NORE1A induces a feedback termination of TNF signaling by antagonizing TNFR1 through ITCH-mediated destruction complex

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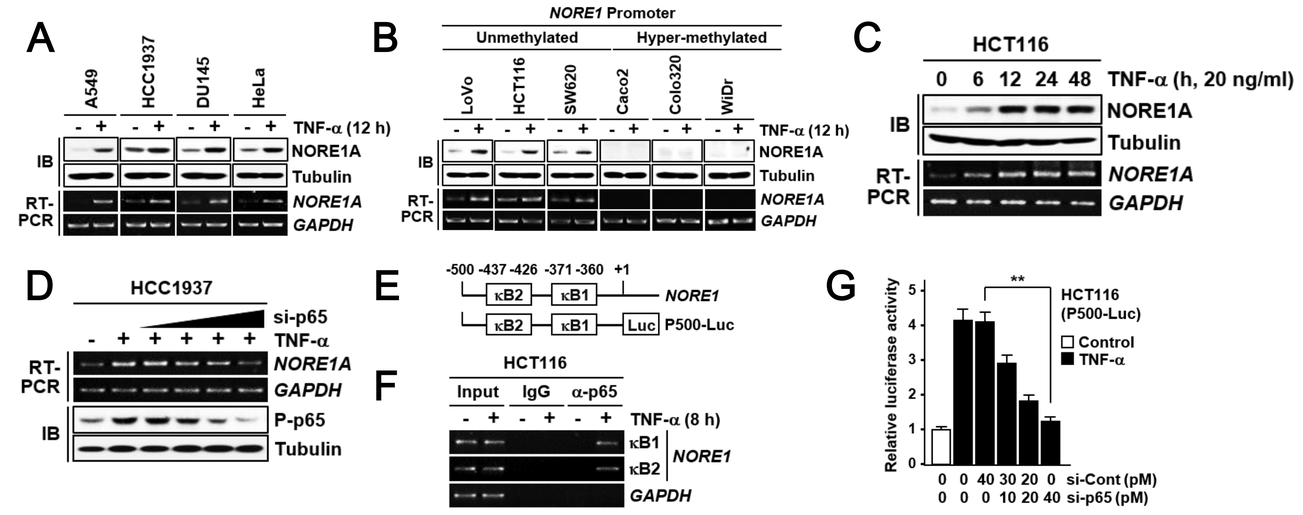

Fig 1. NORE1A is a direct transcription target of TNFα-NFκB signaling.

(A & B) TNFα increases the expression of NORE1A in human cancer cell lines, and this induction occurs only in promoter unmethylated cells.
(C) TNFα treatment induces NORE1A expression in a dose-dependent manner.
(D) Transcriptional activation of NORE1A is inhibited by knock-down of NF-κB subunit p65/RelA.
(E & F) Promoter region of NORE1A has two κB sites within 500 bp length, which are shown to directly bind to p65 after TNFα treatment.
(G) Promoter luciferase assay was performed to prove that NORE1A is a target gene of NF-κB subunit p65/RelA.

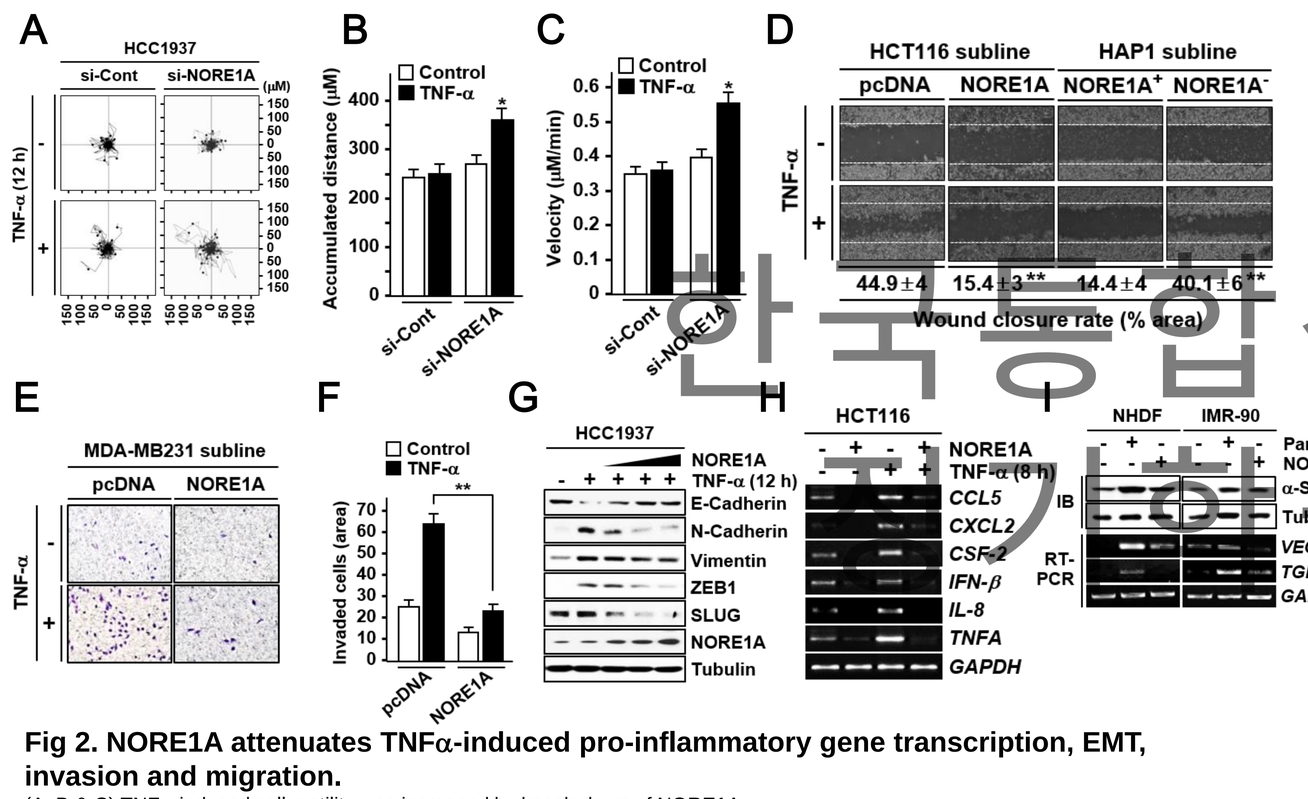

Fig 2. NORE1A attenuates TNFα-induced pro-inflammatory gene transcription, EMT, invasion and migration.

(A, B & C) TNFα-induced cell motility was increased by knock-down of NORE1A
(D, E & F) Wound healing assay and transwell assay showed that cancer cell migration and invasion were suppressed by NORE1A after TNFα treatment.
(G) TNFα-induced EMT was examined by decreasing epithelial marker (E-Cadherin) and increasing mesenchymal marker(N-Cadherin). These transition was inhibited by NORE1A in a dose dependent manner.
(H) Chemokines and cytokines produced by TNFα stimulation were suppressed by NORE1A over-expression.
(I) CAFs activation was evaluated by growing NHDF and IMR-90 in the conditioned media from HCT116 parental or NORE1A-stable cell lines. Induction of α-SMA, the CAFs activation marker, was suppressed in the cells cultured with NORE1A-stable cell conditioned medium.

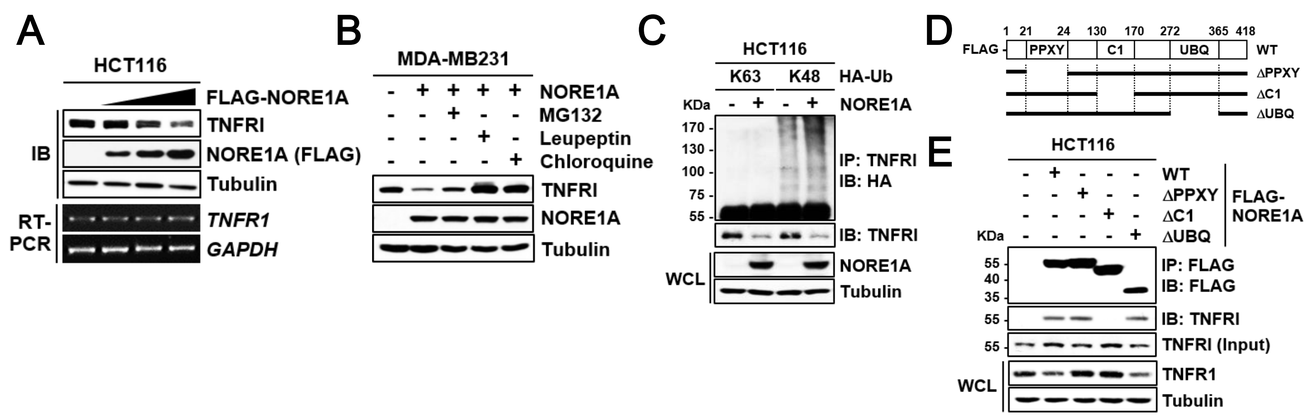

Fig 3. NORE1A destabilizes TNFRI through E3 ligase activity of ITCH

(A) NORE1A suppressed TNFR Ⅰ expression in a dose dependent manner.
(B) TNFR Ⅰ degradation by NORE1A was recovered by Leupeptin and chloroquine which are lysosomal degradation inhibitors.
(C) The K48 linked poly-ubiquitin chains of TNFR Ⅰ were increased by NORE1A
(D & E) The interaction between NORE1A and TNFR Ⅰ was evaluated through deleting several domains of NORE1A. Among the 3 domains, zinc finger containing C1 domain was proved to be essential for the interaction.
(F) NORE1A facilitated TNFR Ⅰ degradation was rescued by knock-down of ITCH, which is a HECT-type E3 ligase.
(G) Ubiquitination-mediated degradation of TNFR Ⅰ by NORE1A is dependent on E3 ligase ITCH.
(H) NORE1A interacted with ITCH, and PPXY motif was shown to be important in this interaction.
(I) NORE1A recruits ITCH to TNFR Ⅰ, while PPXY motif deletion mutant did not.

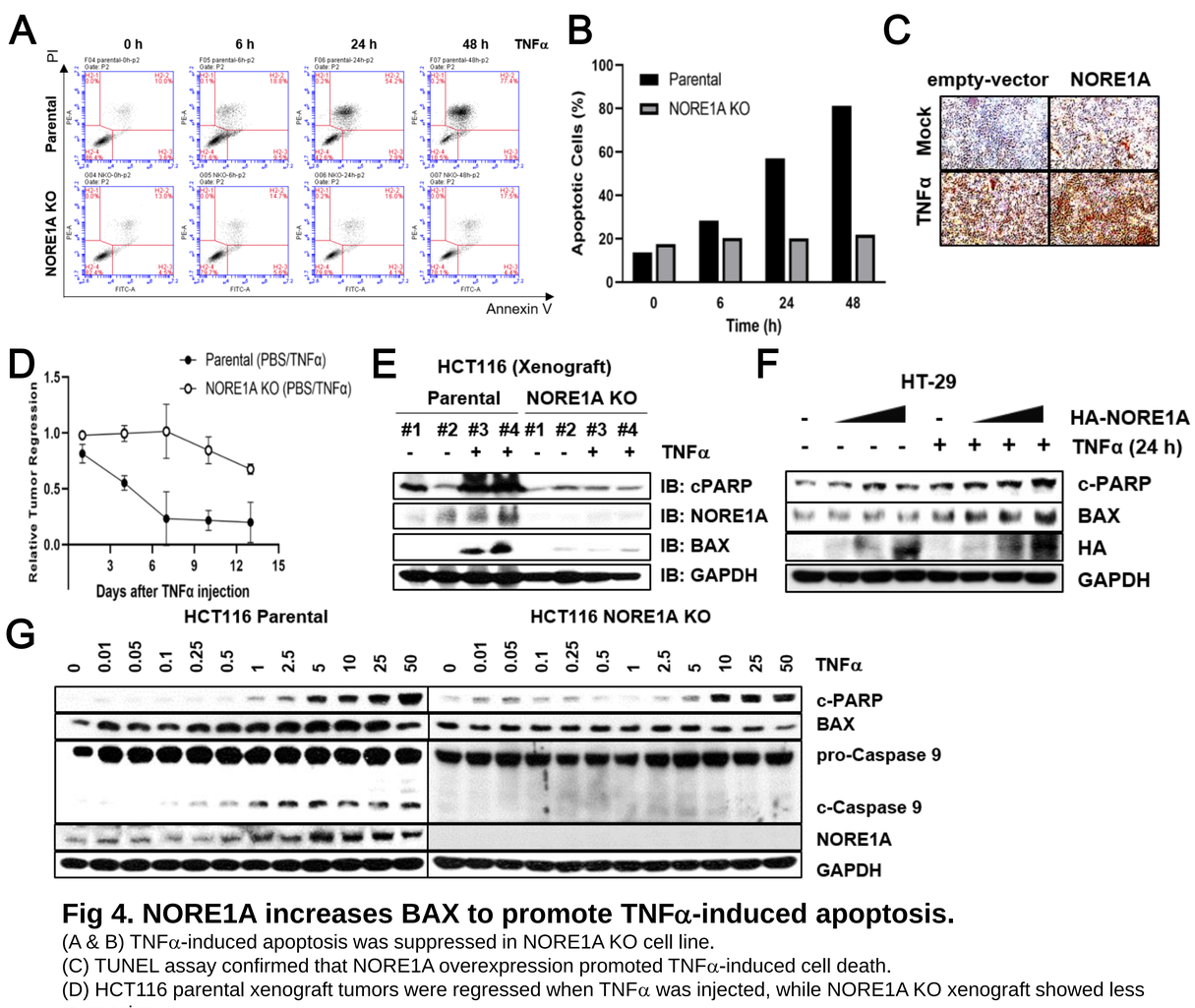

Fig 4. NORE1A increases BAX to promote TNFα-induced apoptosis.

(A & B) TNFα-induced apoptosis was suppressed in NORE1A KO cell line.
(C) TUNEL assay confirmed that NORE1A overexpression promoted TNFα-induced cell death.
(D) HCT116 parental xenograft tumors were regressed when TNFα was injected, while NORE1A KO xenograft showed less regression.
(E) Parental xenografts displayed a high sensitivity to TNFα-induced apoptosis, whereas NORE1A KO xenografts did not.
(F) TNFα-induced apoptosis was increased by NORE1A dose-dependently, which showed tight correlation with BAX expression simultaneously.
(G) NORE1A KO impairs induction of BAX and activation of Caspase 9 after TNFα stimulation, which leads to suppressed apoptosis.

Fig 5. NORE1A protects BAX from ubiquitination by disrupting ITCH-BAX degradation complex.

(A) NORE1A-enhanced TNFα-induced apoptosis was suppressed in HCT116 BAX-/- cell line.
(B) NORE1A abrogated ITCH mediated BAX repression.
(C) BAX expression was increased by intact NORE1A, but not by PPXY motif deleted NORE1A.
(D) ITCH-BAX interaction was increased after TNFα stimulation in NORE1A KO cell line.
(E) NORE1A can block the E3-ligase activity of ITCH, thereby rescuing BAX from degradation.
(F) K48-linked ubiquitination on BAX was obviously increased after TNFα stimulation in NORE1A KO cell line.

Purpose: NORE1A/RASSF5 is a tumor suppressor that is commonly inactivated in human cancers. Although NORE1A was reported to be activated by NF-κB, its role in NF-κB signaling and the underlying mechanism have not been addressed. In this study, we explored the role for NORE1A in the regulation of the TNF-NF-κB pathway.

Methods: NORE1A effect on TNF regulation of inflammation, and apoptosis was examined by flow cytometry, migration and invasion assays. NORE1A regulation of TNF-NF-κB signaling was analyzed by RT-PCR, immunoblot, and immunoprecipitation.

Results: Upon exposure to TNF-α, NORE1A is activated as a transcriptional target of NF-κB while its induction blocks TNF- NF-κB activation, indicating that NORE1A is a feedback terminator of TNF-NF-κB signaling. As predicted, NORE1A suppresses TNF activation of pro-inflammatory gene transcription, EMT, invasion and migration. Mechanistically, NORE1A binds to TNF receptor I (TNFR Ⅰ) and E3 ligase ITCH to facilitate ITCH-mediated K48-linked ubiquitination of TNFR Ⅰ and subsequent lysosomal degradation. Additionally, we found that NORE1A-ITCH interaction protects BAX from ITCH-mediated ubiquitination and thus activates its apoptotic function. Using a series of deletion mutants, we identified that the PPXY motif of NORE1A interacts with ITCH and plays a crucial role for both TNFR Ⅰ degradation and BAX stabilization and activation.

Conclusions: In this study, we demonstrate first that NORE1A is a feedback terminator of TNF-NF-κB signaling, which antagonizes TNFR Ⅰ by facilitating the ITCH-TNFR Ⅰ interaction. Our study also shows that NORE1A binding to ITCH dismantles ITCH from BAX and activates BAX-mediated apoptosis. These data illuminate the mechanistic consequence of NORE1A inactivation in tumorigenesis.