The GIPC1-Akt1 pathway is required for the specification of the eye field in mouse embryonic stem cells

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Abstract

During early patterning of the neural plate, a single region of the embryonic forebrain, the eye field, becomes competent for eye development. The hallmark of eye field specification is the expression of the eye field transcription factors (EFTFs). Experiments in fish, amphibians, birds and mammals have demonstrated largely conserved roles for the EFTFs. Although some of the key signaling events that direct the synchronized expression of these factors to the eye field have been elucidated in fish and frogs, it has been more difficult to study these mechanisms in mammalian embryos. In this study, we have used two different methods for directed differentiation of mouse embryonic stem cells (mESCs) to generate eye field cells and retina *in vitro* to test for a role of the PDZ domain-containing protein GIPC1 in the specification of the mammalian eye primordia. We find that the overexpression of a dominant-negative form of GIPC1 (dnGIPC1), as well as the downregulation of endogenous GIPC1, is sufficient to inhibit the development of eye field cells from mESCs. GIPC1 interacts directly with IGFR and participates in Akt1 activation, and pharmacological inhibition of Akt1 phosphorylation mimics the dnGIPC1 phenotype. Our data, together with previous studies in *Xenopus*, support the hypothesis that the GIPC1-PI3K-Akt1 pathway plays a key role in eye field specification in vertebrates.

INTRODUCTION

The eye development begins at gastrula stages during the regionalization of the anterior neural plate (ANP). The current model proposes that master-regulatory genes expressed in these committed progenitors are sufficient to initiate fate-determining developmental

programs. At a molecular level, *Otx2* is required for the formation of the whole anterior neural region including the eyes [1], and the eye field is subsequently specified by the expression of a network of transcription factors that include *Rax* (retina and anterior neural fold homeobox), *Pax6* (paired box gene 6), *Lhx2* (LIM homeobox-2) and *Six3* (Sine oculis homeobox 3) [2–4].

The regulatory mechanisms that define the domain of EFTF expression are not well understood. Most of what we know about this has been discovered in organisms with accessible embryos like zebrafish and *Xenopus*. These studies have revealed a network of signals upstream of the EFTFs that involve inhibition of BMP and Wnt signaling [5–8]. However, it is not known how much of this model is conserved in mammals. Signaling pathways are difficult to manipulate in mammals at early embryonic stages without affecting other functions of the same molecules. Therefore, *in vitro* model systems that recapitulate key aspects of embryogenesis might provide an approach to understand ANP patterning and retinal specification in mammals.

Recently, embryonic stem cells (ESCs) have emerged as an alternative method to study the earliest steps of mammalian ontogeny. ESCs are pluripotent cells derived from the inner cell mass of pre-implantation blastocysts. These cells behave similarly to those present in the developing embryo and can be differentiated under defined conditions into a broad range of cell types. The differentiation paradigms towards eye field progenitors and mature retinal cells from mouse ESCs (mESCs), human ESCs (hESCs) and induced-pluripotent SCs (iPSCs) are well established [9–16]. Upon differentiation, the cells acquire characteristics of retinal differentiation, progressing through a succession of stages that recapitulates normal *in vivo* development. Hence, ESCs provide a potential model for testing hypotheses concerning forebrain patterning and eye field specification *in vitro*.

Here, we directed mESCs towards eye field and retinal fates, to test whether a specific component of the signaling network in frog embryos, Kermit2, is important in mammalian eye development. In a previous report, Kermit2, the *Xenopus* homolog of GAIP-interacting protein, C terminus (GIPC) was shown to be required for eye formation [17]; morpholino knockdown of this gene led to embryos lacking eyes, but were otherwise apparently normal. GIPC1 is a small adaptor protein that interacts with multiple cytoplasmic proteins and transmembrane receptors and likely plays a role in endosome signaling and membrane recycling [18–21]. In the present study, we use mESC cultures to analyze the function of GIPC in the specification and differentiation of eye field and retinal fates. Our results indicate that GIPC plays a key role in the specification of the eye field, and likely acts through the regulation of PI3K-Akt1 pathway downstream of IGFR.

RESULTS

GIPC1 is expressed in the developing murine retina and upregulated upon retinal differentiation

To determine whether GIPC proteins are required for mouse eye development as has been reported for *XGIPC/Kermit2* in *Xenopus* [17], we analyzed the developmental expression of the three members of this family: *Gipc1, Gipc2* and *Gipc3*. The closest mouse homolog to

Xenopus Kermit2 is the mouse *Gipc1*, with 69.9% total amino-acid identity, but *Gipc2* and *Gipc3* had nearly the same degree of sequence conservation (Gipc2, 69.4% amino-acid identity and *Gipc3*, 67.8% amino-acid identity) (Supplementary Figure 1). All the *Gipc* family members, analogous to *Kermit2*, contain a PDZ domain with a highly conserved carboxylate-binding pocket. We analyzed the expression pattern of *Gipc1,2,3* gene family members in mouse embryos, using *in situ* hybridization (Figure 1). *Gipc1* expression was detected at E8 (Theiler stage 12, 2–7 somite pairs), and persisted until later stages. By contrast, *Gipc2* was not detected at early embryonic stages and we only detected low levels of *Gipc3*. At E8.75 (Theiler stage 14, 8–12 somite pairs), *Gipc1* was widely expressed in the developing embryo. The highest expression levels were detected in the optic vesicles as well as in the telencephalon, the otic vesicle and the branchial arches. Some other regions like the heart and the midbrain also showed moderate levels of *Gipc1* expression (Figure 1).

To further analyze the expression of *Gipc* genes during eye field specification in mouse, we used a previously described protocol for inducing ocular tissues from ESCs [11]. In this protocol, a combination of soluble factors, IGF1, Dkk1 and Noggin1, induces synchronized expression of EFTFs and the undifferentiated mESC colonies undergo a stepwise differentiation process that reproduces the normal retinal developmental timeline (Figure 2A). The mESC initially develop neuroepithelial characteristics (day 3) and, soon after, they yield a highly enriched population of eye field cells (day 5). Subsequently, cells acquire characteristics of retinal differentiation [11], and after 3–7 days of treatment, these cultures contain neuronal rosettes and express multiple eye field markers. To investigate the expression pattern of the *Gipc* genes in these eye field cells, *Gipc1*, *Gipc2* and *Gipc3* were monitored by reverse transcription and quantitative PCR (RT-qPCR). Consistent with the in situ hybridization results, *Gipc1* was expressed in mESC-derived eye field cells, but *Gipc2* and *Gipc3* were not increased after retinal induction (Figure 2B). Western blot analyses detected GIPC1 protein expression as early as day 5 of differentiation in the mESC-derived eye field cells (Figure 2C) and immunofluorescence showed cells co-expressing GIPC1 and Pax6, one of the EFTFs (Figure 2D–F). Together these observations provide evidence that GIPC1 is present in the early stages of eye development in mice.

GIPC1 is required for the development of retinal identity from embryonic stem cells

To determine whether GIPC1 is required for eye development in mice, we used a dominant-negative GIPC1 in mESCs undergoing directed differentiation to retinal cells. Disruption of the carboxylate-binding loop of the GIPC1 PDZ domain (Leu-Gly-Leu, Supplementary Figure 1) prevents the recruitment of PDZ-interacting molecules, causing a dominant negative phenotype [20, 22]. We generated stable mESC lines overexpressing a mutated GIPC1 construct (dnGIPC1) carrying three mutations in the PDZ binding pocket (LGL $\rightarrow$ AAA). We established two clonal lines through transfection and antibiotic selection (dnGIPC#2, dnGIPC#15). Similar to parental R1 cells, the resulting colonies maintained ESC-like morphology with well-defined edges and a high nucleus to cytoplasm ratio. In addition, the pluripotency markers Oct4, Nanog, Sox2 and Lin28 were not affected upon GIPC1 depletion (Supplementary Figure 2) and alkaline phosphate staining indicated that mESCs did not spontaneously differentiate after depletion of GIPC1 activity (data not shown).

We used the retinal differentiation protocol described above to test whether the dnGIPC mESC cell lines would show a reduction in EFTF expression. The eye field differentiation was assessed by RT-qPCR and immunostaining. Remarkably, upon differentiation, dnGIPC lines showed reduced levels of several EFTFs, compared to control lines (Figure 3). *Pax6*, *Rax*, *Six3* and *Lhx2* all showed lower expression levels (Figure 3C, G and M–P) in the dnGIPC derived cells. However, the expression of the transcription factor *Otx2* was not affected in the dnGIPC1 ESC lines (Figure 3J, K and Q). *Otx2* is expressed in the forebrain/midbrain region of the neural plate (Figure 3I), but not in undifferentiated stem cells (Figure 2C), suggesting that the expression of dnGIPC1 did not impair ESC differentiation into more general anterior neuronal fates. These results are very similar to the effects of Kermit2 morpholinos in *Xenopus*, where *Xrx* (*Rax*) and *Pax6* showed reduced expression, but *Otx2* was not affected [17].

To further confirm the GIPC1 loss-of-function phenotype, we generated stable ESC lines that overexpress shRNA against endogenous GIPC1. We tested 5 different shRNAs constructs for their efficiency in downregulating GIPC1 protein in HEK293T cells (Supplementary Figure 3). Three shRNAs (shRNA#1 and shRNA#3 and shRNA#5) were effective in reducing the levels of GIPC1 to 20–30%. Two shRNAs (shRNA#1 and shRNA#3) and control-scrambled shRNA were further used to generate mESC stable lines. After antibiotic selection, these cell lines were indistinguishable from the parental R1 line; the depletion of GIPC1 protein in undifferentiated cells did not affect survival, proliferation or the expression of pluripotency markers (Supplementary Figure 2).

We next tested whether knockdown of GIPC1 would affect retinal differentiation of the mESCs. We found that similar to the dnGIPC1 lines, when subjected to the differentiation protocol, the shRNA-overexpressing mESCs lines showed lower expression levels of all the EFTFs (Figure 3D, H and M–P). Also, similar to the dnGIPC1 lines, the shRNA-overexpressing mESCs also had approximately the same level of *Otx2* expression as the wild type cells, consistent with the hypothesis that inhibiting GIPC1 function does not impair neuronal differentiation in general, but rather has a specific effect on eye field differentiation (Figure 3L and Q). Taken together, these data indicate that GIPC1 is necessary for the acquisition of eye fates, but not for the initial Otx2-dependent neural plate patterning.

dnGIPC1 mESC lines increase expression of telencephalic genes

Upon differentiation, dnGIPC1 cell lines did not express genes that are restricted to the eye field. To assess whether dnGIPC1 cells acquired other ANP identities, we analyzed the expression of Emx1 and Emx2, transcription factors expressed in the telencephalon, and Engrailed (*En1*), which is expressed in the midbrain/hindbrain junction [23]. After 5 days of differentiation, the wild type mESC did not express significant levels of these genes. By contrast, dnGIPC1 and shRNA cell lines exhibited a significant increase in *Emx1* and *Emx2* gene expression (Figure 4A and B). The expression of *En1* was not significantly changed in any of the lines (Figure 4C). The dnGIPC1 cell lines also showed an increase in another cerebral cortical marker, *Tbr2* (Figure 4D, see below). These data indicate that the cells lacking GIPC1 activity can acquire markers of telencephalic identity.

To further confirm this possibility, we cultured mESC for 5 more days *in vitro* (day 10) and analyzed the effects of GIPC1 inhibition by immunolabeling with Pax6 and Otx2 antibodies. This combination of labels allowed us to distinguish different populations of the developing neural plate: while Otx2 and Pax6 are expressed in both the retina and the telencephalon, their expression patterns differ. In the retina, Otx2 is limited to photoreceptors and bipolar cells, while Pax6 is expressed in retinal progenitors, Retinal Ganglion cells (RGCs), amacrine cells and horizontal cells. Thus, in the retina these two transcription factors are expressed in different subsets of cell populations. As expected, as development proceeded in the control cultures, the Otx2$^+$ and the Pax6$^+$ populations segregated (Figure 4E–G). Conversely, in the dnGIPC1 lines, Otx2 and Pax6 did not segregate into distinct populations (Figure 4H–J).

A further way to distinguish telencephalic fates from retinal identity is with a combination of Tbr2/Eomes and Tuj1. In the telencephalon, Tbr2/Eomes is highly expressed in a population of progenitors of the ventricular zone and the subventricular zone of cerebral cortex as well as in the hippocampus and other telencephalic structures [24–26]. By contrast, in the retina, Tbr2/Eomes is expressed in a subpopulation of RGCs [27, 28]. In order to determine the identity of the small percentage of Tbr2$^+$ cells in the control cultures, we coimmunostained these mESC-derived cells using Tbr2 and Tuj1 antibodies. The few Tbr2$^+$ cells present in the control cultures were also Tuj1$^+$ (Figure 4K–M, white arrows) and showed long processes indicating that these cells might be RGCs. In the dnGIPC1 mESCs, Tuj1 and Tbr2 exhibited a large up-regulation comparable to the expression pattern of these proteins in the developing cortex (Figure 4N–P). Taken together these results indicate that after GIPC1 depletion, Otx2$^+$ neural progenitors did not differentiate into retinal progenitor cells, but instead upregulated markers for telencephalic progenitors.

GIPC1 is required for the development of retinal identity in 3D cultures

Recent protocols to differentiate ESCs into a variety of neuronal identities are based on three-dimensional culturing [12, 29–31]. We generated embryoid bodies (EBs) containing retinal neuroepithelium by dissociating mESCs and plating them in 96-well low-attachment plates (3,000 cells/well). In these conditions, cells spontaneously formed EBs, and when grown in the presence of knockout-serum replacement and Matrigel will form optic vesicle and cup-like structures (Figure 5B) [12, 29, 32]. In 13 days, pigmented RPE was observed in about 50% of the EBs and, when kept for longer periods of time, the mESCs-derived retinal progenitors generated the various layers of the retina, including ganglion cells and photoreceptors, recapitulating the normal development of the eye (Figure 5 F, G).

In order to assess the effect of dnGIPC1 on the development of retina in the 3D protocol, we differentiated the dnGIPC1 mESCs using this system (Figure 5A). Compared to control mESCs, only a small fraction of dnGIPC1 EBs developed optic vesicle structures (Figure 5B, C; 32.7% vs 84.7%), which is consistent with the observation that dnGIPC1 cells express reduced levels of EFTFs. In addition, fewer of the EBs exhibited pigmented RPE cells and, when present, the patches of RPE were smaller (Figure 5B, C). Furthermore, the dnGIPC1 mESCS showed a reduction in the levels of Pax6 after 7 days of differentiation (Figure 5D) and a strong upregulation in Tbr2 at day 13 (Figure 5E) when compared with

wild type R1 cells. When maintained for longer periods of time, normal mESCs differentiate into laminar structures with Recoverin[+] photoreceptors, Brn3^{+} RGCs and a clear separation between the Otx2^{+} cells (bipolar cells and photoreceptors) and the Pax6^{+} inner retinal cells (Supplementary Figure 4); however the dnGIPC1 lines did not differentiate into Brn3^{+} RGCs (Figure 5F) nor Recoverin[+] photoreceptors (Figure 5G). These results add further support that GIPC1 activity is necessary for the formation of eye field cells and their subsequent differentiation into mature retina. We verified that the 3D protocol was generating regions of neural retina in the wild type cells by using a newly generated Rax-GFP mESC line (Figure 6A–J) and that GIPC1 is expressed in the Rax-GFP[+] regions by day 10 (Figure 6H).

GIPC1 participates in IGF1R signaling in developing eye field cells

IGF signaling has been linked to the eye development in *Xenopus* and zebrafish embryos [17, 33–35]. Ectopic expression of IGF1R in dorsal positions of the amphibian embryo induced the formation of ectopic eyes and a dominant-negative IGFR construct reduced the expression of eye field markers (*e.g.* Rax) [35]. Additionally, GIPC1 has been shown to interact with IGF1R [17, 36–38] and in *Xenopus*, Kermit2/GIPC is required for IGF1-induced ocular tissues in both embryos and animal caps [17]. We therefore hypothesized that GIPC1 could also be interacting with IGF1R during eye field induction in mouse. To test this possibility, we performed immunoprecipitation using GIPC1 and IGF1R antibodies. HEK 293T cells were transfected with GIPC1 and IGF1R plasmids and immunoprecipitated with either GIPC1, IGF1R antibodies or with an antibody against GFP as a negative control. GIPC1 protein was detected by Western blot in both the GIPC1 and the IGF1R immunoprecipitates, but not in the GFP controls, indicating that GIPC1 and IGF1R proteins can physically interact (Figure 7A, upper panel). We next tested whether GIPC1 and IGFR1 were part of a similar complex in developing retinal progenitors. We carried out immunoprecipitation using cell lysates from either mESC-derived retinal cells after 7 days of differentiation or from freshly dissected E11 mouse retinas. In both cases, we detected GIPC1 protein after immunoprecipitation with IGF1R antibodies (Figure 7A), suggesting that GIPC1 and IGF1R interact in retinal progenitors.

IGF1R signals though several intracellular pathways including Ras/MAPK pathway and PI3K/Akt1 pathway. To determine which of these pathways were affected by inhibition of GIPC1, full-length wild type GIPC1 or the dnGIPC1 were transfected into HEK cells, and the serum-starved cells were treated with 25 ng/ml IGF1 for 10 min, and the IGF1-induced signaling was assayed by measuring levels of ERK1/2 and Akt1 phosphorylation. In control cells, IGF1 induces a significant phosphorylation of ERK1/2 and Akt1 (Figure 7B). By contrast, the expression of the dnGIPC1 inhibited the IGF1 induced activation of both ERK1/2 and Akt1. The inhibition of Akt1 phosphorylation is consistent with results from the *Xenopus* study, where a similar result was obtained with Kermit2 knockdown; however, in *Xenopus*, MAPK phosphorylation was not inhibited [17]. Nevertheless, there does seem to be some difference in the way in which GIPC1 interacts with these two downstream signaling pathways: transfection of full-length wild type GIPC1 was sufficient to activate the PI3K/Akt1 signaling pathway even in the absence of IGF1, but it did not affect ERK1/2 phosphorylation (Figure 7B). Nevertheless, even in the presence of dnGIPC1, IGFR is still

phosphorylated (Figure 7C). Together, these results show that GIPC1 is an important component in IGF1-induced phosphorylation of ERK1/2 and Akt1 downstream of IGF1R.

Since we observed that GIPC1 was required for mESC differentiation into eye field fates and that dnGIPC1 overexpression resulted in a reduction of Akt1 and ERK1/2 phosphorylation, we assayed whether the MAPK and/or the PI3K/Akt1 pathways elicited any roles in the specification of eye field progenitors. To test this possibility, we differentiated mESCs in the 2D protocol. Wild type mESCs were maintained in differentiation media containing DMSO vehicle, a MAPK inhibitor (PD98059), or a PI3K inhibitor (LY294002). Surprisingly, a pharmacological blockade of the MAPK pathway did not have any effects on eye field differentiation at the tested dose (25 μM), and the expression of EFTFs was comparable to the controls (Figure 7D); however, the inhibition of the PI3K partially inhibited retinal specification: *Pax6*, *Rax* and *Lhx2* were significantly reduced. Similar to the dnGIPC1 phenotype, the expression level of *Otx2* was not affected. The expression levels of *Emx1* were slightly increased by RT-qPCR, suggesting a modest increase in telencephalic fates, while the midbrain/hindbrain marker *En1* was not affected in any of the conditions. Immunostaining experiments after control, PD98059 or LY294002 treatments (Supplementary Figure 5) showed consistent effects: PI3K inhibition caused a downregulation in the number of Pax6+ cells and an increase in the telencephalic marker Tbr2, compared to controls and PD98059 treatments.

GIPC1 is known to interact with other signaling pathways in addition to IGFR, such as the TGFβ receptor [39]. To test whether the effects of manipulations in GIPC1 might be mediated in part by this pathway, we differentiated the mESCs using the 3D culture system in the presence or absence of a TGFβ inhibitor, SB431542. In experiments using the Akt inhibitor, LY294002 treatment (Figure 7) resulted in the downregulation of EFTFs except for *Otx2* and *Pax6*. By contrast, inhibition of TGFβ signaling during early stages of retinal differentiation (until day 5) resulted in a slight increase in *Pax6*, *Lhx2*, and *Six3* (Figure 7E) and there was no change in *Rax* expression. These results support the hypothesis that the effects of GIPC1 are independent of the TGFβ signaling pathway.

Discussion

To date, much of what is known about the early steps in eye field formation have been elucidated in frog and zebrafish [40–43]. This is largely due to the accessibility of their embryos to experimental manipulations. It has been more difficult to test hypotheses concerning eye field development in mice, due to the lack of a good experimental system. Thus, we have used the directed differentiation of mESCs to eye field and retinal fates to test whether critical factors in eye field formation in frogs play a similar role in mice. We have found that the normal function of the mouse homolog to Kermit2, GIPC1, is essential for the expression of the EFTFs: overexpression of a dominant-negative form of GIPC1 (dnGIPC1), as well as the downregulation of endogenous GIPC1, is sufficient to inhibit retinal differentiation in two protocols of directed differentiation of mESCs. Moreover, these same manipulations resulted in an increase in markers of telencephalic fates in the cells, consistent with the possibility that cells fated to become eye field take on a telencephalic identity without GIPC1. Functional analyses of GIPC1 in retinal progenitors indicate that it interacts

with IGFR and participates in Akt1 activation. In addition, a pharmacological blockade of Akt1 phosphorylation mimics the dnGIPC1 phenotype suggesting that the GIPC1-PI3K-Akt1 pathway plays a key role in the regional specification of the anterior CNS.

There are now several different protocols for generating eye field and retina from either mouse or human ESCs [9, 11, 12, 32, 44]. We have found eye field specification is dependent on GIPC1 in two different protocols, suggesting that this molecule regulates a key step in this developmental process; at least some of the mechanisms in the progression from ESCs to retinal fates are similar regardless of the specifics of the *in vitro* production method. In one protocol, the addition of anteriorizing factors, such as Dkk1, IGF1 (to antagonize the Wnt pathway) and Noggin (to inhibit BMP signaling) are used to increase the expression of EFTFs in ESC derived EBs in a synchronized manner. Alternatively, EBs maintained in Matrigel and KSR produce optic vesicles without additional factors, leading to laminated neural retinal structures. Our results show that both systems provide models for testing hypotheses about early stages of eye field formation.

The frog homolog to GIPC1, Kermit2, was shown to be important in eye formation. Experimental reductions in Kermit2 expression in *Xenopus* leads to absent or very small eyes in 83% of the embryos; *Pax6* and *Otx2* were reduced in the presumptive eye field, but not in other regions of the neural plate, and *Xrx* and *Xath5* were strongly reduced [17]. We have utilized ESC-derived retinal cells to test the role of the PDZ domain-containing protein GIPC1, and have found that this molecule has a role in mammalian eye development similar to that reported in frog. Another parallel between the findings in *Xenopus* and the present study is that the loss of Kermit2/GIPC1 appears to cause a change in fate from eye field to telencephalon. We found several markers of telencephalic identity were increased in our experiments. However, it is possible that inhibition of GIPC1 causes an increase in cell death of optic fates, rather than a fate shift. We cannot rule this possibility out, though the differentiated cultures of dnGIPC1 mESCs had similar numbers of cells as those derived from wild type mESCs, so cell death was either low or compensated by growth of cells with alternate fates.

Several lines of evidence support the hypothesis that the role of Kermit2 in eye field formation is to modulate the activity of the IGF receptor: (1) Kermit2 binds to the IGFR and (2) eye field expansion induced by IGF1 can be blocked by Kermit2 knockdown. Additional biochemical studies further demonstrated that Kermit2 is required for the maintenance of Akt signaling downstream of IGFR, but is not required for MAPK activity. It is possible that GIPC1 has a similar function in mouse eye field development, since we find that GIPC1 interacts with the IGFR in the early optic structures in mouse embryos. The mechanism by which GIPC1 facilitates Akt signaling in mESCs directed to eye fates is likely to function through its activity in signaling endosomes. One of the most well established functions for GIPC1 is its role in receptor trafficking of endocytic vesicles. In this regard, GIPC1 is important in the endosomal signaling from receptor tyrosine kinases to PI3K-AKT signaling [18, 45] as well as recycling receptors back to the cell surface [46]. Although our data support a role for GIPC1 specifically in IGF1R signaling, since GIPC proteins interact with a large number of signaling receptors, including receptor tyrosine kinases like IGF1R [36, 37], neurotransmitter receptors [47], integrins [21, 48], and TGFβ receptors [39], it is

possible that other signaling molecules are also active in the eye field. However, at least for the TGFβ receptors, our results show that inhibition of this pathway does not mimic the effects of reductions in GIPC1.

The ANP, including the eye fields, is patterned with opposing gradients of Wnt/beta catenin ligands and SFRP inhibitors [49–55] [7]. In addition to the SFRPs, the non-canonical Wnt pathway provides another mechanism for regulating the Wnt activity in the ANP. Wnt11 activates the non-canonical receptor Fzd5 to promote EFTF expression in zebrafish [7], whereas Wnt4 appears to activate the non-canonical Wnt receptor, Fzd3 to promote EFTF expression in *Xenopus* [57]. Several studies have also shown that IGF1 signaling promotes anterior neural fates, and specifically eye field formation [35]. Although the mechanism by which IGF1 promotes EFTF expression is not well understood, Richard-Parpaillon *et al.* (2002) demonstrated that IGF1 can directly antagonize Wnt/beta-catenin mediated transcriptional activation in *Xenopus* [33]. This appears to require the Akt pathway, since Akt activation is downstream of the effects IGF1 has on eye field induction. Our results now extend this model to mammalian eye field specification and suggest that the effects of IGF1, Akt and GIPC1 on EFTF expression are conserved across vertebrates.

Experimental procedures

Animals

All experiments were done in accordance with approved protocols and the animals were housed and bred in the Department of Comparative Medicine at the University of Washington.

mESC culture

Mouse ESCs (R1) were maintained in LIF+ 2 inhibitors (LIF+2i) media: Dulbecco's Modified Eagle's Medium (DMEM) from Invitrogen supplemented with 20% Fetal Bovine Serum (ES qualified, Invitrogen), non-essential amino acids (Invitrogen), sodium pyruvate (Invitrogen) and 0.1 mM of β-mercaptoethanol (Sigma), 100 μl of Leukemia Inhibitory Factor (LIF, ESGRO Millipore ESG1106, 10 million units/ml), 3 μM of GSK3β inhibitor Stemolecule CHIR99021 (Stemgent) and 0.4 μM of MEK inhibitor Stemolecule PD0325901 (Stemgent). All of the cell lines were maintained in feeder-free conditions in growth factor-reduced Matrigel-coated plates.

Retinal differentiation of mESCs

Semi-confluent undifferentiated colonies were dissociated to a suspension of single cells by enzymatic treatment with TrypLE (Gibco) and gentle mechanical dissociation. Next, cells were rinsed with DMEM +10% FBS and plated in retinal differentiation media (DMEM: F12 media, supplemented with non-essential amino acids, sodium pyruvate, BSA (Invitrogen), B27, N2, and 1 ng/ml each of Dkk1, Noggin and IGF1) in low-attachment plates. Floating mESCs spontaneously formed aggregates (EBs) within few hours under these conditions. After three days, EBs were seeded onto Matrigel-coated plates in retinal differentiation media. From day 5–10, media was changed every other day. For the 3D

culture method, we adapted that of Eiraku and Sasai (2012), using DMEM:F12 as above (plus retinoic acid and taurine) as the maintenance media.

qPCR

Total RNA was extracted from the cultures using Trizol (Invitrogen) followed by chloroform extraction as manufacturer's instructions. This was followed by DNase-1 (Qiagen) treatment followed by RNA cleanup using Qiagen RNA mini cleanup kit. cDNA was reverse transcribed using Superscript III RT kit (Invitrogen) as per the manufacturer's instructions. PCR was performed using the primers listed in the Supplementary Table and values were normalized to β-actin.

Immunohistochemistry and microscopy

Cultures were fixed in 4% paraformaldehyde (PFA), and after PBS rinse, non-specific binding was inhibited by incubation in milk powder block solution (5% dried milk powder and 0.5% Triton X-100 in PBS) for 1–2 hours (at room temperature). Cells were incubated in primary antibodies overnight at 4°C, washed in PBS and incubated with appropriate fluorescent conjugated secondary antibodies (Invitrogen) or streptavidin conjugate (Invitrogen) for 1 hour along with DAPI to counterstain nuclei. The coverslips or sections were washed and mounted for microscopy. Primary antibodies were as follows: rabbit anti-Pax6 (1:500, PRB-278P, Covance, Princeton, NJ, USA), goat anti-Otx2-biotin (1:500, BAF1979, R&D Systems, Minneapolis, MN, USA), goat anti-Sox2 (1:100, sc17320, Santa Cruz), goat anti-Lhx2 (1:250, sc-19344, Santa Cruz), Tuj1 (1:3,000, Covance), Tbr2 (1:500, ab23345, Abcam), goat anti-GIPC1 (1:100, Santa Cruz), goat anti-Brn3 (1:500, sc-6026, Santa Cruz), rabbit anti-Recoverin (1:1000, Chemicon), Lin28 (1:100, ab46020, Abcam), chicken anti-GFP (1:250, Abcam), mouse anti-Rhodopsin (1:100), goat anti-S-Opsin (1:150, Santa Cruz), goat anti-Oct3/4 (1:200, sc-8628, Santa Cruz), rabbit Nanog (1:250, ab80892, Abcam).

Whole mount *in situ* hybridization

E8-E9 embryos were dissected in PBS + 2mM EGTA, fixed in 4% PFA, and dehydrated in ethanol. Tissues were rehydrated, treated with Proteinase K, post-fixed with PFA 4% + 0.1% Glutaraldehyde, and incubated 1hr at 68°C in hybridization mix, followed by 0.2 mg/ml of digoxigenin (DIG)-labeled RNA probes for 18 hours. DIG-labeled probes were prepared according to the manufacturer's protocol (Roche). After washing, tissues were incubated overnight at 4°C with anti-DIG-AP antibody (1:2000) (Roche), and developed in NBT/BCIP solution.

HEK 293T transfection

HEK 293T cells were maintained in DMEM media supplemented with 10% FBS. For transfection, the cells were cultured overnight to 40–60% confluence. Transfection was performed using Lipofectamine 2000, following the manufacturer's instructions (Invitrogen).

Western blot and immunoprecipitation assays

HEK 293T, mESC-derived cultures or embryonic retinas were collected in lysis buffer, containing a protease inhibitor cocktail (Roche) and phosphatase inhibitors (10 mM tetra-sodium pyrophosphate, 200 μM sodium orthovanadate and 10 mM sodium fluoride). Cells were centrifuged at 13,000 rpm for 20 min and the supernatant was analyzed either by Western blot or immunoprecipitation. For immunoprecipitation experiments, antibodies were added to 500 μg of total protein of cell lysates or tissue extracts and incubated for 10 h at 4°C. As a control, a GFP antibody was used under the same conditions. Sepharose bead coupled protein G (Sigma) was then added and the suspension was incubated for 2 h at 4°C. Sepharose beads were washed with lysis buffer and the samples were analyzed by a standard Western blot protocol. Membranes were incubated first in 5% BSA in TBS and then with the indicated antibodies. Detection was performed using the ECL system from Amersham. For quantification, films were scanned and then subjected to band densitometry and quantification by ImageJ.

Lentiviral particles production, mESC viral transduction and stable cell line generation

Lentivirus was made using the four-plasmid system. HEK-293T cells were transfected with Lipofectamine 2000 (Invitrogen) and the supernatant was collected 24 and 48 hrs later. R1 mESC were transduced by overnight incubation with virus in 6-well plates on Matrigel at a confluency of 10%. 48 hours after viral transduction, the mESC were selected for with 1μg/ml Puromycin. After 7–10 days, individual colonies (<50 cells) were picked, expanded and analyzed. shRNA lentiviral constructs were obtained from Open BioSystems and tested on HEK cells co-transfected with an expression plasmid for mouse GIPC1. Of the five shRNAs tested, three (sh1, sh3, and sh5) showed substantial reduction in GIPC1 expression when analyzed by Western blotting. Catalog number sh1: RMM3981-9604607 targeted to Exon 3; sh3:RMM3981-9604609, targeted to Exon 2; sh5:RMM3981-9604611, targeted to Exon 7.

Generation of Rax-GFP mESC line

E0.5 Rax-GFP embryos were harvested from naturally mated females. Upon collection, the embryos were plated on gamma-irradiated mouse embryonic fibroblast feeders in mouse ESC culture medium. Once hatched and shortly after plating, the inner cell mass was selectively subcultured by plucking and breaking into 2 or 3 pieces onto a new feeder layer. Once colonies arose with mESC morphology, the new line was passaged using typsin-EdTA. The mouse strain used for this research project, STOCK Tg(Rax-EGFP)25Gsat/Mmcd, identification number 030564-UCD, was obtained from the Mutant Mouse Regional Resource Center, a NCRR-NIH funded strain repository, and was donated to the MMRRC by the NINDS funded GENSAT BAC transgenic project.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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SIGNIFICANCE STATEMENT

In this study, we use the directed differentiation of embryonic stem cells (ESCs) as a model to study key regulatory steps in eye field formation. The regulatory mechanisms that define the eye field are not well understood, and most of what we know about this has been discovered in organisms with accessible embryos like zebrafish and Xenopus. Signaling pathways are difficult to manipulate in mammals at early embryonic stages. ESCs have emerged as an alternative method to study the earliest steps of mammalian ontogeny. The differentiation paradigms towards eye field progenitors and mature retinal cells from ESCs are now well established. Therefore, ESCs provide a potential model for testing hypotheses concerning forebrain patterning and eye field specification in vitro. Here, we directed mESCs towards eye field and retinal fates, to test whether a specific component of the signaling network in frog embryos, Kermit2, is important in mammalian eye development. Our results indicate that GIPC plays a key role in the segregation of the eye field from telencephalic cell fates, and likely acts through the regulation of PI3K-Akt1 pathway downstream of IGFR. Our results show the importance of IGF signaling to patterning of the CNS, and demonstrate the usefulness of ESCs in understanding basic developmental processes in organ formation.
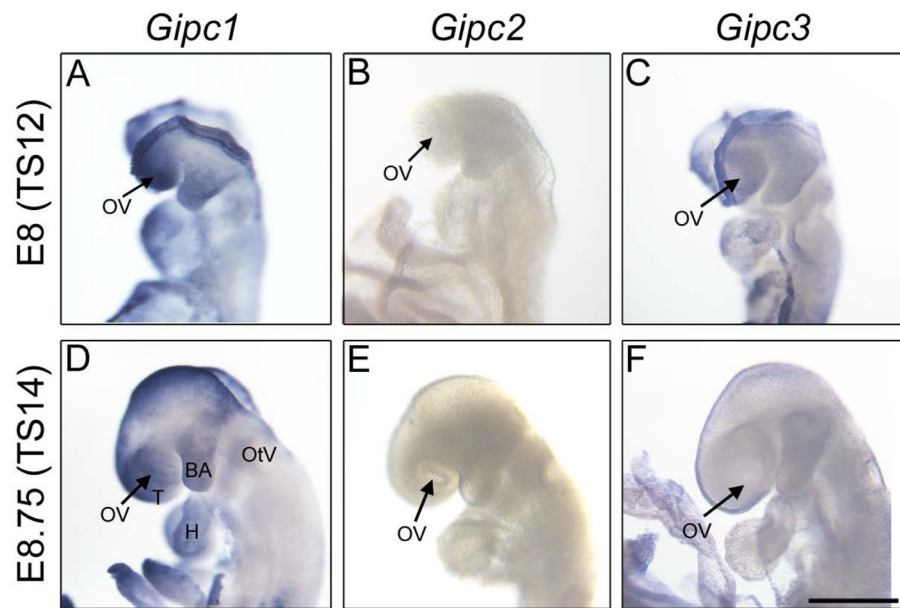
Figure 1. GIPC1, GIPC2, and GIPC3 expression in the developing mouse
Whole mount RNA *in situ* hybridization of GIPC1, GIPC2 and GIPC3 in embryonic day 8 and 8.75 embryos. The arrows indicate eye field/optic vesicle (OV) region. T: telencephalon, BA: branchial arches, OtV: Otic vesicle, H: heart. Scale bar: 300 microns.
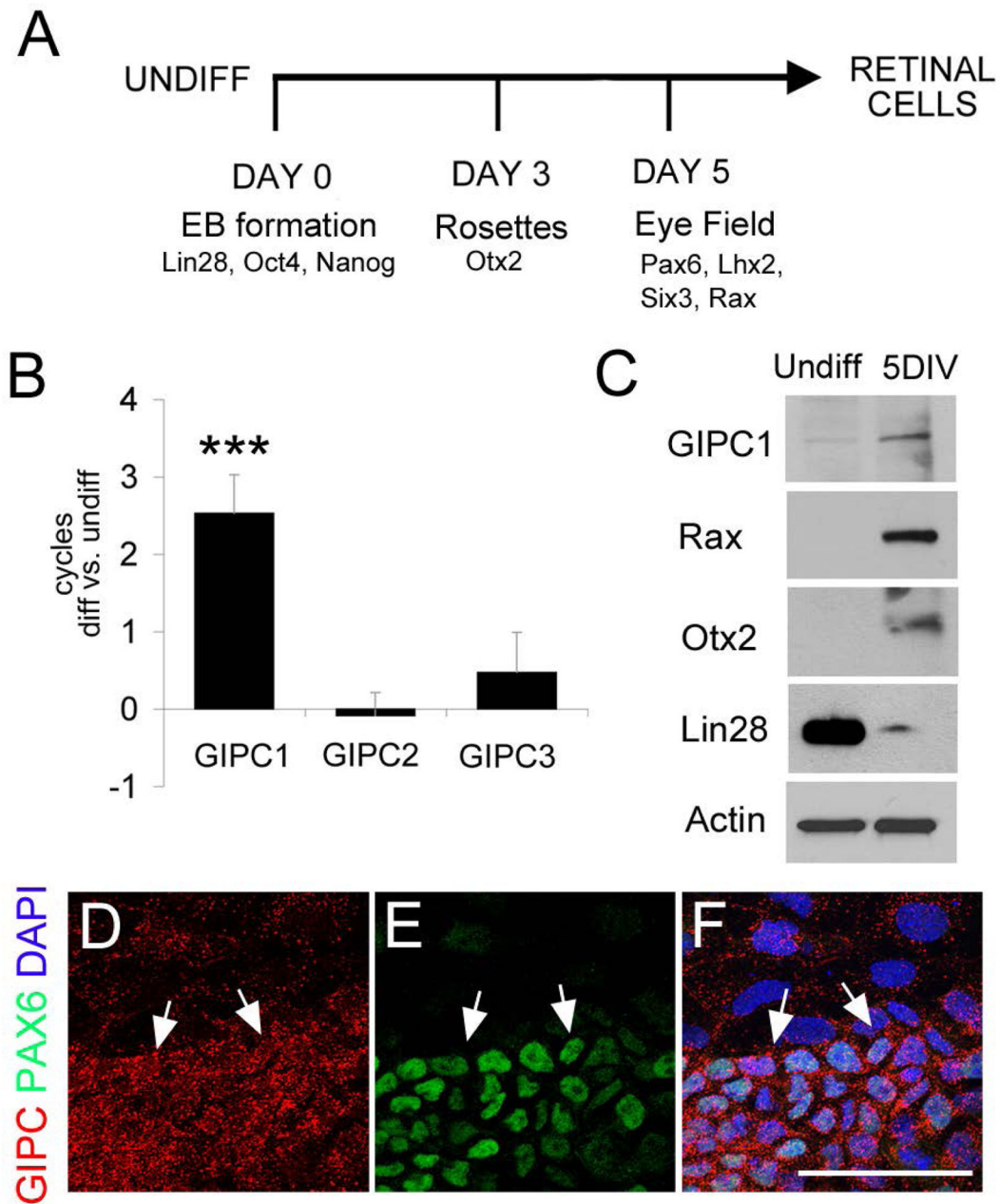
Figure 2. GIPC1 is upregulated upon mESC retinal differentiation

(A) Diagram of the mESC 2D differentiation protocol (B) RT-qPCR analyses of the expression profiles of GIPC1, GIPC2 and GIPC3 in undifferentiated mESCs and cells differentiated for 5 days. Data is represented as the relative change (ddCT) between undifferentiated samples and differentiated cultures and indicates mean ± SEM. *** indicates a p-value < 0.001. (C) Western blot comparison of undifferentiated cells (first lane) and mESCs differentiated for 5 days (second lane). (D–F) Immunostaining of GIPC1 (red) and Pax6 (green, arrows). Arrows indicate cells with higher levels of GIPC1 colocalizing with Pax6+ cells. Scale bar: 50 microns.
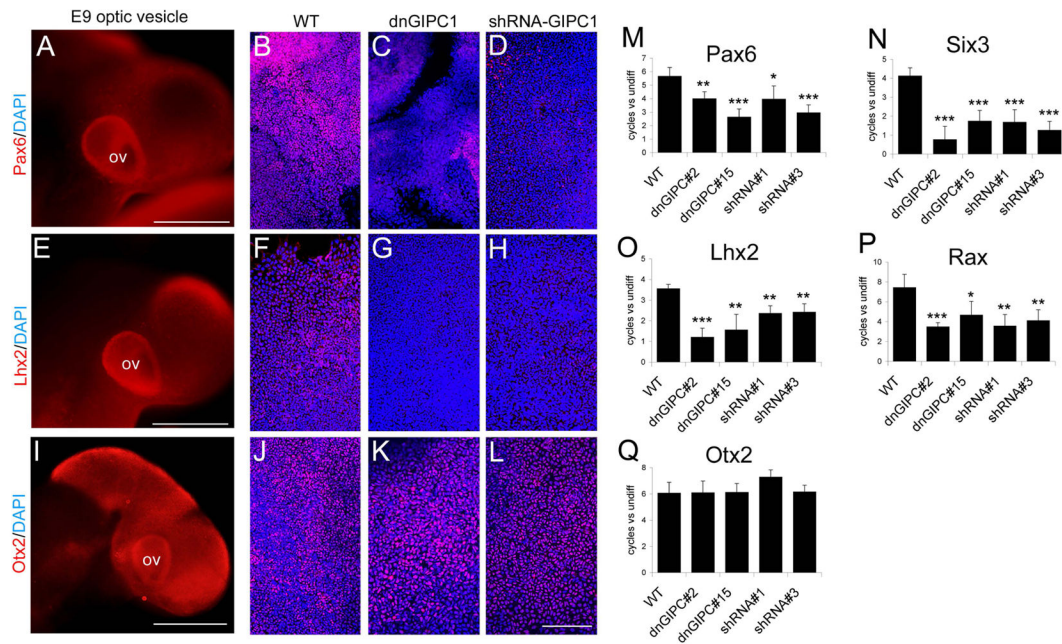
Figure 3. dnGIPC1 and shRNA cell lines show reduced levels of EFTFs using 2D culturing method

Expression of (A) Pax6, (E) Lhx2, and (I) Otx2 in embryonic day 9 embryos. Compared to control cell lines, dnGIPC1 cells and shRNA cells exhibited lower levels of (B–D) Pax6 and (F–H) Lhx2 proteins, but they showed no differences in the number of Otx2$^+$ cells (J–L). RT-qPCR analyses showed a significant decrease in the expression of (M) *Pax6*, (N) *Six3*, (O) *Lhx2,* and (P) *Rax* but not (Q) *Otx2* in both the dnGIPC1 and the shRNA cell lines. RT-qPCR data is represented as the relative change (ddCT) between undifferentiated cells and cultures differentiated for 5 days, and indicates mean ± SEM. OV: optic vesicle. Scale bars: 500 microns in panels A, E and I and 200 microns in panels B–D, F–H, J–L. P-values: * < 0.05, ** < 0.01, *** < 0.001.
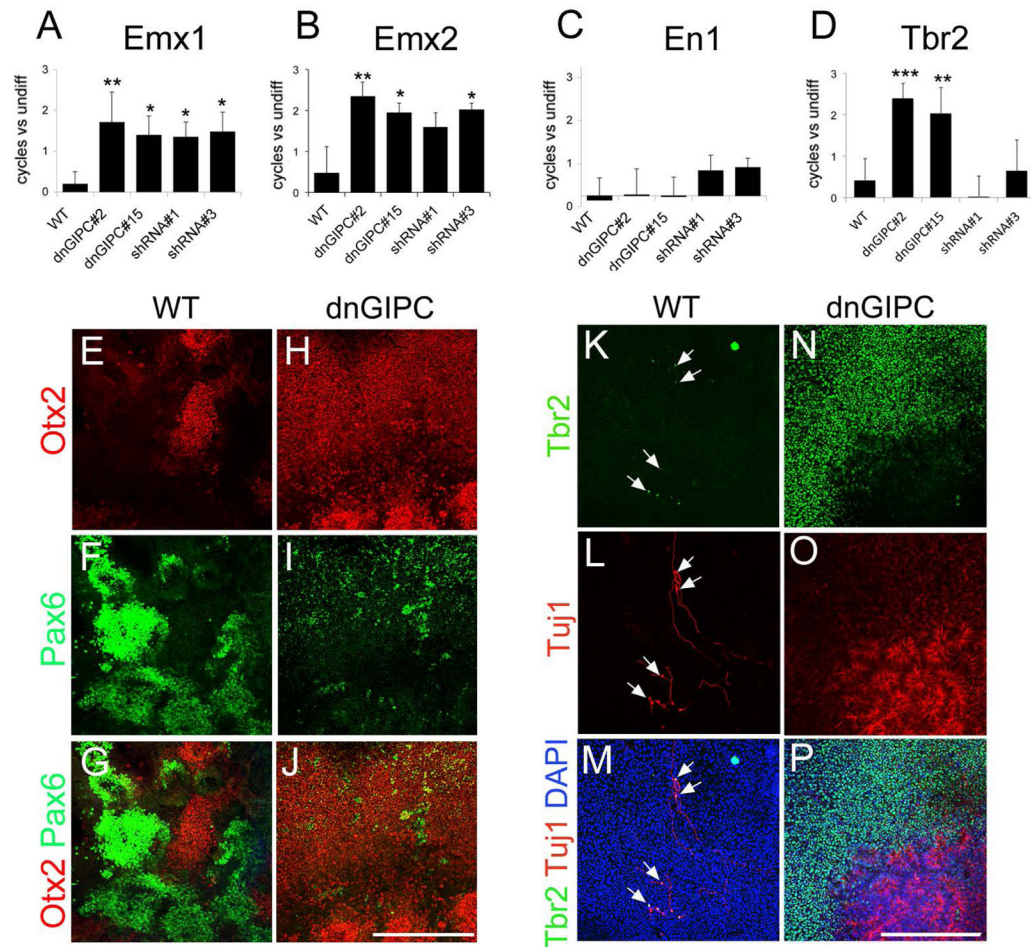
Figure 4. dnGIPC1 cell lines differentiate into telencephalic fates

(A–D) RT-qPCR analyses of the undifferentiated mESCs and cells differentiated under the 2D protocol for 10 days. Data is represented as the relative change (ddCT) between undifferentiated samples and differentiated cultures and indicates mean ± SEM. P-values: * < 0.05, ** < 0.01, *** < 0.001. Immunolabeling experiments at 10 days showing the expression of (E, G, H, J) Otx2 (red), (F, G, I, J) Pax6 (green), (K, M, N, P) Tbr2 (green), and (L, M, O, P) Tuj1 (red).
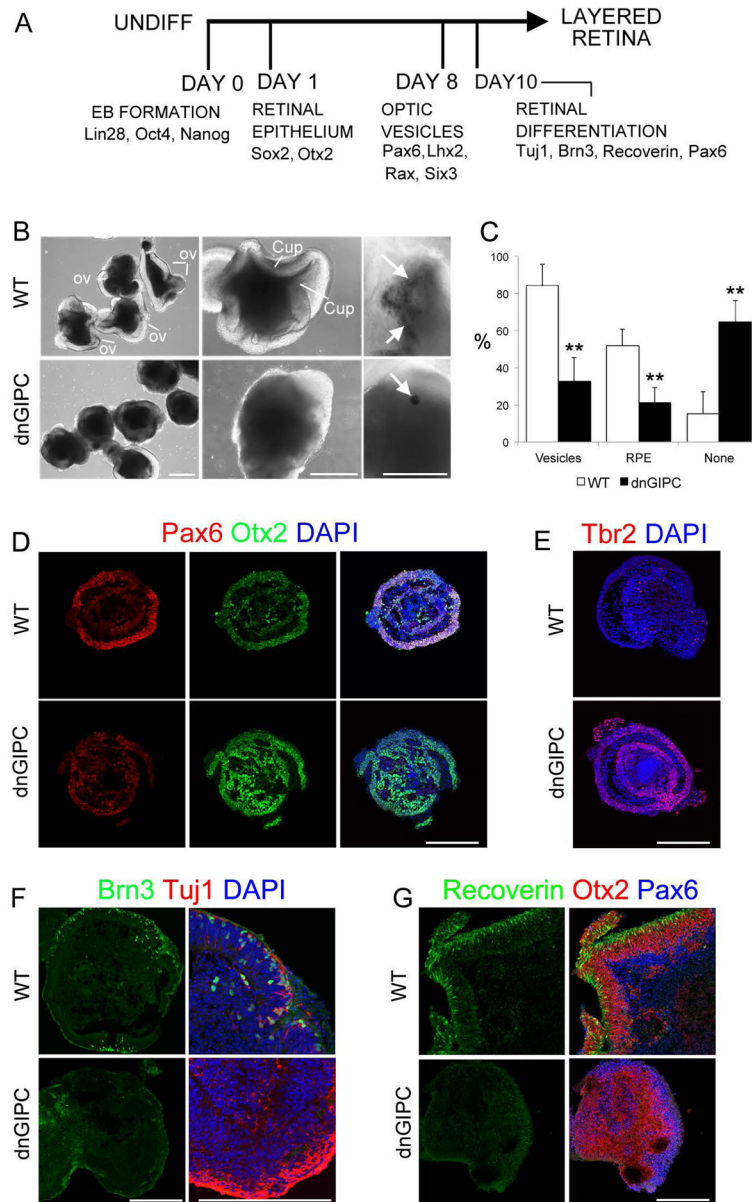
Figure 5. dnGIPC1 cell lines show reduced retinal differentiation in 3D culture systems
(A) 3D differentiation protocol. (B) EBs at day 13 of differentiation. Arrows indicate pigmented RPE. OV: optic vesicle, Cup: cup-like structures. (C) Quantification of the percentage of EBs that exhibited optic vesicle-like structures, RPE or neither. Data is represented as mean ± SEM. (D) Immunostaining of EBs after 5 days of differentiation showing the expression of Pax6 (red) and Otx2 (green). EBs at day 13 showing (E) the expression of the telencephalic marker Tbr2 (red) and (F) the Retinal Ganglion Cell markers, Brn3 (green) and Tuj1 (red). (G) Stainings at day 24 of differentiation showing expression of the photoreceptors marker Recoverin (green), Otx2 (red), and Pax6 (blue). Scale bars: 200 microns. P-Values** < 0.01.
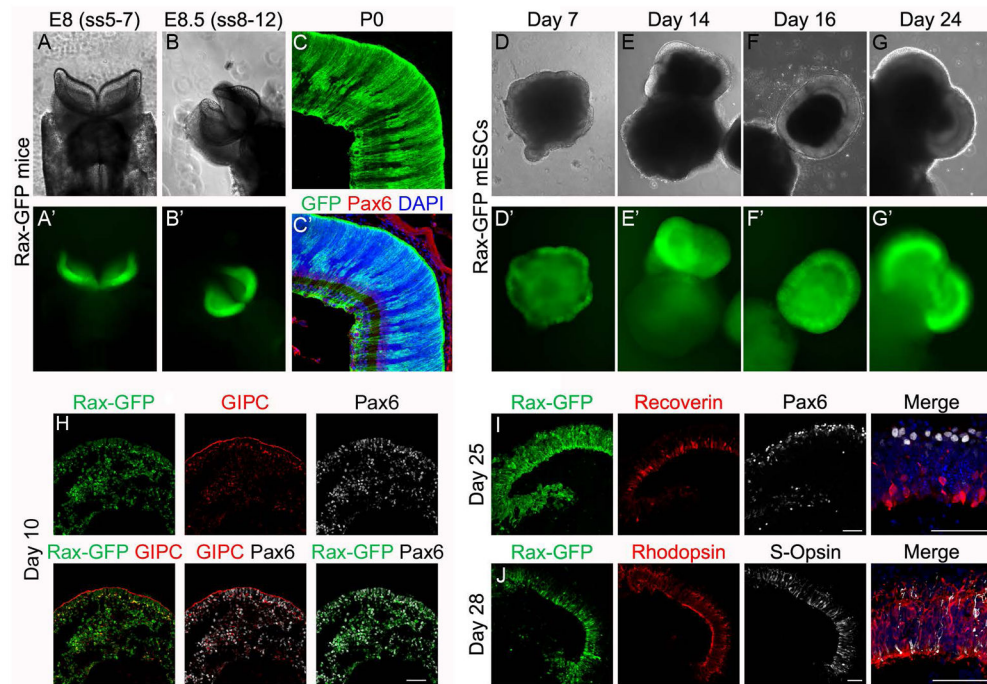
Figure 6. Characterization of Rax expression during early development; mESCs derived from Rax-GFP mice differentiate into laminated retina

Whole mount immunostaining of embryonic day 8 (A, A′) and 8.5 (B, B′), showed Rax promoter driven-GFP expression in the area of the future retina. In postnatal day 0 retinas, Rax was expressed in progenitors, some cones and Müller glia (C–C′). P0 retinas were also immunanostained with Pax6 (red) and DAPI (blue, C′). (D–J) Characterization of Rax-GFP expression in mESCs derived from Rax-GFP mice and differentiated using the 3D protocol. GFP expression was first observed after 7 days of retinal differentiation and was continually expressed throughout differentiation. Live images of EBs were taken at (D, D′) Day 7, (E, E′) Day 14, (F, F′) Day 16, and (G, G′) Day 24. (H) EBs of Rax-GFP mESCs were differentiated for 10 days and immunostained with GFP, GIPC (red), and Pax6 (white). (I) After 25 days of differentiation, GFP⁺ areas expressed Recoverin (red) and Pax6 (white), and were laminated. (J) At Day 28, the GFP⁺ regions (green) also expressed Rhodopsin (red) and S-Opsin (white). Scale bar: 50 microns.
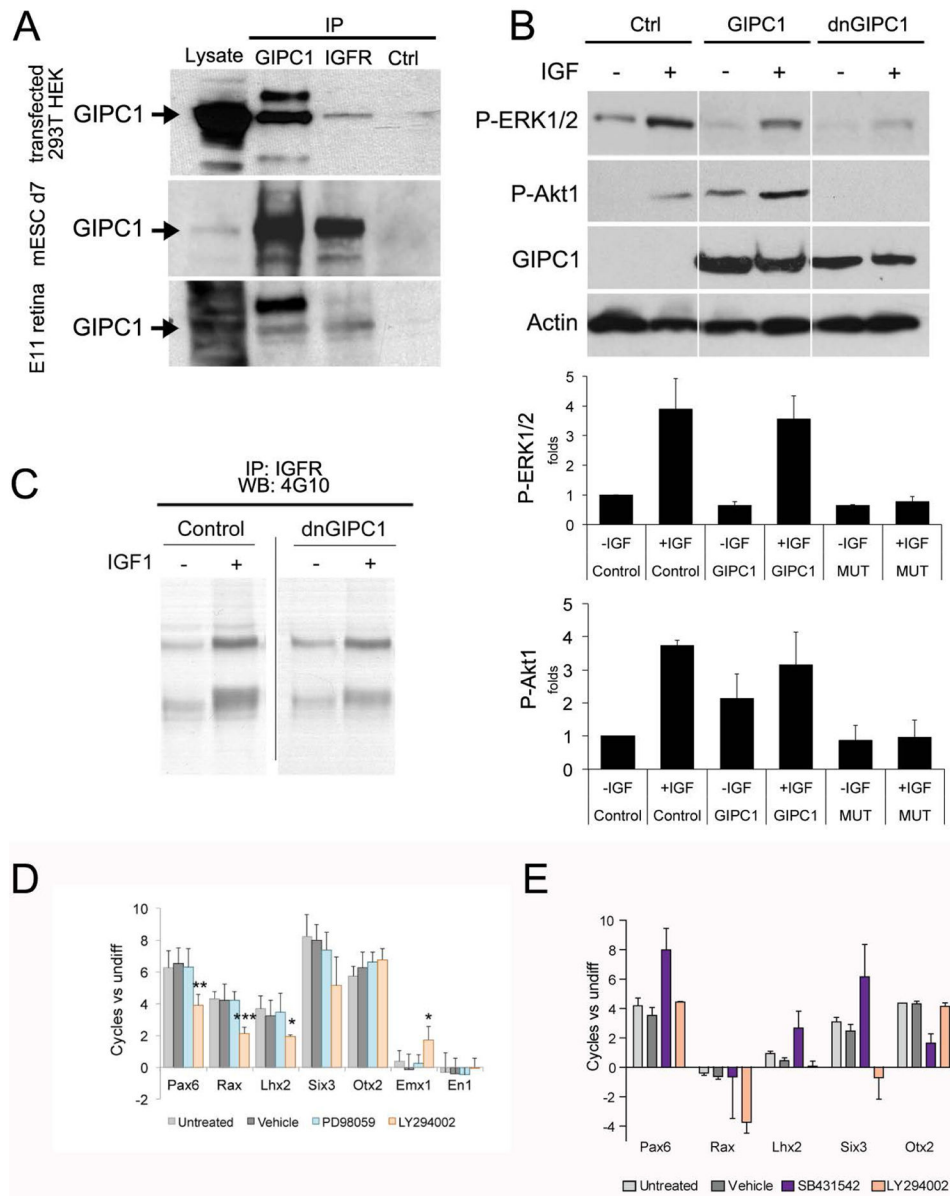
Figure 7. GIPC1 interacts with IGFR in retinal progenitors and inhibition of PI3K-Akt1 pathway reduces mESCs retinal differentiation

(A) Western blot analyses of GIPC1 of cell lysates from either transfected HEK 293T cells (upper panel), mESCs differentiated for 7 days (middle panel) or E11 retinas (bottom panel). Samples were immunoprecipitated with GIPC1, IGF1R antibodies or a GFP antibody as a control. Arrows indicate GIPC1 band. (B) IGF1-dependent phosphorylation of MAPK ERK1/2 and Akt1 proteins in HEK 293T transfected with pRK5 empty vector (lanes 1–2), GIPC1 (lanes 3–4) or dnGIPC1 (lanes 5–6). Western blot analyses of phospho-ERK1/2 (upper panel), phospho-Akt1 (second panel), GIPC1 (third panel) and Actin loading control (bottom panel). Densitometric analyses of 4 independent experiments (normalized to untreated samples) showing IGF1-dependent phosphorylation of ERK1/2 and Akt1. (C) IGF1-dependent phosphorylation of IGFR was analyzed by immunoprecipitation

experiments with IGF1R antibodies followed by Western blot against anti-phosphoTyrosine (4G10) of HEK 293T cells transfected with either a control empty plasmid (pRK5, first two lanes) or dnGIPC1 (lanes 3–4). (D) RT-qPCR analyses of cells differentiated for 5 days using the 2D culturing method in control conditions (light gray bars), treated with DMSO vehicle (dark gray bars), with MAPK inhibitor (blue bars) or Akt1 inhibitor (orange bars). Data is represented as the relative change (ddCT) between undifferentiated cells and cultures differentiated for 5 days and indicates mean ± SEM. P-values: * < 0.05, ** < 0.01, *** < 0.001. (E) RT-qPCR analyses of EFTFs in cells differentiated for 5 days using the 3D culturing method under control conditions (light gray bars), with DMSO vehicle (dark gray bars), with TGF-β receptor inhibitor (purple bars) and Akt inhibitor (orange bars). Data is represented as the relative change (ddCT) between undifferentiated cells and 5 days of retinal differentiation. Mean ± SEM.