Control of endothelial cell tube formation by Notch ligand intracellular domain interactions with activator protein 1 (AP-1)

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Zary Forghany1, Francesca Robertson1,2, Alicia Lundby3, Jesper V. Olsen4, and David A. Baker1,3

From the 1Department of Molecular Cell Biology, Leiden University Medical Center, 2300 RC Leiden, The Netherlands and 2Novo Nordisk Foundation Center for Protein Research and the 3Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, 2200 Copenhagen N, Denmark

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Notch signaling is a ubiquitous signal transduction pathway found in most if not all metazoan cell types characterized to date. It is indispensable for cell differentiation as well as tissue growth, tissue remodeling, and apoptosis. Although the canonical Notch signaling pathway is well characterized, accumulating evidence points to the existence of multiple, less well-defined layers of regulation. In this study, we investigated the function of the intracellular domain (ICD) of the Notch ligand Delta-like 4 (DLL4). We provide evidence that the DLL4 ICD is required for normal DLL4 subcellular localization. We further show that it is cleaved and interacts with the JUN proto-oncogene, which forms part of the activator protein 1 (AP-1) transcription factor complex. Mechanistically, the DLL4 ICD inhibited JUN binding to DNA and thereby controlled the expression of JUN target genes, including DLL4. Our work further demonstrated that JUN strongly stimulates endothelial cell tube formation and that DLL4 constrains this process. These results raise the possibility that Notch/DLL4 signaling is bidirectional and suggest that the DLL4 ICD could represent a point of cross-talk between Notch and receptor tyrosine kinase (RTK) signaling.

The generic Notch signaling network is a central regulator of cell fate (1, 2). This pathway is absolutely necessary for normal development and tissue homeostasis, and corruption of it has been implicated in numerous diseases, including the majority of solid tumors where it plays diverse oncogenic and tumor-suppressive roles (3–5). In vertebrates, the Notch signaling system is composed of four single-pass cell-surface receptors (Notch1–4) and five type 1 transmembrane ligands: Jagged (JAG)4, 1, JAG2, Delta-like (DLL) 1, DLL3, and DLL4 (1, 6, 7). Operationally, the canonical Notch signaling pathway is relatively well characterized (7–9). It is activated by a trans interaction between a Notch receptor on one cell and a ligand expressed by a neighboring cell. This triggers a cascade of proteolytic events terminating in the γ-secretase–mediated cleavage of the Notch intracellular domain, which translocates to the nucleus whereupon it regulates expression of Notch target genes (1, 8). By these means, the molecular and cellular asymmetries required for tissue maintenance and development are established across populations of cells. In recent years, studies have identified manifold, unique facets of Notch signaling (9). These include cis receptor-ligand interactions (because each are expressed in the same cell) (10), ligand-independent signaling (11), endocytosis of Notch and Notch ligands as an essential mediator of signaling (12, 13), and in the case of DLL4 signaling at a distance through the incorporation of DLL4 into exosomes (14, 15). Adding further to the complexity is the extensive cross-talk between the Notch pathway and other major signaling networks such as receptor tyrosine kinase signaling (16); WNT, hedgehog, and transforming growth factor (TGF)-β signaling (17); Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling (18); and hypoxia signaling (19). To understand these mechanisms in greater depth, new studies are beginning to elucidate the role of the Notch ligands in this process. The five ligands share a similar overall architecture: module at the N terminus of Notch ligands (MNNL) domains, a Delta/Serrate/Lag-2 (DSL) domain, between six and 14 EGF-like repeats, a transmembrane segment, and an intracellular domain (ICD) 100–150 amino acids in length (20). The extracellular moiety is essential for establishing the direct contacts with the Notch receptor necessary for eliciting Notch signaling (21). Biochemical and genetic evidence has shown that the intracellular domain is clearly essential for normal functioning of the full-length protein (22–25). Ligand ICDs harbor putative PDZ domains that couple them to membrane-bound proteins required for the maintenance of cell-cell junctions and likely play a central role in assembling those complexes necessary for ligand trafficking (26, 27). Consistent with this, in Dro-
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In common with the Notch receptor, the DLL1 and JAG ligands can be sequentially processed by proteases. Both a disintegrin and metalloprotease (ADAM) metalloendopeptidases (30, 31) and matrix metalloproteinase 14 (in the case of DLL1) (32) mediate ectodomain shedding by cleavage of the ligand close to the transmembrane domain on the extracellular side. Subsequent intramembrane processing by γ-secretase liberates the ICD (33, 34). A growing body of evidence suggests that these ICDs might participate in signaling and downstream transcription, and ectopically expressed ICDs have been localized to the nucleus (35). Moreover, it has been reported that both DLL1 (36) and JAG1 ICDs (37) are able to bind to and disrupt the function of the Notch ICD by mediating its degradation in the case of the JAG ICD. The DLL1 ICD has also been pinpointed as a modulator of TGF-β/activin signaling through binding to SMAD proteins (38). A number of studies have described the effects of ligand ICDs at the cellular level. Inhibition of Notch1 signaling by the JAG1 ICD was shown to regulate cardiac homeostasis in the postnatal heart (39). When ectopically expressed in mesenchymal stromal cells, the JAG1 ICD regulated hematopoietic stem and progenitor cell proliferation (40). Finally, overexpression of the DLL1 ICD brought about the growth arrest of primary endothelial cells (41).

Collectively, the mechanisms described above determine the strength, direction, specificity, and nature of the output of the Notch signaling pathway. The emerging evidence that ICDs are biologically active is thus important for fully understanding Notch signaling in both normal and disease contexts, not least because the Notch pathway in general (42) and the DLL4 ligand in particular (43, 44) have emerged as a potential target for novel therapeutic interventions in cancer. The DLL4 ligand is integral to the development and homeostasis of numerous tissues, most notably the endothelium where it plays a central role in angiogenesis (45–49). It also fundamentally involved in hematopoiesis (50) and the maintenance of the stem cell compartment of intestinal crypts (51). To date, little is known about the function of the DLL4 intracellular domain. Here, we provide evidence that the DLL4 ICD is needed for appropriate DLL4 subcellular localization. We show that it encodes a functional PDZ-binding domain that is necessary for associating to the MUPP1 scaffold protein. We further show that the DLL4 ICD is cleaved. The liberated DLL4 (and DLL1) ICD could interact with the bZIP domain of the JUN proto-oncogene, thereby blocking its binding to the consensus AP-1 DNA-binding site. We found that JUN strongly stimulated endothelial cell sprouting, which was inhibited by the ICDs of both DLL4 and DLL1. These data highlight a previously unreported role for JUN as a potent pro-angiogenic signal and the DLL4 ICD as a potential regulator of Notch signaling. Our evidence suggests that the ICDs of DLL4 (and DLL1) could control endothelial cell sprouting by linking Notch signaling to the action of the AP-1 transcription factor complex.

Results

The intracellular domain of DLL4 is cleaved

The amino acid sequence of the intracellular domain of DLL4 (hereafter referred to as DLL4INTRA) has been highly conserved throughout vertebrate evolution (Fig. 1A). The four C-terminal amino acids (ATEV) encode a putative PDZ-binding domain (52). PDZ-binding domains have been shown to mediate protein-protein interactions, cell adhesion, tight junction integrity, and trafficking (27), and loss of DLL4INTRA led to a significant disruption of normal DLL4 localization in primary human endothelial cells (Fig. 1B). Costaining a specific calnexin antibody showed that DLL4 lacking the intracellular domain (DLL4N) is entrapped in the endoplasmic reticulum/Golgi apparatus. DLL4INTRA harbors a number of additional motifs that suggest it is functionally important, including GSK consensus phosphorylation sites (which are utilized),5 a sumoylation motif, and putative ubiquitination sites. Moreover, ectopically expressed DLL4INTRA is able to accumulate in the nucleus (Fig. 1B), indicating that it may play a role independently of ensuring appropriate DLL4 subcellular distribution. To explore the function of this domain, we raised custom-made antibodies directed against epitopes unique to the C terminus of human DLL4. Fig. 1C shows that these antibodies recognized full-length endogenous DLL4 as well as a number of smaller DLL4 species (predominant forms with masses between ~25 and 35 kDa and a lower molecular mass form of ~12 kDa). Similarly sized fragments could be detected by Western blotting of lysates prepared from cells ectopically expressing full-length DLL4 fused to a C-terminal HA epitope tag (Fig. 1C). Through mass spectrometry, we determined that these fragments constitute the C terminus of DLL4 (Fig. 1C). The 25–35-kDa fragments are predicted to encompass the entire ICD, the transmembrane domain, and sequences of the extracellular domain EGF repeats. The size of the shorter fragment indicates that it is composed uniquely of ICD sequences. Our evidence supports the view that this fragment is generated by post-translational proteolysis of the ICD distal to the transmembrane domain on the intracellular side. First, the molecular mass of the observed endogenous fragment precludes the possibility of it being generated by known mechanisms of alternative splicing alone. Second, mutation of the intracellular domain methionine residues failed to eliminate detection of the band in cells, suggesting that it is not produced through use of internal initiation codons (Fig. 1D). Third, a comprehensive deletion analysis mapped a putative cleavage site to a highly conserved LEVD motif (amino acids 594–597) (Fig. 1E). LEVD closely resembles a consensus caspase cleavage site (53), and the pan-caspase inhibitor Z-VAD-fmk blocked the production of the DLL4ICD (Fig. 1E). It has been reported that the closely related DLL1 ligand undergoes intramembrane processing mediated by γ-secretase to yield an intracellular fragment (31, 33). Although we cannot formally rule out γ-secretase–dependent cleavage of DLL4, we found that release of the observed DLL4INTRA was not blocked by DAPT (Fig. 1F). Together, these observations reveal two unique features of DLL4INTRA: it is necessary for

5 Z. Forghany and D. A. Baker, unpublished data.
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A

Human
VLLVLLGMVAVAVRQLRLRRPDDGS-REAMNNLSDFQKDNLIPAAQLKNTNQKKELEVDC 598
Mouse
VLLVLLGMVAVAVRQLRLRRPDDES-REAMNNLSDFQKDNLIPAAQLKNTNQKKELEVDC 599
Chicken
ALLILFCMIMAMVIIRQMRMQPQQD---LETMNNLSDFQKDNLIPASQLKNTNKNKDLEVDC 712
Zebrafish
ALVVLLCMVVIALRHIHRQASGERTRGEAMNNLSESQRDNLIPTSQLKNTNKQVSLEVDC 595

B

 DLL4FL
 DLL4N
 INTRA

Human
GLDKSNCGKQQNHTLDYNLAPGPLGRGTMPGKFPHS-DKSLGEKAPL-RLHSEKPECRIS 656
Mouse
GLDKSNCGKLQNHTLDYNLAPGLLGRGGMPGKYPHS-DKSLGEKVPL-RLHSEKPECRIS 657
Chicken
GLEKSNY-KPKNHKLDYNLVKDLTSRGTQEDKYYKS-EKCLGEKSPL-RLHSEKPECRIS 769
Zebrafish
TPDKSNY-IHKNCHLDYNSSKEFKDIVSQEDKSSHKYEKCLEEKIPLSRMYREKPECRIS 654

C

 DLL4 FL
 DLL4 N
 DLL4 INTRA

Ectopic
Endogenous

HA                Calnexin             DAPI              MERGE

D

DLL4FL
 DLL4INTRA

Z-VAD (μM) DAPT (μM)

E

Human
VLLVLLGMVAVAVRQLRLRRPDDGS-REAMNNLSDFQKDNLIPAAQLKNTNQKKELEVDC 598
Mouse
VLLVLLGMVAVAVRQLRLRRPDDES-REAMNNLSDFQKDNLIPAAQLKNTNQKKELEVDC 599
Chicken
ALLILFCMIMAMVIIRQMRMQPQQD---LETMNNLSDFQKDNLIPASQLKNTNKNKDLEVDC 712
Zebrafish
ALVVLLCMVVIALRHIHRQASGERTRGEAMNNLSESQRDNLIPTSQLKNTNKQVSLEVDC 595

Human
GLDKSNCGKQQNHTLDYNLAPGPLGRGTMPGKFPHS-DKSLGEKAPL-RLHSEKPECRIS 656
Mouse
GLDKSNCGKLQNHTLDYNLAPGLLGRGGMPGKYPHS-DKSLGEKVPL-RLHSEKPECRIS 657
Chicken
GLEKSNY-KPKNHKLDYNLVKDLTSRGTQEDKYYKS-EKCLGEKSPL-RLHSEKPECRIS 769
Zebrafish
TPDKSNY-IHKNCHLDYNSSKEFKDIVSQEDKSSHKYEKCLEEKIPLSRMYREKPECRIS 654

Human
AICSPRDSMYQSVCLISEERNECVIATEV 685
Mouse
AICSPRDSMYQSVCLISEERNECVIATEV 686
Chicken
AICSPRDSMYQSVFVITEERNECIIATEV 798
Zebrafish
TICSPRDSVYQSVFVIAEERSECVIATEV 683
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DLL4 interacts with the PDZ domain of MUPP1 and the bZIP domain of JUN

To investigate the potential molecular function(s) of the DLL4 intracellular domain, we performed yeast two-hybrid screens to identify partner proteins of DLL4INTA. Three different DLL4 C terminus constructs were used as baits to independently screen a library prepared from primary HUVECs (Fig. 2A). The PDZ domain–containing scaffold protein MUPP1 was obtained in all screens. It is involved in cell-cell junction integrity, including endothelial cell junctions, and protein trafficking (26, 54). Immunoprecipitation studies in tissue culture cells corroborated the interaction between DLL4 and MUPP1 and its dependence on the extreme C terminus (ATEV) of DLL4, lending substance to the view that DLL4INTA has a bona fide PDZ-binding domain (Fig. S1 and see Fig. 1A). Less expected was the interaction with the bZIP-containing transcription factor JUN. We have previously established that JUN levels are dynamically balanced by VEGF receptor signaling in primary endothelial cells (55), and several lines of evidence confirmed that DLL4INTA and JUN can interact both in vitro and in cells. First, we used the sensitive and quantitative proximity ligation assay to visualize the endogenous DLL4-JUN complex. Using custom-made antibodies directed against the DLL4 C terminus and an endogenous JUN antibody, we found that endogenous JUN and DLL4 associated (predominantly in the nucleus) in HUVECs (Fig. 2B). By stably expressing engineered DLL4 mutants in HUVECs, we showed that DLL4INTA was necessary and sufficient for this interaction (Fig. 2C). Second, using purified recombinant JUN protein domains and in vitro translated DLL4 proteins, we demonstrated that DLL4INTA bound efficiently to the bZIP domain of JUN but not to the bZIP domain of CREB (Fig. 2D). Third, Fig. 2, E and G, substantiate our proximity ligation assay (PLA) findings (see Fig. 2, B and C) by showing that DLL4INTA, but not DLL4N, is necessary for binding to JUN in vitro (Fig. 2E) and in tissue culture cells (Fig. 2G). Furthermore, the association between DLL4INTA and JUN requires the bZIP domain of JUN (Fig. 2F) as well as the highly conserved C-terminal 20 amino acids of DLL4, which are shared with the ICD of DLL1 (Fig. 2F and see Fig. S4). The PDZ-binding domain of DLL4INTA was dispensable for this interaction (see Fig. 3C). In these experiments, JUN did not appear to interact with full-length DLL4, which does contain the intracellular domain, suggesting that DLL4 is folded in such a way as to preclude this interaction. It is also possible that DLL4 monomers oligomerize/dimerize, which could render the C terminus inaccessible for JUN interactions. Collectively, these data highlight potentially a dual role for the DLL4ICD: it can interact with the bZIP domain of JUN, and in addition it encodes a PDZ-binding domain that mediates a functional interaction with MUPP1.

DLL4INTA blocks JUN binding to DNA

To determine the mechanistic consequences of DLL4INTA-JUN binding, we established an in vitro assay to recapitulate JUN binding to DNA. For this, we utilized a biotinylated consensus AP-1 DNA-binding site and in vitro translated proteins. Fig. 3A shows that JUN efficiently bound to the AP-1 site. As expected, JUN binding to the AP-1 site required the bZIP domain of JUN because a mutant lacking this domain, but not a mutant lacking amino acids C-terminal of this domain, was unable to bind the consensus sites, thus authenticating the specificity of the assay (Fig. 3A). Compellingly, DLL4INTA, but not DLL4N, inhibited JUN association to the AP-1 site (Fig. 3B) and could impede AP-1 dimerization (Fig. 3B). Both the PDZ-binding motif of DLL4INTA and sequences N-terminal of the LEVD motif were dispensable for this inhibition (Fig. 3C), whereas the highly conserved C-terminal 20 amino acids of DLL4 appear to be necessary for inhibiting in vitro JUN DNA binding (in agreement with our observation that these sequences are needed for binding to the JUN bZIP domain; see Fig. 2F). JUN represses or activates gene expression in context-dependent fashion by docking on target promoters and through the differential recruitment of corepressors and coactivators. To ascertain a more global view of regulation of JUN activity by DLL4INTA, we performed transcriptome profiling of HUVECs ectopically expressing JUN in the presence or absence of DLL4INTA or DLL4N. Ectopic expression of JUN strongly stimulates endothelial cell sprouting (see Fig. 4) and leads to a dramatic alteration in the set of expressed genes (Fig. 3D, compare mock and JUN). Whereas coexpression of DLL4N had relatively more modest effects on the JUN-controlled transcriptome, DLL4INTA significantly altered the expression pattern of JUN-controlled genes (Fig. 3D). These findings were verified by real-time quantitative PCR (qPCR) of a subset of the genes, including DLL4 (Fig. 3E). To test whether these effects could have resulted from DLL4INTA directly occluding JUN DNA binding, we investigated JUN recruitment to the proximal pro-

Figure 1. A, the ICD of DLL4 is evolutionarily conserved. Highlighted are the transmembrane domain (underlined), a putative caspase cleavage site (red), and a PDZ-binding motif (green). *, conserved amino acids; :, partly conserved amino acids (different amino acids are similar); ., partly conserved amino acids (different amino acids are not similar). B, the DLL4 ICD is necessary for appropriate DLL4 subcellular localization, and untethered DLL4INTA is enriched in the nucleus. HUVECs were infected with lentiviruses for stably expressing the indicated HA epitope–tagged DLL4 constructs. DLL4N lacks the ICD and is otherwise identical to wildtype DLL4. DLL4INTA encompasses the ICD alone. The subcellular distribution of DLL4 was visualized by immunofluorescence using an HA antibody. Scale bar, 20 μm. C–F, the ICD of DLL4 is cleaved. C, mass spectrometry of cleaved DLL4 fragments. Endogenous DLL4 or DLL4 expressing a C-terminal HA epitope tag was immunopurified from HUVECs. Samples were separated by SDS-PAGE, and DLL4 ICDs were subjected to in-gel digestion with trypsin followed by on-line nanoflow LC-MS/MS analysis of the peptide mixture on an LTQ Orbitrap Velos mass spectrometer. The MS/MS spectrum of a recovered peptide corresponding to amino acids 608–624 of the DLL4 ICD is shown. D, the indicated ICD methionine residues of HA epitope–tagged full-length DLL4 were mutated to alanine. HA antibody Western blotting was performed on lysates prepared from HUVECs stably expressing the indicated DLL4 ligands. E, a conserved LEVD motif is required for cleavage of the DLL4 ICD. Left panel, the indicated nested deletions of HA epitope–tagged full-length DLL4 were generated by site-directed mutagenesis. HA antibody Western blotting was performed on lysates prepared from HUVECs stably expressing the indicated DLL4 ligands. Corresponding deletions are highlighted above. Right panel, DLL4 ICD cleavage is blocked by the pan-caspase inhibitor Z-VAD but not DAPT. Cells expressing wildtype HA epitope–tagged full-length DLL4 were incubated overnight with or without the indicated concentrations of Z-VAD or DAPT. DLL4 protein species were detected by HA antibody Western blotting. Ab, antibody; DLL4FL, full-length DLL4.
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moterm of DLL4. We have previously shown that DLL4 expression (and JUN expression) is highly attuned to VEGF receptor signaling in HUVECs (55). Fig. 3F shows that short hairpin (sh) RNA-mediated loss of JUN in HUVECs abrogated DLL4 levels and abolished the VEGF-stimulated wave of DLL4 expression. In support of the view that DLL4 expression is governed by JUN, we found that endogenous JUN associated with the proximal promoter of DLL4 and, using an integrated luciferase reporter, that loss of a consensus AP-1 site upstream of the DLL4 TATA box significantly diminished the detectable levels of promoter-bound JUN (Fig. 3G). Accordantly, ectopic expression of DLL4INTRA inhibited VEGF-dependent association of JUN with the DLL4 promoter (Fig. 3H, upper graph) and led to a concomitant inhibition of VEGF-stimulated DLL4 expression (Fig. 3H, lower graphs). Consistent with these findings, stable expression of DLL4INTRA augmented the levels of the repressive H3K27me chromatin methylation mark found at the DLL4 proximal promoter (Fig. 3H, upper graph). These data indicate that DLL4INTRA can negatively regulate JUN binding to DNA and, in consequence, control expression of genes required for endothelial sprouting.

**DLL4INTRA attenuates JUN-mediated endothelial cell tube formation/sprouting**

To elucidate the biological consequences of DLL4INTRA binding to JUN, we used a 3D Matrigel sprouting assay. Fig. 4A shows that enhanced JUN levels hugely increased endothelial sprouting (including filopodia formation), whereas loss of endogenous JUN abolished the ability of HUVECs to sprout. JUN-stimulated sprouting depended on the bZIP domain of JUN and thus presumably relies on functional JUN binding to DNA because a JUN mutant lacking this domain acted as a dominant negative, resulting in strong inhibition of sprouting (Fig. 4A). In this assay, wildtype sprouting networks are relatively short-lived and collapse after ~24 h. By contrast, endothelial cells ectopically expressing JUN were robustly sustained and continued to sprout for several weeks. Additionally, JUN-expressing cells could sprout efficiently in the absence of exogenous VEGF.5 In agreement with our prior findings that DLL4INTRA obstructs JUN DNA binding (see Fig. 3), Fig. 4B shows that coexpression of DLL4INTRA, but not DLL4N, restricted JUN-driven endothelial sprouting. AP-1 transcription factors are characterized by the conserved bZIP domain, which mediates homo- and heterodimerization of AP-1 family members and DNA binding. This begs the question as to whether DLL4INTRA might also influence the activity of other AP-1 family members such as JUNB, which has an established role in vascular morphogenesis (56–58). Our experiments show that JUNB, like JUN, strongly stimulated endothelial tube formation (Fig. 5A), and DLL4INTRA could inhibit this process (Fig. 5B).

**Discussion**

In this study, we have presented evidence that DLL4INTRA could perform a dual function: it is essential for establishing normal DLL4 subcellular localization, and strikingly, the untethered ICD interacted with and inhibited the activity of the JUN transcription factor. By means of chemical inhibitors and targeted mutagenesis, we demonstrated that one mechanism of ICD cleavage is caspase-dependent (see Fig. 1). The pattern of cellular DLL4 protein species indicates that multiple DLL4 cleavage events could occur, but their precise nature is yet to be delineated. What triggers the cleavage event(s) or whether proteolysis is constitutive is also currently unclear and will be important to establish. Certainly, DLL4INTRA is highly conserved and harbors a number of motifs that might control its function (see Introduction) and could influence its fate. We found that endogenous DLL4INTRA accumulated following incubation of cells with VEGF (Fig. S2). Future work will determine whether this simply reflects increased levels of DLL4 and/or specific signaling events. Related to this, unraveling the mechanism(s) of DLL4INTRA turnover could shed further light on its function. The DLL4 ICD contains an intrinsically disordered region terminating in a structured C-terminal PDZ-binding domain. This conformation resembles the proto-oncogene FOS (59, 60) and other signaling proteins and transcription factors (61). We recently found that the C terminus of FOS is composed of a comparable intrinsically disordered region/structured extreme C terminus arrangement that controls intrinsic, rapid proteosomal degradation of FOS, and a similar mechanism might control the activity of DLL4INTRA. Indeed, we found that DLL4INTRA protein levels are strongly stabilized when the proteasome is blocked by MG132, indicating that DLL4INTRA is rapidly turned over.5

**Figure 2. DLL4INTRA interacts with the bZIP domain of JUN.** A yeast two-hybrid baits were constructed by fusing DLL4 ICD fragments in-frame with a LexA DNA-binding domain either at the N or the C terminus of the ICD. Constructs were expressed in yeast to verify expression by Western blotting of lysates using a LexA antibody. A p53-LexA fusion is included as a control (Dualsystems Biotech). Predicted full-length proteins of constructs used to screen a library prepared from HUVECs are marked with an asterisk. A, B, and C are revealed an endogenous DLL4-JUN complex. The graph shows relative complex formation per cell (right upper panel) that was abolished by ablation of either DLL4 or JUN (siRNA efficacies were demonstrated using cells ectopically expressing either DLL4 or JUN; see right lower panels). The endogenous DLL4-JUN complex was undetectable using single antibodies alone. Quantification was performed as described (see "Experimental procedures") with an average of 100 cells scored. Scale bar, 20 μm. D and E, the ICD of DLL4 interacts biochemically with the bZIP domain of JUN. D, recombinant bZIP domains of JUN and CREB3 (and a control protein consisting of the DNA-binding domain of the TEL/ETV6 ETS transcription factor) were incubated with in vitro translated HA epitope-tagged DLL4INTRA. Recombinant proteins were visualized by Coomassie staining. Bound DLL4INTRA was detected with Western bloting. E, recombinant JUN domains (and a control protein) were incubated with in vitro translated HA epitope--tagged full-length DLL4, DLL4N, or DLL4INTRA. Recombinant proteins were visualized by Coomassie staining. Bound DLL4 was detected with Western blotting. F, the C terminus of DLL4INTRA is necessary for binding of DLL4INTRA to the JUN bZIP domain. Experiments were performed as in D and E. DLL4INTRAΔC lacks the N-terminal amino acids 553–567. DLL4INTRAΔC lacks the C-terminal amino acids 666–686. G, the indicated constructs were stably expressed in HUVECs. JUN-DLL4 complexes were immunopurified from cell lysates using a rabbit HA polyclonal antibody and visualized by Western blotting with a FLAG mouse monoclonal antibody. TM, transmembrane domain; IP, immunoprecipitation; Tl, total lysate.
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**A**

**B**

**C**

**D**

**E**

**F**

**G**

**H**

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**VEGF TIME-COURSE (MINS)**

**INTEGRATED LUCIFERASE REPORTER**

**MAKERS, TIME-SERIES EXPERIMENTS**

**قارب، رد فعل الزمن**

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**Input**

**JUN**

**JUN + INTRA**

**JUN + DLL4N**

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**Fold Change in Expression**

**3**

**2**

**1**

**0**

**-1**

**-2**

**-3**

**-4**

---

**VWF**

**DLL4**

**BMP2**

**DUSP5**

---

**Mock**

**C-JUN**

**C-JUN/INTRA**

**C-JUN/DLL4N**

---

**Input**

**JUN Δ DBD**

**ΔC(309-331)**

---

**INTRA**

**Δ N(553-593)**

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**MOCK shRNA**

**JUN shRNA**

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**JUN Enrichment (Normalized to input)**

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**JUN ChIP**

**HA**

**FLAG IP**

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**Evidence for Notch/DLL4 bidirectional signaling**

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JUN is a member of the AP-1 family of transcription factors that plays a pivotal role in cell growth, differentiation, and survival as well as the DNA damage response (62, 63). Our results suggest that DLL4INTRA can block the activity of both JUN and JUNB (see Figs. 4 and 5). A related question is whether other Notch ligand ICDs can function similarly to DLL4INTRA. The DLL1 ligand shares a high degree of amino acid identity with DLL4, including ~40% of the ICD (see Fig. S3). Our results suggest that DLL1INTRA, like DLL4INTRA, can bind to the bZIP domain of JUN (Fig. 3A) and block JUN binding to a consensus AP-1 DNA site (Fig. 3B). Interestingly, unlike DLL4INTRA, DLL1INTRA could also interact with the bZIP domain of CREB3 in vitro (see Fig. S3A), raising the possibility that they have specific as well as overlapping activities. At the cellular level, DLL1INTRA, in common with DLL4INTRA, inhibited JUN-driven endothelial cell sprouting (Fig. S4C). Collectively, these results highlight a potential link between unthethered Notch ligand ICDs and the immediate-early gene AP-1 transcription factor complex. Genome-wide screens have established the importance of ETS and AP-1 transcription factor cooperation by their association to neighboring AP-1 and ETS DNA-binding sites (64). Because ETS transcription factors are essential for angiogenesis (55, 65, 66) and have been reported to control DLL4 expression (55, 67), it is noteworthy that JUN potently stimulates endothelial sprouting and is required for normal expression of DLL4 (see Fig. 3). JUN–DLL4INTRA interactions, therefore, could form part of a feedback loop whereby VEGF-dependent changes in DLL4 expression (as well as other angiogenesis-regulating genes) depend on ETS/AP-1 factors and are regulated by DLL ICDs.

In summary, our data lend support to the idea that biologically active Notch ligand ICDs could help establish appropriate endothelial cell responses through cross-talk with AP-1-dependent signal transduction pathways. They further highlight the possibility that corruption of this mechanism might play a role in disease processes such as tumor angiogenesis and developmental disorders. This is important because the Notch pathway has emerged as a primary target for the design of novel therapeutic interventions in cancer (42). Also, it has recently been reported that heterozygous loss-of-function DLL4 mutations are a potential cause of Adams-Oliver syndrome, a rare congenital disorder characterized by multiple defects, including vascular and cardiac anomalies (68). Two of the identified DLL4 mutations are nonsense mutations that are predicted to lead to complete loss of the DLL4 ICD.

Experimental procedures

Yeast two-hybrid screens

Screens were performed according to the manufacturer’s protocol (Dualsystems Biotech). Bait and prey were rescued from yeast colonies, and genuine interactions were confirmed through retransfection of yeast. Protein-protein interactions were tested quantitatively using a LacZ reporter assay (Dualsystems Biotech).

Proximity ligation assay

For the PLA, cells were seeded into eight-chamber cell culture slides (Lab-Tek II). The next day, cells were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature followed by permeabilization for 5 min with PBS + 0.2% Triton X-100. Following extensive washing, the proximity ligation assay was performed using a Duolink in situ PLA kit (Sigma) according to the manufacturer’s instructions. Briefly, blocking solution was added to each sample, and the slide was incubated for 30 min. All the incubations were performed in a preheated humidity chamber at +37 °C. Subsequently, primary antibodies were used at optimized concentrations in 1× antibody diluent for 1 h. Sequentially, two different PLA probes (anti-mouse MINUS and anti-rabbit PLUS) were mixed and incubated on the slides for 1 h. Ligation and amplification reagents were diluted in water and incubated on the slides for 30 and 100 min, respectively.

Figure 3. DLL4INTRA antagonizes JUN DNA binding. A, JUN binds to a consensus AP-1 DNA-binding site in vitro. A biotinylated double-stranded oligonucleotide harboring three consensus AP-1–binding sites was incubated with the indicated FLAG epitope–tagged JUN and translated proteins. JUNΔ(309–331) lacks the DNA-binding domain. JUNEΔC(309–331) lacks the C-terminal amino acids abutting the DNA-binding domain. DNA-bound JUN was detected by Western blotting. B, DLL4INTRA impedes JUN binding to DNA. As in A, AP-1 DNA-binding sites were incubated with the indicated in vitro translated proteins. DNA-bound JUN was detected by Western blotting. C, the putative PDZ-binding motif of DLL4INTRA is dispensable for DLL4INTRA binding to JUN. In A and B, AP-1 DNA-binding sites were incubated with the indicated in vitro translated proteins. INTRAΔ(ATEV) lacks the putative C-terminal PDZ-binding motif. INTRAΔp53(553–593) lacks the highly conserved N-terminal amino acids. INTRA Δ(566–680) lacks the C-terminal 20 amino acids. DNA-bound JUN was detected by Western blotting. D, DLL4INTRA alters the JUN-controlled transcriptome. A microarray analysis was performed on HUVECs stably expressing the indicated constructs. Protein levels were determined by Western blotting with the indicated antibodies (lower panel). Heat maps show a comparison of global gene expression profiles (upper panels). E, expression levels in HUVECs of the indicated transcripts were determined by real-time qPCR. All values were averaged relative to TBP, SRPR, and CAPNS1. Values were normalized against mock-treated cells. Values represent ±S.D. (n = 3). F, JUN controls DLL4 expression. Real-time qPCR was performed on cDNA prepared from cells stably infected with one of four different JUN shRNA constructs and stimulated with or without VEGF for the shown time course (minutes). All values were averaged relative to TBP, SRPR, and CAPNS1. Values were normalized against the zero time point of mock-infected cells. Values represent ±S.D. (n = 3). G and H, JUN associates with the proximal promoter of human DLL4. G, upper panel, a ChIP analysis was performed on HUVECs incubated with or without 50 ng/ml VEGF for the indicated times. Three different primer sets centered on the illustrated wildtype DLL4 promoter regions highlights the presumed transcription start site (underlined). Conserved consensus AP-1 DNA-binding sites are shown in green. A ChIP analysis was performed on reporter-expressing HUVECs incubated for 30 min with 50 ng/ml VEGF. Equivalent amounts of rabbit IgG were used as a control, and results are presented as -fold changes in recovery (as a fraction of input) relative to the zero time point. Lower panel, expression of a stably integrated luciferase gene. A single representative is shown (both gave comparable results). H, upper graphs, ChIP analyses were performed with the indicated antibodies (as described in G) on control HUVECs and HUVECs stably expressing DLL4INTRA. Lower graphs, DLL4 expression levels in control HUVECs and HUVECs stably expressing DLL4INTRA were determined by real-time qPCR. All values were averaged relative to TBP, SRPR, and CAPNS1. Values were normalized against mock-treated cells. Values represent ±S.D. (n = 3). IP, immunoprecipitation; VWF, von Willebrand factor. Error bars represent S.D.
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**A**
- MOCK
- JUN shRNA #1
- JUN shRNA #2

**B**
- JUN/MOCK
- JUN/DLL4FL
- JUN/INTRA
- JUN/DLL4N

Flag
- Tubulin
- JUN

HA

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Mounting medium was used for preservation of the PLA signals. Images were recorded on a Leica SP8 confocal microscope system using wavelengths of 488 and 561 nm. Statistical analysis of fluorescence signals was performed using BlobFinder software (http://www.cb.uu.se/~amin/BlobFinder/). For each condition, an average of 100 cells was scored. Error bars show the standard error of the mean.

**Mass spectrometry**

Endogenous DLL4 or HA epitope–tagged DLL4 was immunopurified from duplicate lysates, each prepared by lysing HUVECS (20 × 15- and 10 × 15-cm dishes, respectively) in ice-cold lysis buffer (50 mM Tris (pH 7.5), 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl). Protein bands were excised from SDS-polyacrylamide gels and subjected to in-gel tryptic digestion as described previously (69, 70).

**Cell culture, biochemistry, and molecular biology**

Primary HUVECs (Lonza) were cultured in EGM2 medium (Lonza). Human embryonic kidney 293T cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco). 293T cells were typed using short-tandem-repeat analysis of the DNA, and all cell lines were checked for Mycoplasma with the MycoAlert kit (Lonza). Transfections, lentivirus production, and cell infections, Western blotting, and coimmunoprecipitations have been described previously (71). All lysis buffers contained a mixture of protease inhibitors (phenylmethylsulfonyl fluoride, trypsin inhibitor, pepstatin A, leupeptin, and aprotinin).

**Recombinant protein production/in vitro protein-protein interaction**

Domains for recombinant protein production were cloned into the pET 28a vector in-frame to an N-terminal His6 epitope. His epitope–tagged proteins were produced in Escherichia coli BL21(DE3). Following sonication (Misonix Sonicator 3000) in 3 ml of ice-cold buffer/50 ml of bacterial culture (150 mM NaCl, 2.7 mM KCl, Na₂HPO₄, KH₂PO₄, 20 mM imidazole, 10 mM β-mercaptoethanol), proteins were purified onto 50 μl of nickel-agarose beads (Qiagen) by eluting at 4 °C. Beads were washed 10 times with 1 ml of the same buffer. Protein yields were determined by Bradford assay (Bio-Rad), and relative protein integrity and purity were determined by SDS-PAGE and colloidal blue staining (Invitrogen). 5–10 μl of His beads (purified recombinant protein) in 1 ml of buffer were incubated for 2 h at 4 °C with in vitro translated DLL4 proteins made using the TnT coupled reticulocyte in vitro translation system (Promega). Beads were washed 10 times with 1 ml of buffer. Proteins were separated by SDS-PAGE, and associated proteins were detected by Western blotting.

**Plasmid and shRNA construction**

Unless otherwise stated, all cDNAs were fused in-frame with a FLAG or an HA epitope tag and cloned into the pLV lentiviral vector and pCS2 expression plasmid. The luciferase reporter was constructed by cloning 1 kb of the DLL4 proximal promoter downstream of the luciferase gene in the pLV lentiviral vector. All mutants were engineered by site-directed mutagenesis using Phusion high-fidelity DNA polymerase (Thermo Fisher). All constructs were verified by Sanger sequencing (Macrogen). The following Mission shRNA library clones (Sigma) were used: shRNA TRCN0000010366 JUN, shRNA TRCN0000039589 JUN, shRNA TRCN0000009845 JUN, shRNA TRCN0000039588 JUN, shRNA TRCN0000039590 JUN, shRNA TRCN0000039591 JUN, and shRNA TRCN0000039592 JUN. The following siRNA duplexes (Thermo Scientific) were used (sense strands are shown): DLL4 siRNA 1, GCUC-ACUGCGAGAAGAAAU; DLL4 siRNA 2, GCAUGUGGCGAGUGGCUGUUU; JUN siRNA 1, GGAUCAAGGCCGAAGAAAU; and scrambled, ON-TARGETplus Non-targeting siRNA (D-001810-01-05).

HUVECs cells were transfected with duplexes using DharmaFECT transfection reagent according to the manufacturer’s recommendations (Thermo Scientific). Transfected cells were incubated for 72 h prior to PLAs. Efficient delivery of siRNA and subsequent silencing were tested by immunostaining and Western blotting.

**Transcriptome profiling**

Total RNA was isolated using TRIzol (Invitrogen) and further purified using an RNeasy minikit (Qiagen). Total RNA was labeled using an Illumina RNA amplification kit (Ambion). Duplicate samples from two independent experiments were tested. Gene profiles were determined by hybridization to Sentrix HumanHT-12 Expression Beadchips (ServiceXS).

**Analysis of mRNA expression**

RNA isolation, first-strand cDNA synthesis, and analysis of expression of transcripts by quantitative PCR were performed as described previously (55). The following primer sets were used (5’ to 3’ orientation): DLL4FOR, ccttgccaatctgtgat; DLL4REV, tggtgggtcagtta; JUNFOR, gtcccctgataatccagta; BMP2FOR, ccctggcaatgtacttgtgat; BMP2REV, ccctgtggaa; DUSP5REV, cccttttccctgacacagtc; VWFFOR, gtgcacccaacttcacct; JUNREV, ttcttggggcacaggaact; DUSP5FOR, caaatggatgtcagttgatat; VWFREV, ccctgtggaa; BMP2REV, ccctgtggaa; DLL4FOR, ccttgccaatctgtgat; BMP2REV, ccctgtggaa; dll4fl, full-length DLL4.

*Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party-hosted site.*
Figure 5. A, JUNB strongly stimulates endothelial cell tube formation/sprouting. HUVECs ectopically expressing JUN or JUNB were cultured in Matrigel in the presence of 50 ng/ml VEGF. A representative of several independent experiments is shown. After 48 h, in-house computer software was used to quantify the total length of the sprouts, the number of branch points, and the number of loops. Protein levels were determined by Western blotting with the indicated antibodies. Scale bar, 500 μm. B, DLL4INTRA attenuates JUNB-mediated sprouting. HUVECs ectopically expressing the indicated proteins were cultured in Matrigel in the presence of 50 ng/ml VEGF. A representative of several independent experiments is shown. After 24 h, in-house computer software was used to quantify the total length of the sprouts, the number of branch points, and the number of loops. Protein levels were determined by Western blotting with the indicated antibodies. Scale bar, 500 μm.
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(CAPNS1). For each data point, PCRs were performed in triplicate, and error bars show standard deviations from the mean.

HUVEC tube formation/sprouting assay

96-well plates were coated with 60 μl of Matrigel/well 30 min prior to seeding 25000 HUVECs/well. EGM2 medium was supplemented with 50 ng/ml recombinant human VEGF 165 (R&D Systems). Images were taken at multiple time points. Analysis of the sprouting was performed with Stacks (in-house software; Department of Molecular Cell Biology, Leiden University Medical Center). Each individual space bounded by connected tubes constitutes a loop. Branches are the intersections made by connected tubes, and total length is the combined length of all tubes.

Immunofluorescence

Immunostaining was performed as previously described (72) using Alexa Fluor 488 goat anti-mouse secondary antibodies (Thermo Fisher scientific). Imaging was performed with a Leica SP8 confocal microscope.

Protein-DNA interaction assays

In vitro translated protein was made using the TnT coupled reticulocyte in vitro translation system (Promega). 50 pmol of biotinylated double-stranded oligonucleotides harboring three contiguous AP-1 DNA-binding sites were coupled to MyOne streptavidin C1 beads (Invitrogen). Reactions were incubated at 4 °C with vigorous shaking for 30 min in the presence of 1 μg of poly(dI/dC), 4 mM spermidine, 50 mM KCl, 10 mM HEPES (pH 7.6), 5 mM MgCl₂, 10 mM Tris (pH 8), 0.05 mM EDTA (pH 8), 0.05 mM, 0.1% Triton X-100, 20% glycerol. Beads were successively washed three times with the aforementioned buffer. Associated proteins were eluted in Laemmli buffer, and protein-DNA interactions were determined by Western blotting.

ChIP

Confluent 10-cm tissue culture dishes of HUVECs were cross-linked for 10 min with formaldehyde (final concentration, 1%). Glycine was added to a final concentration of 0.125 M and incubated for 5 min. Cells were washed two times with PBS and subsequently lysed in ChIP lysis buffer (1%SDS,10 mM EDTA, 50 mM Tris-HCl (pH 8.1)) supplemented with protease inhibitors. Chromatin was sheared by sonication (Bioruptor UCD-200 ultrasound sonicator, Diagenode), resulting in DNA fragments between 500 and 1000 bp in size. After centrifugation, 10% of the sample was kept as input. Chromatin was diluted 10X in dilution buffer (1%Triton X-100, 2 mM EDTA (pH8.0), 150 mM NaCl, 20 mM Tris–HCl (pH 8.1) supplemented with protease inhibitors). For immunoprecipitation, 2 μg of test or control antibody were added to the diluted chromatin and incubated overnight at 4 °C. Blocked protein A-Sepharose beads (10 μg of sonicated herring sperm DNA) were added for 2 h. Beads were extensively washed (0.1% SDS, 0.1% sodium deoxycholate, 1% TritonX-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES), and complexes were eluted with elution buffer (1% SDS, 0.1 M NaH₂CO₃, 0.2 M NaCl) at 65 °C overnight to reverse cross-linking. Associated DNA was purified by phenol/chloroform extraction and ethanol precipitation (in the presence of 15 μg/ml glycogen (Roche Applied Science)). Real-time qPCR was used to determine recovery of specific DNA fragments. The following primers were used (5’ to 3’ orientation): DLL4promFOR A, tctttttactctggtttgcga; DLL4promREV A, agtcccctgagcctgtcga; DLL4promFOR B, aatggcatggttcagtgaca; DLL4promREV B, cgcgcgtactgaaaccttg; DLL4promFOR C, eggtggccctattacgtt; DLL4promREV C, aaacacgctaggaata; DLL4promFOR D, tcagagttcctctgg; DLL4promREV D, tgattcagctctaagctctg; DLL4promFOR E, acgtcccaacctcttg; DLL4promREV E, cgcaacagctgtatgttcctt; DLL4promFRO F, tcgaagttcttttgctgta; DLL4promREV F, ttcagagttcctaaaggctct; DLL4promFOR G, gaaacagggcaagac; DLL4promREV G, cgcgctctcttctaaactctg; LuciferaseREV1, catgaccgagagatcgcg; LuciferaseREV1, cagcttcttggcggttgta; LuciferaseREV2, tcagttcctagaatgtctccgttac; and LuciferaseREV2, ttgattcagctctaagctctg.

Antibodies and drugs

The following antibodies were obtained from the indicated sources: custom-made rabbit and goat polyclonal antibodies were generated by Eurogentec; FLAG mouse M2 monoclonal (Sigma-Aldrich), anti-HA.11 mouse monoclonal (Covance), anti-HA rabbit polyclonal (Abcam), anti-JUN rabbit (Cell Signaling Technology), anti-JUN mouse (Santa Cruz Biotechnology), anti-FLAG rabbit (Sigma), anti-γ-tubulin (Sigma), H3K27me3 (Bethyl Laboratories), calnexin rabbit polyclonal (Abcam), and FOS rabbit polyclonal (Cell Signaling Technology). Z-VAD was purchased from Sigma.

Author contributions—Z. F. and F. R., assisted by D. A. B., performed the majority of experiments. A. L. and J. V. O. performed mass spectrometry analyses. D. A. B. supervised the study and wrote the paper. All authors read and approved the paper.

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