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Article

IGF-1 Activates a Cilium-Localized Noncanonical Gβγ Signaling Pathway that Regulates Cell-Cycle Progression

Celine Yeh,[1,5] Aiqun Li,[1,5,6] Jen-Zen Chuang,[1] Masaki Saito,[1,3] Alfredo Cáceres,[4] and Ching-Hwa Sung[1,2,*]
[1]Margaret M. Dyson Vision Research Institute, Department of Ophthalmology
[2]Department of Cell and Developmental Biology
Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10065, USA
[3]Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba 6-3, Aramaki, Aoba-ku, Sendai 980-8578, Japan
[4]INIMEC-CONICET, Avenue Friuli 2434, 5016 Cordoba, Argentina
[5]These authors contributed equally to this work
[6]Present address: New York Stem Cell Foundation Research Institute, 3960 Broadway, New York, NY 10032, USA
*Correspondence: chsung@med.cornell.edu
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SUMMARY

Primary cilia undergo cell-cycle-dependent assembly and disassembly. Emerging data suggest that ciliary resorption is a checkpoint for S phase reentry and that the activation of phospho(T94)Tctex-1 couples these two events. However, the environmental cues and molecular mechanisms that trigger these processes remain unknown. Here, we show that insulin-like growth-1 (IGF-1) accelerates G_1-S progression by causing cilia to resorb. The mitogenic signals of IGF-1 are predominantly transduced through IGF-1 receptor (IGF-1R) on the cilia of fibroblasts and epithelial cells. At the base of the cilium, phosphorylated IGF-1R activates an AGS3-regulated Gβγ signaling pathway that subsequently recruits phospho(T94)Tctex-1 to the transition zone. Perturbing any component of this pathway in cortical progenitors induces premature neuronal differentiation at the expense of proliferation. These data suggest that during corticogenesis, a cilium-transduced, noncanonical IGF-1R-Gβγ-phospho(T94)Tctex-1 signaling pathway promotes the proliferation of neural progenitors through modulation of ciliary resorption and G_1 length.

INTRODUCTION

Primary cilia are hair-like sensory organelles that appear on G_1/G_0 cells and are disassembled prior to S phase (Tucker et al., 1979). Although mutations in almost all identified genes involved in ciliary assembly have been associated with ciliopathies (Lee and Gleeson, 2011; Waters and Beales, 2011), relatively little is known about how cilia are disassembled. Recent studies have begun to identify proteins that are involved in ciliary resorption. For example, nuclear distribution gene E homolog 1 (Nde1) negatively regulates ciliary length through its interaction with dynein light chain, LC8 (DYNLL1). Cells depleted of Nde1 develop abnormally long cilia, which leads to a delay in cell-cycle reentry (Kim et al., 2011). Our previous results showed that yet another dynein light-chain protein, Tctex-1 (or DNYLT1), is phosphorylated at Thr94 and recruited to the ciliary transition zone (TZ) prior to S phase reentry (Li et al., 2011). Suppression of phospho(T94) Tctex-1 resulted in the inhibition of both serum-induced ciliary resorption and S phase progression. This reduction in cell-cycle reentry was not observed in nonciliated cells. Conversely, overexpression of a phosphomimic mutant of Tctex-1 (i.e., T94E) accelerated the resorption of cilia and reduced G_1 length (Li et al., 2011). Because phospho(T94)Tctex-1 does not bind to the dynein motor complex (Chuang et al., 2005), the role of Tctex-1 in ciliary resorption and cell cycling is dynein-independent. These data collectively suggest that ciliary resorption is a precursor of S phase entry. Furthermore, the time required for a cilium to resorb is a key determinant of G_1 length.

Radial glia (RG), the proliferating progenitors of the developing neocortex, also have cilia. It has been shown that G_1 length causally influences the fate choice of RG. Shortening G_1 accelerates cell-cycle entry and increases the proliferation rate of RG, whereas lengthening G_1 drives these cells to differentiate into neurons (Calegari and Huttner, 2003; Lange et al., 2009; Pilaz et al., 2009). These findings make the developing cortex a suitable model for investigating the physiological relevance of ciliary dynamics to cell-cycle progression. Our previous results showed that the ciliary TZ expression of phospho(T94)Tctex-1 in RG plays an important role in modulating G_1 length and maintaining the proliferating progenitor population (Li et al., 2011). Inhibiting the expression of phospho(T94)Tctex-1 caused RG to exit the cell cycle prematurely and differentiate into neurons. However, the environmental cues and signal transduction pathways that lead to the expression of phospho(T94)Tctex-1 in the developing brain are unknown.

Platelet-derived growth factor (PDGF) has been shown to be able to induce ciliary resorption by itself (Christensen et al., 2007; Pugacheva et al., 2007; Tucker et al., 1979), but the mechanism by which PDGF receptor activation causes ciliary resorption remains unclear. Previous studies have shown that PDGF-AA-mediated mitogenesis requires functional insulin growth factor 1 receptor (IGF-1R) (Mason and Goldman, 2002;

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Novosyadlyy et al., 2006). IGF-1, a ligand of IGF-1R, is a potent mitogen that accelerates G_1/S progression (Hodge et al., 2004; Popken et al., 2004; Ye and D'Ercole, 2006). Although IGF-1R is conventionally known as a tyrosine kinase receptor (Ullrich et al., 1985), it also has noncanonical G protein coupled receptor (GPCR) activity (Dalle et al., 2001; Hallak et al., 2000; Luttrell et al., 1995). Expression of a scavenger of Gβγ subunits, βARK-ct (Koch et al., 1994), inhibits IGF-1-stimulated mitogenesis (Dalle et al., 2001; Luttrell et al., 1995).

Tctex-1 is synonymous with Activator of G protein Signaling 2 (AGS2), which was first isolated as a GPCR-independent activator of G protein signaling through a functional screen in yeast (Takesono et al., 1999). Activator of G protein Signaling 3 (AGS3), another protein involved in noncanonical Gβγ signaling, was isolated in the same screen (Takesono et al., 1999). Tctex-1 binds directly to the Gβ subunit of heterotrimeric G proteins; Gβγ competes with dynein intermediate chain for Tctex-1 binding in order to regulate the availability of dynein-free Tctex-1 (Sachdev et al., 2007; Takesono et al., 1999). AGS3, on the other hand, binds to $G\alpha_i$. The GoLoco/G protein regulatory (GPR) motifs of AGS3 stabilize the GDP-bound form of $G\alpha_i$, preventing its reassociation with Gβγ (Ghosh et al., 2003; Oner et al., 2010; Peterson et al., 2000). Similar to Tctex-1 silencing (Li et al., 2011), the inhibition of Gβγ activation (via βARK-ct overexpression or AGS3 silencing) pushes cortical progenitors out of division and into differentiation mode (Sanada and Tsai, 2005).

In the present study, we identify and characterize a pathway in which IGF-1 transmits signals through the primary cilium in order to mediate ciliary resorption and cell-cycle entry. This pathway involves the activations of IGF-1R, noncanonical Gβγ signaling, and phospho(T94)Tctex-1 at the base of the cilium.

RESULTS

IGF-1 Induces Ciliary Resorption via the Activation of IGF-1R

Previous studies have confirmed that certain nontransformed cell types (i.e., human retinal pigment epithelial cells [RPE-1], 3T3, and mouse embryonic fibroblasts [MEF]) can be starved to induce quiescence and the formation of cilia. Subsequent readdition of serum triggers biphasic ciliary resorption, which peaks at 2 hr and 24 hr following stimulation (Li et al., 2011; Tucker et al., 1979). The first wave of cilium shortening occurs at mid/late G_1 phase preceding S phase entry, whereas the second wave occurs at the G_2/M transition (Tucker et al., 1979). We first tested whether or not IGF-1 stimulation is able to induce the resorption of cilia in RPE-1 cells. IGF-1 (10 nM) induced ciliary resorption to roughly the same degree as PDGF-AA (50 ng/ml) (Figure 1A), whereas lower concentrations of IGF-1 (1–5 nM) were found to be insufficient (data not shown).

We used three independent methods to verify that IGF-1 mediates ciliary resorption specifically through IGF-1R. First, we demonstrated that IGF-1-induced ciliary resorption was effectively blocked by an IGF-1R neutralizing antibody (Ab), but not by a control (antihemagglutinin) Ab (Figure 1B). Next, we performed ciliary assembly/disassembly assays in cells transfected with constructs encoding small hairpin RNA (shRNA) against IGF-1R and GFP (IGF-1R-sh/GFP), or a construct encoding GFP only (vector control). Fractions of total GFP^+ cells exhibiting cilia were scored at 0 hr, 2 hr, and 24 hr following IGF-1 stimulation (Figure 1C). IGF-1R silencing did not affect the formation of cilia, but significantly inhibited IGF-1-induced resorption of cilia (Figures 1D and 1E). This inhibition was consistently observed in cells transfected with three independent, validated IGF-1R-sh constructs (i.e., confirmed by quantitative RT-PCR (qRT-PCR) to reduce endogenous IGF-1R expression by ~50%), ruling out the possibility of off-target effects (Figure 1E).

Shortly following IGF-1 stimulation, activated IGF-1R (phosphorylated at Tyr1131/1135/1136) (Kato et al., 1994) signals became increasingly enriched at the ciliary transition zone (TZ), located between the γ-tubulin (γ-Tub)-labeled basal body and acetylated-α-tubulin (Ac-Tub)-labeled ciliary axoneme (Figure 1F). Phospho-IGF-1R became prominent at the base of the cilium 2 hr after IGF-1 treatment, a time point that correlates with the first wave of ciliary resorption.

Using an anti-(pan)IGF-1R Ab, we found that IGF-1R localized to both the cell surface (Figure 1G, arrowheads) and primary cilium (Figure 1G, arrows) of almost all RPE-1 cells at various time points before and after IGF-1 stimulation. IGF-1R signals on the cell surface, which often appeared as clusters, were not particularly enriched near the base of the cilium (Figure 1G, arrowheads). The treatment of cells with dynasore blocked dynamin-mediated clathrin-dependent endocytosis (Macia et al., 2006), but did not affect the IGF-1-stimulated appearance of phospho-IGF-1R at the base of cilium (Figure S1 available online). Hence, phospho-IGF-1R at the base of the cilium is not likely to be derived from the translocation of surface IGF-1R via ligand-activated endocytosis. On the other hand, IGF-1R on the ciliary membrane exhibited an uneven spatial distribution pattern, suggesting that these receptors undergo dynamic bidirectional transport (Figure 1G, arrows).

IGF-1 Induces Ciliary Resorption and S Phase Entry by Activating Phospho(T94)Tctex-1 at the Ciliary TZ

The spatial and temporal expression patterns of phospho-IGF-1R described above are strikingly similar to those of phospho(T94)Tctex-1 (Li et al., 2011). This led us to examine whether or not the ciliary and mitogenic effects of IGF-1 require the downstream activation of phospho(T94)Tctex-1. First, we showed that IGF-1 alone is sufficient to induce the expression of phospho(T94)Tctex-1 at the base of the cilium (Figures 2A and 2B). Second, IGF-1R neutralizing Ab, but not control Ab, was able to suppress the activation of phospho(T94)Tctex-1 at the TZ (Figures 2C and 2D). Third, we showed that cells transfected with Tctex-1-sh/GFP were unable to resorb cilia in response to IGF-1 stimulation (Figure 2E).

At the functional level, we found that the ectopic expression of T94E alone reduced the minimum concentration of IGF-1 that was necessary to induce ciliary resorption from 10 nM to 1 nM (data not shown). Coexpression with T94E, but not T94A (the nonphosphorylatable mutant form of Tctex-1), was able to rescue the defects in ciliary resorption caused by reductions in IGF-1R expression (Figure 2F).

The Mitogenic Signals Provided by IGF-1 Are Transmitted through Primary Cilia and Phospho(T94)Tctex-1

Using a 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay (Figure 3A), we found that Tctex-1 suppression significantly blocked

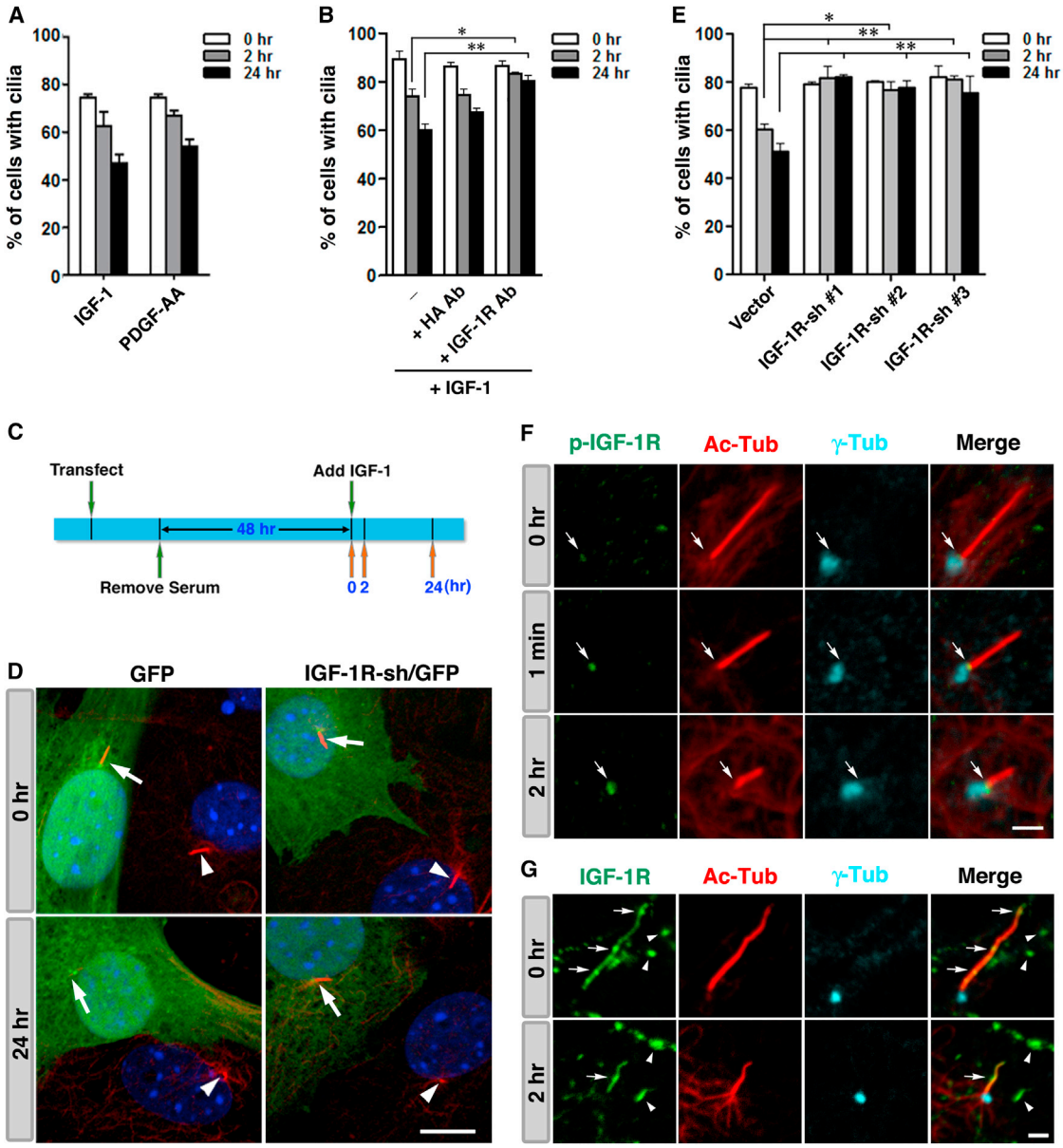


Figure 1. IGF-1 Triggers Ciliary Resorption

(A) Biphasic ciliary resorption profiles of RPE-1 cells treated with IGF-1 or PDGF-AA.

(B) Serum-starved cells were treated with or without 1 μg/ml of anti-IGF-1R αIR3 Ab or antihemagglutinin (HA) Ab 15 min prior to and during IGF-1 treatment.

(C) A timeline depicting the sequence of events in a typical ciliary assembly/disassembly assay involving gene manipulation.

(D) Representative images of 3T3 cells transfected with IGF-1R-sh/GFP or GFP alone at 0 hr and 24 hr following IGF-1 treatment. Arrows and arrowheads point to the cilia in GFP^+-transfected and GFP^--nontransfected cells, respectively. Scale bar represents 10 μm.

(E) Percentages of GFP^+-transfected cells displaying cilia.

(F) Immunolabeling of phospho-IGF-1R (green), Ac-Tub (red), and γ-Tub (cyan) in RPE-1 cells immediately after serum deprivation (0 hr), 1 min, and 2 hr after IGF-1 addition. Arrows point to the ciliary TZ. Scale bar represents 2 μm.

(G) Immunolabeling of (pan)IGF-1R (green), Ac-Tub (red), and γ-Tub (cyan) in RPE-1 cells immediately after serum deprivation (0 hr) and 2 hr after IGF-1 addition. Arrows and arrowheads point to ciliary and plasma membrane localizations of IGF-1R, respectively. Scale bar represents 2 μm. Data are presented as mean ± SEM (**$p < 0.01$; *$p < 0.05$; one-way ANOVA; n = 3 experiments).

See also Figure S1.

IGF-1-induced S phase entry. This phenotype was effectively rescued by coexpression with T94E, but not T94A (Figure 3B), suggesting that IGF-1 triggers S phase entry through a pathway that involves the downstream activation of phospho(T94)Tctex-1.

To assess whether or not IGF-1 transmits its mitogenic signals through primary cilia, we examined the rates of IGF-1-induced S phase progression in two IFT mutant cell lines—RPE-1 stable cell line with *Ift20* suppressed (Follit et al., 2006) and *Ift88* mutant

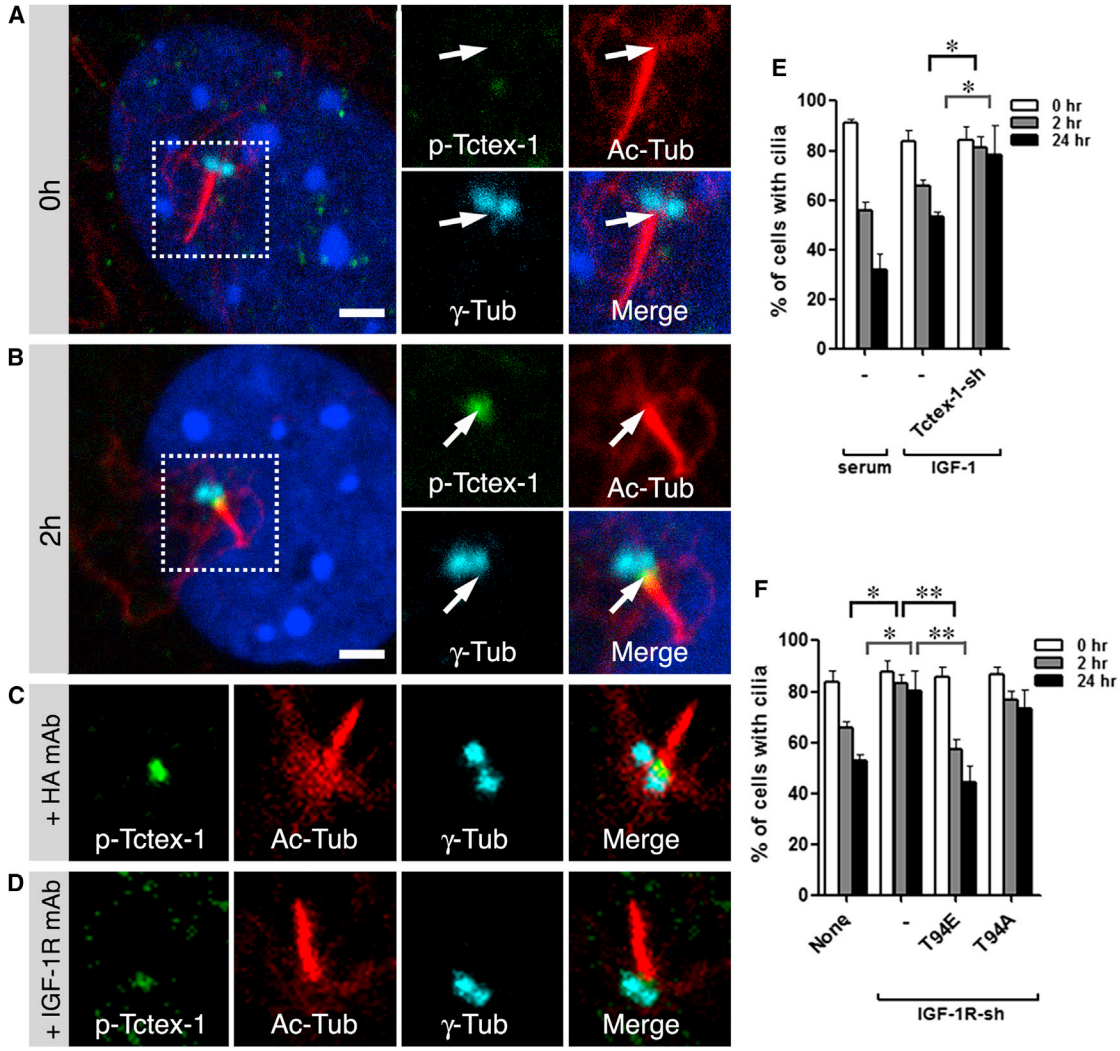


Figure 2. Phospho(T94)Tctex-1 Is a Downstream Effector of IGF-1-Induced Ciliary Resorption

(A and B) Representative images of cells immunolabeled for phospho(T94)Tctex-1, Ac-Tub, and γ-Tub in quiescent RPE-1 cells (A; 0 hr) or IGF-1-treated cells (B; 2 hr). Scale bars represent 2 μm.

(C and D) Representative images of cilia in IGF-1 stimulated RPE-1 cells incubated with control HA Ab (C) or anti-IGF-1R αIR3 Ab (D) and immunolabeled with indicated Abs.

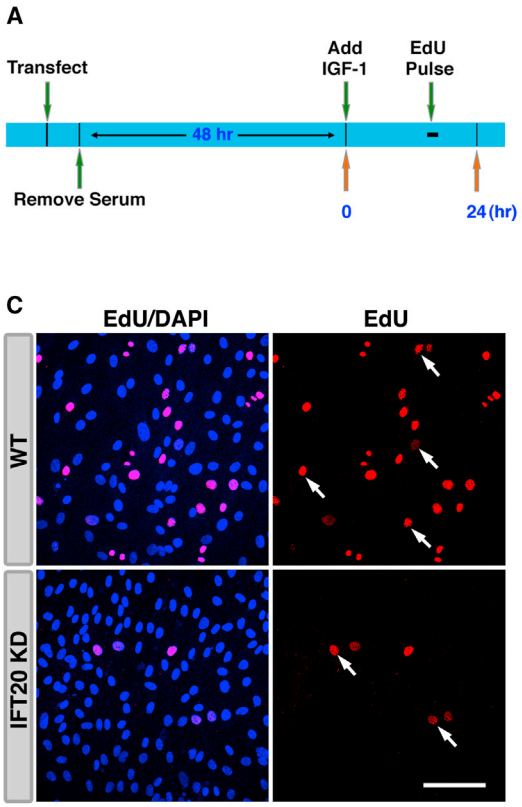
(E) Percentages of control- and Tctex-1-sh/GFP-transfected 3T3 cells displaying cilia after serum or IGF-1 treatments.

(F) Biphasic ciliary resorption profiles of IGF-1-treated cells that were nontransfected, or transfected with IGF-1R-sh/GFP alone (–) or together with Tctex-1 mutant T94E or T94A. Data are presented as mean ± SEM (**p < 0.01; *p < 0.05; one-way ANOVA; n = 3 experiments).

MEF cells derived from *Ift88*$^{\Delta 2-3\beta Gal}$ mice (Jia et al., 2009; Murcia et al., 2000), both of which are unable to form cilia (Jia et al., 2009; Li et al., 2011; Murcia et al., 2000). Significantly fewer IFT mutant cells were able to enter S phase in response to IGF-1 relative to their parental, wild-type counterparts (Figures 3C and 3D). These results suggest that IGF-1-mediated mitogenic signaling requires the presence of a functional cilium.

A Noncanonical Gβγ Signaling Pathway Activates Phospho(T94)Tctex-1 Prior to the Resorption of Cilia

Previous studies have shown that IGF-1 binding leads to an increase in association between IGF-1R and $G\alpha_i^{GDP}$, which sustains the intracellular pool of free Gβγ (Dalle et al., 2001; Hallak et al., 2000; Luttrell et al., 1995). It is possible that this pool of Gβγ is important for generating the dynein-free Tctex-1 that is necessary for Thr94 phosphorylation (Sachdev et al., 2007). Because IGF-1R has no reported guanine nucleotide exchange (GEF) factor activity toward $G\alpha_i$, we surmise that downstream to the activation of IGF-1R, AGS3 might play a role in stabilizing $G\alpha_i^{GDP}$ to maintain free Gβγ (Takesono et al., 1999). In support of this model, GFP-IGF-1R, but not GFP alone, coimmunoprecipitated with AGS3 in transfected 293T cells that coexpressed $G\alpha_i$ (Figure 4A). Furthermore, Gβ (Figure 4B) and AGS3 (Figure 4C) were also concentrated at the base of the cilium (closely apposing the γ-Tub-labeled basal body) at both 0 hr and 2 hr time points in RPE-1 cells.



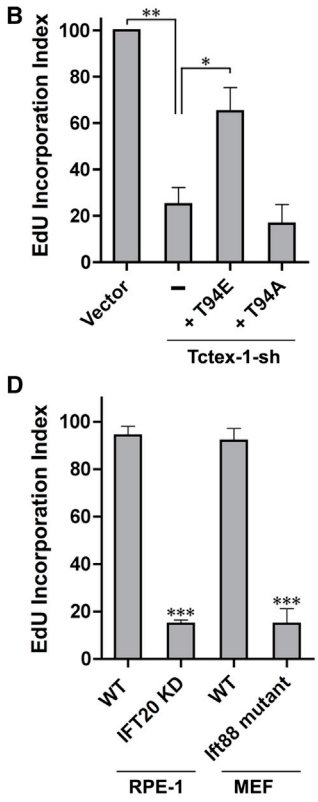


Figure 3. The Mitogenic Signals of IGF-1 Are Transduced through Cilia and Are Phospho(T94)Tctex-1 Dependent

(A) Timeline depicting the sequence of events in a typical S phase entry EdU experiment.

(B) Quantification of EdU incorporation in cells transfected with indicated plasmids. Incorporation of EdU in GFP control-transfected cells was taken to be 100%.

(C) Representative images of EdU-labeled WT and IFT20 KD RPE-1 cells after 16 hr of IGF-1 treatment. Red, EdU; blue, DAPI; arrows, examples of EdU positive nuclei. Scale bar represents 100 μm.

(D) Quantification of EdU incorporation in indicated cell types. For each cell type, incorporation of EdU in parental (WT) cells was taken to be 100%. Data are presented as mean ± SEM (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; one-way ANOVA; n = 3 experiments).

See also Figure S4.

At the functional level, we performed ciliary assembly/disassembly assays in cells depleted of free Gβγ subunits via the transfection of either βARK-ct/GFP or AGS3-shRNA/GFP. Compared to GFP-transfected control cells, fewer cells transfected with either construct were able to form cilia following 48 hr of serum starvation (Figure 4D), indicating that Gβγ signaling is important for ciliogenesis. However, the cilia formed in these cells failed to resorb upon treatment with serum (Figure 4D). Cotransfection with T94E, but not T94A, was able to specifically rescue the defects in biphasic ciliary resorption (but not ciliogenesis) caused by Gβγ removal (Figure 4D).

Finally, we observed that the intensity of phospho(T94)Tctex-1 labeling at the ciliary TZ was significantly weaker in cells transfected with βARK-ct/GFP or AGS3-sh/GFP than in neighboring nontransfected GFP^- cells following serum treatment (Figures 4E–4G). Taken together, these data suggest that depleting free Gβγ inhibits the downstream TZ activation of phospho(T94) Tctex-1 and hence, ciliary resorption.

IGF-1R-Mediated Noncanonical Gβγ Signaling Maintains the Proliferating Progenitor Population during Corticogenesis

Previously, we proposed that RG receive cues from the ventricle via primary cilia, allowing for subsequent recruitment of phospho(T94)Tctex-1, which ensures timely ciliary resorption and proliferation (Li et al., 2011) (Figure 5A). A recent study showed that cerebrospinal fluid (CSF) circulating through the ventricles contains IGF-1R ligands (i.e., IGF-1, IGF-2) (Lehtinen et al., 2011). Furthermore, immunoEM reveals prominent IGF-2 signal on the cilia of RG in the developing neocortex (Lehtinen et al., 2011).

We set out to determine whether or not our proposed IGF-1R-Gβγ-phospho(T94) Tctex-1 signaling pathway exhibits the same functional hierarchy in the developing brain as that seen in vitro. First, we found that phospho-IGF-1R was coenriched with phospho(T94)Tctex-1 near the ciliary TZ region of RG (between Arl13b-labeled cilia and γ-Tub-labeled basal bodies) (Figures 5B and S2A). No significant phospho-IGF-1R signal was found in any other region of the cortex (Figure S2B), suggesting that IGF-1R activation occurs predominantly through cilia in vivo as well. Furthermore, using antibodies validated for specificity, we showed that the immunoreactivity of both Gβ and AGS3 were concentrated at the basal body in RG (Figure 5C).

The majority of RG division occurs at the ventricular zone (VZ) (Figure S2C). Postmitotic neuronal progenies migrate away from the VZ, halt temporarily at the intermediate zone (IZ), and get incorporated into laminated cortical plates according to their birthdates (Figure S2C). We performed knockdown and rescue experiments in E13.5 mouse cortices via in utero electroporation (IUE). Approximately 40 hr posttransfection, the majority of RG transfected with GFP alone produced daughter RG, characterized by the expression of brain lipid-binding protein (BLBP) (Figure 5D) and the possession of nuclei distributed between the VZ and IZ (Figure S2D). Similar to Tctex-1 silencing (Figure S2D) (Li et al., 2011), suppression of Gβγ (via the expression of either βARK-ct or AGS3) caused RG to differentiate into $Tuj1^+$ neurons (Figure 5D) that migrated to the IZ (Figures S2E and S2F). Consistently, significantly fewer cells transfected with βARK-ct-IRES-GFP or AGS3-shRNA-IRES-GFP were mitotic (i.e., phosphohistone-3 [PH3]-positive) compared to cells transfected with control vectors (Figures 5E, S2E, and S2F).

RG transfected with IGF-1R-shRNA/GFP behaved almost identically to RG in which Tctex-1 or Gβγ signaling was suppressed. RG with IGF-1R suppressed exhibited significantly lower mitotic indices (Figure 5F) due to their premature exit

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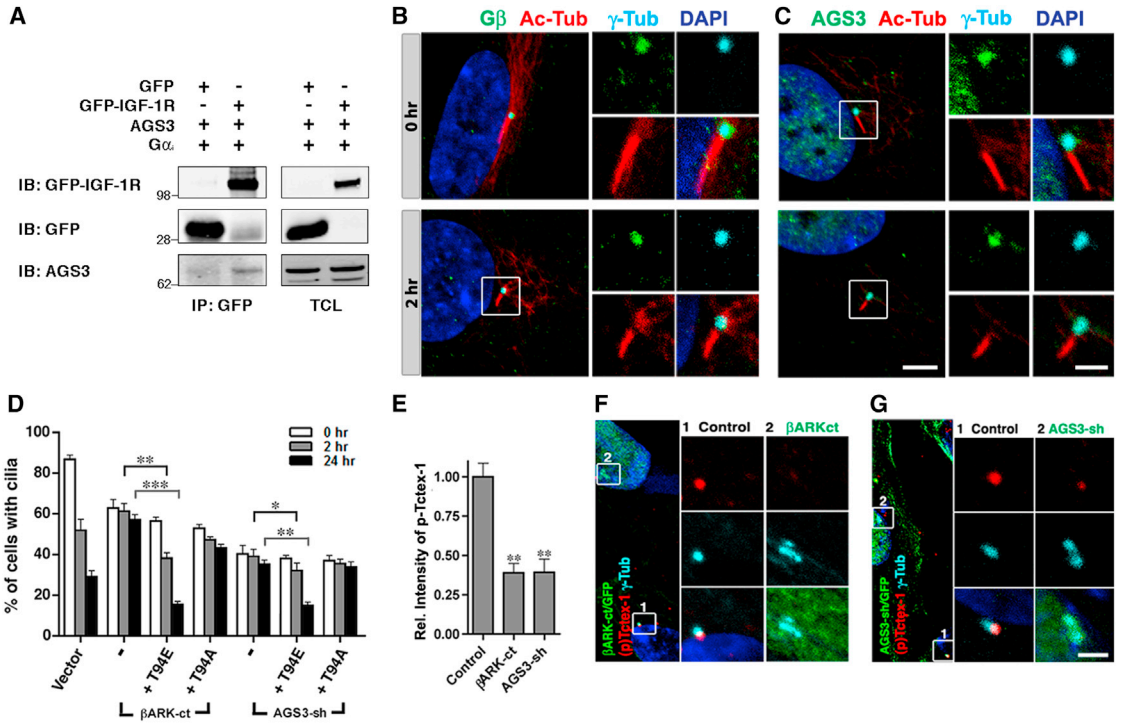

Figure 4. Gβγ Signaling Mediates Ciliary Resorption via the Recruitment of Phospho(T94)Tctex-1 to the Ciliary TZ

(A) Immunoblots of total cell lysates (TCL) or GFP Ab immunoprecipitates of 293T cells transfected with indicated plasmids and then stimulated with IGF-1.

(B and C) Immunolabeling of γ-Tub (cyan), Ac-Tub (red), and Gβ (green in B) or AGS3 (green in C) in RPE-1 cells postserum treatment. Scale bars represent 5 μm (low power panel of C); 2 μm (high power panel of C).

(D) Biphasic ciliary resorption profiles of 3T3 cells transfected with indicated plasmids. Fractions of GFP$^+$ transfected cells displaying cilia at indicated time points following serum addition are shown.

(E) The relative (Rel.) intensity of phospho(T94)Tctex-1 immunolabeling at the ciliary TZ of transfected cells is shown. The immunofluorescence intensity of phospho(T94)Tctex-1 in control nontransfected cells was taken to be 100%.

(F and G) Cells were transfected with βARK-ct/GFP (E) or AGS3-sh/GFP (F), then immunolabeled with phospho(T94)Tctex-1 (red) and γ-Tub (cyan). Columns 1 and 2 are enlarged views of boxed regions 1 and 2, respectively. Scale bar represents 2 μm (G). Data are presented as mean ± SEM (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; one-way ANOVA; n = 3 experiments).

from the cell cycle (Figures S3A and S3C). These cells adopted neuronal fates and migrated to the IZ (Figure S3B).

Remarkably, coexpression of T94E, but not T94A, effectively rescued the majority of phenotypic deviations (e.g., neuron/progenitor ratio, mitotic index) caused by βARK-ct expression (Figures 5D, 5E, and S2E), AGS3 silencing (Figures 5D, 5E, and S2F), or IGF-1R silencing (Figures 5F and S3A–S3C). Finally, we found that the cells with Gβγ or IGF-1R suppressed (that still had their processes contacting the ventricle surface) exhibited significantly weaker phospho(T94)Tctex-1 signals relative to neighboring nontransfected cells (Figures 5G, 5H, and S2G).

DISCUSSION

Our data support a model in which IGF-1 promotes cell-cycle entry by mediating ciliary resorption through a noncanonical Gβγ signaling axis. Figure 6 depicts our proposed stepwise process based on data from our previous and current studies: (1) upon ligand binding, ciliary IGF-1R translocates to the base of the cilium; (2) here, AGS3 binds and stabilizes $G\alpha_i$-GDP bound to activated IGF-1R in order to sustain the release of Gβγ; (3) Gβγ competes with dynein intermediate chain for Tctex-1 binding, generating a pool of dynein-independent Tctex-1; and (4) Tctex-1 is phosphorylated at Thr94 and recruited to the ciliary TZ, where it induces the first wave of ciliary resorption and primes cells to reenter S phase.

Cilia-Dependent versus Cilia-Independent Control of Cell Proliferation

Emerging data suggest that in cycling cells, ciliary disassembly acts as a "checkpoint" to monitor G_1/S progression (Kim et al., 2011; Li et al., 2011). Here, we show that in nontransformed epithelial and fibroblast cells, IGF-1 transmits its proliferative signals primarily through the cilium by modulating its disassembly, which in turn, times the G_1/S transition of the cell. Compromising the formation of cilia in IFT mutant cells eliminates their ability to proliferate in response to IGF-1. This suggests that IGF-1R associated with the ciliary membrane represents the major sensor of IGF-1 in wild-type cells.

Our findings support the theory that cilia are important organelles that transmit mitogenic signals required for cell division (reviewed by Christensen et al., 2007). This leads us to question why IGF-1 induces cell-cycle entry by preferentially activating IGF-1Rs on cilia over those on the cell surface. We speculate

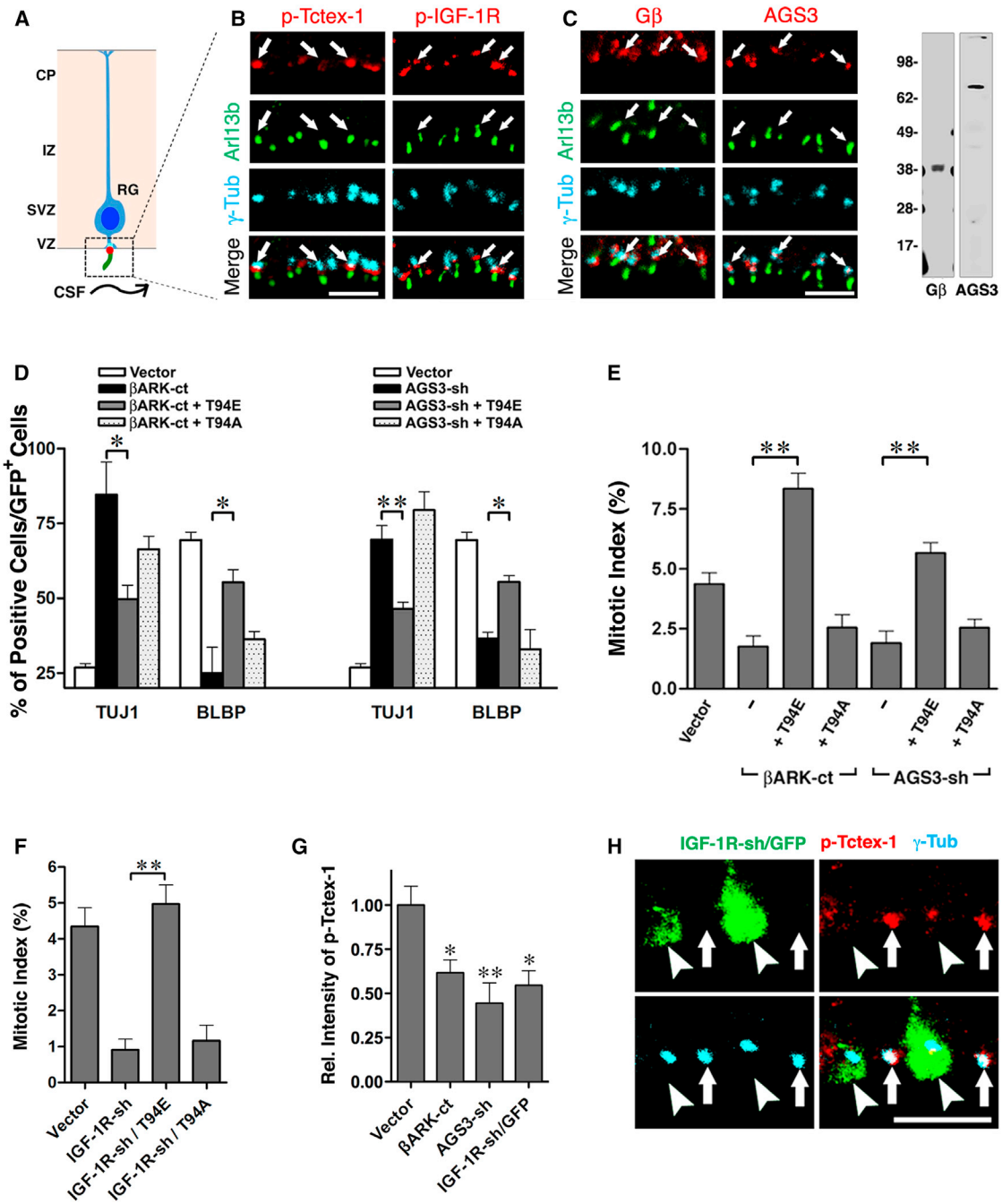

Figure 5. Noncanonical IGF-1R-Gβγ Signaling Pathway Regulates the Recruitment of Phospho(T94)Tctex-1 and Cell Fate Choice of RG

(A–C) Schematic diagram of RG in the developing neocortex. Each RG has a single primary cilium, which projects from its apical endfeet and extends into the ventricles, contacting CSF. Immunolabeling of boxed area is shown in (B) and (C). (B) Enlarged views of ventricle surfaces that are triple labeled with phospho(T94) Tctex-1 (red) or phospho-IGF-1R (red) together with Arl13b (green) and γ-Tub (cyan). (C) Left: enlarged views of the ventricle surfaces immunolabeled with Gβ (red), or AGS3 (red) together with Arl13b (green) and γ-Tub (cyan). Right: Gβ and AGS3 Abs each recognized a single band of expected size in mouse embryonic brain lysates on immunoblots. Scale bars represent 5 μm.

(D) Percentage of total transfected GFP^+ cells that were neurons ($Tuj1^+$) or RG ($BLBP^+$).

(E) Mitotic indices of GFP^+ cells transfected with indicated plasmids. More than 400 cells in four independent animals were scored.

(F) Mitotic indices are presented as fractions of $GFP^+/PH3^+$ out of total GFP^+ cells. More than 600 GFP^+ cells were scored in three independent experiments.

(G) Quantification of the relative (Rel.) intensity of phospho(T94)Tctex-1 of cells transfected with indicated plasmids. The intensity in vector control-transfected cells was taken to be 100%.

(H) Representative images of ventricle surfaces containing IGF-1R-sh/GFP transfected cells. Arrowheads and arrows point to GFP^+ transfected cells and GFP^- nontransfected cells. Scale bar represents 5 μm. Data are presented as mean ± SEM (**$p < 0.01$; *$p < 0.05$; one-way ANOVA).

See also Figures S2 and S3.

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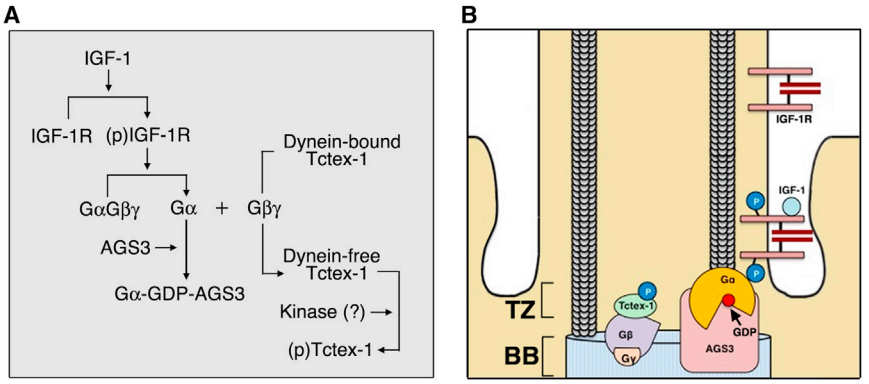

Figure 6. Working Model

(A) Sequential diagram of our proposed signaling pathway. Activated IGF-1R binds to the Gα subunit of the heterotrimeric G protein complex. AGS3 binds and stabilizes GDP-bound Gα. Free Gβγ triggers the release of Tctex-1 from the dynein complex, enabling subsequent phosphorylation of Tctex-1.

(B) Spatial diagram of our working model. BB, basal body.

that the close proximity of ciliary resorption effectors (Li et al., 2011; unpublished data) to centrosomal proteins that are required for S phase entry and that localize to the base of the cilium (Doxsey et al., 2005) is necessary to ensure the temporal coupling of these two cellular events.

Although nonciliated IFT mutant cells lose their ability to respond to IGF-1 (Figures 3C and 3D), it is interesting to note that these mutant cells retain their capacity to enter the cell cycle in response to serum (Figure S4), which contains numerous other growth factors. In fact, these cells entered S phase at a ~2-fold higher rate relative to their wild-type, ciliated counterparts (Figure S4). These results suggest that although IFT mutant cells have lost their ability to sense IGF-1, they respond with higher sensitivity to alternative, serum-derived extracellular cues through signaling pathways that are normally less active in wild-type, ciliated cells. This finding might help to address why cilia have opposing effects on cell growth, depending on the context (Han et al., 2009; Wong et al., 2009).

Finally, many types of cancerous/transformed cells lack cilia (Han et al., 2009; Seeley et al., 2009; Wong et al., 2009; Yuan et al., 2010), but express elevated levels of IGF-1R and its effectors, including insulin receptor substrate and Ras/PI3K/Akt signaling components (Chang et al., 2002; Schubbert et al., 2007; Shimizu et al., 2004). These cells must respond to IGF-1 via cilia-independent signaling pathways transduced by IGF-1Rs on the cell surfaces. In support of this hypothesis, IGF-1 is known to have distinct effects on the growth of normal and transformed cells (Baserga et al., 2003).

Regulation of Ciliary Resorption Is Important for Maintaining Neural Progenitor Population

The cerebral cortex is formed via the coordination between lateral expansion of neural progenitors and their differentiation. During early cortical development, the majority of RG divide to produce identical daughters that expand the mitotic progenitor population, whereas late progenitors tend to adopt neuronal fates. A better understanding of the mechanisms that underlie the switch between self-renewal and neuronal differentiation in RG is essential for elucidating the factors that control brain size (Bond and Woods, 2006). Recent data suggest that CSF-borne IGF-1R ligands are an important source of proliferative cues sensed by receptors on the cilia of RG. Here, a hierarchical signaling cascade involving AGS3 and Gβγ recruits phospho(T94)Tctex-1 to the ciliary TZ in order to control the timing of ciliary resorption and hence, S phase reentry. The expression of IGF-1R ligands in CSF is temporally dynamic, peaking during times of neurogenesis (Lehtinen et al., 2011). This likely represents a mechanism for regulating progenitor population according to stage of development.

Although it remains a technical challenge to quantitatively evaluate the ciliary disassembly of RG in situ, we have shown in the past that inhibiting the expression of proteins known to mediate ciliary resorption (e.g., AurA, HDAC6) (Li et al., 2011) unanimously blocks the S phase re-entry of RG. Furthermore, mouse and human genetics studies have shown that mutations in genes encoding proteins involved in ciliary resorption (e.g., *Nde1*, *Inositol-1,4,5-trisphosphate 5-phosphatase*) are associated with microcephaly (Bakircioglu et al., 2011; Bielas et al., 2009; Jacoby et al., 2009). Microcephaly and mental retardation have also been reported in patients with mutations in *Igf1* or *Igf1R* (Walenkamp et al., 2005; Walenkamp and Wit, 2006). Last, the human Tctex-1 (*TCTEL1*) gene has been mapped to a chromosomal region that is deleted in a subset of patients with mental retardation (Watanabe et al., 1996). Taken together, these findings suggest that the regulation of ciliary dynamics in RG plays an important role in determining the population of proliferating progenitors and hence, the overall size of the brain.

Perspectives

IGF-1R signaling is highly conserved across species and is responsible for a diverse set of physiological roles including lifespan, growth, and metabolism. Interestingly, it has been shown that lifespan regulation of *Caenorhabditis elegans* depends on both the IGF-1R homolog DAF-2 and sensory cilia (Apfeld and Kenyon, 1999). Additionally, cilium-dependent IGF-1R signaling is required to induce the differentiation of preadipocytes (Zhu et al., 2009). Collectively, these and our present studies lead us to speculate that cilia-associated IGF-1R signaling mediates a large and diverse repertoire of cellular functions, some of which have yet to be identified.

EXPERIMENTAL PROCEDURES

Reagents

Primary Abs used were: IGF-1R neutralizing mouse Ab αIR3 (Kull et al., 1983) (EMD Millipore); hemagglutinin Ab (Abcam), IGF-1Rβ subunit rabbit Ab (for immunostaining; Santa Cruz, SC713), phospho-IGF-1R Ab (Tyr1131/1135/1136; Abcam), Ac-Tub mouse IgG_{2b} (Sigma), AGS3 Ab (gift of Joe Blumer) (Blumer et al., 2002), Gβ rabbit Abs (clone T20; Santa Cruz and gift of Tom Sakmar), Arl13b rabbit Ab (gift from Kathryn Anderson) (Caspary et al., 2007), BLBP rabbit Ab (gift from Nathaniel Heintz) (Feng et al., 1994), BrdU rat Ab (Harlan),

dynein intermediate chain mouse Ab (EMD Millipore), GFP rabbit Ab (Invitrogen), GFP chicken Ab (Abcam), GFP mouse Ab (EMD Millipore), γ-Tub mouse Ab (clone GTU-88, IgG_1; Sigma), Ki67 rabbit Ab (Novocastra), Nestin mouse Ab (clone 401, Developmental Studies Hybridoma Bank), P-H3 rabbit Ab (EMD Millipore), antiphospho(T94)Tctex-1 rabbit Ab (Li et al., 2011), and Tuj1 mouse Ab (Covance). Alexa-conjugated streptavidin or secondary Abs (anti-mouse, anti-rabbit, anti-rat, anti-IgG_{2b}) were purchased from Invitrogen; biotinylated anti-IgG_1 and Cy5-conjugated streptavidin were purchased from Jackson Laboratory (used as 1:200). In this study, negative controls involved omission of the primary Ab or use of Ab that had been preabsorbed with antigen. Colabeling of Ac-Tub (mouse IgG_{2b}) and γ-Tub (mouse IgG_1) in this study involved using isotype-specific mouse IgGs.

All plasmids used for overexpression experiments in cultures were under the CMV promoter. The expression constructs used in IUE were cloned into pCAG vector (gift of Dr. C. Cepko). All knockdown constructs were in pU6 vector (gift of Dr. Y. Shi) (Xia et al., 2003). Plasmid encoding human or mouse Tctex-1-sh, control-sh, WT, T94E, or T94A (in pCAG-IRES-GFP) have been described (Li et al., 2011). βARK-ct (gift from R.J. Lefkowitz) (Koch et al., 1994), AGS3 (gift from J. Blumer) (Blumer et al., 2003), GFP-(human)IGF-1R (gift from Drs. Le Roith and Santigo Quiroga) (Buckley et al., 2002), AGS3-sh (gift from Dr. L.-H. Tsai) (Sanada and Tsai, 2005), and IGF-1R-sh-IRES-GFP were also used. IGF-1R shRNA 3 was used for the majority of our experiments. The targeting sequencing of IGF-1R is 5′-GGGAATGGGTCGTGGACAGAT.

Cell Culture, Transfection, Cilium Assembly/Disassembly Assay, EdU Incorporation Index, and Coimmunoprecipitation

IFT20 KD-RPE-1 (Follit et al., 2006) was a gift from G. Pazour (University of Massachusetts Medical School). Wild-type MEF and *Ift88* mutant MEF (*Ift88*$^{\Delta 2-3\beta Gal}$) cells were gifts from A. Liu (Pennsylvania State University) (Jia et al., 2009). All cells were transfected using nucleofection (Amaxa); cells receiving more than one plasmid were routinely immunolabeled to confirm high (>90%) double or triple transfection efficiency. Standard methods were used for immunoprecipitation and immunoblotting. Immunoblots were quantified using the Odyssey Infrared system (LI-COR). A cilium assembly/disassembly assay was performed exactly as previously described (Pugacheva et al., 2007). Briefly, 18 hr posttransfected or nontransfected cells were starved in serum-free medium for 48 hr to induce cilium formation (and gene expression). Assay medium containing either 10% serum, 10 nM IGF-1 (Sigma), or 50 ng/ml PDGF-AA (EMD Millipore) was then added to the cells to induce ciliary resorption. Cells were harvested at various time points for immunolabeling. In neutralization experiments, Abs (1 μg/ml) were added to cells at the time of growth factor addition and replenished once 12 hr postserum addition.

To quantify EdU incorporation, experiments were carried out in synchronized cells. Posttransfected cells (18 hr) were growth-arrested by a 48 hr serum-starvation period. Cells were then cultured in regular medium for 12–16 hr and pulse-labeled with 1 hr EdU (10 μM) followed by a 7–11 hr chase. These cells were subsequently stained using a CLICK-it reaction following manufacturer's instructions (Invitrogen). Coimmunoprecipitation was carried out in 293T cells that were transfected with indicated plasmids, serum starved, and then treated with IGF-1 for 12 hr. Harvested cell lysates were subjected to immunoprecipitation using anti-GFP antibody coupled on Protein G Dynabeads (Invitrogen). Immunoblots of immunoprecipitates were quantified using the Odyssey Infrared system.

IUE, Immunohistochemistry of Mouse Brain Slices, and Quantitative Analyses

IUE procedures were performed on E13.5 CD1 mouse brains as described (Li et al., 2011). At specific time points, electroporated brains were harvested, fixed with 4% paraformaldehyde overnight at 4°C, embedded in low melting agarose, and sectioned by vibratome. Sectioned brain slices were subsequently subjected to immunostaining using standard protocols. For the colabeling of phospho(T94)Tctex-1 and phospho-IGF-1R, sections were first incubated with Ab against phospho(T94)Tctex-1, followed by excess biotinylated goat anti-rabbit Ab. The sections were then paraformaldehyde fixed, incubated with anti-phospho-IGF-1R Ab and γ-Tub Abs, and detected using Alexa488-conjugated anti-rabbit, Cy5-conjugated anti-mouse Abs, and Alexa568 conjugated streptavidin. Analysis of the immunostained sections was carried out on a Leica TCS SP2 spectral confocal system. All the quantification studies were carried out in transfected cells localized within the dorsolateral neocortex to avoid possible variation within particular brain regions. At least three independent brains were analyzed for each DNA construct. In order to improve observer objectivity, image capture and analysis were done at separate times in a double-blind fashion. To score images, the GFP channel was first judged independently, followed by judgments of the other markers. For Ki67 and BrdU nuclear labeling, small punctae or signals that were not compliant with the DAPI nuclear labeling were ignored. All animal manipulations were performed in accordance with the guidelines for animal experiments established by the Institutional Animal Care and Use Committees of Weill Medical College of Cornell University.

Mitotic Index, Cell-Cycle Exit, and Cell-Cycling Analyses in Brain Slices

The mitotic index of treated brain slices was determined by the ratios of GFP and PH3 double-positive cells to total GFP^+ transfected cells in the VZ/subventricular zone. Cell-cycle analysis was performed as described (Li et al., 2011). Briefly, pregnant mice were injected with BrdU (50 mg/kg body weight) 24 hr after IUE, and fetal brains were harvested 24 hr after BrdU treatment. Brain slices were then immunolabeled for GFP, BrdU, and Ki67. The cell-cycle exit index of GFP-transfected cells was determined as the ratio of GFP-labeled cells that exited the cell cycle (GFP^+, $BrdU^+$, and $Ki67^-$) to total GFP-labeled cells with BrdU incorporation (GFP^+ and $BrdU^+$).

Statistical Analyses

Statistical analyses were performed with GraphPad software (GraphPad Prism v4.0, GraphPad Software). Data are presented as the mean ± SEM from at least three representative independent experiments. T test was used for the comparison of two groups. ANOVA was applied for the comparisons in which only one independent variable was being analyzed. The Dunnett's test (as a post hoc test) was used to compare data samples versus control. Statistical significance was defined as $p < 0.05$, 0.01, or 0.001.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.07.014.

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