A Novel Chemical Attack on Notch-mediated transcription by targeting the NACK ATPase

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Abstract

Notch Activation Complex Kinase, NACK, is a component of the Notch transcriptional machinery critical to Notch-mediated tumorigenesis. However, the mechanism by which NACK regulates the Notch-mediated transcription is not well understood. Here we demonstrate that NACK binds and hydrolyses ATP and that only ATP-bound NACK is able to bind to the Notch Ternary Complex (NTC). Considering this we sought to identify inhibitors of this ATP-Dependent function and, using computational pipelines, discovered the first small molecule inhibitor of NACK, Z271-0326, that directly blocks the activity of Notch-mediated transcription and shows potent antitumor activity in PDX mouse models. In conclusion, we have discovered the first inhibitor that holds promise for efficacious treatment of Notch-driven cancers by blocking the Notch downstream NTC activity.

Main Text

The Notch Ternary Complex (NTC) is a nuclear multiprotein complex that orchestrates Notch signal transduction by mediating the formation of a transcriptional activation complex on chromatin. The NTC is composed of the DNA-binding protein CSL [CBF-1/RBP-J, Su(H), Lag-1], Notch intracellular domain (NICD), and Mastermind-like protein (Maml). There are 4 Notch paralogs and 3 Maml paralogs, all of which can form specific NTC species. Upon activation of the Notch pathway, the NICD directly drives the formation of the NTC by binding to CSL followed by the recruitment of Maml. Although detailed knowledge of subsequent events is yet to be elucidated, the NTC appears to serve as a scaffold and lead to the recruitment of additional transcriptional co-activators and epigenetic enzymes resulting in the initiation of Notch transcriptional activation of the target genes¹⁻⁴. Thus, Notch signaling regulates critical processes for cell fate choices, including proliferation, differentiation, apoptosis, and self-renewal by regulation of a transcriptional cascade⁵. However, in many different cancers, Notch signaling is improperly activated, contributing to the initiation and maintenance of the neoplastic phenotype^{6,7}. Therefore, disruption of the assembly or function of the NTC is a desirable therapeutic target. To date, inhibition of Notch signaling has been achieved by preventing the production of the NICD through monoclonal antibodies (mABs) and small-molecule inhibitors of presenilin-dependent g-secretase (GSI). However, these approaches have largely failed due to dose-limiting toxicity^{8,9}. Therefore, there is a great interest in developing alternative therapeutic strategies to directly target the NTC. Previously, we demonstrated that the Notch Activation Complex Kinase (NACK) is an important transcriptional co-activator of Notch-mediated transcription and is essential for Notch-driven tumorigenesis¹⁰. Although NACK encodes a kinase-fold in its C-terminal domain, several motifs consistent with kinase activity are absent and thus NACK falls into the category of pseudokinase. Given this critical role and several examples of druggable pseudokinases, we reasoned that NACK is an intriguing therapeutic target to modulate the Notch signaling pathway in cancer^{11,12}. Herein, we demonstrate that NACK binds and hydrolyzes ATP, and that this activity is required for NACK recruitment to the NTC and engagement of the Notch transcription machinery with RNA polymerase II (RNA pol II). Based on this, using computational and biochemical methods, we have identified a first-in-class potent and functional small-molecule

inhibitor of NACK, Z271-0326. Z271-0326 directly blocks NACK recruitment to the NTC thereby inhibiting the Notch-mediated transcriptional cascade. Moreover, Z271-0326 shows potent antitumor activity in PDX mouse models. Here, we report the discovery of a novel mechanism to directly block the activity of Notch downstream of the NTC via a novel drug-like small molecule with promise for efficacious treatment of Notch-driven cancers.

Results

ATP is required for NACK recruitment to the NTC.

We identified NACK as an integral component of the Notch transcriptional activation complex that is recruited to the NTC by acetylation of Maml by p300. In addition, our previous work demonstrated that ATP enhances the recruitment of NACK to the Notch1 ternary complex in a dose-dependent manner. Although NACK is predicted to be catalytically inactive, we reported that mutation in the putative catalytic Lys residue of NACK prevents NACK binding to the NTC¹³, indicating an intrinsic activity. Therefore, we next sought to determine if the kinase-like domain of NACK binds to and hydrolyzes ATP as a requirement for recruitment to the NTC. To evaluate ATP binding to NACK, the NACK kinase domain was expressed, purified, and subjected to thermal shift assay. This assay monitors the protein melting temperature (T_M), which increases upon ligand binding. We screened three different concentrations of ATP (500 μ M, 1 mM, and 3 mM) and observed that with an ATP concentration of 3 mM, NACK T_M significantly increased by 3.7°C (Fig. 1a), indicating that ATP binds to the NACK kinase domain. To obtain a more accurate measurement of physiological binding constants (K_D), we also used the Surface Plasmon Resonance (SPR). Although we were not able to measure NACK-ATP binding through SPR, we obtained a K_D of approximately 1.5 μ M with a non-hydrolysable ATP analogue AMP-PNP (Fig. 1b). This result suggested that during the experiment NACK could hydrolyze ATP as to significantly reduce the amount of bound ATP. Although we have demonstrated the binding of ATP to NACK, we have never been able to demonstrate kinase activity. That is, we have never been able to demonstrate a phosphotransfer to a substrate of autophosphorylation. Therefore, we sought to determine if NACK had ATPase activity. NACK-WT and the putative catalytic Lys mutant (K1024A) were assayed using the ADP Glo in a time dependent reaction. Unlike the NACK-K1024A mutant, NACK-WT displayed a robust and linear ATPase activity up to 90 min (Fig. 1c). We next determined the K_M value for ATP, which was 62.48 μ M, which is consistent with the average K_M for kinases (58 μ M) (Fig. 1d). To further explore the effect of ATP on NACK binding to the Notch transcription complex, we conducted a CSL-DNA affinity pulldown assay (DAP) by adding ATP or GTP to the binding reaction. We observed that the recruitment of NACK to the NTC was dramatically increased compared to the control. In contrast, NACK-binding was not observed when non-hydrolysable ATP or GTP (AMP-PNP or GTP-rS) was used (Fig. 1e), which suggests that ATP or GTP hydrolysis is required for the recruitment to the NTC. Taken together, these results demonstrate that NACK-ATP binding and is required for NACK recruitment to the NTC.

Discovery and characterization of a NACK small molecule inhibitor.

Initially, we developed a homology model for NACK by using 4 of the most similar kinases with deposited PDB structures as templates (PDB IDs: 2A1A, 4APC, 2BUJ, 2BDW). We utilized this structure model in one of the steps of our virtual screening pipeline to identify the first NACK small molecule inhibitor. Since these initial studies, two apo NACK crystal structures of the kinase domain (human and *Rattus norvegicus*) were deposited in the PDB (PDB IDs: 5VE6 and 6EWX, respectively), as well as a crystal structure of homologue a PEAK1 (human) (PDB ID: 6BHC)^{14,15}. Both deposited PDB structures of NACK were not directly suitable for virtual screening efforts as they lacked resolution of key binding site motifs and were the apo structures. However, we used these structures to optimize our initial structural model using the Schrodinger PRIME homology modeling software^{16,17} by threading 5VE6 as the primary template, and selected 6EWX and 6BHC as homologues. As expected, the optimized structure model aligned well with our initial model displaying over 97% alignment¹⁸. Using our optimized model, ATP was docked into the typical ATP binding site followed by molecular dynamics (MD) simulations. Figure 2a shows the most representative pose of NACK with bound ATP and predicted binding interactions throughout a 200 ns MD simulation. The NACK structure model with docked ATP, the interactions of ATP throughout the MD simulation (2D projection), and the ligand RMSD trajectory and ATP-protein interactions are shown in Supplementary Figs. 1, 2 and 3.

To discover compounds that bind to and inhibit NACK activity among a large space of commercially available compounds, we developed a virtual screening pipeline that uses machine learning followed by docking. We first computed probabilities of activity of small molecules against the most NACK-related kinases (by domain sequence similarity) for over 6 million commercially available compounds (at the time), obtained from eMolecules (ref: <https://www.emolecules.com/>). Specifically, we applied the Laplacien-corrected Naïve Bayes classifiers, that we had previously developed, trained on a total of 12,867 known actives for 19 kinases¹⁹ (Supplementary Fig. 4). After aggregation of the results from the machine learning predictors, we selected 7614 compounds for docking. Six diverse conformational representations of the NACK kinase domain were extracted from the MD simulation of NACK-ATP to account for receptor flexibility. Using the Schrodinger small molecule discovery suite, tautomers, ionization states, and 3D conformation were calculated for each compound (LigPrep) followed by docking into the six different NACK kinase domain structures (Glide SP). The 10 best-scoring compounds were then selected after Z-score transformation of the aggregated docking scores across poses and ligand representations obtained in the different docking models (NACK conformations). To test the effect of the compounds prioritized by our virtual screening pipeline on cell proliferation, we performed a colony-formation assay using Notch-dependent and independent cell lines. Treatment of Notch dependent cell lines with the Z270-0137, Z271-0191, or Z271-0326 compounds displayed a remarkable reduction in a colony formation ability in contrast to the Notch-independent cell line (HC11) (Supplementary Fig. 5a and b). Seeking to discover an inhibitor of NACK recruitment to the NTC, HEK293T cells expressing Notch1, Maml1, and NACK were treated with seven selected compounds and CSL-DAP analysis was performed. Consistent with colony formation results, CSL-DAP revealed that Z271-0191 and Z271-0326 dramatically inhibited NACK binding to the NTC compared to the DMSO control (Fig. 2c). We chose to focus specifically on Z271-0326 as this compound displayed the best activity across several assays and had a

lower cLogP than Z271-0191. We then optimized our NACK structure model docked with Z271-0326 (Fig. 2d and e) and performed a 1 μ s MD simulation to predict key binding interactions between Z271-0326 and NACK (Supplementary Fig. 6).

Z271-0326 enhances NACK ATPase activity and prevents NACK binding to the NTC.

Since ATP is required for NACK binding to the NTC¹³, we investigated if Z271-0326 can disrupt NACK binding to the NTC in the presence of ATP. To this end, we ectopically expressed NACK-WT or K1024A in HEK293T cells. Cells were subsequently treated with 10 μ M of Z271-0326 and CSL-DAP analysis was performed in the presence or absence of ATP (100 μ M). We observed that the Z271-0326 treatment abolished NACK-WT recruitment to the NTC and prevented ATP from enhancing NACK-NTC binding. We further confirmed that mutation at the catalytic Lysine 1024 abolished NACK-recruitment to the NTC (Fig. 3a). We then examined Z271-0326 NACK target engagement by the thermal shift assay. When NACK is assayed in the presence of 10 μ M Z271-0326, we observe a two-stage transition curve. Because the thermofluor displayed a two-stage transition that corresponds to bound and unbound states (Fig. 3b), we calculated the T_M through the analysis of the first derivative of a fluorescence change (Supplementary Fig. 7b) and we observed that NACK melting temperature significantly increased by 8.9°C (Supplementary Fig. 7c). The interaction between inhibitor Z271-0326 and NACK was also characterized by SPR. The dissociation constant associated to NACK/Z271-0326 binding ($K_D = 0.89 \pm 0.07$ μ M) (Fig. 3b) was comparable to the affinity observed for NACK/AMP-PNP binding (Fig. 1b), which suggests that Z271-0326 may mimic AMP-PNP ligand to produce a similar response. The finding that NACK hydrolyzes ATP raised the question whether Z271-0326 could affect NACK ATPase activity. Based on this, we assayed the NACK ATPase activity in the presence of Z271-0326 and found that Z271-0326 enhanced NACK ATPase activity in a time-dependent manner (Fig. 3d). We next performed the NACK ATPase reaction with varying concentrations of ATP, and we observed that Z271-0326 increased NACK ATPase activity in a manner dependent on the ATP concentration. In the presence of Z271-0326, the V_{max} of NACK was increased by 35%, without altering the K_M (Fig. 3e).

Z271-0326 inhibits EAC cell growth by blocking Notch transcriptional activation.

Previously, we demonstrated that Notch drives stemness and tumorigenicity of esophageal adenocarcinoma (EAC)²⁰, and we reported that NACK is also involved in EAC progression by activating Notch-mediated transcription¹⁰. To evaluate the expression level of NACK in esophageal adenocarcinoma (EAC), we analyzed clinical samples derived from surgically resected primary esophageal adenocarcinoma. We detected high levels of NACK and activated Notch1 through RT-qPCR (Fig. 4a) and IHC (Fig. 4b) in chemo-naïve esophageal adenocarcinoma samples derived from the endoscopic ultrasound (EUS) biopsies. We also observed that EAC cell lines (OE33 and OE19) showed high NACK expression compared to normal human primary esophageal epithelial cells (Supplementary Fig. 8a). To test the effect of NACK inhibition on EAC proliferation, we performed a colony formation assay. Treatment cells with Z271-0326 displayed a dose-dependent reduction in colony formation in both OE19 ($EC_{50} = 3.6$ μ M, Fig. 4c) and OE33 ($EC_{50} = 3.14$ μ M, Supplementary Fig. 8b) cell lines. To evaluate the

effect of Z271-0326 in the expression of the Notch target genes, EAC cells were treated with the determined EC_{50} values and were harvested at 6h. RT-qPCR analysis indicated that Z271-0326 treatment significantly inhibited the transcription of Notch target genes in both OE19 (Fig. 4d) and OE33 (Supplementary Fig. 6c) cell lines. In further support of these findings, chromatin immunoprecipitation (ChIP) assay in OE19 cells indicated that Z271-0326 results in less occupancy of Notch1, Maml1, NACK, and the activated form of the RNA pol II (Ser-5) to the HES5 and CCND1 promoters (Fig. 4e and f). Similar results were observed in OE33 cell line (Supplementary Fig. 6d and e). Taken together, these results strongly indicate that Z271-0326 inhibits EAC cell growth by preventing Notch-dependent transcription.

Validation of the Z271-0326 scaffold: UM-74 but not UM-94 demonstrates activity in EAC cell lines and prevents tumor spheres formation.

To demonstrate the activity of Z271-0326 was specific to the small molecule chemotype, we synthesized two closely related analogs based on the predicted binding model of Z271-0326 in NACK. We demonstrated that this activity could be improved (UM-74) or lost (UM-94) with the removal of atoms predicted to be integral for NACK binding. We examined NACK target engagement of UM-74 via thermal shift assay (Supplementary Fig. 9a, b and c), where we observed that UM-74 increased the NACK T_M by 9°C. In contrast, NACK T_M was not changed significantly by UM-94. Next, we tested the efficacy of the analogs to affect EAC cell proliferation. UM-74 inhibited OE19 cell growth more potently than Z271-0326 in the colony formation assays ($EC_{50} = 0.52 \mu M$), whereas the inactive analog UM-94 displayed no effect on OE19 cell growth ($EC_{50} > 30 \mu M$) (Fig. 5a). RT-qPCR analysis of OE19 cells indicated that UM-74 treatment significantly decreased transcription of Notch target genes *HES5* and *CCND1* (Fig. 5b). Furthermore, CSL-DAP analysis in HEK293T, transiently transfected with Notch1ICD-Flag, Maml1-Flag, and NACK-myc, showed that UM-74 also inhibited NACK binding to the NTC, whereas UM-94 did not affect NACK recruitment (Fig. 5c). Since EAC cell lines display CSC features, which are driven by the Notch signaling pathway²⁰, we screened Z271-0326 and analogs by conducting tumor-sphere assays. OE19 cell line treated with UM-74 or Z271-0326 exhibited a decrease in the ability to form secondary tumor-spheres compared to the control (DMSO) and showed more potency than DAPT treatment (Fig. 5d). Treatment of secondary tumor-spheres with Z271-0326 and UM-74 also inhibited signature Notch target-genes transcription (Fig. 5e). Similar results were observed in OE33 cell line (Supplementary Fig. 10a, b and c). Taken together, these data confirm that the NACK inhibitors Z271-0326 and UM-74 affect Notch-dependent cell lines CSC-like properties via the inhibition of Notch-directed transcriptional activation.

Z271-0326 and UM-74 but not UM-94 inhibit tumor growth in PDX model.

To evaluate the effect of NACK inhibitors on tumor growth we employed Notch-dependent cell line (OE19) and EAC patient-derived xenograft (EAC47 PDX) models. Mice harboring established tumors were treated with either Z271-0326, or UM-74 (15 mg/kg), or UM-94 (20 mg/kg) daily via intraperitoneal injections. Z271-0326 and UM-74- treated mice showed a significant attenuation in EAC47 PDX tumor growth (Fig. 6a and b). In contrast, UM-94 treatment had no significant effect on tumor growth (Fig. 6d). No

significant change in body weight was detected throughout the treatment course (Fig. 6c). Z271-0326 also showed similar efficacy in the inhibition of tumor progression in the OE19 xenograft model (Supplementary Fig. 11a and b), causing no observable adverse effects on the animal body weight (Supplementary Fig. 11c). Other pan-Notch inhibitors (such as GSIs) have demonstrated dose limiting toxicity (goblet cell metaplasia) due to inhibition of transcription driven by multiple Notch ICD's²¹⁻²³. To assess this intestinal toxicity, we treated mice daily with 40mg/kg of DZB, Z271-0326, UM-74, or UM-94. Histological analysis of goblet cell metaplasia (PAS staining) after 5 days of treatment revealed a substantial increase of goblet cell numbers in the intestinal crypts of mice treated with the GSI (DBZ). In contrast to the GSI treatment, we found no evidence of GI toxicity after 5 daily treatments with Z271-0326, UM-74, UM-94, or DMSO. Therefore, mice treated with NACK inhibitors (Z271-0326 and UM-74) displayed intact crypts and normal intestinal architecture with no evidence of goblet cell metaplasia, comparable to the vehicle group (DMSO) (Fig. 6e). Together, these results indicate that small molecule NACK inhibitors abrogate tumor growth in clinically relevant models, without displaying goblet cell metaplasia-associated intestinal toxicity. Furthermore, the lack of Goblet Cell metaplasia indicates NACK inhibitors are selective for distinct Notch complexes and may not be pan-Notch inhibitors. Our findings thus suggest that NACK inhibitors are good antineoplastic candidates that abrogate EAC tumor growth with minor side effects, possibly by selectively inhibiting the activation of the Notch1 pathway.

Discussion

In this study we describe Z271-0326 as a first-in-class small molecule inhibitor of NACK function. As NACK is a required co-activator of Notch-mediated transcription, abrogation of NACK function by a small molecule enables a novel, direct, and potentially selective approach to target Notch-dependent cancers.

We identified NACK as a component of the Notch transcriptional machinery by purification of the Notch Ternary Complex (NTC), and we demonstrated that NACK is required for Notch-dependent transcription and tumorigenesis¹⁰. Although we do not have a complete understanding of the role that NACK plays in Notch-mediated transcription, we have some mechanistic details that suggest that NACK links the NTC to RNA Pol II to initiate transcription. A critical step in the recruitment of NACK to the NTC is the acetylation of Maml by p300 on two Lys residues. Mutations in these residues or an inhibition of p300 activity results in the failure of NACK to bind the NTC and uncouples the recruitment of RNA Pol II and initiation of transcription¹³.

We demonstrated that NACK binds and hydrolyzes ATP in several distinct experimental assays and that it is critical for its biochemical role in Notch transcription. NACK, like other kinase-domain proteins, encodes a large loop that separates the N and C-terminal lobes of the kinase domain. Interestingly, in the published low resolution crystal structures, NACK (SGK223) shows a collapsed disorganized ATP pocket that looks unsuitable for binding ATP, with the catalytic lysine (K1024) surrounded by a conserved inhibitory triad (D978, Y981, and Q1021)¹⁵. Nonetheless, our optimized NACK homology model predicted interactions between ATP and the catalytic K1024 through H-bonding and several salt bridges (Fig. 2a,

Supplementary Fig. 3). Lucet *et al*/predicts the gate-keeper residue to be T1092¹⁴. However, according to the Klifs database, the gate-keeper is annotated as R1093²². The disagreement in the predicted residue is likely due to the unusual hinge loop in NACK. If we designate R1093 as the gate-keeper, the dihedral angles of the hinge residues, as displayed by the deposited crystal structures of NACK, the homologue PEAK1, and subsequently our homology model, are typical to most kinases. In our model, the adenine portion of ATP is predicted to H-bond with E1094, which is a common *i* + 1 binding mode displayed by ATP bound kinases. However, assignment of the gatekeeper as a hydrophilic residue with the side chain pointing away from the pocket would be atypical (Supplementary Fig. 12). Presented experimental evidence supports that NACK binds ATP and we have clearly demonstrated that ATP is necessary for NACK to bind the NTC.

One proposition is that ATP is required for the stabilization of the kinase-fold and that hydrolysis of ATP may be required for releasing NACK from the NTC. This, therefore, indicates that NACK may function as a molecular switch regulating Notch-dependent transcription. In this model, we propose that ATP binding is required for the stabilization of the kinase fold which leads to the formation of the Notch transcriptional activation complex. Following the initiation of transcription, a component of this complex may act as an ATPase trigger promoting the release of NACK and disassembly of the NTC from DNA in preparation for another cycle of Notch signaling. This model is interesting in that it would be the first link of Notch signaling to the proliferative state/capacity of the cell.

How does Z271-0326 inhibit NACK function in Notch-mediated transcription? We observed that Z271-0326 appears to enhance ATP hydrolysis. However, we currently do not completely understand the mechanism that underlies this enhancement and how it relates to the inhibition of NACK function. What do we know? Through mutational studies we know that NACK must bind ATP to associate with the NTC. In addition, it also appears that dimerization of NACK is necessary. Therefore, we may be observing a trans-dominant effect wherein Z271-0326 stabilizes the NACK kinase fold in an “inactive” state that prevents dimerization by stimulating ATPase activity in the partner molecule and thereby preventing stable dimer formation and mimicking the inactive monomeric state. Although the full mechanistic details of NACK recruitment and release from the NTC as well as inhibition of this process by a small molecule requires further studies, the development of the first-in-class NACK antagonist represents a breakthrough in the toolbox to modulate Notch signaling and to target Notch-dependent cancers.

At the start of this project, neither a small molecule binder nor any structural information was available for NACK. NACK is currently classified as an understudied Tbio target in the Illuminating the Druggable Genome (IDG) project²³. We knew ATP was required for its role in Notch signaling. Considering this, we thought we should be able to identify an ATP competitive inhibitor by screening, but we also had no high-throughput assay we could utilize and needed a way to perform an *in-silico* screen. We developed a computational pipeline that combines machine-learning and docking, which enabled us to virtually screen millions of compounds. Despite the lack of known inhibitors, we were able to leverage machine learning classifiers for kinases similar to NACK to prioritize compounds likely to be enriched in NACK binders. These compounds were then advanced to docking using an optimized homology model. Using this

pipeline, we identified Z271-0326 as a low micromolar active hit. We validated the initial biological activity after synthesizing Z271-0326 in-house. Based on our computational model, two additional analogs were then synthesized, one we predicted to be inactive (UM-94) and the other predicted to be more active than Z271-0326 (UM-74). In addition to their biochemical and cell-based activity, we demonstrated that Z271-0326 and the more active UM-74 have potent anti-tumor activity and block tumor growth of Notch-dependent PDX models, while UM-94 was inactive.

Taken together, we have developed a novel platform to target Notch-dependent cancers via inhibition of a critical component of Notch dependent transcriptional machinery downstream of the NTC.

Over the last 25 years, there has been great interest in targeting Notch in cancer. However, to date many approaches to block Notch signaling in the clinic have failed. There are several contributing factors to this lack of success in targeting Notch. Of these, pan-inhibition of Notch proteins disrupts normal tissue homeostasis and in the clinical setting provides the dose-limiting toxicity, that is exemplified by goblet cell metaplasia in the intestines. Similarly, the mAb approach suffered from long term exposures likely resulting in a similar effect as pan-Notch inhibition, by depleting the Notch1 positive cells in the crypts^{8,9}. Recently it has been demonstrated that unlike GSIs (pan-Notch inhibitors), a Notch 1 specific inhibitor of the NTC assembly does not direct this toxicity²⁴. Moreover, we present evidence that Z271-0326 and UM-74 do not induce goblet cell metaplasia. Interestingly, we have published that NACK appears to be a selective component of Notch1/Maml1 or Notch1/Maml3 transcriptional activation complexes, but not Notch1/Maml2, indicating a degree of specificity in Notch signaling. Taken together, it would suggest that NACK inhibition does not lead to a pan-Notch inhibitor and inhibits a selective set of Notch-mediated transcriptional programs.

It has been clearly demonstrated that Notch1 plays an essential role in the CSC-like populations in both EAC and TN-Breast cancer²⁰⁻²⁷. In fact, a Notch 1 specific inhibitor can ablate this population selectively, resulting in abrogation of tumor growth in PDX models. We demonstrate in this manuscript that the NACK inhibitors Z271-0326 and UM-74 also dramatically affect the CSC population in EAC and abrogate PDX tumor growth, which supports the model that NACK is a downstream component of the N1 NTCs. Therefore, we now provide a specific inhibitor of NACK that functions to directly inhibit N1-mediated transcriptional activation complexes. In conclusion, we propose NACK as a tumor marker and as pharmacological target for providing efficacy and therapeutic depth in the attack on the activated Notch1 pathway.

Materials And Methods

Cell Lines

Human esophageal adenocarcinoma cells OE33 were obtained from the European Collection of Cell Culture, while OE19 cells were obtained from the Leibniz Institute DSMZ in 2020, authenticated by STR profiling. Both were grown in RPMI medium, supplemented with 10% fetal bovine serum (Gemini Bio), 1%

glutamine and 1% penicillin/streptomycin. HEK293T and HC11 cell lines were obtained from ATCC. HEK293T cells were cultured in the DMEM medium supplemented with 10% fetal bovine serum (Gemini Bio), 1% glutamine and 1% penicillin/streptomycin. HC11 were grown in RPMI supplemented with 10% fetal bovine serum (Gemini Bio), 1% glutamine and 1% penicillin/streptomycin. All cell lines were tested for mycoplasma contamination and propagated in growth media as specified by the providers.

Cloning

A codon-optimized cDNA sequence of human NACK corresponding to amino acid residues 932–1406 (kinase domain) was cloned into the pGEX-6P-1 expression vector using the following primers containing EcoRI and XhoI restriction sites:

Forward 5': cgcgaaattcggtacacacaactgc.

Reverse 5': acactcgagctacagaagttgcagcagcttcag.

Protein Purification

Competent C41DE3 cells were transformed with pGEX-NACK 932–1406 and cells were cultured overnight in LB + ampicillin. 10 L of autoinduction media were prepared and 1 mL of overnight culture was added per liter. Flasks were shake incubated at 37°C for 3 hours and then 20°C overnight. After 24 hours the cells were harvested by centrifugation and the bacterial pellet was resuspended in PBS + lysis buffer. Cells were sonicated and centrifuged at 15,000 G. 60% w/v ammonium sulfate was added to the supernatant to precipitate the protein and then centrifuged again at 15,000 G. The protein pellet was resuspended in PBS + 0.1% triton and incubated overnight with the glutathione affinity resin. GST-NACK tagged protein was eluted from the glutathione affinity resin and then cut with PreScission Protease overnight. A Q Sepharose anion-exchange column was used to remove the free GST from NACK. GST sticks to a Q column while NACK does not at low salt (~ 50 mM NaCl) concentrations. NACK was dialyzed into 20mM Tris buffer, pH8.5, containing 200 mM NaCl, 1% ethylene glycol, and 1 mM TECP. NACK was then concentrated and sized on an S200 26/60 sizing column equilibrated in the same buffer.

Thermal Shift Assay

NACK was prepared in 10 mM HEPES buffer, pH of 7.3 containing 500 mM NaCl at a with a final protein concentration of 1 μ M. The Sypro dye diluted in DMSO was added for a final concentration of 5X. Compounds were all screened at a concentration of 10 μ M in 5% DMSO. 30 μ L of this solution was added to a 96-well PCR plate and the plate was placed into a Bio-Rad CFX96 real-time PCR system. Protein stability was monitored via Sypro orange dye with the FRET channel selected and heated at 0.5°C per 30 seconds from 25°C to 75°C. Fluorescence measurements were collected at every interval. T_M was determined using a Boltzmann distribution fit for one stage melting transitions. For two stage transitions, the T_M was determined by picking the temperature that corresponds with the largest change in fluorescence using first derivative analysis.

RT-qPCR Analysis

OE33 and OE19 cell lines (5x10⁶) were seeded in cell culture dish (60 mm) and exposed, after 24h, to either Z271-0326, or UM-74, or UM-94 (3 μ M) or DAPT (15 μ M) treatment for 6h. At the end of the treatment, total mRNA was isolated with Qiagen RNA extraction kit, and cDNA was synthesized according to the manufacturer's protocol (4368814; life Technologies). Gene expression was determined using RT-qPCR analysis using TaqMan probes (Applied Biosystems), followed by normalization to the expression of HPRT. As a control, TATA-binding protein ("TBP") gene expression was also monitored. Data analysis was performed using the GraphPad Prism software (Version 8.4.3).

CSL-DNA Affinity Pulldown (DAP) and Western Blot

In the CSL-DAP assay, HEK293T cells were co-transfected with N1ICD-FLAG, Maml1-FLAG and NACK-myc. Transfections were performed using the Lipofectamine2000 transfection reagent (Invitrogen) according to the manufacturer recommended protocol. After two days of transfection, cells were treated with compounds for 2 hours prior to collecting the cells. Nuclear cell lysates were incubated with DNA streptavidin beads, previously incubated with annealed 47 mer biotinylated dsDNA containing two high-affinity CSL binding sites facing forward or two mutated CSL binding sites³⁴. Proteins that bound to the beads were resolved by SDS-PAGE and blotted onto the nitrocellulose membrane. Blocking was performed for 1h at 4°C in TBST containing 5% non-fat dry milk. Membranes were then incubated with the primary antibodies: α -RBPSUH (CSL, Cell Signaling Technology, D104A), α -Maml1 (Cell Signaling Technology, D3K7B), α -Notch1val1744 (Cell Signaling Technology, 4147S), α -Myc-Tag (71D10) (Cell Signaling Technology, 22778), α -Flag-Tag (Millipore, F7425). Membranes were washed 3 times with TBST and incubated with HRP-linked secondary antibodies (Cell Signaling, mouse no. 7076/rabbit no. 7074) for 1 h at room temperature. The ECL- Western blotting substrate kit (Thermo Fisher, 32106) was used to detect HRP activity.

Chromatin Immunoprecipitation Assay

OE33 and OE19 cells were treated with either DAPT as a positive control, or DMSO (vehicle) as a negative control, or compounds for screening for 6 hours. After the treatment, cells were cross-linked with 1% formaldehyde for 10 min at 37°C and cross-linking was quenched by adding glycine to a final concentration of 0.125 M. Nuclear pellet was digested using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher). Nuclear pellet was digested using Micrococcal Nuclease (NEB) to yield chromatin fragments of approximately 300 to 800 bp and lysed in ChIP buffer (1% Triton, 2mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl pH 8 and 0.01% SDS) containing a protease inhibitor cocktail (Roche). Lysates were immunoprecipitated with either α -Notch1 (Bethyl Laboratories, Montgomery, TX; A301-894A), α -Maml1 (Cell Signaling, 12166s), α -NACK (Bethyl Laboratories, A302-675A), α -Pol II pSer5 (Abcam, ab5131) antibodies and a-IgG (Abcam) and were reverse cross-linked overnight at 65°C in 200 mM NaCl and proteinase K. DNA immunoprecipitates were cleaned using the PCR purification Kit (Qiagen) and HES1, HES5 or CCND1 promoter were amplified by qPCR. Primer sequences *HES1*:

forward: 5'CGTGTCTCCTCCTCCCATT3'.

reverse: 5'GGGGGATTCCGCTGTTAT3'.

HES5:

forward: 5'-GGGAAAAGGCAGCATATTGA-3'.

reverse: 5'-CACGCTAAATTGCCTGTGAA-3'

CCND1:

forward: 5'-GAAACTTGACAGGGGTTGT-3'

reverse: 5'-ATTTTCCAAGAACGCCACGAG-3'

Colony Formation Assay and Cell Viability Assays

Cells were cultured at low density (500 cells/well) under treatment in 6-well plates. Media containing inhibitor was changed every 48 hours. After 10–12 days, colonies were stained with 0.01% crystal violet and counted.

Tumor Sphere Formation Assay

EAC cell lines were cultured in DMEM/F12 with 2% B-27 serum-free supplement (17604-044; Invitrogen), 20 ng/mL epidermal growth factor (EGF; PHG0311L; Invitrogen), and 20 ng/mL basic fibroblastic growth factor (bFGF; PHG0266; Invitrogen) for 14 days. Resulting tumor spheres were examined and counted under the microscope. The tumor spheres were collected for further RT-qPCR analysis to test the effect of hit-to-lead compounds on the stem like target genes.

ADP-Glo Kinase Assay

ADP-Glo kinase assay was performed using the ADP-Glo™ Kinase Assay Kit (Promega, V9101) according to the manufacture's recommended protocol. Corresponding compounds (concentrations as indicated) or DMSO (2.5%) were co-incubated with recombinant NACK (0.3 μM). The in vitro ADP-Glo™ kinase assay was carried out in 384-well plate with 5μL total reaction volume to determine kinase activity. The primary kinase reaction was carried out for 90 minutes at 37°C. The reaction was terminated by adding an equal volume of the ADP-Glo Reagent. After the incubation for 40 minutes at room temperature, 10 μL of Kinase Detection Reagent was added. Luminescence was recorded after an additional incubation at room temperature for 30 minutes.

Surface Plasmon Resonance

A GST capture kit (GE Healthcare) was utilized to covalently immobilize an anti-GST antibody, provided in the kit, to the sensor chip surface (CM5 chip, GE Healthcare) by following the manufacturer's instructions.

Capture was performed by injecting GST-NACK (1 µg/µL) over the immobilized anti-GST antibody in Hepes buffer (10 mM Hepes, pH 7.5, 150 mM NaCl, 3 mM EDTA, 0.0005% NP-40) for 7 min at 10 µL/min. A reference flow cell was prepared by capturing GST following the procedure described above. The GST capture resulted in a stable baseline. Experiments were performed on a Biacore T200 instrument (GE Healthcare) at 25 °C. Small molecule binding to NACK was performed in 50 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 10 mM MgCl₂ and 5% DMSO (running buffer). The SPR signal arising from the sample was corrected for its respective control containing DMSO. Data visualization and analysis were performed using Biacore T200 software (GE Healthcare) and Origin 8.0 (OriginLab).

Animal Experiments

Five-to-six- week- old nude female mice were purchased from Charles River Laboratories. Procedures involving animals and their care conformed to institutional guidelines that comply with national and international laws and policies. To evaluate the effect of inhibitors on tumor growth, PDX cancer models and xenografts were established as described³⁵. When the tumor size reached 150–200 mm³, the mice were treated with daily dose of 15 or 25mg/kg for intraperitoneal injection (i.p.). 5 mice per treatment/5 mice per negative control. Vehicle used for i.p.: DMSO. According to the IACUC-approved protocol, the stop/end point criteria are based on the days it takes for tumors to reach 2000 mm³. Tumor volume was measured by the formula: volume = (S × S × L)/2. Animal experiments were reviewed and approved by the University of Miami Institutional Animal Care and Use Committee (Miami, FL, USA).

Tissue Sample Preparation, PAS Staining Immunohistochemistry

C57BL/6 mice were injected intraperitoneally with 40mg/kg of NACK inhibitors and DZB, daily for 5 days. Tissue sample preparation and PAS staining immunohistochemistry were performed as previously described²¹.

Statistics

Statistical Analyses Graphs and statistics were generated using the GraphPad Prism 7 with default parameters. The significance of differences between groups was evaluated by using, unpaired t-test, one- or two-way analysis of variance (ANOVA). A p-value of < 0.05 was considered statistically significant. The figure legend specifies the method used and p-values. All cell-based experiments were performed at least three times in triplicate.

Computational Methods

All computational experiments were performed using the Schrodinger 2019-1 software suite via the Maestro user interface²⁸. Homology modeling was accomplished using PRIME²⁹. Two models were generated using 5VE6 as the template and 6EWX and 6BHC were selected as homologues. Each model was evaluated for stability across a 1 µs all atom explicit water MD simulation. The model that demonstrated the best stability throughout the experiment was selected for additional studies. ATP was

docked into this model through alignment of CASK (PDBID: 3MFS) complexed with AMP-PNP, followed by Induced Fit Docking of ATP into the NACK structure model^{30, 31}. Viable poses were subjected to 200 ns MD simulations where ligand pose stability as well as number of predicted conserved putative interactions and length of subsequent reactions was evaluated to determine the best predictive pose of ATP bound to NACK.

A type-II NACK model was generated using PRIME where the activation loop “DFG out” conformation of LCK bound with imatinib (PDBID:2PL0) was aligned with the NACK virtual model and threaded into the NACK template using the Splice tool. This model was then minimized and a 1 μ s MD simulation was performed to evaluate model stability. Several frames were clustered and utilized for docking of Z271-0326 and analogues. All docked poses of compounds in NACK were evaluated for stability through a 200 ns MD simulation.

Molecular Dynamic simulations were performed using GPU accelerated Desmond version 2019-1 (DE Shaw Research) on Tesla M40 GPUs³². Systems were created with a 10 Å orthorhombic box with SPC solvation model and the OPLS3e force field. MD simulations were performed as isothermal isobaric (NPT) ensemble at 300K and 1.01325 bar over a production time frame between 200 ns to 1 μ s. Prior to the production run the Desmond multi-sim workflow included a series of minimization and short simulation steps to relax the system.

General Chemistry Methods

All general solvents were purchased from VWR international and used as is without further purification. Anhydrous solvents and deuterated solvents were purchased from Sigma-Aldrich and utilized directly. Building blocks were sourced from VWR international, Sigma Aldrich, and Enamine. Analytical thin layer chromatography was performed on glass backed silica gel 60 F254 plates purchased from VWR international. Flash column chromatography was performed on Biotage Isolera One or Biotage Isolera Selekt automated silica gel chromatography. Analytical LC was performed on a semi-preparative Shimadzu prominence UFLC with a C18 column (5 micron, 4.6 x 150 mm). ¹³C NMR spectra were recorded on a Bruker AVIII HD 600 MHz spectrometer located at The Scripps Research Institute Florida NMR facility. ¹H NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer at The University of Miami NMR laboratory. ¹H and ¹³C chemical shifts are reported in δ values in ppm utilizing deuterated trace solvent signal as an internal standard. Data are reported as follows: chemical shift, multiplicity, coupling constant (Hz), integration. Splitting patterns multiplicities and are annotated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad), dd (doublet of doublet), dt (doublet of triplet), qd (quartet of doublet). Low-resolution mass spectra were obtained on a Shimadzu LCMS 2020 system with electrospray ionization. High-resolution mass spectrometry analysis was conducted at Florida International University Mass Spectrometry Facility on a Bruker ultrOTOF-Q high resolution mass spectrometer operating at full scan (200–950 Da) in positive (+) and negative (-) mode. Internal calibration was used for each sample using ions from the Agilent ESI-L tuning solution.

Declarations

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Competing interests: AJC is a co-founder of StemSynergy Therapeutics, Inc. which has licensed the Z271-0326 scaffold from the University of Miami.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from

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Figures

Figure 1. ATP is required for NACK recruitment to the NTC.

Figure 1

NACK binds and hydrolyses ATP molecule, which enhances its recruitment to the NTC. a, Determination of NACK/ATP binding affinity using isothermal analysis of thermal unfolding data. The thermal unfolding of NACK is monitored using SYPRO Orange. A significant increase in T_m is demonstrated when NACK is treated with 3 mM ATP. T_m Vehicle control = 44.05 °C $\pm$ 0.5 °C, T_m NACK with 3 mM ATP = 47.82 °C $\pm$ 0.4 °C. b, The binding between NACK and ATP analog AMP-PNP using surface plasmon resonance (SPR). c, NACK ATPase activity was determined by ADP-Glo assay. Kinase reactions were performed with 0.3 μ M of NACK WT and K1024A protein, at 37 degrees, in a time-dependent and, d, ATP dependent experiment. Error bars indicate the range of kinase activity in triplicate reactions. Data are displayed as averages and standard error of the mean. e, DNA pull-down of nuclear lysate from HEK293T transiently transfected with Notch1ICD-Flag, MAML1-Flag, and NACK-myc in presence of ATP, GTP, AMP-PNP or GTP-rs (100 μ M).

Figure 2

Virtual Screening Approach to Identify Z271-0326. a, The most representative structure of NACK and ATP during a 200 ns MD simulation. b, Structure-based virtual screening pipeline utilized to discover hit compound Z271-0326. c, Potential NACK inhibitors were screened in DNA pull-down, using nuclear lysate from HEK293T transiently transfected with Notch1ICD-Flag, MAML1-Flag and NACK-Flag. d, The

predicted 3-D binding pose of Z271-0326 in NACK. e, Predicted 2-D interactions of Z271-0326 residues with NACK.

Figure 3. Z271-0326 binds to the NACK protein, affects NACK ATPase activity, and prevents NACK binding to the NTC.

Figure 3

Z271-0326 binds to the NACK protein, affects NACK ATPase activity, and prevents NACK binding to the NTC. a, DNA pull-down of nuclear lysate from HEK293T, transiently transfected with Notch1ICD-Flag, MAML1-Flag, and NACK-myc, pretreated for two hours with Z271-0326 10μM. The lysate was then

either incubated or not with 100μM of ATP. b, Determination of NACK/Z271-0326 binding affinity using Thermal shift assay and c SPR. d, Kinase assays were performed with 0.3μM of NACK WT, at 37 degrees at the indicated time points and e ATP concentrations. *P < 0.05. Statistical analysis was performed using two-way ANOVA.

Figure 4. Effect of small molecule inhibitors in EAC cell lines.

Figure 4

Effect of small molecule inhibitors in EAC cell lines. a, mRNA levels of NACK and Notch1 of clinical samples derived from surgically resected primary EAC. Tumor tissue is compared to its normal tissue. b, High levels of NACK and activated Notch1 expression are both observed in chemo-naïve esophageal adenocarcinoma samples from endoscopic ultrasound (EUS) biopsies. c, Colony formation assays were performed in OE19 EAC cell lines under treatment of different concentration of Z271-0326 every other day for ten days. The colony number was quantified and compared to the control (DMSO). d, mRNA levels of HES5, CCND1, or TBP gene expression of OE19 treated for 6h with DMSO, DAPT, or Z271-0326 were analyzed and normalized to HPRT1. e and f, ChIP assay on the HES5 and CCND1 promoter using the indicated antibodies (n=3) Error bars are representative of three independent experiments, and values indicate mean $\pm$ SD. p values ≤ 0.05 are considered statistically significant and indicated by an asterisk. *P ≤ 0.05 ; **P ≤ 0.01 ; ***P ≤ 0.001 .

Figure 5. Validation of analogs 74 and 94 in cell based-assays.

Figure 5

Validation of analogs UM-74 and UM-94 in cell-based assays. a, Colony formation assays were performed in OE19 EAC cell lines under treatment of different concentration of Z271-0326, UM-74 and UM-94 every other day for ten days. The colony number was quantified and compared to the control (DMSO). b, mRNA levels of HES5, CCND1, or TBP gene expression of OE19 treated for 6h with DMSO, DAPT, Z271-0326, UM-74 and UM-94 were analyzed and normalized to HPRT1. c, Tumor sphere formation assays were

performed in OE19 treated every other day with DAPT (15 μ M), Z271-0326, UM-74, and UM-94 (3 μ M). d, mRNA levels of Notch targets were then determined in qPCR. Unpaired two-tailed t-test was used to determine significance. Error bars are representative of three independent experiments, and values indicate mean $\pm$ SD. p values $\leq$ 0.05 are considered statistically significant and indicated by an asterisk. *P $\leq$ 0.05; **P $\leq$ 0.01; ***P $\leq$ 0.001; ns (no significant difference). e, DNA pull-down of nuclear lysate from HEK293T, transiently transfected with Notch1ICD-Flag, MAML1-Flag, and NACK-myc, pretreated for two hours with 10 μ M of Z271-0326, UM-74, UM-94 or DMSO. The lysate was then either incubated or not with 100 μ M of ATP.

Fig 6. Validation of our scaffold in the inhibition of tumor growth in PDX model.

Figure 6

Validation of Z271-0326 scaffold in the inhibition of tumor growth in a PDX model. a, Tumor volume over time upon treatment (treatment for 34-32 days) of EAC PDX with DMSO (vehicle), Z271-0326 and UM-74 (15 mg/kg). Drugs were given daily by intraperitoneal injection. Statistical analysis was performed using two-way ANOVA, * $P < 0.05$. b, Tumor weights collected at end point ($n = 5$). c, Body weights of EAC47 PDX during treatment. d, PDX EAC47 treated with UM-94 (40 mg/kg). Data are displayed as averages and

standard error of the mean. Unpaired two-tailed t-test was used to determine significance * $P < 0.05$). e, Immunohistochemical analysis of the small intestine from mice treated with DMSO, DBZ, Z271-0326, UM-74 and UM-94 at 40mg/kg for 5 days. The absence of goblet cells is shown by Periodic acid-Schiff staining (PAS) (upper panel). H&E staining in the lower panel.

Supplementary Files

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