga2b	25.9	3.3	27.8	0.5	6.35	7.80	1.2E-02	4.5E-03	2.72	0.3553	2.72
Itga3	25.8	0.6	26.1	0.3	6.29	6.10	1.3E-02	1.5E-02	0.88	0.3063	-1.14
Itga4	28.6	2.0	28.7	0.4	9.05	8.71	1.9E-03	2.4E-03	0.79	0.6975	-1.26
Itga5	25.8	0.9	27.0	0.2	6.31	7.00	1.3E-02	7.8E-03	1.62	0.0221	1.62
Itga6	21.1	1.0	21.2	0.3	1.60	1.24	3.3E-01	4.2E-01	0.78	0.1222	-1.29
Itga7	30.4	1.1	31.6	1.2	10.91	11.58	5.2E-04	3.3E-04	1.58	0.4286	1.58
Itga8	29.3	0.2	29.5	0.7	9.76	9.46	1.2E-03	1.4E-03	0.81	0.4565	-1.23
Itga9	26.8	0.6	26.5	0.2	7.30	6.46	6.3E-03	1.1E-02	0.56	0.0167	-1.80
Itgal	27.1	0.9	26.0	2.5	7.56	6.05	5.3E-03	1.5E-02	0.35	0.3509	-2.83
Itgam	27.0	0.8	26.5	1.3	7.46	6.53	5.7E-03	1.1E-02	0.52	0.2689	-1.91
Itgav	22.5	0.5	23.4	0.2	2.99	3.37	1.3E-01	9.7E-02	1.30	0.2398	1.30
Itgax	32.9	2.0	30.6	8.0	13.34	10.61	9.6E-05	6.4E-04	0.15	0.3560	-6.63
Itgb1	20.5	0.9	20.9	0.1	0.97	0.93	5.1E-01	5.3E-01	0.97	0.9591	-1.03
Itgb2	29.5	1.5	29.0	3.4	9.97	8.96	1.0E-03	2.0E-03	0.49	0.3753	-2.02
Itgb3	27.8	2.3	27.5	1.3	8.27	7.54	3.2E-03	5.4E-03	0.60	0.7755	-1.66
Itgb4	31.1	1.3	30.4	2.0	11.56	10.43	3.3E-04	7.3E-04	0.45	0.3435	-2.20
Itgb5	21.5	1.1	21.8	0.5	2.01	1.77	2.5E-01	2.9E-01	0.84	0.5279	-1.19
Itgb6	31.6	0.5	32.0	1.2	12.03	11.96	2.4E-04	2.5E-04	0.95	0.7012	-1.05
Pak1	27.1	0.8	28.3	0.4	7.56	8.28	5.3E-03	3.2E-03	1.65	0.0254	1.65
Pak2	22.8	0.9	23.0	0.3	3.30	3.04	1.0E-01	1.2E-01	0.84	0.3997	-1.19
Pak3	25.5	1.4	25.5	1.5	5.93	5.55	1.6E-02	2.1E-02	0.77	0.4274	-1.30
Pak4	24.4	1.0	23.9	0.3	4.89	3.90	3.4E-02	6.7E-02	0.50	0.0086	-1.98
Parva	22.8	0.6	23.2	0.2	3.26	3.23	1.0E-01	1.1E-01	0.98	0.8412	-1.02
Parvb	26.2	2.9	27.7	2.5	6.72	7.74	9.5E-03	4.7E-03	2.03	0.3674	2.03

Gene Symbol	ILK10 (n=4)		Wt (n=4)		AVG ΔC_t		$2^{\Delta C_t}$		Ratio	t-test	Fold Change
	AVG Ct	SD	AVG Ct	SD	ILK10 (n=4)	WT (n=4)	ILK10	Wt	ILK10 /WT	p value	ILK10 /Wt
Parvg	25.6	4.8	28.2	2.7	6.08	8.23	1.5E-02	3.3E-03	4.45	0.3634	4.45
Pdpk1	23.5	1.4	23.6	0.3	4.00	3.56	6.2E-02	8.5E-02	0.73	0.4163	-1.36
Pip5k1c	24.5	3.1	27.3	0.6	5.03	7.31	3.1E-02	6.3E-03	4.85	0.1795	4.85
Plec	22.3	0.5	23.4	2.3	2.76	3.40	1.5E-01	9.4E-02	1.56	0.8244	1.56
Prkca	26.3	5.8	22.9	0.8	6.78	2.95	9.1E-03	1.3E-01	0.07	0.1199	-14.20
Prkcb	26.3	0.4	27.2	0.5	6.73	7.20	9.4E-03	6.8E-03	1.39	0.1717	1.39
Prkcc	30.9	0.3	32.2	1.2	11.43	12.24	3.6E-04	2.1E-04	1.76	0.2493	1.76
Pten	22.7	1.3	22.2	0.2	3.22	2.19	1.1E-01	2.2E-01	0.49	0.0026	-2.04
Ptk2	25.3	0.8	25.4	0.1	5.82	5.36	1.8E-02	2.4E-02	0.73	0.0055	-1.37
Pxn	25.0	0.6	24.9	0.4	5.45	4.95	2.3E-02	3.2E-02	0.71	0.0566	-1.42
Rac1	20.0	1.1	20.4	0.4	0.51	0.36	7.0E-01	7.8E-01	0.90	0.6175	-1.11
Rac2	27.3	1.1	29.5	3.8	7.80	9.48	4.5E-03	1.4E-03	3.21	0.9749	3.21
Raf1	23.4	0.9	24.1	0.5	3.86	4.10	6.9E-02	5.8E-02	1.18	0.5330	1.18
Rap1a	22.6	0.6	22.8	0.2	3.07	2.78	1.2E-01	1.5E-01	0.82	0.0389	-1.22
Rap1b	22.5	1.8	22.5	0.3	3.00	2.48	1.3E-01	1.8E-01	0.70	0.8273	-1.43
Rapgef1	25.9	1.0	22.2	4.1	6.41	2.18	1.2E-02	2.2E-01	0.05	0.3506	-18.71
Rasgrf1	28.2	7.6	31.4	3.9	8.67	11.39	2.5E-03	3.7E-04	6.58	0.3582	6.58
Rhoa	20.5	0.8	24.5	7.0	0.97	4.47	5.1E-01	4.5E-02	11.33	0.3893	11.33
Rock1	24.2	0.8	24.7	0.3	4.68	4.66	3.9E-02	3.9E-02	0.99	0.8294	-1.01
Rock2	30.2	0.9	30.8	1.3	10.68	10.82	6.1E-04	5.5E-04	1.10	0.7274	1.10
Shc1	20.6	1.0	20.6	0.4	1.06	0.64	4.8E-01	6.4E-01	0.75	0.1859	-1.34
Sos1	24.3	0.7	24.8	0.2	4.81	4.81	3.6E-02	3.6E-02	1.00	0.9522	-1.00
Sos2	24.7	0.9	25.1	0.4	5.17	5.11	2.8E-02	2.9E-02	0.96	0.7504	-1.04
Src	24.9	0.7	25.4	0.5	5.41	5.43	2.3E-02	2.3E-02	1.01	0.9142	1.01
Tln1	24.7	0.4	25.3	0.1	5.20	5.34	2.7E-02	2.5E-02	1.11	0.4212	1.11
Tns1	26.8	1.0	27.1	1.1	7.25	7.07	6.6E-03	7.4E-03	0.89	0.6427	-1.13
Vasp	24.7	0.6	25.4	0.5	5.15	5.44	2.8E-02	2.3E-02	1.22	0.4532	1.22

Gene Symbol	ILK10 (n=4)		Wt (n=4)		AVG ΔC_t		$2^{\Delta C_t}$		Ratio	t-test	Fold Change
	AVG Ct	SD	AVG Ct	SD	ILK10 (n=4)	WT (n=4)	ILK10	Wt	ILK10 /WT	p value	ILK10 /Wt
Vav1	29.8	0.8	30.9	2.8	10.28	10.95	8.1E-04	5.1E-04	1.59	0.5814	1.59
Vav2	24.3	0.9	24.5	0.1	4.76	4.53	3.7E-02	4.3E-02	0.85	0.2274	-1.17
Vcl	24.6	1.5	23.5	1.5	5.06	3.48	3.0E-02	9.0E-02	0.33	0.1722	-2.99
Zyx	22.9	1.3	24.2	0.4	3.36	4.18	9.7E-02	5.5E-02	1.77	0.1550	1.77
Actb	22.9	1.0	23.3	0.1	3.40	3.27	9.5E-02	1.0E-01	0.92	0.5922	-1.09
B2m	22.7	0.7	23.4	0.1	3.21	3.39	1.1E-01	9.5E-02	1.13	0.1484	1.13
Gusb	17.1	0.7	17.8	0.1	-2.38	-2.20	5.2E+00	4.6E+00	1.13	0.1628	1.13
Hsp90ab1	17.5	0.8	18.0	0.2	-2.06	-2.01	4.2E+00	4.0E+00	1.04	0.7018	1.04