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Molecules and Cells

Regulation of Notch1 Signaling by Delta-like Ligand 1 Intracellular Domain through Physical Interaction

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Notch signaling involves the proteolytic cleavage of the transmembrane Notch receptor after binding to its transmembrane ligands. The Delta-like ligand 1 also undergoes proteolytic cleavage upon Notch binding, resulting in the production of a free intracellular domain. In this study, we have demonstrated that the Delta-like 1 intracellular domain (Dll1-IC) specifically binds to Notch1-IC in the nucleus, thereby disrupting the association of the Notch1-IC-RBP-Jk-MAM transcription activator complex. Additionally, the Notch1-mediated blockage of the induction of MyoD is abolished by the co-expression of Dll1-IC. Collectively, our results show that Dll1-IC functions as a negative regulator in Notch signaling via the disruption of the Notch1-IC-RBP-Jk complex.

INTRODUCTION

The Notch signaling pathway performs a critical role in the determination of cell fate during development in organisms ranging from invertebrate to vertebrate species (Artavanis-Tsakonas et al., 1995; Weinmaster, 1998). Interactions between Notch and its ligands, Delta or Serrate/Jagged, promote two proteolytic cleavage steps (Nye and Kopan, 1995). Upon ligand binding, the Notch receptor is cleaved and translocated into the nucleus and functions as a transcriptional activator. An important nuclear target of Notch-IC is the DNA binding protein, RBP-Jk/CBF-1, the *Suppressor of Hairless* [Su(H)] (Mumm and Kopan, 2000). Notch1-IC interacts with RBP-Jk/Su(H) and its co-activator Mastermind (Mam), resulting in the activation of target gene transcription (Kovall, 2007). Several downstream targets of Notch signaling have also been identified, including Enhancer of split [E(spl)] complex genes and the mammalian homologues of the Hairy and E(spl) genes, Hes1 and Hes5 (Mumm and Kopan, 2000). These basic helix-loop-helix (bHLH) proteins antagonize other bHLH factors, such as MyoD, which induces muscle differentiation (Hirsinger et al., 2001; Jin et al., 2009; Kageyama et al., 2000; Kopan et al., 1994; Kuroda et al., 1999).

Delta is a major transmembrane ligand for the Notch receptor and performs an important function in Notch signaling. It was recently demonstrated that many single transmembrane-spanning proteins--including Notch, Delta and amyloid precursor protein (APP)--are substrates for gamma-secretase (Koo and Kopan, 2004). Delta has also been shown to be cleaved by ADAM protease and gamma-secretase to release an intracellular domain and thereby generate Dll1-IC, which is trafficked to the nucleus (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Qi et al., 1999; Six et al., 2003). Indeed, several groups have produced evidence of the nuclear localization of the intracellular fragments of Notch ligands. Evidence has recently been accumulating in support of a functional role of the intracellular domains of Notch ligands, which would imply downstream signal transduction. Dll1-IC has been shown to bind to smad2/3, thereby mediating transforming growth factor-b (TGF-b)/Activin signaling (Hiratochi et al., 2007). Thus, it has been previously reported that the lacking intracellular domain of Delta (X-Delta) affects normal development in *Xenopus* (Chitnis, 1995; Hiratochi et al., 2007; Sun and Artavanis-Tsakonas, 1996). It has also been demonstrated that Dll1-IC functions as an antagonist of Notch signaling in *Drosophila* (Sun and Artavanis-Tsakonas, 1996). Dll1-IC also triggers cell growth arrest via p21 expression (Kolev et al., 2005). Dll1 in a subset of cells during myogenic differentiation *in vitro* contributes to the downregulation of Notch signaling in neighboring cells and facilitates their progression into differentiation (Sun et al., 2008). Furthermore, although it has been well established that the proteolytic processing of Notch1 ligands downregulates Notch1 signaling in neighboring cells, the effects of ligand cleavage on the Notch1 pathway within the same cells remain less clear. Thus, the most important and critical question in this regard is whether or not Dll1-IC affects Notch1 signaling in the same cell.

In this study, our data demonstrate that Dll1-IC is an inhibitor of the Notch1 signaling pathway, and a component of the mechanism by which Dll1-IC suppresses Notch1 signaling.

MATERIALS AND METHODS

Plasmid constructs

The mouse Notch1-IC and mouse Dll1 deletion mutants con-

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structed in the present study were as follows: pCMV-3Flag-Notch1-IC-N (amino acid residues 1744~2110), pCMV-3Flag-Notch1-IC-ΔNΔC (amino acid residues 2110~2369), pCMV-3Flag-Notch1-IC-C (amino acid residues 2369~2531), and pEGFP-Dll1-IC (amino acid residues 567-722). The 4× CSL-Luc, Hes1-Luc, Hes5-Luc, pMyoD-Luc promoter reporter, pCS2-Myc-MyoD and Myc tagged Notch1-IC were a kind gift from Raphael Kopan (Washington University, USA). The N-terminal Flag tagged human RBP-Jk constructed via standard PCR, and inserted into *Bam*HI and *Xba*I sites of pcDNA3-Flag mammalian expression vector.

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells and C2C12 mouse myoblasts were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS) and fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified incubator under an atmosphere of 95% O_2, and 5% CO_2. For plasmid DNA transfection, cells were plated at a density of 2×10^6 cells/100-mm dish, grown overnight, and transfected with appropriate expression vectors in the presence of the indicated combinations of plasmid DNAs via the calcium phosphate method.

Luciferase reporter assay

The cells were lysed in chemiluminescent lysis buffer (18.3% of 1 M K_2HPO_4, 1.7% of 1 M KH_2PO_4, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT) and assayed for luciferase activity using a luciferase assay kit (Promega). The activity of the luciferase reporter protein in the transfected cells was normalized relative to the β-galactosidase activity in the same cells.

Immunoblot analysis

After 48 h of transfection, the cultured HEK293 cells were harvested and lysed in RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM DTT, and 2 μg/ml each of leupeptin and aprotinin] for 30 min at 4°C. The cell lysates were then subjected to 15 min of centrifugation at 12,000 × *g* at 4°C, and the resultant soluble fraction was subjected to SDS-PAGE. The separated proteins were transferred to a PVDF membrane, which was subsequently incubated for 1 h at room temperature with Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and 5% nonfat dried milk. The blots were probed with various antibodies including Immune complexes and detected with horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse IgG (Amersham Biosciences, Inc.) and an enhanced chemiluminescence system (Pierce).

Coimmunoprecipitation assays

The cells were lysed in 1 ml of RIPA buffer for 30 min at 4°C. Following 20 min of centrifugation at 12,000 × *g*, the supernatants were subjected to immunoprecipitation with appropriate antibodies coupled to protein A-agarose beads (Peptron). The resultant immunoprecipitates were then washed three times in phosphate-buffered solution (PBS, pH 7.4). Laemmli's sample buffer was subsequently added to the immunoprecipitated pellets; the pellets were heated for 5 min at 95°C and analyzed via SDS-PAGE. Western blotting was conducted with the indicated antibodies.

Immunofluorescence staining

Assays were conducted as described previously with HEK293 cells plated at 1×10^5 cells per well onto cover slips (Fisher). A total of 0.5 μg of appropriate DNA per well was then transfected with Lipofectamine reagent (Invitrogen). The transfected cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), and then permeabilized with 0.1% Triton X-100 in PBS. Mouse anti-FlagM2 antibody (Sigma) and anti-Myc mAb (Novus Biologicals) were employed as primary antibodies at a dilution of 1:100, and washed three times in PBS. Alexa-488 (Invitrogen) or Alexa-546 conjugated anti-mouse secondary antibody (1:100) was added, and then the DNA dye ToPro-3 was used for nuclear localization (blue). The stained cells were evaluated for localization via confocal microscopy (Leica TCS SPE). Each image is a single Z section at the same cellular level. The final images were acquired and analyzed via confocal microscopy with LAS AF software (Leica). The scale bars represent 25 μm, as indicated (Mo et al., 2011).

RESULTS

Dll1-IC suppresses the transcriptional activity of Notch1 target genes

In order to determine whether Dll1-IC is involved in regulating the transcriptional activation of Notch1 target genes, a reporter assay was performed in HEK293 cells using luciferase reporter genes. In this study, three types of luciferase reporter genes were evaluated under the control of Hes1 promoter (Hes1-Luc), Hes5 promoter (Hes5-Luc), and artificial 4× CSL (4× CSL-Luc). We investigated the effect of Dll1-IC on Notch1-IC transcriptional activity. As expected, the Notch1-IC induced 4× CSL luciferase reporter activity increased by 9 ± 2.3 (S.D.) fold compare to control. Cotransfection of Dll1-IC with Notch1-IC, the Notch1-IC induced reporter activity was inhibited to 2.5 ± 0.5 (S.D.) fold (Fig. 1A). We also found similar results using Hes1-Luc and Hes5-Luc reporter systems (Figs. 1B and 1C). Cotransfection of Dll1-IC with Notch1-IC, Hes1-Luc and Hes5-Luc activity were reduced to 2 ± 0.3 (S.D.) and 5.7 ± 1.2 (S.D.) fold, respectively. These results indicated that Dll1-IC suppresses the transcriptional activity of Notch1 in intact cells.

Dll1-IC prevents the physical interaction between Notch1-IC and RBP-Jk

To observe the effects of Dll1-IC on the molecular interactions between Notch1-IC and RBP-Jk, coimmunoprecipitation was conducted in HEK293 cells via the cotransfection of Myc-tagged Notch1-IC, Flag-tagged RBP-Jk, and GFP-tagged Dll1-IC. Notch1-IC and RBP-Jk were coimmunoprecipitated, but when cotransfected with Dll1-IC, the band of Notch1-IC that interacted with RBP-Jk disappeared (Fig. 2A). We subsequently evaluated the formation of the complex in an effort to define more precisely the role of Dll1-IC in the negative regulation of Notch1-IC-RBP-Jk-MAML-mediated signaling. Remarkably, the formation of the Notch1-IC-RBP-Jk-MAML complex was prevented in the presence of Dll1-IC (Fig. 2B), thereby suggesting that Dll1-IC may perform a critical role with regard to the downregulation of Notch1IC-mediated transcription activity, by disrupting the formation of the active complex.

Physical interaction of Dll1-IC with Notch1-IC in intact cells

Given that our results implicate Notch1-IC as a target of Dll1-IC, we next attempted to determine whether these two proteins interact physically in intact cells. HEK293 cells were cotransfected with vectors encoding for GFP-tagged Dll1-IC and Myc-tagged Notch1-IC, and were then subjected to co-immunoprecipitation analysis. Immunoblot analysis using the anti-HA antibody of Myc immunoprecipitates from the transfected cells showed that GFP-Dll1-IC associated physically with Myc-

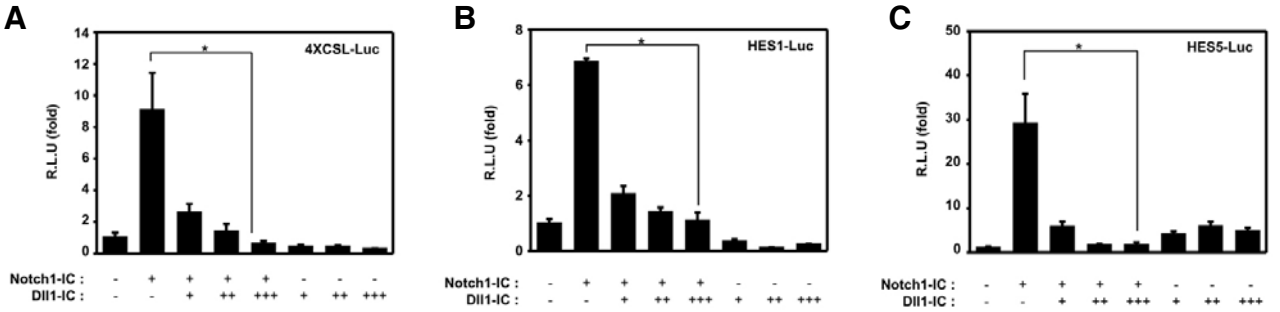

Fig. 1. Dll1-IC suppresses the transcriptional activity of Notch1 target genes. (A, B, and C) HEK293 cells were transfected with expression vectors encoding for Notch1-IC and Dll1-IC with the luciferase reporter plasmids 4× CSL-Luc, Hes1-luc, and Hes5-luc along with pCMV-β-galactosidase as indicated. After 48 h of transfection, cells were lysed and assayed for luciferase activity. The luciferase activity of each sample was normalized to the β-galactosidase activity measured in the same sample. The data are expressed as the means ± S.D. of triplicates from one of three independent experiments. The data were evaluated for significant differences via student's *t*-test. *ANOVA, $P < 0.001$.

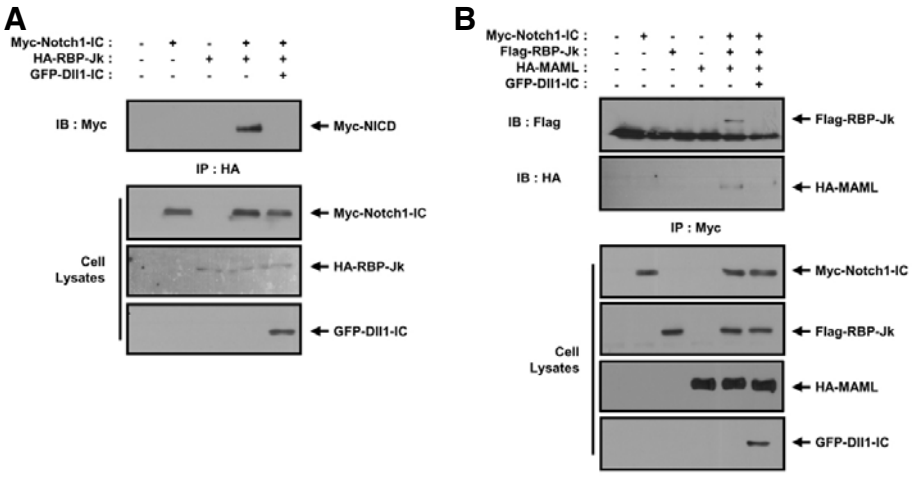

Fig. 2. Dll1-IC prevents the physical interaction between Notch1-IC and RBP-Jk. (A) HEK293cells were transfected with the indicated combinations of expression vectors for Myc-Notch1-IC, HA-RBP-Jk and GFP-Dll1-IC. The cell lysates were lysed and immunoprecipitated against anti-Myc monoclonal antibody. The immunocomplexes were analyzed via SDS-PAGE and immunoblotting against anti-HA monoclonal antibody. The expression of Notch1-IC or RBP-Jk was analyzed via immunoblotting using anti-Myc or anti-HA monoclonal antibody. (B) HEK293 cells were transfected with the indicated combinations of expression vectors for Myc-Notch1-IC, Flag-RBP-Jk, HA-Mastermind and GFP-Dll1-IC. The cell lysates were then lysed and immunoprecipitated against anti-Myc monoclonal antibody. The immunocomplexes were analyzed via SDS-PAGE and immunoblotting against anti-Flag or anti-HA monoclonal antibody. Notch1-IC, RBP-Jk and Mastermind-like (MAML) expression were analyzed via immunoblotting using anti-Myc anti-Flag or anti-HA monoclonal antibodies.

Notch1-IC in the cells (Fig. 3A). The structure of Notch1-IC features a CDC domain that includes six ankyrin repeats, an OPA domain, and a PEST domain. We attempted to determine which of these domains might be involved in the interaction between Notch1-IC and Dll1-IC. We employed a variety of Flag-tagged Notch1 deletion mutants: Notch1-IC-N (CDC domain), Notch1-IC-ΔNΔC (OPA domain) or Notch1-IC-C (PEST domain). We conducted coimmunoprecipitation experiments using three Notch1-IC deletion mutants and GFP-tagged Dll1-IC. Our results demonstrate that Dll1-IC bound to Notch1-IC-N, but not to Notch1-IC-ΔNΔC and Notch1-IC-C (Fig. 3B). Previous studies have demonstrated that Dll1-IC was present in the nuclei of developing neural stem cells (NCS) after being released from the cell membrane by gamma-secretase (Hiratochi et al., 2007). Additionally, Notch1-IC is located predominantly within the nucleus. Therefore, in this study, we performed immunofluorescence staining analyses to determine whether Dll1-IC was colocalized with Notch1-IC and RBP-Jk or was not in the nucleus. In order to identify the cellular compartment, Flag-Notch1-IC, Myc-RBP-Jk or GFP-Dll1-IC was expressed in HEK293 cells. Notch1-IC and RBP-Jk were localized mainly in the nucleus, and the intercellular distribution of Dll1-IC is in both cytoplasm and nucleus (Fig. 3C). Dll1-IC was predominantly localized in the nucleus. Coexpression of Notch1-IC enhanced the translocation of Dll1-IC into the nucleus (Fig. 3C). Interestingly, RBP-Jk and Dll1-IC were both detected in the nucleus and RBP-Jk facilitated nuclear distribution of Dll1-IC (Fig. 3C).

The effect of Dll1-IC on the suppression of the MyoD gene promoter activity by Notch1-IC

To evaluate the role of Dll1-IC in the Notch1-IC induced suppression of MyoD transcriptional activity, we conducted transient transfection studies using a luciferase reporter gene linked to the promoter/regulatory region of the MyoD gene (MyoD-Luc). MyoD is a central regulator in the determination of somatic cells into the myogenic program. The MyoD mediated luciferase reporter activity increased by 2.7 ± 0.3 (S.D.) fold. Cotransfection with Notch1-IC, MyoD transcriptional activity was suppressed to 1.4 ± 0.1 (S.D.) fold. Furthermore, the Notch1-IC mediated suppression of MyoD transcriptional activity was restored to 3 ± 0.4 (S.D.) fold via coexpression of Dll1-IC (Fig. 4). Moreover, MyoD transcriptional activity was increased to 3.9 ± 0.8 (S.D.) fold by Dll1-IC alone, thereby suggesting that Dll1-IC might play a role in the regulation of muscle

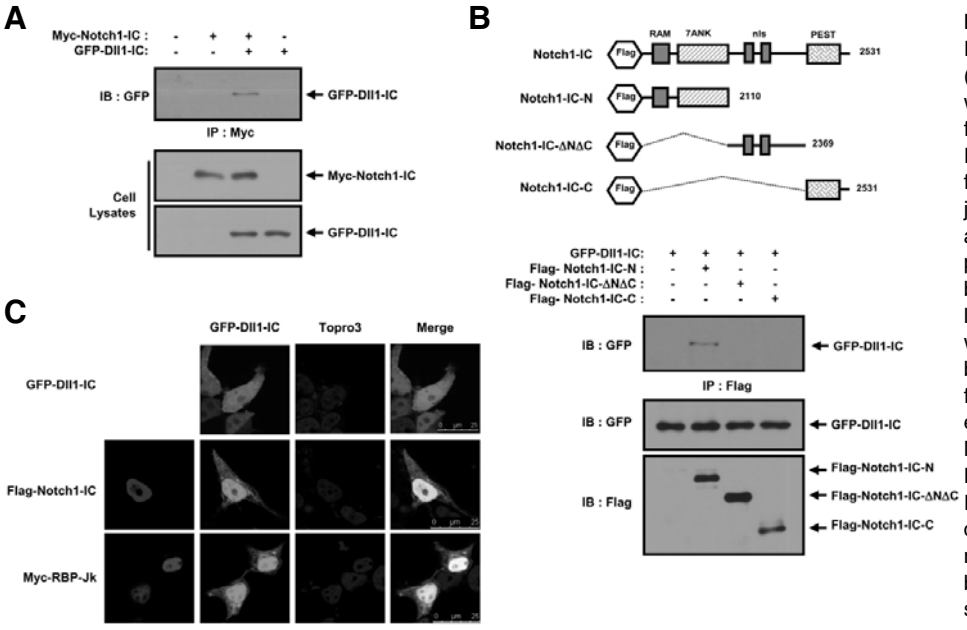

Fig. 3. Physical interaction of Dll1-IC with Notch1-IC in intact cells. (A) HEK293 cells were transfected with expression vectors encoding for Myc-Notch1-IC and GFP-Dll1-IC as indicated. After 48 h of transfection, the cell lysates were subjected to immunoprecipitation with anti-Myc antibody. The immunoprecipitates were then immunoblotted with anti-GFP antibody. Cell lysates were also immunoblotted with anti-Myc and anti-GFP antibody. (B) HEK293 cells were transfected with the expression vector encoding for Flag-Notch1-IC, Flag-Notch1-IC-N, Flag-Notch1-IC-NC, Flag-Notch1-IC-C, and GFP-Dll1-IC. After 48 h of transfection, the cell lysates were subjected to immunoprecipitation with anti-Flag antibody. The immunoprecipitates were subsequently immunoblotted with anti-GFP antibody. Cell lysates were also immunoblotted with anti-Flag or anti-GFP antibody. (C) HEK293 cells were transfected with Flag-Notch1-IC, Myc-RBP-Jk or GFP-Dll1-IC. After 48 h, Flag-Notch1-IC and Myc-RBP-Jk were stained with Alexa-546. The cells were observed with a confocal microscope.

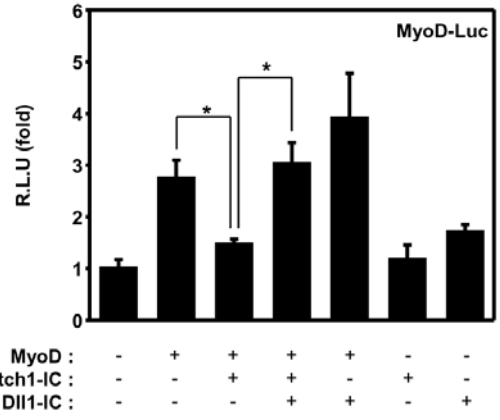

Fig. 4. The effect of Dll1-IC on the suppression of the MyoD gene promoter activity by Notch1-IC. HEK293 cells were transfected with expression vectors encoding for Notch1-IC and Dll1-IC with the luciferase reporter plasmid MyoD-Luc along with pCMV-β-galactosidase as indicated. After 48 h of transfection, the cells were lysed and assayed for luciferase activity. The luciferase activity of each sample was normalized to the β-galactosidase activity measured in the same sample. These results represent the means ± S.D. of three independent experiments. The data were evaluated for significant differences by student's *t*-test. *ANOVA, $P < 0.05$.

differentiation. These results demonstrated that the transcriptional activity of MyoD was upregulated by Dll1-IC via the suppression of Notch1 signaling.

DISCUSSION

Delta is cleaved sequentially by proteases including ADAM and gamma-secretase, and the processing of Drosophila Delta was shown to be upregulated in co-cultures with Notch-expressing S2 cells. It has been previously reported that a truncated intracellular isoform of Delta evidences prominent nuclear localization (Bland et al., 2003; Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six et al., 2003). The intracellular domain of Delta1 results in a non-proliferating senescent-like cell phenotype that depends on the expression of the cell cycle inhibitor, p21, and is suppressed by constitutively active Notch1 (Kolev et al., 2005). However, the direct relationship between Dll1-IC and Notch1-IC remains poorly understood.

In this report, we determined that Notch1-IC-mediated target gene induction is downregulated by Dll1-IC, via the disruption of the formation of the Notch1-IC and RBP-Jk complex; this suggests that after cleavage by gamma-secretase, Notch and Delta-like 1 signaling can be interconnected under certain conditions. Dll1-IC, the gamma-secretase cleavage of Delta-like1, has received particular attention because it resembles the S3 Notch cleavage generating Notch-IC and is then translocated into the nucleus, inducing the transcription of several genes. This transcription activity of Dll1-IC appears to mediate important physiological functions. The functional involvement of Dll1-IC in Notch1 signaling, then, remains a matter of some controversy. Our results demonstrated that Notch1-IC transcriptional activity was inhibited by the presence of Dll1-IC, which suggests that Dll1-IC may also involve the suppression of Notch1-IC transcriptional activity. In previous reports, it has been determined that Notch1-IC and RBP-Jk are the key components in the Notch1-IC transcription complex (Tamura et al., 1995). Then Notch1-IC recruits coactivator, Mastermind like-protein MAML. MAML protein was capable of binding with the intracellular domain of Notch1 and RBP-Jk *in vivo* (Wu et al., 2002). We demonstrated that the expression of Dll1-IC results in a down-regulation of Notch1-IC-RBP-Jk-MAML complex-mediated transcriptional activity through disrupt the formation of the Notch1-IC-RBP-Jk-MAML trimeric complex. However, the dissociation sequence and mechanisms relevant to this phenomenon remain obscure. The intracellular domain of Notch1 functions as a constitutively activated repressor of myogenesis

in both cultured cells and frog embryos (Kopan et al., 1994; Nye et al., 1994). Notch1-IC has been previously suggested to exert a regulatory effect on MyoD expression (Kopan et al., 1994). In this study, we concluded that the overexpression of Notch1-IC reduced MyoD transcriptional activity. This reduced MyoD transcriptional activity is rescued by co-expression with Dll1-IC.

In summary, we have demonstrated the suppression of Notch signaling by Dll1-IC. Delta is cleaved sequentially by the RIP mechanism probably including ADAM and gamma-secretase (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Qi et al., 1999; Six et al., 2003), and the cleaved Dll1-IC is released from the cell membrane and translocates to the nucleus, where it suppresses notch signaling through the disruption of Notch1-IC transcriptional complex. Henceforth, the results of this study may begin to shed some light on what may be a signal cross-talk mechanism of Notch and Delta after cleavage by gamma-secretase.

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