Glycogen synthase kinase 3β inhibition enhances Notch1 recycling

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ABSTRACT **The Notch signaling pathway is essential throughout development and remains active into adulthood, where it performs a critical role in tissue homeostasis. The fact that defects in signaling can lead to malignancy illustrates the need to control Notch activity tightly. GSK3β is an established regulator of the Notch signaling pathway, although its mechanism of action remains unclear. Given the emerging role for GSK3β in receptor trafficking, we tested the idea that GSK3β controls signaling by regulating Notch transport. Consistent with published reports, we find that GSK3β inhibition enhances Notch1 signaling activity. Immunolocalization analysis reveals that Notch1 localization within a tubulovesicular compartment is altered when GSK3β activity is disrupted. We also find that receptor cell surface levels increase following acute GSK3β inhibition. This is followed by elevated Notch intracellular domain (NICD) production and a corresponding increase in signaling activity. Moreover, Notch transport assays reveal that receptor recycling rates increase when GSK3β activity is inhibited. Collectively, results presented here support a model where GSK3β regulates signaling by controlling postendocytic transport of Notch1. Given that GSK3β activity is suppressed following stimulation by multiple signal transduction pathways, our findings also suggest that cells can modulate Notch1 activity in response to extracellular signals by mobilizing Notch1 from endosomal stores.**

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INTRODUCTION

The evolutionarily conserved Notch signaling pathway performs a critical role in development, where it regulates processes such as stem cell maintenance, cell differentiation, and maintenance of cell viability (Bray, 2016). The pathway is stimulated when Notch binds one of several ligands belonging to the Delta, Serrate, and Lag-2 family of integral membrane proteins that are expressed on the surfaces of neighboring cells (Kopan and Ilagan, 2009). Once bound by ligands, Notch undergoes a series of regulated proteolytic cleavage events that ultimately result in release of the Notch intracellular domain (NICD) into the cytoplasm (De Strooper *et al.*, 1999; Brou *et al.*, 2000; van Tetering *et al.*, 2009). After being liberated from the membrane, NICD enters the nucleus and interacts with members of the CSL (CBF1, Su(H), Lag-1) family of transcription factors to coordinate gene expression (Bray and Bernard, 2010; Kovall and Blacklow, 2010).

The Notch pathway must be tightly controlled, given that signaling defects can lead to disease (Joutel *et al.*, 1996; Rangarajan *et al.*, 2001; Weng *et al.*, 2004). Emerging evidence implicates a critical role for endosomal transport genes in controlling Notch activity (Baron, 2012; Kandachar and Roegiers, 2012; Conner, 2016). While it is likely that cells possess mechanisms to regulate Notch activity in response to a dynamic extracellular environment, it remains unclear if extracellular cues can directly impact Notch transport decisions that lead to changes in signaling capacity. In this regard, we were particularly interested in GSK3β. GSK3β is a dual-specificity kinase that serves as a nexus for multiple signaling pathways, including those initiated by Wnt, EGF, and various other growth factors (Medina and Wandosell, 2011). Although GSK3β is an established regulator of the Notch signaling pathway (Foltz *et al.*, 2002; Espinosa *et al.*, 2003; Jin *et al.*, 2009; Kim *et al.*, 2009; Guha *et al.*, 2011), it remains unclear how it controls Notch1 activity. In addition to its role

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Abbreviations used: EGF, epidermal growth factor; GSK3β, glycogen synthase kinase 3β; JAG1, jagged1.

in integrating information from multiple signal transduction pathways, GSK3β is also known to regulate endosomal transport of integrins (Roberts *et al.*, 2004), the prolactin receptor (Plotnikov *et al.*, 2008), and the mannose-6-phosphate receptor (Adachi *et al.*, 2010). Thus, GSK3β may serve as an important link between extracellular signaling events and changes in Notch trafficking.

Here we investigate the relationship between GSK3β function and Notch1 transport within the endosome. In doing so, we discovered that GSK3β is a critical regulator of Notch1 trafficking, where it inhibits receptor recycling. Intriguingly, our findings also suggest that signaling pathways that inhibit GSK3β activity can modulate Notch1 activity by regulating the rates at which endosome-stored receptors are delivered to the cell surface.

RESULTS AND DISCUSSION

GSK3β regulates Notch1 endosomal transport

Conflicting reports suggest that GSK3β regulates Notch signaling either positively (Foltz *et al.*, 2002; Guha *et al.*, 2011) or negatively (Espinosa *et al.*, 2003; Jin *et al.*, 2009; Kim *et al.*, 2009) in rodent tissue culture and animal models. Thus, to resolve how GSK3β controls Notch activity in human cells, we measured Notch1 signaling with a dual-luciferase assay in HeLa cells following GSK3β inactivation by small interfering RNA (siRNA) or treatment with XXVII, a highly specific inhibitor of GSK3β (Berg *et al.*, 2012). We find that GSK3β silencing or pharmacologic inhibition increases Notch1 signaling (Figure 1, A–C), demonstrating that in this system, GSK3β regulates the signaling pathway negatively.

We next tested the hypothesis that GSK3β might regulate Notch1 sorting decisions, similarly to what is observed for other integral membrane proteins. Notch1 immunolocalization analysis in U2OS control cells reveals that the receptor is enriched within a tubulovesicular compartment and present at the cell surface at cell–cell contact sites (Figure 2, A and E). By contrast, when GSK3β is silenced with siRNA (Figure 2B) or inhibited by pretreating cells with XXVII for 18 h (Figure 2F), we observed a marked decrease in Notch1 associated with tubulovesicular endosomes that radiate from the perinuclear region. Instead, Notch1 was found on vesicles that were either broadly distributed throughout the cytoplasm or enriched in a perinuclear region that lacked prominent tubular projections. We quantified this observation by cell counting and found that in the majority of cells, Notch associates with perinuclear vesicles when GSK3β is silenced or pharmacologically inhibited (Figure 2, I and J). On the basis of these findings, we conclude that GSK3β controls Notch1 activity negatively by regulating receptor transport decisions within cells.

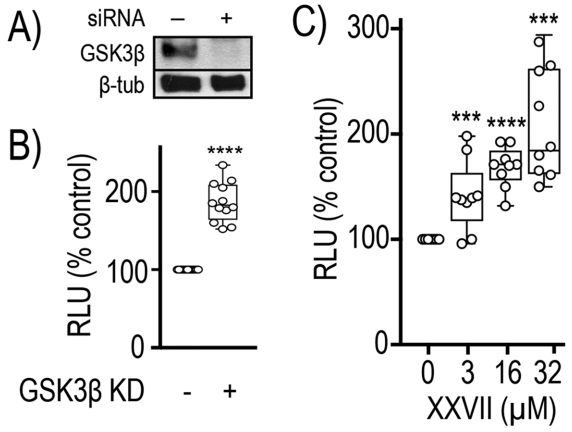

FIGURE 1: GSK3β inactivation promotes Notch1 signaling. (A) Immunoblot analysis of U2OS cells treated with control (-) or GSK3β-specific (+) siRNA. β-tubulin (β-tub) was used as a loading control. (B, C) Notch signaling was measured using the dual luciferase assay in tTA HeLa cells expressing the CD8-NΔE Notch1 chimera that were treated with control (–) or GSK3β (+) siRNA (B) or with DMSO or increasing concentrations of XXVII to inhibit GSK3β activity (C). Box-and-whisker plots show each data point from at least three independent experiments. Asterisks indicate a *p* value (see *Materials and Methods*).

GSK3β inactivation enhances Notch1 recycling rates

We previously established that Notch1 accumulation at the cell surface increases signaling capacity (Sorensen and Conner, 2010). Thus, given that GSK3β inhibition elevates Notch1 signaling and alters receptor localization, we postulated that GSK3β might increase the amount of Notch1 at the plasma membrane. To test this, we quantified Notch1 cell surface levels using a cloned single-chain variable fragment antibody to the Notch1 extracellular domain (Falk *et al.*, 2012), which we fused in tandem to superfold GFP and *Gaussia* luciferase (scFv-N1) which enables both visualization and enzymatic quantification. To minimize the risk that extended periods of GSK3β inactivation by siRNA knockdown or XXVII treatment (see Figure 1) might result in indirect effects on Notch1 transport and signaling, we evaluated Notch1 cell surface levels at earlier time points following GSK3β inactivation with XXVII. In doing so, we observed an ~50% increase in Notch1 cell surface levels in XXVII-treated cells within 2 h relative to control cells (Figure 3A). By 4 h, Notch1 cell surface levels had increased greater than twofold when GSK3β was inactivated. Given that cells used in this study (HeLa and U2OS) express JAG1, a ligand that activates Notch1 (Lindsell *et al.*, 1995), we anticipated that increased Notch1 presence at the cell surface should also increase endogenous signaling. We tested this prediction by evaluating NICD production by immunoblot and signaling using the dual-luciferase signaling assay. Immunoblot analysis reveals a time-dependent increase in NICD production relative to controls when GSK3β is acutely inactivated (Figure 3, B and C). Consistently, we observed a corresponding increase in downstream signaling activity (Figure 3D).

Taken together, these findings argue that GSK3β down-regulates signaling by limiting the presence of Notch1 at the cell surface, possibly by promoting receptor endocytosis or inhibiting its recycling. To distinguish between these possibilities, we first visualized Notch1 internalization following the scFv-N1 uptake. We reasoned that if GSK3β promoted Notch1 internalization, acute inactivation of the kinase should lead to diminished antibody uptake. In doing so, we found that scFv-N1 antibody is effectively internalized and targeted to tubulovesicular endosomes following a 10-min antibody pulse in both control cells and those pretreated with XXVII for 1 h (Figure 4, A and B). From this observation, we conclude that GSK3β activity is not essential for robust Notch1 endocytosis. Therefore, we next evaluated Notch1 recycling kinetics using an antibody pulse-chase approach. To do so, we first pulsed cells with scFv-N1 antibody for 12 min and then quantified a time course for antibody recycling to the cell surface by measuring *Gaussia* luciferase activity. In this case, we discovered that pretreating cells for 1 h with XXVII significantly increased antibody recycling rates relative to those in controls (Figure 4C). On the basis of this, we conclude that GSK3β activity inhibits Notch1 recycling from endosomal stores.

We recently observed that Notch1 recycles from tubulovesicular endosomes via a rab4a-mediated transport route (Zheng and Conner,

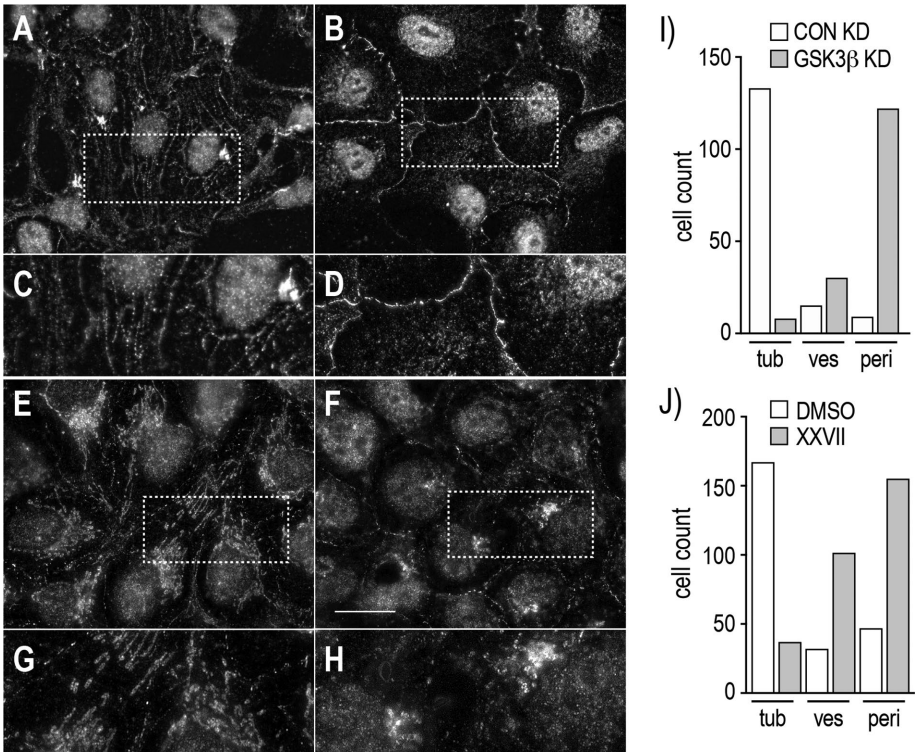

FIGURE 2: Notch1 localization is disrupted by GSK3β inhibition. Notch1 immunolocalization in U2OS cells treated for either 72 h with control (A) or GSK3β-specific siRNA (B) or 18 h with DMSO (E) or 32 μM XXVII to inactivate GSK3β (F). 2× magnified regions of boxed areas are shown in C, D, G, and H. Bar = 10 μm. Notch1 localization pattern quantification is shown in cells treated with control siRNA ($n = 157$), GSK3β-specific siRNA ($n = 160$, I), DMSO ($n = 311$), or XXVII ($n = 295$, J). Notch1 localization patterns for each condition were grouped into three general categories: 1) cells containing prominent Notch1-positive tubular endosomes (tub), 2) cells where Notch1 was associated with vesicles distributed throughout the cytoplasm (ves), and 3) cells where Notch accumulated in a perinuclear region that lacked prominent tubular endosomes (peri).

unpublished data). Therefore, we next asked whether GSK3β inhibition leads to Notch1 recycling via this route, using the dual luciferase signaling assay. We reasoned that if GSK3β prevents Notch1 recycling via a rab4a-mediated route, the signaling increases that arise when GSK3β activity is perturbed should be suppressed when rab4a is also silenced. Following rab4a depletion, Notch signaling is reduced by ~50%, indicating that receptor recycling by this route is critical to maintaining robust signaling capacity. Similarly, we observed that the increases in Notch1 signaling, which result following GSK3β depletion, are partially suppressed when both rab4a and GSK3β are silenced concurrently (Figure 4D). We interpret this finding as indicating that when GSK3β activity is lost, Notch1 is directed to the cell surface via a rab4a-dependent transport route. Consistent with this conclusion, immunolocalization analysis reveals that scFv-N1 antibody transits rab4a-positive tubulovesicular endosomes following internalization (Figure 4G). However, given that silencing both GSK3β and rab4a leads to a partial suppression of signaling relative to GSK3β knockdown alone (Figure 4D), we cannot rule out the possibility that GSK3β inactivation leads to Notch1 recycling via multiple routes. Collectively, these findings argue that GSK3β inactivation stimulates Notch signaling capacity by enhancing Notch1 recycling via recycling routes that include the rab4a-dependent trafficking pathway.

GSK3β dynamically associates with recycling endosomes

To gain insight into how GSK3β regulates Notch1 trafficking within cells, we next performed coimmunolocalization analysis between GSK3β and Notch1. We found that GSK3β localized to vesicles distributed throughout the cytoplasm that lacked Notch1 in a subpopulation of cells (Figure 5A). In contrast, in other subpopulations, GSK3β extensively colocalized with Notch1 on tubulovesicular endosomes (Figure 5D). To quantitate variation in the extent to which GSK3β is found on Notch1-positive endosomes, we employed an unbiased approach using methodologies developed by Manders *et al.* (1993) to establish an overlap (colocalization) coefficient between GSK3β and Notch1 in a larger population of cells ($n = 251$). Our analysis revealed that the extent of colocalization is highly variable where ~60% of GSK3β was found to colocalize with Notch1 on tubular endosomes in most cells (Figure 5G). On the basis of ergodic principles (Kafri *et al.*, 2013; Wheeler, 2015), we interpret this variation in colocalization as suggesting that GSK3β recruitment to endosomes is dynamically regulated. Moreover, these localization studies, combined with transport data, centrally position GSK3β as a key regulator of Notch1 endosomal sorting decisions, possibly in response to environmental cues.

Regulation of Notch1 transport by GSK3β

Results presented here reinforce the conclusion that GSK3β is a negative regulator of the Notch1 signaling pathway, in agreement with several published observations (Espinosa *et al.*, 2003; Jin *et al.*, 2009; Kim *et al.*, 2009). Our finding that GSK3β inhibition enhances signaling by promoting Notch1 recycling through a rab4a-mediated transport route is consistent with results where 1) GSK3β inactivation leads to misrouting of the mannose 6-phosphate receptor to the cell surface (Adachi *et al.*, 2010) and 2) constitutively active forms of GSK3β suppress αvβ3 and α5β1 integrin recycling (Roberts *et al.*, 2004). Collectively, these observations, combined with findings presented here, argue that GSK3β performs a general postendocytic role in directing receptor transport for Notch1 and other signaling receptors. In contrast, pharmacologic inhibition of GSK3β was also recently found to enhance transferrin receptor internalization (Reis *et al.*, 2015). The latter result suggests that GSK3β inhibits nutrient receptor endocytosis. Thus, it is possible that GSK3β functions at multiple receptor transport steps and/or differentially regulates transport depending on receptor type and/or downstream targets.

What is the mechanism by which GSK3β regulates Notch1 transport? Previous studies reveal that GSK3β binds directly to and phosphorylates Notch2 to down-regulate signaling in NIH-3T3 cells (Espinosa *et al.*, 2003). Similar observations were reported for Notch1 in GSK3β-null mouse embryonic fibroblasts (Foltz *et al.*, 2002). Thus, it is possible that Notch phosphorylation by GSK3β is a critical step in controlling receptor transport decisions. However, in contrast to our findings, Notch signaling is reduced in GSK3β-null embryonic fibroblasts, and GSK3β overexpression was found to stabilize the activated form of Notch1 (NICD1), suggesting that GSK3β regulates Notch1 positively (Foltz *et al.*, 2002). The nature of

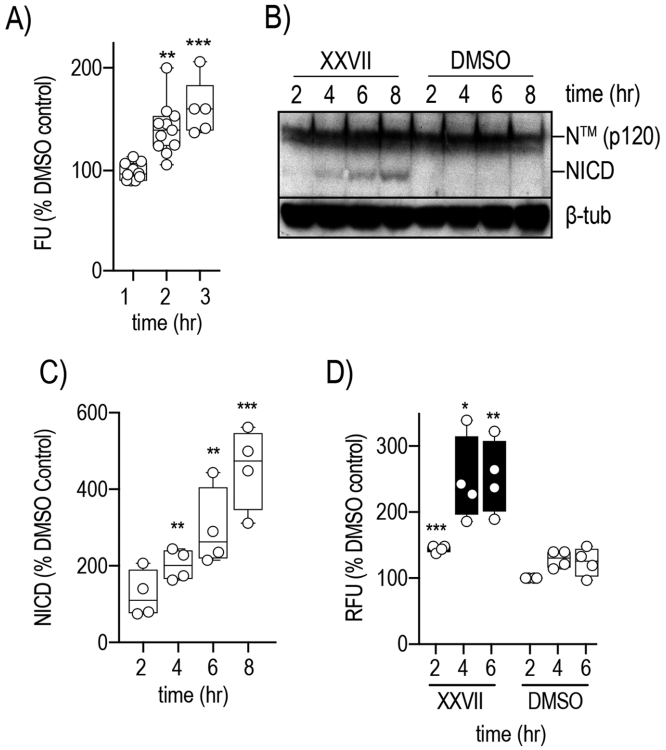

FIGURE 3: Acute GSK3β inhibition enhances Notch activity. (A) Endogenous Notch1 cell surface levels were quantitated by measuring the *Gaussia* luciferase activity of scFv-Notch1-sfGFP-GLuc bound to receptors on cells pretreated with DMSO and 32 μM XXVII for the indicated time period. Data are shown as a percentage of the time-matched DMSO control. (B) A representative immunoblot from cells pretreated with DMSO or XXVII for the indicated time period is shown. (C) NICD levels were quantified from four independent experiments by densitometry. (D) Endogenous Notch1 signaling was measured using the dual-luciferase signaling assay in tTA HeLa cells following pretreatment with DMSO or XXVII for the indicated time period. Box-and-whisker plots show each data point from three to five independent experiments. Asterisks indicate *p* values.

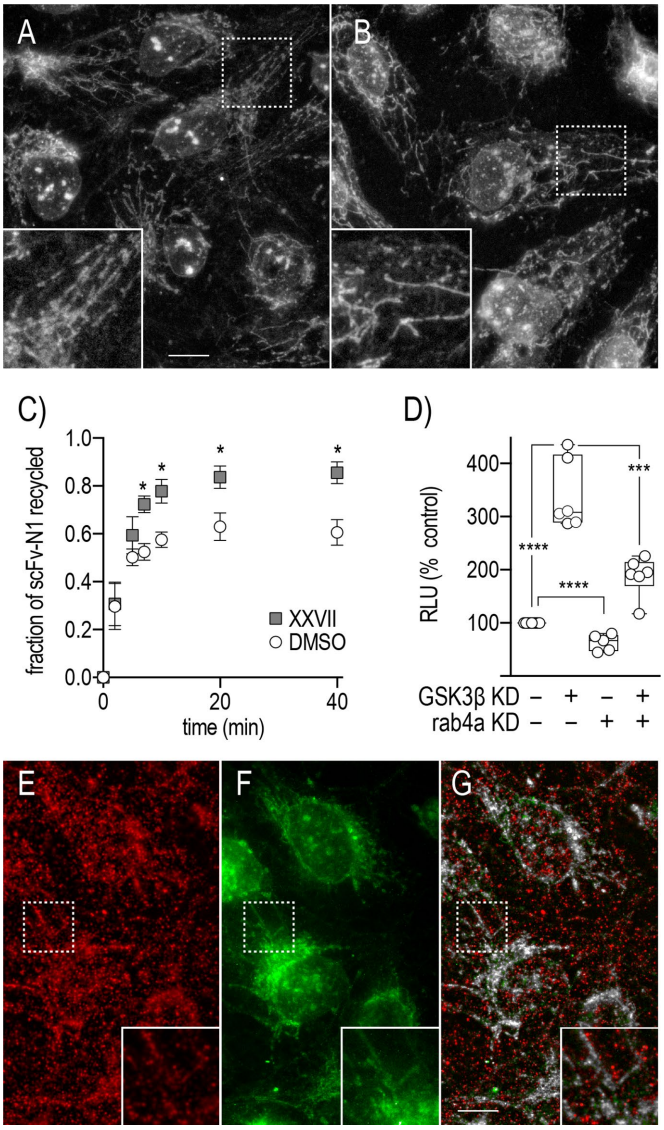

FIGURE 4: GSK3β inhibits Notch1 recycling. Immunofluorescence analysis of U2OS cells incubated for 10 min with scFv-N1-sfGFP-GLuc to allow antibody endocytosis following pretreatment with DMSO (A) or 32 μM XXVII (B) for 3 h. (C) Kinetics of scFv-N1-sfGFP-GLuc recycling in U2OS cells pretreated with DMSO or XXVII for 1 h. Error bars indicate ±SEM; asterisks indicate the *p* value from three independent experiments. (D) Notch1 signaling measured with the dual-luciferase signaling assay in cells following treatment with the indicated siRNA for 72 h. Box-and-whisker plots show each data point from at least three independent experiments. Immunolocalization analysis of rab4a (red, E) in U2OS cells that were allowed to internalize scFv-N1-sfGFP-Gluc antibody (green, F) for 10 min. A colocalized pixel map (G), obtained using the Colocalization Threshold plug-in for ImageJ, shows colocalized pixels in gray scale. Insets show 2× magnified regions of boxed areas. Bar = 10 μM.

this discrepancy between cell culture systems remains unclear, although more recent genetic studies in mouse animal models demonstrate that multiple signaling pathways, including Notch, are markedly up-regulated when GSK3β is conditionally removed in the brain (Kim *et al.*, 2009). Given the cross-talk and reciprocal regulation between Notch and other signaling pathways (Hurlbut *et al.*, 2007; Ammeux *et al.*, 2016), it is possible that chronic loss of GSK3β leads to changes in Notch activity depending on which other signaling pathways are also disrupted.

Importantly, our discovery that GSK3β association with Notch1-positive endosomes appears to be dynamic and that GSK3β inhibition enhances Notch1 recycling from endosomal stores supports a model where cells can directly modulate Notch1 signaling capacity and bypass the need for additional transcription of the receptor. For example, GSK3β activity is inhibited when cells are stimulated with Wnt, insulin, or other growth factors (Cross *et al.*, 1995; Metcalfe and Bienz, 2011). Thus, when GSK3β activity is suppressed, Notch1 is mobilized and recycled to the cell surface from endosomal stores. This, in turn, would increase Notch signaling capacity by increasing receptor exposure to ligands on the surfaces of neighboring cells. This proposed mechanism would enable cells to up-regulate Notch activity in acute response to local environmental conditions. Establishing which signaling pathways impact Notch mobilization from endosomal stores will be the focus of future studies.

MATERIALS AND METHODS

Cells and culture conditions

tTA HeLa and U2OS cells were obtained from Sandra Schmid (University of Texas, Southwestern) and Wendy Gordon (University of

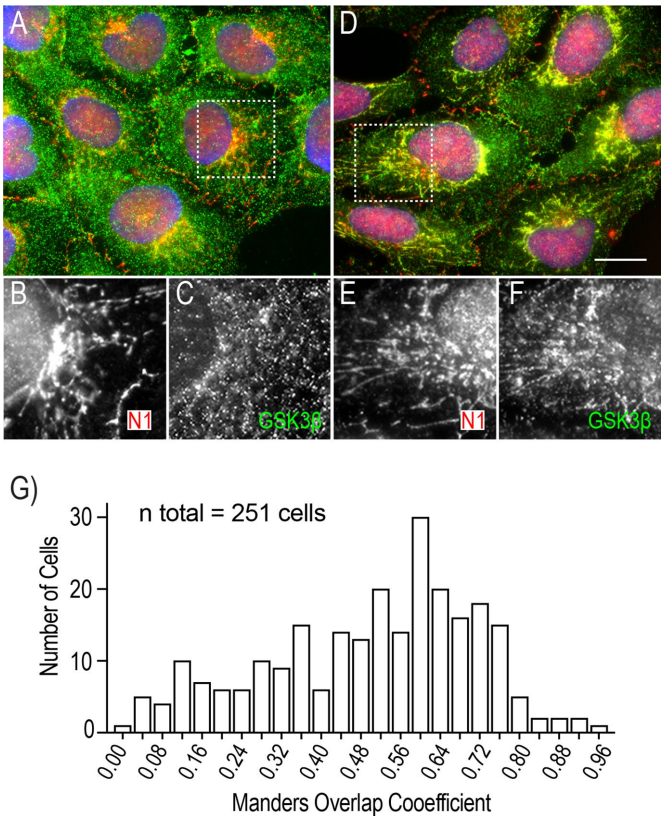

FIGURE 5: GSK3β recruitment to Notch1-positive endosomes is dynamic. Coimmunolocalization analysis of endogenous Notch1 (N1, red) and GSK3β (green) in U2OS cells. Images were obtained from multiple regions of a single coverslip. Single channel images of magnified, boxed regions in A and D are shown in B and C and E and F, respectively. (G) The extent of colocalization between GSK3β and Notch1 was quantified from at least four independent immunolocalization experiments ($n = 251$ cells) using the ImageJ Colocalization Threshold plug-in. A histogram of cell counts is shown. Rabbit pAb against the cytoplasmic tail was used to detect Notch1, mAb 3D10 was used to detect GSK3β, and DAPI is in blue to mark nuclei. Bar = 10 μm.

Minnesota), respectively. Cells were cultured in modified DMEM containing 10% fetal bovine serum (FBS), 4.5 g/l glucose, and 100 U/ml penicillin–streptomycin at 37°C with 5% CO_2. tTA HeLa cells were maintained in the presence of 400 mg/ml G418 to maintain expression of the tetracycline *trans*-activator (tTA). Each culture was maintained at a subconfluent density for no more than 20 passages.

Antibodies and other reagents

Polyclonal antibody against mammalian Notch1 was generated in rabbit against a GST-fused Notch1 cytoplasmic tail (mouse amino acids 1759-2306) by Covance. The monoclonal antibody (mAb) E7 was used to identify β-tubulin. DTX1 pAb (OAGA00965) was purchased from Aviva Systems Biology. Monoclonal antibodies targeting Rab4a (4E11, sc-517263) and GSK3β mAb (3D10, 9832) were purchased from Santa Cruz Biotechnologies and Cell Signaling Technology, respectively. XXVII (361570) was purchased from EMD Millipore.

Constructs

The activated Notch1 mimic CD8-NΔE was previously described (Sorensen and Conner, 2010). Anti-Notch1-E6 in pBIOCAM5 was a gift from John McCafferty (University of Cambridge) (Addgene plasmid #39344) and was used to generate scFv-Notch1-sfGFP-GLuc in pBIOCAM5. The Notch dual luciferase reporter construct was generated by cloning firefly luciferase behind 10 repeating RBPJκ transcription factor binding sites for Notch1-induced expression in pAD-Tet. In the same plasmid backbone, *Renilla* luciferase was cloned in the opposite direction behind two repeating tTA binding sites to provide low-level constitutive expression in tTA HeLa cells. All constructs were sequence-verified.

scFv-Notch1-sfGFP-GLuc antibody production

Recombinant Notch1 antibody was produced by transfecting HEK293 cells with scFv-Notch1-sfGFP-GLuc pAD-CMV(+) plasmid for 48 h. Culture media containing secreted antibody was then concentrated 50× with a Centricon concentrator (UFC710008; EMD Millipore) with a 10-kDa cutoff by centrifugation. Concentrated antibody was stored at 4°C. For cell surface binding studies, scFv-Notch1-sfGFP-GLuc antibody was used at a 1:100 dilution on fixed, nonpermeabilized cells.

Immunolocalization and image quantification

For imaging studies, U2OS cells were grown on glass coverslips, transferred to ice, and washed with Dulbecco's phosphate-buffered saline (DPBS). Cells were fixed with ice-cold acetone for 2 min, followed by methanol for 2 min. Coverslips were then washed with DPBS containing 0.1% Tween (PBST) before antibody addition. Cells were incubated with primary antibody for 1 h at room temperature (RT), washed with PBST, and incubated for 1 h at RT with the appropriate secondary antibody conjugated to either Alexa 488 or Alexa 555. Samples were visualized by epifluorescence using a Zeiss Axio Imager M1 (Zeiss) and captured with a 12-bit monochrome Jenoptik CCD camera (Jena). Images were imported, cropped, and assembled into panels using Photoshop CS6 and Illustrator CS6 (Adobe Systems). ImageJ (v1.51q) was used to quantify colocalization between Notch1 and GSK3β. Each image was background-subtracted using a 50-pixel rolling ball radius. The perinuclear region containing tubular endosomes was selected as a region of interest in the Notch1 channel. The extent of colocalization was then determined using the Colocalization Threshold plug-in, which automatically determines the Manders overlap coefficient (Manders *et al.*, 1993), where data are presented as a fraction of a total.

Notch signaling assay and statistical analysis

Signaling was evaluated with the dual reporter plasmid (see *Constructs*) using a dual-luciferase RBP-Jk reporter assay (SA Biosciences) and assessed according to the manufacturer's protocols (Promega). In each case, RBP-Jk–promoted firefly luciferase activity was normalized to constitutively expressed *Renilla* luciferase under the control of a tetracycline-regulatable (TRE) promoter driven by tTA. Relative luciferase units (RLU) represent signaling expressed as a ratio of Notch-promoted firefly luciferase activity to *Renilla* luciferase activity. Notch signaling was measured using the Notch chimera CD8-NΔE (Sorensen and Conner, 2010), which mimics the activated receptor. This chimera was expressed under the control of a TRE promoter that limits protein expression to near-endogenous levels, as previously described (Zheng *et al.*, 2013). All data were statistically analyzed using a paired *t* test (two-tailed) to calculate a *p* value with Prism (v7.0b, Graphpad). *p* values are represented in each figure with asterisks; $p < 0.05$, 0.005, 0.0005, and 0.0001 are shown as *, **, ***, and ****, respectively. NS (not significant) indicates $p > 0.5$.

siRNA silencing

To silence protein expression, cells were transfected twice with siRNA using RNAiMax (Thermo Fisher) on the first day and 24 h later following manufacturer protocols. Cells were incubated an additional 18–24 h before functional analysis. Previously published and validated siRNAs were purchased from Qiagen or Gene Pharma: GSK3β (CCCAAATGTCAAACTACCAAA; Krueger *et al*., 2007), rab4a (ACGGCCATGTCCGAAACCTACGATT; Barbarin and Frade, 2011), negative control (TTCTCCGAACGTGTCACGTTT). Where indicated, protein expression silencing was validated by immunoblot analysis.

scFv-N1-sfGFP-GLuc recycling assay

For recycling assays, cells grown in 35-mm dishes were pretreated with either DMSO or 32 μM XXVII in DMEM/10%FBS for 1 h at 37°C. Media were replaced with fresh growth media containing identical drug concentrations and scFv-N1-sfGFP-GLuc antibody (1:100 dilution). Cells were then incubated at 37°C for 12 min to allow antibody internalization. Antibody uptake was terminated by transferring dishes to ice, aspirating antibody-containing media, and washing with ice-cold DPBS. Cells were then incubated on ice for 10 min in DPBS containing 5 mM EDTA and 20 mM TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) to resuspend cells and irreversibly inactivate surface-bound scFv-N1-GLuc–*Gaussia* luciferase activity relies on disulfide bonds and is inactivated with reducing agents (Inouye and Sahara, 2008). Cells were then pelleted and resuspended in 0.5-ml ice-cold DMEM/10% FBS containing DMSO or XXVII. Aliquots of 40 μl were transferred into 1.5-ml tubes for each time point. All tubes, except for the zero time point (total), were then transferred to 37°C for the indicated time period before being returned to ice to stop recycling. scFv-N1sfGFP-GLuc antibody, which returned to the cell surface, was then inactivated by addition of 1 μl 0.5 M TCEP. Cells were washed with 1.0 ml DPBS and gently pelleted by centrifugation at 4°C, supernatant was aspirated, and cells were lysed with 20 μl DPBS containing 1% TX-100. A quantity of 5 μl of lysate was then used to measure the luciferase activity retained within cells. Luciferase activity at each time point was then divided by the total and subtracted from 1 to determine the fraction of recycled scFv-N1-GLuc.

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