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O-Fucose and Fringe-modified NOTCH1 extracellular domain fragments as decoys to release niche-lodged hematopoietic progenitor cells

Shuiliang Yu[2], Weihuan Wang[2], Marwah Albakri[2], Xiaoxing Yu[3], Gurnoor Majihail[4], Seunghwan Lim[5], Rachel K Lopilato[6], Atsuko Ito[6], John Letterio[5], Robert S Haltiwanger[6], and Lan Zhou[1,2]

[2]Department of Pathology, Case Western Reserve University, Cleveland, OH 44106, USA, [3]Beachwood High School, Beachwood, OH 44122, USA, [4]Hathaway Brown School, Shaker Heights, OH 44122, USA, [5]Department of Pediatrics, Case Western Reserve University, Cleveland, OH 44106, USA, and [6]Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, USA

[1]To whom correspondence should be addressed: Tel: (216)3681671; Fax: (216)3680494; e-mail: Lxz47@case.edu

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Abstract

Successful hematopoietic progenitor cell (HPC) transplant therapy is improved by mobilizing HPCs from the bone marrow niche in donors. Notch receptor–ligand interactions are known to retain HPCs in the bone marrow, and neutralizing antibodies against Notch ligands, Jagged-1 or Delta-like ligand (DLL4), or NOTCH2 receptor potentiates HPC mobilization. Notch–ligand interactions are dependent on posttranslational modification of Notch receptors with *O*-fucose and are modulated by Fringe-mediated extension of *O*-fucose moieties. We previously reported that *O*-fucosylglycans on Notch are required for Notch receptor–ligand engagement controlling hematopoietic stem cell quiescence and retention in the marrow niche. Here, we generated recombinant fragments of NOTCH1 or NOTCH2 extracellular domain carrying the core ligand-binding regions (EGF11–13) either as unmodified forms or as *O*-fucosylglycan-modified forms. We found that the addition of *O*-fucose monosaccharide or the Fringe-extended forms of *O*-fucose to EGF11–13 showed substantial increases in binding to DLL4. Furthermore, the *O*-fucose and Fringe-extended NOTCH1 EGF11–13 protein displayed much stronger binding to DLL4 than the NOTCH2 counterpart. When assessed in an in vitro 3D osteoblastic niche model, we showed that the Fringe-extended NOTCH1 EGF11–13 fragment effectively released lodged HPC cells with a higher potency than the NOTCH2 blocking antibody. We concluded that *O*-fucose and Fringe-modified NOTCH1 EGF11–13 protein can be utilized as effective decoys for stem cell niche localized ligands to potentiate HPC egress and improve HPC collection for hematopoietic cell therapy.

Key words: decoys, hematopoietic progenitor cells, Notch, *O*-fucosylglycans

Introduction

Hematopoietic progenitor cell (HPC) transplant is a potentially curative therapy for blood and nonhematologic diseases. A successful outcome of HPC transplant depends on infusion of an adequate number of functionally active mobilized HPCs. Successful improvement in mobilization or enhancement of reconstitution of mobilized HPCs is highly desired to improve hematopoietic cell therapy (HCT) outcome. Hematopoietic stem cells (HSCs) in the bone marrow niche are controlled by the balanced regulation of diverse niche supporting cells and signaling pathways (Calvi and Link 2015; Morrison and Scadden 2014). Among these, Notch is a conserved receptor with important roles in regulating HSC biology through direct cell–cell interaction. An important feature of Notch is its adhesive nature, which was first described by cell aggregation assays in *Drosophila* studies and involves the engagement of Notch receptors with Notch ligands (Fehon et al. 1990; Rebay et al. 1991). There are four Notch receptors (NOTCH1–4) and two families of Notch ligands, Jagged (JAG1, 2) and Delta-like ligands (DLL1, 4). We and others reported that NOTCH2 is the major isotype expressed on HSCs and nonlymphoid progenitor cells (Oh et al. 2013; Varnum-Finney et al. 2011; Wang et al. 2015; Yao et al. 2011). In addition, JAG1 and DLL4 are expressed by bone marrow endothelial cells and osteoblasts to regulate homeostasis and regenerative hematopoiesis (Poulos et al. 2013; Yu et al. 2015). We showed that HSPC adhesion to the marrow osteoblasts relies on Notch–ligand interaction and is further enhanced by the glycosylation of Notch receptor, specifically *O*-fucosylation of epidermal growth factor (EGF)-like repeats present on the ligand-binding region of Notch receptor (Wang et al. 2015).

Notch transactivation is the result of receptor and ligand interaction that causes release of the intracellular domain of Notch receptors after several events of protease digestion (Kovall et al. 2017). This interaction is dependent on posttranslational modification of Notch receptors with *O*-glucose and *O*-fucose, added to serine in the consensus C^1-X-S-X-(P/A)-C^2, or to serine or threonine residue in the consensus sequence C^2-X-X-X-X-(S/T)-C^3, by protein *O*-glucosyltransferase 1 or protein *O*-fucosyltransferase 1 (POFUT1), respectively, on the EGF modules of Notch extracellular domain (Harvey and Haltiwanger 2018; Pandey et al. 2020; Varshney and Stanley 2018). *O*-Fucose modification enhances Notch affinity for JAG or DLLs and regulates Notch signaling transactivation (Okajima and Irvine 2002; Okajima et al. 2003; Okamura and Saga 2008; Shi and Stanley 2003; Taylor et al. 2014). This is supported by recent crystal structural determinations showing that *O*-fucose attached to Thr466 within the POFUT1 consensus sequence of NOTCH1 EGF-like repeat 12 (EGF12) acts as a surrogate amino acid to make functional contact with a specific domain on DLL4, thus directly affecting Notch–ligand binding (Luca et al. 2015). Subsequently, the same group showed that *O*-fucose attached to the Thr residues in the POFUT1 consensus sequences of NOTCH1 EGF8 and EGF12 interacts with a specific domain on JAG1 (Luca et al. 2017). Elongation of *O*-fucose to a disaccharide (GlcNAcβ1-3Fucose) by any of the Fringe enzymes (*N*-acetylglucosaminyltransferases), Lunatic fringe (Lfng), Manic fringe or Radical fringe, further influences Notch signaling by enhancing Notch activation in response to DLLs (Chen et al. 2001; Kakuda and Haltiwanger 2017; Kakuda et al. 2020; Moloney, Panin, et al. 2000a; Rampal et al. 2005; Shao et al. 2003; Shimizu et al. 2001; Taylor et al. 2014). In mammals, the GlcNAcβ1-3Fucose disaccharide can be further elongated to a tetrasaccharide: Siaα2-6Galβ1-4GlcNAcβ1-3Fuc (Moloney, Panin, et al. 2000a; Moloney, Shair, et al. 2000b), although addition of the galactose and sialic acid has little additional effect on Notch–ligand binding (Taylor et al. 2014).

We previously reported that the posttranslational modification of Notch by *O*-fucosylation regulates myelopoietic homeostasis, HSC quiescence and HSC niche location (Murata et al. 2014; Wang et al. 2015; Yao et al. 2011; Zhou et al. 2008). We then reported that neutralizing Notch ligand (DLL4 or JAG1) or NOTCH2 receptor with blocking antibodies potentiates HSPC mobilization (Wang et al. 2015, 2017). In this study, we asked if the addition of *O*-fucose and its extension by Fringe could substantially enhance the binding of recombinant Notch protein bearing the core ligand-binding domain (EGF11–13) with two HSC niche ligands, DLL4 and JAG1, and whether the glycosylated forms of recombinant Notch protein could function as decoys for Notch ligands, thus blocking the interaction of Notch with the ligand and effectively releasing lodged HPCs from an in vitro model of osteoblastic niche.

Results

Expression of NOTCH1 and NOTCH2 fragments with or without *O*-fucose and Fringe modifications

In our previous studies, we showed that lack of Notch *O*-fucosylation in mice or blocking Notch–ligand engagement with antibodies led to enhanced HPC mobilization (Wang et al. 2015; Yao et al. 2011). Here, we generated recombinant fragments of the NOTCH1 and NOTCH2 extracellular domains containing the core ligand-binding region, EGF11–13, to assess if the homologous ligand-binding domain of NOTCH1 and NOTCH2 shows similar enhancement of HPC mobilization.

The coding sequences of both mouse NOTCH1 and NOTCH2 EGF11–13 fragments, referred as $N1_{11–13}$ and $N2_{11–13}$ in our data analysis and figure legends, respectively, were cloned and fused with C-terminal human immunoglobulin G (IgG) fragment crystallizable (Fc)- or His_6-tags. All these constructs were transfected into Chinese hamster ovary (CHO) cells or HEK293T cells and cultured in media with or without *L*-fucose to generate unmodified and *O*-fucose-modified proteins. To generate Fringe-extended *O*-fucose glycans, recombinant protein expression constructs were cotransfected with Lfng expression plasmids and cultured in media with *L*-fucose. As growing cells in media without *L*-fucose did not prevent *O*-fucosylation, we mutated the fucosylation sites in EGF12 of both NOTCH1 and NOTCH2 from Thr to Val (NOTCH1: T466V; NOTCH2: T470V) to generate unfucosylated protein. Previous studies showed that the T466V substitution of a NOTCH1 EGF11–13 protein bound DLL4 and JAG1 like the wild-type protein and did not affect the native fold of EGF12 (Taylor et al. 2014). The *O*-fucosylation-modified and Fringe-extended glycoproteins were generated using the wild-type constructs. The unfucosylated (mutant) proteins are referred to as $N1_{11–13}$ Mut and $N2_{11–13}$ Mut; the *O*-fucose monosaccharide-modified proteins are $N1_{11–13}$ + Fuc and $N2_{11–13}$ + Fuc; and the proteins with Fringe-extended *O*-fucose (di-, tri- and tetrasaccharide) are $N1_{11–13}$ + Fuc + Lfng and $N2_{11–13}$ + Fuc + Lfng. The purified secreted recombinant His_6-tagged NOTCH1 and NOTCH2 proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue (Figure 1A). Compared with the unmodified mutant proteins, the fucosylated and Fringe-extended glycoproteins migrated slightly slower presumably because

of the sugar modifications. To confirm the fucosylation and Fringe modification in these recombinant proteins, the three forms of $N1_{11–13}$-His_6 and $N2_{11–13}$-His_6 proteins (Figure 1B and C, respectively) were analyzed by nano-liquid chromatography (LC)–electrospray ionization (ESI)–tandem mass spectrometry (MS/MS) spectra analysis (Supplementary Figure S1). The MS glycoproteomic analysis confirmed the absence of any sugar moiety on the tryptic peptide derived from EGF12 of mutated $N1_{11–13}$ and $N2_{11–13}$ protein ($N1_{11–13}$ with T466V substitution and $N2_{11–13}$ with T470V, left panel, Figure 1B and C), the presence of *O*-fucose monosaccharide on >80% of the same EGF12 tryptic peptide-derived wild type (WT) NOTCH1/2 EGF11–13 ($N1_{11–13}$ + Fuc and $N2_{11–13}$ + Fuc, middle panel, Figure 1B and C) and the presence of elongated *O*-fucose as di-, tri- and tetrasaccharide glycoform on 18.3, 6.4 and 62.4%, respectively, of the EGF12 peptide derived from EGF11–13 coexpressed with Lfng ($N1_{11–13}$ + Fuc + Lfng) (Figure 1B, right panel). The elongated *O*-fucose (di-, tri- and tetrasaccharide) glycoform was also found in >80% of the EGF12 peptides derived from Fringe-modified NOTCH2 EGF11–13 ($N2_{11–13}$ + Fuc + Lfng, right panel, Figure 1C). Similar modification patterns were revealed for the three forms of IgG Fc fusion NOTCH1 proteins analyzed by nano-LC-ESI-MS/MS spectra analysis (Supplementary Figure S2).

Flow-based binding comparison of NOTCH1 fragments and NOTCH2 fragments

The recombinant His-tagged Notch fragments modified with *O*-fucose alone or *O*-fucose with Fringe were first tested for binding to OP9 cells stably transfected with full-length murine DLL4 in a flow cytometry-based assay, and the level of binding with increasing concentrations of each protein tested was compared with the unmodified protein. We found that the mutated NOTCH1 fragment ($N1_{11–13}$ Mut) binds better to DLL4 than the NOTCH2 mutant fragment ($N2_{11–13}$ Mut) (Figure 2). *O*-Fucose modification of NOTCH1 fragments ($N1_{11–13}$ + Fuc) enhanced the binding to DLL4 more than *O*-fucose modification of NOTCH2 fragments (Figure 2C). Finally, Lfng extension of *O*-fucose on $N1_{11–13}$ caused a much larger increase in binding to DLL4 than $N2_{11–13}$ fragments (Figure 2C). Neither NOTCH1 nor NOTCH2 fragments showed significant binding to DLL4 in the presence of EDTA (not shown), suggesting the interaction is calcium dependent. The IgG-Fc NOTCH1 and NOTCH2 fragments showed a similar binding pattern to OP9-DLL4 as the His-tagged fragments (Supplementary Figure S3).

We also compared the binding of His-tagged NOTCH1 and NOTCH2 fragments to OP9 cells expressing full-length JAG1. The Lfng-extended and *O*-fucosylated fragments of $N1_{11–13}$ (300 nM) and $N2_{11–13}$ proteins (300 nM) showed a much weaker binding to JAG1 than to DLL4 (Supplementary Figure S4A). The nonmodified fragment and the *O*-fucosylated fragment failed to show significant binding to JAG1 (Supplementary Figure S4B; methods described below).

O-Fucose and Fringe modification enhance binding affinity between NOTCH1 EGF11–13 and DLL4 by surface plasmon resonance analysis

To further define the contribution of *O*-fucose and Fringe-mediated extension of *O*-fucose to the enhanced binding of Notch to the ligand, we applied surface plasmon resonance (SPR) to determine the binding affinity of the Notch fragment–ligand interaction. The recombinant mouse DLL4-Fc was immobilized on a CM5 chip surface through primary amine coupling on a BioCore 3000 system. Different concentrations of His-tagged Notch fragments were added to the chip in the solution phase. Corroborating the results of flow cytometry, we found that $N1_{11–13}$ + Fuc and $N1_{11–13}$ + Fuc + Lfng bound DLL4 with increasing affinity compared with $N1_{11–13}$ Mut and displayed dissociation constants (K_d) of 347 ± 95 and 36 ± 5.1 nM, respectively, when the binding curves were fitted with 1:1 Langmuir binding with baseline shift model. However, the interaction of the mutated $N1_{11–13}$ ($N1_{11–13}$ Mut) with DLL4 yielded a K_d >10 μM K_d (Figure 3A, left panel). Therefore, the addition of *O*-fucose on EGF12 enhances the binding of $N1_{11–13}$ to DLL4, and the extension of the monosaccharide by Fringe further enhances its binding to DLL4 (Figure 3A, middle and right panel, and Figure 3B). We then analyzed NOTCH2 protein interaction with DLL4 using SPR. The mutated $N2_{11–13}$ fragment showed very low binding, whereas the proteins modified by the *O*-fucose and Fringe bound poorly to DLL4, precluding a reliable quantification of K_d (Figure 4A and B). Consistent with the observations obtained from the flow-based binding assay, SPR analysis did not generate meaningful K_d values for both NOTCH1 (Supplementary Figure S4B) and NOTCH2 proteins (not shown) bound to JAG1 due to the weak interactions (not shown).

Fc-NOTCH1 EGF11–13 released lodged HPCs better than NOTCH2 blocking antibodies

To test whether the Notch proteins bearing the core ligand-binding domain could function as decoy molecules for Notch ligands, we first tested the ability of Notch proteins to block the adhesion of HPCs ($Lin^-c\text{-}kit^+$; LK) to the bone marrow stroma cells expressing Notch ligand, JAG1 or DLL4. We previously reported that HPCs display stronger adhesion to Notch ligand-expressing OP9 cells (OP9-JAG1, OP9-DLL1 and OP9-DLL4) than to OP9 cells without ligand, and this form of adhesion could be completely inhibited by recombinant ligand proteins (Wang et al. 2015). In addition, adhesion of HPCs to OP9 cells expressing Notch ligand is completely abolished in *Pofut1*-deficient HSPCs due to the loss of the *O*-fucosylglycans on Notch EGF-like repeats that are important for the binding of Notch ligands (Yao et al. 2011). Because the concentrated IgG-Fc NOTCH1 and NOTCH2 fragments had a similar binding pattern to OP9-DLL4 by flow-based assay (Figure 2 and Supplementary Figure S3) and by SPR analysis (not shown) as the His-tagged fragments, we used concentrated IgG-Fc $N1_{11–13}$ proteins in the subsequent HPC adherence assay and the HPC release assay. Consistent with these findings, we found that HPC adhesion to OP9-JAG1 and OP9-DLL4 was significantly blocked by the Fc-tagged $N1_{11–13}$ modified by *O*-fucose ($N1_{11–13}$ + Fuc) or extended by Fringe ($N1_{11–13}$ + Fuc + Lfng) (Figure 5A). In comparison, Fc-tagged $N2_{11–13}$ proteins did not exhibit significant blockade of HPC adhesion (not shown).

We then used an in vitro model of 3D osteoblastic (OB) "spheroid niche" to assess the ability of these proteins to release lodged HPCs. In this setting, artificial osteoblastic spheroids are formed by human fetal osteoblasts (hFOB), which express Delta and JAG1 ligands, in ultra-low attachment (ULA) 96-well U-bottom plates. Forty-eight hours after the addition of 2×10^4 carboxy fluorescein succinimidyl ester (CFSE)-labeled mouse bone marrow HPCs, when ~15–20% of added cells homed to the spheroid (Figure 5B) without significant proliferation, cells were cocultured with blocking anti-NOTCH2 or the three glycoforms of Fc-tagged $N1_{11–13}$. The NOTCH2 blocking antibody, but not the control antibody, caused a 42% decrease in cells remaining in the spheroids. Coculture with three glycoforms of Fc-tagged $N1_{11–13}$ ($N1_{11–13}$ Mut, $N1_{11–13}$ + Fuc and

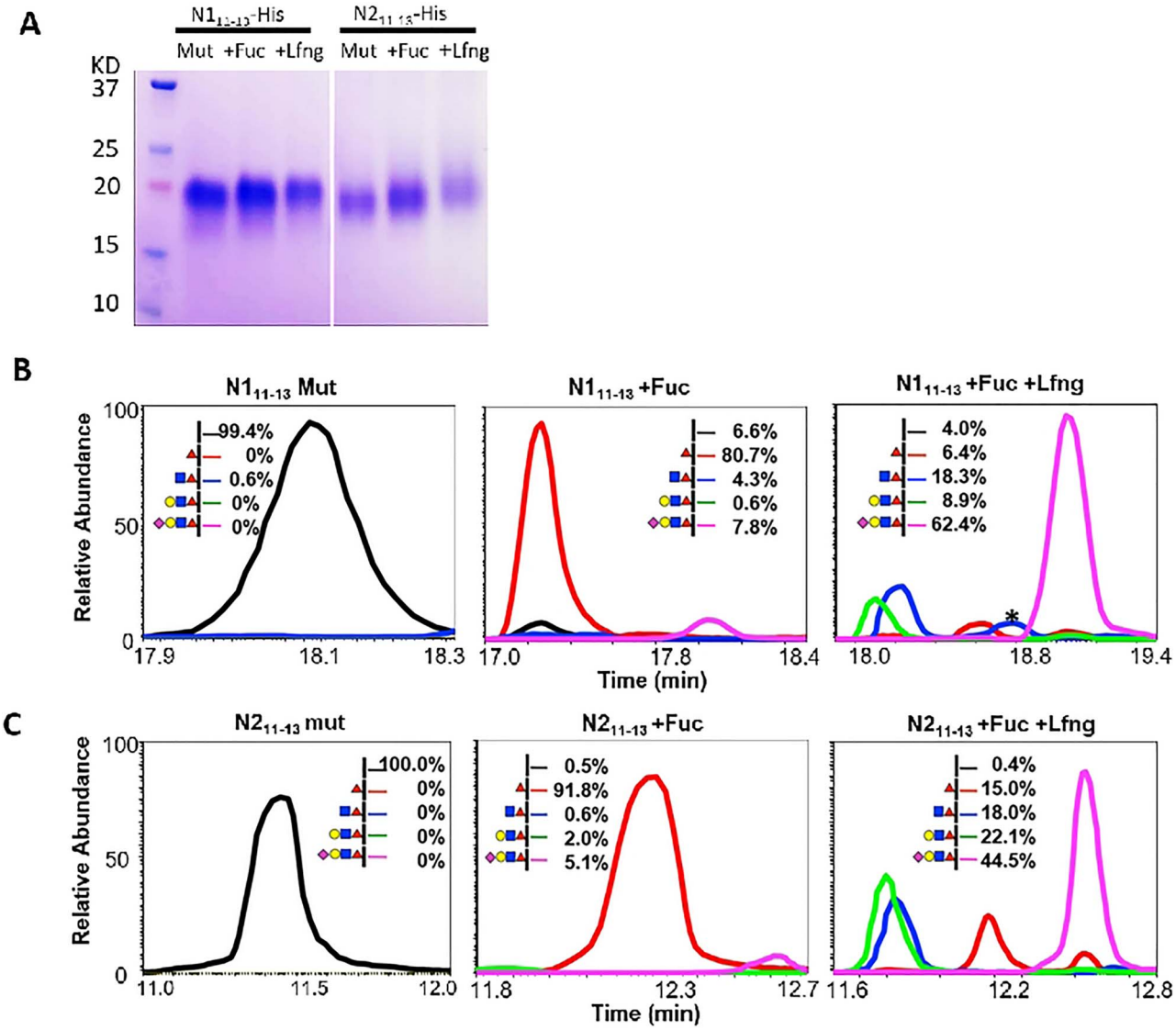

Fig. 1. Glycosylation status of His-tagged NOTCH1 and NOTCH2 EGF11–13 proteins. (**A**) Coomassie blue staining of His-tagged Notch fragments. These recombinant proteins were produced in HEK293T cells and purified as described in *Materials and Methods*. The unmodified (naked, Mut) forms were generated by mutating the fucosylation site in EGF12 of both NOTCH1 and NOTCH2 from Thr to Val (N1: T466V; N2: T470V). The Lfng-modified forms (+Lfng) were generated in cells cotransfected with Lfng. (**B**) Mass spectral analysis of His-tagged N1$_{11-13}$ proteins. Extracted ion chromatograms (EICs) are shown for the ions corresponding to the potential glycoforms of the peptide (unmodified, black line; fucose monosaccharide, red line; fucose disaccharide, blue line; fucose trisaccharide, green line; and fucose tetrasaccharide, purple line) containing the *O*-fucose consensus sequence from EGF12, TGPRCEIDVNECISNPCQNDATCLDQIGEF (or TGPRCEIDVNECISNPCQNDAVCLDQIGEF for the mutant protein), derived from chymotryptic digests of N1$_{11-13}$ Mutant, N1$_{11-13}$ + Fuc and N1$_{11-13}$ + Fuc + Lfng proteins. Asterisk indicates an ion that matches the mass of the EGF12 peptide modified with a fucose disaccharide, but MS2 demonstrates it is not the EGF12 peptide. Spectra for the major glycoforms are shown in Supplementary Figure S1A–C. (**C**) Mass spectral analysis of N2$_{11-13}$ proteins as in (**B**). EICs are shown for the ions corresponding to the potential glycoforms of the peptide containing the *O*-fucose consensus sequence from EGF12, CEMDINECHSDPCQNDATCLDK (or CEMDINECHSDPCQNDAVCLDK for the mutant protein), derived from tryptic digests of N2$_{11-13}$ Mutant, N2$_{11-13}$ + Fuc and N2$_{11-13}$ + Fuc + Lfng proteins. Spectra for the major glycoforms are shown in Supplementary Figure S1D–F.

N1$_{11-13}$ + Fuc + Lfng) caused a 61, 72 and 77% decrease of cells remaining in the spheroids, respectively (Figure 5C–F). In comparison, coculture of Fc-tagged N2$_{11-13}$ led to <10% egress of cells when compared with nontreated cells (not shown). This finding suggests that N1$_{11-13}$, but not N2$_{11-13}$, could function as decoys for niche-expressing ligands and induce the release of lodged HPCs by outcompeting with NOTCH2 receptor, the major receptor expressed on HSCs and progenitor cells.

Discussions

In this study, using flow cytometry and SPR analysis, we found that Fringe-mediated extension of O-fucose of murine N1$_{11-13}$ resulted in a substantial increase in binding to DLL4. This is consistent with the crystal structure analysis showing that *O*-fucose on EGF12 T466 mediates a direct interaction with the ligand-binding region of DLL4 (Luca et al. 2015), and other reports that the O-fucose and

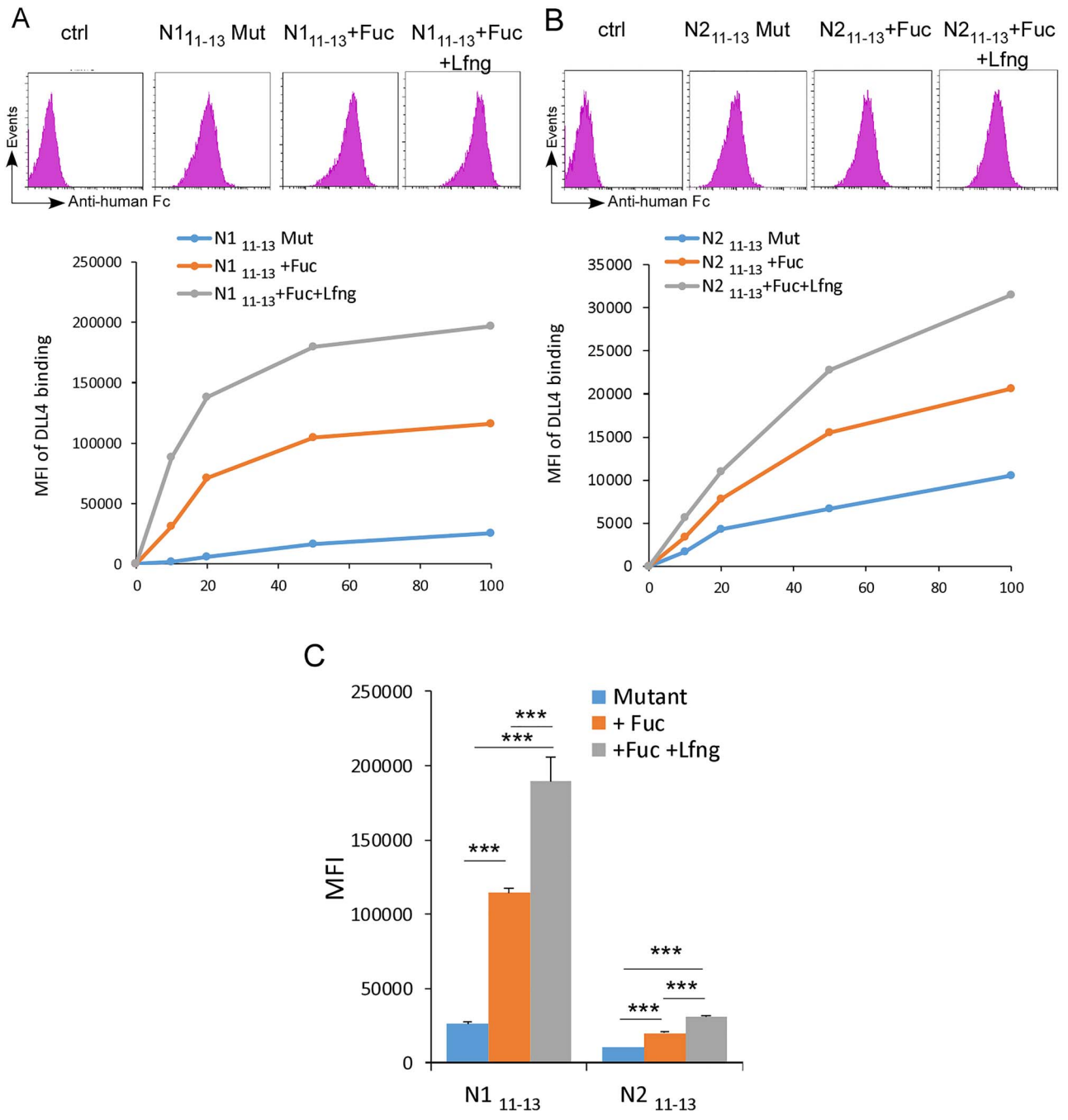

Fig. 2. Binding of His-tagged NOTCH1 and NOTCH2 fragments with DLL4 by FACS analysis. Binding of increasing concentrations of unmodified and *O*-fucosylglycan forms of $N1_{11–13}$ (**A**) and $N2_{11–13}$ (**B**) to OP9-DLL4 cells were analyzed by FACS. Mean fluorescence values (MFI) determined by each protein (100 nM) were shown for N1/2 11–13 fragments (**C**).

the GlcNAc-fucose modifications enhances the binding of $N1_{11–13}$ with DLL1 and DLL4 (Taylor et al. 2014). We report a K_d value of 347 ± 95 nM for the binding of DLL4-Fc to *O*-fucose-modified $N1_{11–13}$, which lacks Fringe modification ($N1_{11–13}$ + Fuc). This is similar to the K_d value for the association of DLL4 with NOTCH1 EGF 6–15 without Fringe extension (Andrawes et al. 2013). However, insect cell expressed $N1_{11–13}$ showed a much lower affinity (K_d value of several micrometer) to a truncated DLL4 without an Fc tag where the NOTCH1 protein was immobilized on the chip in the SPR analysis (Luca et al. 2015). The methodology and construct differences might cause the divergent K_d values observed. We found that the elongation at the *O*-fucose site of NOTCH1 EGF12 ($N1_{11–13}$ + Fuc + Lfng) yielded a much stronger binding to DLL4 with a K_d value of 36 ± 5.1 nM. Mass spectral analysis showed that EGF12 of $N1_{11–13}$ was modified at high stoichiometry with *O*-fucose, and that Fringe-mediated elongation of the *O*-fucose resulted in >90% elongation of the *O*-fucose to di-, tri- and tetrasaccharide forms. Prior studies suggest that elongation past the disaccharide has

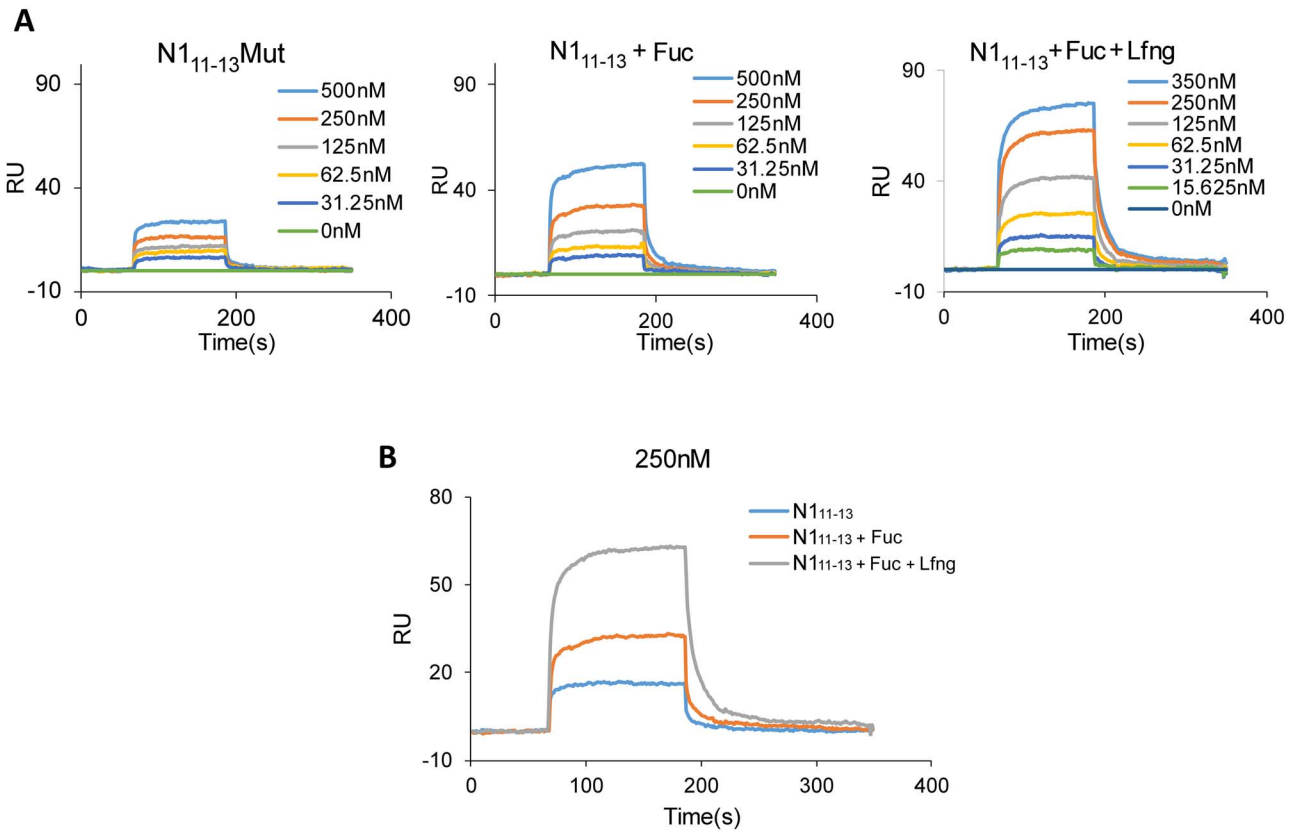

Fig. 3. SPR Analysis of His-tagged NOTCH1 EGF11–13 fragments bound to DLL4. (**A**) Representative SPR traces for binding of increasing concentrations of unmodified mutant (left), *O*-fucose-modified (middle) and Lfng-modified (right) $N1_{11–13}$ fragments in response units (RU). (**B**) Combined SPR traces for binding of three forms of proteins at 250 nM.

little or no additional enhancement to $N1_{11–13}$ binding to ligands (Taylor et al. 2014).

Although overall murine NOTCH2 is homologous to murine NOTCH1, we found that nonmodified $N1_{11–13}$ protein or its modification by *O*-fucose alone or Fringe-mediated extension of *O*-fucose has a higher binding affinity to DLL4 than their NOTCH2 counterparts. Such differences in the interaction between NOTCH1 and NOTCH2 with DLL4 are presumably contributed by different residues of each receptor lining the ligand-binding interface (Kakuda et al. 2020; Luca et al. 2015, 2017; Tveriakhina et al. 2018). Interestingly, modification of full-length NOTCH1 by any Fringe has little or no effect on activation from DLL4 in cell-based assays, whereas Lfng causes a small but significant enhancement of NOTCH2 activation from DLL4 (Kakuda et al. 2020). As NOTCH1 has a much higher binding affinity for DLL4 than DLL1 in the absence of Fringe modifications, the level of binding is sufficient to fully activate NOTCH1 (Andrawes et al. 2013; Kakuda et al. 2020). Thus, Fringe-mediated enhancement of NOTCH1 binding to DLL4 has no additional effect on activation, but it should enhance cell-to-cell adhesion.

When assessed in the HPC adhesion and an in vitro 3D osteoblastic niche model, we found that Fringe and *O*-fucose-modified $N1_{11–13}$ protein more effectively blocked HPC adhesion to bone marrow stroma cells and released lodged HPC cells than nonmodified protein or NOTCH2 blocking antibody. This compelling evidence suggests that the Fringe and *O*-fucose-modified $N1_{11–13}$ may be used as decoys for various Notch ligands to mobilize HPCs from HSC niche. Until recently, filgrastim (G-CSF), either alone or in combination with chemotherapy, was associated with unsuccessful mobilization of an "optimal" $CD34^+$ dose (5×10^6/kg) in up to 40% of patients (Pusic et al. 2008). AMD3100 (a CXCR4 antagonist; plerixafor) increases total $CD34^+$ mobilized when combined with filgrastim, and this combination is often used in myeloma and non-Hodgkin lymphoma patients (Mohty et al. 2014). Unfortunately, this approach does not always lead to adequate HPC collection in patients who have been previously exposed to extensive cytotoxic therapy, and multiple HCT procedures may be considered in some cases (Keating 2011; Moreb et al. 2011; Moskowitz et al. 1998). In addition, in HPT mobilization for sickle cell disease (SCD) patients undergoing transplant, the administration of proinflammatory G-CSF to donors who are frequently siblings for SCD patients and present sickle cell trait carries the risk of provoking sickling or other complications (Al-Khabori et al. 2015; Grigg 2001). Therefore, developing more efficacious strategies is highly desirable for HPC cell therapy. In summary, similar to NOTCH2 blocking antibody and Notch ligand neutralizing antibodies, the Fringe and *O*-fucose-modified $N1_{11–13}$ protein has the potential to enhance the mobilization of HSPC cells when used in combination with the current mobilizing reagents.

Materials and methods

Plasmids and cells

To construct NOTCH1 or NOTCH2 EGF11–13 expression plasmids, the coding sequence of N-terminal signal peptide and the sequence of EGF11–13 were fused by extension overlapping polymerase chain reaction technology and inserted into Xho I/BamH

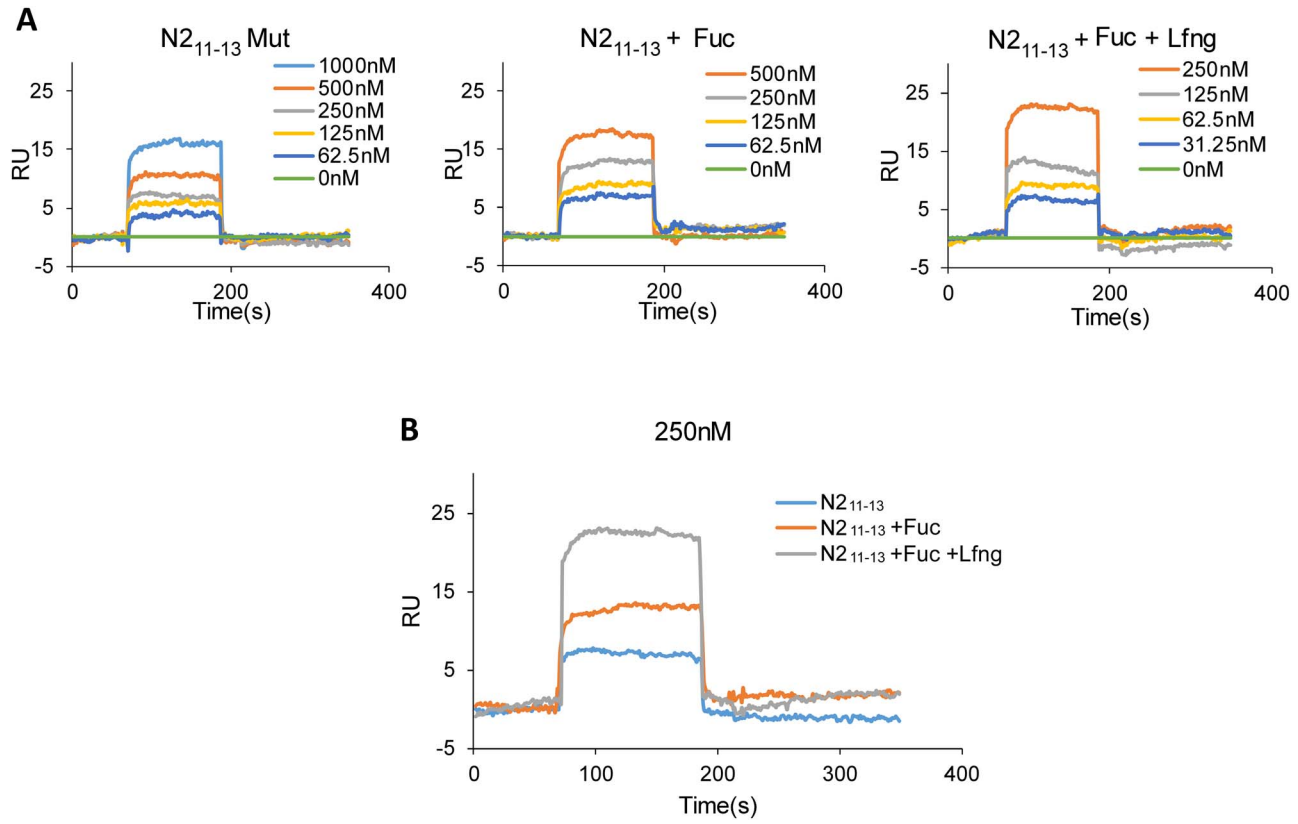

Fig. 4. SPR analysis of His-tagged NOTCH2 EGF11–13 fragments bound to DLL4. (**A**) Representative SPR traces for binding of increasing concentrations of unmodified mutant (left), *O*-fucose-modified (middle) and Lfng-modified (right) $N2_{11–13}$ fragments in response units (RU). (**B**) Combined SPR traces for binding of three forms of proteins at 250 nM.

I site on the backbone of pcDNA1.1, which contains the human IgG Fc coding region. All fucosylation site mutants (Thr to Val; ACT to GTT or ACC to GTC) were generated with QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Tech, Santa Clara, CA). To generate C-terminal Myc-His_6-tagged NOTCH1 or NOTCH2 EGF11–13 expression constructs, both the wild-type and mutant sequences of EGF11–13 were amplified and inserted into Hind III/Xho I site of pSec-Tag2/HygroC vector (Invitrogen, Carlsbad, CA). HEK293T cells were obtained from American Type Culture Collection (ATCC) and maintained in Dulbecco's-Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS; Gibco). CHO cells were maintained in Minimum Essential Medium alpha (MEM alpha) and supplemented with 2.0 mM glutamine, 1.0 g/L glucose, 10 mg/L ribonucleosides and deoxyribonucleosides, 10% FBS and100 IU/mL penicillin/streptomycin (Penn/Strep). The NOTCH2 blocking antibody and the control antibody were kindly provided by Dr. Christian Siebel (Genentech, Inc., South San Francisco, CA) (Wu et al. 2010).

Recombinant protein expression

The expression plasmids encoding the Fc-tagged $N1_{11–13}$ or $N2_{11–13}$ proteins were transfected into CHO cells with lipofectamine 3000 and cells were split and cultured in pro CHO-AT serum-free medium (Lonza, Basel, Switzerland) with or without 1 mM *L*-fucose supplemented with 2.0 mM GlutaMAX-I (Gibco, Waltham, MA), 100 μM sodium hypoxanthine and 16 μM thymidine (Lonza, Basel, Switzerland). For Fringe modification, the expression constructs (Fc-tagged $N1_{11–13}$ or $N2_{11–13}$) were cotransfected with Lfng expression plasmid (from Dr. John Lowe) at a 2:1 ratio. Cells were cultured for 6–7 days and the supernatants were collected, filtered and concentrated. Concentrated culture supernatants were used directly for all assays. The concentration of Fc-tagged proteins was determined by sandwich enzyme-linked immunosorbent assay. Anti-human IgG antibody (Sigma, St. Louis, MO) was coated and used to capture Fc fused Notch proteins for 1 h at 25°C. HRP conjugated anti-human Fc antibody was used to detect the binding proteins. Purified recombinant CD14-Fc protein (R&D System, Minneapolis, MN) was used for standard curve. Recombinant proteins were also identified by immunoblotting with anti-human IgG Fc-HRP antibody (Sigma, St. Louis, MO).

His_6-tagged Notch proteins were expressed and purified as previously described (Kakuda and Haltiwanger 2014) In brief, HEK293T cells were transfected with expression plasmids encoding $N1_{11–13}$ or $N2_{11–13}$ using Polyfect reagent (Qiagen, Hilden, Germany) and cultured in Opti-MEM I Reduced Serum Media (Gibco, Waltham, MA) for 4 days. For Fringe modification, the expression plasmids (His-tagged $N1_{11–13}$ or $N2_{11–13}$) were cotransfected with Lfng expression plasmid (from Dr. John Lowe) at a 2:1 ratio. The secreted proteins were harvested and incubated with Nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Thermo Fisher Scientific, Waltham, MA) at 4°C overnight. After washes, the proteins were eluted with 250 mM imidazole and dialyzed against phosphate-buffered saline. The purity of concentrated proteins was examined by Coomassie blue

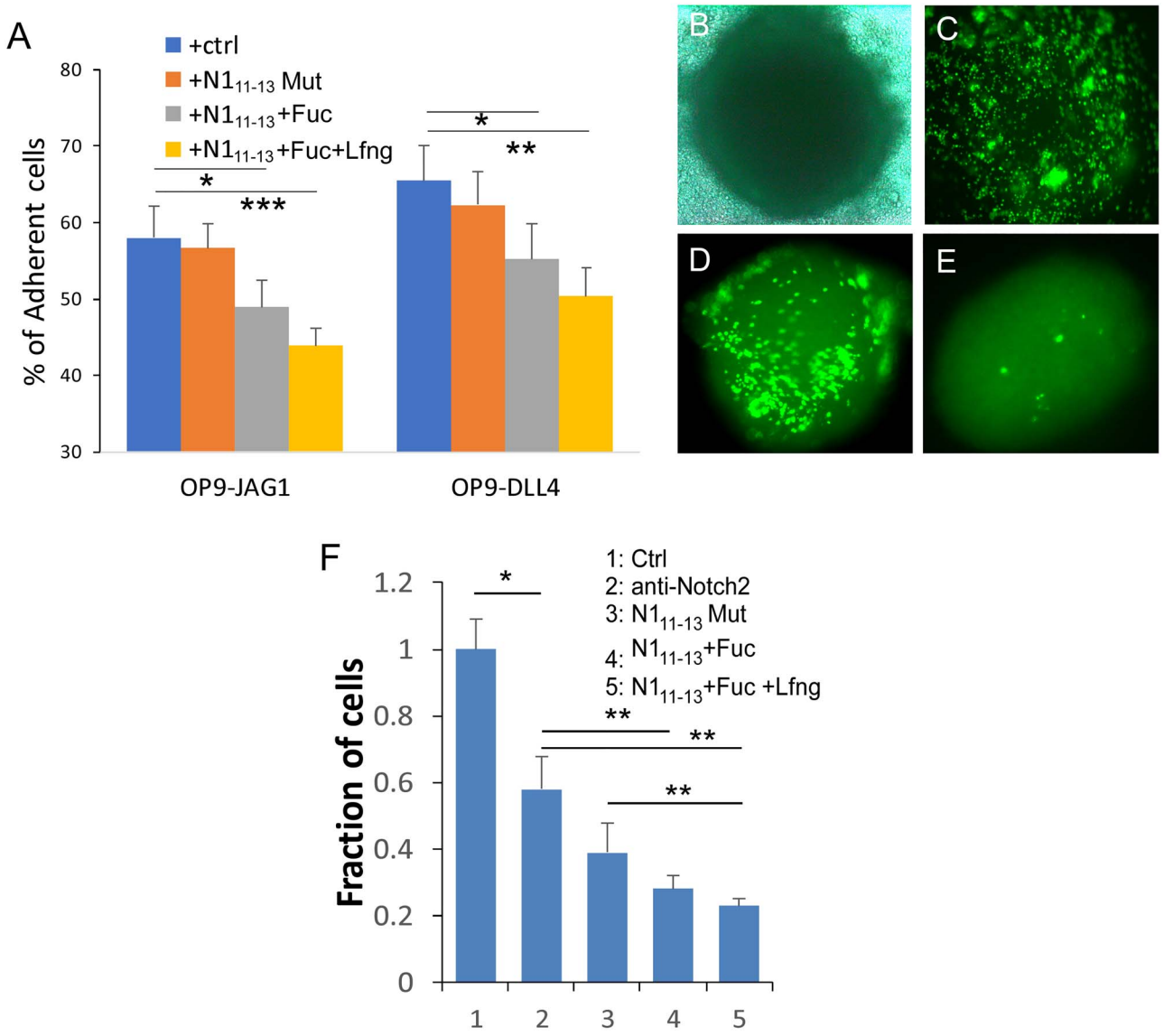

Fig. 5. Fc-tagged NOTCH1 EGF11–13 blocked HPC adhesion and released HPCs from osteoblastic niche. (**A**) OP9-JAG1 and OP9-DLL4 cells were preincubated with concentrated culture supernatant from CHO cells transfected with empty vector (Control), or constructs encoding Fc-tagged unmodified mutant $N1_{11-13}$, $N1_{11-13}$+ Fuc or $N1_{11-13}$ + Fuc + Lfng, and then cocultured with 2.0×10^5 LKs. Nonadherent cells were counted and compared with those from control cocultures. Phase contrast (**B**) and (**C**) fluorescent microscopy of CFSE-labeled HPC (LK) cells remained in FOB spheroids after 48 h coculture. Fluorescent microscopy of HPC cells remaining in spheroids after 24 h culture with (**D**) anti-NOTCH2 or (**E**) $N1_{11-13}$+ Fuc + Lfng. (**F**) Cell fractions remaining in FOB spheroids in the presence of anti-NOTCH2 or Fc-tagged NOTCH1 fragments after 48 h ($n = 3$/each). Student's *t*-test $^*P < 0.05$, $^{**}P < 0.01$.

staining and immunoblotting with HRP-His tag antibody (Thermo Fisher Scientific, Waltham, MA). The concentration of proteins was determined using Bradford 1X Dye Reagent Assay (Bio-Rad, Hercules, CA).

Mass spectrometry

Concentrated supernatant from CHO cells expressing Fc-tagged $N1_{11-13}$ (mutant, fucosylated and Fringe modified) was separated by SDS-PAGE, and the band migrating at the position of EGF11–13 (Supplementary Figure S2A) was cut out and subjected to in-gel reduction/alkylation and digestion with V8 protease as described (Kakuda and Haltiwanger 2014). Briefly, excised bands were cut into 1 mm^2 pieces and reduced in 100 µL reduction solution (0.02 M Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in 50 mM Tris-HCl, pH 8.0) for 5 min at 100°C. After removing the supernatant, gel pieces were alkylated in 150 µL alkylation solution (100 mM iodoacetamide in 50 mM Tris-HCl, pH 8.0) in the dark at room temperature (RT) for 30 min. The supernatant was removed, and gel pieces were rotated in 1 mL gel washing solution (20 mM diammonium phosphate pH 8.0 in 50% methanol) overnight at 4°C. Gel washing solution was replaced two times the next morning in 30 min cycles with rotating at RT. The remaining gel wash was removed and replaced with 1 mL acetonitrile. Gel pieces were rotated for 30 min at RT or until appearing white in color. The supernatant was removed, and the gel pieces were placed in the hood for 1 h until completely dry. The gel pieces were digested with 200 ng V8 protease in 100 µL 20 mM diammonium phosphate at 37°C for 4 h. The samples were sonicated for 20 min, and the supernatant was collected

in a protein LoBind tube. Remaining peptides were extracted from gel pieces with two cycles of 50 µL 5% formic acid and 20 min bath sonication. The pooled supernatants were cleared of impurities by 0.2 µm nylon centrifugal filter (VWR, Radnor, PA) before zip-tip protocol with Pierce C18 tip (Thermo Fisher Scientific, Waltham, MA). The tip was activated by two volumes of 95% acetonitrile in 0.1% acetic acid and equilibrated with three volumes of 0.1% acetic acid. Peptides were applied twice and washed six times with 0.1% acetic acid before elution with 50% acetonitrile in 0.1% acetic acid. The elution was diluted two times with 0.1% formic acid for a final concentration of 25% acetonitrile.

Purified $N1_{11–13}$ and $N2_{11–13}$-Myc-His_6 proteins (~1 µg each of fucosylation mutant, fucosylated and Fringe modified) were subjected to in-solution reduction/alkylation and digestion with chymotrypsin or trypsin, respectively, as described previously (Kakuda and Haltiwanger 2014, 2017; Rana et al. 2011).

The resulting peptides from these digests were injected into an EASY-nLC 1000 HPLC system (Thermo Fisher Scientific, Waltham, MA) for peptide separation using a C_{18} reverse-phase column attached to a Q-Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) for peptide detection. Separation of (glyco) peptides was performed with a binary gradient solvent system that consists of solvent A (5% acetonitrile in 0.1% formic acid) and solvent B (80% acetonitrile in 0.1% formic acid) with a constant flow rate of 300 nL/min. Spectra were recorded with a resolution of 70,000 in the positive polarity mode over the range of *m/z* 400–2000 and automatic gain control target value was 1×10^6. The 10 most prominent precursor ions in each full scan were isolated for higher energy collisional dissociation-MS/MS fragmentation with normalized collision energy of 27%, automatic gain control target of 1×10^5, an isolation window of *m/z* 1.2, dynamic exclusion enabled, and fragment resolution of 17,500. Raw data files were analyzed using Proteome Discoverer v.2.1.0.81 (Thermo Fisher Scientific, Waltham, MA) with Byonic v.2.10.5 (Protein Metrics, Cupertino, CA) as a module for automated identification of (glyco) peptides. Extracted ion chromatograms of (glyco) peptides from EGF12 were generated using Xcalibur v.4.0.27.19 (Thermo Fisher Scientific, Waltham, MA).

Flow cytometry binding assay

Different concentrations of Notch proteins were preclustered with PE-conjugated anti-Myc antibody (R&D, Minneapolis, MN) or PE-conjugated anti-human Fc antibody (BD, Franklin Lakes, NJ) in 100 µL binding buffer (Hank's balanced salt solution supplemented with 1 mM $CaCl_2$ and 1% bovine serum albumin) at 4°C for 30 min as described (Kakuda et al. 2020), then added to DLL4 or JAG1 expressing OP9 cells. After incubating at 4°C for 1 h, cells were washed with the binding buffer and analyzed on flow cytometry. For the Fc-tagged proteins, concentrated supernatants collected from CHO cells transfected with protein-coding vectors and empty vector was used in controls.

SPR assay

Recombinant mouse DLL4-Fc and JAG1-Fc protein (Adipogen, San Diego, USA) was immobilized to a CM5 chip surface by amine coupling and the binding of His-tagged $N1_{11–13}$ and $N2_{11–13}$ proteins to DLL4 or JAG1 were assessed by multiple injections of different concentrations of each protein, including His-tagged $N1_{11–13}$ and $N2_{11–13}$ without fucosylation (mutants), fucosylated and with Fringe modification. All experiments were carried out at 25°C in HBS-P+ buffer, pH 7.4 (Biacore, Uppsala, Sweden), supplemented with 1 mM $CaCl_2$ on B-3000 Biacore instrument. Collected data were analyzed by using BIA evaluation (Version 4.0.1) and traces were corrected for refractive index by subtraction of a control trace. To calculate K_d values, a series of concentrations of $N1_{11–13}$ and $N2_{11–13}$ proteins were injected and bound to DLL4 or JAG1 immobilized chip surface and the corrected kinetic trace curves were fitted by using 1:1 (Langmuir) binding with baseline shift model. SPR analysis were similarly performed by using concentrated supernatants collected from CHO cells transfected with constructs encoding Fc-tagged $N1_{11–13}$ and $N2_{11–13}$ proteins without fucosylation, fucosylated and with Fringe modification.

HPC adherence assay

A modified HPC assay was performed as described previously (Wang et al. 2015). Briefly, confluent OP9-JAG1 and OP9-DLL4 cells were seeded in a 48-well plate. HPCs were magnetically isolated first by negative lineage selection followed by c-kit positive selection as described (Wang et al. 2015). HPC ($Lin^-c\text{-}kit^+$; LK) cells (1.5×10^5) suspended in 200 µL RPMI supplemented with 10% FBS were seeded into each well and incubated for 1 h at 37°C. After controlled agitation on a plate mixer (Gyrotory shaker G2, 120 rpm) for 30 s, nonadherent cells were removed by pipetting and a gentle wash of the coculture plate and counted using a hemacytometer. Ratios of adherent LK cells to those initially added were calculated. To determine the efficacy of Fc-tagged Notch proteins to block HSPC adhesion, OP9 cells were preincubated with concentrated supernatants collected from CHO cells transfected with plasmids encoding empty vector (Control) or Fc-tagged $N1_{11–13}$ and $N2_{11–13}$ proteins at 0.05 mg/mL (or equivalent amounts of control supernatants) for 30 min before the addition of LK cells.

HPC release assay from 3D osteoblastic niche

To generate 3D osteoblastic spheroids, human preosteoblasts immortalized with SV40 large T antigen, hFOB1.19 (ATCC® CRL-11372) (FOB hereafter), were cultured in DMEM/F-12 (5% FBS with Pen-n/Strep, 2.5 mM glutamine, 0.3 mg/mL G418) at 33°C. These cells grow rapidly at 33–34°C and can differentiate to mature osteoblasts at 37°C (Harris et al. 1995). For spheroid formation, FOB cells (4×10^4/100 µL per well) were seeded in ULA U-bottom 96-well plates (Costar, Washington, D.C.). Seeded cells were cultured at 32°C for 4 days and then moved to 37°C for 4 days followed by coculture with 2×10^4 of mouse CFSE-labeled HPC cells. About 15–20% of cells homed to the spheroid without significant proliferation during the coculture period, while cells actively migrated in and out of spheroids in the next 48 h of culture (de Barros et al. 2010). After 48 h of coculture in the presence of NOTCH2 blocking antibody (1 µg/mL) or concentrated supernatants (0.05 mg/mL) collected from CHO cells transfected with plasmids encoding empty vector (Control) or Fc-tagged $N1_{11–13}$ unmodified mutant (Mut), $N1_{11–13}$ + Fuc and $N1_{11–13}$ + Fuc + Lfng, cells that migrated outside of spheroids and those maintained in the spheroids were collected and enumerated by flow cytometry.

Statistics

Data are presented as means ± SD, unless otherwise stated. Statistical significance was assessed by Student's *t*-test.

Supplementary data

Supplementary data are available at *Glycobiology* online.

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Conflict of interest statement

None declared.

Abbreviations

DLL, Delta-like ligand; DMEM, Dulbecco's-Modified Eagle Medium; ESI, electrospray ionization; FBS, fetal bovine serum; hFOB, human FOB; HSCs, hematopoietic stem cells; IgG, immunoglobulin G; JAG, Jagged; LC, liquid chromatography; Lfng, Lunatic fringe; LK, $Lin^{-}c\text{-}kit^{+}$; MS/MS, tandem mass spectrometry; Penn/Strep, penicillin/streptomycin; POFUT1, protein *O*-fucosyltransferase 1; SCD, sickle cell disease; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPR, surface plasmon resonance.

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