Positive selection CRISPR screens reveal a druggable pocket in an oligosaccharyltransferase required for inflammatory signaling to NF-κB
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Graphical abstract

Highlights
- Loss of OST-A subunit STT3A, but not its paralog STT3B, blocks LPS signaling to NF-κB
- STT3A is required for N-glycosylation and membrane localization of the LPS receptor TLR4
- CRISPR base editing and cryo-EM defined a drug-binding pocket in STT3A
- NGI-1 prevents a productive binding pose of the glycan donor in the active site of STT3A

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In brief
Innovative approaches identify druggable glycosylation processes that regulate TLR4-mediated activation of the pro-inflammatory transcription factor NF-κB.
Positive selection CRISPR screens reveal a druggable pocket in an oligosaccharyltransferase required for inflammatory signaling to NF-κB

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SUMMARY

Nuclear factor κB (NF-κB) plays roles in various diseases. Many inflammatory signals, such as circulating lipopolysaccharides (LPSs), activate NF-κB via specific receptors. Using whole-genome CRISPR-Cas9 screens of LPS-treated cells that express an NF-κB-driven suicide gene, we discovered that the LPS receptor Toll-like receptor 4 (TLR4) is specifically dependent on the oligosaccharyltransferase complex OST-A for N-glycosylation and cell-surface localization. The tool compound NGI-1 inhibits OST complexes in vivo, but the underlying molecular mechanism remained unknown. We did a CRISPR base-editor screen for NGI-1-resistant variants of STT3A, the catalytic subunit of OST-A. These variants, in conjunction with cryo-electron microscopy studies, revealed that NGI-1 binds the catalytic site of STT3A, where it traps a molecule of the donor substrate dolichyl-PP-GlcNAc2-Man9-Glc3, suggesting an uncompetitive inhibition mechanism. Our results provide a rationale for and an initial step toward the development of STT3A-specific inhibitors and illustrate the power of contemporaneous base-editor and structural studies to define drug mechanism of action.

INTRODUCTION

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signal transduction pathway regulates many physiologic processes in mammals, such as inflammation, immunity, and development.1-3 NF-κB signaling can be initiated by extracellular stimuli that bind to specific cell-surface receptors,1 such as bacterial lipopolysaccharide (LPS) and its cognate receptor Toll-like receptor 4 (TLR4),4-9 CpG oligonucleotide and its receptor TLR9,10 and tumor necrosis factor alpha (TNF-α) and the TNF receptor.11-13 These diverse upstream signaling events eventually converge on the NF-κB family of inducible transcription factors (p65 [RelA], c-Rel, RelB, p50/p105 [NF-κB1], and p52/p100 [NF-κB2]) that are sequestered in the cytoplasm at baseline.14 Upon pathway activation, they are released, translocate to the nucleus, dimerize, and activate transcription after binding to genomic NF-κB response elements.

Given these above roles, it is unsurprising that hyperactivation of the NF-κB pathway is implicated in many human disease states, including sepsis,15 cancer (particularly lymphoma),16 and autoimmune disease.17 Multiple drugs inhibit NF-κB signaling, including anti-TNF therapies, which are mainstays for treating multiple autoimmune diseases,18; Bruton’s tyrosine kinase inhibitors, which are used to treat various lymphomas and leukemias;19; and steroids/non-steroidal anti-inflammatory drugs (NSAIDs), which are used to manage certain symptoms caused by infections or autoimmune diseases.20,21

To ask whether any potential therapeutic targets remain to be discovered in this important pathway, we did a forward genetic screen to identify all genes whose loss abrogates LPS signaling to NF-κB. Such “pathway screening,” which can potentially identify all genes in a signaling pathway from the stimulus/cell membrane receptor to the effector/transcription factor, has been done in the past, such as for TNF-α signaling to NF-κB.22 Most of these prior genetic screens, however, used loss-of-function assays in which hits were identified based on a decrease of a cellular readout (e.g., decreased transcriptional activity or decreased viability). Such screens can be confounded if the perturbants tested (whether chemical or genetic) cause, either as on-target or off-target effects, a general decrease in cellular
fitness. Positive selection screens typically have better signal-to-noise characteristics and statistical power than negative selection (e.g., single-guide RNA [sgRNA] dropout) screens and help mitigate the risk of identifying non-specific assay positives that globally impair cellular fitness. We therefore endeavored to create a dual reporter system in which NF-κB transcription factors drive the expression of a modified suicide gene and green fluorescent protein (GFP), allowing this system to be used bidirectionally in positive selection screens for perturbants that either abrogate or activate NF-κB signaling.

RESULTS

Development of a positive selection screen for genes required for NF-κB activation

Toward this end, we made a lentivirus that encodes (1) a modified deoxycytidine kinase (DCK*) and (2) GFP from a bicistronic mRNA driven by an artificial NF-κB-responsive promoter (Figure 1A). DCK* converts the non-natural and non-toxic nucleoside bromovinyl deoxyuridine (BVdU) into a toxin.24 We reasoned that increasing NF-κB activity would sensitize cells bearing this reporter to BVdU, whereas decreasing NF-κB activity, such as by inactivating genes required for signaling to NF-κB, would have the opposite effect.

We introduced our reporter (NF-κB-DCK*) or, as a control, a bicistronic reporter containing a constitutive promoter (human ubiquitin C [UBC]) instead of the NF-κB-responsive promoter (UBC-DCK*) (Figure 1A), into NALM-6 lymphoblastic leukemia cells by lentiviral infection. We also infected these cells to stably produce Cas9 to facilitate subsequent gene editing. Successfully infected subclones were isolated by drug selection, followed by fluorescence-activated cell sorting (FACS) in the presence of LPS, which activates NF-κB and therefore induces GFP expression.

As expected, treatment of NALM-6 cells harboring the NF-κB-DCK* reporter with LPS increased the expression of both DCK* and GFP, as determined by immunoblot and flow cytometry, respectively, in contrast to the constitutive expression of DCK* and GFP in NALM-6 cells harboring the UBC-DCK* reporter (Figures 1B and 1C). To further enhance the dynamic range of this assay, we inactivated the endogenous NF-κB inhibitor TNFAIP325 using CRISPR-Cas9, which led to an approximately 2-log difference in GFP intensity between the unstimulated and stimulated NF-κB-DCK* cells (Figure 1B). The induction of DCK* in the NF-κB-DCK* cells by LPS was NF-κB-dependent because inactivation of MYD88, which is required for signaling from the LPS receptor TLR4 to NF-κB,26 largely abolished this effect (Figure 1C). Differences in DCK* expression mirrored changes in BVdU sensitivity. Specifically, LPS increased the sensitivity of the NF-κB-DCK* cells, but not the UBC-DCK* cells, to BVdU. Upon exposure to LPS, the shift in the BVdU IC50 value in the TNFAIP3−/− NF-κB-DCK* cells approached 2 logs (Figure 1D).

In a proof-of-concept experiment, we lentivirally infected both the TNFAIP3−/− NF-κB-DCK* cells and TNFAIP3−/− UBC-DCK* cells so that they would either produce mCherry and an MYD88 sgRNA or produce blue fluorescent protein (BFP) and a control sgRNA (sgctrl). We mixed the mCherry-positive and BFP-positive cells at a ratio of approximately 1:99, treated them with LPS in the presence or absence of BVdU, and monitored the ratio of mCherry cells to BFP cells by FACS. Treatment of the TNFAIP3−/− NF-κB-DCK* cells for 20 days with BVdU and LPS led to an approximately 60-fold enrichment of the mCherry cells, consistent with loss of NF-κB activity conferring a fitness advantage in this setting (Figure 1E). This was specific because virtually no enrichment was seen in the cells treated with LPS alone, and virtually no enrichment was observed in the TNFAIP3−/− UBC-DCK* cells treated with LPS, irrespective of BVdU exposure (Figures 1E and 1F).

Identification of genes required for activation of NF-κB by LPS

Encouraged by these findings, we infected the TNFAIP3−/− NF-κB-DCK* cells and TNFAIP3−/− UBC-DCK* cells with a lentiviral whole-genome sgRNA library (76,441 guides targeting 19,114 genes with approximately four guides per gene)27 and, after puromycin selection, treated them with LPS and BVdU. We monitored sgRNA abundance over time by next-generation sequencing (NGS), with a particular focus on genes whose sgRNAs were positively selected (“hits”) (Figure 2A; Tables S1 and S2). Gratifyingly, the TNFAIP3−/− NF-κB-DCK* screen, but not the TNFAIP3−/− UBC-DCK* screen, identified over a dozen genes encoding proteins in the canonical signaling pathway used by LPS to activate NF-κB (Figure 2B). Intriguingly, the rank order of these hits loosely approximately whether they acted proximally or distally to LPS, with TLR4 scoring as the top hit and NF-κB scoring near the bottom of the hits using a p value of 10−5 as a cutoff (Figure 2A). Conceivably, this pattern reflects an increasing diversity of TLR4-independent functions as one moves downstream of TLR4, including functions that partially compromise cellular fitness when lost. Whether this pattern will be seen with similar screens for other signal transduction pathways awaits further study.

We next infected the TNFAIP3−/− NF-κB-DCK* cells with a custom lentiviral sgRNA library targeting the top 500 hits from the primary TNFAIP3−/− NF-κB-DCK* screen (10 sgRNAs/hit). This library also included 50 guides targeting five essential genes, 500 non-targeting guides, and sgRNAs against all human TLRs and both TNF-α receptors (TNFRSF1A and TNFRSF1B). After puromycin selection, the cells were treated with BVdU and one of three NF-κB agonists (LPS, CpG, or TNF-α) for 16 days, after which sgRNA enrichment was again detected by NGS (Figure 2C; Table S3). CNPY3, which acts as a co-chaperone for TLR4, was the top-scoring hit in the LPS screen, followed closely by TLR4 itself. All genes that tested positively in the original LPS screen at a p value cutoff of 10−5 were retested positively with the custom library in the LPS-treated cells.

As expected from knowledge of their respective signal transduction pathways, some hits from the CpG and TNF-α screens were unique to these NF-κB agonists, while others were shared by two or more of the three NF-κB agonists tested (Figure 2C). Gratifyingly, the CpG receptor TLR9 and the TNF receptor were among the top hits in the CpG and TNF-α screens, respectively (Figure 2C). We elected to focus further on the oligosaccharyltransferase A (OST-A) catalytic subunit STT3A, which was the top druggable hit in the LPS primary and secondary screens not previously linked to TLR4-mediated signaling. Mammalian cells express two OST enzymes (OST-A and
OST-B) that have distinct catalytic subunits (STT3A or STT3B) and perform distinct cellular functions. OST-A catalyzes co-translational N-glycosylation of N-x-S/T sequons and is responsible for the majority of N-glycans attached to acceptor proteins, whereas OST-B catalyzes post-translational N-glycosylation of those sequons skipped by OST-A, including sequons in loops connecting transmembrane helices, in proximity to disulfide bridges, or at the C terminus. We also further studied two additional hits from the LPS screens: DC2 (which encodes an adaptor subunit that interacts directly with STT3A and the translocation channel Sec61 during OST-A-catalyzed co-translational glycosylation) and CCDC134 (which encodes a coiled-coil domain protein that itself is glycosylated and reportedly exists in both secreted and intracellular forms). In secondary validation assays, inactivation of STT3A, DC2, or CCDC134 in the TNFAIP3−/− NF-κB-DCK* cells engineered to produce hemagglutinin (HA)-tagged TLR4 and in parental NALM-6 cells. In both cases, inactivation of either STT3A or CCDC134 decreased cell-surface TLR4 levels, which was corroborated by immunoblot analysis of proteins captured on streptavidin agarose after cell-surface biotinylation. HSP90B1, which is a chaperone required for folding and trafficking of TLR4 to the cell surface, served as a control. HSP90B1 scored positively in the primary LPS screen but not as strongly as STT3A and CCDC134, presumably because it, unlike STT3A and CCDC134, is a common essential gene according to the Cancer Dependency Map (DepMap). STT3A or CCDC134 loss downregulated HSP90B1, which is known that STT3A loss causes the paradoxical hyperglycosylation and destabilization of HSP90B1.

Regulation of TLR4 glycosylation and surface localization by OST-A

TLR4 contains nine N-glycosylation sequons, and two of them (Asn526 and Asn575) are essential for cell-surface localization of TLR4. In our immunoblot analysis of whole-cell lysates, we observed two bands for HA-TLR4. To confirm that the
upper band corresponds to glycosylated TLR4, we treated the cell lysates with two different glycosidases: PNGase F, which cleaves all types of N-glycans at the site of attachment to asparagine, and endoglycosidase H (Endo H), which cleaves the glycosidic bond between the two N-acetylglucosamine (GlcNAc) units of high-mannose N-glycans. PNGase F, but not Endo H, partially cleaved the N-glycans attached to TLR4 (Figure 3C), as revealed by its enhanced electrophoretic mobility. Inactivation of STT3A, CCDC134, or HSP90B1 also resulted in hypoglycosylation of TLR4 (Figures 3B and 3C). Although CCDC134 has been reported to be O-glycosylated,40,41 the data in Figures 3C and S1 argue that it is also N-glycosylated on Asn148, although this N-glycosylation does not appear to be required for CCDC134 function in the NF-κB signaling pathway. The residual glycosylation of TLR4 observed in Figure 3C in cells lacking CCDC134 or HSP90B1 could imply that CCDC134, like HSP90B1, primarily affects TLR4 folding, which indirectly affects TLR4 glycosylation.

Notably, HA-TLR4 glycosylation was dramatically reduced in HEK293T cells lacking the catalytic subunit of OST-A (STT3A), but not of OST-B (STT3B), by virtue of CRISPR-Cas9-based gene editing (Figure 3D). This, and the fact that STT3B did not score in our screen, suggests that control of TLR4 glycosylation and NF-κB signaling is a specific attribute of OST-A. By contrast, loss of STT3B, but not STT3A, impaired the glycosylation of the glucose transporter glucose transporter 1 (GLUT1), in keeping with earlier studies (Figure 3D).30,42 Knockout of STT3B did not abrogate LPS-mediated induction of NF-κB activity in the TNFAIP3/C0/C0 NF-κB-DCK* NALM-6 cell line (Figure 3E). Furthermore, knockout of Stt3a in murine macrophages using CRISPR-Cas9 also led to the hypoglycosylation of endogenous Tlr4 and reduced the induction of the endogenous NF-κB target inducible nitric oxide synthase (iNOS) by LPS, with the most effective Stt3a sgRNAs having the greatest effects on Tlr4 glycosylation and NF-κB activation (Figures 3F and 3G). Therefore, STT3A regulates both endogenous and exogenous TLR4 in multiple species.

Loss of TLR4, as expected, blocked signaling by LPS to NF-κB (Figure 2D). A chimera of the CD16 extracellular domain fused to
The transmembrane and intracellular domains of TLR4 localize to the cell surface (Figure S2) and activates NF-κB signaling (Figure 3H). Activation of NF-κB by the CD16-TLR4 fusion protein was STT3A independent (Figure 3H). Therefore, diminished induction of NF-κB by LPS in STT3A/C0/C0 cells is specifically due to loss of TLR4 on the cell surface.

TLR4 hypoglycosylation and abrogated NF-κB signaling upon treatment with pan-OST- and OST-A-specific inhibitors

Reintroduction of wild-type STT3A, but not a catalytic-dead version of STT3A, rescued TLR4 glycosylation in HEK293T cells in which endogenous STT3A was eliminated by CRISPR-Cas9 (Figure 4A). N-linked glycosylation inhibitor 1 (NGI-1) is a compound that inhibits the catalytic activity of OST enzymes. It inhibited TLR4 glycosylation and induction of iNOS by LPS in a time- and dose-dependent manner (Figures 4B and 4C). NGI-1 also blocked the LPS-induced activation of the NF-κB-responsive reporter in TNFAIP3/C0/C0 NF-κB-DCK* cells with an IC50 between 1 and 10 μM (Figure 4D). Remarkably, NGI-1, despite being a tool compound that reduces N-glycosylation of multiple proteins and that may still carry potentially harmful off-target effects on as yet unknown targets, substantially protected LPS-treated TNFAIP3/C0/C0 NF-κB-DCK* cells from BVdU-induced cell death (Figure 4E).

NGI-1 partially inhibits both OST-A and OST-B and, perhaps as a result, did not phenocopy genetic ablation of STT3A (Figure 4F). In particular, at higher doses (10 μM), NGI-1, like the toxin tunicamycin, decreased the glycosylation and cell-surface localization of TLR4, TNF-R1, and CD19, in contrast to STT3A ablation, which only affected TLR4. At lower doses (1 μM), however, NGI-1, like STT3A ablation, preferentially affected the glycosylation and cell-surface localization of TLR4 relative to TNF-R1 and CD19 (Figure 4F). These findings, together with the data in Figure 3D, suggested that TLR4 glycosylation is particularly sensitive to loss of STT3A activity relative to loss of STT3B activity. We therefore sought an OST-A-specific inhibitor.
NGI-1 analogs with OST-B-specific activity have been identified; however, OST-A-selective inhibition has remained elusive. In hopes of achieving the latter, we synthesized and tested NGI-1 analogs with substitutions to the thiazole (R1), pyrrolidine (R2), or sulfonamide (R3) groups, followed by testing for OST-A or OST-B preference, using wild-type, STT3A knockout, or STT3B knockout cells expressing the Halo3N glycoprotein as a readout. Among these analogs, NGI-235 preferentially inhibited OST-A compared with OST-B. We confirmed this apparent selectivity by examining the glycosylation of various endogenous dual STT3A/B substrates (SSR1, LAMP1, GP130, and integrin β1), with NGI-235, in contrast to NGI-1, having little or no effect on their glycosylation in STT3A knockout cells.

We compared the pharmacodynamic effects of NGI-235 to NGI-1 in our NALM-6 cells overexpressing TLR4. NGI-1 caused the hypoglycosylation of both TLR4 and GLUT1, in keeping with its dual activity against OST-A and OST-B. By contrast, NGI-235 selectively inhibited TLR4 glycosylation and cell-surface localization without altering GLUT1 glycosylation. To further validate this selectivity with additional substrates, we confirmed, using appropriate knockout cells, that prosaposin is a specific STT3A substrate and that a GFP variant, GFP Q185N,N186C, with an N-terminal endoplasmic reticulum (ER) retention signal sequence attached (ER-GFP Q185N,N186C), is a specific STT3B substrate. NGI-235 inhibited the glycosylation of prosaposin and TLR4, but not the glycosylation of GLUT1 and ER-GFP Q185N,N186C, while NGI-1 impaired the glycosylation of prosaposin, TLR4, GLUT1, and ER-GFP Q185N,N186C. Therefore, NGI-235 can be viewed as a prototype OST-A inhibitor that can blunt NF-κB signaling.

Identification of drug-resistant mutants in STT3A using a CRISPR base-editor-screening approach

How NGI-1 and NGI-235 physically interact with OST-A was not known, which would delay or prevent OST-A from being pursued as a therapeutic target. In this regard, neither NGI-1 nor NGI-235...
have been optimized for in vivo studies. Moreover, it has not been proven that these compounds directly bind to the STT3A catalytic subunit. We therefore pursued both genetic and biochemical approaches, using NGI-1 to address these knowledge gaps.

For the genetic approach, we aimed to mutagenize the endogenous STT3A gene using CRISPR-Cas9 base editing in an effort to identify drug-resistant mutants. We designed a custom sgRNA library that tiled the entire coding sequence of STT3A (encoding 705 amino acids) with 1,299 sgRNAs (Table S4). We paired this sgRNA library with a protospacer adjacent motif (PAM)-flexible Cas9 variant that (1) only requires an NG PAM site (NG-Cas9) and (2) was linked to either an APOBEC moiety allowing for cytosine-to-thymidine (C/T) base editing or to an ABE moiety allowing for adenine-to-guanine (A/G) base editing. We introduced either the A/G or C/T libraries into TNFAIP3/−−/ NF-κB-DCK∗ NALM-6 cells that, unlike the cells used for our previous screens, lacked wild-type Cas9 (Figure 5A).

After puromycin selection, the cells were treated with either DMSO or NGI-1 for 24 h, followed by LPS. 24 h later, we sorted for the top 1% persistently GFP bright cells (indicative of NF-κB activation). These cells were recovered, expanded, and subjected to a second round of positive selection as above. The sgRNAs in the persistently bright cells were then identified by NGS. Analysis of relative sgRNA abundance in the A → G base-editor screen identified three clusters of sgRNAs that scored strongly compared with the DMSO-treated control (cluster 1: N217–L224, cluster 2: F256–E266, and cluster 3: F330–Y338) (Figure 5B; Table S4). The data from the C → T base-editor screen did not produce such obvious clusters, although individual guides that scored positively in this screen did fall within the regions that scored highly in the A → G screen (Figure 5C; Table S4). We hypothesized that sgRNAs enriched in the NGI-1-treated arm mutated STT3A such that it remained active in the presence of NGI-1 and thereby preserved NF-κB signaling.

To empirically determine which mutation(s) they created, we reintroduced a subset of the top-scoring guides from the two screens (A → G and C → T), one sgRNA at time, into TNFAIP3/−−/ NF-κB-DCK∗ NALM-6 cells, along with the NG-Cas9 base editor. We then sequenced the endogenous STT3A locus. When the top-scoring guide in the A → G screen was
reintroduced into cells, 62.1% of sequenced reads revealed nucleotide substitutions (Figure 5D). The most common substitutions, leading to F330S and Y331H mutations in STT3A, were predicted based on the editing window of this NG-Cas9-ABE base editor, but we also identified less abundant substitutions that caused F330P, F330L, and S332P mutations due to editing outside of the predicted window (Figure 5D). The top-scoring guide from the C → T screen was a less efficient editor (4.4% of reads demonstrating nucleotide substitutions) (Figure 5E). This guide was predicted and confirmed to introduce an E266K mutation into STT3A (Figure 5E). Analysis of additional sgRNAs in this fashion (Table S5) showed that most of them introduced their predicted mutations. Accordingly, we tested a subset of the top-scoring STT3A mutants, whether empirically determined (Figures 5E and 5F) or predicted (Table S5), in functional assays.

Specifically, we stably reintroduced wild-type STT3A or the putative drug-resistant STT3A mutants into HEK293T cells in which endogenous STT3A was deleted, using CRISPR-Cas9, and which also overexpressed HA-tagged TLR4. Both wild-type STT3A and the drug-resistant STT3A mutants robustly restored the surface expression of TLR4 in the absence of NGI-1 (Figure S5), indicating that the mutations did not affect catalytic activity. As expected, treating HEK293T cells expressing exogenous wild-type STT3A with 10 μM NGI-1 for 48 h profoundly decreased cell-surface TLR4 levels (Figure 5F) and TLR4 glycosylation (Figure 5G). By contrast, the glycosylation and cell-surface localization of TLR4 in NGI-1-treated cells was rescued by STT3A mutants in each of the three clusters identified in our screens, with the F256P, Q260R, E266K, and Y331H conferring the greatest NGI-1 resistance (Figures 5F and 5G). These results were specific for TLR4, as these STT3A mutants did not rescue the NGI-1-induced glycosylation defects of GLUT1, consistent with GLUT1 being a substrate of OST-B but not of OST-A. The identified clusters were located in proximity to the proposed lipid-linked oligosaccharide (LLO) binding site and to the external loop 5 (EL5) that connects TM9 and TM10—regions that are essential for OST function.

**Structure of OST-A with bound NGI-1**

To determine the binding site and precise interaction of NGI-1 with STT3A, we pursued structural studies of human OST-A bound to NGI-1 (Figure S6). We purified the endogenous OST-A complex using a Flp-In-HEK293T cell line carrying a tagged copy of the DC2 gene, a subunit of OST-A. OST-A was purified in the presence of the detergent digitonin and 5 μM NGI-1, which was increased to 100 μM NGI-1 prior to cryo-grid preparation.

We obtained an electron microscopy (EM) density map resolved at an overall resolution of 3.6 Å, with the local resolution of the transmembrane regions and of the active site reaching ~3.0 Å (Figure S6). We observed well-defined density for a single molecule of NGI-1 bound to the active site of STT3A, where it interacts with residues of the EL5 that connects TM9 and TM10, and with a single molecule of the native LLO (dolichyl-PP-GlcnAc2-Man9-Glc3) co-purified with OST-A (Figures 6A and 6B). The EM density for bound LLO was of excellent quality and covered eight isoprenoid units (forty carbons) of the dolichyl tail, the pyrophosphate group, and the A-branch of the oligosaccharide, which provides the main recognition elements of the glycan moiety (Figure 6B). We also observed density close to the pyrophosphate group, which we assigned to a manganese ion, an essential cofactor of OST (Figure 6A).

The structural findings allowed us to rationalize the drug-resistant phenotype displayed by most of the mutants identified in our base-editor screen. Clusters 2 and 3 contain residues that form the binding pocket of the sulfonamide group of NGI-1 (Figure 6C). They include Phe256, Phe330, Gln260, and Glu266 (Figure 6D), residues whose mutation resulted in the strongest drug-resistant phenotypes in the base-editor screen (Figures 5F and 5G). We performed mutagenesis of these residues and found that all mutations of Glu266, as well as mutations introducing charged side chains replacing Phe256, rendered STT3A drug resistant (Figure 6E), suggesting that these residues play an important role in NGI-1 recognition. For Glu260, only the mutation identified in the base-editor screen (Q260R) conferred drug resistance, possibly because the side chain of the arginine could reach into the binding pocket of NGI-1 (Figures 6D and 6E). Residues Ile346 and His352 are close to the 5-methyl-aminothiazole and the N-pyroridoline groups of NGI-1 (Figure 6D). Mutations of Ile346 caused a drug-resistance phenotype, whereas mutations of His352 did not. Notably, the H352Y mutant was less active than wild-type STT3A, which suggests that His352 has a previously unrecognized role in catalysis (Figures 6E and 5C). Our structure could not readily explain the phenotype of some of the mutants resulting from the base-editor screen, including residues in cluster 1 or the mutation Y331H in cluster 2. We speculate that these mutations alter binding pocket topology and thus indirectly impair NGI-1 binding. Despite this, the base-editor screen proved highly successful in mapping the drug-binding pocket of NGI-1 within OST-A.

**Inhibitory mechanism of NGI-1**

We previously showed that the catalytic cycle of OST can either be initiated ("primed") by binding of the sequon of an acceptor protein ("peptide-primed") or by binding the donor LLO dolichyl-PP-GlcNAc2-Man9-Glc3 ("LLO-primed"). In the LLO-primed state, the interactions with the catalytic STT3 subunit entail a specific geometry of bound LLO, including electrostatic interactions with the pyrophosphate group and polar interactions with the GlcNAc2 group at the reducing-end of the glycan (Figure 7A). Our structure of NGI-1-bound OST-A revealed that the pyrophosphate group is partially displaced compared with the LLO-primed structure of the yeast enzyme. In addition, the first five sugar units (two GlcNAc and the first three mannose units of the A-branch) are shifted and tilted, whereas the other mannoses and terminal glucoses superimpose well with those of the LLO-primed conformation (Figure 7B). This change in LLO geometry and binding pose has functionally important consequences. First, Tyr530 (corresponding to S. cerevisiae Y521) cannot form a hydrogen bond with the acetamido group of the reducing-end GlcNAc. This interaction is of key importance and conserved not only in eukaryotic OST enzymes but also in the bacterial PglB, where it is essential for activity (Figures 7A and 7B). Second, the electrostatic interaction between Arg329 (corresponding to S. cerevisiae R328) and the
pyrophosphate group of bound LLO, as observed in the ternary complex structure of yeast OST, is precluded by the positioning of the NGI-1 molecule. Arg329 instead interacts with the second GlcNAc unit via hydrogen bonding, stabilizing bound LLO (Figures 7A and 7B). Third, although an acceptor peptide can still bind to the inhibited OST-A, the amide group and an acceptor asparagine residue could not be positioned sufficiently close to the C1 atom of the reducing-end GlcNAc to allow for an efficient nucleophilic attack. This suggests that NGI-1 might act like an uncompetitive inhibitor that binds the LLO-primed state of OST-A, alters its geometry, and thus traps the enzyme in an LLO-bound state incompatible with catalysis (Figure 7C). Given that NGI-1 facilitates the co-purification of native LLO, it likely enhances the affinity of LLO to the OST-A complex. NGI-1 could therefore also be considered an LLO-cooperative inhibitor.

Figure 6. Cryo-EM structure of OST-A with NGI-1 bound
(A) OST-A structure is shown in ribbon representation. Subunits are colored individually and labeled. Bound NGI-1 is shown as orange spheres. Bound LLO (Dol-PP-GlcNAc-Man3Glc) is shown as black sticks. The inset shows a close-up view of NGI-1 and LLO binding sites, with STT3A shown in ribbon. NGI-1 is shown in orange sticks, and bound LLO is shown in black sticks. The dolichyl tail and the glycan moiety of the LLO are indicated and labeled. Manganese (II) ion is shown as a pink sphere.
(B) Close-up view of the bound LLO in the presence of NGI-1. OST subunits are shown in ribbon representation with subunits colored as in (A). NGI-1 and LLO are shown as in (A). Transmembrane helix 9 (TM9) is represented as a green cylinder and labeled.
(C) Ribbon representation of STT3A with bound LLO and NGI-1. The three clusters identified by the base-editor screen are indicated in boxes and labeled. NGI-1 and LLO are shown as in (A). Transmembrane helix 9 (TM9) is represented as a green cylinder and labeled.
(D) Close-up of NGI-1 binding to the STT3 subunit. Residues involved in binding are shown as sticks. EM density, NGI-1, LLO, water, and manganese (II) ion are shown as in (B) and labeled.
(E) Anti-TLR4 FACS to detect cell-surface TLR4 after 24 h of NGI-1 treatment of STT3A/C0/293T cells exogenously expressing TLR4-HA and the indicated STT3A variants. Error bars represent standard deviation. See also Figures S6 and S7.

DISCUSSION
We found that genetic or pharmacologic ablation of STT3A, and hence OST-A, impairs NF-κB pathway activation in response to LPS. This was specific because inhibiting STT3B did not impair the NF-κB response to LPS. Mechanistically, we found that the LPS cell-surface receptor TLR4 is particularly dependent on OST-A relative to OST-B for its proper glycosylation and trafficking to the cell surface. We show that the previously identified OST inhibitor NGI-1 functions by binding directly to a pocket within STT3A that is contiguous with the binding site of the LLO substrate. This region is highly conserved within STT3B, consistent with NGI-1’s activity against both OST complexes. NGI-1 acts as an uncompetitive inhibitor, causing a shift in the binding pose of the donor LLO substrate, resulting in an OST-LLO-inhibitor complex that is not compatible with catalysis. Drug-resistant STT3A mutants, identified either genetically via base-editor screens or predicted structurally from our cryoelectron microscopy (cryo-EM) studies, functionally corroborate our structural observations regarding the binding site of NGI-1 to STT3A.
Our suicide-gene-based CRISPR screen for genes required for NF-κB activation demonstrates the power of combining
whole-genome CRISPR libraries with positive selection assays, as does a recent report of a CRISPR screen that used cells expressing the suicide gene HSV-TK under the control of the transcription factor NRF2. Our primary screen was sensitive because it correctly identified multiple genes in the canonical LPS-driven NF-κB pathway, from TLR4 and its co-chaperones to the REL/NF-κB transcription factors. It was also highly specific because it was not, unlike many negative selection screens, plagued by hits that reflect a non-specific loss of cellular fitness and because there were very few false positives based on subsequent subgenomic validation CRISPR screens. Our subgenomic screens also revealed that some of our true positives were specific for LPS, while others also affected signaling from alternative NF-κB agonists such as TNF-α and CpG. Given our experience and the importance of NF-κB in health and disease, it would be of interest to rerun our primary screen with these and other NF-κB agonists.

In addition to STT3A, our screen identified CCDC134 as being required for NF-κB signaling by LPS. CCDC134 is a poorly characterized protein that contains an N-terminal signal peptide and a C-terminal KDEL ER retention signal sequence and is reported to be both localized to the ER and also secreted. Interestingly, its top co-dependencies in DepMap include STT3A and HSP90B1, suggesting that these three proteins are intimately linked. Moreover, loss of CCDC134, like loss of STT3A, downregulated HSP90B1, although the biochemical explanation for this finding is not yet clear.

Two other groups reported CRISPR screens for genes required for NF-κB signaling, both of which were based on downregulation of a transcriptional readout. Parnas and coworkers used endogenous TNF as a readout, as measured by FACS, in murine dendritic cells exposed to LPS. Among the sgRNAs enriched in the TNF-low population were sgRNAs targeting various OST components. They did not, however, identify OST-A-specific components. Sato and coworkers used Ba/F3 cells expressing exogenous TLRs and an NF-κB-driven GFP reporter treated with the NF-κB ligands Flagellin, R848, or CpG to discover that OST-A is required by TLR5, TLR7, and TLR9. Their finding with respect to TLR9 is consistent with our own findings and with a recent report that NGI-1 blocks TLR9 glycosylation and processing.

A structural understanding of how drugs interact with their targets facilitates their optimization. NGI-1 was identified in cell-
based assays as a potent inhibitor of N-glycosylation that targets both OST-A and OST-B complexes, and has since been widely used as a tool compound for diverse applications, including the investigation of glycan occupancy in mammalian cells, as an anti-cancer therapeutic, and as a potential antiviral drug. However, the inhibitory mechanism of NGI-1 remained unknown. The unbiased identification of drug-resistant mutants is a powerful tool for validating the targets of new drugs and their mode of binding. The high signal-to-noise ratio of our screening system, along with its ability to be run in positive selection mode, whether the desired result is to turn a pathway off or keep it on, lent itself to it being coupled to newer gene perturbation technologies, such as CRISPR base-editing technology. Our experience with base-editor technology yields some important conclusions. First, although base-editor technology does not achieve saturation mutagenesis (in contrast to methods such as the mutagenesis by integrated tiles [MITE] approach), its coverage is broad enough to find drug-resistant mutants. Second, although determination of the exact mutations introduced by an sgRNA requires time-consuming cloning of individual sgRNAs, their re-introduction into cells, genomic DNA isolation, and NGS, the mutations introduced by a given sgRNA and base editor combination are predictable enough that one could potentially bypass these steps and directly test the list of candidate mutations in secondary biological assays. Third, CRISPR base-editor screens have specific advantages over other mutagenesis screening approaches (MITE, error-prone PCR, replication in error-prone bacteria) because they leverage existing CRISPR workflows, meaning they can be easily adopted by labs already experienced in CRISPR screens. Moreover, they mutagenize the endogenous gene, thus avoiding artifacts of exogenous overexpression (including drug sequestration).

The structure of NGI-1-bound OST-A revealed a binding pocket that is highly conserved in OSTB, the catalytic subunit of OST-B, rationalizing why NGI-1 can inhibit both OST complexes (Figures 6 and S7). The inhibitory mechanism of NGI-1 potentially explains why some glycosylation sequons remain unaffected by NGI-1 treatment in vivo. Because NGI-1 traps an LLO-primed state, OST activity is probably unaffected by NGI-1 if the reaction is initiated by peptide binding (peptide priming). The structure of NGI-1 bound to OST-A therefore provides a fundamental insight into the biologic effects of OST inhibitors and should facilitate their potential development for the treatment of human diseases.

Despite the undisputed importance of protein glycosylation in the ER, neither STT3A nor STT3B are essential genes, presumably due, at least in part, to partial redundancy between them. In fact, STT3A ablation conferred a fitness advantage to our reporter cells engineered to express a suicide gene upon treatment with LPS. Nonetheless, hypomorphic germline mutations in either STT3A or STT3B can cause congenital disorders of glycosylation with various clinical abnormalities. Whether these clinical abnormalities reflect a requirement for STT3A during development or in adult cells remains to be determined.

Using genetics to predict the eventual toxicity of small-molecule inhibitors is challenging, however, as most drugs are useful precisely because they can be titrated with respect to the degree and duration of target inhibition they achieve in the hope of exploiting a therapeutic window. Moreover, drug-bound targets often have different biological effects than does their genetic ablation. NGI-1, which inhibits both STT3A and STT3B, is far less toxic than the pletropic glycosylation inhibitor tunicamycin in vivo and is apparently safe when administered to rodents—such as in a nanoparticle formulation—in short-term studies. A compound that was specific for STT3A would be predicted to have a larger therapeutic window than a dual STT3A/B inhibitor, but the ultimate safety of such a compound will have to be determined empirically.

An STT3B-specific inhibitor has been reported, and we describe here a compound, NGI-235, that preferentially inhibits STT3A. Our findings should motivate and facilitate the development of STT3A-specific inhibitors with better drug-like properties. In general, positive selection screens such as the ones we describe here could prove to be useful to find drug targets for indirectly inhibiting undruggable proteins.

**Limitations of the study**

We have not yet proven that the catalytic function of STT3A is necessary for cell-surface localization of TLR4 and subsequent downstream NF-κB signaling in vivo, partly because NGI-1, and especially NGI-235, have not been optimized for animal studies. It is likely that specific inhibition of STT3A, either genetically or pharmacologically, causes the misfolding of additional proteins that can affect other signaling pathways that were not examined here. Nonetheless, these other hypothetical consequences of STT3A inactivation are compatible with cell viability and did not mitigate the fitness advantage that STT3A loss conferred upon our reporter cells treated with NF-κB agonists. NGI-235 preferentially inhibits the glycosylation of the STT3A-specific substrates we examined, but there may be additional context- or sequon-dependent characteristics of protein substrates that determine whether NGI-235 inhibits their glycosylation; such characteristics could be revealed by future studies examining large-scale effects of NGI-235 on the proteome. Finally, our structural data do not explain why NGI-235 displays isoform selectivity compared with NGI-1, based on our *in silico* modeling to date. Future structural studies of NGI-235 and other isoform-selective inhibitors bound to the OST complex will be needed.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
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  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
  - Cell lines
- **METHOD DETAILS**
  - Chemicals
**Supplemental Information**

Supplemental information can be found online at https://doi.org/10.1016/j.cell.2024.03.022.

**Acknowledgments**

We thank members of the Kaelin laboratory and James DeCaprio for helpful discussions, Samuel McBrayer and Matthew Oser for technical guidance, Matthew Li for assistance with molecular biology assays, and the DFCI Flow Cytometry Core. We thank Anna-Lena Schinke for help with protein expression and cell culture work and the staff at the Scientific Center for Optical and Cytometry Core. We thank Anna-Lena Schinke for help with protein expression and cell culture work and the staff at the Scientific Center for Optical and Cytometry Core. We thank Anna-Lena Schinke for help with protein expression and cell culture work and the staff at the Scientific Center for Optical and Cytometry Core. We thank Anna-Lena Schinke for help with protein expression and cell culture work and the staff at the Scientific Center for Optical and Cytometry Core.

**Author Contributions**

B.L.L. and W.G.K. led experimental design and execution of cell-based assays, and A.S.R. and K.P.L. led experimental design, execution, and analysis of all cryo-EM studies. B.L.L., A.S.R., K.P.L., and W.G.K. assembled and wrote the manuscript with input from all authors. M.B. and J.N.C. conceived and oversaw the manuscript with input from all authors. M.B. and J.N.C. conceived and oversaw the manuscript with input from all authors.

**Declaration of Interests**

B.L.L. is currently a paid employee of Blueprint Medicines. M.H. is currently a paid employee of Thermo Fisher Scientific. W.G.K. is a paid advisor to Casdin Capital, Circle Pharma, FibroGen, Nextech Invest, and Tango Therapeutics. W.G.K. receives compensation for serving as a board director for Eli Lilly and Company, IconOvir Bio, and LifeMine Therapeutics.

**References**


**STAR METHODS**

**KEY RESOURCES TABLE**

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## Reagents and Resources

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### Deposited data

- Atomic coordinates of the model for OST-A/NGI/LLO: This study PDB: 8PN9
- 3D cryo-EM map for OST-A/NGI/LLO: This study EMD-17779

### Experimental models: Cell lines

- NALM-6: DSMZ Cat# ACC 128
- RAW264.7: ATCC Cat# TIB-71
- HEK293T: ATCC Cat# CRL-11268
- Flp-In™-293 Cell Line: Invitrogen Cat# R75007

### Oligonucleotides

- See Table S6 for primer sequences: This study N/A

### Recombinant DNA

- p1242 3x-KB-L: Provided by Bill Sugden Addgene, Cat# 26699
- pLK304-gateway-DCK^*-IRES-GFP: Koduri et al. Addgene, Cat# 176291
- pLenti HRE-Luc PGK hygro: Briggs et al. Addgene, Cat# 118706
- WT PBRM1-TRIPZ-neo: Gao et al. Addgene, Cat# 107406
- Hu-TLR4 cDNA wt pDEST40: Provided by Scott Friedman Addgene, Cat# 42646
- pLVX-M-puro: Provided by Boyi Gan Addgene, Cat# 125839
- plenti UBC-gate-3XHA-PKG-hygro: McBrayer et al. Addgene, Cat# 125839
- pDONR223_FCGR3B_WT: Provided by Jesse Boehm, Matthew Meyerson, and David Root Addgene, Cat# 82896
- lentCRISPR v2-puro: Provided by Feng Zhang Addgene, Cat# 52691
- lentCRISPR v2-Blast: Provided by Mohan Babu Addgene, Cat# 83480
- lentiguidue puro: Provided by Feng Zhang Addgene, Cat# 52963
- lentiguidue puro P2A BFP: Koduri et al. N/A
- lentiguidue puro P2A mCherry: Koduri et al. N/A
- nCas9 NG-ABE plus sgRNA: Sangree et al. Addgene, Cat# 179098
- nCas9 NG-APOBEC-1 plus sgRNA: Sangree et al. Addgene, Cat# 179095
- psPAX2: Provided by Didier Trono Addgene, Cat# 12260
- pMD2.G: Provided by Didier Trono Addgene, Cat# 12259
- SHRE/GFP: Provided by Martin Brown and Thomas Foster Addgene, Cat# 46926

### Software and algorithms

- GraphPad Prism 9.3.0: GraphPad N/A
- BioRender: N/A https://www.biorender.com/
- FlowJo v10.7.1: Becton Dickinson & Company N/A
- Pooled Screen Analysis Tool: Broad Institute https://portals.broadinstitute.org/gpp/public/
- cryoSPARC v4.2.1: Punjani et al. https://cryosparc.com/

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, William G. Kaelin, Jr. (william_kaelin@dfci.harvard.edu).

Materials availability
Plasmids generated in this study have been deposited to Addgene. There are restrictions to the availability of NALM-6 cells and derivatives due to a Materials Transfer Agreement with Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Data and code availability
Atomic coordinates of the model for OST-A/NGI/LLO have been deposited at the RCSB Protein Data Bank (PDB) and are publicly available as of the date of publication. The three-dimensional cryo-EM map of OST-A/NGI/LLO has been deposited in the Electron Microscopy Data Bank (EMDB) and is publicly available as of the date of publication. Accession numbers are listed in the key resources table.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines
NALM-6 cells (male acute lymphoblastic leukemia cells) were directly obtained from DSMZ (ACC 128) and were cultured in RPMI-1640 (Gibco 11875-093) containing 10% FBS and 1% penicillin/streptomycin (Gibco, catalog no. 15070-063). RAW 264.7 cells (male murine macrophage cell line containing the Abelson murine leukemia virus) were obtained from American Type Culture Collection (ATCC TIB-71) and were cultured in DMEM (Gibco, catalog no. 11995-065) containing 10% FBS and 1% penicillin/streptomycin. HEK293T cells were originally obtained from ATCC (CRL-11268) and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The Flp-In\textsuperscript{Tm}/C212-293 Cell Line was obtained from Invitrogen (catalog no. R75007) and cultured in DMEM supplemented with 10% FBS. All cells were maintained in the presence of 5% CO\textsubscript{2} at 37\degree C. Cell line identities were verified using STR profiling through the Dana-Farber Molecular Diagnostics Laboratory core facility, which uses the GenePrint 10 system (Promega, catalog no. B9510). Cell lines were verified to be mycoplasma negative before each CRISPR screen using the MycoAlert Mycoplasma Detection Kit (Lonza, catalog no. LT07-318).

METHOD DETAILS

Chemicals
Lipopolysaccharides from Escherichia coli O111:B4 that were purified by trichloroacetic acid extraction were obtained from Sigma (catalog no. L4130), resuspended to a concentration of 2 mg/mL in sterile water, and used at a final concentration of 10 µg/mL in all experiments with NALM-6 cells and at a concentration of 100 ng/mL in all experiments with RAW 264.7 unless otherwise noted. (ε)-5-(2-Bromovinyl)-2'-deoxyuridine (bromovinyldeoxyuridine, BVdU) was obtained from Chem Impex (catalog no. 27735) and resuspended to a stock concentration of 1 M in DMSO. CpG oligonucleotide (ODN 2006) was obtained from InvivoGen (catalog no. tirl-2006) and resuspended to a stock concentration of 1 mg/mL in sterile water. Recombinant human TNFα (Peprotech 300-01A), corresponding to the C-terminal extracellular domain of the full-length transmembrane protein, was reconstituted to a stock concentration of 10 µg/mL in PBS. NGI-1 was obtained from Sigma-Aldrich (catalog no. SML1620) and resuspended to a stock concentration of 10 mg/mL in DMSO. NGI-235 was synthesized as described below.

NF-κB reporter plasmid
The NF-κB reporter plasmid was made using an In-Fusion HD cloning kit (Takara Bio 638910). The included CloneAmp HiFi PCR pre-mix was used to amplify the promoter-less Gateway destination vector pLentiX1 puro DEST (template: Addgene #17297, F primer: 17297-F, R primer: 17297-R, see Table S6 for primer sequences). These primers created a linearized backbone that excluded the gateway cassette as well as the attR-WPRE-PGK-puroR cassette of the original vector. Overlap PCR was then used to create an
For the CD16-TLR4 overexpression experiment, the CD16-TLR4 chimera was created by using overlap PCR (F primer: humanTLR4-F, R primer: humanTLR4-R) followed by Gateway cloning to introduce TLR4 into the plenti-Ubc-3XHA-PGK-hygro

For experiments where TLR4 was overexpressed, the cDNA for human TLR4 (Addgene #42646) was subcloned into a pDONR vector cDNA expression plasmids

For the catalytic-dead STT3A mutation, this same approach was used but with four fragments, ultimately containing E63A, D167A, and E351Q mutations.33 After PCR amplification, the STT3A cDNA was then cloned, using NEBuilder HiFi DNA Assembly Master according to the manufacturer’s instructions (New England Biolabs Catalog No. E2621X), into pLVX-M-puro (Addgene #125839) that had been digested with BamHI and EcoRI. The matched full-length TLR4 control for this fusion experiment was amplified with an HA tag (template: Addgene #42646, F primer TLR4-HA>F, R primer: 42646-R). This amplified product was cloned and directly tested for base editing efficiency were as detailed in Table S6.

CRISPR/Cas9 sgRNA knockout plasmids

The vectors lentCRISPRv2-puro (Addgene plasmid #52961) and lentCRISPRv2-blast (Addgene plasmid #83480) were used to express sgRNAs with Cas9, and lentGuide (Addgene plasmid #52963) was used to express sgRNAs without Cas9. Complementary oligonucleotides corresponding to each sgRNA were designed to generate overhangs, once annealed, that were compatible with cleavage of the BsmBI sites in the lentCRISPRv2-puro, lentCRISPRv2-blast, lentGuide puro-BFP, and lentGuide puro-mCherry vectors. The lentCRISPR and lentGuide vectors were digested with FastDigest BsmBI (Esp3I) (Thermofisher FD0454), gel-purified, and ligated with annealed oligonucleotides. The sgRNA sequences used are reported in Table S6.

CRISPR/Cas9 sgRNA base editor plasmids

The vector pRDA 429 (Addgene plasmid #179098) was used to express an sgRNA simultaneously with a broad specificity Cas9-D10A nickase fused to an adenosine base editor (ABE8e). The vector pRDA 336 (Addgene plasmid #179095) was used to express an sgRNA simultaneously with a broad specificity Cas9-D10A nickase fused to a cytosine base editor (APOBEC-1). Cloning of guides was performed as described above using BsmBI sites, gel purification, and ligation of annealed oligonucleotides. Guides were cloned into both the A->G base editor plasmid (RDA 429) and the C->T base editor plasmid (RDA 336). The sgRNA sequences individually cloned and directly tested for base editing efficiency were as detailed in Table S6.

cDNA expression plasmids

For experiments where TLR4 was overexpressed, the cDNA for human TLR4 (Addgene #42646) was subcloned into a pDONR vector (F primer: humanTLR4-F, R primer: humanTLR4-R) followed by Gateway cloning to introduce TLR4 into the plenti-Ubc-3XHA-PGK-hygro vector.32 For the CD16-TLR4 overexpression experiment, the CD16-TLR4 chimera was created by using overlap PCR to combine the N terminal portion of CD16 (template: Addgene #82896, F primer: 82896-F, R primer: 82896-R) with the C terminal portion of TLR4 with an HA tag appended (template: Addgene #42646, F primer: 42646-F, R primer: 42646-R). This amplified PCR product was cloned, using NEBuilder HiFi DNA Assembly Master according to the manufacturer’s instructions (New England Biolabs Catalog No. E2621X), into pLVX-M-puro (Addgene #125839) that had been digested with BamHI and EcoRI. The matched full-length TLR4 control for this fusion experiment was amplified with an HA tag (template: Addgene #42646, F primer TLR4-HA>F, R primer: 42646-R) and cloned into the same backbone vector as the CD16-TLR4 chimera. For STT3A overexpression experiments, a synthetic human STT3A cDNA with a C-terminal HiBiT tag was constructed by assembly PCR of 33 overlapping oligonucleotides. The resulting synthetic cDNA lacked recognition sites for the sgRNAs used in our study and lacks common restriction enzyme sites (see Table S6). For generation of STT3A mutants, a two-step overlap PCR approach was taken where overlapping internal primers were used to amplify, separately, N-terminal and C-terminal portions of STT3A, each containing the desired mutation. These were then used as a template for a second reaction containing external flanking primers (5’ forward: STT3A-F, 3’ reverse: STT3A-R) to amplify full-length STT3A. The catalytic-dead STT3A mutation, this same approach was used but with four fragments, ultimately containing E63A, D167A, and E351Q mutations.33 After PCR amplification, the STT3A cDNA was then cloned, using NEBuilder HiFi DNA Assembly Master according to the manufacturer’s instructions (New England Biolabs Catalog No. E2621X), into pLVX-M-puro (Addgene #125839) that had been digested with BamHI and EcoRI. The matched full-length TLR4 control for this fusion experiment was amplified with an HA tag (template: Addgene #42646, F primer TLR4-HA>F, R primer: 42646-R) and cloned into the same backbone vector as the CD16-TLR4 chimera. For STT3A overexpression experiments, a synthetic human STT3A cDNA with a C-terminal HiBiT tag was constructed by assembly PCR of 33 overlapping oligonucleotides. The resulting synthetic cDNA lacked recognition sites for the sgRNAs used in our study and lacks common restriction enzyme sites (see Table S6). For generation of STT3A mutants, a two-step overlap PCR approach was taken where overlapping internal primers were used to amplify, separately, N-terminal and C-terminal portions of STT3A, each containing the desired mutation. These were then used as a template for a second reaction containing external flanking primers (5’ forward: STT3A-F, 3’ reverse: STT3A-R) to amplify full-length STT3A. The catalytic-dead STT3A mutation, this same approach was used but with four fragments, ultimately containing E63A, D167A, and E351Q mutations.33 After PCR amplification, the STT3A cDNA was then cloned, using NEBuilder HiFi DNA Assembly Master according to the manufacturer’s instructions (New England Biolabs Catalog No. E2621X), into pLVX-M-puro (Addgene #125839) that had been digested with BamHI and EcoRI. For CCDC134 overexpression experiments, the human CCDC134 cDNA corresponding to the coding sequence from transcript variant GenBank: NM_024821.5 was purchased from the Harvard Medical School PlasmID Repository. The CCDC134 cDNA was subcloned into a pDONR vector (F primer: CCDC134-F, R primer: CCDC134-R). Site-directed mutagenesis was then performed using the QuikChange II XL site-directed mutagenesis kit (Agilent Catalog #200521) according to manufacturer’s instructions on the vector (using primers 5’ forward: CCDC134res-F and 3’ reverse CCDC134res-R) to create a cDNA resistant to the first CCDC134 sgRNA listed above. Further mutations to investigate its glycosylation were also introduced with the QuikChange II XL site-directed mutagenesis kit. Gateway cloning was then used to introduce CCDC134 into the plenti-Ubc-3XHA-PGK-hygro vector mentioned above. For experiments using ER-GFPQ185,N186C, an ER-retention signal sequence was appended to the GFP cDNA from Addgene #176291 as it was subcloned into the pDONR vector using Gateway cloning (5’ forward: 176291-F, 3’ reverse: 176291-R). The Q185N and N186C mutations were introduced
Lentiviral synthesis and transduction
Lentiviral particles were made by Lipofectamine 2000-based co-transfection of HEK293T cells with the desired expression vector and the packaging plasmids psPAX2 (Addgene #12260) and pMD2.G (Addgene #12259) in a ratio of 8:3:1, respectively, for lentiviral plasmids, or 4:3:1 for all others. Virus-containing media was collected 48 and 72 hours after transfection, passed through a 0.45 μM filter, divided into aliquots, and frozen at -80 °C until use. For virus stocks made from the base editor plasmids (Addgene, Cat# 179095 and Addgene, Cat# 179098), the viral supernatant was concentrated 100-fold prior to infection by mixing the supernatant with Lenti-X Concentrator (Clontech Cat #631231) in a 1:3 ratio and following the manufacturer’s instructions. For infections, NALM-6 cells were plated at a density of 1 x 10^6 cells per milliliter in 6-well plates. Virus containing media (500 μL) was added to 2 mL of cells; polybrene was also present at a final concentration of 8 μg/mL. HEK293T cells to be infected were plated at a density of 500,000 cells per well in a 6-well plate and infected the following day with 500 μL of virus plus 2 mL of media containing polybrene at a final concentration of 8 μg/mL. RAW 264.7 cells were plated at a density of 400,000 cells per well in a 6-well plate and infected with the same protocol as used for HEK293T. For infection all cells were centrifuged at 2000 x g for 2 hrs at room temperature to enhance infection efficiency. The next day, cells were placed in fresh media. The following day, cells were placed under drug selection. For NALM-6, stable cell lines were established by selection in media containing 400 μg/mL hygromycin, 1 μg/mL puromycin, 600 μg/mL neomycin, or 3 μg/mL blasticidin as appropriate for the drug resistance cassette present in each virus. HEK293T stable cell lines were established by selection in media containing puromycin 1 μg/mL and RAW 264.7 cells were selected in 4 μg/mL puromycin.

Nucleofection of cell lines
To generate an NALM-6 cell line with TNFAIP3 knockout but without constitutive expression of Cas9, NALM-6 parental cells were nucleofected with Cas9:RNA ribonucleoprotein complex targeting TNFAIP3. An alt-R crRNA targeting TNFAIP3 using the guide sequence in Table S6 was synthesized by IDT. This crRNA was then annealed with an alt-R tracrRNA (IDT catalog no. 1072532) in a 1:1 ratio. In one reaction, 120 pmol of the crRNA:tracrRNA duplex, 104 pmol of alt-R S.p. Cas9 nuclease (IDT catalog no. 1081058), and 2.1 μL of sterile PBS were incubated at room temperature for 20 minutes. This 5 μL reaction was then added to 20 μL of SF solution (Lonza catalog no. V4XC-2032) containing 400,000 NALM-6 cells. The cells were nucleofected using protocol CM-150 on a 4D-Nucleofector X Unit (Lonza, catalog no. AAF-1003X). After recovery and expansion in culture media, the cells were later infected with a lentivirus containing the NF-kB-DCX*-IRESeGFP reporter, selected in neomycin, and sorted for individual clones with high dynamic range in GFP expression.

To generate the STT3A- and STT3B-knockout cells used to interrogate the function of STT3A in Figures 3D, 3H, 4A, 4F, 5F, 5G, and 6E, either NALM-6 or HEK293T cells were nucleofected with guides and then single cell cloned to generate a homogenous population of knockout cells. An alt-R crRNA targeting STT3A (see Table S6), an alt-R crRNA targeting STT3B (see Table S6), and a control alt-R crRNA (IDT catalog no. 1072544) were purchased from IDT, annealed to the alt-R tracrRNA, and loaded on Cas9 as above. The 5 μL Cas9:RNA ribonucleoprotein complex was mixed with 20 μL of SF solution containing 200,000 HEK293T cells and nucleofected using protocol DS-150 on the Lonza catalog no. V4XC-2032, containing 400,000 NALM-6 cells. The cells were nucleofected using protocol CM-150 on a 4D-Nucleofector X Unit (Lonza, catalog no. AAF-1003X). After recovery and expansion in culture media, the cells were later infected with lentiviruses containing the NF-kB-DCX*-IRESeGFP reporter, selected in puromycin, and sorted for individual clones with high dynamic range in GFP expression.

Immunoblot analyses
Cells were lysed in EBC lysis buffer (50 mM tris-HCl [pH 8.0], 250 mM NaCl, 0.5% NP-40, and 5 mM EDTA) supplemented with a protease inhibitor cocktail (Roche, Catalog no. 11836170001) and phosphatase inhibitor cocktail (Roche, Catalog no. 4906845001). Whole cell extracts were quantified using the Bradford protein assay. Prior to loading, equivalent amounts of protein for each sample were mixed with NuPAGE 4X LDS Sample Buffer (Novex by Life Technologies, Catalog no. NP0007) supplemented with 2-mercaptoethanol (Sigma-Aldrich, Catalog no. 152472500) to a final concentration of 1X. Protein lysates were resolved via SDS-PAGE and transferred onto 0.2 μM nitrocellulose membranes using the BioRad Trans-Blot Turbo Transfer System. Membranes were blocked with 5% non-fat milk in tris-buffered saline with 0.1% Tween 20 (TBS-T) for a minimum of 1 h and then probed with the indicated primary antibodies overnight at 4 °C as detailed in the key resources table. Membranes were then washed three times in TBS-T, probed with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature, and washed three additional times in TBS-T. Bound antibodies were detected with the chemiluminescent immunoblotting detection reagents Immobilon (Thermo Fisher Scientific, #WBKLS0500) or SuperSignal West Pico (Thermo Fisher Scientific, #PI34078). For HiBIT blots, proteins were visualized using the Nano-Glo HiBIT Blotting System (Promega, Catalog no. N2410). Briefly, immediately after transfer, unblocked nitrocellulose membranes were incubated in Nano-Glo Blotting Buffer and LgBiT protein overnight; the
following day Nano-Glo Luciferase Assay Substrate was added to the mixture for 5 minutes. Hi-BIT protein were then visualized by exposing the membranes to autoradiography film (Denville Scientific, Catalog No. E3018).

**BVDU kill curves**
For the experiment in Figure 1D, NALM-6 cells with the indicated genetic modifications were first seeded into 6-well plates at 40,000 cells per well in 2 mL of media, which, where indicated, contained 12.5 μg/mL LPS (final concentration 10 μg/mL). On the same day, 1 M BVDU was diluted into media to prepare 5X stock solutions of BVDU at concentrations of 5 mM, 50 μM, 5 μM, and 500 nM. For each stock solution, DMSO concentration was adjusted to a final concentration of 0.5%. Each well in the 6-well dish received 0.5 mL of the 5X stock solution of BVDU to achieve final concentrations of 1 mM, 100 μM, 10 μM, 1 μM, and 100 nM, respectively. A total of 0.5 mL of media with 0.5% DMSO was added to the sixth well as a control. Seven days later, viable cells were counted using a Vi-Cell XR cell counter. For the experiment in Figure 4E, NALM-6 cells were plated at 40,000 cells per well in 2 mL of media with either DMSO, NGI-1 at 1 μM, or NGI-1 at 10 μM for 24 hours before the simultaneous addition of LPS (final concentration 10 μg/mL) and/or BVDU in a total of 0.5 mL as above. Cells were counted on day 8.

**FACS-based mCherry-BFP competition assay**
Cas9-expressing TNFAIP3-/- NFκB-DCK* cells and the UBC-DCK* expressing control cells were stably transduced with lentivirus encoding either (i) sgMYD88 and mCherry or (ii) sgCTRL and BFP. These cells were pooled together and analyzed by FACS to achieve a final ratio of mCherry-positive to BFP-positive cells of 1:99. The pool of infected cells was plated at 40,000 cells/mL in a 6-well plate and then cultured in media containing LPS at 10 μg/mL and either 10 μM BVDU or DMSO for 20 days. Cells were collected for FACS analysis on the final day of the experiment. Data analysis was performed with FlowJo v10.7.1 software (Becton Dickinson & Company).

**Creation of NALM-6 cell lines for screening**
The NALM-6 clonal cell line used for the CRISPR knockout screen was generated by first transducing the NALM-6 parental line with plentiCRISPR-blast containing either a control sgRNA or an sgRNA to TNFAIP3 (thus simultaneously introducing Cas9 at the same time). These polyclonal lines were then stably infected with lentiviruses containing the NFκB-DCK*-IRES-GFP or UBC-DCK*-IRES-GFP reporters as well as a neomycin resistance marker. The cells were then grown in the presence of neomycin for 7 days. To identify a homogenous population of cells with robust induction of GFP (and thus DCK*) upon stimulation with LPS, flow cytometry was used to sort, into a 96 well plate, single cells with high GFP fluorescence after stimulation. These clones were grown for approximately 3 weeks and then each was individually examined by high-throughput flow cytometry for its level of GFP induction upon LPS exposure to identify a clone with the maximum dynamic range. The generation of the NALM-6 clonal cell line used for the CRISPR base editor screen followed the same procedure as above, except instead of stably introducing Cas9 and an sgRNA to TNFAIP3 with plentiCRISPR, a Cas9 ribonucleaseprotein complex targeting TNFAIP3 was nucleofected into the NALM-6 cell line before introduction of the NFκB reporter plasmid (see section on nucleofection of cell lines). This allowed for brief expression of Cas9 to avoid interference with later introduction of a nickase Cas9 linked to a base editor moiety.

**Subgenomic library synthesis**
Gene-targeting sgRNAs and appropriate controls were designed using the rule set described at the Genetic Perturbation Program (GPP) portal (http://portals.broadinstitute.org/gpp/public). Oligonucleotides were flanked by PCR primer sites, and PCR was used to amplify DNA using NEBNext kits (New England Biolabs, catalog no. E7645). The PCR products were purified using Qiagen PCR cleanup kits and cloned into pXPR_BRD003 using Golden Gate cloning reactions. Pooled libraries were amplified using electrocompetent Stbl4 E. coli cells. Viruses were generated as outlined at the GPP portal. The sgRNA library was custom-designed to target the top scoring 500 genes from the whole genome screen performed with LPS/BVDU, as well as additional controls including 5 essential genes, 500 non-targeting guides, and other controls (such as guides targeting all human TLR genes). It consisted of 5111 sgRNAs targeting 508 genes (~10 sgRNAs targeting each gene) and 500 nontargeting sgRNAs as controls (Table S3) for a total of 5611 guides.

**STT3A targeting library synthesis**
For the design of an NG-base editor library tiling STT3A, we first identified all possible guides, regardless of PAM sequence, with an editing window (nts 4 – 8 of the guide) in the coding sequence, allowing the editing window to extend into flanking introns, for a total of 5,222 guides. We then retained guides utilizing PAMs with high and intermediate activity, as identified in Sangree et al.52; we removed guides with constraint violations (a run of 4 or more Ts, BsmBI sites), resulting in 1,327 guides remaining. For off-target filtering, guides with 3 or more off-target sites in the genome with 0 mismatches were removed. This filtering brought the total number of guides to 1,299. An additional 75 non-targeting guides and 75 intergenic guides were added as negative controls and 32 guides targeting splice donor sites in pan-lethal genes were added as positive controls (for lethality screens), bringing the total number of guides in this library to 1,481 (Table S4).

**CRISPR knockout screens**
For the whole genome screen, 1.25 x 10^6 cells of the NALM-6 clonal reporter cell lines were infected with the Brunello sgRNA library (approximately 1600 cells per sgRNA, or about 480 cells per sgRNA after selection) on day 0. To do this, the cells were mixed with...
polybrene (8 μg/mL) and library virus (purchased from the Broad Institute) at an MOI of 0.3. The cells were then plated into 2 mL aliquots onto 6-well plates and centrifuged at 139 x g in a tabletop clinical centrifuge for 2 hours at room temperature. The following day, cells were collected, pooled, centrifuged to remove the virus and polybrene, and then the cell pellet was resuspended in RPMI plus 10% FBS plus penicillin/streptomycin at a concentration of 1 x 10^6 cells/mL. On day 2, the cells were placed under selection with puromycin (1 μg/mL) at a density of 145,000 cells/mL. The cells from this point forward were maintained in 3 L disposable spinner flasks with vented caps (Corning catalog no. CLS3581) continuously rotating on a Thermolyne Cellgro Stirrer 45600. On day 7 after infection, 240 x 10^6 cells were pelleted and resuspended in 1.2 L of fresh media containing puromycin 1 μg/mL; the remaining cells were discarded. On day 11 after infection three aliquots of 1 x 10^7 cells per arm were pelleted, washed with PBS, and frozen for later genomic DNA isolation (baseline time point). Additionally, 80 x 10^6 (approximately 1000 cells/guide) were resuspended at a concentration of 40,000 cells/mL in 2 L of media containing 10 μg/mL LPS and 10 μM BVDU. Thus, at least 1000 cells per sgRNA were subjected to BVDU selection. No media changes or additional exposure to LPS and/or BVDU were done until day 25 (day 14 after LPS/BVDU exposure), when two aliquots of 1 x 10^7 cells per arm were pelleted, washed with PBS, and frozen for later genomic DNA isolation (end time point).

Following completion of the screen, genomic DNA was isolated using the NucleoSpin Blood XL kit (Macherey-Nagel, catalog no. 740950.50) according to the manufacturer’s protocol. Genomic DNA was subsequently purified by processing it through the OneStep PCR Inhibitor Removal Kit (Zymo Research, catalog no. D6030). DNA was then quantified using the Qubit 1X dsDNA HS Assay Kit (Invitrogen, catalog no. Q33231) on a Qubit 4 Fluorometer (Invitrogen). Raw Illumina reads were normalized between samples using log₂[sgRNA reads/total reads for sample] x 1 x 10^6 + 1]. The initial time point data (day 11) were then subtracted from the end time point after BVDU selection (day 25) to determine the relative enrichment of each individual sgRNA after BVDU treatment using hypergeometric analysis publicly available through the Broad Institute GPP Web Portal (https://portals.broadinstitute.org/gpp/public/). The averaged data from the two biological replicates for each reporter were used for all analyses.

For the subgenomic screens, 2.7 x 10^7 cells (about 4800 cells/guide, or about 1440 cells/guide after selection) were infected with the cells with polybrene (8 μg/mL) and library virus at an MOI of 0.3 on day 0. The cells/polybrene/virus mixture was split into 2 mL aliquots onto 6-well plates and centrifuged at 139 x g for 2 hours at room temperature. The following day, as with the whole genome screen, the cells were spun down and resuspended in fresh media, followed by puromycin selection at 1 μg/mL beginning on day 2. From this point forward, the cells were carried in a 500 mL disposable spinner flask with vented caps (Corning, catalog no. 3578) on a Thermolyne Cellgro Stirrer 45600. On day 6 after infection, 30 x 10^6 cells were pelleted and resuspended in 300 mL of fresh media containing puromycin 1 μg/mL; the remaining cells were discarded. On day 12 after infection, the cells were simultaneously exposed to BVDU and each individual NFκB stimulant. For quantification of baseline sgRNA abundance, 3 x 10^7 cells at this time were washed with PBS and frozen. Additionally, 7.5 x 10^6 cells were taken for each NFκB stimulant and resuspended in 250 mL media containing 10 μM BVDU plus either LPS at 10 μg/mL, TNFα at 1 ng/μL, or CpG at 1 μg/mL. No media changes or additional exposure to stimulants and/or BVDU were done until day 28 (day 16 after BVDU exposure), when pellets of 3 x 10^7 cells were washed with PBS and frozen for subsequent genomic DNA isolation. Genomic DNA isolation and quantification of sgRNA abundance followed the protocol as outlined above for the whole genome knockout screen, except that the NucleoSpin L Midi kit (Macherey-Nagel, catalog no. 740954) was used due to the smaller size of the cell pellets.

**Quantitative reverse-transcription PCR**

For the experiments in Figure 2E, the NALM-6 reporter cells stably infected with lentiviruses expressing the indicated sgRNAs were plated to a density of 100,000 cells/mL in 10 mL of media with or without LPS 10 μg/mL for 24 hours. RNA was extracted using a QiAshredder (Qiagen, catalog no. 79656) followed by the RNeasy mini kit (Qiagen, catalog no. 74106) according to the manufacturer’s instructions. RNA concentration was determined using the NanoDrop 8000 (Thermo Fisher Scientific). cDNA was generated by reverse transcription from 1 μg starting RNA using the AffinityScript qPCR cDNA Synthesis kit (Agilent, catalog no. 600559) according to the manufacturer’s instructions using random hexamers as primers. cDNA was diluted 1:5 for subsequent use in quantitative PCR (qPCR) reactions. qPCR was performed using the LightCycler 480 (Roche) in a multiplex fashion with two TaqMan probes (Thermofisher Scientific) interrogating the abundance of two genes— a gene of interest and the housekeeping gene beta actin (ACTB)— per reaction. Each reaction contained 1 μL diluted cDNA, 2.8 μL water, 0.35 μL of a FAM-conjugated TaqMan probe to the gene of interest, 0.35 μL of a VIC-conjugated primer-limited TaqMan probe to ACTB, and 3.5 μL of TaqMan Multiplex Master Mix (Applied Biosystems, catalog no. 4461882) in a 384-well plate (Roche, catalog no. 04729749001) covered with Lightcycler 480 sealing foil (Roche, catalog no. 04729757001). All quantitative calculations were performed using the 2^ΔΔCt method using ACTB as the reference gene. The Ct values for each probe were normalized to the Ct value of ACTB for that individual well. The data from each experiment were then normalized to the unstimulated (no LPS) control to determine the relative fold change in mRNA expression. The following TaqMan probes were used: Hs00540548_s1 (human CXCR5), Hs00179139_m1 (human PIM2), Hs00174128_m1 (human TNFA), and Hs01060665_g1 (human ACTB).

**Flow cytometry for surface proteins**

For flow cytometry of surface TLR4, 200,000 cells were centrifuged at 68 x g for 4 minutes at room temperature, washed once in PBS, resuspended in 200 μL flow cytometry staining buffer (FACS Buffer, eBioscience catalog no. 00-4222-26), and then moved to a V-bottom plate. Cells were again pelleted by centrifugation at 400 x g for 4 minutes at room temperature, resuspended in 100 μL...
FACS buffer containing 0.2 μL anti-human TLR4 antibody conjugated to PE (clone TF901, BD Biosciences catalog no. 564215) for 30 min at 4°C. Cells were then washed twice with FACS Buffer. For small scale experiments, cells were immediately analyzed by flow cytometry. For larger scale experiments, such as those in Figures 5F and 6E, the cells were fixed by resuspending them in 2% para-formaldehyde (Electron Microscopy Sciences, Catalog No. 15710) in PBS for 30 min at 4°C after incubation with the anti-TLR4 antibody, washed twice in FACS buffer, and resuspended in FACS Buffer prior to analysis. FACS was performed with a BD LSRFortessa Cell Analyzer.

Cell surface biotinylation

The Pierce Cell Surface Biotinylation and Isolation Kit (ThermoScientific Catalog No. A44390) was used according to the manufacturer’s protocol with the following modifications. Approximately 40 x 10⁶ NALM-6 cells with the genetic modifications as indicated in Figure 4F were pre-treated for 24 hours with DMSO, NGI-1 (1 μM or 10 μM), or tunicamycin 1 μM. Then, 10 x 10⁶ cells were set aside and frozen as pellets for the eventual analysis of whole cell lysate via the immunoblot protocol above. The remaining 30 x 10⁶ cells were washed once with PBS and then resuspended in 30 mL ice cold PBS containing 5 vials of EZ-link sulfo-NHS-SS-biotin from the kit for 30 minutes at 4°C with continuous rocking. The labeling reaction was quenched by washing the cells twice with TBS. Cells were then lysed in 500 μL of the kit-supplied lysis buffer and 300 μL of the clarified supernatant was mixed with NeutrAvidin Agarose slurry. After 4 washes with supplied Wash Buffer, proteins were directly eluted from the beads by incubation for 10 minutes at 100°C in a 1:1 mixture of EBC lysis buffer and NuPAGE 4X LDS Sample Buffer (Novex by Life Technologies, Catalog No. NP0007) supplemented with 2-mercaptoethanol (Sigma-Aldrich, Catalog No. 125472500) at a final concentration of 5%.

Endoglycosidase treatment of lysates

After lysis of cells in EBC lysis buffer, 160 μg of protein was brought to a final volume of 40 μL using lysis buffer and the 10X Glyco-protein Denaturing Buffer supplied with the glycosidases enzymes. The sample was heated to 100°C for 10 min and rapidly cooled to 10°C in a thermocycler. For PNGase F treatment (New England Biolabs, Catalog No. P0704S), NP-40 was then added to a final concentration of 1% and GlycoBuffer 2 was added to a final concentration of 1X. Four microliters of PNGase F was added to the final reaction volume and the reaction was incubated at 37°C for 1 hour. For Endo H treatment (New England Biolabs, Catalog No. P0702S), GlycoBuffer 3 was added to a final concentration of 1X and then 4 μL of Endo H was added to the final reaction volume and the reaction was incubated at 37°C for 1 hour. Finally, NuPAGE 4X LDS Sample Buffer (Novex by Life Technologies, Catalog No. NP0007) was added in a 1:1 ratio to the reaction volume (final concentration of sample buffer was 2X) and the reaction was heated at 70°C for 10 min, cooled, and loaded onto an SDS-PAGE gel for electrophoresis.

NGI-235 synthesis

The synthesis of NGI-235 was completed in three steps from commercially available 5-(chlorosulfonyl)-2-fluorobenzoic acid (1) as illustrated in Figure S3A. Reaction with N-methylbenzylamine and triethylamine in dichloromethane gave sulfonamide 2 in 83% yield. Subsequent coupling with 5-ethylthiazol-2-amine using HATU and diisopropylamine in DMF gave the corresponding amide (3) in 88% yield. Finally, nucleophilic aromatic substitution of the fluoride with 1-cyclopropyl-N-methylmethylamine at 80°C afforded NED-4086 (NGI-235) in 39% yield.

CRISPR base editor screens

For the base editor screens, 7.5 x 10⁶ cells (about 5000 cells/guide, or about 1500 cells/guide after selection) were infected by mixing the cells with polybrene (8 μg/mL) and library virus at an MOI of 0.3 on day 0. The cells/polybrene/virus mixture was split into 2 mL aliquots onto 6-well plates and centrifuged at 139 x g for 2 hours at room temperature. Four hours after the spin had completed, the wells were pooled, and the cells were spun down and re-plated in fresh media. On day 3 after infection, 12 x 10⁶ cells were placed under selection with 0.125μg/mL puromycin at a density of 100,000 cells/mL. From this point forward, cells were carried in a vented 175cm² flask ( Falcon, catalog no. 353112). On day 10, pellets of 20 x 10⁶ cells were frozen down for baseline genomic DNA analysis (these pellets were ultimately not necessary for the analysis of the screen). On the same day, 15 x 10⁶ cells were treated with NGI-1 at 10 μM in 150 mL or, as a control, DMSO. Two days later (day 12), LPS was added to each flask to a final concentration of 10 μg/mL. Twenty-four hours later, on day 13, the top 1% GFP positive cells were sorted and placed into fresh RPMI + 10% FBS + penicillin/streptomycin; anywhere from 110,000-158,000 cells were collected for each arm for each replicate. These cells were then expanded. On day 25, 15 x 10⁶ were again treated with either 10 μM NGI-1 or DMSO, followed by LPS stimulation 48 hours later, followed by sorting for the top 1% GFP positive cells on day 28. For this second sort anywhere from 60,000-130,000 cells were collected for each arm of the replicate. After expansion, the cells were then frozen on day 33 after being washed with PBS and aliquoted into pellets of 20 x 10⁶ cells.

Following completion of the screen, genomic DNA was isolated using the NucleoSpin L Midi kit (Macherey-Nagel, catalog no. 740954.20) according to the manufacturer’s protocol. Genomic DNA was subsequently purified by processing it through the OneStep PCR Inhibitor Removal Kit (Zymo Research, catalog no. D6030) and quantified using Qubit. Raw Illumina reads were normalized between samples using log₂([sgRNA reads/total reads for sample] × 1 x 10⁶ + 1]. The log normal reads for each guide at the end of the screen in the DMSO-treated arm were subtracted from the log normal reads for the same guide in the NGI-1 treated arm to arrive at the relative guide abundance depicted in Figures 5B and 5C. The screen was performed for each base editor (C->T and A->G) in parallel and in duplicate. Averaged data from the two biological replicates were used for all analyses.
Analysis of DNA for editing efficiency
The QIAamp DNA Blood Mini Kit (Qiagen, Catalog No. 51104) was used to extract genomic DNA from cell pellets of the NALM-6 reporter cell line after stable lentiviral infection with the viruses encoding the base editors and sgRNAs. Subsequently, approximately 100 ng of genomic DNA from each cell line was used in separate PCR reactions to generate ~200bp amplicons surrounding the site of the intended edit using primers as detailed in Table S6. PCR was performed using KAPA HiFi HotStart ReadyMix (Roche, Catalog No. KK2601) with the following conditions: 2 min at 94 °C, 40 cycles of 10 s at 98 °C, 30 s at 56 °C, and 30 s at 68 °C, followed by a final extension step for 5 min at 68 °C. Amplicons were gel-purified and subjected to next generation sequencing (approximately 100,000 reads) through the MGH CCIB DNA Core. Output fastq files were analyzed using the web-based CRISPResso2 program (http://crispresso2.pinellolab.org/). 12

Purification of human OST-A complex
A stable HEK293 cell line enabling the tetracycline-inducible expression of Flag-DC2 was used for OST-A purification. DC2 expression was induced by addition of 1 μg/mL tetracycline (Sigma) for 48 h at 37 °C. Cells were harvested, washed twice with phosphate-buffered saline, and collected by centrifugation before being frozen in liquid nitrogen and stored at −80 °C. All steps of protein purification were performed either on ice or at 4 °C. Purification of OST-A was performed with small modifications of the previously described protocols. 53,81 Briefly, cells were resuspended in lysis buffer containing 50 mM HEPES pH 7.5, 200 mM NaCl, 5 mM EDTA, 3 mM EGTA and 10% (w/v) glycerol, supplemented with cComplete™, EDTA-free Protease Inhibitor Cocktail (Roche), and 0.5 mM phenylmethylsulfonylfluoride (PMSF). After cell lysis using a Dounce homogenizer, the membrane fraction was collected by high-speed centrifugation at 100,000 x g for 30 min, and resuspended in solubilization buffer containing 50 mM HEPES, pH 7.5; 400 mM NaCl; 5 mM EDTA; 3 mM EGTA and 10% (w/v) glycerol. Solubilization was performed by incubation with 1% (w/v) digitonin (Merck) for two hours. The supernatant was collected after high-speed centrifugation at 100,000 x g for 30 min and incubated with anti-Flag M2 affinity agarose gel (Sigma) for two hours. The gel was washed with washing buffer containing 25 mM HEPES, pH 7.5; 150 mM NaCl; 5 mM EDTA; 3 mM EGTA and 10% (w/v) glycerol and 0.1% (w/v) digitonin, and subsequently eluted with 0.4 mg/mL Flag peptide (Sigma) in the same buffer and concentrated and further purified by size exclusion chromatography using a Superose 6 column (GE Life Sciences) with a buffer containing 25 mM HEPES, pH 7.5; 150 mM NaCl; 2 mM DTT and 0.1% (w/v) digitonin. 5 μM NGl-1 (Sigma) was added to all purification buffers.

Grid preparation and cryo-EM data acquisition
Cryo-EM grids were prepared using a Vitrobot Mark IV (FEI) with an environmental chamber set to 95% humidity and 4 °C. Purified OST-A complex at 2.5 mg/mL was supplemented with 100 μM NGl-1 (Sigma) and incubated for 15 min prior to vitrification. Aliquots of 4 μL of sample were placed onto glow-discharged Quantifoil carbon grids (R1.2/1.3, copper, 300 mesh). Grids were blotted with filter paper for 2.5–4.0 sec and flash-frozen in a mixture of liquid ethane and propane cooled by liquid nitrogen. Grids prepared with different blotting times were screened for ice thickness and particle distribution. The best grid was imaged with a Titan Krios (FEI) electron microscope operated at 300 kV, equipped with a Gatan K3, direct electron detector and Gatan Imaging Filter, with a slit width of 20 eV. Movies were recorded semi-automatically with EPU2 software (Thermo Fisher Scientific) with a defocus range between −0.6 and −2.4 μm and in super-resolution mode with a super-pixel size of 0.324 Å/pixel.

Cryo-EM data processing and model building
Data processing was performed entirely using cryoSPARC v4.2.1. A detailed pipeline of cryo-EM data processing is shown in Figure S6 and further details are in Table S7. Briefly, movies were corrected for beam-induced motion using patch motion correction and binned twice. Contrast transfer function (CTF) parameters were estimated using patch CTF estimation. Particle-picking was performed using blob picking, and binned by a factor of 2 during particle extraction. Several rounds of 2D classification were performed and the best classes were selected. A small set of particles was used to generate an initial map, which was subsequently used as reference for two rounds of heterogeneous 3D refinement. Particles in the best class were re-extracted to 0.648 Å/pixel, and subjected to an additional round of heterogeneous 3D refinement. Good classes were combined and further refined using non-uniform refinement and local refinement with mask yielding a 3D reconstruction at 3.6 Å resolution. Model building was performed in Coot 0.9,78 using the DoiP-bound OST-A model PDB ID:67SO as initial model. Final model was refined in PHENIX version 1.17.1.79

Figure preparation and data analysis
Structural figures were prepared using PyMOL 4.6.0 and UCSF ChimeraX 0.9.30

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical information for individual experiments can be found in the corresponding figure legends. Statistical analyses were carried out using GraphPad Prism software. Significance of all comparisons was calculated by unpaired two-tailed t test using a cutoff of p < 0.05.
Figure S1. CCDC134 is N-glycosylated at Asn148, related to Figure 3

(A) Immunoblot of TNFAIP3−/− NF-κB-DCK− NALM-6 cells stably infected with either a control sgRNA (sgctrl) or an sgRNA to CCDC134 (sgCCDC134) and then stably infected with a lentivirus encoding an HA-tagged, sgRNA-resistant form of the indicated CCDC134 variants. Cells were treated with 10 μg/mL LPS for 24 h prior to collection for immunoblotting.

(B) Immunoblot analysis of cell extracts from HEK293T cells stably expressing an HA-tagged CCDC134 after the extracts were treated with the N-glycosidase PNGase F (PNGase) or endoglycosidase H (EndoH) ex vivo.
Figure S2. The CD16-TLR4 chimera localizes to the cell surface, related to Figure 3

FACS for surface TLR4 (left) and surface CD16 (right) in NALM6 TNFAIP3−/− NF-κB-DCK* reporter cells, with or without intact STT3A, after stable infection with lentiviruses encoding TLR4 or CD16-TLR4, as indicated. Note that the CD16-TLR4 chimera does not contain the TLR4 extracellular domain recognized by the anti-TLR4 antibody used for the left panels.
Figure S3. NGI-235 is a derivative of NGI-1 that selectively inhibits OST-A, related to Figure 4

(A) The three-step synthesis of the NGI-1 derivative NGI-235 began with commercially available 5-(chlorosulfonyl)-2-fluorobenzoic acid (compound 1). Reaction with N-methylbenzylamine and triethylamine in dichloromethane gave sulfonamide 2 in 83% yield. Subsequent coupling with 5-ethylthiazol-2-amine using hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) and diisopropylamine in DMF gave the corresponding amide (3) in 88% yield. Finally, nucleophilic aromatic substitution of the fluoride with 1-cyclopropyl-N-methylmethylamine at 80°C afforded NED-4086 (NGI-235) in 39% yield.

(B) Wild-type, STT3A knockout, or STT3B knockout HEK293T cells stably expressing the Halo 3N glycoprotein were treated with the indicated concentrations of NGI-235 for 24 h and analyzed by immunoblot. Arrows on the right indicate the different glycoforms of Halo 3N (0–3n). The hypo-glycosylated form of the Halo protein only appears in NGI-235-treated cells that retain STT3A as the sole OST catalytic subunit.

(C) Wild-type, STT3A knockout, or STT3B knockout HEK293T cells were treated with the indicated concentrations of NGI-1 or NGI-235 for 24 h, and the glycosylation of multiple endogenous proteins was analyzed by immunoblot. NGI-235 has minimal effects on glycosylation of proteins in an STT3A-knockout background.
Figure S4. NGI-235 has effects on prosaposin, an STT3A-dependent substrate, but not on ER-GFP\textsuperscript{Q815N,N186C}, an STT3B-dependent substrate, related to Figure 4.

(A) Immunoblot of clonal HEK293T cells with the indicated STT3A/B genotypes that were stably infected with a lentivirus encoding ER-GFP, ER-GFP\textsuperscript{Q815N,N186C}, or with the empty vector.

(B) GFP intensity, as measured by FACS, of clonal HEK293T cells stably expressing ER-GFP\textsuperscript{Q815N,N186C} as in (A). Error bars represent standard deviation.

(C) Immunoblot of HEK293T cells stably expressing TLR4-HA and either ER-GFP or ER-GFP\textsuperscript{Q815N,N186C} after treatment with DMSO, NGI-1 (10 \( \mu \)M), or NGI-235 (10 \( \mu \)M) for 24 h. TNF-R1 is a glycosylated cell-surface receptor minimally affected by STT3A knockout (see Figure 4F) that is also minimally affected by NGI-235 treatment.

(D) GFP intensity, as measured by FACS, of clonal HEK293T cells stably expressing ER-GFP\textsuperscript{Q815N,N186C} and treated with DMSO, NGI-1 (10 \( \mu \)M), or NGI-235 (10 \( \mu \)M) for 24 h, as in (C). Error bars represent standard deviation.
Figure S5. Putative drug-resistant mutants of STT3A have baseline activity comparable to wild-type STT3A in the absence of NGI-1, related to Figure 5.

(A and B) (A) Anti-TLR4 FACS to detect cell-surface TLR4 and (B) anti-HA immunoblot analysis of either STT3A+/+ or STT3A−/− HEK293T cells expressing exogenous TLR4-HA that were rescued by stable retroviral infection of the indicated STT3A variants. Error bars represent standard deviation.

(C) Anti-TLR4 FACS to detect cell-surface TLR4 in STT3A−/− HEK293T cells stably expressing TLR4-HA and then rescued by stable retroviral infection with the indicated STT3A variants. Error bars represent standard deviation.
Figure S6. Purification of OST-A and cryo-EM data analysis, related to Figure 6
(A) Preparative size exclusion chromatography profile of purified OST-A in a Superose 6 10/300 GL column.
(B) SDS-PAGE of purified OST-A after preparative SEC.
(C) Pipeline of the EM data processing.
(D and E) (D) Local resolution map and (E) Fourier shell correlation (FSC) curve for the final map.
Figure S7. NGI-1 binding site in human OST complexes, related to Figure 6
Left: binding site of NGI-1 in STT3A as observed in the structure of NGI-1-bound OST-A (PDB: 8PN9). NGI-1 is shown as sticks and colored orange. Right: predicted binding site of NGI-1 in STT3B, based on the structure of a ternary complex of OST-B (PDB: 6S7T).