Dual Regulation of Notch1 Signaling Pathway by Adaptor Protein Fe65*

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Background: The importance of Notch and Fe65 function during development has been well recognized.

Results: Adaptor protein Fe65 attenuates Notch1 signaling via the accelerated degradation of the membrane-tethered Notch1.

Conclusion: Dual regulation of Notch1 signaling pathway by adaptor protein Fe65.

Significance: The findings of this study may begin to shed some light onto what may be a signal cross-talk mechanism of Notch1 signaling and the Fe65 adaptor protein.

Notch1 receptor functions as a critical controller of cell fate decisions and also as a key regulator of cell growth, differentiation, and proliferation in invertebrates and vertebrates. In this study, we have demonstrated that the adaptor protein Fe65 attenuates Notch1 signaling via the accelerated degradation of the membrane-tethered Notch1 in the cytoplasm. Fe65 also suppresses Notch1 transcriptional activity via the dissociation of the Notch1-IC-recombining binding protein suppressor of hairless (RBP)-Jk complex within the nucleus. Fe65 is capable of forming a trimeric complex with Itch and membrane-tethered Notch1, and Fe65 enhances the protein degradation of membrane-tethered Notch1 via an Itch-dependent proteasomal pathway. Collectively, our results demonstrate that Fe65 carries out different functions depending on its location in the regulation of Notch1 signaling.

Notch1 is a single-pass type I transmembrane receptor that performs a key role in the determination of cell fate, differentiation, cell proliferation, cell survival, and cell death (1–4). Notch1 receptor is produced by furin in the endoplasmic reticulum Golgi apparatus (S1 cleavage) during transport to the cell surface, where it is expressed in heterodimeric form (5, 6). After binding to the specific ligands, Jagged and Delta, the transmembrane C-terminal fragment of Notch is generated via proteolytic cleavage by a disintegrin and metalloprotease domain metalloprotease (S2 cleavage) (7, 8). The cleavage of this fragment by γ-secretase (S3 cleavage) subsequently induces the release of the Notch intracellular domain from the membrane (9). Then, the Notch1 intracellular (Notch1-IC)2 domain is translocated into the nucleus and binds to the cofactors CBF1/suppressor of hairless/Lag-1 (CSL), MAML-1 (mastermind-like-1), and p300/CBP, leading to the transcriptional activation of downstream target genes such as Hes1, Hes5, Hes7, Hey1, Hey2, and HeyL (8, 10–14).

After the transcriptional regulation of the target genes, Notch1-IC undergoes proteasomal degradation in the nucleus via the ubiquitin-proteasome system (15–20). Several E3 ubiquitin ligases have been implicated in the half-life of Notch1-IC, including Fbw7, which promotes proline-, glutamate-, serine-, threonine-rich domain-dependent Notch1 intracellular degradation in the nucleus (15, 17–20). Additionally, the results of a recent genetic study demonstrated that Itch regulates the PEST-independent degradation of cytoplasmic Notch protein (21). The E3 protein ubiquitin ligase (E3) Itch, a novel E3 Ub ligase, or atrophin-1 interacting protein 4 (AIP4, hereafter referred to as Itch), is absent in the non-agouti-lethal 18H or Itchy mice (22). The Itch gene encodes for 854 amino acids with a relative molecular weight of 113 kDa (23). Itch is a monomeric protein that belongs to the homologous to E6-AP carboxyl terminus-type family of E3s, whose modular structural organization consists of four WW domains and a unique proline-rich motif (22). In Drosophila, genetic evidence suggests that the homologous to E6-AP C terminus domain harboring the E3 ubiquitin ligase suppressor of deltex negatively regulates Notch receptor signaling (24). The results of a recent study have demonstrated that Itch ubiquitiniates membrane-tethered Notch1 under both in vivo and in vitro conditions (21). They concluded that the Notch-IC domain functions as a direct substrate for Itch (21). However, the step regulated by Itch has yet to be clearly elucidated.

Fe65 is a neuronal adaptor protein that mediates the assembly of multimolecular complexes through a variety of protein-protein interaction domains: the WW domain, which binds to proline-rich sequences, and two C-terminal phosphotyrosine-binding domains (25, 26). Fe65 is a brain-enriched protein of unknown function that binds to the cytoplasmic domain of amyloid precursor protein (27). We also demonstrated previously that Notch1-IC inhibits AICD transcriptional activity via physical binding with amyloid precursor protein intracellular domain, Fe65, and Tip60 (28). Fe65 is localized within the cytoplasm and nucleus of the cell (29), although moderate amounts

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2 The abbreviations used are: Notch1-IC, Notch1 intracellular; CSL, CBF1/suppressor of hairless/Lag-1; NES, nuclear export sequence; NEXT, Notch1 extracellular truncation.
of Fe65 are tethered to membranes via binding with APP (30, 31). Fe65 expression can lead to the stabilization and nuclear translocation of AICD, where it may induce apoptosis through Tip60 (32). The functions of Fe65 remain to be clearly elucidated, but it is found within both the cytoplasm and nucleus and has been shown to play roles in cell motility and nuclear signaling. Whereas the importance of Notch and Fe65 function during development has been well recognized, the molecular and biochemical mechanisms via which Fe65 exerts its regulatory effect on Notch signaling remain incompletely understood.

Herein, we have evaluated the mechanism underlying the Fe65-mediated dual regulation of Notch1 signaling. Our data show that Fe65 inhibits the transcriptional activity of Notch1 via an induced reduction in the protein stability of membrane-tethered Notch1, and that the level of the membrane-tethered Notch1 protein was markedly down-regulated in the presence of Fe65 via the proteasomal degradation of membrane-tethered Notch1 through its interaction with Notch1-IC and disrupts the Notch1-IC-recombining binding protein suppressor of hairless (RBP)-Jk transcription complex within the nucleus.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—HEK293 cells and NIH3T3 cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator with an atmosphere containing 5% CO2. The cultured cells were transiently transfected via the calcium phosphate method for human embryonic kidney 293 cells. For plasmid DNA transfection, the cells were grown to ~80% confluence and transfected with the plasmids (18, 33).

** Luciferase Reporter Assay**—Human embryonic kidney 293 cells were cotransfected with 4XCSL-Luc (a four-time repeating section of the RBP-Jk target sequence, CGTGGGAA, with the luciferase gene), Hes1-Luc, Hes5-Luc, and β-galactosidase coupled with the indicated vector constructs. After 48 h of transfection, the cells were lysed in chemiluminescent lysis buffer at room temperature. Protein levels were analyzed via immunoblotting.

**Ubiquitination Assay**—The HEK293 cells were transfected with indicated expression vectors and then harvested 48 h after transfection. After 42 h of transfection, the cells were treated with 10 μM of MG132 for 6 h, and the cells were lysed for 10 min in 1 ml of radioimmune precipitation assay lysis buffer at room temperature. After 20 min of centrifugation at 12,000 × g, the supernatants were subjected to immunoprecipitation with specific antibodies. After overnight incubation, protein A-agarose was added, and the samples were incubated for 3 h at 4 °C on the rotator. The beads were then again washed three times in ice-cold PBS, and any proteins that remained bound to the beads were eluted by boiling in 5× protein sample buffer. The samples were separated via SDS-PAGE, and visualized by immunoblotting.

**Immunoblot Analysis**—After 48 h of transfection, the cultured cells were harvested and lysed in radioimmune precipitation assay 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, 2 μg/ml each of leupeptin and aprotinin) for 30 min at 4 °C. The resultant soluble fraction was boiled in Laemmli buffer and subjected to SDS-PAGE. After gel electrophoresis, the separated proteins were transferred via electrophoresis from PVDF membranes (Millipore). The membranes were blocked with Tris-buffered saline solution (pH 7.4) containing 0.1% Tween 20 and 5% nonfat milk. The blotted proteins were subsequently probed with anti-Myc antibody (9E10), anti-HA (12CA5) antibody, or anti-FLAG M2 antibody (Sigma), followed by incubation with anti-mouse horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Inc.). The blots were then developed via ECL.

**Coimmunoprecipitation**—After 48 h of transfection, the cells were lysed for 10 min in 1 ml of radioimmune precipitation assay lysis buffer at room temperature. After 20 min of centrifugation at 12,000 × g, the supernatants were subjected to immunoprecipitation with specific antibodies. After overnight incubation, protein A-agarose was added, and the samples were incubated for 3 h at 4 °C on the rotator. The beads were then again washed three times in ice-cold PBS, and any proteins that remained bound to the beads were eluted by boiling in 5× protein sample buffer. The samples were separated via SDS-PAGE, and visualized by immunoblotting.

**Protein Accumulation Assay**—Cells were treated with the proteasomal inhibitor MG-132 (Sigma-Aldrich), or the translational inhibitor cycloheximide (Sigma-Aldrich). MG-132 was used at 0, 5, and 10 μM for 6 h for the dosage assay of the proteasomal inhibitors. Half-life experiments employing the cycloheximide-mediated inhibition of protein synthesis were conducted as described previously (18). Cycloheximide was used at 100 μM for 0, 1, 2, 4, and 6 h for the time-course assay of the translational inhibitors. Protein levels were analyzed via immunoblotting.

**Negative Regulation of Notch1 Signaling by Fe65**

In this study, we also investigated the molecular and biochemical mechanisms via which Fe65 inhibits the transcriptional activity of Notch1. Using a four-time repeating section of the RBP-Jk target sequence, CGTGGGAA, with the luciferase gene, Hes1-Luc, Hes5-Luc, and β-galactosidase coupled with the indicated vector constructs. These constructs were coexpressed in HEK293 cells along with the indicated expression vectors for Fe65 and Notch1-IC. After 48 h of transfection, the cells were harvested and lysed in radioimmune precipitation assay 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, 2 μg/ml each of leupeptin and aprotinin) for 30 min at 4 °C. The resultant soluble fraction was boiled in Laemmli buffer and subjected to SDS-PAGE. After gel electrophoresis, the separated proteins were transferred via electrophoresis from PVDF membranes (Millipore). The membranes were blocked with Tris-buffered saline solution (pH 7.4) containing 0.1% Tween 20 and 5% nonfat milk. The blotted proteins were subsequently probed with anti-Myc antibody (9E10), anti-HA (12CA5) antibody, or anti-FLAG M2 antibody (Sigma), followed by incubation with anti-mouse horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Inc.). The blots were then developed via ECL.
and 3′-dTdTGAUUAACGUAGAUAUGA-5′ (lower strand) (GenePharma). The nucleotide sequences for siRNA targeting human Itch were as follows. Itch, 5′-AAGGAGCAACAUGUGGAUAAUAdTdT-3′ (upper strand) and 3′-dTdTU-UCCUCGUUGUAGACCUAAUUAU-5′ (lower strand) (GenePharma). siRNA was transfected into HEK293 cells using Lipofectamine according to the supplier’s manual.

RESULTS

Fe65 Suppresses Notch1 Transcriptional Activity—To evaluate the possible functions of Fe65 in Notch1 signaling, a reporter assay was conducted with NIH3T3 and HEK293 cells using luciferase reporter genes. The NIH3T3 cells or HEK293 cells were transfected with 4XCSL-Luc or Hes1-Luc or Hes5-Luc as well as either the active Notch1 mutant ΔEN1 (membrane-tethered Notch1) or an empty vector. As anticipated, ΔEN1-mediated transcription activity was found to have increased in these samples. We determined that Fe65 attenuated the ability of membrane-tethered Notch1 to stimulate transcription (Fig. 1, A and B). The basic helix-loop-helix proteins Hes1 and Hes5, both of which harbor multiple RBP-Jk-binding DNA sequences on their promoters, were identified as essential targets of Notch1 in epithelial cells (36). Therefore, we confirmed the effects of Fe65 on the Notch1 signaling pathway using the Hes1 or Hes5 reporter systems. ΔEN1 expression significantly induced the activation of the Hes1 or Hes5 reporter systems (Fig. 1, A and B). Fe65 coexpression inhibits ΔEN1-induced natural Hes1 or Hes5 promoter transcriptional activity (Fig. 1, A and B).

Tip60 Does Not Affect Fe65-mediated Inhibition of Notch1 Signaling Pathway—Previous reports have demonstrated that the Fe65 adaptor protein forms a complex with the Tip60 histone acetyltransferase (32, 34). We demonstrated previously that the expression of Tip60 down-regulates Notch1-IC-mediated transcriptional activity through Tip60-dependent acetylation of Notch1-IC (34). To determine the function of Tip60 in the Fe65-mediated suppression of Notch signaling, we introduced a Tip60 mutant (Tip60*) that cannot bind to Fe65. ΔEN1 expression was found to significantly induce the activation of the 4XCSL-Luc reporter system (Fig. 2A). When Fe65 was transiently coexpressed in HEK293 cells, constitutively active ΔEN1-mediated transcriptional activity was suppressed to a substantial degree (Fig. 2A). However, the forced expression of Tip60* exerted no effect on the negative role of Fe65 in ΔEN1-mediated transcriptional activity. We also confirmed the effects of Fe65 on the ΔEN1-mediated transcriptional activity using Notch1-IC. When Fe65 was transiently coexpressed in HEK293 cells, Notch1-IC-mediated transcriptional activity was apparently suppressed (Fig. 2B). However, coexpression of Tip60* exerted no effect on the negative role of Fe65 in Notch1-IC-mediated transcriptional activity.
activity (Fig. 2B). These results show that Fe65 involved in the regulation of Notch1 signaling in a Tip60-independent manner.

**Fe65 Facilitates the Degradation of Membrane-tethered Notch1 by the Proteasome-dependent Pathway, but Notch1-IC Does not Affect Fe65-mediated Degradation**—We carried out immunoblot analysis on HEK293 cells to determine whether Fe65 performs a function in the regulation of the ΔEN1 (membrane-tethered Notch1) protein level. Cells were cotransfected with ΔEN1-Myc and Myc-Fe65. We found that the ΔEN1 protein level (membrane-tethered Notch1) was reduced upon cotransfection of Fe65 (Fig. 3A). To determine whether the degradation of ΔEN1 protein level is mediated by the proteasome pathway, the proteasome inhibitor MG132 was employed for the treatment of ΔEN1-expressing and Fe65-expressing cells. MG132 can reversibly block all activities of the 26 S proteasome (37). The cells were treated with proteasome inhibitor for 6 h, and ΔEN1 protein was detected via an immunoblot assay. The ΔEN1 protein level was reduced in the presence of Fe65 but was restored to a significant extent by treatment with MG132 (Fig. 3B). Next, we transfected two expression vectors encoding for Fe65 fused at the COOH terminus to the nuclear export signal (NES) of the MAPKK or Fe65 fused to a mutated, nonfunctional version of this NES. The transfection of the Fe65-NES had the same effect as the wild-type Fe65, whereas the loss of function mutant NES conjugated Fe65 (Fe65-mNES) has no effect on the ΔEN1 protein level (Fig. 3C). To determine the possible role of Fe65 in the regulation of Notch1-IC protein stability, HEK293 cells were transfected with Myc-Notch1-IC and Myc-Fe65. Our studies showed that Fe65 could not regulate the steady-state level of Notch1-IC proteins (Fig. 3, D and E). We determined that the ΔEN1 protein level was reduced upon cotransfection of Fe65, but Notch1-IC protein was not reduced upon cotransfection of Fe65. These results showed that the stability of the ΔEN1 protein was down-regulated by Fe65 via the proteasome-dependent pathway. Our results demonstrate that Fe65 carries out different functions in Notch1 signaling, depending on its location.
Negative Regulation of Notch1 Signaling by Fe65

An antibody analysis with anti-Notch1 antibody. These results imply that Fe65 negatively regulated the transactivation of Notch1-IC target genes via the suppression of the interaction between Notch1-IC and RBP-Jκ in intact cells (Fig. 4D).

Notch1-IC Interacts Directly with Fe65 in Intact Cells—During our previous investigations, we have noticed that Fe65 may interact with Notch1-IC (28). In an effort to delineate more precisely the manner in which Fe65 prevents Notch1-IC and RBP-Jκ-mediated transcription, we conducted a series of in vitro binding and coimmunoprecipitation experiments. In the in vitro binding studies, purified GST or GST-Fe65 proteins were immobilized onto GSH-agarose. FLAG-RBP-Jκ and Myc-Notch1-IC expressing the cell lysates were incubated with either GST or GST-Fe65, both of which had been immobilized onto GSH-agarose. The interaction between GST-Fe65 and Notch1-IC has been detected on bead complexes in the absence of RBP-Jκ (Fig. 5A). However, despite the performance of repeated experiments, no interaction was determined to have occurred between GST-Fe65 and RBP-Jκ (Fig. 5A). To characterize the physical interaction occurring between Fe65 and Notch1-IC or RBP-Jκ, coimmunoprecipitation was performed with HEK293 cells via the cotransfection of FLAG-Notch1-IC, HA-RBP-Jκ, and Myc-Fe65. The transfected cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FLAG or anti-HA antibody (Fig. 5B). The results indicated that Fe65 interacts with Notch1-IC but not with RBP-Jκ, and no trimeric complex was detected in this case (Fig. 5B).

Next, we attempted to ascertain whether Fe65 was capable of physically associating with Notch1-IC in intact cells. HEK293 cells were transfected with Fe65 siRNA. The cells were subjected to immunoprecipitation with anti-Notch1-IC, and the resultant immune pellets were examined via immunoblotting analysis with anti-Fe65 antibody. The immunoblot data demonstrated that the pellets interacted directly with endogenous Notch1-IC in the intact cells (Fig. 5C). These findings strongly indicate that a physical interaction between the two endogenous proteins Fe65 and Notch1 does indeed occur in intact cells. Notch1-IC harbors a CDC domain that harbors a RBP-J association module domain, seven ankyrin repeats, an opa-repeat region domain, and a PEST domain within its structure. We attempted to determine which, if any, of these domains might be involved in the interaction between Notch1-IC and Fe65. We utilized a variety of FLAG-Notch1 deletion mutants: 

FIGURE 3. Fe65 facilitates degradation of membrane-tethered Notch1 by the proteasome-dependent pathway. A, HEK293 cells were transfected for 48 h with the indicated combinations of expression vectors encoding for ΔEN1-Myc and Myc-Fe65. B, HEK293 cells were transfected for 42 h with the indicated combinations of expression vectors encoding for ΔEN1-Myc and Myc-Fe65. The cells were then treated with the indicated quantity of MG132 for 6 h. C, HEK293 cells were transfected for 48 h with the indicated combinations of expression vectors encoding for ΔEN1-Myc, Myc-Fe65 NES, and Myc-Fe65 mNES. D, HEK293 cells were transfected for 48 h with the indicated combinations of expression vectors encoding for Myc-Notch1-IC and Myc-Fe65. E, HEK293 cells were transfected for 42 h with the indicated combinations of expression vectors encoding for Myc-Notch1-IC and Myc-Fe65. The cells were then treated with the indicated amounts of MG132 for 6 h. A–E, the cell lysates were also subjected to immunoblotting (IB) analysis with the indicated antibodies.
Notch1-IC-N, Notch1-IC-ΔNΔC, and Notch1-IC-C. We conducted coimmunoprecipitation assays using the same three Notch1-IC deletion mutants and Myc-Fe65. As shown in Fig. 5D, the RAM-ankyrin repeat domains of Notch1-IC interact with Fe65. These data, therefore, suggest that the RAM-ANK domain of Notch1-IC was critically important to the interaction of Fe65 with Notch1-IC.

Fe65 Negatively Regulates Notch1 Signaling via an E3 Ligase, Itch—We evaluated the involvement of Fe65 in the membrane-tethered Notch1 proteasome-dependent degradation pathway by conducting luciferase reporter gene assays and Western blot analysis. The transcriptional activity of ΔEN1 was inhibited by Fe65 but recovered in the presence of MG132 (Fig. 6A). The ΔEN1 protein level was reduced in the presence of Fe65 but was restored significantly by treatment with MG132 (Fig. 6A). After ligand binding, it is cleaved (S2 cleavage) by tumor necrosis factor α-converting enzyme to yield a membrane-tethered intermediate fragment referred to as Notch1 extracellular truncation (NEXT). This fragment is then cleaved by presenilin-dependent γ-secretase to release the intracellular domain from the membrane. Moreover, the endogenous Notch1 NEXT protein level was lower in the wild-type cells than in the Fe65 siRNA-transfected cells (Fig. 6B, first and third lanes), and treatment with MG132 enhanced the endogenous Notch1 NEXT protein level by inhibiting proteasomal degradation (Fig. 6B, second and fourth lanes). These results showed that the stability of the Notch1 NEXT protein was down-regulated by Fe65 via the proteasome-dependent pathway. Generally, the ubiquitination of proteins results in their rapid degradation, and membrane-tethered Notch1 is degraded by the ubiquitin-proteasome system in the cytoplasm (16). Membrane-tethered Notch1 is ubiquitinylated by Itch (24). We expected that Itch might function as a media-
tor for the negative regulation of membrane-tethered Notch1 by Fe65. Therefore, we evaluated the involvement of Itch using the Itch siRNA. When Itch siRNA was cotransfected with ΔEN1 and Fe65, the transcriptional activation of ΔEN1 was increased (Fig. 6C). According to Western blot analysis, the membrane-tethered Notch1 protein level was shown to have been reduced by Fe65 and was restored markedly by the cotransfection of Itch siRNA (Fig. 6C). These results demonstrated that Itch siRNA could recover and enhance the transcriptional activity and protein level of membrane-tethered Notch1 in the presence of Fe65.

Unlike the levels observed for the control short interference plasmid-transfected cells, the cells transfected with short interference Itch harbored low levels of Itch (Fig. 6C). Accordingly, we suggest that Fe65 negatively regulates membrane-tethered Notch1 through Itch.

Fe65 Facilitates Stable Association between Membrane-tethered Notch1 and E3 Ligase Itch via the Formation of a Trimeric Complex—We attempted to determine whether ΔEN1 could be subjected to proteasome-mediated proteolysis, as reported previously. We transfected the HEK293 cells with ΔEN1-Myc and Myc-Fe65, and the quantity of remaining ΔEN1 was evaluated...
after various periods of cycloheximide treatment. We deter-
mined the protein stability of EN1 in HEK293 cells by cyclo-
heximide treatment with or without Fe65. Cycloheximide
interacts with the translocase enzyme and blocks protein syn-
thesis in eukaryotic cells. After cycloheximide treatment, the
level of EN1 protein declined gradually, with approximately
half of the protein degraded after 3 h without Fe65 (Fig. 7
A). Upon cycloheximide treatment, the reduced level of EN1 protein declined rapidly, with approximately half of the protein being degraded after 1.5 h in the presence of Fe65 (Fig. 7
B). No reduction was noted in the level of actin employed as a control (Fig. 7
A). This result demonstrates that EN1 is rapidly turned
over in the presence of Fe65. To characterize the ubiquitination
of EN1-Myc, HA-Ub and Myc-Fe65 were cotransfected in
HEK293 cells. Precipitation with anti-Notch1 antibody and
immunoblotting with EN1 demonstrated that the polyubiq-
uitinated EN1 appeared as bands. The ubiquitinated EN1 levels increased with the coexpression of Fe65 (Fig. 7
B). We also attempted to determine whether Fe65 could regulate the level of EN1 ubiquitination through Itch. Immunoblot analysis of the EN1 precipitated with anti-Notch1 antibody showed that the polyubiquitinated EN1 levels were increased with the coexpression of Itch with wild-type Fe65 (Fig. 7
C). However, the ubiquitination of EN1 was decreased substantially by coexpression of Itch and Fe65-mNES (Fig. 7
C).

Previous reports have shown that Numb binds to the intracellular domain of Notch1 and then recruits the Itch to the complex (21, 38–40). Itch polyubiquitinates Notch1-IC, leading to its degradation in a proteasome-dependent man-
er (21, 39, 41–43). We also attempted to determine whether Fe65 could regulate the physical interaction between Itch and Notch1-IC or form a trimeric complex. We
subsequently evaluated the involvement of Fe65 in the phys-
ical association between Itch and Notch1-IC via a coimmu-
noprecipitation experiment. HEK293 cells were cotrans-
fected with vectors encoding for Flag-Notch1-IC, HA-Itch,
and Myc-Fe65, and were then subjected to coimmunoprecipitation analysis. Immunoblot analysis using the anti-FLAG antibody of anti-HA antibody immunoprecipitates from the transfected cells demonstrated that Fe65 facilitates the physical association between Itch and Notch1-IC in the cells (Fig. 7D). These results revealed that the down-regulation of the membrane-tethered Notch1 protein by Fe65 occurred via an Itch-dependent pathway. At this point, we evaluated the formation of a trimeric complex between Fe65 and Notch1-IC or Itch in an effort to define more precisely the role of Fe65 in the negative regulation of Notch1 signaling. We detected binding between Notch1-IC and Fe65 but not between Fe65 and Itch, although the trimeric complex was detected in this case (Fig. 7E). Therefore, the results demonstrated that Fe65 interacts with Itch in the presence of Notch1-IC, thereby forming a trimeric complex.

**Fe65 Prevent Notch1-IC-inhibited Cell Death and Notch1-IC-mediated Signaling**—Next we evaluated the effect of Fe65 on Notch1-IC-inhibited cell death. H2O2 induced incremental cell death in HEK293 cells, and this cell death might be repressed by Notch1-IC and Fe65 siRNA, as shown by DAPI staining (Fig. 8A). These results suggest that Fe65 prevents Notch1-IC-inhibited cell death.

Furthermore, we also attempted to confirm the effects of Fe65 on Notch1-mediated Notch target gene expression (Fig. 8B). HEK293 cells were transfected with Myc-Notch1-IC and Fe65 siRNA. The cells were immunoblotted with anti-Hes1 and anti-Hes5 antibody (Fig. 8B). We found that Fe65 siRNA increased the Notch target gene expression. These results revealed that Fe65 negatively regulated the transactivation of Notch1-IC target genes via the suppression of the interaction between Notch1-IC and RBP-Jk.

**FIGURE 7.** Fe65 facilitates stable association between Notch1 and E3 ligase Itch through the formation of a trimeric complex. A, HEK293 cells were transfected for 48 h with the indicated combinations of expression vectors encoding for ΔEN1-Myc and Myc-Fe65. The cells were then treated with 100 μM of cycloheximide (CHX) for the indicated time periods. Antibody to β-actin was used as a loading control. B, HEK293 cells were transfected with expression vectors encoding for ΔEN1-Myc, HA-Ub, and Myc-Fe65 in the indicated combinations. After 42 h of transfection, the cells were treated with 10 μM of MG132 for 6 h, the cell lysates were immunoprecipitated (IP) with anti-Notch1 antibody, and the immunoprecipitates were immunoblotted (IB) with anti-HA antibody. C, HEK293 cells were transfected with expression vectors for ΔEN1-Myc, HA-Ub, Itch-Myc, Itch-DN-Myc, Myc-Fe65, and Myc-Fe65 mNES, as indicated. After 42 h of transfection, the cell lysates were treated with 10 μM of MG132 for 6 h, the cell lysates were immunoprecipitated with anti-Notch1 antibody and the immunoprecipitates were immunoblotted with anti-HA antibody. Cell lysates were also subjected to immunoblotting analysis with the indicated antibodies. D, HEK293 cells were transfected with expression vectors encoding for FLAG-Notch1-IC, HA-Itch, and Myc-Fe65 in the indicated combinations. After 48 h of transfection, the cells were lysed, the cell lysates were immunoprecipitated with anti-HA antibody, and the immunoprecipitates were immunoblotted with anti-FLAG antibody. The cell lysates were also subjected to immunoblotting analysis with the indicated antibodies. E, HEK293 cells were transfected with expression vectors encoding for FLAG-Notch1-IC, HA-Itch, and Myc-Fe65 in the indicated combinations. After 48 h of transfection, the cells were lysed, the cell lysates were immunoprecipitated with anti-Myc antibody, and the immunoprecipitates were immunoblotted with anti-FLAG or anti-HA antibody. Cell lysates were also subjected to immunoblotting analysis with the indicated antibodies.
DISCUSSION

In this study, we have demonstrated that Fe65 promotes the degradation of membrane-tethered Notch1. The nuclear localization of Fe65 inhibits Notch1 transcription activity via the dissociation of the Notch1-IC and RBP-Jk complex. Furthermore, Notch1-IC is capable of forming a trimeric complex with Fe65 and Itch. Fe65 thereby enhanced the protein degradation of membrane-tethered Notch1 via an Itch-dependent proteasomal pathway.

FIGURE 8. Fe65 prevents Notch1-IC-inhibited cell death and Notch1-Ic-mediated signaling. A, HEK293 cells were transfected for 48 h with expression vector encoding GFP, Notch1-IC, siControl, or siFe65. The transfected cells were treated with the indicated quantity of H$_2$O$_2$ for 20 min. The cells were stained with DAPI, and TUNEL and GFP-positive cells were scored for apoptotic nuclei with a fluorescence microscope. Data are mean ± S.D. of values from three independent experiments. B, HEK293 cells were transfected with expression vectors encoding for Myc-Notch1-IC and Fe65 siRNA. The cell lysates were also subjected to immunoblotting (IB) analysis with anti-Hes1 and anti-Hes5 antibody. Antibody to β-actin was used as a loading control.

FIGURE 9. Proposed model for the inhibition of Notch1 signaling pathway by Fe65. Fe65 suppresses Notch1 signaling via an acceleration of the degradation of NEXT in the cytoplasm. Notch1 is capable of forming a trimeric complex with Itch and Fe65. Fe65 enhanced the protein degradation of NEXT via the Itch-dependent proteasomal pathway. Additionally, Fe65 suppresses Notch1 transcriptional activity via the dissociation of the Notch1-IC-RBP-Jk complex in the nucleus.
We demonstrated previously that the expression of Notch1-IC down-regulates AICD-Fe65-Tip60 complex-mediated transcriptional activity, reactive oxygen species generation, and cell death through physical binding with AICD, Fe65, and Tip60 (28). The neuronal adaptor protein Fe65 consists of several protein interaction domains, including one WW and two phosphotyrosine interaction/binding domains. Fe65 interacts with APP and nuclear protein (32). Fe65 expression is also developmentally regulated, with levels declining after embryonic day 15 and increasing again progressively from postnatal day 10 to adulthood (44). Previous reports have suggested the possibility of cross-talk between the Fe65 and Notch1 signaling pathway (28, 34). Fe65 belongs to a family of WW domain proteins, among which many have been shown to be involved in transcriptional regulation (45, 46). Our results demonstrated that Notch1 transcriptional activity was attenuated by the presence of Fe65, which indicates that Fe65 may also involve the suppression of Notch1 transcriptional activity. We also found that Fe65 is involved in the regulation of Notch1 signaling via a Tip60-independent manner.

The nuclear localization of Fe65 has also been reported (47), and several studies have suggested that one potential function for Fe65 in the nucleus may involve gene transactivation (32, 47, 48). However, the precise function of Fe65 in the nucleus and the mechanisms that regulate the translocation of Fe65 into the nucleus remain unclear. We have determined that Notch1-IC interacts directly with Fe65. Intriguingly, our results showed that the inhibitory mechanism functioned via the suppression of the interaction of Notch1-IC and RBP-Jk.

Itch belongs to the Neddd4/Rsp5 family of E3 ubiquitin ligases, which have been reported to control the down-regulation of both transmembrane proteins through mono-ubiquitination, and that of cytoplasmic proteins by polyubiquitination and proteasome degradation (39). E3 ubiquitin ligases that target membrane-associated Notch1 for ubiquitination have been described recently described (39). The RING finger containing E3 ligase Cbl regulates the ubiquitination of membrane-bound Notch1 and has also been reported to result in the lysosomal degradation of Notch1 (49). The HECT type E3 ligase Itch also targets a membrane-tethered form of Notch1 for ubiquitination (23). In this study, we determined that Fe65 stimulated the proteasomal degradation of membrane-tethered Notch1 via interaction with Itch. We have demonstrated that membrane-tethered Notch1 interacts with Itch, and that together Fe65 and Itch cooperate to increase the ubiquitination of Notch1. The Fe65 NES mutant does not enhance membrane-tethered Notch1 ubiquitination. Our results suggest that Fe65 may function as an adaptor for the recruitment of the E3 ligase Itch and components of the ubiquitination machinery to the membrane-tethered Notch1 receptor, thus promoting Notch1 ubiquitination and protein degradation (Fig. 9).

In summary, the results of this study reveal that Fe65 functions as a negative regulator in Notch1 signaling via the promotion of membrane-tethered Notch1 protein degradation. Fe65 also dissociates from the Notch1-IC-RBP-Jk complex in the nucleus without affecting Notch1-IC protein turnover. Henceforth, the findings of this study may begin to shed some light onto what may be a signal cross-talk mechanism of Notch1 signaling and the Fe65 adaptor protein.

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