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NACK is an integral component of the Notch transcriptional activation complex and is critical for development and tumorigenesis

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Abstract

The Notch signaling pathway governs many distinct cellular processes by regulating transcriptional programs. The transcriptional response initiated by Notch is highly cell context dependent, indicating that multiple factors influence Notch target gene selection and activity. However, the mechanism by which Notch drives target gene transcription is not well understood. Herein, we identify and characterize a novel Notch-interacting protein, NACK, which acts as a Notch transcriptional co-activator. We show that NACK associates with the Notch transcriptional activation complex on DNA, mediates Notch transcriptional activity, and is required for Notch-mediated tumorigenesis. We demonstrate that Notch1 and NACK are co-expressed during mouse

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development and that homozygous loss of NACK is embryonic lethal. Finally, we show that NACK is also a Notch target gene, establishing a feed forward loop. Thus, our data indicate that NACK is a key component of the Notch transcriptional complex and is an essential regulator of Notch-mediated tumorigenesis and development.

Keywords

Notch; tumorigenesis; atypical kinase; esophageal adenocarcinoma; pancreatic ductal adenocarcinoma

Introduction

The Notch signaling pathway acts as a critical regulator during development by governing cell fate determination through direct regulation of transcriptional programs that drive cellular processes including proliferation, differentiation, self-renewal, and apoptosis. These same cellular processes are also driven by aberrant activation of Notch in tumorigenesis. Notch was first implicated in human cancer in T cell acute lymphoblastic leukemia (T-ALL), where activating mutations result in ligand-independent proteolytic cleavage of Notch1 and increased stability of the intracellular domain (N^{ICD}) (1). Evidence for genetic alterations in Notch genes can be found in solid tumors; however, the major mechanism for aberrant Notch activity in many non-hematological human malignancies appears to be elevated expression of Notch pathway components and/or loss of negative regulators (2–4).

This mechanism is relevant in solid tumors because Notch signaling is mediated through direct cell-to-cell contact between a Notch-expressing cell and a cell expressing a DSL (Delta, Serrate, Lag-2) ligand. This binding initiates a series of proteolytic cleavages ultimately leading to release of N^{ICD} from the plasma membrane and its translocation to the nucleus (5–7). What follows is a step-wise recruitment of Notch and co-activators of the Mastermind-like (Maml) family to the DNA (8–10). This complex binds to CSL (CBF1-Su(H)-LAG1), displacing the co-repressor complex and initiating transcription of target genes (11–13). Although a prevailing scheme for Notch signaling has been accepted, little is known regarding the regulation of transcription initiation by the Notch/Maml/CSL complex. In an effort to identify novel components that are involved in Notch-mediated transcription, we carried out a biochemical screen in Notch-dependent lymphomas and defined a new interacting partner of Notch. We identified and characterized NACK (Notch Activation Complex Kinase) as a new protein interacting with $N1^{ICD}$ and implicated in Notch transcriptional regulation.

Materials and Methods

Subcellular Fractionation

Subcellular fractionation was performed as described previously (14).

Cell Culture and Transfection

All cell culture reagents were purchased from Invitrogen, unless otherwise indicated. Mouse embryonic fibroblasts (MEFs) were prepared from wild-type C57Bl/6 embryos at day E13.5 following a standard MEF isolation protocol (15). Transfections were performed using Lipofectamine 2000.

Western Blotting

Cell lysates were resolved by SDS-PAGE, transferred onto Immobilon-P membranes (Millipore), blocked in 5% milk, and incubated with the appropriate primary antibody overnight. Primary antibodies were α-Flag (1:5000; Sigma), monoclonal α-NACK (1:1000; directed against aa209–287 and aa1051–1152; affinity purified using a NACK-GST fusion protein), polyclonal α-NACK (1:1000; directed against aa1051–1152; affinity purified using a NACK-GST fusion protein), α-CSL (1:1000; polyclonal), α-p27 (1:1000; Santa Cruz Biotechnology, Dallas, TX), α-β-actin (1:5000; Abcam), α-HeyL (1:1000; Abcam), and α-tubulin (1:15000; Sigma). After incubation with HRP-conjugated secondary antibodies, proteins were detected using an enhanced chemiluminescence reaction (Amersham Biosciences, Pittsburgh, PA) according to the manufacturer's specification.

DNA Pull-down

Streptavidin agarose beads (Pierce, Rockford, IL) were incubated with previously annealed 47mer biotinylated dsDNA containing two high affinity CSL binding sites facing forward (2× CSL binding DNA) or 2 mutated CSL binding sites (Mut. DNA; for oligo sequences see Table S1). For the *in vitro* DNA pull-down, recombinant Flag-tagged proteins were incubated with DNA streptavidin beads and bound proteins were analyzed by western blot. For DNA pull-down experiments from cells, 293T cells were transfected with N1^{ICD}, Maml1, and NACK and lysates were incubated with DNA streptavidin beads. Protein bound to the beads was analyzed by western blot.

Luciferase Reporter Assay

H1299 cells were transfected with 8× CSL luciferase reporter vector (10), SV40 β-galactosidase (internal transfection control; Clontech, Mountain View, CA), and N1^{ICD}, Maml1, and NACK expression plasmids. Luciferase activity in the lysates was analyzed using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Control siRNA, siRNA against human Maml1, and siRNA against human NACK were purchased from Dharmacon (Lafayette, CO).

N1^{ICD} Lymphoma

N1^{ICD} T cell lymphomas were generated as described previously (16).

Viral Infections

NACK shRNA and control (scrambled) shRNA were purchased in the pLKO vector from Open Biosystems (Thermo Scientific, Pittsburgh, PA). Lentivirus was packaged using psPAX2 packaging vector and pMD2.G envelope plasmid. Retrovirus was packaged using SV40 psi$^-$ packaging vector. Virus was collected 48 h post-transfection. Cells were infected

overnight with virus-containing medium in the presence of 8 μg/mL hexadimethrine bromide (Polybrene, Sigma), and infected cells were selected with 2.5 μg/mL puromycin.

RT-PCR

RNA was isolated from cells using Trizol reagent (Invitrogen) following the manufacturer's instructions. RNA was isolated from tumors using the RNeasy Mini Kit (Qiagen, Germantown, MD). cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following the manufacturer's directions. qPCR reactions were carried out in the Bio-Rad CFX96 thermal cycler using Sybr Green Master Mix (Bio-Rad, Hercules, CA). Gene expression in human and mouse was normalized to *GAPDH* and *Hprt*, respectively. Primer sequences are available upon request.

Chromatin Immunoprecipitation

OE33 cells were cross-linked with 1% formaldehyde and cross-linking was quenched by adding glycine to a final concentration of 0.125 M. Cells were resuspended in SDS lysis buffer and sonicated to yield chromatin fragments of approximately 300 to 800 bp. Lysates were immunoprecipitated with α-Notch 927 (polyclonal), α-Notch (ab27526, Abcam), or α-Pragmin (Bethyl Laboratories, Montgomery, TX) antibodies and were reverse cross-linked at 65°C in 200 mM NaCl for 4 h followed by incubation with RNase A and proteinase K. DNA was cleaned using PCR purification kit (Qiagen) and *Hes1* and *GAPDH* were amplified by qPCR. Primer sequences are available upon request.

β-galactosidase Staining of Embryos

Whole embryos were extracted and washed in PBS at room temperature. Embryos were then fixed at 4°C in cold fixative for 60 min, then washed and stained for 24–36 h.

In situ Hybridization

In situ hybridization of Notch1 and NACK was performed as previously described (17,18). The Notch1 probe was designed in the ANK repeat domain and the NACK probe was designed in the kinase domain.

Immunohistochemistry

IHC was performed on 5 μm paraffin sections prepared from paraffin-embedded tissue arrays. Tissue sections were rehydrated, pre-treated with antigen unmasking solution (1:100 dilution; Vector Laboratories, Burlingame, CA), and then treated with 3% H_2O_2 and blocked with protein block serum-free (Dako, Carpinteria, CA). Sections were incubated with polyclonal antibodies against NACK (α-Pragmin, 1:50) or cleaved Notch1 (1:200 dilution; Abcam, ab-8925), then with biotinylated secondary antibodies (Vector Laboratories). Immunoreactivity was detected using the ABC Elite kit (Vector Laboratories) with AEC as the final chromogen and hematoxylin as the nuclear counterstain.

Soft Agar Experiments

HC11 cells were infected with shRNA against NACK and N1[ICD] and cells were plated in soft agar (base agar 0.5%, top agar 0.35%). Plates were incubated at 37°C until colonies were visible by eye and then colonies were stained with 1% MTT (Sigma).

Colony Formation

EAC cells were seeded in 6-well plates at a density of 10,000 cells/well and allowed to attach overnight. Cells were then infected with lentiviruses expressing control shRNA or shRNA against NACK. Seven days post-infection, colony formation was quantitated by staining cells with Crystal Violet (Millipore) and counting the number of colonies.

Xenografts

OE19 cells were infected with lentivirus expressing shRNA against NACK, and then were mixed 1:1 with Matrigel (BD Biosciences; 5 mg/mL) and injected into the flanks of nude mice (*nu/nu*, Jackson Laboratory, Bar Harbor, ME). Xenografts were measured weekly.

Knockout Mouse

Embryonic stem cells harboring a knock-out first (KOF) allele with a promoter-driven cassette inserted between exons 2–3 of the *D8Ertd82e* gene were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (19). Chimeras were produced in C57Bl/6 mice by the Transgene Facility of Sylvester Comprehensive Cancer Center at the University of Miami (Miami, FL). Germline transmission was verified by PCR using primers specific to the common loxP site and the neomycin selection marker (Neo). *NACK*[KOF] denotes mice harboring the targeted *D8Ertd82e*[tm1a(KOMP)Mbp] allele. To remove the KOF cassette, *NACK*[KOF/+] mice were bred to heterozygous Tg(ACTFLPe)9205Dym mice (Jackson Laboratory), which have ubiquitous expression of FLPe recombinase and the resulting pups were heterozygous for the floxed allele (*NACK*[flox/+]). Primer sequences are available upon request. All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Miami.

Results

NACK is a novel protein with kinase fold that interacts with Notch1[ICD]

In order to gain a better understanding of the molecular machinery involved in Notch-mediated transcription, Notch complexes were affinity purified from Notch-induced T cell lymphomas and analyzed by liquid chromatography-mass spectrometry (LC-MS/MS) to identify novel binding partners of N1[ICD] (Figure S1A) (20). LC-MS/MS analysis revealed that all 3 Maml family proteins as well as CSL were co-purified with Notch, thereby authenticating our purification protocol (Figure S1B). Other proteins that were co-purified with Notch included an uncharacterized protein annotated as hypothetical protein D8Ertd82e. Since this protein was co-purified with N1[ICD] and core components of the transcriptional activation complex, and sequence analysis predicted the presence of a kinase domain at the C-terminus, we refer to it as Notch Activation Complex Kinase (NACK).

In order to validate the interaction between NACK and N1^{ICD}, co-immunoprecipitation studies were performed from 293T cells co-expressing Myc-tagged NACK and Flag-tagged N1^{ICD} (Figure S1C, lane 10). These studies recapitulated the purification results and therefore demonstrate that NACK is a novel interacting partner of N1^{ICD}.

NACK is part of the Notch transcriptional activation complex

To determine the specificity of the relationship between Notch and NACK, we used a monoclonal NACK antibody to pull down NACK from lysates derived from the Notch-driven T cell lymphomas 4084 and NA1 (Ikaros-null), and the Myc-driven T cell lymphoma 6780 (16,21,22). Expression of NACK was robust in 4084 and NA1 cells compared to 6780 cells, demonstrating co-expression of Notch and NACK (Figure 1A). Subcellular fractionation of 4084 cells revealed that NACK and Notch co-localize to the nucleus (Figure 1B). To determine whether NACK interacts with the ternary Notch complex on DNA, we performed DNA pull-down experiments by incubating beads coupled to DNA containing 2 CSL sites (2× CSL) with nuclear lysate from 4084 lymphoma cells and wt mouse thymocytes. Beads coupled to DNA containing 2 mutated CSL sites (2× mut) were used as a control for specificity. We found that NACK is pulled down concomitantly with N1^{ICD} and Maml1 in a CSL-dependent manner (Figure 1C). We achieved similar results when we applied the same technique to lysates from 293T cells transfected with different combinations of N1^{ICD}, Maml1, and NACK (Figure 1D, lanes 9 and 16). Furthermore, the binding of NACK to the Notch complex on DNA appears to be Maml1-dependent, as Notch and CSL alone are not sufficient to recruit NACK to the DNA (Figure 1D, lanes 15 and 16).

Previously, we reported mutations in Notch1 and Maml1 that inhibit the transcriptional activity of the Notch complex on a Notch-responsive promoter. Notch1 $^{\Delta2105}$ has a deletion of amino acids 2105–2114 (which lie in the 7th Ank repeat) (23). Maml1–305 contains only the first 305 amino acids of Maml1, which include the Notch binding domain, and acts in a dominant negative manner (10). Gel filtration studies revealed that both mutated proteins can form the ternary complex with CSL on DNA; however, both the activity and the size of the complexes are dramatically reduced, suggesting that the mutated components fail to recruit a necessary co-activator (10,23). Based on our findings that NACK is a co-activator of Notch signaling, we hypothesized that these mutants of Notch and Maml might fail to recruit NACK to the ternary complex. We transfected cells with wt or mutant Notch1 and Maml1, plus NACK, and performed a DNA pull-down. The results show that mutation of either Notch1 (Notch1 $^{\Delta2105}$) or Maml1 (Maml1–305) is sufficient to eliminate binding of NACK to the transcription activation complex on DNA (Figure 1D, lanes 8–10). This result suggests that these mutated Notch and Maml proteins are unable to activate transcription due to their failure to recruit NACK to the transcriptional activation complex.

NACK is a co-activator of Notch transcription

Given that NACK is part of the Notch transcriptional activation complex on DNA, we reasoned that NACK should play a role in Notch-mediated transcriptional activity. To address this, CSL-dependent transcriptional reporter assays were performed by transfecting different combinations of N1^{ICD}, Maml1, and NACK into H1299 cells and assaying for luciferase driven by an artificial promoter containing 8 CSL binding sites (8× CSL).

Transfection of N1^{ICD} in H1299 cells increased CSL-directed transcription by about 26-fold when compared with vector alone (Figure 2A). When NACK was included, there was a marked increase in activity, similar to the increase seen when Maml1, a bona fide co-activator of Notch transcription, was transfected with N1^{ICD}. Including both NACK and Maml1 in the transfection with N1^{ICD} resulted in a robust cooperative effect on Notch activity, compared to Notch + Maml1 (Figure 2A), indicating that NACK, like Maml1, is a co-activator of Notch transcriptional activity. Titration of NACK suggests that NACK is a rate-limiting component of the transcription activation complex, because at every concentration of Maml, addition of NACK increases transcriptional activity (Figure 2B).

To assess the role of endogenous NACK on Notch-mediated transcriptional activity, we used targeted siRNA pools to reduce protein levels of hNACK in H1299 cells and examined the effects on luciferase reporter activity. Transfection of H1299 cells with hNACK siRNA resulted in a 74% knockdown of NACK mRNA expression (data not shown) and decreased the transcriptional activity of N1^{ICD} (Figure 2C). Similarly, a siRNA-mediated decrease in hMaml1 mRNA expression (84% knockdown) also reduced N1^{ICD} transcriptional activity, providing further evidence that NACK, like Maml, is a co-activator of Notch-mediated transcriptional activity.

The involvement of NACK in the Notch transcriptional complex suggests that NACK associates with N1^{ICD} on chromatin. To explore this, we carried out chromatin immunoprecipitation (ChIP) experiments using chromatin extracts derived from OE33 esophageal adenocarcinoma cells, which are dependent on Notch activity (data not shown). Results demonstrate that NACK and N1^{ICD} specifically co-localize on the promoter region of Hes1, a known Notch target gene. In contrast, there was no enrichment of either N1^{ICD} or NACK on the GAPDH promoter, which is not a Notch target. (Figure 2D, upper panel). Immunohistochemistry on OE33 cells infected with shRNA targeting NACK shows the specificity of the NACK antibody used for ChIP (Figure 2D, lower panel). Additionally, treatment of OE33 cells or 786-0 renal adenocarcinoma cells with the γ-secretase inhibitor DAPT abrogated the binding of Notch1, Maml1, and NACK to the Hes1 promoter (Figure 2E), supporting the association of NACK with the Notch transcription complex on DNA. Further studies in these cells demonstrate that knockdown of NACK expression using shRNA results in a decrease in Hes1 expression similar to that induced by treatment with DAPT (Figure 2F), indicating that NACK is required for Notch-mediated transcription. To further explore the effect of NACK on transcription of Notch target genes, we showed that ectopic expression of Notch in HC11 mammary epithelial cells induced expression of the Notch target genes Hes1, Hey1, and Hey2, and knockdown of NACK in these cells attenuated the level of expression (Figure 2G). Taken together, these experiments indicate that NACK is an integral component of the Notch transcriptional activation complex on DNA, acting as a co-activator of Notch-mediated transcription.

NACK is a transcriptional target of Notch signaling

In the course of examining NACK gene expression in T-ALL, we found higher expression in tumor samples compared to normal thymus (Figure 3A). This led us to ask whether NACK is a target of Notch signaling. To test this, we used mouse embryonic fibroblasts

(MEFs) as a cell culture system devoid of measurable Notch activity. MEFs were infected with retrovirus expressing N1 [2444] or GFP as an infection control. Results show that NACK mRNA expression is dramatically increased when N1^{ICD} is introduced into the system, as seen by RT-PCR (Figure 3B). As expected, mRNA expression of HeyL, a canonical target of Notch signaling, is also increased. In contrast, mRNA expression of p27, a gene down regulated by Notch signaling, is decreased. These data suggest that Notch1 can induce NACK expression.

In order to determine whether other Notch family members drive NACK transcription, HC11 mouse mammary epithelial cells were infected with retroviruses expressing N1^{ICD}, N2^{ICD}, N3^{ICD}, or N4^{ICD}. Results show that all Notch family members increase NACK mRNA levels when compared to control (Mig) infection, as shown by qPCR analysis (Figure 3C).

To demonstrate that NACK is a direct target of Notch, we analyzed the DNA sequence of the NACK promoter and found two predicted CSL binding sites at −1981 bp and −1790 bp (Figure 3D), suggesting that Notch regulates NACK transcription by binding to its promoter. ChIP assay of the GAPDH and NACK promoters showed specific binding of Notch only to the NACK promoter (Figure 3E). Taken together, these results show NACK as a transcriptional target of Notch signaling.

NACK is co-expressed with Notch during development and in tumors

Since Notch is important during many stages of early development, we asked if NACK is also expressed during development, and whether the expression co-localizes with Notch. We generated a knock-in mouse by inserting the β-galactosidase gene under control of the NACK promoter and inhibitor methionine without disrupting the expression of the wt NACK protein. Staining of an E16.5 embryo showed widespread β-galactosidase expression throughout the embryo (Figure 4A). I*n situ* hybridization demonstrated co-expression of NACK and Notch1 in the central nervous system of E12.5 and E16.5 wt mouse embryos (Figure 4B). These results indicate that NACK is expressed in the developing embryo and shares a high degree of expression overlap with Notch1.

Since many solid tumors display aberrant expression of Notch receptors and ligands, we hypothesized that NACK, as a specific co-activator, would be expressed at higher levels in cancer tissues compared to normal tissues. Using clinical samples derived from surgically resected pancreatic ductal adenocarcinoma and esophageal adenocarcinoma, we analyzed expression of N1^{ICD} and NACK in tumor versus normal tissues by immunohistochemistry (IHC) and qPCR (Figure 4C and D). Higher levels of N1^{ICD} and NACK protein expression where observed by IHC in pancreatic ductal adenocarcinoma tumor samples when compared to normal pancreas (Figure 4C, representative sample). Validation of the NACK antibody used for IHC is shown in Figure 2E. Expression of NACK, Notch1, and the Notch target gene Hey1 were all elevated in esophageal adenocarcinoma tissues compared to normal tissue (Figure 4D). These data demonstrate that expression of Notch and NACK are linked and that they are co-expressed in development and in tumors.

NACK is required for Notch1-mediated transformation and tumorigenesis

HC11 mammary epithelial cells have little to no endogenous Notch activity but can be transformed by the addition of exogenous N1[ICD], as evidenced by the acquisition of anchorage-independent growth in soft agar. To determine if Notch-mediated transcription is dependent on NACK, we examined the effect of NACK on Notch-mediated transformation by knocking down endogenous expression of NACK in HC11 cells and analyzing the outcome by soft agar and qPCR assays. HC11 cells were infected with lentivirus expressing control shRNA or shRNA against NACK. After selection, cells were infected with N1[ICD] or empty control vector. While NACK knockdown had no effect on the growth of normal mouse mammary epithelial HC11 cells (Figure 5A, top panel), the number of soft agar colonies formed by HC11 cells infected with N1[ICD] was greatly reduced in the shRNA-infected samples (Figure 5A, bottom panel). Knockdown of NACK was confirmed by qPCR (Figure 5B). Moreover, the mRNA levels of the Notch target genes *Hey1* and *Cyclin D1* (24) were also decreased in the presence of shNACK, further supporting the role of NACK as a co-activator of Notch-mediated transcriptional activity (Figure 5B). These results demonstrate the requirement for NACK in Notch-mediated transformation.

We next examined the effect of NACK on Notch-mediated tumorigenesis. Knockdown of NACK in esophageal adenocarcinoma cells (OE19 and OE33), which was verified by western blot, resulted in dramatic inhibition of the clonogenic potential of these cells (Figure 5C). OE19 and OE33 cells are dependent on Notch activity for survival and have detectable levels of activated N1[ICD] protein and NACK (data not shown). To address the effect of NACK on tumor growth, OE19 cells infected with control shRNA or shNACK were injected subcutaneously into the flanks of nude mice and tumor size was measured every 4 days for 24 days. Knockdown of NACK, again verified by western blot, resulted in decreased tumor growth (Figure 5D), indicating a major role for NACK in Notch-mediated tumorigenesis.

NACK knockout is embryonic lethal

Embryonic stem (ES) cells harboring a targeted allele of *D8Ertd82e* were obtained from the International Knockout Mouse Consortium (KOMP). The targeted *D8Ertd82e*[tm1a(KOMP)Mbp] allele (denoted in this manuscript as *NACK*[KOF]) carried a knockout first (KOF), promoter-driven cassette inserted between the second and third exons of the gene (Figure 6A, top panel). The presence of the splice acceptor site in the cassette is predicted to generate a non-functional truncated transcript. We determined that knockout of *NACK* is embryonic lethal based on the absence of homozygous *NACK*[KOF/KOF] pups among 114 live births from *NACK*[KOF/+] breedings (Figure 6A, middle panel). In fact, we were unable to recover *NACK*[KOF/KOF] embryos even as early as E9.5. Representative genotyping results are shown in Figure 6A, bottom panel.

Crossing *NACK*[KOF/+] mice with transgenic mice expressing FLPe (the enhanced version of the site-specific recombinase FLP) (25) resulted in recombination between the FRT sites, generating a floxed allele (Figure 6B, top panel). This recombination reverts the allele to the wild-type (but floxed) configuration. Genotyping results showed that the segregation of the floxed allele follows Mendelian inheritance, indicating that FLPe recombination rescues the

embryonic lethality of the KOF allele (Figure 6B, middle panel). We confirmed the recombination by demonstrating the loss of Neo without loss of the common LoxP site (Figure 6B, bottom panel). Characterization of this NACK knockout first mouse model clearly demonstrates that *NACK* is an essential gene in development.

In conclusion, we have demonstrated that NACK is a novel integral component of the Notch transcriptional activation complex, acts as a co-activator of Notch signaling, and participates in a feed-forward loop whereby NACK increases the transcriptional activity of Notch, leading to increased transcription of NACK and other target genes (Figure 7A). Taken together, these data suggest that NACK is required for Notch-mediated transcription and that NACK knockout is embryonic lethal, highlighting the critical importance of this protein in both cancer and development.

Discussion

The importance of Notch signaling in hematologic malignancies and solid tumors has made Notch an attractive target for therapeutic intervention in cancer (2,4,26). Currently, therapeutic approaches have been limited to inhibition of Notch^{ICD} production either by blocking γ-secretase inhibitors or by antibody antagonists. A key feature of Notch signaling is rapid recruitment of pathway-specific co-activators to initiate a burst of target gene transcription and subsequent degradation and turnover of N1^{ICD} transcription complexes. To better understand the mechanisms of Notch signaling during tumorigenesis and to identify novel druggable components of Notch transcription complexes, we performed a protein screen from Notch-induced lymphoma. Herein we report the identification of a novel binding partner of the Notch transcriptional activation complex, which we termed NACK. This protein has been previously identified as Pragmin and has been shown to interact with Rho family GTPases (27) and C-terminal Src kinase (28). Using both *in vivo* and *in vitro* approaches we demonstrated that NACK acts as a regulator of Notch transcription, is necessary for Notch-driven tumorigenesis, and is required for embryonic development. Taken together, this study demonstrates that NACK is critical for Notch function.

We propose that NACK is recruited to the Notch complex by interactions with Notch and Maml on chromatin. Two pieces of data suggest that the requirement for NACK in Notch transcriptional activation serves a role in the timing of Notch signaling: first, that NACK is rate limiting in transcription and second, that NACK is a target gene of Notch and thus establishes a feed-forward relationship.

Analysis of NACK expression in various cells and tissues reveals that its steady state level is low, but is exquisitely sensitive to activation of Notch. That is, in cells that lack activation of Notch we find low levels of NACK, but when Notch is activated, levels of NACK are rapidly induced. Therefore, we argue that NACK functions as a "reostat" for Notch transcriptional activity. Therefore, recruitment of NACK to create functional Notch transcriptional activation complexes is a mechanism to temporally regulate Notch signaling. Although we propose that NACK is required for basal Notch transcriptional activity, the possibility exists that NACK instead plays a role in modulating the strength and duration of the transcriptional activity of Notch complexes.

NACK is in a class of kinases annotated in the kinome as atypical kinases (29). Proteins in this class are predicted to have kinase folds, but they harbor substitutions in amino acid residues that are thought to be critical for catalysis. While it was originally thought that these substitutions rendered atypical kinases enzymatically inactive, there are several recent reports assigning kinase activity to previously identified "inactive" atypical kinases (30–32). NACK has several major protein kinase features, including the VAIK and HRD motifs; however, it lacks the conserved DFG motif that functions in metal binding. Substitution in the highly conserved Mg^{2+}-binding DFG motif is also noted in the atypical kinase CASK, which has demonstrable kinase activity (33). These findings suggest that NACK might have kinase activity as well; however, no targets have yet been identified. Identification of proteins phosphorylated by NACK will provide further insight into the functional role of this protein and may highlight potential targets for therapeutic intervention.

Underscoring a role for NACK in Notch signaling, we observed a striking overlap in the expression of Notch1 and NACK during mouse development and in human tumor samples (Figure 4). Notch signaling acts as a central axis for cell proliferation and lineage specification during embryonic development, and also plays a pivotal role during neurogenesis (34). This supports our finding that NACK is co-expressed with Notch1 in the central nervous system of E12.5 and E16.5 wt mouse embryos (Figure 4B). Further evidence for the role of NACK during development comes from our NACK knockout-first mouse, which shows that loss of NACK is embryonic lethal (Figure 6). We were unable to detect $NACK^{KOF/KOF}$ embryos even as early as E9.5, suggesting that embryos with this genotype either do not implant in the uterine wall or are reabsorbed very early in gestation.

Knockout of either Notch1 or Notch2 is lethal by E11.5 (35–37), while knockout of Notch3 or Notch4 has no effect on development (38–40). Interestingly, CSL knockout results in death before E10.5 (41), likely due to the combined effect on all 4 Notch receptors. The severity of NACK knockout is similar to that of CSL knockout, and both are more severe than loss of a single Notch receptor, suggesting that loss of NACK might also impact signaling through all 4 Notch receptors. This is supported by our finding that each of the Notch receptors is able to induce NACK expression (Figure 3C). Alternatively, NACK could play a role in other signaling pathways that are important during development. This has been shown for Maml1, which is also a co-activator of the muscle-specific transcription factor MEF2C (42) as well as β-catenin signaling (43). Since it appears that NACK is recruited to the Notch ternary complex by Maml, it is plausible and perhaps likely that NACK is also participating in other developmental pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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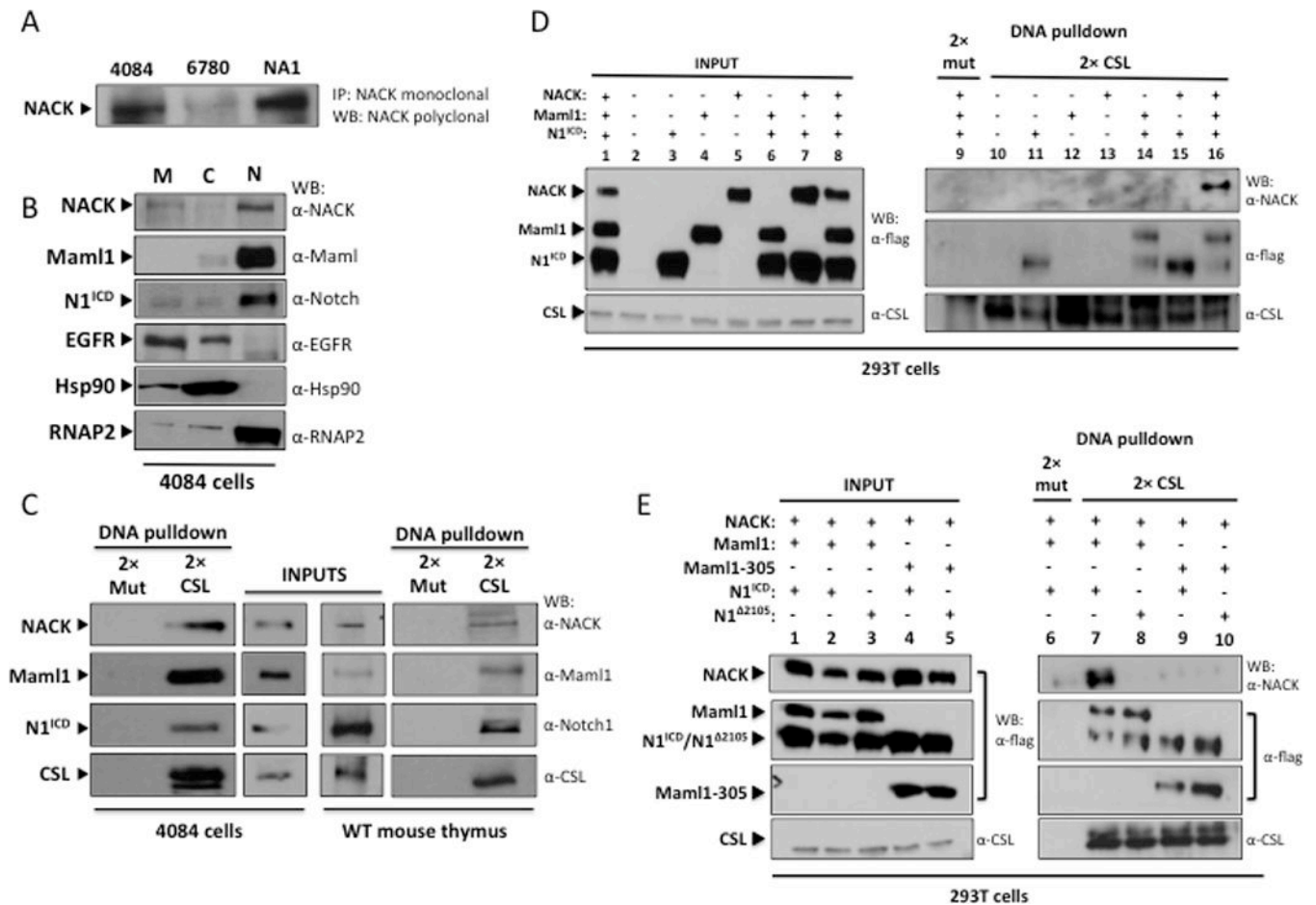
Figure 1. NACK forms a complex with N1^ICD and Maml1
A. IP/western analysis of lysates from T-ALL cell lines. 4084 – Notch-induced lymphoma; 6780 – Myc-induced lymphoma; NA-1 – Ikaros-null lymphoma. **B.** Western analysis of subcellular fractions of 4084 cells. M – membrane; C – cytoplasm; N – nuclear. **C.** DNA pull-down and western analysis of nuclear lysates from 4084 cells and wt mouse thymocytes using beads conjugated to 2× CSL binding DNA or 2× mutant CSL binding DNA (2× mut). Input lanes represent 5% of total nuclear lysate. **D.** DNA pull-down and western analysis of lysates from 293T cells transfected with different combinations of N1^ICD, Maml1, and NACK. Input lanes represent 10% of total lysate. **E.** DNA pull-down and western analysis of lysates from 293T cells transfected with different combinations of N1^ICD, N1^2105, Maml1, Maml1–305, and NACK. Input lanes represent 10% of total lysate.
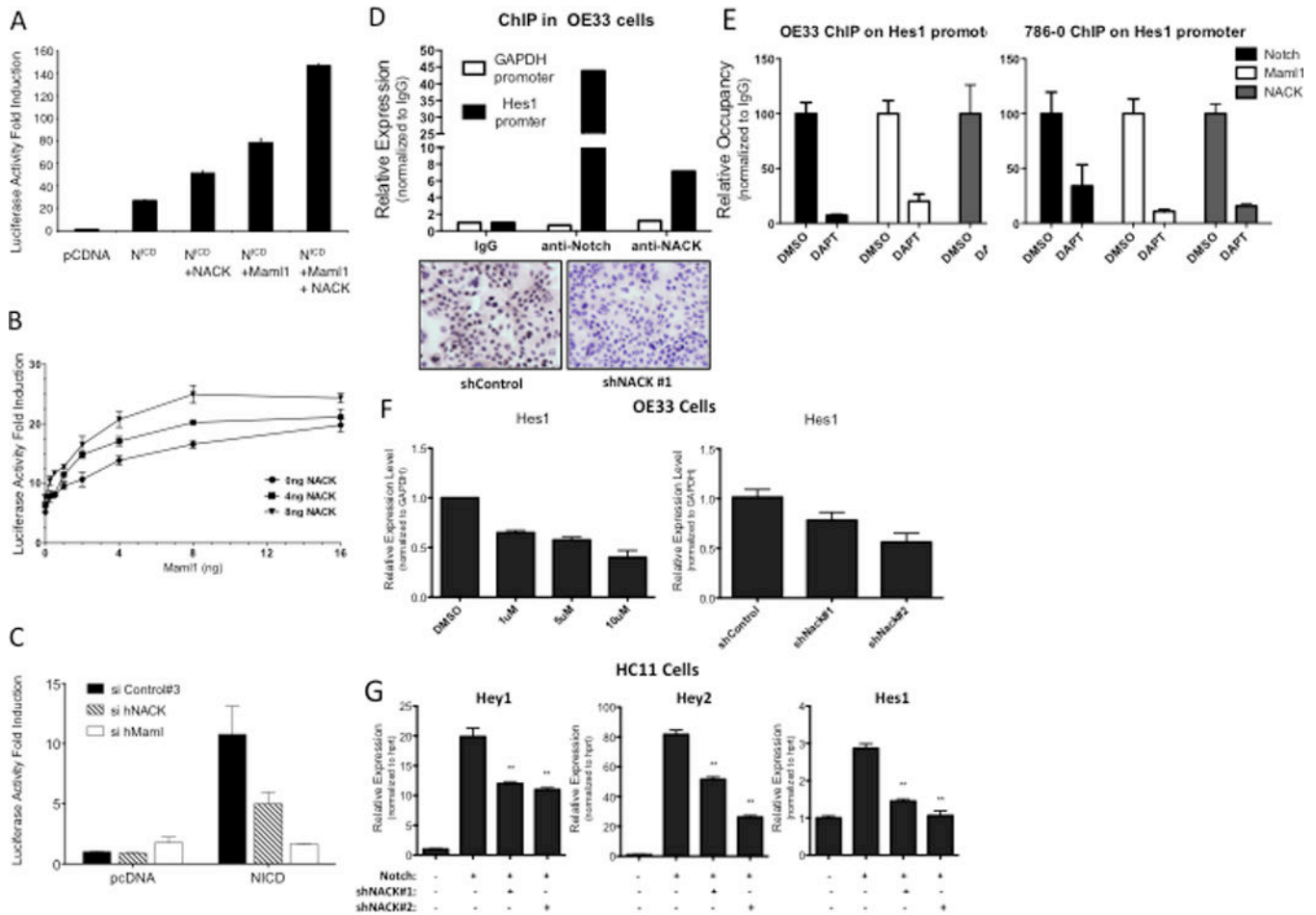
Figure 2. NACK is a co-activator of Notch signaling

A–C. 8× CSL luciferase reporter assays in H1299 cells transfected with different combinations of plasmids. Experiments were performed in triplicate and bars represent mean (SEM). **A.** Histogram of luciferase activity. **B.** Titration curve showing luciferase activity with different quantities of Maml1 and/or NACK over a fixed quantity of N1^{ICD} (2 ng). **C.** Histogram of luciferase activity after siRNA-mediated knockdown of Maml or NACK. **D.** ChIP of Notch and NACK on the Hes1 promoter in OE33 cells. IHC validates specificity of the α-NACK antibody. **E.** ChIP of Notch, Maml, and NACK on the Hes1 promoter in OE33 and 786-0 cells after treatment with DAPT. **F.** Hes1 expression in OE33 cells treated with DMSO or DAPT, or infected with shRNA against NACK. Bars represent mean (SEM) of 3 samples. **p<0.01 versus shControl. **G.** Expression of Notch target genes in HC11 cells infected with N1^{ICD} and shRNA against NACK. Bars represent mean (SEM) of 3 samples. **p<0.01 versus Notch.
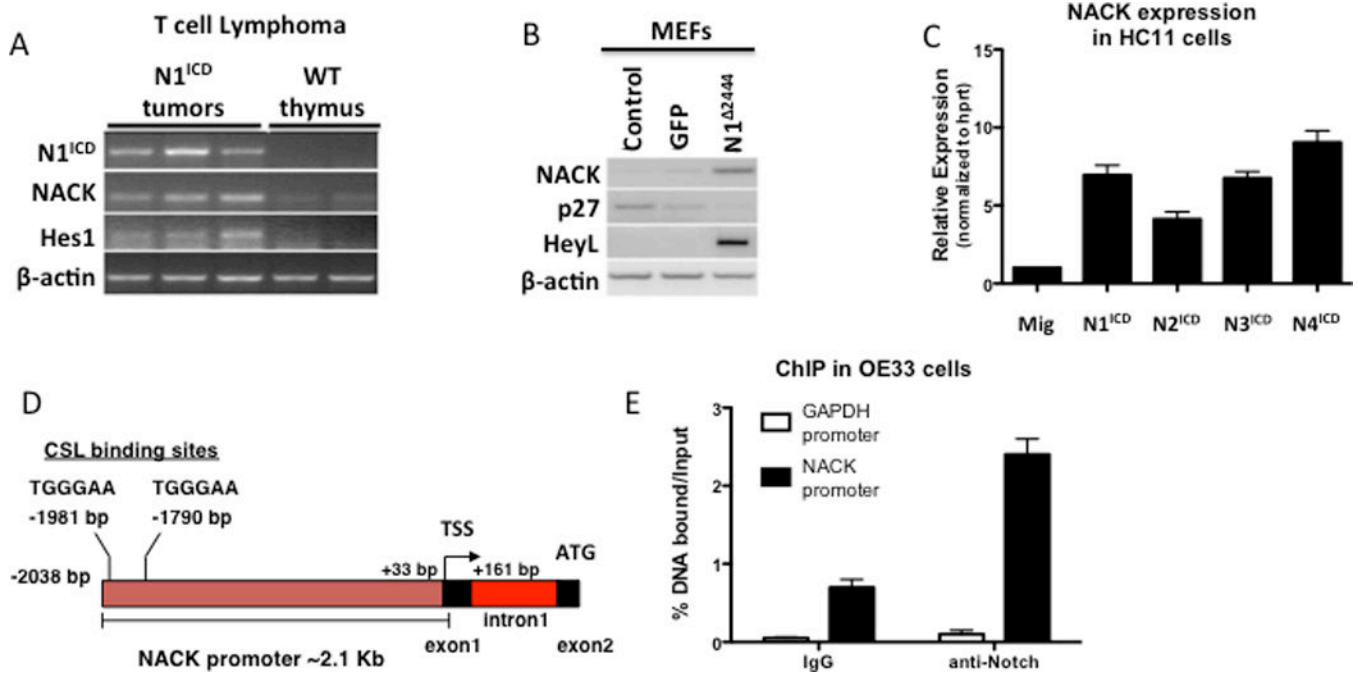
Figure 3. NACK is a transcriptional target of Notch signaling
A. mRNA expression in mouse lymphoma cells expressing exogenous N1^{ICD} or wt mouse thymocytes. **B.** mRNA expression in MEFs infected with N1^{ICD}. **C.** NACK expression in HC11 cells infected with N1^{ICD}, N2^{ICD}, N3^{ICD}, or N4^{ICD}. Histogram shows mean (SEM). **D.** Schematic representation showing architecture of the NACK promoter. Numbers denote genomic sequence from − to + with respect to the translation initiation codon ATG. TSS, transcription start site. **E.** ChIP of Notch on the NACK promoter in OE33 cells.
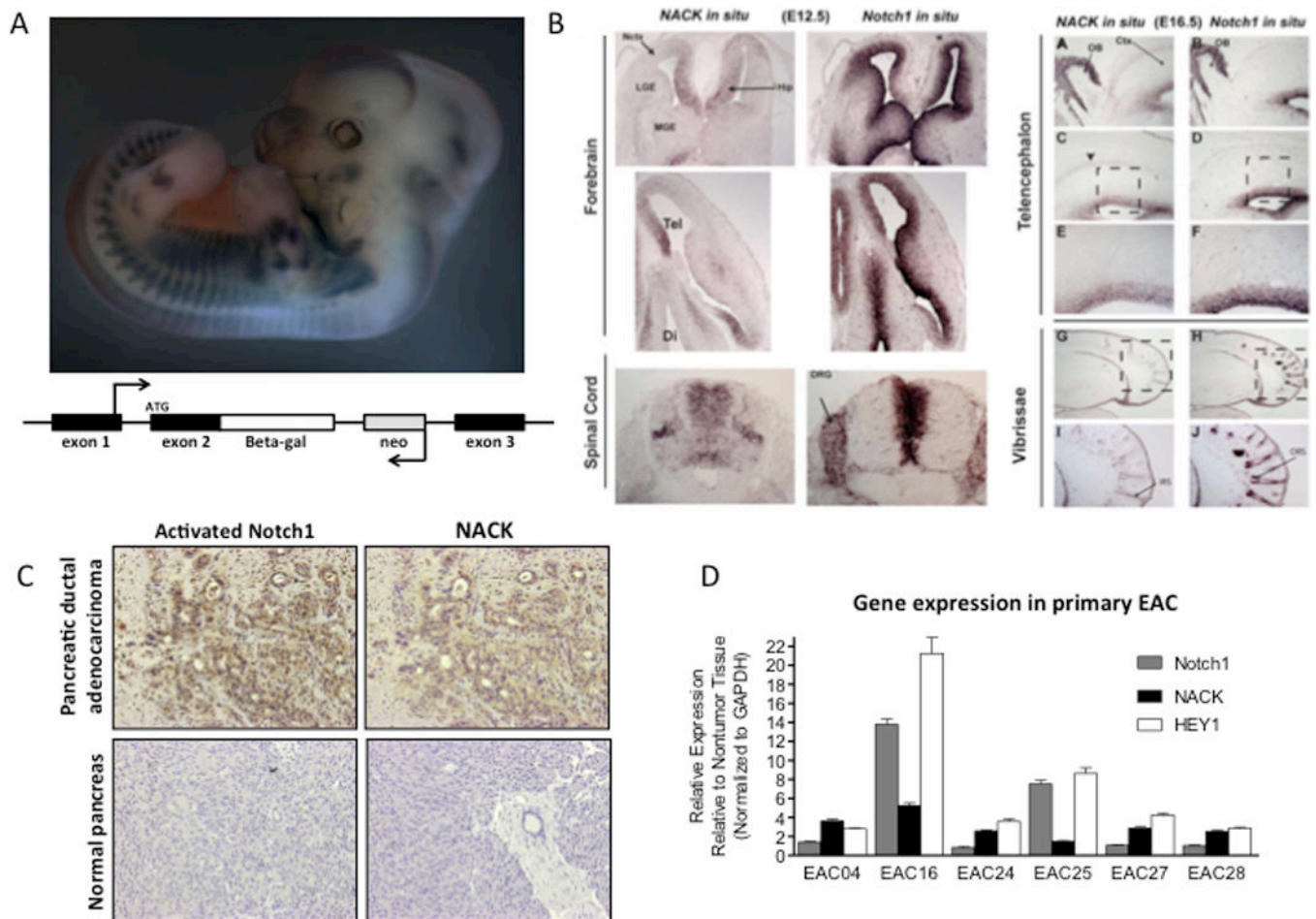
Figure 4. NACK is co-expressed with Notch during development and in tumors
A. Upper panel, β-galactosidase staining of transgenic knock-in mouse E16.5 embryo. Bottom panel, schematic representation showing knock-in construct. **B.** *In situ* hybridization of NACK and Notch1 in mouse central nervous system during development. **C.** IHC of activated N1^{ICD} and NACK in pancreatic ductal adenocarcinoma and normal pancreas (representative samples). **D.** Expression of Notch1, NACK, and Hey1 in esophageal adenocarcinoma and adjacent esophageal mucosa. Histogram shows mean (SEM).
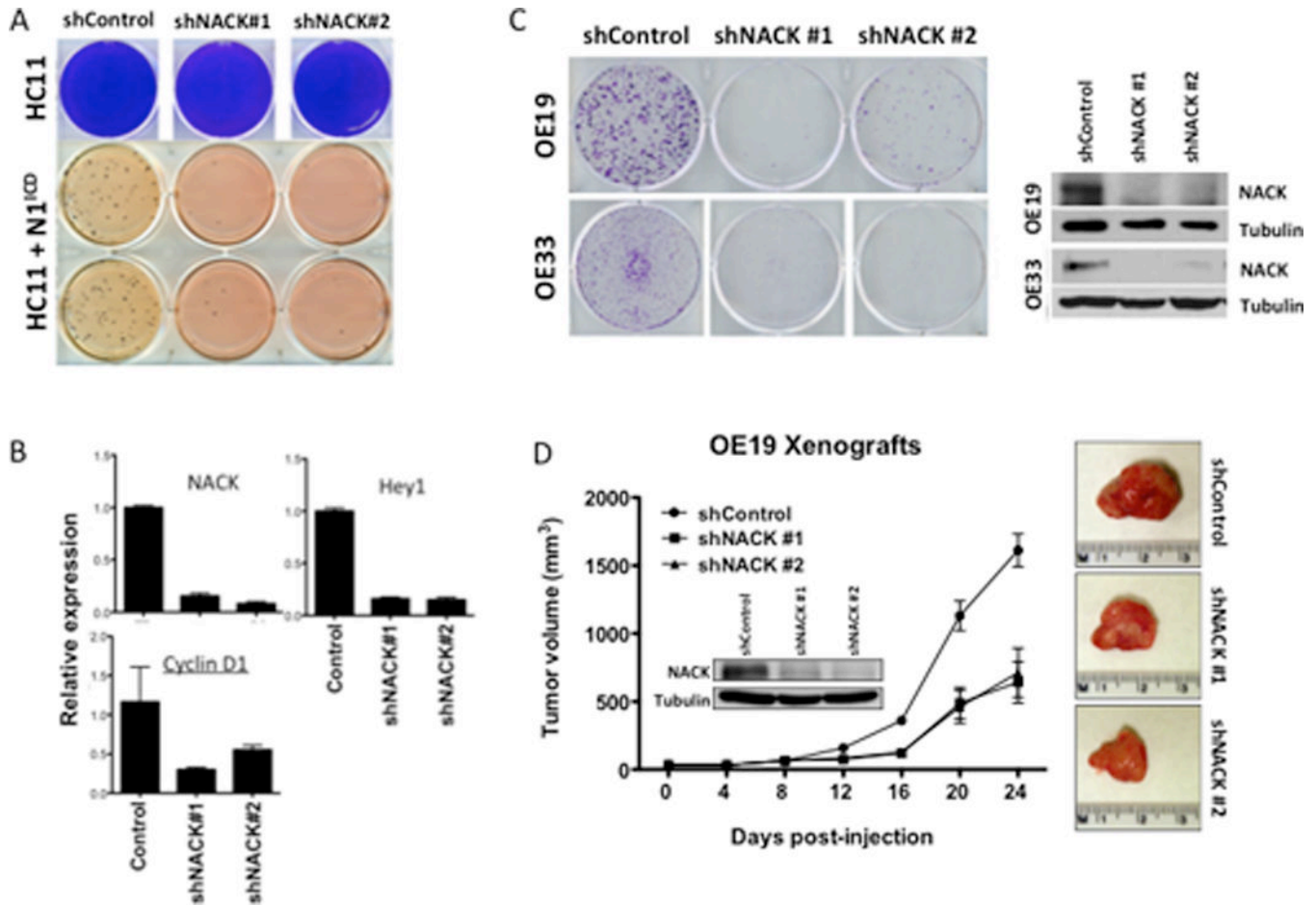
Fig. 5. NACK is required for Notch-mediated transformation and tumorigenesis
A. Upper panel, colony growth of HC11 cells infected with shRNA against NACK. Lower panel, growth of HC11 cells infected with N1^{ICD} and shRNA against NACK in soft agar. **B.** Expression of NACK, Hey1, and Cyclin D1 in HC11 cells infected with shRNA against NACK. Bars represent mean (SEM) of fold-change relative to control shRNA. **C.** Colony formation in OE19 and OE33 cells infected with shRNA against NACK. NACK knockdown was verified by western blot. **D.** Xenograft formation from OE19 cells infected with shRNA against NACK. Tumor volume was measured weekly and error bars indicate SEM. n=8 per group. At 24 d post-injection, tumors were harvested and representative images are shown from each group.
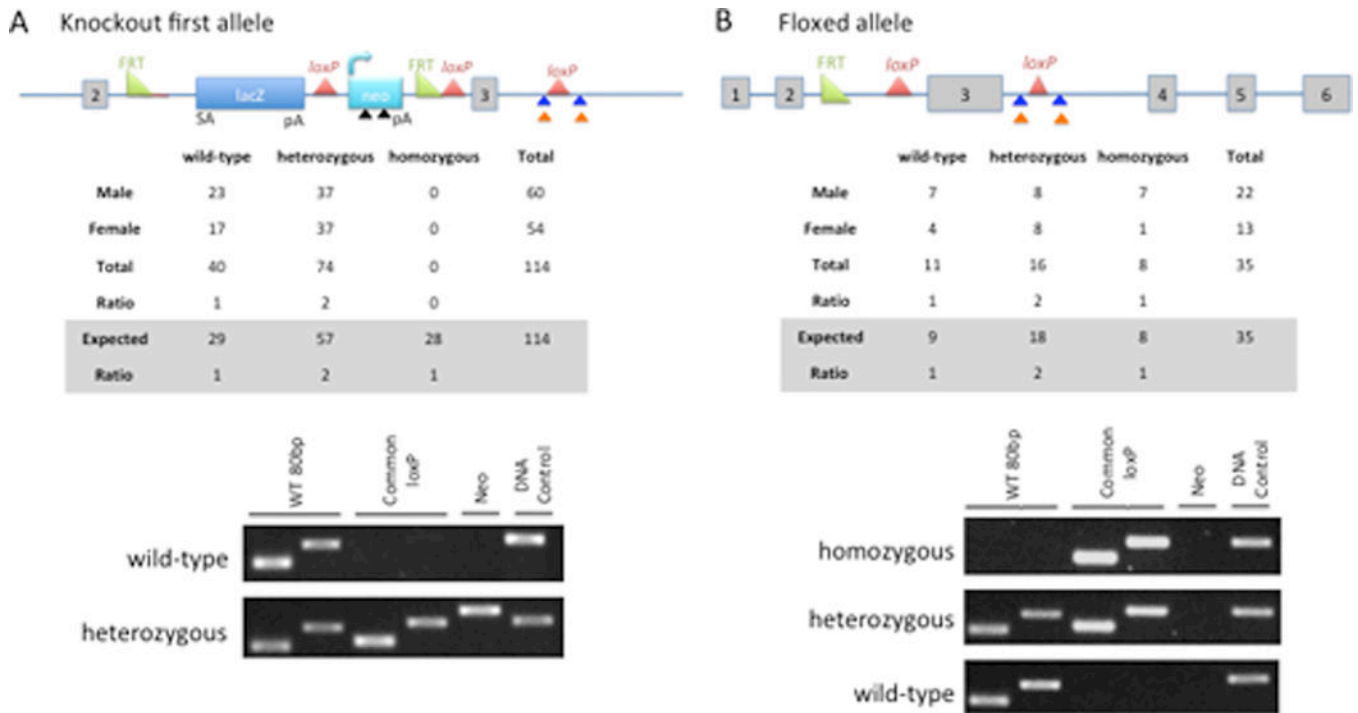
Figure 6. NACK knockout is embryonic lethal
A. Upper panel, schematic of knockout first allele. Colored arrows mark the locations of the primers used for genotyping: black triangle, Neo primers; blue triangle, common loxP primers; orange triangle, WT 80bp primers. Middle panel, summary of live births achieved from mating two *NACK*[KOF/+] mice. Bottom panel, genotyping results from wt and heterozygous pups. **B.** Top panel, schematic of floxed allele resulting from FLPe recombination. Middle panel, summary of live births achieved from mating two *NACK*[flox/+] mice. Bottom panel, representative genotyping results from wt, heterozygous, and homozygous pups.