

Defining the BTK-dependent chromatin-informed gene regulatory network in chronic lymphocytic leukemia

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Abstract

Bruton tyrosine kinase (BTK) is central to chronic lymphocytic leukemia (CLL) biology, yet the regulatory programs that depend on BTK remain poorly defined. Here, we used acute NX-5948 (Bexobrutideg)-mediated BTK degradation to define BTK-dependent regulatory circuitry in CLL. Integrating early transcriptional and chromatin responses with chromatin-state annotation, target-gene linkage, and transcription factor assignment, we resolved a chromatin-informed framework linking BTK loss to downstream gene-regulatory output. This framework is separated into two directional programs: a dominant BTK-maintained network that collapsed after BTK degradation and a smaller BTK-released network that emerged upon BTK loss. BTK-responsive elements were concentrated in promoter- and enhancer-linked chromatin compartments, consistent with a model in which BTK-dependent transcriptional output is mediated through specific active regulatory elements. Functional validation in a CLL-like cell model provided orthogonal support for the NFAT-linked component of the BTK-maintained regulatory circuitry. BTK-dependent regulon activity was associated with clinical outcome, and projection of bulk-derived programs into longitudinal single-cell RNA-seq resolved malignant CLL cells into distinct regulatory states with maintained-like and released-like features. Together, this work defines BTK-dependent regulatory programs in CLL through a chromatin-informed framework linking BTK activity to gene-regulatory output. These findings lay the foundation for understanding how BTK-dependent circuitry contributes to malignant cell-state diversity, clinical outcome, and remodeling during therapy.

Introduction

Bruton tyrosine kinase (BTK) is a central mediator of B-cell receptor (BCR) signaling and a major determinant of chronic lymphocytic leukemia (CLL) biology and other B-cell malignancies, influencing malignant B-cell survival, proliferation, and microenvironmental interactions.[1, 2] BTK-targeted therapies have transformed the management of CLL, yet relapse and resistance remain common, arising through both genetic lesions and adaptive remodeling of signaling networks.[1] Despite the central role of BTK in CLL pathogenesis and therapy, the regulatory programs that depend on BTK remain incompletely defined. Our recent work used NX-5948-mediated BTK degradation to define an acute BTK-loss transcriptional signature in primary CLL, highlighting clinically relevant transcriptional outputs of BTK depletion.[3] However, transcriptional response alone does not reveal how BTK-dependent gene expression is wired through regulatory chromatin and transcription factor networks. Defining that layer requires reconstruction of a gene regulatory network (GRN), because a GRN organizes pathway-responsive genes and cis-regulatory elements into connected TF–regulatory element–gene relationships that can distinguish core signaling-dependent circuitry from secondary or compensatory responses.[4, 5] In cancer, this level of organization is particularly important because it provides a mechanistic framework for identifying regulatory dependencies, interpreting malignant cell states, and understanding how therapeutic perturbation reshapes the underlying circuitry.[6]

Epigenomic studies have shown that CLL is characterized by extensive remodeling of regulatory chromatin.[7–10] Reference epigenome analyses established that CLL harbors widespread *de novo*

active and accessible regulatory elements, with differences across major clinico-biological subtypes.[8] Complementary enhancer-focused studies integrating H3K27ac and chromatin accessibility defined core regulatory circuitry in CLL and identified lineage-linked transcription factor dependencies, including a prominent role for PAX5-centered enhancer logic.[11] Our work has shown that BCR signaling can regulate specific CLL transcriptional outputs through direct enhancer control.[12] Longitudinal studies of ibrutinib-treated CLL patients demonstrated that BTK inhibition induces reproducible chromatin and transcriptional remodeling, including reduced NF- κ B activity and progressive erosion of CLL cell identity.[13] Together, these studies define important aspects of baseline regulatory architecture and therapy-associated response, but they do not resolve a chromatin-informed framework linking BTK activity to transcription factor occupancy, regulatory elements, and target-gene output.

Acute targeted degradation provides a useful way to interrogate this missing layer. In contrast to traditional ATP-competitive BTK inhibitors, which suppress catalytic activity while leaving non-catalytic BTK functions intact, BTK degraders can specifically deplete the BTK protein and reduce confounding from prolonged adaptive remodeling.[14] In primary CLL and patient-derived models, degrader approaches can induce rapid and sustained depletion of wild-type and mutant BTK while suppressing BCR-linked signaling outputs. This creates an opportunity to use acute BTK loss not simply as a therapeutic perturbation, but as a mechanistic tool for defining BTK-dependent regulatory circuitry. Recent work in other oncogenic signaling systems has highlighted the value of a similar strategy. In KRAS-mutant cancers, acute pathway-directed analyses were used to define the KRAS- and ERK-dependent transcriptome and the ERK-regulated phosphoproteome, thereby resolving missing regulatory layers that could not be inferred from steady-state measurements alone and linking pathway dependence to system-level network architecture.[15, 16] By analogy, acute BTK degradation provides a principled framework for reconstructing the BTK-dependent gene regulatory network in CLL, because it captures the proximate consequences of pathway loss before chronic adaptation obscures the underlying circuitry.

Here, we integrated RNA-seq and ATAC-seq responses to acute NX-5948 exposure with baseline chromatin-state annotation, regulatory-element-to-gene linkage, and motif-informed transcription factor assignment to define a chromatin-informed BTK regulatory framework in CLL. This analysis resolved two directional programs, a dominant BTK-maintained network and a smaller BTK-released network, and linked these programs to promoter- and enhancer-associated regulatory elements. We then extended this framework beyond the discovery cohort by testing its association with clinical outcome and by projecting bulk-derived programs into longitudinal single-cell RNA-seq to determine whether BTK-dependent circuitry resolves into distinct malignant cell states *in vivo*.

Materials and methods

Detailed methods are provided in the Supplementary Information.

Primary CLL samples

All patients provide research blood samples by signing and submitting written informed consent according to the Declaration of Helsinki to the Mayo Clinic Institutional Review Board, which approved these studies. Informed consent was also obtained from healthy donors to obtain their B cells. Clinical annotations, including [treatment status, IGHV status, cytogenetics], are provided in Table S1. Primary CLL B cells and normal B cells were purified from blood samples using the RosetteSep B cell enrichment kit (Stem Cell Technologies). The typical purification of CD5+/CD19 + CLL B cells was > 95%, as determined by flow cytometric analysis. Normal B cells were purified from healthy donor blood samples using the RosetteSep B cell enrichment kit (Stem Cell Technologies) with CD19 + CLL B cells at > 95%.

Cell treatment and acute BTK degradation

CLL B cells were cultured in AIM V medium with NX-5948 (MedChemExpress) (1nM) or DMSO for 6 hours. Then, anti-IgM (10 µg/mL) or vehicle control was added to the culture for an additional 18 hours before the cells were harvested. BTK degradation was verified by immunoblot.

Data and code availability

The bulk RNA-seq, ATAC-seq, and CUT&Tag datasets generated in this study have been deposited in a public repository and will be released upon publication. Accession numbers will be provided before final acceptance (links will be available to reviewers). Processed data underlying the chromatin-informed BTK regulatory atlas, including differential-expression tables, differential-accessibility tables, peak-to-gene linkage tables, TF–peak–gene network tables, and program gene sets, will be made available in a public data repository. Custom code used for analysis and figure generation will be deposited in a public repository and made available upon publication. Public single-cell RNA-seq data analyzed in this study were obtained from GSE111014.

Results

Acute BTK degradation reveals a proximate BTK-dependent regulatory state distinct from chronic ibrutinib-associated remodeling in CLL

To define the BTK-dependent molecular state in CLL, we used acute NX5948-mediated BTK degradation as a mechanistic perturbation and profiled primary CLL cells by RNA-seq and ATAC-seq (n = 8, **Table S1**), while using baseline histone profiles as a chromatin prior for downstream network reconstruction (Fig. 1A). This design was intended to capture the immediate consequences of BTK loss rather than the remodeled states that emerge under prolonged inhibition. Acute BTK degradation produced a strong and directional response at both the transcriptional and chromatin levels. RNA-seq identified 711 significantly changed genes, with a predominance of decreased over increased transcripts (442 down versus 269 up) (Fig. 1B, **Table S2**).^[3] ATAC-seq identified 1,336 significantly changed accessible regions among 83,036 tested, with a marked excess of closing over opening peaks (1,100 closing versus 236

opening) (Fig. 1C, **Table S3**). MA plots and heatmaps showed that these responses were coherent across samples in both assays (**Fig. S1A-D**). Genomic annotation of NX5948-responsive ATAC peaks showed that both opening and closing regions were distributed predominantly across intronic and intergenic compartments, consistent with regulation through non-promoter cis-regulatory elements (**Fig. S1E**). Integration of linked RNA and ATAC changes further showed that concordant responses were strongly biased toward chromatin closing with gene downregulation, consistent with collapse of a BTK-supported transcriptional state (**Fig. S1F, Table S4**).

We next placed this acute degrader-defined state in the context of prolonged BTK inhibition. Same-direction overlap between acute NX5948 response and longitudinal 1-year ibrutinib response from CLL patients was limited at both the RNA and chromatin levels,[10, 12] with 34 shared downregulated genes and 13 shared upregulated genes, together with 85 shared closing regions and only 1 shared opening region (Fig. 1D, E). Pathway analysis reinforced this distinction. (Fig. 1F and **Fig. S1G**). Genes decreased after acute NX5948 treatment were enriched for TNF α signaling via NF- κ B, MYC targets, mTORC1 signaling, unfolded protein response, and IL2/STAT5 signaling, whereas genes increased after 1 year of ibrutinib were enriched for inflammatory response, TNF α signaling via NF- κ B, KRAS signaling, IL6/JAK/STAT3 signaling, complement, coagulation, and related remodeling programs (Fig. 1F). Thus, acute BTK degradation does not simply reproduce a chronic inhibitor-associated state. Instead, it presents a coordinated BTK-dependent transcriptional and chromatin state that is masked by long-term therapeutic adaptation and therefore provides an appropriate entry point for reconstruction of BTK-dependent regulatory circuitry. These results indicate that most available BTK-linked transcriptional datasets reflect chronic inhibition and therefore do not fully capture the immediate consequences of BTK loss.[3]

BTK-responsive chromatin changes localize to active promoter- and enhancer-linked regulatory elements

Having defined an acute BTK-dependent transcriptional and chromatin state, we next asked where in the regulatory genome this response resides. To provide chromatin context, we intersected NX5948-responsive ATAC peaks with baseline histone-mark maps generated from H3K4me1, H3K4me3, H3K27ac, H3K27me3, H3K9me3, and H3K36me3 CUT&Tag (Fig. 2A). Both opening and closing peaks overlapped active chromatin features, but the dominant closing arm showed particularly strong overlap with H3K4me1 and H3K27ac, supporting disruption of active cis-regulatory elements after acute BTK loss. By contrast, repressive chromatin features contributed a smaller fraction of the response.

Chromatin-state classification further validated this interpretation. NX5948-responsive peaks were enriched in active promoter and enhancer states, whereas polycomb-associated, heterochromatic, and transcribed gene-body states only made smaller contributions (Fig. 2B). Collapsing these states into broader regulatory classes highlighted the same conclusion: BTK-responsive chromatin changes were focused primarily within promoter- and enhancer-linked regulatory compartments rather than being

diffusely distributed across the accessible genome (**Fig. S2A**). Notably, the dominant closing-peak arm was heavily enriched in enhancer-linked chromatin, indicating that acute BTK degradation preferentially destabilizes active cis-regulatory architecture that supports transcriptional output in CLL.

We next linked BTK-responsive peaks to candidate target genes to connect regulatory-element behavior to downstream transcription. For this purpose, we generated a scored peak-to-gene framework based on peak annotation, distance to the transcription start site, promoter/distal/gene-body assignment, and consistency between ATAC and RNA responses (see methods for details) (**Figs. S2B and S2C**).^[5] This yielded 1,288 linked peak-gene relationships involving 1,112 genes (**Fig. 2C**) (**Table S5**). These links were dominated by distal and gene-body assignments, with a smaller promoter-linked subset, thereby establishing a broad substrate for downstream TF-peak-gene reconstruction. We also showed that linked peak-gene relationships displayed the expected distance-to-TSS structure and were more often concordant than discordant with RNA response, particularly within the dominant closing arm (**Fig. S2C**). Together, these results show that the acute BTK-dependent state exposed by NX5948 is anchored in active promoter- and enhancer-linked chromatin and can be propagated to target-gene output, providing the chromatin-informed framework for reconstructing BTK regulatory circuitry in CLL.

A chromatin-informed TF-peak-gene atlas resolves BTK-maintained and BTK-released regulatory circuitry in CLL

We next used these linked peak-gene relationships to build a chromatin-informed TF-peak-gene atlas of BTK response in CLL. In this framework, candidate TFs were first assigned to BTK-responsive peaks based on motif support, and those TF-associated peaks were then connected to linked target genes through the peak-to-gene map. We retained directional relationships in which TF-associated peak behavior and linked gene behavior were coherent with the response to acute BTK degradation, and we then grouped these edges into two directional subnetworks: a BTK-maintained arm and a BTK-released arm as described above (**Fig. 3A**). The resulting atlas was strongly asymmetric in size, with the BTK-maintained arm containing far more regulatory links, TF hubs, and target genes than the BTK-released arm (**Fig. 3B and 3C**, **Fig. S3A**, **Table S5**).

At the hub level, we defined each TF hub by aggregating all TF-assigned peak-to-gene links connected to that TF and then ranking TFs by the number of linked target genes in the resulting network. By this measure, the BTK-maintained network was centered on TFs linked to canonical BCR-responsive transcriptional output, including JUN, NFATc1, FOSL2, ISL1, and NFKB1, whereas the BTK-released network was organized around a smaller set of hubs led by TCF3, PTF1A, TCF12, ASCL1, and ASCL2 (**Figs. 3D, S3B and S3C**). The target-gene structure of the atlas exhibited substantially broader connectivity in the maintained arm than in the released arm (**Fig. S3C**). Together, these features indicate that BTK-maintained circuitry is larger and more internally connected than the BTK-released circuitry that emerges after BTK loss.

Mapping these links onto baseline chromatin states showed that both subnetworks were localized at enhancer-like regulatory elements, with the strongest enrichment in active enhancers and only limited representation at active promoters (Fig. 3C and 3E). In contrast, the promoter-centered component was smaller and compositionally distinct, with a broader contribution from active-promoter and non-enhancer states (Fig. 3E). Representative browser tracks illustrated these principles at specific loci. At the IGSF3 locus, an ISL1 or NFATc1-assigned BTK-maintained peak lay within an enhancer-like chromatin context at baseline and showed reduced accessibility after NX-5948 despite anti-IgM stimulation (Fig. 3F). Similarly, at the TGFBR3 locus, NFATC1- and ATF3-assigned BTK-maintained peaks were embedded within enhancer-like baseline chromatin and also lost accessibility after BTK degradation (**Fig. S3D**). Together, these analyses reveal a chromatin-informed GRN in which acute BTK degradation preferentially drives the collapses of a large enhancer-centered regulatory network while also releasing a smaller secondary program.

NFATc1 perturbation preferentially disrupts BTK-maintained chromatin features in a CLL-like model

To test whether a key TF node emerging from the chromatin-informed atlas could functionally recapitulate the BTK-maintained regulatory arm, we selected NFATc1 for orthogonal perturbation in the CLL like MEC1 cell line.[17] We chose NFATc1 for validation for three reasons. First, NFATc1 ranked among the prominent hubs in the BTK-maintained network identified after acute BTK degradation, placing it within the dominant circuitry that collapsed after BTK loss (Fig. 3D). Second, NFATc1 represented the NFAT-linked branch of the BTK-maintained atlas and therefore provided a tractable way to test whether disruption of one major maintained TF module was sufficient to erode BTK-linked chromatin structure. Third, our previous work showed NFATc1 has an important role in BCR signaling-regulated enhancers.[12]

Because MEC1 is a CLL-like model rather than a primary-patient system, we first placed its chromatin landscape in the context of the atlas framework.[17] MEC1 shared a substantial subset of accessible chromatin with the broader CLL regulatory landscape and the GRN atlas (**Fig. S4A**). Within this structure, 483 combined-union regions were shared across MEC1, the GRN atlas, and the full CLL master set, whereas 805 GRN-atlas regions were present within the CLL master set but not detected in MEC1 (**Fig. S4A**). Thus, MEC1 partially recapitulates the atlas-defined CLL chromatin landscape and provides a tractable model for functional validation of selected regulatory nodes.

We next used CRISPR-mediated depletion of NFATc1 in MEC1 cells and confirmed effective reduction of NFATc1 protein in both sgNFATc1 perturbation groups relative to sgGFP controls (Fig. 4A). To determine whether NFATc1 loss affected the atlas-defined BTK-maintained chromatin program, we profiled ATAC signal across the 598 BTK-maintained peaks. Metagene plots and heat maps showed broadly reduced accessibility in two NFATc1 depleted samples relative to sgGFP across these BTK-maintained regions (Fig. 4B). The result that sgNFATc1/2 showed less reduction compared to sgNFATc1/1 was consistent to

the less effective depletion of this guide RNA. Region-level quantification confirmed this effect: compared with sgGFP, accessibility across BTK-maintained peaks was significantly reduced in both sgNFATc1/1 and sgNFATc1/2 (**Fig. S4B**; Wilcoxon $P = 1.17 \times 10^{-76}$ and 6.85×10^{-96} , respectively). Standardized paired effect sizes were moderate to large for both perturbations, with Cohen's $d_z = -0.68$ for sgNFATc1/1 versus sgGFP and Cohen's $d_z = -0.72$ for sgNFATc1/2 versus sgGFP. Browser-track views at representative atlas loci supported the same conclusion. At the TGFBR3 locus, highlighted BTK-maintained elements showed reduced accessibility after NFATc1 depletion across both replicate pairs (Fig. 4C). Similarly, at the IGSF3 locus, accessibility at the highlighted maintained element was attenuated in sgNFATc1 cells relative to sgGFP (Fig. 4D).

Taken together, these data provide orthogonal support for the NFAT-linked branch of the BTK-maintained atlas. Although MEC1 captures only part of the full CLL regulatory landscape, CRISPR-mediated NFATc1 depletion was sufficient to reduce accessibility at atlas-defined BTK-maintained elements globally and at representative loci (Fig. 4B–D; **Fig. S4A, B**). These findings support a model in which NFATc1 is not merely a correlated output of BTK signaling, but a functional component of the maintained chromatin circuitry that helps sustain active regulatory elements in CLL-like cells.

BTK-maintained regulatory circuitry is preferentially associated with progression-free outcome in CLL

We next asked whether the BTK-responsive TF programs resolved by the chromatin-informed atlas were associated with clinical outcome in the ICGC CLL cohort.[18, 19] To do this, we projected the atlas-derived TF program gene sets into the curated ICGC CLL cohort and quantified each program in each sample as the mean expression of its member genes in the preferred untreated primary tumor-specimen expression matrix with matched audited clinical annotations. In the validated untreated primary overall survival (OS) cohort, no BTK-maintained or BTK-released TF program remained significant after multiple-testing correction. Given the much smaller number of OS events than progression free survival (PFS) events in the audited cohort, this negative result is most consistent with limited OS sensitivity in this setting rather than with a lack of biological relevance (**Fig. S5A**).

We therefore focused the main clinical analysis on strict PFS, which is more closely aligned with active disease behavior. In the untreated primary PFS cohort, BTK-maintained programs dominated the prognostic landscape: most significant associations arose from the BTK-maintained arm, whereas only a minority came from the BTK-released arm (Fig. 5A). This fits the biological structure of the atlas, in which the BTK-maintained network is the larger and more coherent branch that collapses after BTK degradation, while the BTK-released branch is smaller and likely reflects secondary or compensatory rewiring.

To define how this clinical signal mapped onto the atlas, we examined representative TF programs that span the major transcriptional outputs of the BTK-maintained network together with a comparator from the BTK-released arm. FOSL2, NFATc1, and NFkB1 represent AP-1-, NFAT-, and NF-kB-linked branches of

the BTK-maintained circuitry, respectively, whereas ASCL2 was included as a representative BTK-released program. In this framework, progression-associated signal localized predominantly to the same AP-1-, NFAT-, and NF- κ B-linked network that collapsed after BTK degradation, whereas the BTK-released arm contributed a smaller set of associations overall (Fig. 5B-F).

Taken together, the outcome analysis supports a model in which the BTK-maintained regulatory arm, rather than the BTK-released arm, is the principal clinical correlate of disease progression. This strengthens the central interpretation of the atlas that acute BTK degradation preferentially dismantles a transcriptional network that is both mechanistically downstream of BCR/BTK signaling and clinically relevant to ongoing CLL progression.

Single-cell profiling reveals heterogeneous remodeling of BTK-maintained and BTK-released states in CLL

We next asked whether the BTK-responsive programs identified by the chromatin-informed atlas were preserved in a published longitudinal single-cell RNA-seq dataset from CLL during ibrutinib treatment. [13] Using marker-based annotation, we found that this compartment partitioned into multiple transcriptionally distinct states. Subsequent cluster-selection analyses resolved clusters 0, 4, 5, and 8 as the principal CLL states (Fig. 6A; **Fig. S6A, B**). At the level of the CLL compartment, BTK-maintained program activity generally declined across treatment, whereas BTK-released activity was more variable and remained stable or increased in several patients (Fig. 6B **and C**). Thus, the directional asymmetry seen in the bulk atlas was preserved at single-cell resolution.

We therefore asked how these BTK-responsive programs were distributed across the key CLL states. Baseline program balance differed substantially across clusters 0, 4, 5, and 8 (Fig. 6D, E, **and F**). Cluster 5 showed the highest baseline BTK-maintained activity, whereas cluster 8 showed the strongest BTK-released signal. By contrast, clusters 0 and 4 were relatively depleted for maintained activity, and cluster 4 was also low for released activity. These data indicate that the malignant compartment contains pre-existing transcriptional states that differ in their relationship to the maintained and released BTK programs before treatment.

Longitudinal analysis showed that treatment remodeled these states asymmetrically rather than uniformly. BTK-maintained activity decreased across all key clusters, with the largest drops in clusters 0 and 4 (Fig. 6G and S6C). By contrast, BTK-released activity increased in clusters 0, 4, and 5, but not in cluster 8, where it instead declined slightly from baseline (Fig. 6H and S6C). This indicates that the emergence of the released program is state-dependent and does not simply mirror the collapse of the maintained arm across all malignant subpopulations.

At the level of individual TF hubs, the same asymmetry was preserved. Maintained TF programs, including NFATc1, JUN, FOSL1, FOSL2, NFKB1, and TCF12, showed broadly negative treatment-associated shifts across clusters 0, 4, 5, and 8, whereas released TF programs, including ASCL1, TCF3,

TCF12, PTF1A, and ASCL2, showed positive shifts that were strongest in cluster 0 and progressively weaker across clusters 4 and 5 (Fig. 6I). Pathway analysis further distinguished the biological character of these states, separating an MHC II-high cluster from BCR-associated states within the CLL compartments (Fig. 6J). Together, these single-cell analyses validate the central conclusion of the atlas: BTK loss is dominated by the collapse of a maintained regulatory network, while a smaller released program emerges in a state-restricted manner across malignant CLL cells.

Discussion

BTK is a central effector of B-cell receptor signaling and a major determinant of CLL biology, yet the direct regulatory circuitry that depends on ongoing BTK activity has yet to be defined. A major reason is that most available BTK-linked transcriptional and chromatin datasets come from chronic inhibitor exposure or remodeled steady states, conditions in which adaptive rewiring and compensatory programs are already superimposed on the primary pathway response.[12, 20] In this study, acute NX-5948-mediated BTK degradation enabled resolution of the immediate chromatin and transcriptional consequences of BTK loss in CLL. By integrating early RNA and ATAC responses with baseline chromatin-state annotation, peak-to-gene linkage, and TF assignment, we defined a chromatin-informed regulatory framework that connects BTK loss to downstream gene-regulatory output. Rather than producing a uniform response, acute BTK degradation separated into two directional programs: a dominant BTK-maintained network that collapsed after BTK loss and a smaller BTK-released network that emerged upon degradation. This organization provides a regulatory view of BTK signaling in CLL that extends beyond individual genes and instead resolves the structure of the downstream malignant program.

A central conclusion of this work is that the BTK-maintained arm is the major regulatory consequence of acute BTK loss. BTK-responsive elements were concentrated in promoter- and enhancer-linked regulatory space, indicating that ongoing BTK signaling sustains both proximal and distal chromatin activity at genes that help define the CLL state. Integration of motif enrichment with linked target-gene behavior further showed that this maintained arm is organized around TF modules connected to canonical BCR-responsive outputs, including AP-1, NFAT, and NF- κ B linked programs.[21] These findings are consistent with the established role of BTK as a core signaling node downstream of the BCR but extend that framework by showing how BTK dependence is distributed across a linked regulatory architecture. In contrast, the BTK-released arm was smaller and less coherent, suggesting that the primary effect of acute BTK degradation is not activation of an alternative dominant network, but dismantling of a pre-existing regulatory system that supports the malignant state.

The value of the chromatin-informed framework is that it resolves this organization across regulatory layers that are often studied separately. Differential accessibility alone does not distinguish promoter-linked from enhancer-linked responses, and transcriptional changes alone do not identify the regulatory elements and TFs through which those changes are structured. By integrating these layers, we could move from responsive peaks to linked genes, from linked genes to candidate TF hubs, and from TF hubs

to directional subnetworks within the broader BTK response. In this sense, the atlas provides not only a catalog of BTK-responsive features but also a mechanistic model of how acute BTK loss is propagated through the CLL regulatory landscape.

Our orthogonal validation supports this interpretation. NFATc1 was prioritized because it emerged as a prominent hub within the BTK-maintained atlas and represented the NFAT-linked branch of the dominant maintained circuitry. In MEC1 cells, which partially recapitulated the atlas-defined CLL chromatin landscape, CRISPR-mediated NFATC1 depletion reduced accessibility across atlas-defined BTK-maintained elements, including representative maintained loci. These data provide functional support for the NFAT-linked branch of the maintained atlas and argue that NFATc1 is not simply a correlated output of BTK signaling, but a component of the chromatin circuitry that helps sustain BTK-maintained regulatory elements.

Our single-cell analyses further showed that this remodeling is not uniformly distributed across malignant cells. Instead, the maintained and released programs were partitioned across distinct CLL states and changed differently across those states during treatment. Broad loss of BTK-maintained activity across malignant clusters supports the view that this arm reflects core BTK-dependent circuitry, whereas the more selective emergence of BTK-released activity indicates that secondary responses are state-restricted. These observations strengthen the conclusion that the regulatory consequences of BTK loss are shaped not only by pathway logic but also by malignant cell state. More broadly, they suggest that the CLL response to BTK loss is organized around the collapse of a shared core network together with more heterogeneous adaptive changes superimposed on specific cellular states.

Together, these findings support a model in which ongoing BTK activity sustains a dominant chromatin-linked transcriptional network that underlies the CLL state, and acute BTK loss preferentially dismantles this maintained circuitry while exposing a smaller, state-restricted released program. This distinction is important because it separates the core regulatory architecture that appears to support disease biology from secondary responses that emerge after pathway collapse. More broadly, our findings argue that the major consequence of BTK loss in CLL is the collapse of a disease-relevant maintained regulatory network, establishing this circuitry as the central downstream substrate of BTK dependence in the leukemia.

Declarations

Conflict of interest

NEK: Advisory Board for: AbbVie, AstraZeneca, BeOne, Janssen, Pharmacyclics. DSMC (Data Safety Monitoring Committee) for: AstraZeneca, BMS-Celgene, Dren Bio. Research funding from: AbbVie, Acerta Pharma, AstraZeneca, Genentech, Pharmacyclics/Janssen, Merck. All funds are directed to the Mayo Clinic and are not retained personally.

SAP

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Author Contribution

ZW conceived the study. ZW and NEK designed and supervised the study. SS, WH, and HCD contributed to the sample collection. ZW and QG performed the experiments, acquired and analyzed the data. CY provided constructive advice and reagents. SAP provided the patient information and the clinical samples. ZW, SAP, and NEK wrote the manuscript. All authors read and edited the manuscript.

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Data Availability

The bulk RNA-seq, ATAC-seq, and CUT&Tag datasets generated in this study have been deposited in a public repository and will be released upon publication. Accession numbers will be provided before final acceptance (links will be available to reviewers). Processed data underlying the chromatin-informed BTK regulatory atlas, including differential-expression tables, differential-accessibility tables, peak-to-gene linkage tables, TF–peak–gene network tables, and program gene sets, will be made available in a public data repository. Custom code used for analysis and figure generation will be deposited in a public repository and made available upon publication.

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Figures

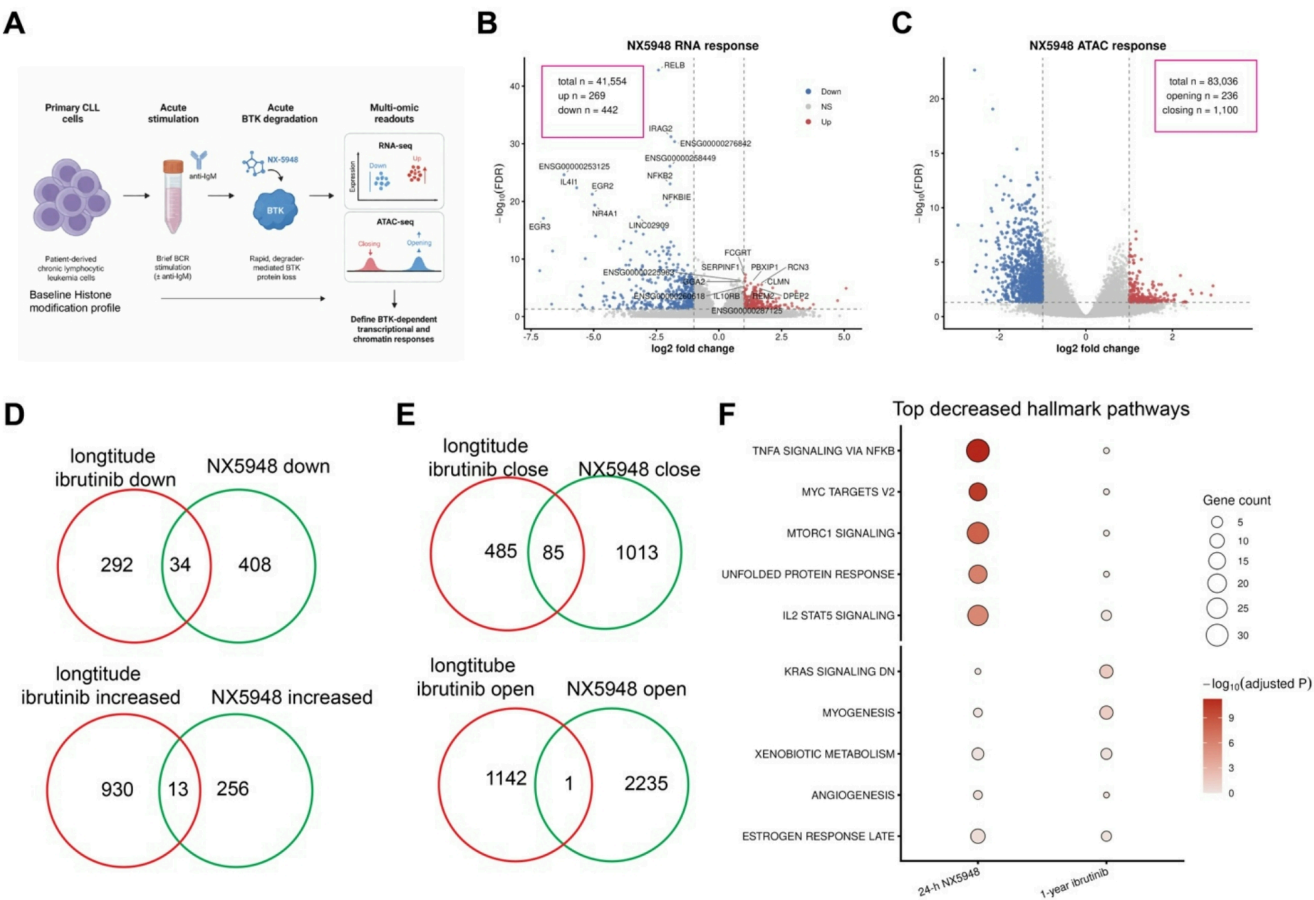


Figure 1

Acute BTK degradation reveals a coordinated BTK-dependent transcriptional and chromatin state in CLL.

(A) Experimental and analytical overview. Primary CLL cells were subjected to BCR stimulation and acute NX5948-mediated BTK degradation, followed by RNA-seq and ATAC-seq profiling; baseline histone modification profiles were incorporated as a chromatin prior for downstream regulatory-network analysis.

(B) Volcano plot showing differential gene expression after acute NX5948 treatment (NX-5948 + anti-IgM vs anti-IgM). Significantly increased genes are shown in red, significantly decreased genes in blue, and non-significant genes in gray. Selected representative genes are labeled.

(C) Volcano plot showing differential chromatin accessibility after acute NX5948 treatment. Opening peaks are shown in red, closing peaks in blue, and non-significant peaks in gray.

(D) Venn diagrams showing same-direction overlap between longitudinal 1-year ibrutinib RNA-seq gene sets and NX5948-responsive genes for downregulated genes (top) and upregulated genes (bottom).

(E) Venn diagrams showing same-direction overlap between longitudinal 1-year ibrutinib ATAC-seq peak sets and NX5948-responsive peaks for closing peaks (top) and opening peaks (bottom).

(F) Signed decreased Hallmark pathway comparison between acute NX5948 response and 1-year ibrutinib response.

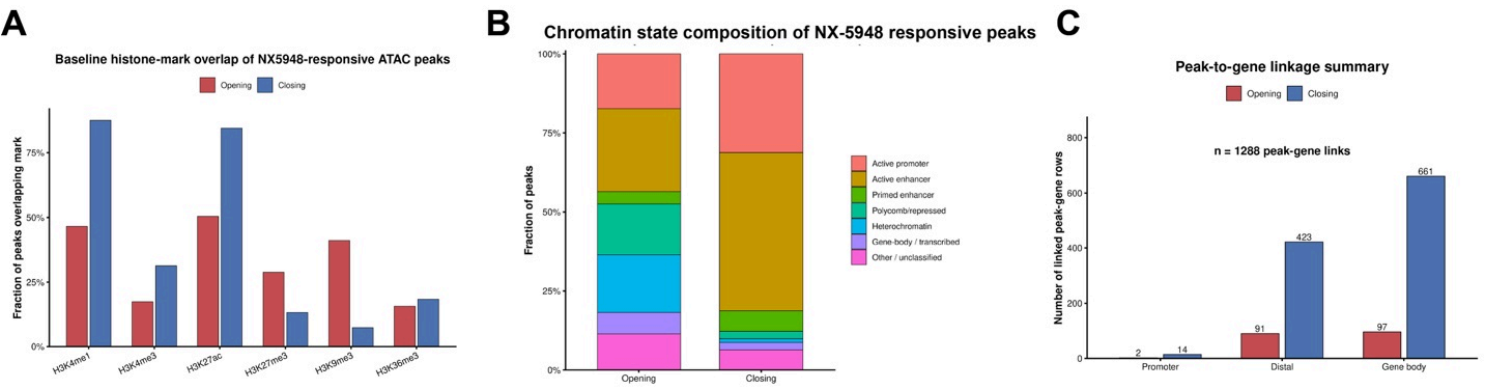


Figure 2

BTK-responsive chromatin changes localize to active promoter- and enhancer-linked regulatory elements.

(A) Baseline histone-mark overlap of NX5948-responsive ATAC peaks. Fractions of opening and closing peaks overlapping baseline H3K4me1, H3K4me3, H3K27ac, H3K27me3, H3K9me3, and H3K36me3 peak sets are shown.

(B) Chromatin-state composition of NX5948-responsive ATAC peaks. Peaks were assigned to active promoter, active enhancer, primed enhancer, polycomb/repressed, heterochromatin, gene-body/transcribed, or other/unclassified states using the baseline histone-mark framework.

(C) Summary of scored peak-to-gene links for NX5948-responsive chromatin elements. Candidate links were prioritized using genomic context, distance to the transcription start site, link class, and

consistency between ATAC and RNA responses, and are shown here stratified by link class and accessibility direction.

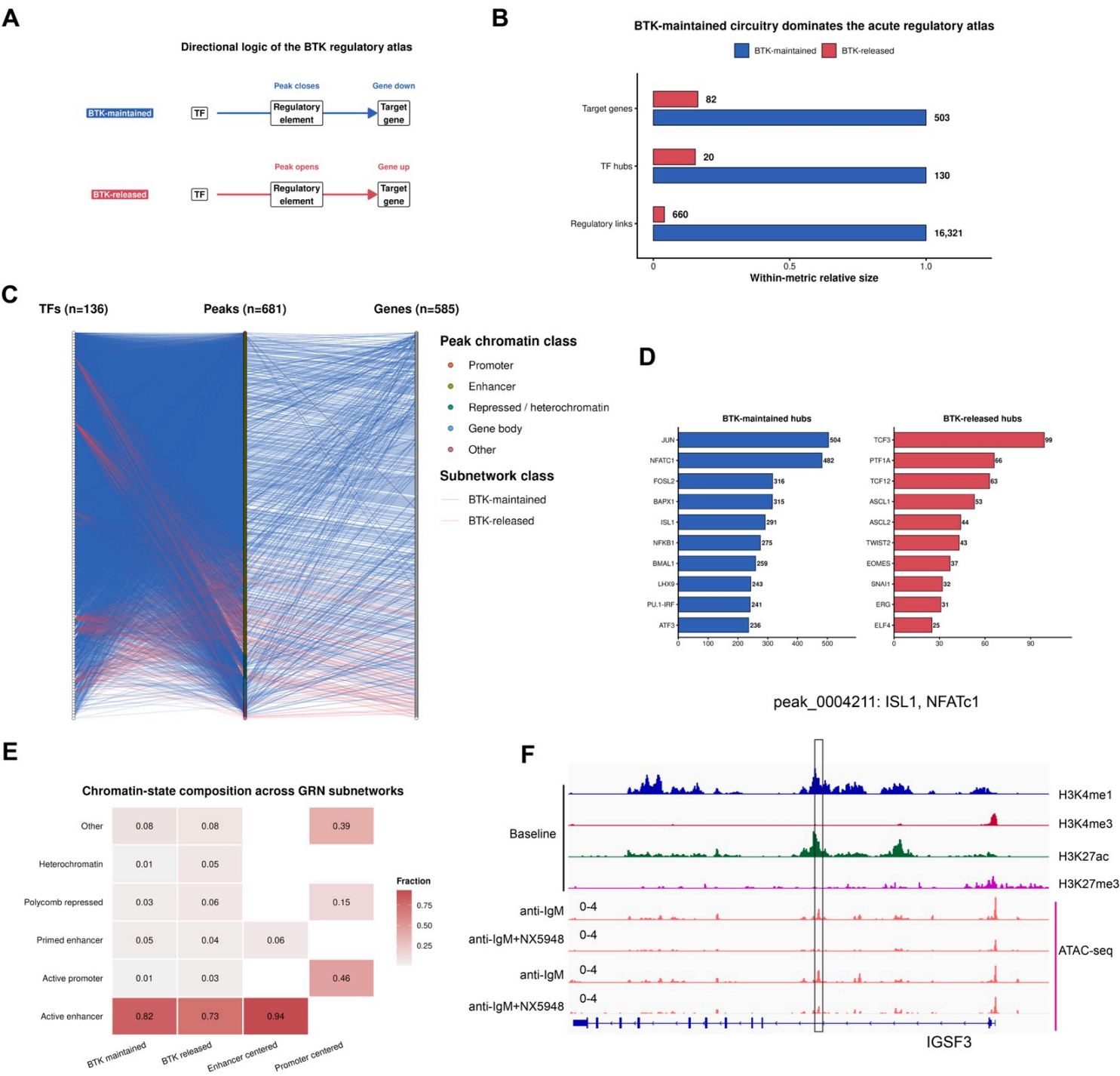


Figure 3

A chromatin-informed regulatory atlas identifies dominant BTK-maintained circuitry.

- (A) Schematic illustration of the chromatin-informed regulatory atlas.
- (B) Relative size of the BTK-maintained and BTK-released subnetworks, shown by number of regulatory links, TF hubs, and target genes. Values indicate absolute counts.

(C) Alluvial representation of the chromatin-informed GRN connecting TFs, BTK-responsive peaks, and target genes. Peak nodes are colored by baseline chromatin class; links are colored by subnetwork class.

(D) Top TF hubs in the BTK-maintained and BTK-released subnetworks, ranked by number of linked target genes after aggregating all TF-assigned peak-to-gene edges connected to each TF.

(E) Baseline chromatin-state composition of GRN subnetworks. Fractions are shown for BTK-maintained and BTK-released links, as well as enhancer-centered and promoter-centered subnetworks.

(F) Representative browser-track view of a BTK-maintained regulatory element at the IGSF3 locus. The highlighted ISL1/NFATc1-assigned peak is shown with baseline histone marks and ATAC-seq signal under anti-IgM and anti-IgM plus NX-5948 conditions. The locus illustrates loss of accessibility at an enhancer-like BTK-maintained element after BTK degradation.

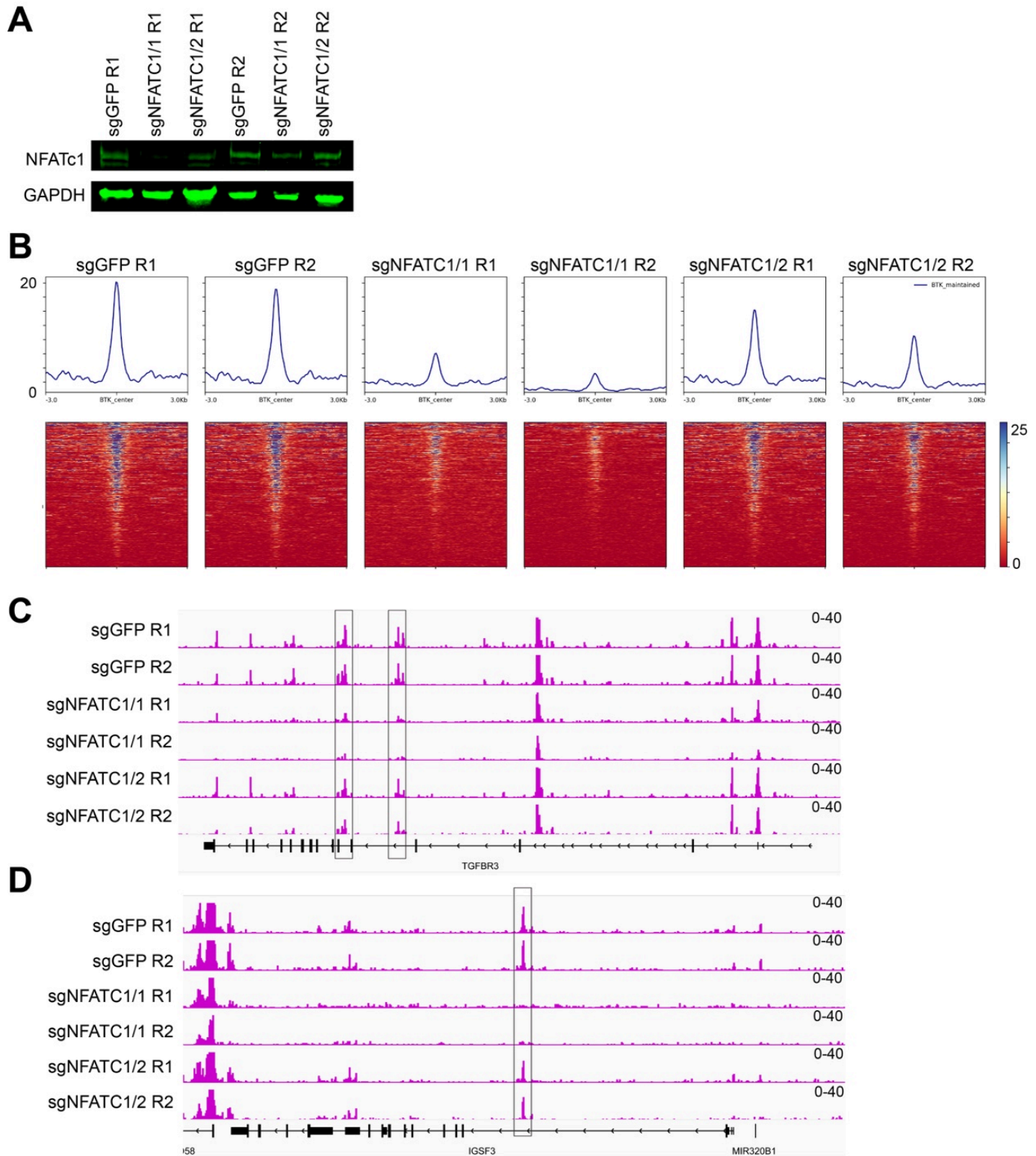


Figure 4

NFATc1 putatively regulates the BTK-maintained chromatin program in MEC1 cells.

(A) Immunoblot confirming NFATc1 depletion in sgNFATc1_1 and sgNFATc1_2 MEC1 cells relative to sgGFP controls across two replicate pairs (R1 and R2).

(B) Metagenome profiles (top) and corresponding heat maps (bottom) of ATAC signal centered on the 598 atlas-defined BTK-maintained peaks across sgGFP, sgNFATc1_1, and sgNFATc1_2 samples. Signal is

shown across ± 3 kb from peak center.

(C) Browser-track view of representative atlas-defined BTK-maintained elements at the **TGFB β 3** locus. Highlighted regions show reduced accessibility after NFATC1 depletion relative to sgGFP.

(D) Browser-track view of a representative atlas-defined BTK-maintained element at the **IGSF3** locus, showing reduced accessibility after NFATC1 depletion relative to sgGFP.

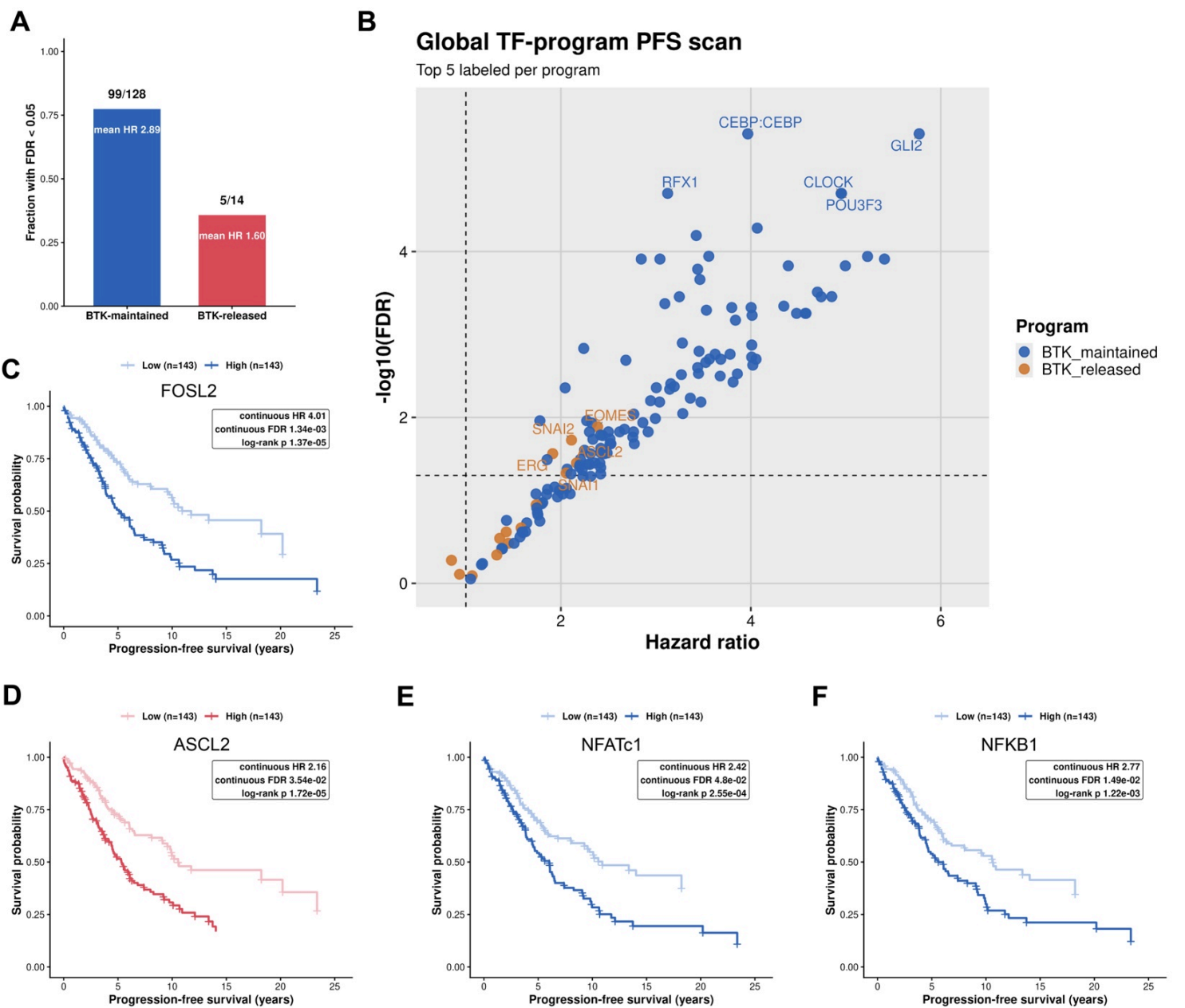


Figure 5

BTK-maintained TF programs preferentially associate with disease progression in CLL.

(A) Summary of TF-program progression-free survival (PFS) associations in the primary ICGC CLL cohort, shown as the fraction of BTK-maintained and BTK-released programs with FDR < 0.05. Numbers above bars indicate significant/tested programs; text within bars indicates mean hazard ratio.

(B) Global TF-program PFS scan across the chromatin-informed atlas. Each point represents one TF

program, plotted by hazard ratio and $-\log_{10}(\text{FDR})$; selected representative programs are labeled. Blue, BTK-maintained; red, BTK-released. Dashed lines indicate $\text{HR} = 1$ and $\text{FDR} = 0.05$.

(C–F) Kaplan-Meier analyses for representative TF programs. Patients were stratified into low and high program-score groups by median split. Annotations show the continuous Cox-model hazard ratio and FDR, together with the log-rank P value for the dichotomized comparison. (C) FOSL2 as a representative AP-1-linked BTK-maintained program. (D) ASCL2 as a representative BTK-released program. (E) NFATc1 as a representative NFAT-linked BTK-maintained program. (F) NFkB1 as a representative NF- κ B-linked BTK-maintained program. Across the cohort, progression-associated signal was concentrated in the BTK-maintained arm, consistent with the dominant collapse of this regulatory branch after acute BTK degradation.

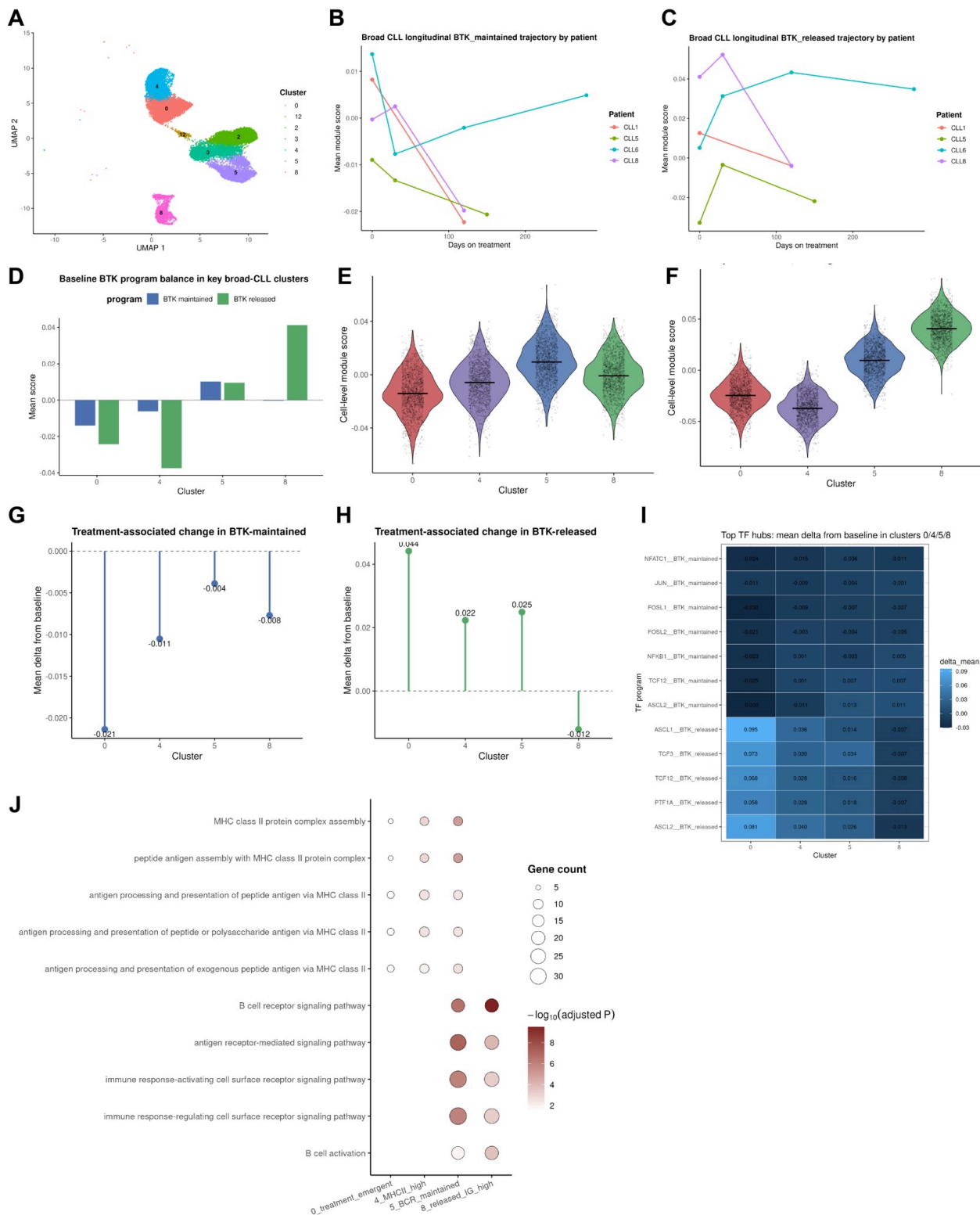


Figure 6

Single-cell validation resolves state-specific remodeling of BTK-maintained and BTK-released programs in CLL.

(A) UMAP of the CLL compartment, colored by cluster identity. Clusters 0, 4, 5, and 8 were selected as the principal malignant states for downstream analysis.

(B and C) Longitudinal patient-level mean module scores for the BTK-maintained program (B) and BTK-

released program (C) across treatment time in the broad CLL compartment.

(D) Baseline comparison of BTK-maintained and BTK-released activity across clusters 0, 4, 5, and 8.

(E and F) Cell-level distributions of baseline BTK-maintained (E) and BTK-released (F) scores across clusters 0, 4, 5, and 8.

(G and H) Treatment-associated change from baseline for BTK-maintained (G) and BTK-released (H) activity across clusters 0, 4, 5, and 8.

(I) Heat map of treatment-associated changes in representative maintained and released TF programs across clusters 0, 4, 5, and 8.

(J) Pathway-enrichment analysis across the selected CLL states, highlighting biologic differences among clusters, including separation of an MHC II-high state from BCR-associated states. Together, these analyses show that the collapse of the BTK-maintained arm is broad across malignant CLL states, whereas the induction of the BTK-released arm is more state-restricted.

Supplementary Files

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