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HB-EGF Signaling is Required for Glucose-Induced Pancreatic β-Cell Proliferation in Rats

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ABBREVIATIONS

3C	Chromosome conformation capture
BTC	betacellulin
ChIP	Chromatin immunoprecipitation
ChREBP	Carbohydrate response element binding protein
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FACS	Fluorescent activated cell sorting
GIR	Glucose infusion rate
HB-EGF	Heparin-binding epidermal growth factor-like growth factor
mTORC1	Mammalian target of rapamycin complex 1
TSS	Transcriptional start site

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ABSTRACT

The molecular mechanisms of β-cell compensation to metabolic stress are poorly understood. Previously we observed that nutrient-induced β-cell proliferation in rats is dependent on epidermal growth factor receptor (EGFR) signaling. The aim of this study was to determine the role of the EGFR ligand heparin-binding epidermal growth factor-like growth factor (HB-EGF) in the β-cell proliferative response to glucose, a β-cell mitogen and key regulator of β-cell mass in response to increased insulin demand. We show that exposure of isolated rat islets to HB-EGF stimulated β-cell proliferation, whereas inhibition of EGFR or HB-EGF blocked the proliferative response not only to HB-EGF but also to glucose. Furthermore, knockdown of HB-EGF in islets blocked β-cell proliferation in response to glucose ex vivo and in vivo in transplanted glucose-infused rats. Mechanistically, we demonstrate that HB-EGF mRNA levels were increased in β cells in response to glucose in a carbohydrate response element binding protein (ChREBP)-dependent manner. In addition, chromatin-immunoprecipitation studies identified ChREBP binding sites in proximity to the HB-EGF gene. Finally, inhibition of Src family kinases, known to be involved in HB-EGF processing, abrogated glucose-induced β-cell proliferation. Our findings identify a novel glucose/HB-EGF/EGFR axis implicated in β-cell compensation to increased metabolic demand.

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INTRODUCTION

In obesity, the maintenance of glucose homeostasis is critically dependent on the capacity of the pancreatic β cell to meet the increased insulin requirements that arise due to insulin resistance. Failure of this mechanism leads to type 2 diabetes (1). Hence, understanding how the β cell compensates for insulin resistance is a critical prerequisite to defining the pathogenesis of type 2 diabetes.

B-cell compensation involves both an increase in the capacity to secrete insulin and an increase in mass. In adult rodents, β-cell expansion arises primarily from the replication of existing β cells (2; 3). Over the last decade, modelling metabolic stress in rodents has led to the identification of an array of factors including insulin receptors (4), neurotransmitters (5), epidermal growth factor receptors (EGFR) (6), serpinB1 (7) and nutrients (8) that control β-cell proliferation. Prominent among these factors, glucose controls β-cell replication in both rodent (9-12) and human (13) islets. Glucose-induced β-cell proliferation requires glucokinase (10; 11), the enzyme catalyzing the first committed step in glucose metabolism. Although ATP-sensitive potassium channel closure and membrane depolarisation are also involved (11), the mitogenic effects of glucose appear to be independent of insulin receptor signaling (12). Instead, evidence supports a role of Insulin Receptor Substrate 2 (IRS2), Mammalian Target of Rapamycin (mTOR) (12) and the Carbohydrate-responsive element–binding protein (ChREBP) isoforms α/β (14; 15). ChREBP is a glucose sensing transcription factor that binds DNA with its partner, Mlx, at carbohydrate response elements to stimulate glucose-responsive genes (16). Despite this progress, the precise mechanisms underlying glucose-induced β-cell proliferation remains debated.

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We established an *in vivo* model of nutrient excess in rats, in which a 72-h co-infusion of glucose and a lipid emulsion triggers a marked increase in β-cell proliferation and mass (17). Subsequent studies identified a signaling cascade involving the EGFR-mTOR-FoxM1 that underlies the β-cell response to nutrient infusion (18). In support of these finding, EGFR loss-of-function prevents compensatory β-cell mass expansion in adult rodents under conditions of physiological (pregnancy) and pathophysiological (high fat feeding) insulin resistance (6) as well as following partial pancreatectomy (19). However, the identity of the EGFR ligand mediating this effect remains unknown. In previous studies we discovered that expression of the heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) is up-regulated in islets from nutrient-infused rats, and that exogenous HB-EGF stimulates replication of MIN6 cells and primary rat β cells (18). HB-EGF is a member of the EGF family synthesized as a membrane-anchored precursor that can be processed by the action of a disintegrins and metalloproteinases (ADAM) to release the soluble active form (20). HB-EGF induces phosphorylation of EGFR and subsequent activation of a downstream signaling cascade including MAPK and PI3K/AKT.

The aim of this study was 1) to determine the role of HB-EGF in the β-cell proliferative response to glucose in rat islets ex vivo and in vivo and 2) to investigate the mechanisms linking glucose to an HB-EGF/EGFR signaling pathway promoting β-cell proliferation.

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RESEARCH DESIGN AND METHODS

Reagents and solutions

RPMI-1640 and qualified FBS were from Invitrogen (Carlsbad, CA). Recombinant HB-EGF and betacellulin (BTC) were from R&D Systems (Minneapolis, MN). The HB-EGF inhibitor, CRM197, and the Src family kinase inhibitor, PP1, were from Sigma-Aldrich (St. Louis, MO). The EGFR tyrosine kinase inhibitor AG1478 and the mammalian target of rapamycin complex 1 (mTORC1) inhibitor rapamycin were from LC Laboratories (Woburn, MA). Adenoviruses expressing shRNAs against HB-EGF (AdV-shHB-EGF) and control scrambled shRNA (AdV-shCTL) were from Vector Biolabs (Malvern, PA). SmartPool small interfering RNA (siRNA) duplexes against rat ChREBP and control siRNA were obtained from Dharmacon (Lafeyette, CO).

Islet isolation and adenoviral infection of islets

Islets were isolated from 2-month-old male Wistar or Lewis rats (Charles River, Saint-Constant, QC, Canada) by collagenase digestion and dextran density gradient centrifugation as described (21). For adenoviral infections, isolated islets were partially dissociated and then infected with 100 plaque-forming units (pfu) of adenoviruses/cell overnight as described (22), after which the medium was replaced with complete medium and cultured for an additional 24 h prior to stimulation ex vivo or transplantation.

Ex vivo islet proliferation

Isolated islets were cultured in RPMI-1640 with 10% (vol./vol.) qualified FBS (complete medium) for 72 h in the presence of glucose, 100 ng/ml HB-EGF or 50 ng/ml BTC as indicated in the Figure legends. EdU (10 μM) was added as indicated. The media were changed every 24 h. At the end of treatment, islets were embedded in optimal cutting temperature (OCT) compound, frozen,

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sectioned at 8 μm and mounted on Superfrost Plus slides (Life Technologies Inc., Burlington, ON, Canada). Sections were immunostained for insulin (INS) or Nkx6.1 and the proliferative marker Ki67 or EdU using the Click-iT™ EdU Imaging Kit (Life Technologies Inc.). Primary antibodies and dilutions are listed in Supplemental Table S1. Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Images were taken with a fluorescence microscope (Zeiss, Thornwood, NY). Proliferation was calculated as the percentage of double-positive Ki67+ (or EdU+) and INS+ (or Nkx6.1+) cells over the total INS+ (or Nkx6.1+) population. At least 1500 β cells from 7-17 individual islets were manually counted per condition.

Ex vivo islet insulin secretion and content

Triplicate batches of ten islets each were sequentially incubated twice with KRB solution containing 0.1% (wt/vol.) BSA and 2.8 mM glucose for 20 min at 37 °C, then incubated for 1 h with 2.8 or 16.7 mM glucose. Intracellular insulin content was measured following acid–alcohol extraction. Insulin was measured by radioimmunoassay using a rat insulin RIA kit (Millipore, Billerica, MA).

Islet transplantation and glucose infusion in rats

All procedures were approved by the Institutional Committee for the Protection of Animals at the Centre Hospitalier de l'Université de Montréal. Male Lewis rats weighing 250–350 g (~2-month-old) (Charles River) underwent catheterization of the jugular vein for infusion and the carotid artery for sampling as described (23). For islet transplantation, 500 islets isolated from 2-month-old male Lewis rats were infected with AdV-shHB-EGF or AdV-shCTL, as described above, and injected via a cannula under the left kidney capsule during the catheterization surgery. Animals were allowed to recover for 72 h followed by intravenous infusions of either saline (Sal) (0.9%

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wt/vol. NaCl; Baxter, Mississauga, ON, Canada) or 70% (wt/vol.) glucose (Glu) (McKesson, Montreal, QC, Canada) for an additional 72 h. The glucose infusion rate (GIR) was adjusted to maintain plasma glucose at 13.9–19.4 mmol/l throughout the 72 h infusion.

Immunostaining of tissue sections

Transplanted kidneys and pancreata were fixed for 4 h in 4% paraformaldehyde and cryoprotected overnight in 30% sucrose. Tissues were then embedded in OCT, frozen, sectioned at 8 μm and mounted on Superfrost Plus slides (Life Technologies Inc.). Antigen retrieval was performed using sodium citrate buffer and β-cell proliferation was assessed as described above.

Flow cytometric sorting of β-cells

Islets were isolated from male RIP7-RLuc-YFP transgenic rats (24), washed in PBS and dispersed in accutase (Innovative Cell Technologies, Inc., San Diego, CA) for 10 min at 37°C. At the end of the digestion, cells were washed, resuspended in PBS, and passed through a 40-μm filter prior to sorting. Flow cytometric sorting of YFP-positive and -negative cells was carried out using a FACSAria II flow cytometer with FACSDiva software (BD Biosciences, San Jose, CA). YFP-expressing cells were detected using the 488-nm laser and 530/30-nm BP filter.

Quantitative RT-PCR

Total RNA was extracted from 150-200 whole islets or 100,000 sorted islet cells using the RNeasy micro kit (Qiagen, Valencia, CA). RNA was quantified by spectrophotometry using a NanoDrop 2000 (Life Technologies Inc.) and 1 ug of RNA was reverse transcribed. Real-time PCR was performed by using QuantiTect SYBR Green PCR kit (Qiagen). Results were normalized to cyclophilin A RNA levels.

Chromatin immunoprecipitation (ChIP) and chromatin confirmation capture (3C)

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INS1 832/13 cell culture, siRNA treatment and RNA isolation and RT-PCR were performed as described (14). ChIP was performed as previously described (15). Briefly, INS-1 cells were cultured for 16 h in 2 mM glucose followed by 6 h at 2 or 20 mM glucose. An anti-ChREBP or normal rabbit IgG were used for immunoprecipitation and a genomic region 30 kb downstream from the transcription start site of the HB-EGF gene known to bind ChREBP (25) was amplified by RT-PCR. Primary antibodies are listed in Supplemental Table S1. 3C was performed essentially as described in (26). INS-1 cells were treated as for ChIP. The sequences of the primers used for RT-PCR, ChIP and 3C are shown in Supplemental Table S2.

Immunoblotting

Proteins were extracted from INS-1 cells and subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with primary antibodies against phospho-EGFR, phospho-S6 ribosomal protein and α-tubulin in 5% (wt/vol) milk. Primary antibodies and dilutions are listed in Supplemental Table S1. Signals were revealed using horseradish peroxidase–conjugated anti-rabbit IgG secondary antibodies (Bio-Rad, Richmond, CA) in 5% (wt/vol) milk and visualized using Western Lighting Plus-ECL (Perkin Elmer Inc., Waltham, MA). Band intensity was quantified using ImageJ software (National Institutes of Health).

Statistical analyses

Data are expressed as mean ± SEM. Significance was tested using one-way ANOVA with Tukey or Dunnett post hoc test, or two-way ANOVA with post hoc adjustment for multiple comparisons, as appropriate, using GraphPad Instat (GraphPad Software, San Diego, CA). $P<0.05$ was considered significant.

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RESULTS

HB-EGF induces β-cell proliferation via EGFR-mTOR signaling.

We previously showed that HB-EGF stimulates β-cell proliferation in dispersed rat islets (18). To confirm and extend these findings, we assessed the β-cell proliferative response to HB-EGF in intact rat islets using either Ki67 or EdU-labeling to mark proliferating cells and insulin or Nkx6.1 to mark β cells (Fig. 1). In the presence of 2.8 mM glucose, HB-EGF and BTC increased the percentage of Ki67-positive β cells to levels comparable to those detected in response to 16.7 mM glucose (Fig. 1A-D). Similar results were obtained when using EdU as a proliferative marker (Fig. 1E&F). Exposing islets to the EGFR tyrosine kinase inhibitor AG1478 or the mTORC1 inhibitor rapamycin abrogated HB-EGF induced β-cell proliferation (Fig. 1G&H). We then asked whether HB-EGF affects insulin secretion. Neither acute (1 h) nor chronic (24 h) exposure to HB-EGF affected insulin secretion or insulin content, although glucose-stimulated insulin secretion as a percentage of insulin content was slightly reduced following chronic HB-EGF treatment (Supplemental Fig. S1). These results show that exogenous HB-EGF promotes rat β-cell proliferation via EGFR and mTOR without significantly affecting insulin secretion.

Glucose-induced β-cell proliferation in isolated rat islets requires HB-EGF/EGFR signaling.

Given that glucose is a rodent beta-cell mitogen (9-12), we next examined the contribution of EGFR signaling to glucose-induced β-cell proliferation. Treatment of islets for 72 h with 16.7 mM glucose led to an approximately 3-fold increase in Ki67 staining compared to 2.8 mM glucose (Fig. 2A&B). Addition of AG1478 completely prevented the glucose-induced increase in β-cell proliferation (Fig. 2A&B). Similarly, addition of the HB-EGF inhibitor CRM197 (Fig. 2C&D) or adenoviral-mediated HB-EGF knockdown (Fig. 2E&F) blocked the stimulatory effect of glucose

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on β-cell proliferation. These results show that HB-EGF/EGFR signaling is required for glucose-induced β-cell proliferation in isolated rat islets.

Glucose-induced β-cell proliferation in transplanted islets requires HB-EGF.

To test whether islet-derived HB-EGF is necessary for glucose-induced β-cell proliferation in vivo, Lewis rats were transplanted under the kidney capsule with islets infected with either Adv-shHBEGF or Adv-shCTL, and infused with Sal or Glu for 72 h (Fig. 3A). Average blood glucose levels and glucose infusion rates were not significantly different between both groups (Supplemental Fig. S2). As expected, glucose infusion increased the percentage of Ki67-positive β cells in the endogenous pancreas to the same extent in Adv-shCTL and Adv-shHBEGF transplant recipients (Fig. 3B&C). Adv-shCTL-infected islet grafts showed increased β-cell proliferation in response to glucose infusion (Fig. 3D&E). In contrast, Adv-shHBEGF-infected islets were unresponsive to the mitogenic effects of glucose (Fig. 3D&E). These data demonstrate that, as observed in isolated islets (Fig. 2), HB-EGF/EGFR signaling is required for glucose-induced β-cell proliferation in vivo.

HB-EGF gene expression is up-regulated in β cells in response to glucose.

As we previously showed that infusion of glucose and lipids in rats increases HB-EGF mRNA levels in islets (18), we asked whether glucose alone was sufficient to stimulate HB-EGF expression in isolated islets. Indeed, 2-month-old rat islets exposed to 16.7 mM glucose for 24 h displayed a 1.5-fold increase of HB-EGF mRNA in comparison to 2.8 mM glucose (Fig. 4A). To determine whether the increase in islet HB-EGF gene expression was primarily in β cells, we made use of a transgenic rat expressing yellow fluorescent protein (YFP) under the control of the Ins2 promoter (RIP7-RLuc-YFP) (24) to enrich for β cells by flow cytometry after glucose treatment.

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Interestingly, glucose augmented HB-EGF mRNA levels in the YFP-positive (β-cell enriched; Fig. 4B), but not the YFP-negative (Fig. 4C), fraction. We conclude that glucose stimulates HB-EGF gene expression in rat β cells.

Glucose stimulates HB-EGF gene expression via ChREBP.

ChREBP is a key mediator of glucose-induced transcriptional changes (25). Therefore, we asked whether HB-EGF is a direct target of ChREBP. Consistent with the results shown in Fig. 4, glucose increased HB-EGF expression in untransfected INS-1 cells and in cells transfected with a control siRNA (Fig. 5A). In contrast, siRNA-mediated knockdown of ChREBP abolished the glucose response (Fig. 5A). ChREBP ChIP-seq and DNase-seq analyses of INS-1 cells exposed to glucose identified putative enhancer elements containing canonical ChREBP binding sites located approximately 30 kb downstream of the HB-EGF transcriptional start site (TSS) (25). ChIP analysis for one of these elements showed that a 6 h exposure to 20 mM glucose significantly increased ChREBP binding, whereas binding to a control region was unchanged (Fig. 5B). Furthermore, 3C analysis revealed increased interactions between these enhancers and the HB-EGF promoter in the presence of 20 mM glucose (Fig. 5C). These results strongly suggest that glucose-induced HB-EGF gene expression is mediated by direct binding of ChREBP to enhancers located 3' to the HB-EGF gene.

Glucose-induced β-cell proliferation is dependent on Src upstream of EGFR activation, but glucose-induced mTOR activation does not require HB-EGF.

Processing of proHB-EGF by ADAM proteins releases the active form that binds and activates EGFR (20). Previous studies in mesangial cells suggest that glucose-induced proteolytic processing of HB-EGF requires Src activation (27). Therefore, we investigated the role of Src

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family kinases in glucose-induced β-cell proliferation. Addition of the Src inhibitor PP1 abrogated the β-cell proliferative response to 16.7 mM glucose but not to HB-EGF (Fig. 6A&B), consistent with the possibility that glucose promotes proHB-EGF cleavage via Src followed by HB-EGF activation of EGFR.

Previous studies showed that glucose-induced β-cell proliferation is dependent on mTOR activation (28). As the mitogenic effect of HB-EGF was also dependent on mTOR in rat islets (Fig. 1G&H), we asked whether mTOR activation by glucose is dependent on HB-EGF. Exposing islets for 24 and 48 h to 16.7 mM glucose led to a significant increase in phosphorylation of the mTOR substrate S6 ribosomal protein (S6RP) (Fig. 6C&D). However, at these time points HB-EGF did not increase S6RP phosphorylation, and blocking HB-EGF with CRM197 did not affect glucose-induced mTOR activation ((Fig. 6C&D). Hence, glucose-induced mTOR activation is independent of HB-EGF.

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DISCUSSION

The results of this study demonstrate a critical role for HB-EGF in glucose-induced β-cell proliferation in rat β cells. Exposing isolated islets to exogenous HB-EGF induced β-cell proliferation, whereas blocking HB-EGF signaling by inhibiting either EGFR or HB-EGF completely prevented the proliferative response. In vivo, silencing HB-EGF prevented the increase in β-cell proliferation in islets transplanted under the kidney capsule of glucose-infused rats. Taken together, these results reveal that a glucose/HB-EGF/EGFR axis controls β-cell proliferation. Mechanistically, we showed that HB-EGF gene expression was induced by glucose in the β cell through the action of ChREBP. In addition we found that glucose-, but not HB-EGF-induced β-cell proliferation is blocked by Src inhibition. As Src family kinases are involved in EGFR transactivation via ADAM metalloproteases, we propose a mechanism whereby glucose activates ChREBP and Src to promote HB-EGF gene expression and HB-EGF membrane shedding, respectively, and subsequently EGFR downstream signaling and cell cycle activation (Fig. 7).

Our previous (18) and current results are in agreement with studies showing that overexpression of HB-EGF by retrograde injection of adenoviruses into the pancreatic duct leads to proliferation of pre-existing β-cells in adult mice (29). In contrast, an increase in β-cell proliferation was not described following precocious HB-EGF expression in developing mouse β cells (30). However, the presence of pancreatic fibrosis, stromal expansion and islet dysfunction in this model may have precluded such an effect. Interestingly, overexpression of HB-EGF (29) or BTC (31) in pancreatic ducts promotes β-cell neogenesis, and EGF gain-of-function studies in human duct cells (32; 33) support a similar conclusion. Hence, we propose that the major effect of HB-EGF is to promote proliferation of existing β cells in rat islets ex vivo and in vivo but that β-cell neogenesis from

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exocrine tissue could also contribute to the overall beneficial effects of HB-EGF on β-cell mass. In contrast to its effects on β-cell proliferation, acute and extended (24 h) exposure to HB-EGF did not alter insulin secretion or insulin content in rat islets ex vivo. However, positive, anti-diabetic, effects of HB-EGF on the β cell were demonstrated in multiple low-dose streptozotocin diabetic mice whereby combined treatment of gastrin and HB-EGF led to improved glucose tolerance and islet function due in part to a reduction in insulitis (34). Further studies will be required to fully elucidate the pleotropic effects of HB-EGF on pancreatic islets.

We found that AG1478, a specific inhibitor of EGFR with minimal activity towards other ErbB isoforms, completely abrogates HB-EGF induced β-cell proliferation. As HB-EGF signals via EGFR (ErbB1) and ErbB4 but not ErbB2 or ErbB3 (35) and EGFR is expressed in β-cells, whereas ErbB4 is only weakly expressed in rodent islets (36), we propose that HB-EGF acts predominantly via EGFR to promote β-cell proliferation.

EGFR inhibition, loss-of-function and dominant negative studies in adult rodents in the context of pathophysiological and physiological metabolic stress (6; 18; 37), and partial pancreatectomy (19) all suggest that β-cell EGFR underlies the maintenance of glucose homeostasis by transducing signals that increase β-cell proliferation and mass. Notwithstanding a role for BTC downstream of GLP-1 (38), however, attempts to investigate the role of EGFR ligands in the regulation of β-cell mass and function have been limited to gain-of-function approaches (29; 31; 39; 40), whereas the identification of endogenous ligands contributing to β-cell compensation have until now not been addressed. We found that glucose, a natural β-cell mitogen and key effector of regulation of β-cell mass in the face of increased insulin demand (11), requires HB-EGF signaling. When rat islets were exposed to glucose ex vivo or in vivo, the β-cell mitogenic response was dependent on both

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EGFR and HB-EGF. Although HB-EGF was essential for the glucose response, whether HB-EGF is the sole endogenous EGFR ligand acting during β-cell compensation to metabolic stress remains an open question. BTC (39), epiregulin (EPGN) (41), TGFalpha and EGF (39; 42) exert mitogenic effects on the β cell and are expressed in developing (43) and adult (36) rodent islets and during β-cell neogenesis (44). Hence, different EGFR ligands likely contribute to β-cell compensation in a context-dependent manner.

In previous studies we showed that HB-EGF gene expression is upregulated in islets following nutrient-infusion in rats (18) and a similar trend was found in obese, diabetes-resistant (B6) mice (45). Our present results suggest that the increase in HB-EGF gene expression is due, at least in part, to the direct action of glucose and are consistent with the time- and dose-dependent increase in HB-EGF gene expression observed in response to glucose in INS-1 cells (25) and rat islets (46). In addition we found that ChREBP is necessary for HB-EGF gene expression and that ChREBP binds a 3' HB-EGF gene enhancer element. Primary targets of ChREBP in the β-cell include RORg and Myc (25), whereas the cell cycle regulatory cyclins and cyclin dependent kinases (cdks), which lack ChREBP binding sites, respond to glucose in a delayed manner due to their dependency on first-phase factors (14; 25). Hence, downstream of ChREBP, HB-EGF/EGFR signaling could play a role alongside first-phase transcription factors to drive cell cycle regulators and initiate β-cell cycle progression in response to glucose (Fig. 7).

Although membrane anchored proHB-EGF may be involved in juxtacrine signaling (47), the major effects of HB-EGF in the β cell are likely mediated by the soluble form generated by proteolytic processing of proHB-EGF. In mesangial cells, glucose promotes HB-EGF shedding and EGFR transactivation through Src-dependent activation of metalloproteases (27). Our results showing that Src inhibition blocked glucose- but not HB-EGF-induced β-cell proliferation suggest that this

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phenomenon is also operative in β cells. Consistent with this possibility, short-term exposure of MIN6 and human islets to glucose leads to phosphorylation of the Src family kinase YES (48). Overall, our data are consistent with the model proposed in Fig. 7 whereby glucose promotes Src-dependent proHB-EGF processing leading to HB-EGF shedding and stimulation of β-cell proliferation via paracrine/autocrine signaling through the EGFR.

mTOR is an essential mediator of mitogen induced β-cell proliferation (49). Blocking mTOR activity prevents the mitogenic effects of glucose (28) and, as we showed in the present study, also mitigates HB-EGF-induced proliferation. Surprisingly however, blocking HB-EGF had no effect on the increase in mTOR activity in response to glucose yet HB-EGF inhibition completely prevented glucose-induced β-cell proliferation. Hence, we postulate the existence of a parallel signal emanating from EGFR acting alongside the mTOR pathway that is necessary for β-cell cycle engagement. A number of signaling effectors are known to act downstream of EGFR including, MAPK, PI3K/AKT and IRS2 (50) that could contribute to the mitogenic response to HB-EGF (Fig. 7).

In conclusion, this study reveals a critical role of HB-EGF/EGFR signaling in glucose-induced β-cell proliferation in rat islets. Future studies will focus on further elucidating the underlying mechanism and assessing the importance of this pathway in human islets.

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H.M. and M.R.M. designed the experiments and acquired the data. H.M., M.R.M., D.K.S., J.G. and V.P. researched data, analyzed the results, and wrote the manuscript. All authors revised the manuscript and approved the final version. V.P. is the guarantor of this work and, as such, takes full responsibility for the work.

The authors have no relevant conflict of interest to disclose.

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FIGURE LEGENDS

Figure 1. HB-EGF stimulates β-cell proliferation via the EGFR. (A-F) Isolated rat islets were exposed to 2.8 or 16.7 mM glucose or to HB-EGF (100 ng/ml) or betacellulin (BTC; 50 ng/ml) in the presence of 2.8 mM glucose for 72 h. (G, H) Isolated rat islets were exposed to 2.8 mM glucose and left untreated or treated with HB-EGF (100 ng/ml) with or without AG1478 (300 nM) or rapamycin (Rap; 10 nM) for 72 h. Proliferation was assessed by Ki67 (A-D, G, H) or EdU (E, F) staining and Nkx6.1 (A, B) or insulin (INS) (C-H) to mark β cells. Representative images of Nkx6.1 (red), Ki67 (green) and nuclei (blue) (A) or insulin (green), Ki67 or EdU (red) and nuclei (blue) (C, E, G) staining. Arrows show positive nuclei for Ki67 and EdU. (B, D, H) The percentage of Ki67+ insulin+ (or Nkx6.1+) cells of total insulin+ (or Nkx6.1+) cells. (F) Percentage of EdU+ insulin+ cells of total insulin+ cells. Data represent individual values and are expressed as mean ± SEM (n=4–6). Scale bars, 50 μm. *p < 0.05, **p<0.01, ***p<0.001, as compared to the 2.8 mM glucose control condition. ns, not significant.

Figure 2. Glucose stimulates β-cell proliferation via HB-EGF/EGFR signaling. (A-D) Isolated rat islets were exposed to 2.8 or 16.7 mM glucose with or without AG1478 (300 nM) (A, B) and 2.8 or 16.7 mM glucose or HB-EGF (100 ng/ml) in the presence of 2.8 mM glucose with or without CRM197 (10 mg/ml) (C, D) for 72 h. (E, F) Isolated rat islets were infected with Adv-shHBEGF or Adv-shCTL and exposed to 2.8 or 16.7 mM glucose for 72 h. Proliferation was assessed by Ki67 staining and insulin (INS) staining to mark β-cells. (A, C, E) Representative images of insulin (green), Ki67 (red) and nuclei (blue) staining. Arrows show positive nuclei for Ki67. (B, D, F) The percentage of Ki67+ insulin+ cells of total insulin+ cells. Data represent individual values and means ± SEM (n=4–6). Scale bars, 50 μm. **p<0.01, ***p<0.001, ****p<0.0001, as compared to the 2.8 mM glucose condition. ns, not significant.

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Figure 3. HB-EGF is required for glucose-induced β-cell proliferation *in vivo*. (A) Isolated rat islets were infected with Adv-shHBEGF or Adv-shCTL and transplanted under the kidney capsule of two-month-old Lewis rats infused with saline (Sal) or glucose (Glu) for 72 h. (B-E) Proliferation was assessed by Ki67 staining and insulin (INS) staining to mark β-cells. (B, D) Representative images of insulin (green), Ki67 (red) and nuclei (blue) staining in the pancreas (B) or transplanted islets (D). Arrows show positive nuclei for Ki67. (C, E) The percentage of Ki67+ insulin+ cells of total insulin+ cells in the pancreas (C) and transplanted islets (E). Data represent individual values and means ± SEM (n=4–6). Scale bars, 50 μm. ***p<0.001, ****p<0.0001, as compared to the saline condition. ns, not significant.

Figure 4. Glucose increases HB-EGF gene expression in the β-cell. (A-C) HB-EGF mRNA was measured in isolated intact rat islets (A) or in FACS-sorted YFP-positive (B) and YFP-negative (C) cells from isolated RIP7-RLuc-YFP islets following exposed to 2.8 or 16.7 mM glucose for 24 h. mRNA was determined by quantitative RT-PCR and normalized to cyclophilin A. Data are presented as the fold-increase over the 2.8 mM glucose condition and represent individual values and means ± SEM (n=5-6). *p < 0.05, as compared to the 2.8 mM glucose condition. ns, not significant.

Figure 5. ChREBP mediates glucose-induced HB-EGF gene expression in INS-1 cells. (A) HB-EGF RNA was measured in INS-1 cells exposed to 3 or 15 mM glucose for 18 h in the presence of a control siRNA (SiCon) or an siRNA directed against ChREBP (SiChR) (n=3). mRNA was determined by quantitative RT-PCR and normalized to β-actin. NT, non transfected. (B) ChREBP binding to a genomic region 30 kb downstream from the transcription start site of the HB-EGF gene known to bind ChREBP (black bar in C, upper) was assessed in INS-1 cells exposed to 2 or 20 mM glucose for 6 h followed by ChIP using an antibody against ChREBP or control IgG (n=3).

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Data indicate the percent binding after subtraction of the IgG control. Inset, Pklr coding region serves as a negative control. (C) Upper, genome browser view of 38,000 bp of the genomic locus spanning the TSS of the HB-EGF gene showing the ChREBP binding (ChREBP ChIP) and DNAse hypersensitivity sites (DNAse HS) downstream of the gene (25). Black bar, region amplified in (B). Lower, chromatin confirmation capture (3C) data from INS-1 cells treated as in (B), aligned to the genome browser and expressed as interaction frequency normalized to maximum interaction (n=3). Black line, anchor primer. Shaded gray added for clarity represents interaction frequency after 20 mM glucose treatment. Data are expressed as mean ± SEM.*$p < 0.05$, ***$p<0.001$, as compared to the control condition. ns, not significant.

Figure 6. Src is required for glucose- but not HB-EGF-induced β-cell proliferation and glucose-induced mTOR activation does not require HB-EGF. (A, B) Isolated rat islets were exposed to 2.8 or 16.7 mM glucose or HB-EGF (100 ng/ml) in the presence of 2.8 mM glucose for 72 h with or without the Src inhibitor PP1 (1 μM). Proliferation was assessed by Ki67 staining and insulin (INS) to mark β-cells. (A) Representative images of insulin (green), Ki67 (red) and nuclei (blue). Arrows show positive nuclei for Ki67. (B) The percentage of Ki67+ insulin+ cells of total insulin+ cells. Scale bars, 50 μm. (C, D) Isolated rat islets were exposed to 2.8 or 16.7 mM glucose or HB-EGF (100 ng/ml) in the presence of 2.8 mM glucose with or without CRM197 (10 mg/ml) for 24 and 48 h. Representative Western blot (C) of phospho-S6RP (pS6RP) and α-tubulin and densitometric quantification (D) of pS6RP normalized to α-tubulin. Data represent individual values and means ± SEM (n=4-6). *$p < 0.05$, **$p<0.01$, ***$p<0.001$, as compared to the 2.8 mM glucose condition or as indicated in the graph (B). ns, not significant.

Figure 7. Proposed mechanism of glucose/HB-EGF/EGFR axis controlling β-cell proliferation. An increase in the soluble, active form of HB-EGF is mediated by glucose-induced

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ChREBP, which increases HB-EGF gene expression, and by glucose-induced Src, which is coupled to metalloprotease (ADAM)-dependent proHB-EGF processing. Subsequent binding of HB-EGF to the β-cell EGFR activates signaling pathways including mTOR but also possibly MAPK, PI3K/AKT and IRS2 that together promote β-cell proliferation. Specific inhibitors used in this study to block glucose- and HB-EGF-induced β-cell proliferation are indicated.

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Figure 1

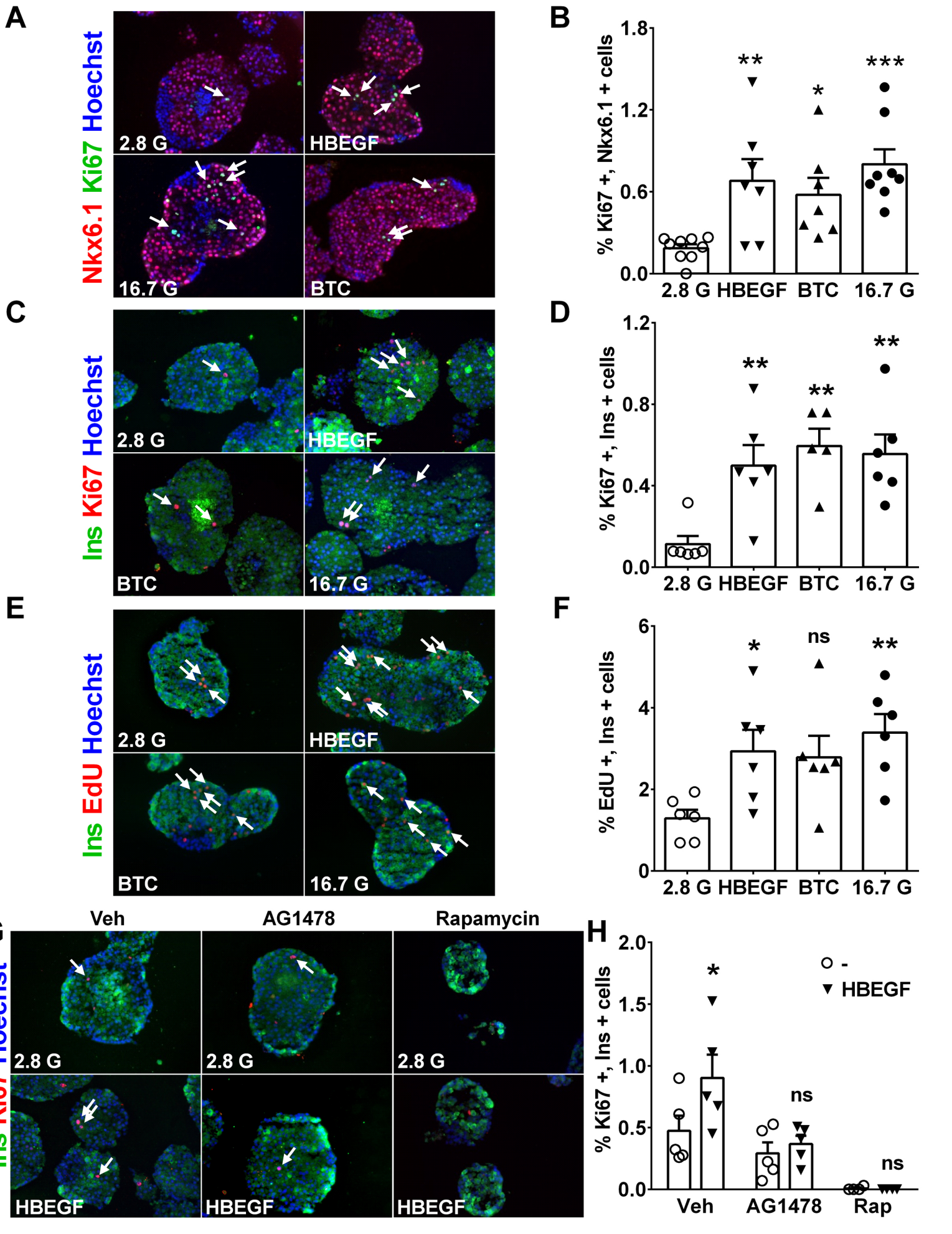

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Figure 2

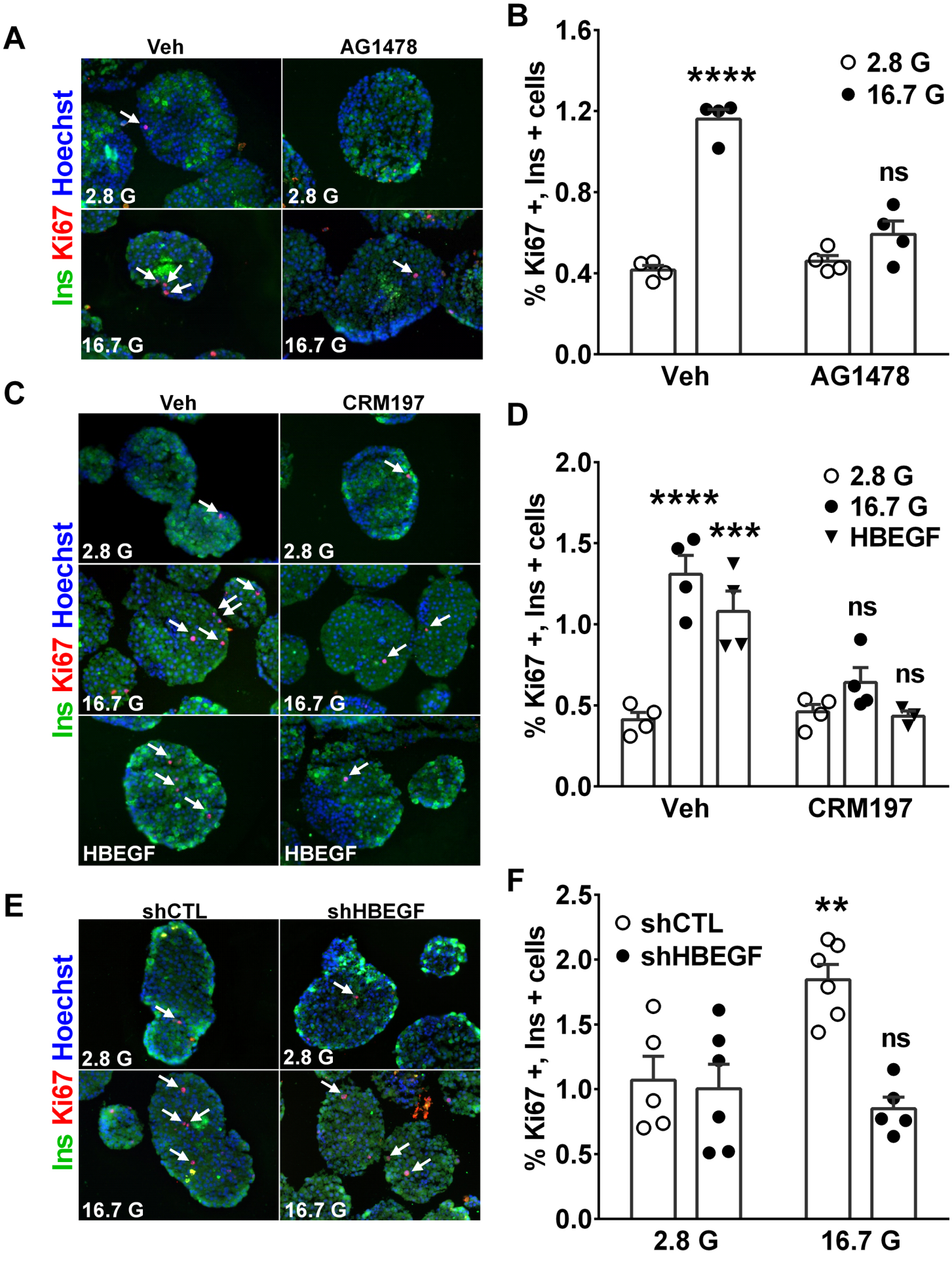

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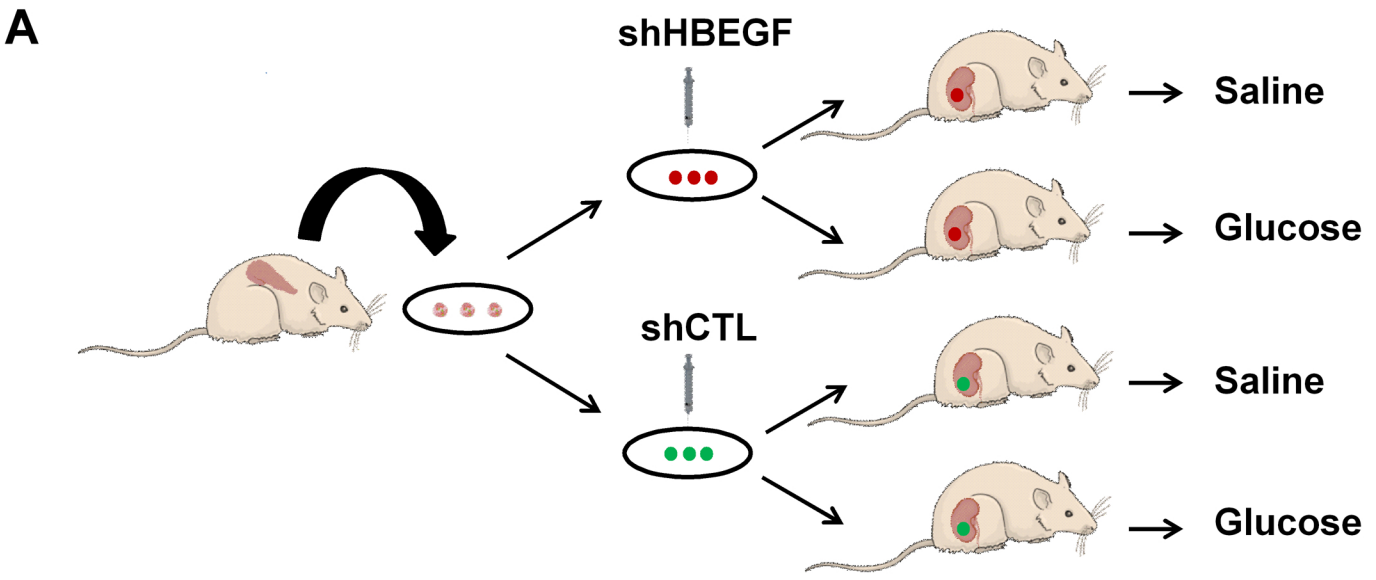
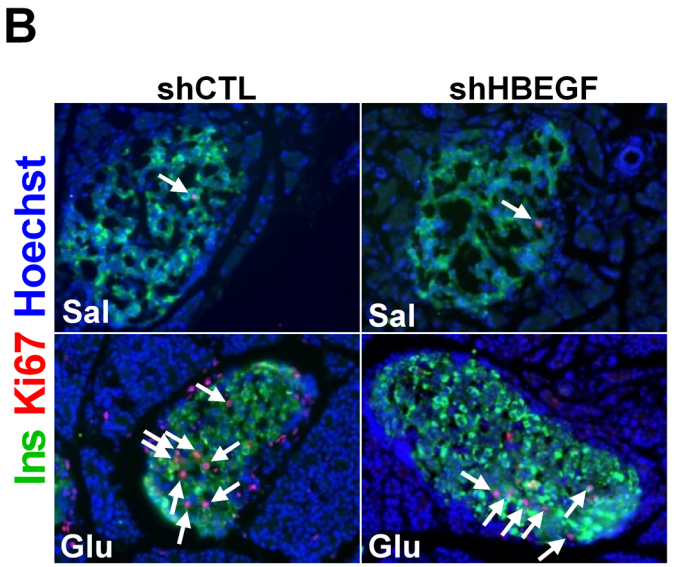
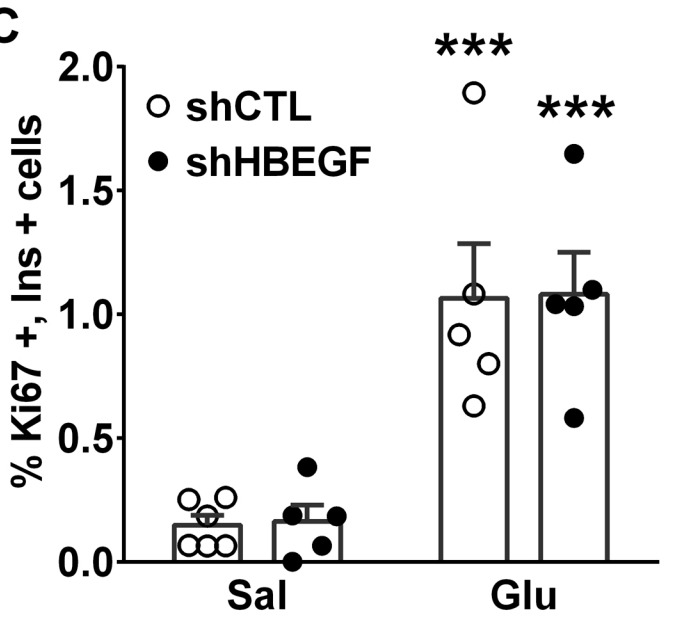
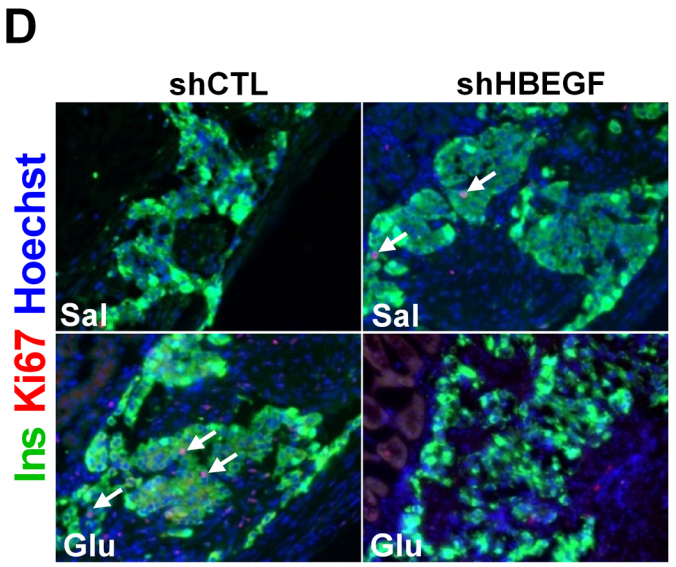
Figure 3









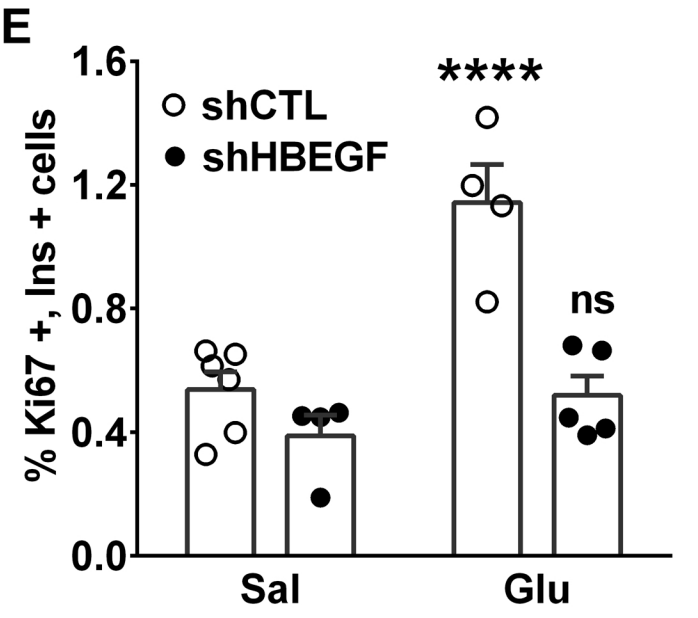


Figure 4

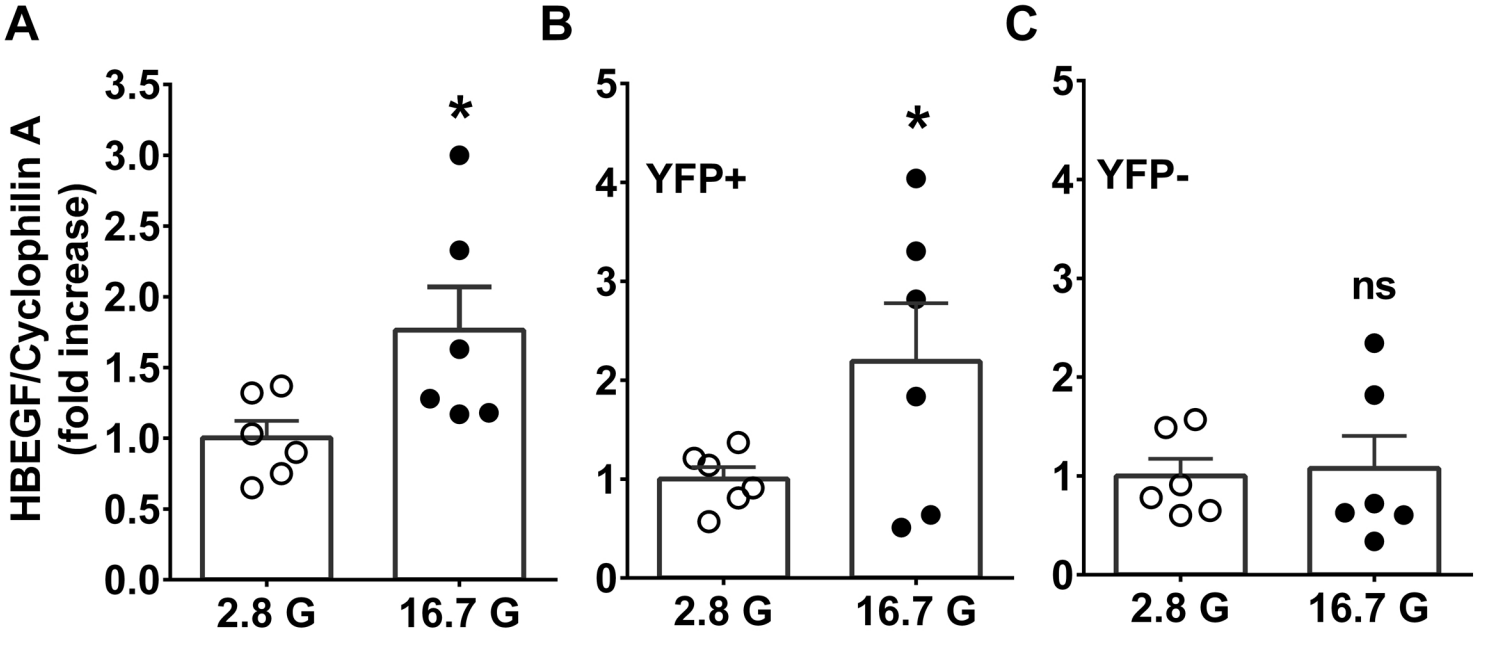

A
B
C
HBEGF/Cyclophilin A
(fold increase)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
2.8 G
16.7 G
5
4
3
2
1
0
YFP+
*
2.8 G
16.7 G
5
4
3
2
1
0
YFP-
ns
2.8 G
16.7 G

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Figure 5

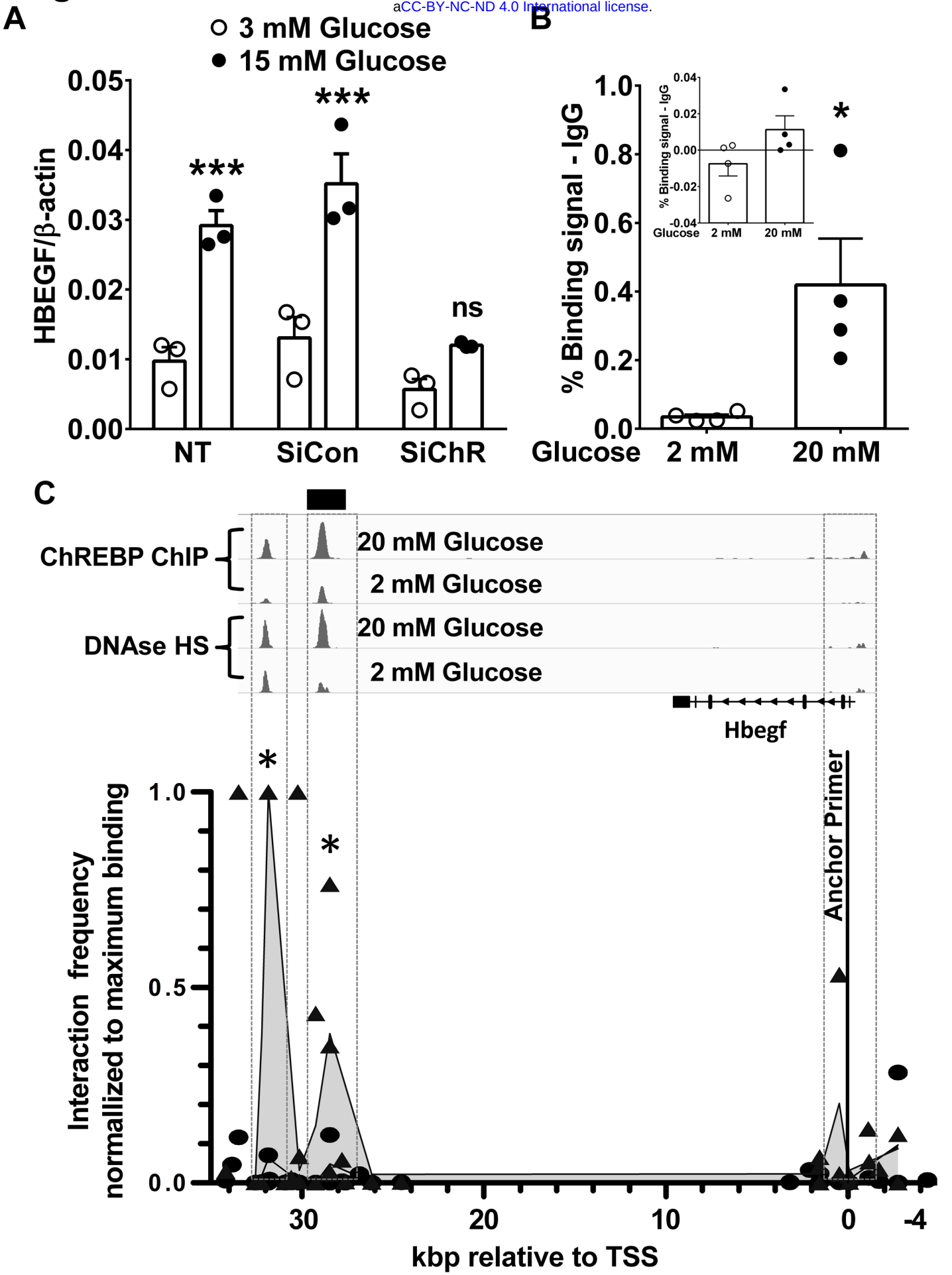


Figure 6

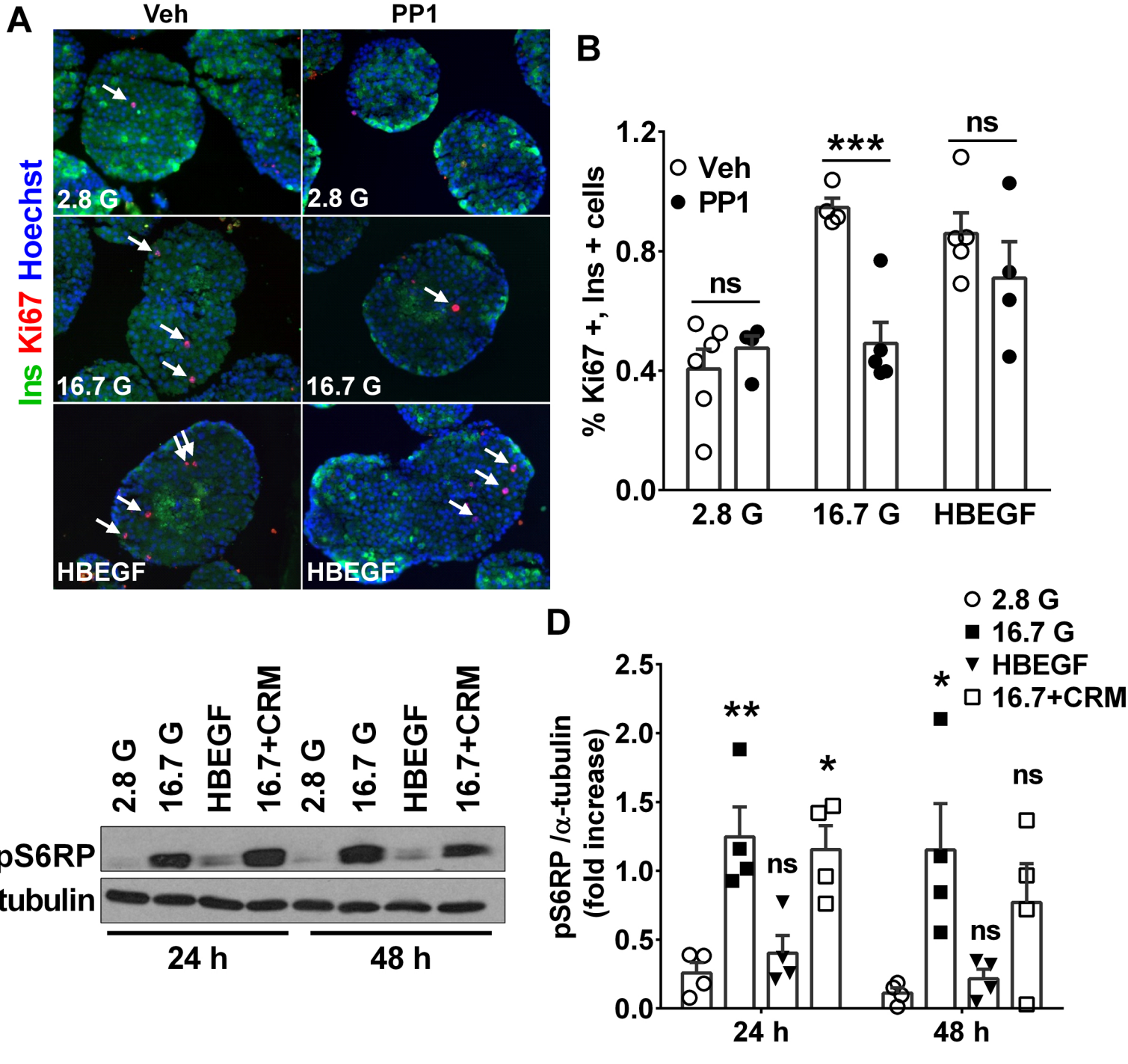
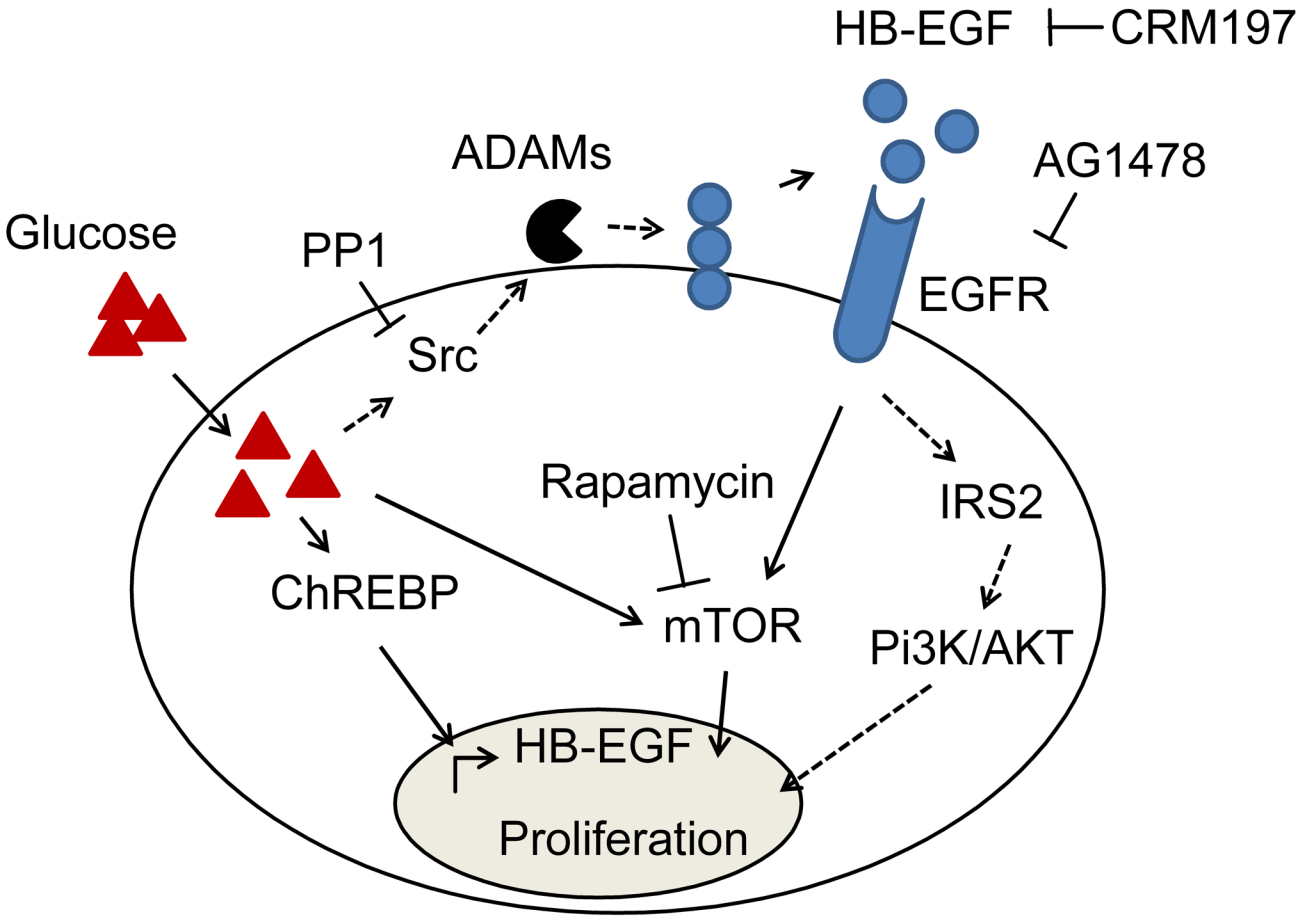

A
Veh
PP1
Ins Ki67 Hoechst
2.8 G
2.8 G
16.7 G
16.7 G
HBEGF
HBEGF
B
% Ki67 +, Ins + cells
1.2
0.8
0.4
0.0
Veh
PP1
ns
***
ns
2.8 G
16.7 G
HBEGF
C
2.8 G
16.7 G
HBEGF
16.7+CRM
2.8 G
16.7 G
HBEGF
16.7+CRM
pS6RP
α-tubulin
24 h
48 h
D
2.8 G
16.7 G
HBEGF
16.7+CRM
pS6RP /α-tubulin
(fold increase)
2.5
2.0
1.5
1.0
0.5
0.0
**
ns
*
*
ns
ns
24 h
48 h

Figure 7


HB-EGF
CRM197
ADAMs
AG1478
Glucose
PP1
EGFR
Src
Rapamycin
IRS2
ChREBP
mTOR
Pi3K/AKT
HB-EGF
Proliferation