Antibodies against the extracellular domain of human Notch1 receptor reveal the critical role of epidermal-growth-factor-like repeats 25–26 in ligand binding and receptor activation

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INTRODUCTION

The Notch signalling pathway is implicated in a wide variety of cellular processes throughout metazoan development. Although the downstream mechanism of Notch signalling has been extensively studied, the details of its ligand-mediated receptor activation are not clearly understood. Although the role of Notch ELRs [EGF (epidermal growth factor)-like-repeats] 11–12 in ligand binding is known, recent studies have suggested interactions within different ELRs of the Notch receptor whose significance remains to be understood. Here, we report critical inter-domain interactions between human Notch1 ELRs 21–30 and the ELRs 11–15 that are modulated by calcium. Surface plasmon resonance analysis revealed that the interaction between ELRs 21–30 and ELRs 11–15 is ∼10-fold stronger than that between ELRs 11–15 and the ligands. Although there was no interaction between Notch1 ELRs 21–30 and the ligands in vitro, addition of pre-clustered Jagged1Fc resulted in the dissociation of the preformed complex between ELRs 21–30 and 11–15, suggesting that inter-domain interactions compete for ligand binding. Furthermore, the antibodies against ELRs 21–30 inhibited ligand binding to the full-length Notch1 and subsequent receptor activation, with the antibodies against ELRs 25–26 being the most effective. These results suggest that the ELRs 25–26 represent a cryptic ligand-binding site which becomes exposed only upon the presence of the ligand. Thus, using specific antibodies against various domains of the Notch1 receptor, we demonstrate that, although ELRs 11–12 are the principal ligand-binding site, the ELRs 25–26 serve as a secondary binding site and play an important role in receptor activation.

Key words: Abruptex, antibody, epidermal-growth-factor-like repeat (ELR), Jagged/Delta-like, ligand-binding site, Notch1.

Recent studies from our laboratory have shown that a mAb (monoclonal antibody) against the hNotch (human Notch) 1 ELRs 11–12 inhibits binding of both Jagged and Delta-like ligands to the receptor [6]. Though it is well documented that ELRs 11–12 are necessary and sufficient for ligand binding [5], involvement of other ELRs that may facilitate or impair ligand binding cannot be ruled out. Furthermore, how ligand binding to the ELRs 11–12 leads to a conformational change at a distant S2 site is difficult to envisage without invoking roles of other ELRs in the ligand-mediated receptor activation.

In addition to the explicit role of ELRs 11–12 in the Notch receptor–ligand interactions, various studies have suggested involvement of other regions of the Notch ECD in ligand binding. For example, studies in Drosophila suggest importance of the Abruptex region (ELRs 24–29) of Notch in ligand binding. For example, studies in Drosophila suggest importance of the Abruptex region (ELRs 24–29) of Notch in ligand-dependent receptor activation [10–13]. Previously it has been demonstrated that in Drosophila Notch, the Abruptex domain competes for ligand binding by interacting with ELRs 11–14 [14]. Furthermore, the Abruptex region has been shown to harbour multiple sites for post-translational modifications, namely glycosylation and fucosylation, which modulate the functions of the Notch receptor, thus highlighting the importance of this region along with the ELRs 11–12 in ligand-mediated receptor activation [15,16]. However, the molecular intricacies of the mechanisms by which the Abruptex region modulates the ligand–receptor interactions have not been clearly elucidated.

In the absence of strong biochemical or biophysical evidence, structural organization of Notch1 ECD remains unknown. The

Abbreviations used: ADAM, a disintegrin and metalloproteinase; cbELR, calcium-binding ELR; DPBS, Dulbecco’s PBS; ECD, extracellular domain; EGF, epidermal growth factor; ELR, EGF-like repeat; PBS, fetal bovine serum; GST, glutathione transferase; HEK, human embryonic kidney; hNotch, human Notch; HRP, horseradish peroxidase; LNR, Lin-12 Notch repeat; mAb, monoclonal antibody; mNotch1, murine Notch1; NRR, negative regulatory region; pAb, polyclonal antibody; SPR, surface plasmon resonance.

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crystal structure of a smaller receptor fragment (ELRs 11–13) suggested a rigid extended rod-like arrangement of Notch ECD [17]. Bioinformatic predictions suggest that the calcium-binding motifs within the linker region of two adjacent ELRs contribute to this structural rigidity and the absence of such motifs from most ELRs in the Abruptex region perhaps provides the required flexibility in the Notch1 ECD [18,19]. A previous study also suggested plausible inter- or intra-domain interactions between ELRs of the Notch extracellular domain [20].

The leading model for ligand-mediated receptor activation proposes that endocytosis of the membrane-bound ligand generates a mechanical force on Notch, which in turn pulls the LNRs, thus exposing the S2 cleavage site [21,22]. However, the existence of soluble ligands in Caenorhabditis elegans and identification of YB-1 as a soluble ligand for hNotch3 poses several perplexing questions on the force-based activation model [23,24]. It has been hypothesized that the soluble ligands may activate receptors allosterically [25]. However, such a mechanism lacks experimental evidence. In the present study, we have investigated interactions between various domains of hNotch1 ECD and demonstrate their possible involvement in the Notch receptor–ligand interactions. Using antibodies as a novel tool to study the Notch structure–function relationship, we propose the existence of a cryptic ligand-binding site in the hNotch1 ECD within ELRs 25–26.

MATERIALS AND METHODS

Generation of stable cell lines

The cell lines overexpressing hNotch1 [HEK (human embryonic kidney)-293 hN1] and the ligands Jagged1, Jagged2, Delta-like1 and Delta-like4 used in the present study were reported previously [6].

Expression of Notch1 receptor fragments

Fragments of the hNotch1 ECD were expressed as GST (glutathione transferase) fusion, His-tag or Fc-fusion proteins. Primers for these fragments are given in Supplementary Table S1 (at http://www.biochemj.org/bj/449/bj4490519add.htm). The cDNAs encoding the ELRs 1–5 (amino acids 20–216), 11–15 (amino acids 412–601) [6], and NRR (amino acids 1448–1725) were amplified from hNotch1, cloned into pGEX-4T1 (GE Healthcare) and purified from the soluble cell lysate using a glutathione affinity matrix. Similarly, the ELRs 21–30 fragment (amino acids 791–1181) was expressed as a His-tag fusion protein using the pET28 vector (Novagen) and purified by Ni2+ affinity chromatography. The hNotch1 receptor fragments of ELRs 1–12 (amino acids 20–488), ELRs 10–20 (amino acids 372–789) and ELRs 21–30 were also expressed as Fc-fusion protein using the expression vector pFUSE-Fc-IgG1 (InvivoGen) and purified from the culture supernatant of (Chinese hamster ovary) CHO cells by Protein-A affinity chromatography (GE Healthcare) [6] (Figure 1 and Supplementary Figure S1 at http://www.biochemj. org/bj/449/bj4490519add.htm).

pAbs (polyclonal antibodies) and mAbs

pAbs were raised against ELRs 1–5, 11–15, 21–30 and NRR in rabbits using well-established immunization protocols [6,26,27]. mAbs against ELRs 11–15 of hNotch1 were described previously [6].

Figure 1 Schematic illustration of the hNotch1 receptor fragments

Various hNotch1 receptor fragments were expressed in bacterial or mammalian expression systems and affinity purified using glutathione, Ni2+ or Protein A affinity chromatography.

Construction of hNotch1 mutants

Single point mutations in the evolutionarily conserved cbELR12 (calcium-binding ELR 12; D469G) and the divergent cbELR 25 (N956G) (Supplementary Figure S2 at http://www.biochemj.org/bj/449/bj4490519add.htm) were generated by primer-based site-directed mutagenesis using the Phusion mutagenesis kit (Thermo Fisher, USA). The hNotch1 receptor fragments, pGEX-4T1 ELRs 11–15 and pET28 ELRs 21–30 were used as templates for D469G (ELR 12D/G) and N956G (ELR 25N/G) mutations respectively. To generate the full-length hNotch1 with the single point mutants ELR12 D469G and ELR25 N956G, the pcDNA3.1 hNotch1 was used as the template. Furthermore, pCDNA3.1 ELR12 D469G DNA was used as the template to generate the double mutant ELR 12D469G, 25N956G. Similarly, the full-length hNotch1/Delta1 EGF25-26 cDNA was generated by PCR based protocol using the Phusion mutagenesis kit and pCDNA3.1 hNotch1 as the template. Primers for these mutants are listed in Supplementary Table S1.

Solid phase assay for ligand-binding and inter-domain interactions

The purified hNotch1 receptor fragments (200 ng) were coated on a plastic surface and incubated with purified Fc-fusion soluble Notch ligands [6] followed by the addition of anti-human Fc HRP (horseradish peroxidase)-conjugate (Sigma–Aldrich) and the peroxidase activity was determined. To study the inter-domain interactions, GST-fusion proteins ELRs 1–5, 11–15 and NRR
were coated on a plastic surface followed by the addition of His-tag ELRs 21–30 protein and the interaction was determined using an anti-His-tag antibody (GE Healthcare). The effect of single point mutations on inter-domain interactions was also examined in the same assay. All solid-phase assays were performed in triplicate and repeated three times.

**GST pull-down assay**

ELRs 11–15 were coupled to GSH beads (GE Healthcare) and allowed to interact with the lysate of cells expressing ELRs 21–30 in the presence and absence of 1.26 mM Ca2+. The complex was further pulled down by centrifugation and electrophoresed on reducing SDS/PAGE (12.5% gels). All pull-down experiments were performed in calcium/magnesium-free TBS buffer, and EGTA was used to demonstrate the specificity of the interactions. The inter-domain interactions were probed by determining the bound ELRs 21–30 using an anti-His-tag antibody. The effect of single point mutations on these interactions was also determined in the same assay. All the pulldown experiments were performed in triplicate and repeated three times.

**SPR (surface plasmon resonance) assay**

The hNotch1 ELRs 21–30 and ELRs 11–15 proteins were dissolved in 50 mM sodium acetate buffer, pH 5.5, and immobilized on an EDC/NHS [N-ethyl-N‘-(3-dimethyl amino propyl)-carbo-di-imide-hydrochloride/N-hydroxy succinimide]-activated CM5 sensor chip as recommended by the manufacturer (Biacore AB), yielding a surface density of approximately 7000 resonance units. The Notch ligand Fc-fusion proteins Jagged1, Jagged2, Delta-like1, and Delta-like4 were diluted in HBSS buffer and allowed to bind at 25°C using a flow rate of 10 μl/min as previously described [17]. The dissociation constant, $K_d$, was calculated from the ratio of the dissociation rate ($k_{off}$) to the association rate ($k_{on}$) determined from three sensorgrams for the analyte concentration ranging from 5 to 10 μM using the curve-fitting BIAevaluation software, version 3.0 (Biacore AB) and the 1:1 Langmuir model. The experiment was repeated three times.

**Flow cytometry assay**

The cells were harvested using DPBS (Dulbecco’s PBS)-EDTA, resuspended in DPBS containing 2% FBS (fetal bovine serum; Invitrogen) (FBS/DPBS) and incubated with the primary antibody for 40 min at room temperature (25°C) following was washing, resuspension and incubation in 0.1 ml of FBS/DPBS containing FITC-conjugated anti-rabbit or mouse secondary antibodies for 30 min at room temperature. The cells were washed, resuspended in DPBS and analysed using the Becton Dickinson FACScanto. The median values were calculated using the ‘Stat’ program of CellQuest by Becton Dickinson. The flow-cytometry-based ligand-binding assay was performed on ice as described previously [6]. All the flow cytometry assays were performed in triplicate and repeated three times.

**Luciferase reporter assay**

To determine the effect of anti-Notch1 antibodies on Notch signalling, a functional assay for Notch signalling was developed. HEK-293 hN1 cells were seeded (5 × 10^4 cells/ well) in a 24 well plate (Nunc) and transfected with 790 ng of 12×CSL-Luc and 10 ng pGL3 Basic or 800 ng pGL3 control along with 1 ng pRL-Tk (Promega) using Lipofectamine™ 2000 (Invitrogen) as per the manufacturer’s instructions. The ligands were provided by pre-coating the wells with purified Jagged1Fc or Delta-like4Fc (10 μg/well). The transfected cells were incubated with or without anti-Notch1 antibodies. The luciferase reporter activity was estimated after 36 h using the Dual Luciferase assay kit following the manufacturers’ protocol (Promega) and a TD-20 Luminometer (Turner Design). All transfection experiments were performed in triplicate and repeated three times.

**RESULTS**

**Characterization of hNotch1 fragment-specific antibodies**

Generation and characterization of rabbit pAbs against hNotch1 ELRs 1–5, 11–15, 21–30 and NRR is shown in Supplementary Figures S3 and S4 (at http://www.biochemj.org/bj/449/bj4490519add.htm) [6]. The antibodies raised against GST fusion proteins were passed through the GST affinity matrix to remove GST-specific antibodies and used in subsequent experiments. As shown in Supplementary Figure S4, the antibodies were specific for the cognate antigens with very little or no cross-reactivity with any other receptor fragment, highlighting the specificity of these antibodies.

**Identification of inter-domain interactions between hNotch1 ELRs**

The biochemical properties and importance of hNotch1 ELRs 11–15 in ligand binding have been reported previously [6]. Previous studies have suggested the existence of Notch receptor intra- or inter-domain interactions [14,18,20]. To investigate possible interactions between various hNotch1 receptor fragments, His-tagged ELRs 21–30 were incubated with ELRs 1–5, 11–15 and NRR (GST-fusion proteins) immobilized on a plastic surface, and the complex formation was determined using an anti-His-tag antibody. As shown in Figure 2(A), significant interactions of ELRs 21–30 were detected only with ELRs 11–15, but not with ELRs 1–5 or NRR, clearly demonstrating the specificity of the interactions between these two domains. These results are in agreement with a previous report on Drosophila Notch [14]. Furthermore, there was a significant increase in the interaction between ELRs 21–30 and 11–15 in the presence of Ca2+, which was decreased by EGTA, indicating the importance of calcium in this process (Figure 2B). Similar interactions could be demonstrated between ELRs 21–30Fc and ELRs 1–12Fc, as well as between ELRs 21–30Fc and ELRs 10–20Fc expressed using a mammalian expression system (Supplementary Figure S5 at http://www.biochemj.org/bj/449/bj4490519add.htm). Taken together, these data demonstrate inter-domain interactions between ELRs 21–30 and ELRs 11–15 in the hNotch1 receptor.

**In vitro interactions between ligands and hNotch1 ELRs**

Direct interactions between different fragments of hNotch1 receptor with the ligands were investigated by adsorbing the receptor fragments on a plastic surface followed by incubation with Notch ligands Jagged1Fc and Delta-like4Fc and determining the ligand binding. As shown in Figure 2(C), only ELRs 11–15 showed significant ligand binding, with no other fragments (ELRs 1–5 and ELRs 21–30) exhibiting any binding at all. This is in agreement with our previous results that ELRs 11–15 constitute the primary ligand-binding site [6].

The effect of the ligands on inter-domain interaction was next investigated by adsorbing ELRs 11–15 on a plastic surface followed by addition of ELRs 21–30 in the presence of increasing concentrations of Jagged1Fc or Delta-like4Fc and determining ELRs 21–30 and 11–15 complex formation. As shown in
Figure 2 Inter-domain interactions between ELRs of hNotch1 receptor

(A) GST-tagged ELRs 11–15, 1–5 and NRR and GST were coated on a plastic surface and incubated with ELRs 21–30 (His-tagged protein) and the complexes formed were determined using an anti-His-tag antibody. (B) ELRs 11–15 were coupled to GSH beads and incubated with cell lysates expressing ELRs 21–30 in the presence and absence of 1.26 mM CaCl₂ and the complex formed was quantified using an anti-His-tag antibody. (C) ELRs 1–5, 11–15 and 21–30 were separately coated on a plastic surface and incubated with the Notch ligands Jagged1/Delta-like4-Fc and ligand binding was determined using an anti-human-Fc HRP conjugate. (D) ELRs 11–15 were coated on a plastic surface and incubated with a fixed concentration (12.5 μg/ml) of ELRs 21–30 in the presence of various concentrations of Notch ligands Jagged1/Delta-like4-Fc and binding of ELRs 21–30 was determined using an anti-His-tag antibody. Results are means ± S.D., n = 3.

Figure 2(D), this complex formation was inhibited by the ligands in a dose-dependent manner, demonstrating that ELRs 21–30 compete with ligands for binding to ELRs 11–15. Thus these data suggest that even though ELRs 21–30 do not bind the ligand directly, it is likely to influence the ligand–receptor interactions by interacting with ELRs 11–15.

SPR analysis of hNotch1 receptor–ligand and ELR interactions

SPR experiments were performed to determine the affinities of inter-domain interactions as well as binding affinities between ELRs 11–15 and ligands. The ELRs 11–15 fragment was coated on a CM5 chip and the binding of Notch ligands was determined. As shown in Figures 3(A)–3(D), and Table 1, all four ligands exhibited binding to ELRs 11–15, out of which Jagged1 and Jagged2 appeared to have relatively higher affinities (Kₐ values of 2.93 μM and 3.26 μM respectively) in comparison with those of Delta-like1 and Delta-like4 (Kₐ values of 16.89 μM and 39.98 μM respectively). ELRs 21–30 and ELRs 1–5 did not show any ligand binding (results not shown) which is in agreement with the data shown in Figure 2(C). Interestingly, the inter-domain interactions between ELRs 21–30 and 11–15 (Kₐ = 0.22 μM) were found to be stronger than those between the ligands and ELRs 11–15 (Figure 3E and Table 1).
Figure 3 Interactions between ELRs and Notch ligands

(A-D) ELRs 11–15 were coated on a CM5 chip and allowed to interact with the Notch ligands Jagged (JAG) 1, Jagged2, Delta-like (DLL) 1 and Delta-like4. SPR was determined under different experimental conditions. (E) The ability of ELRs 21–30 to interact with ELRs 11–15 was also determined in the same assay. All experiments were repeated three times. RU, relative units.

Table 1 Quantification of the data obtained from SPR

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$K_d$ ($\mu$M) ($n = 3$)</th>
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<tr>
<td>Jagged1 Fc</td>
<td>2.93 ± 0.34</td>
</tr>
<tr>
<td>Jagged2 Fc</td>
<td>3.26 ± 0.56</td>
</tr>
<tr>
<td>Delta-like1 Fc</td>
<td>16.89 ± 0.82</td>
</tr>
<tr>
<td>Delta-like4 Fc</td>
<td>39.98 ± 0.76</td>
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<tr>
<td>ELRs 21–30 His-tag</td>
<td>0.22 ± 0.03</td>
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Effect of anti-(ELRs 21–30) antibodies on hNotch1 receptor–ligand interactions

To understand further the role of ELRs 21–30 in receptor–ligand interactions, a pAb was generated against this region of hNotch1. This antibody could bind ELRs 21–30 in ELISA (Supplementary Figure S3A) and Western blot (Supplementary Figure S3C), and also recognized the full-length hNotch1 receptor on the cell surface by flow cytometry (Supplementary Figure S3B). The antibodies exhibited marginal cross-reactivity with ELRs 11–15, and
hence were passed through an ELRs 11–15 affinity matrix to remove any cross-reacting antibodies. As shown in Supplementary Figure S3(A), these affinity-purified antibodies were completely devoid of anti-(ELRs 11–15) antibodies but retained those specific for anti-ELRs 21–30. Furthermore, the affinity-purified antibodies did not bind to any other hNotch1 ECD fragments or their ligands (Supplementary Figure S4A), demonstrating their specificity to ELRs 21–30, and were used in subsequent experiments.

The effect of anti-(ELRs 21–30) antibodies on hNotch1 ligand–receptor interactions was investigated next. HEK-293 hN1 cells were pre-incubated with different anti-Notch1 antibodies, and ligand binding was determined by flow cytometry. As shown in Figure 4(A) and Supplementary Figure S6A (at http://www.biochemj.org/bj/449/bj4490519add.htm), ligand binding was significantly inhibited by anti-(ELRs 21–30) antibodies, suggesting ELRs 21–30 may play a role in ligand binding. In a reverse experiment, pre-incubation of HEK-293 hN1 cells with Jagged1Fc inhibited anti-(ELRs 21–30) antibody binding (Figure 4B and Supplementary Figure S6B), suggesting that ligand binding to full-length Notch receptor may alter or obstruct the ELRs 21–30 region. Furthermore, all anti-Notch1 antibodies could bind to HEK-293 hN1 cells in a dose-dependent manner (Figure 4C). However, only ELRs 11–15- and 21–30-specific antibodies inhibited Jagged1Fc binding in a dose-dependent manner (Figure 4D). Although these data confirmed the role of ELRs 11–15 in ligand binding as previously reported [5,6], ELRs 21–30 emerged as a new player in Notch ligand–receptor interactions.

**Effect of anti-(ELRs 25–26) antibodies on hNotch1 receptor–ligand interactions**

Since anti-(ELRs 21–30) antibodies recognize a large epitope (390 amino acids), it was essential to identify the key segment within this region critically important for the ligand–receptor interactions. For this purpose, the anti-(ELRs 21–30) antibodies were further dissected into those specific for ELRs 21–24, 25–26 and 27–30 by passing them sequentially through affinity matrices of ELRs 21–24 and 27–30 (Supplementary Figure S7A at http://www.biochemj.org/bj/449/bj4490519add.htm). The antibodies specific for ELRs 21–24 and 27–30 were eluted from the columns by glycine/HCl, pH 2.8, and neutralized immediately (positive immunoaffinity purification). The antibody pool that did not bind to either affinity matrix was designated as the antibody specific for ELRs 25–26 (negative immunoaffinity purification). As shown in Supplementary Figures S7(B) and S7(C), the immunoaffinity purified antibodies recognized the respective fragments while exhibiting no cross-reactivities with any other receptor fragment, particularly the ‘principal’ ligand-binding domain of ELRs 11–12.

When the effect of these antibodies on ligand-binding was investigated by flow cytometry, the anti-(ELRs 25–26) antibodies were most effective in inhibiting ligand binding to hNotch1, whereas those specific against ELRs 21–24 and 27–30 had marginal effects (Figure 4E). A similar pattern was observed when the ligand-mediated receptor activation was investigated in the presence of these immunoaffinity-purified antibodies. As shown in Figure 4(F), the anti-(ELRs 25–26) antibodies, as well as the parent [anti-(ELRs 21–30)] antibodies, inhibited response to the ligand in a dose-dependent manner, whereas those against ELRs 21–24 and 27–30 had no effect, once again emphasizing the importance of ELRs 25–26 in ligand–receptor interactions. Interestingly, the anti-(ELRs 25–26) antibodies appeared to be more effective than the parent [anti-(ELRs 21–30)] antibodies, suggesting an increase in the specific activity after immunoaffinity purification (Figure 4F).

Furthermore, to rule out any possible discrepancies in the functional characteristics between the positive and negative immunoaffinity purified antibodies, the negative immunoaffinity purified anti-(ELRs 25–26) antibodies were additionally purified using the ELRs 25–26 affinity matrix (Supplementary Figure S7A). As shown in the Supplementary Figure S8(A) (at http://www.biochemj.org/bj/449/bj4490519add.htm), the inhibitory properties of the positive immunoaffinity purified antibodies were the same as those shown in Figure 4(F). Taken together, these data revealed the significance of ELRs 25–26 in the ligand–receptor interactions.

**Effect of ligands on the Notch1 inter-domain interactions**

The SPR data suggested that ELRs 21–30 and ELRs 11–15 may form a relatively high affinity complex in the absence of the ligand. Since, as shown in Figure 2(D), the ligand and ELRs 21–30 compete for binding to ELRs 11–15, the question that needed to be addressed was whether the ligand can dissociate the pre-formed inter-domain complex. This was investigated by incubating the pre-formed complex of ELRs 21–30 and ELRs 11–15 with increasing concentrations of Jagged1Fc and the amount of remaining inter-domain complex was determined by estimating ELRs 21–30 using an anti-His-tag antibody. As shown in Figure 5, the ligand led to a modest decrease in the inter-domain interactions in a dose-dependent manner, suggesting that the ligand can dissociate the complex of ELRs 21–30 and 11–15.

It has previously been demonstrated that Notch signalling is enhanced upon the clustering of the ligands by addition of anti-Fc antibody [28]. In view of that, whether addition of the ligand (Jagged1Fc) in a pre-clustered form (obtained by pre-incubating Jagged1Fc with anti-Fc antibodies) would enhance dissociation of the inter-domain complex was next investigated. Addition of this pre-clustered ligand significantly enhanced the dissociation of the inter-domain complex in a dose-dependent manner with a decrease in EC50 values of the ligand (Figure 5). This observation demonstrates that the ligated ligands are more efficient in disengaging the inter-domain complex.

**Effect of mutations in ELRs 11–15 and 21–30 on inter-domain interactions, ligand binding and receptor activation**

Although there was no in vitro interaction between the hNotch1 receptor fragment ELRs 21–30 and the ligands, the specific antibodies against ELRs 21–30 inhibited ligand binding to full-length hNotch1 receptor, suggesting that this segment is a cryptic ligand-binding site that perhaps emerges after initial interaction of the ligand with ELRs 11–12. The data also indicated that the ELRs 21–30 and 11–15 may form a calcium-dependent complex (Figures 2A and 2B). The involvement of calcium in these interactions was further established by mutating the cbELR in the hNotch1 receptor fragments (ELRs 11–15 and 21–30) as well as the full-length receptor. The mutant hNotch1 receptor fragment ELRs 11–15 (cbELR 12 D469G) exhibited significantly reduced binding to the wild-type ELRs 21–30 protein (Figures S2A and S2B), whereas a similar mutation in ELRs 21–30 (cbELR 25 N956G) had no such effect. Similarly, the cbELR12 (D469G) mutation in full-length hNotch1 receptor abolished the ligand binding and subsequent receptor activation whereas the mutation in the cbELR 25 (N956G) in full-length hNotch1 had no effect (Figures S6C and S6D) [29]. Furthermore, the double mutant ELR 12 (D469G), ELR 25 (N956G) in full-length hNotch1 receptor exhibited properties similar to cbELR12 (D469G) mutation, clearly demonstrating the
Secondary ligand-binding site in Notch1 ECD

Figure 4  Effect of anti-Notch antibodies on hNotch1 receptor–ligand interactions

(A–E) Flow-cytometry based experiments using HEK-293 hN1 cells. (A) The cells were pre-incubated with anti-(ELRs 21–30) antibodies followed by incubation with Jagged1Fc (JAG1 Fc; 10 μg/ml) and ligand binding was determined using anti-human Fc-specific FITC conjugate. (B) The cells were pre-incubated with Jagged1Fc protein followed by incubation with the anti-(ELRs 21–30) antibodies and the antibody binding was determined using the anti-rabbit IgG–FITC conjugate. (C) The cells were incubated with increasing concentrations of anti-Notch1 antibodies and binding was determined using an anti-rabbit IgG FITC conjugate. RMFI (relative median fluorescence intensity) was calculated after normalizing with the control IgG. (D) The cells were pre-incubated with increasing concentrations of anti-Notch1 antibodies followed by incubation with Jagged1Fc protein and the ligand binding was determined. (E) The cells were pre-incubated with immunoaffinity purified (iAP) anti-(ELRs 21–24), -(ELRs 21–24), -(ELRs 25–26) and -(ELRs 27–30) antibodies followed by incubation with Jagged1/Delta-like4 Fc and the ligand binding was determined. (F) HEK-293 hN1 cells transfected with 12×CSL-Luc reporter plasmid were cultured for 36 h on plates pre-coated with Jagged1 Fc in the presence of increasing concentrations of anti-Notch1 antibodies and the luciferase reporter activities were determined by dual luciferase assay. The ratio of firefly luciferase to Renilla luciferase was calculated for normalization. Results are means ± S.D., n = 3. The experiments were repeated three times.

role of cbELR12 in inter-domain interaction, ligand binding and receptor activation.

In view of the effect of anti-(ELRs 25–26) antibody on ligand–receptor interactions, the consequences of deleting ELRs 25–26 from the full-length Notch1 receptor was next investigated. As shown in Figures 6(C) and 6(D), ELRs 25–26 deletion (ΔELRs 25–26) led to a partial decrease in Jagged1Fc and Delta-like4-Fc binding and a consequent decrease in the receptor activation. These results are in agreement with the antibody data, suggesting that ELRs 25–26 play a critical role in the secondary ligand-binding events. The proper folding of the mutant hNotch1 receptors was further examined by determining the binding of...
highlighting the specificity of the antibody (Supplementary Figure 5). Furthermore, the anti-(ELRs 25–26) antibodies inhibited ligand binding to both wild-type and mutant mNotch1 receptors in the flow-cytometry-based ligand-binding assays. As shown in Figures 7(B) and 7(C), anti-(ELRs 25–26) antibodies inhibited ligand binding of anti-(ELRs 25–26) antibodies. As shown in Figures 7(B) and 7(C), anti-(ELRs 25–26) antibodies inhibited ligand binding to both wild-type and mutant mNotch1 receptors in the flow-cytometry-based ligand-binding assays. Furthermore, the anti-(ELRs 25–26) antibodies also inhibited the ligand-mediated Notch receptor activation (Figures 7D and 7E). Interestingly, the inhibitory effects of anti-(ELRs 25–26) antibodies were more profound on the ELR 26 mutant receptor, suggesting the involvement of this region in the ligand–receptor interactions.

**DISCUSSION**

Despite extensive studies on the downstream consequences of Notch signalling, the initial events of ligand–receptor interactions have not been clearly elucidated. Specific antibodies against different domains of the receptor are exceptional tools to investigate the receptor–ligand interactions [32]. Recently, we reported antibodies against the hNotch1 receptor that can inhibit ligand binding and consequent receptor activation [6]. The present study is an extension of our previous study with the aim of investigating the roles of various domains of hNotch1 in ligand binding and receptor activation. In the absence of any biochemical or biophysical evidence, it is not clear whether the Notch ECD is a rigid rod-like structure or a relatively flexible globular molecule [33]. The mechanism of Notch receptor activation has not been clearly elucidated and the questions that needed to be answered are whether the endocytosis of the ligands leading to receptor activation is the only mechanism or whether there is an allostery involved with a series of conformational changes in the Notch ECD leading to receptor activation. In the present study, we demonstrate a specific inter-domain interaction between the ligand-binding domains ELRs 11–15 and ELRs 21–30 of the hNotch1 receptor. These interactions are evolutionarily conserved, as the same domains were found to take part in Drosophila Notch inter-domain interactions [14]. Although the hNotch1 receptor fragment ELRs 21–30 does not bind to the Notch ligands in vitro, its interaction with ELRs 11–15 may fine-tune the receptor activation by modulating the ligand–receptor interactions indirectly. Our results also suggest that both inter-domain interactions and ligand binding are regulated by calcium binding to the ELR 12. This also indicates that the ligands, as well as the ELRs 21–30, interact with the calcium-bound “stiff” conformation of the ELRs 11–12.

In spite of extensive evidence of the ligand binding to ELRs 11–12, limited information is available on the relative affinities of the ligands for these repeats and whether the ligands bind to these repeats exclusively. The in vitro binding assays demonstrated that all four ligands bind to the ELRs 11–15 domain with no detectable binding to either the ELRs 21–30 or ELRs 1–5 receptor fragments, suggesting specificity, with the affinities of Jagged1 and Jagged2 being higher than those of the Delta-like ligands. SPR analysis also revealed that the inter-domain interactions between the ELRs 21–30 and ELRs 11–15 were stronger than those of the ligand with ELRs 11–15. In vitro binding assays suggest that, in the basal state, both ELRs 21–30 and ELRs 11–15 form a tight complex that may keep the receptor in an inactive state. We demonstrate further that the presence of a ligand (in this case Jagged1Fc) can dissociate this inter-domain complex in a dose-dependent manner, thereby disengaging the two domains of the hNotch1 receptor. It is intriguing how the inter-domain complex that is formed with much higher affinity can be dissociated by the ligand that has a relatively lower affinity for ELRs 11–15. Clustering of the ligands on the cell surface may facilitate such a process. Recently Ilagen et al. [28] demonstrated that clustering can enhance ligand-mediated Notch signalling. In the present study, ligands that are Fc–fusion proteins were used which can be easily clustered by an antibody against the Fc domain. Addition of an anti-Fc antibody not only facilitates the dissociation of the complex, but also

![Figure 5](image.jpg)

*Figure 5. Ligand-mediated dissociation of hNotch1 inter-domain interactions*

GST-tagged ELRs 11–15 protein was coated on to a plastic surface, incubated with 12.5 μg/ml of His-tagged ELRs 21–30 for 1 h followed by washing with TBST (20 mM Tris, 137 mM NaCl and 0.1% Tween-20, pH 7.6). The complex formed was incubated with increasing concentrations of Jagged1Fc (broken line) or Jagged1Fc that was pre-clustered using different amounts of anti-Fc antibodies (Ab) for 1 h at room temperature and the amount of the inter-domain complex retained was determined using the anti-His-tag antibody. The results are means ± S.D., n = 3. The experiment was repeated three times.

Various mAbs or pAbs against hNotch1 in flow cytometry and ELISA-based assays. As shown in Supplementary Figures S9, S10 and S11 (at [http://www.biochemj.org/bj/449/bj4490519add.htm](http://www.biochemj.org/bj/449/bj4490519add.htm)), there was no difference in binding of these antibodies to the wild-type or mutant hNotch1 receptors, clearly indicating overall normal folding of the mutant receptors. Importantly, binding of anti-(ELRs 25–26) antibodies to ΔELRs 25–26 was abolished, highlighting the specificity of the antibody (Supplementary Figure S9).

**Effect of anti-(ELRs 25–26) antibodies on the O-fucosylation impaired the gain-of-function phenotype**

It has been demonstrated that O-fucosylation and O-glucosylation on ELRs are important for Notch signalling [16,30,31]. A single point mutation affecting O-fucosylation in the mNotch1 (murine Notch1) ELR 26 led to a ligand-dependent gain-of-function with a 4- and 2-fold increase in ligand-mediated signalling by the Jagged and Delta-like ligands respectively [31]. Significant sequence similarity between hNotch1 and mNotch1 signalling by the Jagged and Delta-like ligands respectively [31]. Although the hNotch1 receptor fragment ELRs 21–30 does not bind to the Notch ligands in vitro, its interaction with ELRs 11–15 may fine-tune the receptor activation by modulating the ligand–receptor interactions indirectly. Our results also suggest that both inter-domain interactions and ligand binding are regulated by calcium binding to the ELR 12. This also indicates that the ligands, as well as the ELRs 21–30, interact with the calcium-bound “stiff” conformation of the ELRs 11–12.

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enhanced the sensitivity of the complex to Jagged1Fc as indicated by a decrease in EC\textsubscript{50} value. The in vivo consequence of ligand density on Notch receptor activation and cell fate decision has been previously demonstrated by Delaney et al. [34]. The results from the present study provide a plausible explanation for ligand-clustering-mediated Notch receptor activation.

Notch receptors exhibit significant sequence similarity across species, especially in domain organization and in cbELRs. Although the cbELRs are conserved between hNotch1 and Drosophila Notch, the Abruptex domain exhibits subtle variations in these repeats. Mutations in calcium-binding sites within the hNotch1 receptor fragments ELRs 11–15 and ELRs 21–30 demonstrated that cbELR 12 within ELRs 11–15 modulates both ligand binding to hNotch1 as well as inter-domain interactions with ELRs 21–30. These results suggest that ELRs 21–30 interact with the calcium-bound conformation of ELRs 11–15, similar to the ligand. On the basis of bioinformatic predictions, the hNotch1 ELRs 11–19 are cbELRs and in the presence of

Figure 6  Effect of mutations in the Notch1 ELRs 11–15 and 21–30 on inter-domain interactions, ligand binding and receptor activation

Wild-type or mutant (12D/G) ELRs 11–15 proteins were coated on to a plastic surface (A) or GSH beads (B) and their ability to form an inter-domain complex with the wild-type or mutant (25N/G) ELRs 21–30 was determined using the anti-His-tag antibody as described in Figure 2(A). HEK-293 cells were transfected with the wild-type or mutant full-length hN1 along with 12\times CSL-Luc reporter plasmid, and harvested 36 h post-transfection. (C) Ligand binding was determined using Jagged1/Delta-like4-Fc proteins by flow cytometry as described in Figure 4(A). RMFI, relative median fluorescence intensity; Wt, wild-type. (D) Ligand-mediated Notch1 activation was determined using Jagged1 (JAG1)/Delta-like4 (DLL4)-Fc and the luciferase reporter activities were determined by dual luciferase assay as described in Figure 4(F). Results are means ± S.D., n = 3. The experiments were repeated three times.
calcium may take a rigid rod-like structure, whereas the relative absence of such cbELRs within ELRs 22–29 provides structural flexibility [19]. This may explain the striking effect of the cbELR 12 mutation and the negligible effect of the cbELR 25 mutation on Notch1 receptor structure/function.

Our results from the present study and the previous studies convincingly suggest that the initial ligand-binding events take place at ELRs 11–12 [5,6,30,35]. However, a large number of mutational arrays in the Drosophila Notch Abruptex domain have been shown to result in the ligand-dependent gain-of-function phenotype [12]. O-fucosylation and O-glucosylation in the Abruptex region also affects receptor function [15,16,36], suggesting a possible cryptic role of this region in the ligand–receptor interactions. We used highly characterized and specific antibodies as a novel tool to investigate the functional properties of the Abruptex domain in the hNotch1 receptor. The results of the present study show that antibodies against a part of the Abruptex region (ELRs 25–26) impede ligand binding, whereas antibodies against ELRs 1–5 have no such effect. Furthermore, using immunoaffinity-purified antibodies, we demonstrated that the anti-(ELRs 25–26) antibodies were more potent, whereas the anti-(ELRs 21–24) and anti-(ELRs 27–30) antibodies have only a marginal effect on the ligand–receptor interactions. The anti-(ELRs 25–26) antibodies also inhibited the gain-of-function phenotype associated with mutation in the O-fucose site in ELR 26. It has been proposed that O-fucosylation at the ELRs may modulate the flexibility of the Notch extracellular domain [31]. Thus it is tempting to hypothesize that these mutations may modulate the ligand–receptor interactions by altering the interplay between ELRs 21–30 and 11–15. Most interestingly, deletion of ELRs 25–26 from the hNotch1 receptor partially abolished ligand binding. These results are remarkably different

Figure 7 Effect of anti-(ELRs 25–26) antibodies on O-fucose mutation in mNotch1

(A) Sequence alignment of hNotch1 (hN1) and mNotch1 (mN1) ELRs 25–26. (B–E) HEK-293 cells were transfected with the wild-type (Wt) or mutant mNotch1 receptors and harvested 36 h later. (B and C) Ligand binding was determined in the presence and absence of anti-(ELRs 25–26) antibodies followed by incubation with (B) Jagged1Fc (JAG1 Fc) or (C) Delta-like4Fc (DII4 Fc). The ligand binding was determined by flow cytometry as described in Figure 4(A). (D and E) Jagged1/Delta-like4-Fc-mediated receptor activation was determined in the presence and absence of anti-(ELRs 25–26) antibodies using the luciferase reporter activities as described in Figure 4(F). The results are means ± S.D., n = 3. The experiments were repeated three times.
The receptor by exposure of the S2 site. These domains and may lead to an altered conformation of ELRs 21–30, leading to generation of the secondary ligand-binding site at ELRs 25–26. These changes in ELRs may allosterically activate the receptor by exposure of the S2 site.

The results of the present study suggest that the ligand-binding domain (LBD) and ELRs 21–30 interact in vitro and can keep the Notch receptor in an auto-inhibitory state. Ligand binding dissociates these domains and may lead to an altered conformation of ELRs 21–30, leading to generation of the secondary ligand-binding site at ELRs 25–26. These changes in ELRs may allosterically activate the receptor by exposure of the S2 site.

Overall, the results of the present study provide an alternative allosteric model for Notch receptor activation. As the ligand binding competes for inter-domain interactions, it displaces ELRs 21–30 from the ligand-binding domain. We propose that this altered conformation of the receptor creates a secondary ligand-binding site and these events may facilitate the exposure of the S2 cleavage site in the NRR (Figure 8). As previously reported, EDTA-mediated receptor activation requires ADAM17, whereas the ligand-mediated mechanical pulling requires ADAM10 metalloproteases [37]. It will be interesting to investigate whether the mechanical pulling or allosteric mode of the receptor activation requires the same array of metalloproteases. The allosteric model of receptor activation can explain the mechanism of Notch activation mediated by the soluble ligands in C. elegans and humans [23,24]. A differential response of Notch to the cis and trans ligands has been demonstrated [38]. However, how these inter-domain interactions preside over the cis-inhibition remains to be elucidated. It is tempting to speculate that the ligand in cis might stabilize these inter-domain interactions, whereas the trans-ligands destabilize them by competition. An autosomal dominant disease, CADASIL (cerebral autosomal dominant arteriopathy with sub-cortical infarcts and leukoencephalopathy) is associated with mutations in the extracellular domain of hNotch3 [39,40]. It has been speculated that these mutations modulate Notch function similar to the Aburstex gain-of-function phenotype [41]. Furthermore, it has been demonstrated that the above mutations also impair fringe-mediated O-fucose elongation on Notch3 and may alter the tertiary structure of the receptor [31]. The ability of the anti-(ELRs 25–26) antibody to recuperate the O-fucose impairment gain-of-function phenotype suggests that the kind of antibodies characterized in the present study can serve as a potential therapeutic tool to combat the Notch pathologies associated with the ligand-dependent gain-of-function phenotypes. It has been demonstrated that mutation in the O-glucosyltransferase Rumi can suppress the ligand-independent gain-of-function mutations in the NRR domain and affect the S2 cleavage [42,43], further indicating the interplay of ELRs in Notch receptor activation.

The results of the present study emphasize the critical involvement of the inter-domain interactions in Notch receptor activation as hypothesized in Figure 8. The proposed model, although exploratory, provides an explanation for the importance of the Abruptex region in Notch receptor–ligand interactions. The oligomeric status of Notch still remains to be elucidated. However, in light of the proposed dimeric organization of the Notch receptor [20,44], it will be interesting to determine whether ELRs 11–15 and ELRs 21–30 form either intra-molecular interactions in the same receptor molecule or form inter-molecular complexes with two independent receptors. Overall, our results provide novel insights into the Notch receptor–ligand interactions. Implications of such interactions in various developmental fates need to be explored further.

AUTHOR CONTRIBUTION
Ankur Sharma planned the study, conducted the experiments, analysed the data and co-wrote the paper. Annapoorni Rangarajan co-wrote the paper. Rajan Dighe supervised the experiments, analysed the data and co-wrote the paper.

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REFERENCES


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SUPPLEMENTARY ONLINE DATA

Antibodies against the extracellular domain of human Notch1 receptor reveal the critical role of epidermal-growth-factor-like repeats 25–26 in ligand binding and receptor activation

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Supplementary Figures S1–S11 and Table S1 are on the following pages

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Figure S1 Expression and purification of Notch receptor fragments

(A) ELRs 1–5, (B) ELRs 11–15, (C) ELRs 21–30, (D) NRR, (E) ELRs 21–24, (F) ELRs 25–26 and (G) ELRs 27–30 were expressed and purified using either glutathione or Ni²⁺ affinity chromatography. The purified proteins were examined by SDS/PAGE and confirmed using either anti-GST or anti-His-tag antibodies. a/s, antiserum; hN1, hNotch1.
Figure S2  Phylogenic analysis of calcium-binding ELRs in Notch receptors

The ELRs in the Notch ECD were classified as calcium-binding (cb) or non-calcium binding as predicted by UniProtKB/Swiss-Prot. hNotch1 cbELRs 11–19 are highly conserved throughout the species, whereas cbELRs in the Abruptex region (ELRs 24–29) show considerable divergence across the species.
Figure S3  Characterization of anti-ELR 21–30 antibodies

(A) ELRs 21–30 or 11–15 proteins were coated on to a plastic surface and binding of anti-(ELRs 21–30) polyclonal antibodies to these proteins was determined by ELISA. (B) Binding of anti-(ELRs 21–30) antibodies to the full-length hNotch1 receptor in HEK-293 hN1 cells was determined using flow cytometry. (C) Ability of anti-Notch1 antibodies to detect the full-length hNotch1 receptor in the cell lysate of untransfected HEK-293 cells or HEK-293 hN1 cells was determined by immuno-blot analysis using (i) anti-(ELRs 21–30); (ii) anti-NRR; and (iii) anti-Notch1 (c-20) sc-6014 antibodies (Santa Cruz Biotechnology); (iv) anti-β-actin antibody was used as the control. a/s, antiserum; IaP, immunoaffinity purified; N-, Notch; N-FL, Notch full-length; N-TMD, Notch transmembrane.

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Cross reactivities of immunoaffinity purified (IaP) anti-Notch1 antibodies were determined by incubating the antibodies with different receptor fragments or ligands (only for anti-(ELRs 21–30)). Notch1 receptor fragments ELRs 1–5, 11–15, 21–30 and NRR were coated on to a plastic surface and binding of (A) anti-(ELRs 21–30), (B) anti-(ELRs 1–5) and (C) anti-NRR antibodies to these receptor fragments was determined by ELISA. a/s, antiserum; hN1, hNotch1.
Figure S5  Inter-domain interactions between ELRs
ELRs 1–12, 10–20 and 21–30 were expressed as Fc fusion protein from the culture supernatant of CHO (Chinese hamster ovary) cells by Protein A affinity chromatography. ELRs 1–12Fc, ELRs 10–20Fc and Fc alone were coated on a plastic surface, incubated with ELRs 21–30Fc and the complex formed was determined using anti-(ELRs 21–30) immunoaffinity purified antibodies.

Figure S6  Quantification of the data shown in Figures 4(A) and 4(B) of the main text
IaP, immunoaffinity purified; JAG, Jagged; RMFI, relative median fluorescence intensity.
Figure S7  Characterization of the immunoaffinity purified anti-\{(ELRs 21–24), -(ELRs 25–26) and -(ELRs 27–30) antibodies\}

(A) Schematic illustration of immunoaffinity purification (IaP) protocol. (B) Notch1 ELRs 11–15, 21–30, 21–24, 25–26 and 27–30 proteins were coated on to a plastic surface and the specificity of the immunoaffinity purified anti-Notch1 antibodies to the cognate receptor fragment was determined by ELISA. (C) Ability of IaP anti-Notch1 antibodies to recognize specific receptor fragment by immunoblotting.
Figure S8 Functional characterization of Immunoaffinity purified anti-(ELRs 21–24), -(ELRs 25–26) and -(ELRs 27–30) antibodies

(A) HEK-293 hN1 cells were transfected with 12×CSL-Luc reporter plasmid and cultured on plates pre-coated with Jagged1Fc in presence and absence of Immunoaffinity purified (IaP) anti-Notch1 antibodies, and the luciferase reporter activities were determined using a dual luciferase assay. (B) HEK-293 hN1 cells were pre-incubated with Jagged1Fc (JAG1Fc) followed by addition of anti-Notch1 antibodies and antibody binding was determined using the anti-rabbit FITC conjugate by flow cytometry. RMFI, relative median fluorescence intensity.
Figure S9  Cell-surface expression of wild-type and ELRs Δ25–26 hNotch1 receptor

HEK-293 cells were transfected with the wild-type or mutant (ΔELR 25–26) hNotch1 cDNAs. Cells were harvested 36 h post-transfection and incubated with anti-Notch1 mAbs or pAbs. The antibody binding was determined using an appropriate FITC-conjugated secondary antibody by flow cytometry. The traces represent observations from three independent experiments.
Figure S10  Cell surface expression of the wild-type and ELR 12 D469G hNotch1 receptor

HEK-293 cells were transfected with the wild-type or mutant (cbELR 12 D469G) hNotch1 cDNAs, cells were harvested 36 h post-transfection and incubated with anti-(ELRs 11–15) antibody, and the antibody binding was determined using anti-rabbit FITC conjugate by flow cytometry. The traces represent observations from three independent experiments.
Secondary ligand-binding site in Notch1 ECD

Figure S11  Anti-(ELRs 11–15) monoclonal antibodies binding to ELRs 11–15 wild-type or ELR 12 D469G receptor fragment

Wild-type or mutant Notch1 receptor fragment ELRs 11–15 was coated on to a plastic surface and binding of anti-(ELRs 11–15) monoclonal antibodies was determined in the presence of 1.26 mM CaCl₂ by ELISA.

Table S1  Primer nucleotide sequences

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