

# Insulin Receptor Substrate 2 Is Essential for Maturation and Survival of Photoreceptor Cells

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Insulin receptor substrates (Irs-proteins) integrate signals from the insulin and insulin-like growth factor-1 (IGF1) receptors with other processes to control cellular growth, function, and survival. Here, we show that *Irs2* promoted the maturation and survival of photoreceptors in the murine retina immediately after birth. *Irs2* was mainly localized to the outer plexiform layer as well as to photoreceptor inner segments. It was also seen in ganglion cells and inner plexiform layer but in smaller amounts. Compared with control littermates, *Irs2* knock-out mice lose ~10% of their photoreceptors 1 week after birth and up to 50% by 2 weeks of age as a result of increased apoptosis. The surviving photoreceptor cells developed short organized segments, which displayed proportionally diminished but otherwise normal electrical function. However, IGF1-stimulated Akt phosphorylation was barely detected, and cleaved/activated caspase-3 was significantly elevated in isolated retinas of *Irs2*<sup>-/-</sup> mice. When diabetes was prevented, which allowed the *Irs2*<sup>-/-</sup> mice to survive for 2 years, most photoreceptor cells were lost by 16 months of age. Because apoptosis is the final common pathway in photoreceptor degeneration, pharmacological strategies that increase *Irs2* expression or function in photoreceptor cells could be a general treatment for blinding diseases such as retinitis pigmentosa.

**Key words:** *Irs2*; IGF1 signaling; retina; photoreceptor degeneration; apoptosis; knock-out mice

## Introduction

Development of the retina, like other regions in the CNS, is regulated by transcription factors, growth factors, and neuropeptides that coordinate proliferation, differentiation, and maturation of neuroepithelial precursor cells (Frade et al., 1999; Levine et al., 2000; Kumar, 2001; Takatsuka et al., 2004). Diabetic retinopathy, the most frequent complication of diabetes and the leading cause of blindness in humans, involves vascular damage accompanied by neural apoptosis in the retina (Bronson et al., 2003). Many reports show that insulin and insulin-like growth factors (IGFs) play an important role in retinal growth and survival (Hernandez-Sanchez et al., 1995; Frade et al., 1996; Alarcon et al., 1998; Barber et al., 2001). Insulin and IGF1 receptors are found in the retina of several species, including *Drosophila*, chick, mice, and humans (Rodrigues et al., 1988; Bassnett and Beebe,

1990; Bronson et al., 2003). IGF1 stimulates the generation of differentiated neurons in neuroepithelial cell cultures from embryonic chick neural retina (Alarcon et al., 1998). Antibodies that inhibit IGF1 signaling reduce production of differentiated retinal neurons in chick embryos (Frade et al., 1996), and antibodies that inhibit insulin signaling promote apoptosis in the neuroretina (Diaz et al., 2000). Thus, a failure of the insulin/IGF signaling cascades in photoreceptor cells themselves together with defects in the vasculature might contribute to retinopathy.

The receptors for insulin and IGF1 are tyrosine kinases that recruit and phosphorylate various proteins to generate physiological responses (White, 2003). The direct association between the insulin receptor and Dock/Nck guides axons from the retina to the developing fly brain (Song et al., 2003). The insulin receptor stimulates tyrosine phosphorylation of Chico, the fly ortholog of the mammalian insulin receptor substrates (IRS1, IRS2, IRS3, and IRS4), which promotes brain and body growth (Oldham et al., 2002). In mice, *Irs1* generally controls body growth and peripheral insulin action, whereas *Irs2* integrates insulin action with body weight control, pancreatic  $\beta$ -cell growth and survival, and female fertility (Tamemoto et al., 1994; Withers et al., 1998, 1999; Burks et al., 2000). Disruption of the insulin receptor in neurons does not impair brain development, and retinal degeneration has not been described in these mutant mice (Bruning et al., 2000; Schubert et al., 2004), although the size of photoreceptors is reduced in insulin receptor mutant *Drosophila* (Brogiolo et al.,

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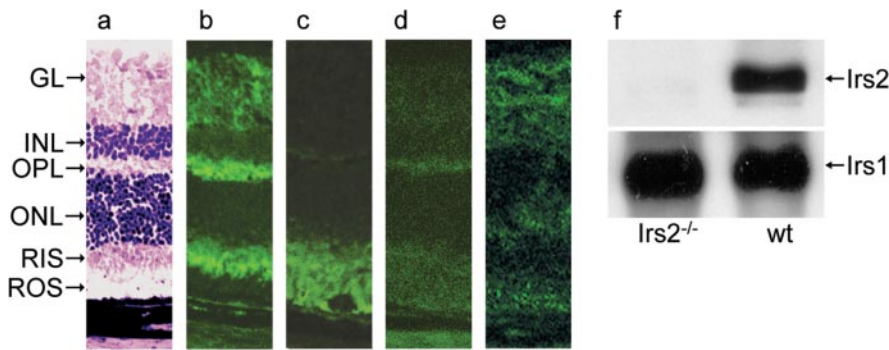
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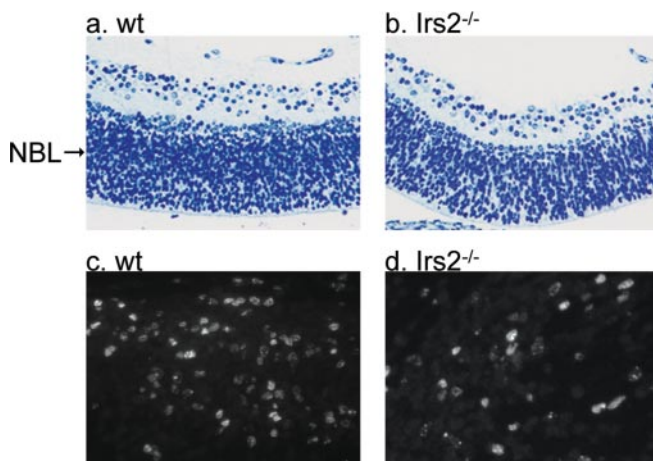
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**Figure 1.** *Irs2* expression in the retina of adult WT mice. *a*, Eyes were collected from 9-week-old mice, and frozen sections were prepared for immunofluorescent staining (original magnification, 25 $\times$ ). *a*, WT retina with H&E staining; *b*, WT retina with antibody against *Irs2*; *c*, WT retina with antibody against rhodopsin; *d*, WT retina without primary antibody against *Irs2*; *e*, *Irs2*<sup>-/-</sup> retina with antibody against *Irs2*. *f*, The retinas were dissected from 9-week-old WT and *Irs2*<sup>-/-</sup> mice, and whole retinal lysates (50  $\mu$ g) were separated by 7.5% SDS-PAGE gels. Western blotting was used to detect *Irs1* and *Irs2*. GL, Ganglion cell layer; RIS, rod inner segments; ROS, rod outer segments.



**Figure 2.** Proliferation of retinal precursors in WT and *Irs2*<sup>-/-</sup> retina. BrdU (100  $\mu$ g/g of body weight) was injected into pregnant mice at E12. Six hours later, the mice were killed, and embryos were collected. The genotype of embryos was identified by PCR. Adjacent sections were stained with 1% toluidine blue (*a*, *b*; original magnification, 40 $\times$ ), or immunofluorescent staining using anti-BrdU antibody was performed (*c*, *d*; original magnification, 80 $\times$ ). BrdU-labeled cells were counted per area using the Improvise OpenLab software (4 embryos per genotype). NBL, Neuroblast layer.

2001); however, *Irs2* signaling mediates IGF1-stimulated brain growth during mouse development (Schubert et al., 2003).

*Irs1* and *Irs2* mediate many physiological functions of insulin and insulin-like growth factors through the direct activation of the PI 3-kinase  $\rightarrow$  AKT cascade, including the inhibition of apoptosis (Alessi and Downes, 1998; Kermer et al., 2000; Barber et al., 2001; Vincent and Feldman, 2002). IGF1-stimulated PI 3-kinase protects axotomized rat retinal ganglion cells from death, whereas constitutive activation disrupts retinal morphogenesis as a result of increased survival of photoreceptors at developmental stages in which cell division has ceased (Kermer et al., 2000; Pimentel et al., 2002). Here, we show that deletion of the *Irs2* gene in mice facilitates photoreceptor cell apoptosis, resulting in a 50% loss of cells by 2 weeks of age and almost a complete loss by 16 months of age.

## Materials and Methods

**Animals.** The generation of *Irs1*<sup>-/-</sup>, *Irs2*<sup>-/-</sup>, *Irs2*<sup>-/-</sup>;*Pdx1*<sup>tg</sup>, and *Irs2*<sup>-/-</sup>;*rat insulin promoter-Irs2*<sup>tg</sup> mice has been described previously (Withers et al., 1998, 1999; Kushner et al., 2002; Hennige et al., 2003). All

lines were maintained on a C57BL/6 background, and wild-type (WT) littermates were used as controls. Genotyping of the animals was performed by PCR as described previously (Withers et al., 1998, 1999; Kushner et al., 2002; Hennige et al., 2003). Mice were maintained on normal 12 h light/dark cycle and handled in accordance with protocols approved by the local Institute Animal Care and Use Committees.

**Histology.** Eyeballs were either fixed in 4% paraformaldehyde for 4 h, processed for paraffin sections, and stained with hematoxylin and eosin (H&E) or overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, processed for Epon sections, and stained with 1% toluidine blue. Both paraffin and Epon sections were used for light microscopic observation. Ultrathin sections were cut from the Epon-embedded samples and stained with uranyl acetate and lead citrate. These stained ultrathin sections were viewed and photographed using a Philips (Eindhoven, The Netherlands) 301

transmission electron microscope. For quantitation, H&E-stained paraffin retinal sections from 1- and 2-week-old WT and *Irs2*<sup>-/-</sup> mice were viewed using a Zeiss (Oberkochen, Germany) Axiovert S100 microscope and video camera. The thickness of different layers of retina was measured using OpenLab software (Improvisation, Lexington, MA). In each individual mouse studied, we measured a total of six retinal cross sections made through the optic nerve head. These data were then averaged, and similar results obtained from three animals of each genotype on each time point were analyzed statistically using Student's *t* test.

**5-Bromo-2-deoxyuridine labeling.** 5-Bromo-2-deoxyuridine (BrdU) (Roche, Indianapolis, IN), 100  $\mu$ g/g of body weight, was injected intraperitoneally into pregnant mice [embryonic day 12 (E12)] and 1- or 2-week old pups. Tissues were harvested after 6 h and fixed in 2.5% glutaraldehyde, dehydrated, and embedded in Epon 502. Immunostaining was performed in these sections using anti-BrdU antibodies (1:1000; Roche). For quantitation, stained sections were viewed using a Zeiss Axiovert S100 microscope and video camera. Counts (of anatomical equal parts of the neuroblast layer of the retina) and analysis were done using OpenLab software (Improvisation). The data were statistically analyzed using Student's *t* test.

**Immunostaining and terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling assay in retinal sections.** Eyeballs were either freshly frozen in OCT for cryostat sections or fixed in 4% paraformaldehyde for 4 h and processed for paraffin sections. Immunohistochemical staining in cryostat sections was used to localize *Irs2* (1:200; Upstate Biotechnology, Charlottesville, VA) and rhodopsin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) in the retina. Terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL) assay was performed in paraffin sections using the ApopTag Peroxidase *In Situ* Apoptosis Detection kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. The number of TUNEL-positive nuclei in the inner nuclear layer (INL) or the outer nuclear layer (ONL) was counted under the microscopy. At 1 and 2 weeks of age, six sections spanning the entire retina were counted, and the data were averaged for each of three animals for each genotype. The data were statistically analyzed using Student's *t* test.

**TUNEL analysis in whole-mount retina.** TUNEL analysis in whole-mount retina was performed according to published methods with minor modifications (Barber et al., 1998). Briefly, the eyeballs from both WT and *Irs2*<sup>-/-</sup> mice at the age of 10 d (four eyes of two animals from each genotype) were enucleated and fixed in 4% paraformaldehyde for 30 min, and then retinas were dissected. Isolated retinas were flat mounted on microscope slides, dried at room temperature for 30 min, and then incubated in a humidified box at 4 $^{\circ}$ C overnight. On the next day, the retinas were dehydrated through graded alcohols and defatted in xylene overnight. The retinas were rehydrated and permeabilized with 0.3% Triton for 20 min and digested with proteinase K (Chemicon) for

30 min in the room temperature. TUNEL assay was performed using the ApopTag Plus Fluorescein *In Situ* Apoptosis Detection kit (Chemicon) according to the manufacturer's instructions.

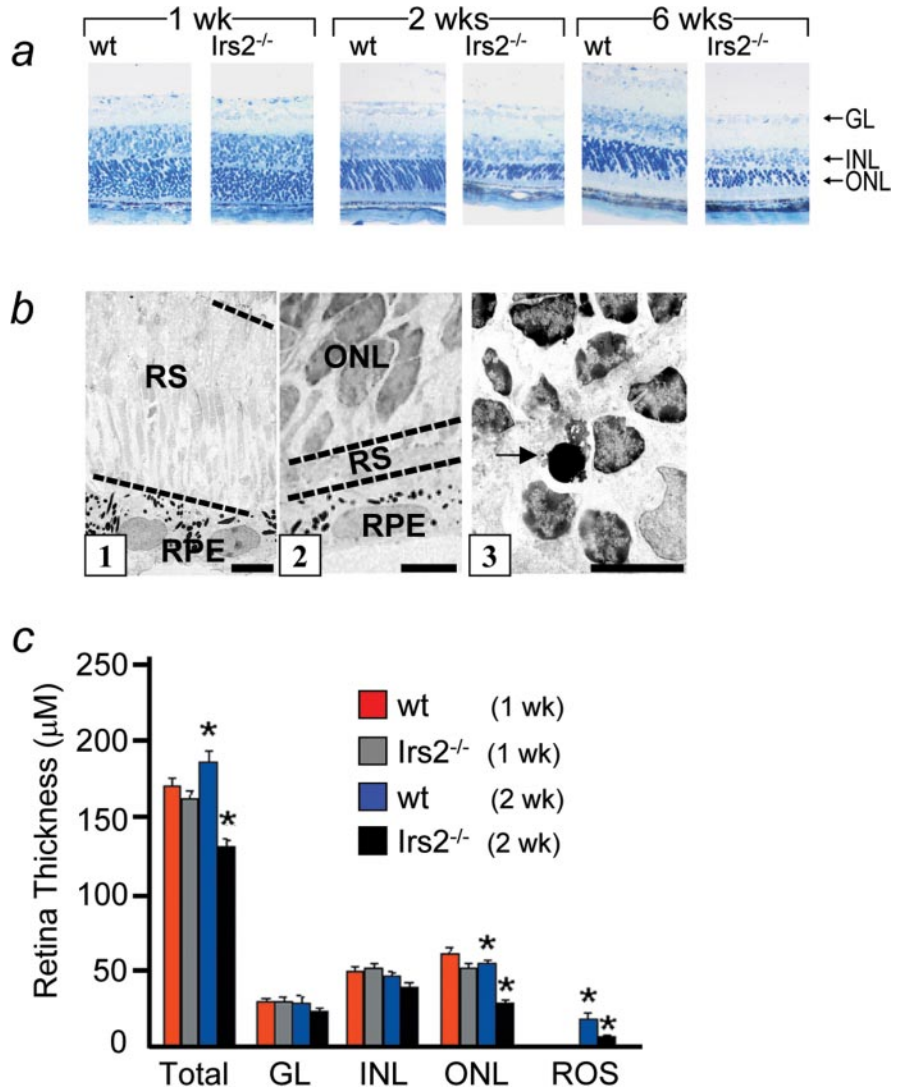
**Immunoblotting.** For immunoblotting, retinas were dissected from 9-week-old *Irs2*<sup>-/-</sup> and WT mice using a dissecting microscope and then treated with human IGF1 (100 nM; R&D Systems, Minneapolis, MN) for 20 min. Retinas were then lysed in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% (v/v) NP-40, 5 mM EDTA, 5% (v/v) glycerol, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na<sub>3</sub>VO<sub>4</sub>. Protein expression was determined from whole lysates (50 μg) dissolved in Laemmli buffer and resolved on 7.5% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes, blocked with 3% dry milk solution, and incubated with the indicated antisera. Primary antibodies against Irs1, Irs2, p85, caspase-3, Akt, phospho-Akt (Ser<sup>473</sup>) (Cell Signaling Technology, Beverly, MA), or IGF1-receptor (Santa Cruz Biotechnology) were detected using ECL chemiluminescence reagents and exposure to x-ray film.

**Electroretinography.** After overnight dark adaptation, 10-week-old mice were anesthetized (ketamine, 80 mg/kg; xylazine, 16 mg/kg), and the pupils were dilated (1% tropicamide, 2.5% phenylephrine HCl, 1% cyclopentolate). The cornea was anesthetized (0.5% proparacaine HCl), and electroretinograms (ERGs) were recorded from the corneal surface using a coiled stainless steel wire; needle electrodes placed in the cheek and tail served as reference and ground leads, respectively. Responses were elicited by strobe flash stimuli, differentially amplified (bandpass, 0.5–1500 Hz), averaged, and stored using an LKC (Gaithersburg, MD) UTAS E-3000 signal averaging system. After a dark-adapted series of responses was recorded, using flash intensities that ranged from -3.6 to 2.1 log cd s/m<sup>2</sup>, cone ERGs were obtained to stimuli ranging from -0.1 to 1.2 log cd s/m<sup>2</sup> superimposed on a steady 1.5 log cd/m<sup>2</sup> adapting field. The results were statistically analyzed using two-way repeated measures ANOVAs.

## Results

### The role of *Irs2* in maturation and survival of photoreceptor cells

Immunofluorescent staining was used to locate the *Irs2* protein in the retina of normal adult mice (Fig. 1). The different layers of the retina were shown in the section stained with H&E (Fig. 1*a*). Immunofluorescent staining with an antibody against *Irs2* revealed that the *Irs2* protein was primarily restricted to the outer plexiform layer (OPL) and photoreceptor inner segments but was also seen in ganglion cells (GCL) and inner plexiform layer in less intensity (Fig. 1*b*). *Irs2* was not detected in photoreceptor outer segments and the retinal pigment epithelium (Fig. 1*b*). The specificity of *Irs2* immunostaining was confirmed by omitting the specific antibody entirely or by immunostaining *Irs2*<sup>-/-</sup> retinal sections (Fig. 1*d,e*). By comparison, rhodopsin was localized in both inner and outer segments of photoreceptors (Fig. 1*c*). To confirm the expression of *Irs2*, retina from WT or *Irs2*<sup>-/-</sup> mice



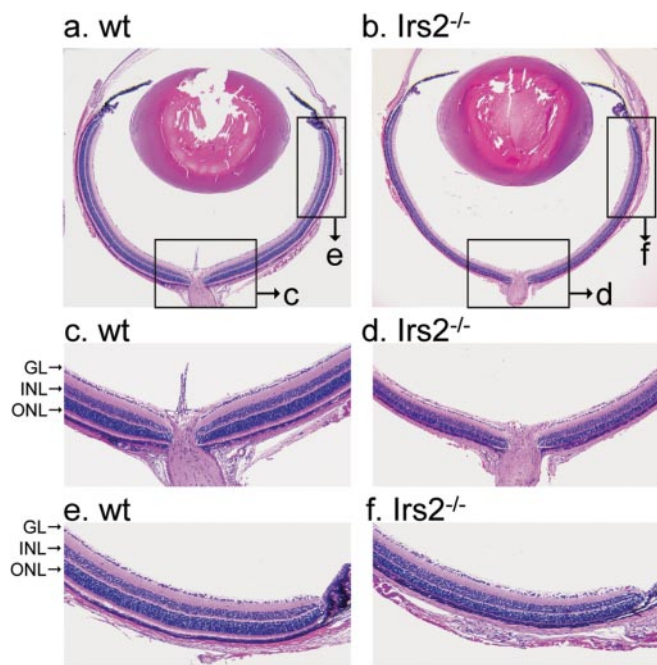
**Figure 3.** Morphological analysis of *Irs2*<sup>-/-</sup> retina. Eyes of *Irs2*<sup>-/-</sup> and WT mice at 1, 2, and 6 weeks of age were fixed in 2.5% glutaraldehyde overnight and embedded in Epon 502. Sections of 1 μm thickness from central retina were stained with 1% toluidine blue and used for light microscopic observation (*a*; original magnification, 20×). Ultrathin sections from WT and *Irs2*<sup>-/-</sup> mice retina were stained with uranyl acetate and lead citrate for electron microscopic observation (*b*). Segments of photoreceptors in WT (*b1*) and *Irs2*<sup>-/-</sup> (*b2*) and apoptotic photoreceptor in *Irs2*<sup>-/-</sup> (*b3*, arrow) at 2 weeks of age. Scale bars, 5 μm. *c*, Quantitative study on the thickness of total and different layers of *Irs2*<sup>-/-</sup> mice and WT at 1 and 2 weeks of age. Six sections through optic nerve head were measured and averaged from each eye (6 eyes from 3 mice per genotype at each time point). GL, Ganglion cell layer; RS, rod segments; RPE, retinal pigment epithelium. \**p* < 0.01. Error bars represent SD.

were homogenized, and the proteins were resolved by SDS-PAGE. Western blotting revealed both *Irs1* and *Irs2* proteins in the neuroretinae of WT mice, whereas *Irs2* was absent from the neuroretinae of *Irs2*<sup>-/-</sup> mice (Fig. 1*f*).

To determine the effect of *Irs2* on the proliferation of retinal precursors, BrdU incorporation was determined at E12 and at 1 and 2 weeks of age. Consistent with reduced brain growth of *Irs2*<sup>-/-</sup> mice (Schubert et al., 2003), the number of BrdU-labeled retinal precursors in *Irs2*<sup>-/-</sup> mice at E12 was decreased by ~40% (WT, 77.6 ± 5.8; *Irs2*<sup>-/-</sup>, 45.4 ± 6.2; *p* < 0.01) (Fig. 2). However, BrdU incorporation was nearly identical in WT and *Irs2*<sup>-/-</sup> retina at 1 or 2 weeks of age (data not shown), suggesting that *Irs2* signaling was not involved in the cell proliferation of postnatal retina.

To investigate the role of *Irs2* in postnatal development of the retina, we examined retinal histology 3 d, 1 week, 2 weeks, and 6





**Figure 4.** Morphological analysis of *Irs2*<sup>-/-</sup> retina. Eyes of *Irs2*<sup>-/-</sup> and WT mice at 2 weeks of age were fixed in 4% paraformaldehyde for 4 h, and paraffin sections were stained with H&E and used for light microscopic observation (original magnification: *a, b*, 4×; *c–f*, 20×). GL, Ganglion cell layer.

weeks of age (Fig. 3). These ages were selected to minimize the detrimental effects of hyperglycemia and hyperinsulinemia that develops after 6 weeks of age in *Irs2*<sup>-/-</sup> mice (Withers et al., 1998, 1999). At 3 d of age, there were no significant differences in the morphology of retina between *Irs2*<sup>-/-</sup> and WT controls (data not shown). Four days later, the *Irs2*<sup>-/-</sup> retina contained all cellular and plexiform layers, but the ONL was reduced ~10%, and the outer segments were shorter than normal (Fig. 3*a,c*). By 2 weeks of age, the *Irs2*<sup>-/-</sup> retina was 50% thinner than WT as a result of a loss of cells in the ONL (Fig. 3*a,c*). Thinning of the ONL was uniform throughout the retina (Fig. 4). In contrast, the thickness of the INL or GCL were not significantly decreased in the *Irs2*<sup>-/-</sup> mice (Fig. 3*a,c*). In addition to ONL thinning, both inner and outer segments of *Irs2*<sup>-/-</sup> mice were shorter than those of WT littermate controls (Fig. 3*b*). The thickness of the ONL in *Irs2*<sup>-/-</sup> mice changed relatively little from 2 to 6 weeks of age (Fig. 3*a*).

#### Maintenance of photoreceptors during aging

*Irs2*<sup>-/-</sup> mice normally die from extreme hyperglycemia at ~15 weeks of age as a result of  $\beta$ -cell death and inadequate compensatory insulin secretion (Withers et al., 1998). To examine the retina in older *Irs2*<sup>-/-</sup> mice, we restored pancreatic  $\beta$ -cell function in *Irs2*<sup>-/-</sup> mice by transgenic expression in  $\beta$ -cells of the nuclear transcription factor Pdx1 (*Pdx1*<sup>tg</sup> mice) or *Irs2* (*Irs2*<sup>tg</sup> mice) (Kushner et al., 2002; Hennige et al., 2003). Previous results show that the compound *Irs2*<sup>-/-</sup>::*Pdx1*<sup>tg</sup> or *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> mice were insulin resistant as a result of systemic disruption of *Irs2*; however, these mice do not develop diabetes as a result of compensatory pancreatic  $\beta$ -cell function (Kushner et al., 2002; Hennige et al., 2003).

At 1, 2, and 6 weeks of the age, *Irs2*<sup>-/-</sup>::*Pdx1*<sup>tg</sup> mice displayed similar reductions in retinal thickness as noted in *Irs2*<sup>-/-</sup> mice (data not shown). At older ages (Fig. 5), *Irs2*<sup>-/-</sup>::*Pdx1*<sup>tg</sup> mice displayed a progressive loss of photoreceptors (Fig. 5*b,c,f*), which

was not observed in WT mice (Fig. 5*a,d*) or *Pdx1*<sup>tg</sup> mice (Fig. 5*e*). Seven *Irs2*<sup>-/-</sup>::*Pdx1*<sup>tg</sup> mice were studied at 16 months of age. The ONL was reduced to one row of nuclei in three mice or two rows of nuclei in two mice; the ONL was completely absent in two mice. At these later ages, we also noted moderate cell loss in the inner retinal layers. Because these changes were of late onset, these are likely to reflect a secondary response to photoreceptor degeneration (Marc et al., 2003). However, because *Irs2* was also expressed in the inner retina, the changes of the inner retina may reflect a primary role of *Irs2* in these neurons. Resolution of this issue will require the use of conditional knock-out mice. Parallel experiments using *Irs1*<sup>-/-</sup> mice, which develop glucose intolerance but not diabetes, displayed no changes in the retina (Fig. 5*h*). These results indicate that the *Irs2* branch of the insulin/IGF signaling cascade was specifically required for survival of retinal neurons, particularly photoreceptor cells immediately after birth and for their long-term maintenance as mice age.

#### Photoreceptor cell apoptosis in the absence of *Irs2*

Apoptosis occurs during normal retinal development (Pequignot et al., 2003). To determine whether apoptosis was dysregulated in the retina of *Irs2*<sup>-/-</sup> mice, TUNEL was performed (Fig. 6). TUNEL staining in whole-mount retinas demonstrated that apoptosis occurred uniformly throughout the retina (Fig. 6*a,b*). Apoptotic cells in the different layers of the retina were localized by TUNEL staining in retinal cross sections. TUNEL-positive cells were observed equally in the GCL and in the INL of WT and *Irs2*<sup>-/-</sup> mice at 1 week of age but were barely detected in these layers by 2 weeks (Fig. 6*c,e*). However, the number of TUNEL-positive cells was significantly increased in the ONL of *Irs2*<sup>-/-</sup> mice, particularly between 1 and 2 weeks of age, but also at later time points (Fig. 6*d,f,g*). Electron microscopic analysis revealed the hallmarks of apoptosis including nuclear condensation in the dying photoreceptor cells (Fig. 3*b*).

#### *Irs2* signaling in the retina

Insulin/IGF1 activates the PI3-kinase  $\rightarrow$  Akt cascade, which promotes cell survival by inhibiting apoptosis in a wide range of cells and tissues (Lawlor and Alessi, 2001; Vincent and Feldman, 2002). To establish a role for *Irs2* in the regulation of Akt in the retina, isolated retina were stimulated with IGF1, and Akt phosphorylation was measured by phospho-specific immunoblotting (Fig. 7). IGF1 strongly stimulated Akt phosphorylation in WT retina, whereas basal and IGF1-stimulated phosphorylation of Akt was not detected in *Irs2*<sup>-/-</sup> retina (Fig. 7*a*). The absence of Akt phosphorylation was attributed to defective signaling, because total Akt was expressed equally in WT and *Irs2*<sup>-/-</sup> mice (Fig. 7*a*). Moreover, IGF1 receptor, *Irs1*, and p85 were expressed normally in the *Irs2*<sup>-/-</sup> retina, suggesting that *Irs2* was required to activate the PI3-kinase  $\rightarrow$  Akt cascade in the mouse retina (Fig. 7*b*).

Caspase-3 exists as an inactive proenzyme that undergoes proteolysis to produce two subunits that dimerize to form the active enzyme, which plays a central role in the execution phase of cell apoptosis. Caspase-3 is the predominant caspase involved in the cleavage of amyloid- $\beta$  4A precursor protein, which is associated with neuronal death in Alzheimer's disease, and caspase-3 promotes apoptosis of retinal neurons (Kermer et al., 2000; Barber et al., 2001). Because the PI 3-kinase  $\rightarrow$  Akt cascade strongly inhibits caspase-3 cleavage, we examined the status of caspase-3 in the *Irs2*<sup>-/-</sup> retina. The cleaved form of caspase-3 was strongly detected by immunoblotting in the extracts of *Irs2*<sup>-/-</sup> retina, whereas it was not detected in retinal extracts from WT mice (Fig. 7*c*). These results suggest that the *Irs2* signaling promoted pho-

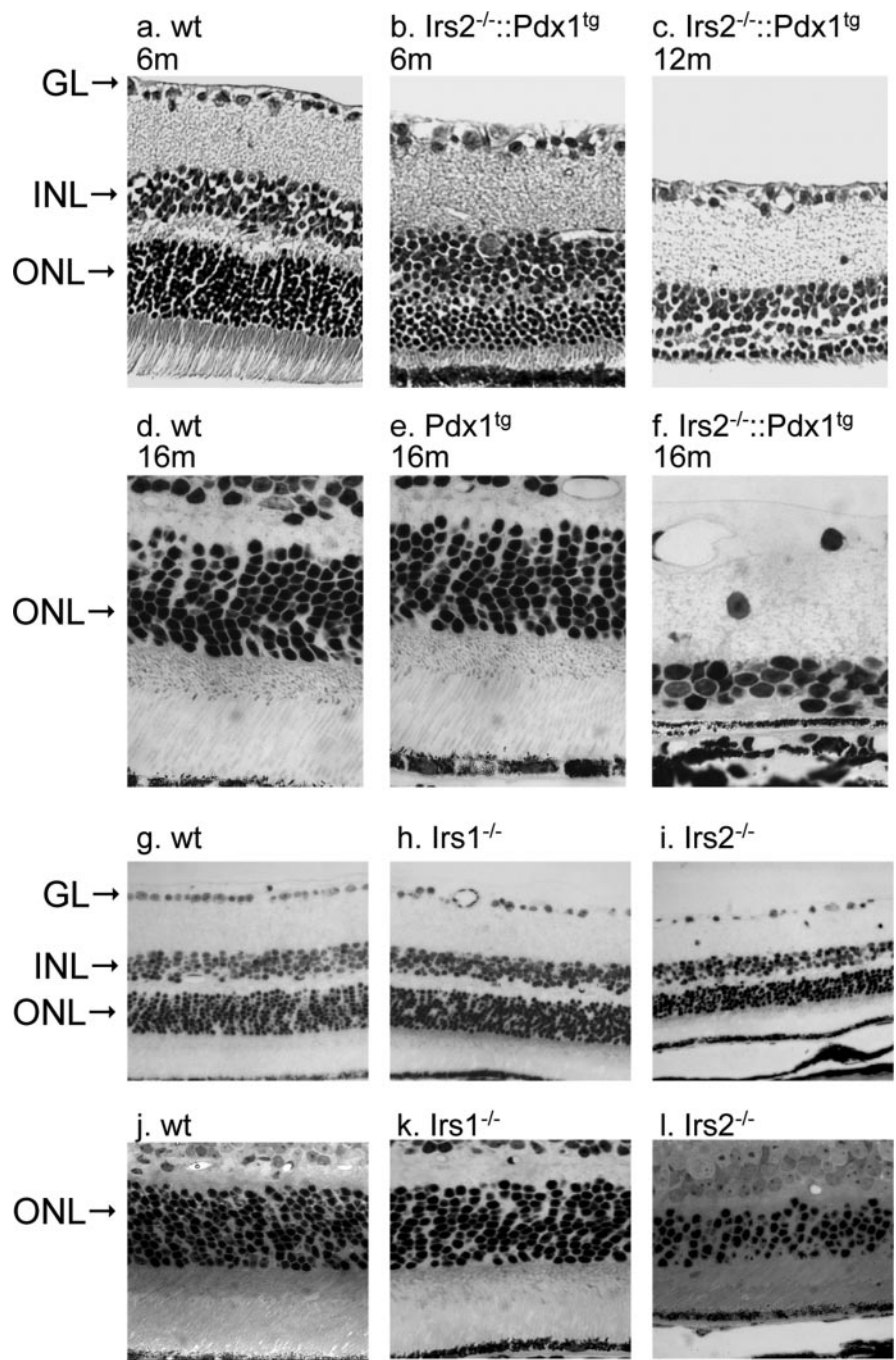
photoreceptor cell survival, at least in part, by inhibiting the caspase activation cascade.

### Retinal function in *Irs2*<sup>-/-</sup> mice

Rhodopsin is a transmembrane protein that initiates the visual transduction cascade. Mutations in this gene are known to cause photoreceptor degeneration in retinitis pigmentosa (Dryja et al., 1991). We immunostained retinal sections from 1-, 2-, and 6-week-old WT and *Irs2*<sup>-/-</sup> mice with antibody against rhodopsin. Rhodopsin staining was reduced in the *Irs2*<sup>-/-</sup> retina, consistent with the decreased number of photoreceptor cells; however, there was no difference between the location of rhodopsin in WT and *Irs2*<sup>-/-</sup> retina (Fig. 8). These results show that *Irs2* signaling is not involved in the synthesis or translocation of rhodopsin, which is important for normal function of photoreceptors.

To directly evaluate photoreceptor function in *Irs2*<sup>-/-</sup> mice, ERGs were recorded from *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> mice and WT littermates using stimulus conditions that isolate rod- and cone-driven activity (Peachey and Ball, 2003) (Fig. 9). The *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> mice were used for these experiments to avoid the physiological complication of hyperglycemia, although these mice develop progressive hyperinsulinemia (Hennige et al., 2003). Although the overall waveforms were similar, ERG amplitudes were reduced in *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> mice (Fig. 9*a*). Dark-adapted ERGs, reflecting activity of the rod pathway, were significantly reduced in *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> mice (Fig. 9*a*, top two rows of traces). Light-adapted responses, reflecting cone-driven retinal activity, were also reduced (Fig. 9*a*, bottom traces). The amplitude of the a-wave was significantly reduced in *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> mice at all flash intensities ( $F_{(1,4)} = 44.9$ ;  $p < 0.01$ ), which was approximately proportional to the loss of photoreceptors in *Irs2*<sup>-/-</sup> mice.

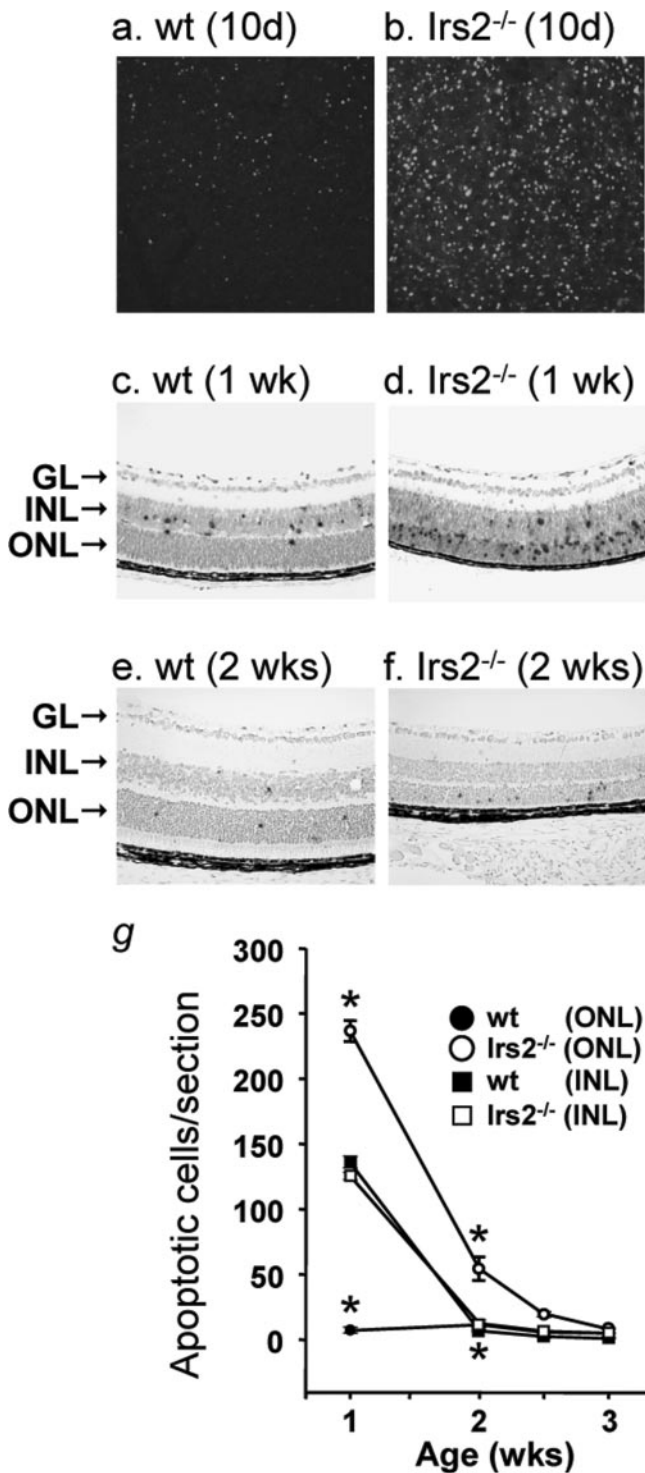
To determine whether *Irs2* signaling affects the kinetics of phototransduction, the leading edges of the a-waves evoked by a high-intensity flash of WT and *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> mice were compared after normalization (Hood and Birch, 1997). When normalized, there was substantial overlap between the leading edges of the a-waves of WT and *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> mice (Fig. 9*b* inset), indicating that the remaining outer segments retain normal phototransduction gain (Hood and Birch, 1997). Rod-driven b-waves of *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> mice were also significantly reduced in amplitude (Fig. 9*c*) ( $F_{(1,4)} = 10.4$ ;  $p < 0.05$ ) and delayed in implicit time (Fig. 9*d*) ( $F_{(1,4)} = 10.1$ ;  $p < 0.05$ ). Although the amplitude reduction is likely to reflect a decreased input from photoreceptors, this factor alone does not account for the implicit time delays (Hood and Birch, 1992) and may reflect an additional defect in *Irs2*<sup>-/-</sup> mice. The position of the a- and b-wave response functions along the intensity



**Figure 5.** Morphological changes of retina in adult *Irs2*<sup>-/-</sup> and *Irs1*<sup>-/-</sup> mice. Eyes were collected from older *Irs2*<sup>-/-</sup>::*Pdx1*<sup>tg</sup> at 6, 12, and 16 months of age, and cross sections were prepared for light microscopic observation (3 mice at 6 and 12 months, respectively; 7 mice at 16 months; *a–f*). *g–l*, Retinal cross sections obtained from 9-week-old WT, *Irs1*<sup>-/-</sup>, and *Irs2*<sup>-/-</sup> mice (original magnification is 20 $\times$  for *a, b, c, g, h, i* and 40 $\times$  for *d, e, f, j, k, l*). GL, Ganglion cell layer.

axis were not shifted from control, indicating that cells that remain in the *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> retina retain near normal light sensitivity. Cone ERGs, isolated by a steady adapting field, were also significantly reduced in *Irs2*<sup>-/-</sup>::*Irs2*<sup>tg</sup> mice (Fig. 9*e*) ( $F_{(1,4)} = 30.9$ ;  $p < 0.01$ ); however, the implicit time was not changed (Fig. 9*f*) ( $F_{(1,4)} = 1.3$ ; NS). Across all stimulus conditions, cone ERGs were reduced on average to  $64.9 \pm 11.3$  (SD) percent of control responses, whereas rod responses were reduced to  $34.9 \pm 10.7$  (SD) percent of the control values. This result suggests that *Irs2* expression is of greater importance to rod than cone photoreceptors.

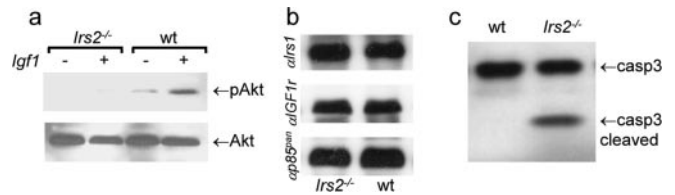




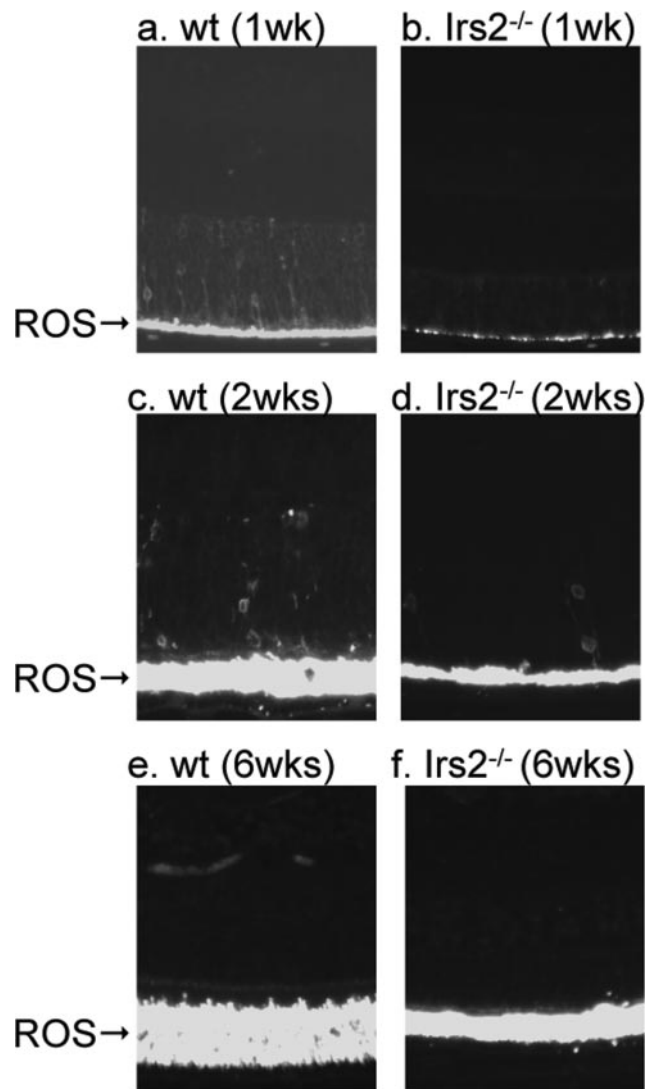
**Figure 6.** Apoptosis in the retina of *Irs2*<sup>-/-</sup> mice. TUNEL staining in whole-mount retina from 10-d-old WT and *Irs2*<sup>-/-</sup> mice using fluorescent method (*a, b*) (original magnification, 20×); TUNEL assay in paraffin retinal cross sections of 1- and 2-week-old WT and *Irs2*<sup>-/-</sup> mice using peroxidase method (*c–f*) (original magnification, 20×). Positive nuclei were counted and plotted as a function of age. Six retinal cross sections were measured and averaged from each mouse; data points indicate the average ± SD of three mice (*g*). GL, Ganglion cell layer. \**p* < 0.01.

**Discussion**

Our results reveal an important but unexpected requirement for neonatal photoreceptor cell survival on the *Irs2* branch of the insulin/IGF signaling cascade. During the first 3 weeks after birth,

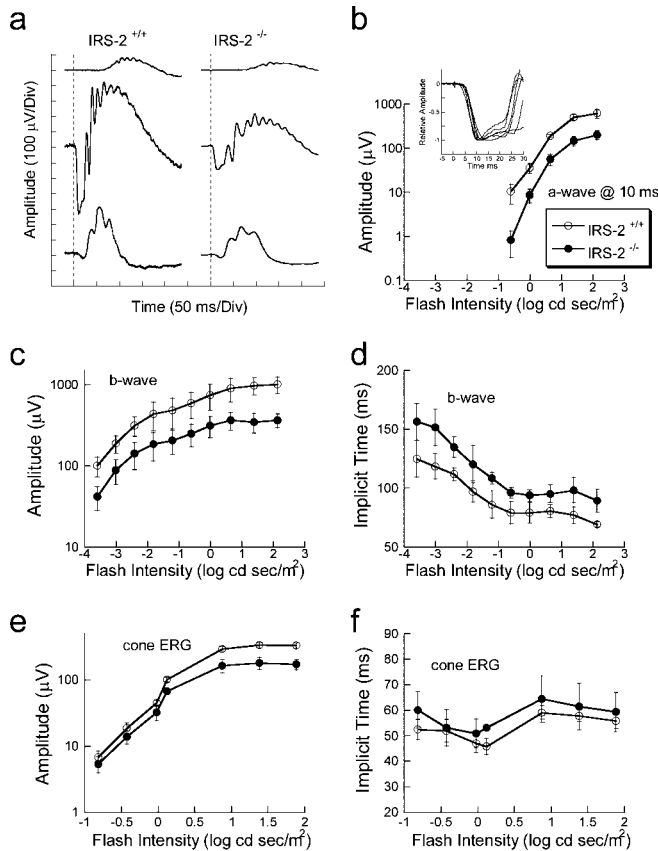


**Figure 7.** Akt phosphorylation and status of caspase-3 in *Irs2*<sup>-/-</sup> retina. The retinas were dissected from 9-week-old WT and *Irs2*<sup>-/-</sup> mice. The whole retinal lysates (50 μg) were separated by 7.5% SDS-PAGE gels. Western blot was used to determine *Irs1*, IGF1r, p85, and caspase-3 (*casp3*) (*b, c*). For analysis of phosphorylation of Akt, the dissected retinas were treated with or without human IGF1 *in vitro* (100 nM for 20 min), and Western blot was used to detect Akt phosphorylation using antibody against phospho-Akt (pAkt; Ser<sup>473</sup>) (*a*). Four retinas were pooled from two mice of each genotyping for each experiment, and two independent experiments were performed.



**Figure 8.** Immunofluorescent staining of rhodopsin. Eyeballs from 1, 2, and 6-week-old WT and *Irs2*<sup>-/-</sup> mice were fixed in 4% paraformaldehyde. Five-micrometer paraffin sections were made for immunofluorescent staining using anti-rhodopsin antibody. ROS, Rod outer segments (original magnification, 20×).

a very low level of apoptosis of photoreceptor cells is seen in the retina of WT mice. In contrast, one-half of the photoreceptor cells in the *Irs2*<sup>-/-</sup> mice undergo apoptosis during this period. After this apoptotic phase, the remaining *Irs2*<sup>-/-</sup> photoreceptor



**Figure 9.** Electrorinography of *Irs2*<sup>-/-</sup> mice. *a*, Representative ERG signals recorded from the corneal surface of *Irs2*<sup>+/+</sup> (left) or *Irs2*<sup>-/-</sup> (right) mice to strobe stimuli presented to the dark-adapted eye (top and middle waveforms) or superimposed on a steady rod-desensitizing adapting field (bottom waveforms). *b*, Amplitude of the dark-adapted a-wave measured at 10 ms after flash onset. Inset, Comparison of the leading edge of the dark-adapted ERG a-wave in three *Irs2*<sup>+/+</sup> and three *Irs2*<sup>-/-</sup> mice. *c*, Amplitude of the dark-adapted b-wave. *d*, Implicit time of the dark-adapted b-wave. *e*, Amplitude of the cone ERG. *f*, Implicit time of the cone ERG. Data points indicate the average  $\pm$  SD of three mice.

cells survive despite the development of extreme hyperglycemia and life-threatening diabetes caused by the loss of functional pancreatic  $\beta$ -cells (Withers et al., 1998). When diabetes is prevented in *Irs2*<sup>-/-</sup> mice by restoring compensatory  $\beta$ -cell function, which includes relative hyperinsulinemia, photoreceptor cell apoptosis progresses slowly until most of the cells are lost by 16 months of age. Because phototransduction measured by ERG is relatively normal in *Irs2*<sup>-/-</sup> mice, we conclude that *Irs2* signaling contributes to normal vision by promoting survival of photoreceptor cells in the retina.

The important role of *Irs2* in the neonatal retina might be related, at least in part, to its relatively high expression in the OPL and photoreceptor inner segments. *Irs2* is required in these cells for IGF1-stimulated activation of the PI 3-kinase  $\rightarrow$  Akt cascade. Akt signaling strongly inhibits caspase cleavage cascades (Kermer et al., 2000; Barber et al., 2001; Vincent and Feldman, 2002). Activated caspase-3 correlates with photoreceptor degeneration in various rodent models (Liu et al., 1999; Yoshizawa et al., 2002). Activated caspase-3 accumulates in the *Irs2*<sup>-/-</sup> retina, which may explain the observed increase in photoreceptor cell apoptosis.

*Irs1* is widely distributed throughout the retina, including the GCL, INL, and photoreceptor outer segments (Gosbell et al., 2000). Although both *Irs1* and *Irs2* exist in the retina, the morphology of the retina in *Irs1*<sup>-/-</sup> mice is normal up to two years of

age. These results suggest that *Irs1* does not contribute to photoreceptor survival and are consistent with previous results showing that *Irs2*, but not *Irs1*, associates with the insulin receptor and PI 3-kinase activation in bovine photoreceptor outer segments (Rajala and Anderson, 2001; Reiter et al., 2003). The inability of *Irs1* to activate the Akt and inhibit caspase-3 cleavage in the retina remains to be resolved.

Although photoreceptor apoptosis occurs in the diabetic retina (Barber et al., 1998; Park et al., 2003), it is unlikely that photoreceptor loss in *Irs2*<sup>-/-</sup> mice is caused by diabetes. Normalizing glucose homeostasis in *Irs2*<sup>-/-</sup> mice by transgenic expression of Pdx1 or *Irs2* in the pancreatic  $\beta$ -cells did not restore normal retinal growth or survival. Moreover, there was no obvious photoreceptor loss in mice with a conditional knock-out of *Irs2* in pancreatic  $\beta$ -cells that develop diabetes (data not shown) (Lin et al., 2004). Together, it appears that insulin/Igf1 signaling via *Irs2* rather than negative effects of hyperglycemia is critical for photoreceptor survival.

Before birth, the proliferation of photoreceptor cell precursors is reduced in proportion to the retarded brain growth of the *Irs2*<sup>-/-</sup> mice (Schubert et al., 2003). Although the brain of the *Irs2*<sup>-/-</sup> mice is 50% smaller than normal at birth, and remains small throughout life, the neonatal retina displays a nearly normal number of photoreceptor cells at birth. Furthermore, differentiation of retinal precursors is not affected by the deletion of *Irs2*, because all layers of the retina in *Irs2*<sup>-/-</sup> mice form in a normal manner, except that photoreceptor outer segments never achieved a normal length. Thus, *Irs2* signaling is required for early retinal precursor proliferation and photoreceptor survival after birth.

The biphasic pattern of photoreceptor degeneration that occurs in the *Irs2*<sup>-/-</sup> mice is not ordinarily seen in other mouse models of retinal degeneration. In other mouse models of early onset and severe photoreceptor degeneration, virtually all the photoreceptors cells are lost by 1 month of age (Keeler, 1924; Hagstrom et al., 1999; Ramamurthy et al., 2004). The sensitivity of *Irs2*<sup>-/-</sup> photoreceptor cells to apoptosis during the postnatal period might reflect the high metabolic rate of these cells as they elaborate outer segments and become electrically active (Blanks et al., 1974; Ames et al., 1992; Bakall et al., 2003). Whether a similar requirement for *Irs2* exists in human retina will be important to establish.

Rare autosomal recessive diseases reveal an association between retinal degeneration and peripheral metabolic disorders, including type 2 diabetes. Patients with Alstrom syndrome develop neurosensory hearing loss and progressive retinal degeneration accompanied by insulin resistance that progresses to type 2 diabetes (Benso et al., 2002; Iannello et al., 2004). Bardet-Biedl syndrome is characterized by many systemic disorders, including retinal dystrophy or pigmentary retinopathy, which is accompanied by abdominal obesity and type 2 diabetes (Iannello et al., 2002). Mutations in the *Tubby* gene family reveal a relationship between retinal function and insulin action (Ikeda et al., 2002); mutations in the mouse *Tubby* gene causes obesity, deafness, and retinal photoreceptor degeneration (Heckenlively et al., 1995). In addition, mutations in tubby-like protein 1 (TULP1), a member of the human *Tubby* gene family, are associated with autosomal recessive retinitis pigmentosa (Banerjee et al., 1998; Hagstrom et al., 1998). Moreover, disruption of the *Tulp1* gene in mice causes early onset and severe photoreceptor degeneration (Hagstrom et al., 1999). Future experiments focusing on the role of *Irs2* signaling in these disorders might reveal new connections between these signaling cascades.

The development of specific treatments for every cause of retinal degeneration is complex, because mutations in over 100 genes are associated with degeneration of the human retina (Dryja, 1997; Pacione et al., 2003). Strategies to prevent retinal degeneration are under evaluation in animal models and include transplantation of retinal pigment epithelium or stem cells, gene therapy, and growth factor treatment (Milam, 1993; Ali et al., 2000; Bok et al., 2002; Kicic et al., 2003). Apoptosis appears to be the final common pathway of photoreceptor degeneration regardless of the genetic defect (Chang et al., 1993; Portera-Cailliau et al., 1994; Ren et al., 2001). Thus, strategies to inhibit photoreceptor cell apoptosis can preserve visual function, although the underlying gene defects are not corrected (Chen et al., 1996; Davidson and Steller, 1998; Nir et al., 2000). Because *Irs2* expression is strongly induced by phosphorylated cAMP response element-binding protein in  $\beta$ -cells and neurons, activation of this transcription factor might provide a general approach to prevent or retard photoreceptor cell degeneration (Jhala et al., 2003; Brubaker and Drucker, 2004).

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