

Functional epigenomic liquid biopsy identifies potential therapeutic vulnerabilities after enfortumab vedotin–pembrolizumab resistance in metastatic urothelial carcinoma (mUC)

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Abstract

Enfortumab vedotin plus pembrolizumab (EV/P) is the standard first-line treatment for advanced urothelial carcinoma (UC), yielding unprecedented clinical benefit; however, resistance ultimately develops in many patients, and longitudinal liquid biopsy offers an opportunity to monitor tumor evolution and emerging therapeutic vulnerabilities. We collected 96 serial plasma samples from 48 patients with advanced UC treated with first-line EV/P, of which 79 samples from 41 patients passed predefined quality-control criteria for H3K4me2 cfChIP-seq profiling. Progression during EV/P was accompanied by regulatory reprogramming characterized by recurrent increases in ERBB3-associated H3K4me2 activity together with DNA damage response, replication stress, hypoxia and metabolic adaptation programs. Longitudinal patient-level analyses identified heterogeneous increases in potentially therapeutically relevant loci, including ERBB2, FGFR3, TACSTD2, WEE1, CD276 and PPARG, that were not evident at baseline in selected patients. Among three platinum-treated patients with longitudinal samples, the two patients deriving clinical benefit showed declining ERCC2, ERBB3 and BRCA2 regulatory activity, whereas the patient with progressive disease showed increased ERCC2 and ERBB3 activity. In one illustrative patient, increased ERBB2 regulatory activity at EV/P progression preceded a durable response to trastuzumab deruxtecan. These findings support prospective evaluation of longitudinal functional epigenomic profiling as a complementary approach for characterizing resistance and informing biomarker-guided treatment sequencing after EV/P.

INTRODUCTION

Enfortumab vedotin, an antibody-drug conjugate (ADC) targeting Nectin-4, combined with the immune checkpoint inhibitor pembrolizumab has transformed the treatment of locally advanced or metastatic urothelial carcinoma (UC) and is now the standard first-line regimen following the pivotal EV-302 trial¹². EV/P nearly doubled median progression-free survival and overall survival compared with platinum-based chemotherapy², establishing a new therapeutic benchmark in advanced UC. As EV/P also moves into perioperative disease settings³⁴, understanding the biology that determines primary and acquired resistance is becoming increasingly important.

Despite durable responses in a subset of patients, many tumors ultimately progress on EV/P^{5,6,7}. The biological determinants of this resistance remain incompletely characterized, and post-progression treatment is generally selected empirically rather than according to molecular features acquired during therapy. This represents a growing clinical challenge because resistance to combination therapy is likely to be dynamic and heterogeneous, and therefore may not be adequately captured by static pretreatment biomarkers.

Following EV/P progression in first line metastatic disease, platinum-based chemotherapy remains commonly used, while other options include molecularly matched therapy for FGFR3-altered tumors, trastuzumab deruxtecan in HER2-expressing disease, taxane-based therapy and clinical trials⁸⁻¹⁴. Subsequent-treatment analyses from EV-302 and emerging real-world series indicate that platinum

chemotherapy retains activity in a subset of patients, but no validated biomarker currently identifies those most likely to benefit^{8–11}. In parallel, multiple ADCs and other targeted agents are entering clinical development in the post-EV/P setting¹⁵. The expanding number of therapeutic options therefore creates a need not only to define mechanisms of resistance, but also to identify evolving tumor states that could inform rational treatment selection.

Repeat tissue biopsy at EV/P progression is often clinically impractical and may incompletely capture spatially heterogeneous disease. Liquid biopsy provides a minimally invasive strategy for serial assessment of tumor evolution. Although circulating tumor DNA can define genomic alterations and changes in tumor burden, epigenomic profiling of circulating chromatin can provide an additional functional layer by measuring histone modifications associated with active regulatory regions^{16,17}. Such an approach may be particularly relevant for ADCs and other targeted therapies, for which the presence and dynamic regulation of therapeutic targets may change under treatment pressure.

Here, we hypothesized that longitudinal plasma epigenomic profiling could capture treatment-associated regulatory evolution, identify biological states associated with EV/P resistance and reveal emergent therapeutic vulnerabilities not apparent before treatment. We therefore profiled H3K4me2-marked circulating chromatin longitudinally in patients receiving first-line EV/P and integrated target-focused and genome-wide analyses with clinical outcomes. Our findings provide a framework for prospective evaluation of functional plasma epigenomics as a complementary biomarker strategy for monitoring tumor-state evolution and informing therapeutic sequencing after EV/P resistance.

RESULTS

Forty-eight patients with locally advanced or metastatic UC treated with first-line EV/P were included in the analysis, contributing 96 longitudinal plasma samples collected at predefined pretreatment, on-treatment and progression time points, of which 79 were evaluable for chromatin profiling. The median age was 68 years (interquartile range, 57–71), and the cohort included 36 male and 12 female patients. At treatment initiation, most patients had an ECOG performance status of 0–1. Forty patients presented with metastatic disease and eight with locally advanced disease. Visceral metastases were present in 24 patients, including liver, bone, and lung involvement, whereas the remaining patients had non-visceral disease, predominantly involving lymph nodes. Most tumors were classified as urothelial carcinoma with predominant conventional histology; however, 16 samples exhibited divergent histologies, including micropapillary (n = 8), sarcomatoid (n = 2), squamous differentiation > 50% (n = 3), glandular differentiation > 50% (n = 1), plasmacytoid (n = 1), and poorly differentiated carcinoma harboring a SMARCA4 mutation (n = 1) (Table 1).

The overall response rate (ORR) to EV/P was 56.2% (27/48), including 7 complete responses (CR) and 20 partial responses (PR); 11 patients achieved stable disease (SD), corresponding to a disease control rate of 79% (Fig. 1a). Although the ORR was numerically lower than that reported in the phase III EV-302 trial (67.7%), the substantial antitumor activity observed in our cohort was broadly consistent with the clinical

activity of EV/P in the pivotal trial. Ten patients had PD as their best response, whereas an additional 11 patients developed progression after an initial period of clinical benefit and subsequently received second-line systemic therapy.

Following progression on EV/P, 21 patients (44%) received subsequent systemic therapy. Twelve patients were treated with platinum-based combination chemotherapy, of whom 8 were evaluable for response: 4 achieved a partial response (PR), 1 had a 20% tumor reduction, and 3 experienced disease progression. Subsequent targeted therapies included trastuzumab deruxtecan (T-DXd) in 6 patients. One patient received subsequent erdafitinib and 2 patients received other treatments. Among 4 patients evaluable for response to T-DXd, the ORR was 50%, including 2 PRs and 1 SD, while 1 patient experienced progressive disease.

To investigate circulating tumor chromatin dynamics during treatment, we performed longitudinal plasma chromatin profiling across the cohort. We profiled the H3K4me2 histone mark, which is associated with transcriptionally active promoters and enhancers (Fig. 1b). Seventy-nine samples from 41 of the 48 patients (7 CR, 15 PR, 9 SD and 10 PD) met predefined peak-enrichment and signal-to-noise quality thresholds and were carried forward into downstream analyses. Paired pretreatment and post-progression samples were available for 16 patients, all three time points were available for 10 patients, and 13 patients contributed a single time point. Longitudinal comparisons were therefore restricted to patients with evaluable paired samples (Table 2).

To explore potential therapeutic vulnerabilities emerging during EV/P treatment and resistance, we designed a predefined panel of therapeutically relevant target and biomarker candidate genes (Table 3) and quantified regulatory activity within promoter-associated regions (± 2 kb around transcription start sites) across longitudinal samples (Fig. 1a,b). Because H3K4me2 marks active promoters and enhancer elements, its genomic distribution is broader than that of H3K4me3, which is largely confined to promoter regions immediately surrounding the transcription start site (TSS). To capture promoter-associated H3K4me2 together with proximal regulatory activity, we quantified H3K4me2 signal within a ± 2 -kb window centered on the TSS¹⁸.

We first evaluated baseline (pre-treatment) regulatory activity across actionable targets, focusing initially on genes directly related to the EV/P mechanism of action. Baseline levels of H3K4me2 at the *NECTIN4* locus did not differ significantly across response groups (Fig. 1c). cfChIP signal at *CD274* (PD-L1) likewise showed no significant association with response at baseline (Fig. 1d).

Overall, most actionable targets at baseline were not associated with response (Suppl. Figure 1). Only *CD70* and *TACSTD2* differed significantly across response groups, with lower baseline regulatory activity in complete responders than in patients with partial response, stable disease or progressive disease (Fig. 1e,f). Because activity at these loci was comparable in partial responders and progressors, this pattern separates complete responders from all other response groups rather than defining a resistance-specific pretreatment state.

Beyond the predefined target panel, genome-wide differential chromatin profiling of pretreatment samples revealed distinct regulatory programs in complete responders and patients with primary progressive disease (Fig. 1g). Baseline progressors exhibited enrichment of chromatin states associated with epithelial-mesenchymal transition (EMT), including activation of extracellular matrix genes such as COL7A1 and COL12A1 together with the EMT-associated transcription factor SNAI2. Gene set enrichment analysis further demonstrated enrichment of EMT, TGF- β signaling, angiogenesis, hypoxia and IL6-JAK-STAT3 programs in progressors. These findings indicate that pretreatment chromatin states contain biological information associated with primary resistance, although baseline activity across the predefined therapeutic target panel did not robustly discriminate resistant disease^{19,20}.

Therapeutic targets emerging at progression on EV/P

We next investigated longitudinal chromatin dynamics by comparing promoter-associated activity across pretreatment, on-treatment, and post-progression samples and evaluating clinically actionable genes within a curated therapeutic panel (Fig. 2a; Suppl. Figure 2).

Among patients with progressive disease and paired longitudinal samples, recurrent target-associated changes included increased ERBB3 and ERCC2 regulatory activity, each observed in five of six patients with available paired longitudinal samples (four pretreatment/progression pairs and two pretreatment/on-treatment pairs; Fig. 2b,e). Additional recurrent changes involved replication-stress programs, including WEE1, together with MTAP-associated metabolic alterations (Fig. 2b). These observations nominate recurrent ERBB3 regulatory activation and broader stress-response programs as candidate features of the evolving resistant state rather than establishing any single alteration as a causal mechanism of EV/P resistance.

To further assess the activation of actionable genes in progressive disease (PD), we corrected for tumor fraction by modeling the signal as the combined contributions of tumor and non-tumor components, as described in the Methods section, and performed longitudinal analyses at the patient level. Tumor-fraction-corrected results confirmed substantial interpatient heterogeneity in chromatin evolution (Fig. 2c, 2d). Individual tumors acquired distinct combinations of HER-family signaling, DNA damage response, immune-associated pathways, and lineage plasticity programs, giving rise to diverse, therapeutically actionable regulatory states. These included activation of *FGFR3* and *ERBB2* regulatory regions in tumors already known to have corresponding genomic alterations, as well as increased activity at *TACSTD2* (Trop-2), *WEE1*, *PPARG* and *CD276* in selected cases, highlighting the ability of longitudinal plasma epigenomic profiling to uncover individualized therapeutic vulnerabilities that were not evident at baseline.

Taken together, these findings suggest that EV/P resistance is accompanied by recurrent biological programs superimposed on patient-specific regulatory evolution, generating heterogeneous potentially therapeutically relevant states that warrant prospective evaluation for treatment selection following disease progression.

Association of ERCC2, ERBB3, and BRCA2 dynamics with platinum response

Second-line treatment after EV/P progression was administered to 21 of 48 patients (44%) and was based on physician preference (12 platinum-based, 6 trastuzumab deruxtecan, 1 erdafitinib, 2 other). Among the 12 patients treated with platinum-based chemotherapy, eight were evaluable for response, and longitudinal samples spanning EV/P treatment and preceding platinum therapy were available for three. In this limited subset, *ERCC2* and *ERBB3* trajectories differed according to subsequent platinum response. The patient with progressive disease (P26) demonstrated increases in both *ERCC2* and *ERBB3* regulatory activity (Fig. 3a,b). In contrast, the two patients with marked clinical responses (Patients 48 and 35) showed substantial declines in both markers (Fig. 3a,b).

Notably, both platinum responding patients also showed a reduction in *BRCA2*-associated regulatory activity (Fig. 3b). In Patient 48, *BRCA2*-associated signal decreased, consistent with an exceptional response to platinum-based therapy. Likewise, in Patient 35, *BRCA2* levels declined to undetectable levels. Post-treatment samples were not available for the remaining platinum treated patients, limiting broader longitudinal analyses.

HER2 regulatory dynamics and response to trastuzumab deruxtecan

Six patients received trastuzumab deruxtecan (T-DXd) as second- or third-line therapy. Baseline *HER2* regulatory activity in Patients 4, 5, 14, and 27 measured < 100, 300, 300 and 418 RPM counts, respectively, spanning the cohort median baseline *HER2* activity of 275 RPM counts (Supplementary Fig. 1). In Patient 14, for whom a post-treatment sample was available, *HER2*-associated regulatory activity increased from 300 to 500 RPM between the pre-treatment sample and disease progression on EV/P. This patient subsequently achieved a durable partial response to T-DXd. Because this observation rests on a single patient and a single measurement at each time point, it should be regarded as hypothesis-generating.

Genome-wide chromatin remodeling identifies proliferative and hypoxia-associated programs in resistant disease

To comprehensively characterize the regulatory landscape associated with treatment response, we performed genome-wide differential chromatin analysis comparing post-treatment samples from patients with complete response (CR) and progressive disease (PD). This analysis identified 8,918 genes meeting the differential-activity threshold ($\text{padj} < 0.01$) and absolute $\log_2$ fold change greater than 1 (Fig. 4a), revealing widespread regulatory remodeling beyond the actionable loci described above.

Gene set enrichment analysis demonstrated significant activation of proliferation- and stress-associated programs in progressive disease, including MYC targets (V2), E2F targets, oxidative phosphorylation,

DNA repair, and hypoxia, consistent with the emergence of intrinsic resistance states. Differential H3K4me2-associated regulatory activity further highlighted activation of canonical MYC-regulated metabolic genes, including *CAD*, *LDLR*, and *SLC16A1* (*MCT1*), involved in nucleotide biosynthesis, lipid uptake, and metabolic rewiring. Progressive tumors also exhibited increased cfChIP signal at multiple hypoxia-associated genes, including the canonical HIF target *EGLN3* (*PHD3*) together with *P4HA2*, *ENO3*, *ITGA5*, *COL5A1*, and *TGFBI*, supporting activation of hypoxia-driven extracellular matrix remodeling and invasive programs (Fig. 4a). Collectively, these findings show that progressive disease after EV/P was associated with MYC-driven proliferation and metabolic adaptation together with HIF-mediated responses to hypoxic stress, biological programs previously associated with therapeutic resistance and tumor progression.

Chromatin evolution during therapy is associated with clinical outcome

We next investigated whether chromatin evolution correlated with progression-free survival (PFS) by comparing patients with PFS above or below the cohort median.

At baseline, differential analysis of actionable chromatin programs did not identify significant differences between PFS groups (Fig. 4b). However, genome-wide analysis revealed enrichment of proliferation-associated pathways, including MYC and E2F target programs, in tumors from patients with shorter PFS, suggesting that tumors destined to relapse early already possess a highly proliferative chromatin landscape before treatment initiation (Fig. 4b).

Following treatment, the chromatin landscape diverged substantially according to clinical outcome. Among actionable loci, *PSCA* and *ERBB2* showed higher regulatory activity in patients with shorter PFS, with a similar but non-significant trend for *PPARG* (Fig. 4c), consistent with adaptive HER-family signaling, luminal lineage plasticity, and immune-resistant tumor states^{21–23}. No actionable locus reached statistical significance in favor of the longer-PFS group.

Genome-wide analysis further revealed that tumors from patients with shorter PFS acquired chromatin programs associated with aggressive tumor evolution (Fig. 4c). Gene set enrichment demonstrated activation of epithelial-mesenchymal transition (EMT), hypoxia, and KRAS signaling, indicating extensive phenotypic reprogramming during therapy (Fig. 4c). Consistent with these pathway-level changes, H3K4me2-associated regulatory activity increased at genes involved in extracellular matrix remodeling and invasion, including *LAMC2*, *COL5A1*, and *CRLF1*, supporting the emergence of a highly invasive phenotype.

Overall, these findings suggest that poor clinical outcome is associated with both pre-existing proliferative chromatin programs before therapy and treatment-induced acquisition of mesenchymal, hypoxia-adapted regulatory states, characterized by increased cellular plasticity, invasive potential, and adaptive signaling that may facilitate therapeutic escape.

DISCUSSION

Resistance to combination therapies is an evolutionary process that may not be adequately captured by static pretreatment biomarkers. Here, longitudinal profiling of H3K4me2-marked circulating chromatin in patients with advanced urothelial carcinoma treated with enfortumab vedotin plus pembrolizumab revealed dynamic regulatory remodeling associated with therapeutic resistance. Whereas baseline activity across the predefined therapeutic target panel showed limited ability to distinguish primary resistance, serial profiling identified recurrent ERBB3 regulatory activation together with broader proliferative, DNA damage-response, hypoxia and metabolic programs emerging during treatment. Superimposed on these shared resistance-associated states, individual tumors acquired distinct potentially therapeutically relevant programs, suggesting that longitudinal functional profiling may complement genomic analyses by revealing biological vulnerabilities as they emerge under treatment pressure.

The distinction between baseline and longitudinal information is important. Pretreatment genome-wide profiling identified EMT, TGF- β , angiogenesis, hypoxia and IL6-JAK-STAT3 programs associated with primary progressive disease, indicating that baseline chromatin states contain features of intrinsic resistance. However, baseline activity across the predefined therapeutic target panel did not robustly distinguish primary resistance, whereas dynamic analyses revealed recurrent changes during therapy. Among progressing tumors with paired samples, ERBB3 showed one of the most consistent gains in promoter-associated H3K4me2 activity. These findings nominate reactivation of ERBB3 regulatory elements as a candidate feature of acquired resistance and suggest that serial liquid biopsy may capture information that is not apparent from a single pretreatment assessment. Independent validation will be required to establish the reproducibility and clinical significance of this observation.

EV/P is currently administered without biomarker-based patient selection, and defining the molecular determinants of response and resistance remains important as EV-based regimens expand into earlier disease settings^{3,4,13,14,24–26}. Resistance is likely multifactorial, potentially involving reduced NECTIN4 expression²⁷, altered ADC processing or drug efflux, resistance to microtubule-directed cytotoxicity, and immune escape within the tumor microenvironment^{7,25}. EV may also enhance antitumor immunity through immunogenic cell death and improved antigen presentation²⁸, further emphasizing that resistance to the combination may reflect adaptation across both tumor-intrinsic and immune-associated pathways. These mechanisms may have therapeutic implications: loss of NECTIN4 could favor agents targeting alternative tumor antigens, whereas resistance related to ADC architecture or payload processing could provide a rationale for evaluating Nectin-4–directed agents using alternative drug-conjugate platforms, such as BT8009²⁹. In parallel, genomic alterations including *TP53* and *KMT2D* have been associated with inferior outcomes after EV/P^{30,31}.

Within this complex landscape, increased post-treatment ERBB3 regulatory activity was a recurrent target-associated event among progressing tumors with paired samples. ERBB3 changes occurred alongside DNA damage-response and replication-stress pathways, metabolic rewiring and proliferative

MYC/E2F programs, consistent with coordinated adaptation to therapeutic pressure rather than isolated pathway activation. These observations nominate ERBB3 as a candidate marker of the resistant state and provide a rationale for prospective investigation of ERBB3-directed strategies after EV/P progression. As ERBB3-directed therapies enter clinical evaluation, longitudinal assessment of circulating ERBB3 regulatory activity could test whether this dynamic signal has predictive value for treatment selection.

Importantly, the resistant state was not uniform across patients. Longitudinal profiling identified increased regulatory activity at ERBB2, FGFR3, TACSTD2, CD276, PPARG and WEE1 in selected tumors, illustrating substantial interpatient heterogeneity. These findings support a model in which recurrent higher-order resistance programs coexist with patient-specific regulatory states. Such heterogeneity may become increasingly relevant as targeted agents, ADCs and other therapeutic classes expand in the post-EV/P setting, but the functional dependence and therapeutic sensitivity associated with these signals require prospective validation.

Genome-wide analyses further showed that progressive disease was associated with proliferative and metabolically adaptive programs characterized by MYC and E2F signaling, hypoxia, glycolysis, mTORC1 signaling and extracellular matrix remodeling. These programs have been implicated in treatment resistance across tumor types and suggest that adaptation to EV/P may involve coordinated proliferative, metabolic and microenvironmental remodeling. Interpretation of post-treatment comparisons nevertheless requires consideration of differences in circulating tumor content and cellular composition, particularly when comparing samples from patients with complete response and progressive disease ³².

A further hypothesis generated by this study is that functional chromatin trajectories may contain information relevant to subsequent therapy. Among only three platinum-treated patients with available longitudinal samples, the two patients deriving clinical benefit showed reductions in ERCC2, ERBB3 and BRCA2 regulatory activity, whereas the patient with progressive disease showed increased ERCC2 and ERBB3 activity. In a separate illustrative case, increased ERBB2 regulatory activity at EV/P progression preceded a durable response to trastuzumab deruxtecan. These small numbers preclude claims of predictive performance; rather, they illustrate treatment-specific hypotheses that can now be tested prospectively in larger, predefined cohorts.

Plasma functional epigenomic profiling may therefore complement conventional ctDNA approaches rather than replace them. ctDNA can quantify tumor-derived material and identify genomic alterations, including potentially actionable variants ^{33,34}, whereas cfChIP can provide a readout of regulatory activity and its evolution under therapeutic pressure ³⁵. Integration of these complementary layers, ideally with transcriptomic and protein-level measurements, may help distinguish the presence of a genomic alteration from the functional state of the corresponding pathway or therapeutic target.

As the number of therapies available after EV/P expands, a central challenge will be matching evolving tumor biology to subsequent treatment. Prospective studies should therefore incorporate predefined serial sampling and treatment-specific biomarker hypotheses to determine whether dynamic plasma epigenomic signals can define clinically meaningful subgroups and improve therapeutic allocation beyond genomic biomarkers alone.

Several limitations should be acknowledged. The cohort was modest, longitudinal sampling was incomplete, and analyses of subsequent therapy were exploratory because few treated patients had paired samples. Accordingly, the ERCC2, ERBB3 and BRCA2 trajectories observed in relation to platinum response and the ERBB2 trajectory preceding response to trastuzumab deruxtecan should be considered hypothesis-generating. H3K4me2-associated regulatory activity is an epigenomic readout and does not by itself establish target expression, pathway dependence or therapeutic sensitivity; orthogonal genomic, transcriptomic and protein-level validation will therefore be important. Because circulating chromatin signal is influenced by the abundance of tumor-derived material, we additionally performed tumor-fraction-adjusted analyses using matched low-pass whole-genome sequencing and ichorCNA estimates. These analyses supported the patient-specific regulatory changes observed in the unadjusted data, but estimates become increasingly uncertain at low tumor fractions and rely on assumptions regarding the hematopoietic background and relative immunoprecipitation efficiency. Larger independent cohorts with predefined sampling and integrated molecular measurements will be required to establish whether these dynamic regulatory states are reproducible and can prospectively guide therapy.

Together, these findings support a model in which resistance to EV/P is not represented by a single molecular alteration but by dynamic regulatory evolution comprising both recurrent adaptive programs and patient-specific acquired states. Longitudinal cfChIP profiling provides a minimally invasive approach to capture this evolution and may complement genomic liquid biopsy by identifying functional changes that arise under therapeutic pressure. Prospective validation will be required to determine whether these evolving regulatory states predict sensitivity to subsequent therapies, but our results provide a framework for moving precision oncology from static molecular classification toward longitudinal assessment of tumor state.

Methods

Patient cohort and study design

This prospective translational study enrolled patients with histologically confirmed locally advanced or metastatic urothelial carcinoma (mUC) treated with first-line enfortumab vedotin plus pembrolizumab (EV/P) according to institutional standards of care. Eligible patients were required to have measurable disease based on RECIST v1.1 criteria and adequate organ function at treatment initiation. Clinical data, including baseline demographic characteristics, prior treatments, sites of metastatic involvement, and radiographic response assessments, were collected from electronic medical records using standardized case report forms.

Peripheral blood samples were collected longitudinally at predefined clinical time points, including prior to treatment initiation (baseline), at the first radiographic response assessment during therapy, and at treatment discontinuation or confirmed disease progression. Radiographic response was determined using RECIST v1.1 criteria. Progression-free survival was defined as the time from treatment initiation to radiographic or clinical disease progression or death from any cause. Overall survival was defined as the time from treatment initiation to death from any cause. Patients without an event were censored at the date of last clinical follow-up.

The primary translational objective was to identify plasma-derived epigenomic biomarkers associated with resistance to EV/P and to characterize dynamic regulatory programs that could inform subsequent therapeutic selection. Secondary objectives included evaluation of associations between longitudinal regulatory activity changes and clinical outcomes, including objective response and progression-free survival.

Ethics approval and consent

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the institutional review board or ethics committee at each participating center. All patients provided written informed consent for blood collection, molecular analyses, and use of clinical data for research purposes prior to study participation. Data were de-identified prior to analysis to ensure patient confidentiality and compliance with applicable data protection regulations.

Definition of theoretical predictive or actionable targets

Theoretical predictive or actionable targets were predefined based on their potential therapeutic or biomarker relevance in urothelial cancer. Candidate genes were selected a priori on the basis of existing biological and clinical evidence and included molecular targets of approved therapies, targets of investigational agents evaluated in phase I-III clinical trials, and genes with evidence supporting a potential role as predictors of treatment sensitivity or resistance. Selection was performed independently of the subsequent clinical outcomes of the corresponding investigational therapies. We therefore refer to this set as a predefined therapeutically relevant target and biomarker candidate panel rather than implying established clinical actionability for every gene.

The full predefined therapeutically relevant target and biomarker candidate panel is provided in Table 3. Selected genes prioritized for focused analyses included *NECTIN4*, *TACSTD2* (*TROP2*), *FGFR3*, *ERBB1–3*, *SLITRK6*, *MTAP*, *PRMT5*, *PPARG*, *CD274* (*PD-L1*), *CD276* (*B7-H3*), *CCR8*, *ERCC2*, *ITGB6*, *CD47*, *CD70*, *WEE1*, *GPRC5D*, *BRCA2*, and *PSCA*. The CancerSurfaceome Atlas³⁶ was not used to select or prioritize these targets. However, several genes included in our independently predefined set were also identified or characterized in the Cancer Surfaceome Atlas, providing an independent overlap between our clinically driven selection and a systematic pan-cancer analysis of potentially actionable cell-surface targets.

Target-focused analyses subsequently quantified regulatory activity at these predefined genes to investigate whether their longitudinal molecular profiles were associated with treatment response or

could generate hypotheses regarding emergent therapeutic vulnerabilities. These analyses were exploratory and were not designed to establish target expression, pathway dependence or treatment-specific predictive validity.

Plasma processing, chromatin immunoprecipitation and sequencing

Peripheral blood was collected in cell-stabilizing tubes and processed within standardized time frames to minimize leukocyte lysis and background genomic contamination. Plasma was isolated by sequential centrifugation steps and stored at -80°C until analysis.

Chromatin immunoprecipitation was performed targeting histone H3 lysine 4 dimethylation (H3K4me2) using the Millipore antibody (reference 07-030), enabling enrichment of genomic regions corresponding to transcriptionally active promoters and enhancers. Immunoprecipitated DNA libraries were prepared using indexed adapters and amplified under conditions minimizing amplification bias. Libraries underwent high-throughput paired-end sequencing on Illumina platforms to achieve sufficient genome-wide coverage for regulatory state inference.

Quality control procedures included assessment of library complexity, fragment size distribution, sequencing depth, and signal-to-noise enrichment metrics at canonical active regulatory regions. Samples failing predefined quality thresholds were excluded from downstream analyses. Seventy-nine of the profiled samples met these criteria and were retained for analysis (Table 2).

Bioinformatic processing and regulatory signal quantification

Sequencing reads were aligned to the human reference genome hg19 using the Burrows-Wheeler Aligner (BWA)³⁷ with duplicate read removal and stringent quality filtering. Genome-wide binding sites were identified using Model-Based Analysis of ChIP-Seq (MACS v2.1.1.20160309) with a q-value (FDR) threshold of 0.01³⁸.

Regulatory activity scores were calculated by integrating signal intensity across gene-linked regulatory domains using multiBamSummary within promoter-associated regions (± 2 kb around transcription start sites) across longitudinal samples. Regulatory activity is reported throughout as RPM (Reads Per Million) counts, as reads-per-million-normalized read counts within the ± 2 -kb window centered on the transcription start site.

Longitudinal regulatory trajectories were constructed for each patient by integrating time-resolved activity scores, enabling identification of resistance-associated regulatory reprogramming events. All computational analyses were conducted using reproducible workflows, CHIPS and CoBRA pipeline^{39,40}, to ensure analytical transparency and portability.

Tumor-fraction correction of cfChIP-seq signal

Each plasma library from the CR and PD patients was modelled as a two-component mixture of tumour-derived and hematopoietic cell-free DNA with mixing weight T , taken as the ichorCNA tumour fraction from the matched low-pass whole-genome sequencing input of the same draw:

$$x_{gs} = (1 - T_s)B_g + T_s U_{gs} \quad (1)$$

where x_{gs} is the gene-averaged H3K4me2 signal for gene g in library s , B_g the hematopoietic background and U_{gs} the tumor-derived component. B_g was estimated from the ten libraries with undetectable tumor fraction ($T = 0$; nine from complete responders, one pre-treatment sample from a patient who subsequently progressed), computed as the median across the constituent loci of each gene and then the median across control libraries. Solving (1) for the tumor component gives

$$U_{gs} = [x_{gs} - (1 - T_s)B_g] / T_s \quad (2)$$

which is reported as the ratio U_{gs}/B_g , where 1 denotes a tumor compartment indistinguishable from background. Genes were filtered to remove mitochondrial, unannotated, pseudogene, antisense and read-through loci and those with zero background signal (14,661 of 18,429 retained), and libraries were placed on a common scale using a median-of-ratios size factor computed against B_g . Values are reported for all libraries with $T > 0$. Because (2) divides by T , the variance of U scales as $1/T^2$ and the uncertainty of each estimate is given alongside it; negative values, arising when $x_{gs} < (1 - T_s)B_g$, were retained rather than truncated at zero. The method assumes B_g does not differ systematically between response groups and that H3K4me2 immunoprecipitation efficiency per genome is equal in tumor and leukocyte chromatin.

Genome-wide regulatory annotation and target-focused analyses

Genome-wide analyses prioritized regulatory regions linked to genes in the predefined therapeutically relevant target and biomarker candidate panel. These included targets associated with antibody–drug conjugates, targeted therapies, and immunomodulatory approaches, such as *NECTIN4*, *TACSTD2* (*Trop-2*), *FGFR3*, *ERBB1–3*, *SLITRK6*, *MTAP*, *PRMT5*, *PPARG*, *PD-L1* (*CD274*), *B7-H3* (*CD276*), *CCR8*, *ERCC2*, *WEE1*, *GPRC5D*, *ITGB6*, *CD47*, *CD70* as well as *BRCA2* and *PSCA* (Table 3). This predefined panel included targets of approved therapies, targets of investigational agents in clinical development, and candidate biomarkers with potential relevance to treatment sensitivity or resistance.

Statistical analysis

Comparisons of regulatory activity between response groups were performed using two-sided Wilcoxon rank-sum tests for pairwise comparisons, with significance annotated as * $P < 0.05$ and ** $P < 0.01$. Genome-wide differential regulatory activity was assessed using DESeq2. Genes were considered differentially active at an absolute log2 fold change > 1 with Benjamini-Hochberg-adjusted $P < 0.01$ for the principal genome-wide analyses reported in the Results, unless otherwise specified. Gene set

enrichment analysis was performed against the MSigDB Hallmark collection together with a bladder-cancer-specific hypoxia gene set described in the Supplementary Methods. All statistical tests were two-sided. Analyses were performed in R (version 4.5.3).

Declarations

Data availability

Raw and processed H3K4me2 cell-free chromatin immunoprecipitation sequencing (cfChIP-seq) data generated from longitudinal plasma samples of patients with locally advanced or metastatic urothelial carcinoma treated with enfortumab vedotin plus pembrolizumab have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number **GSE334331**. The study included 96 plasma samples from 48 patients, of which 79 samples from 41 patients met quality-control criteria and were included in downstream analyses. Publicly available datasets and reference resources used in this study are described in the Methods section.

Competing Interests

J.B. Serves on advisory boards for Pfizer, Astra-Zeneca, Merck, BMS, and MSD and has received honoraria from Merck and MSD. T.K.C.: Alkermes, Arcus Bio, AstraZeneca, Aravive, Aveo, Bayer, Bristol Myers-Squibb, Bicycle Therapeutics, Calithera, Caris, Circle Pharma, Deciphera Pharmaceuticals, Eisai, EMD Serono, Exelixis, GlaxoSmithKline, Gilead, HiberCell, IQVA, Infinity, Institut Servier, Ipsen, Jansen, Kanaph, Lilly, Merck, Moderna, Nikang, Neomorph, Nexomic, Nuscan/PrecedeBio, Novartis, Oncohost, Pfizer, Roche, Sanofi/Aventis, Scholar Rock, Surface Oncology, Takeda, Tempest, Up-To-Date, CME and non-CME events (Mashup Media, Elite Elements, Peerview, OncLive, MJH, CCO, Pulse Health and others), Xencor, outside the submitted work. Institutional patents filed on molecular alterations and immunotherapy response/toxicity, rare genitourinary cancers, and ctDNA/liquids biopsies. Equity: Tempest, Osel, Precede Bio, CureResponse, Primium, Abalytics, Faron Pharma, Open Medicine. Committees: NCCN, GU Steering Committee, ASCO (BOD 6/2024-), ESMO, ACCRU, KidneyCan. Medical writing and editorial assistance support may have been funded by Communications companies in part. No speaker's bureau. Mentored several non-US citizens on research projects with potential funding (in part) from non-US sources/Foreign Components. The institution (Dana-Farber Cancer Institute) may have received additional independent funding of drug companies or/and royalties potentially involved in research around the subject matter.

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Author contributions

J.B., H.W.L. and P.C. conceived and designed the study and jointly supervised the work. J.B. led the clinical investigation, including patient recruitment, clinical data collection and interpretation of clinical outcomes. H.W.L. and P.C. oversaw the experimental and computational components of the study. R.L., S.E.S., Y.X., G.S.G., S.K., L.R., R.N. and S.A.B. contributed to sample processing, sequencing, bioinformatic analyses and interpretation of epigenomic data. I.B.E., C.M., B.A.M. and M.D.K. contributed to patient characterization, clinical data collection and interpretation of clinical findings. S.C.B., T.K.C. and M.L.F. contributed to scientific interpretation and critical revision of the manuscript. J.B., H.W.L. and P.C. drafted and revised the manuscript with contributions from all coauthors. All authors reviewed and approved the final manuscript.

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Tables

Tables 1 to 3 are available in the Supplementary Files section.

Figures

Figure 1

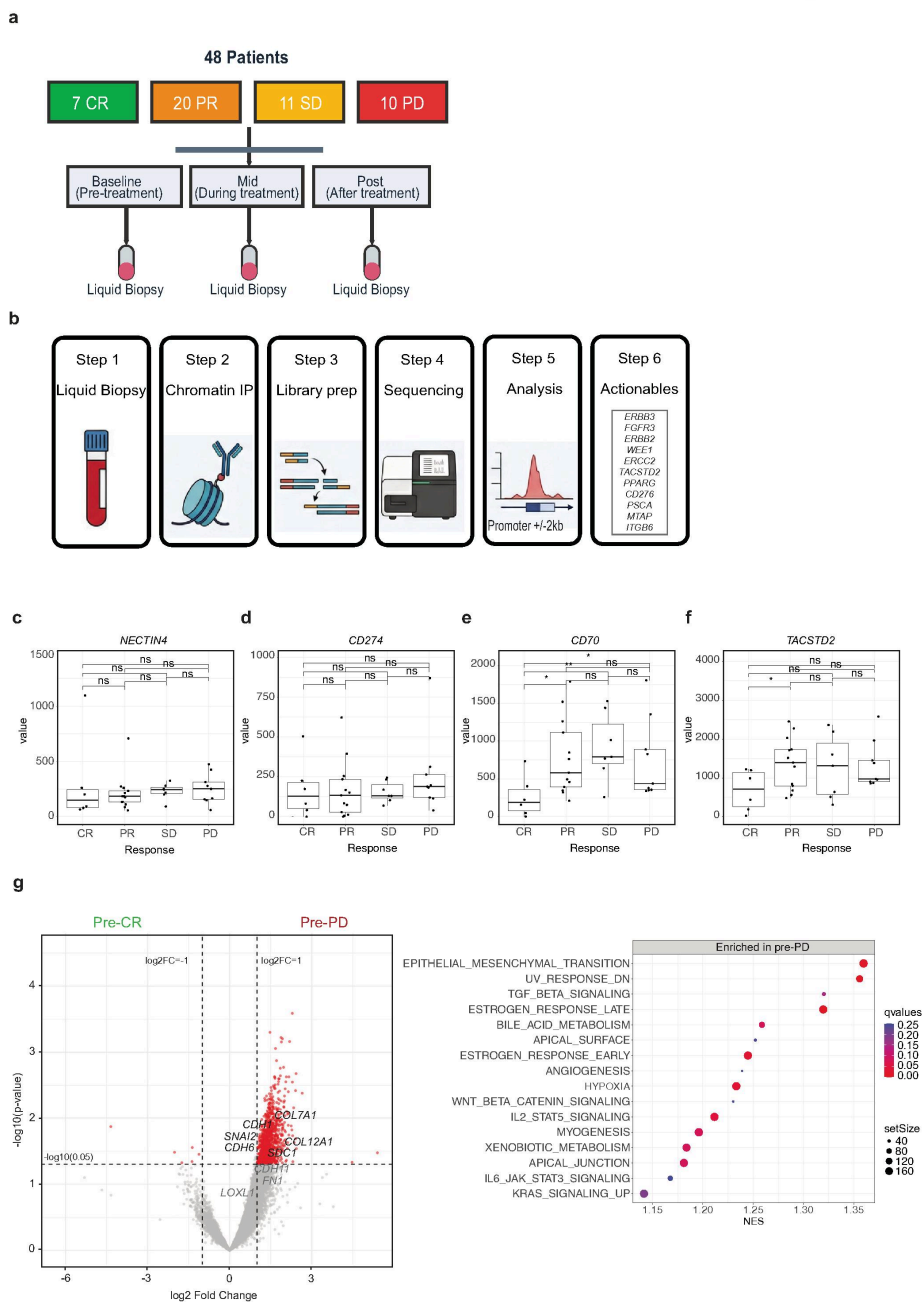


Figure 1

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Figure 2

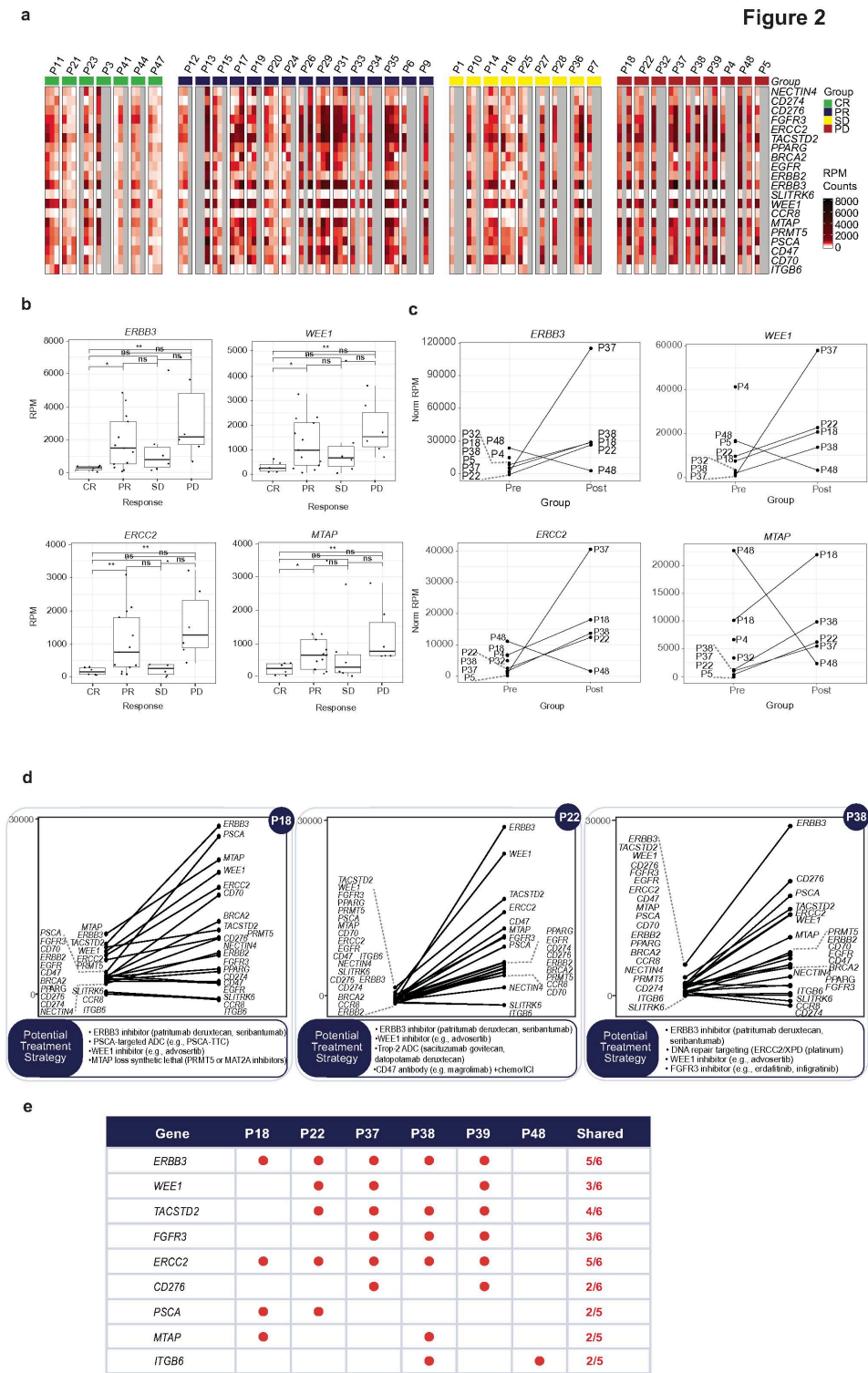
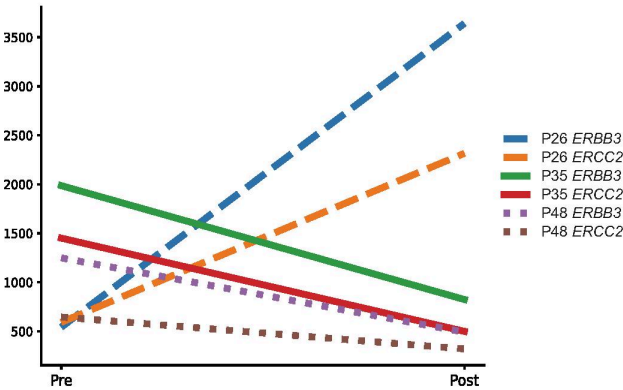


Figure 2

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Figure 3

a



b

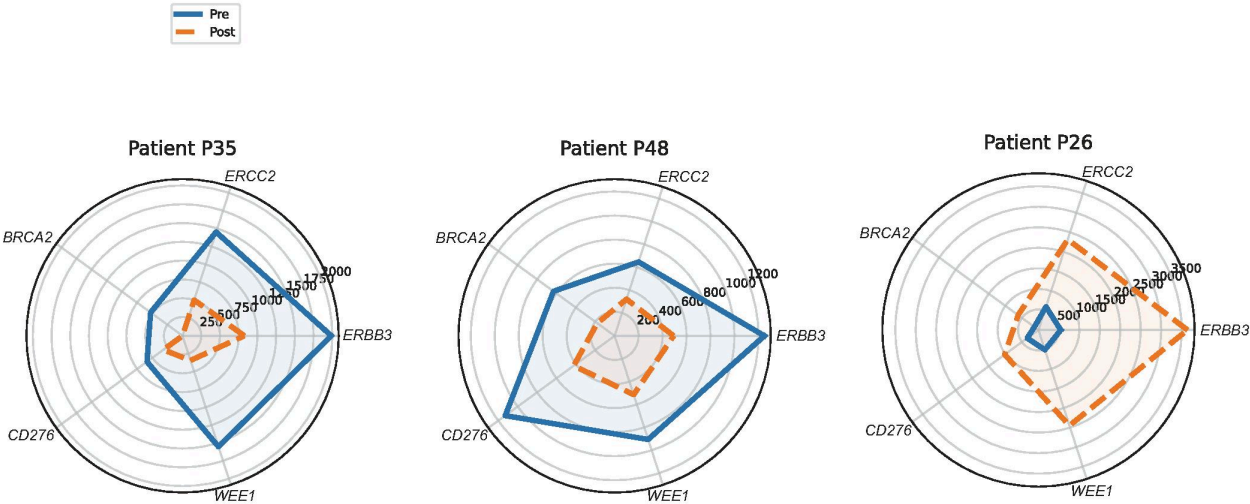


Figure 3

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Figure 4

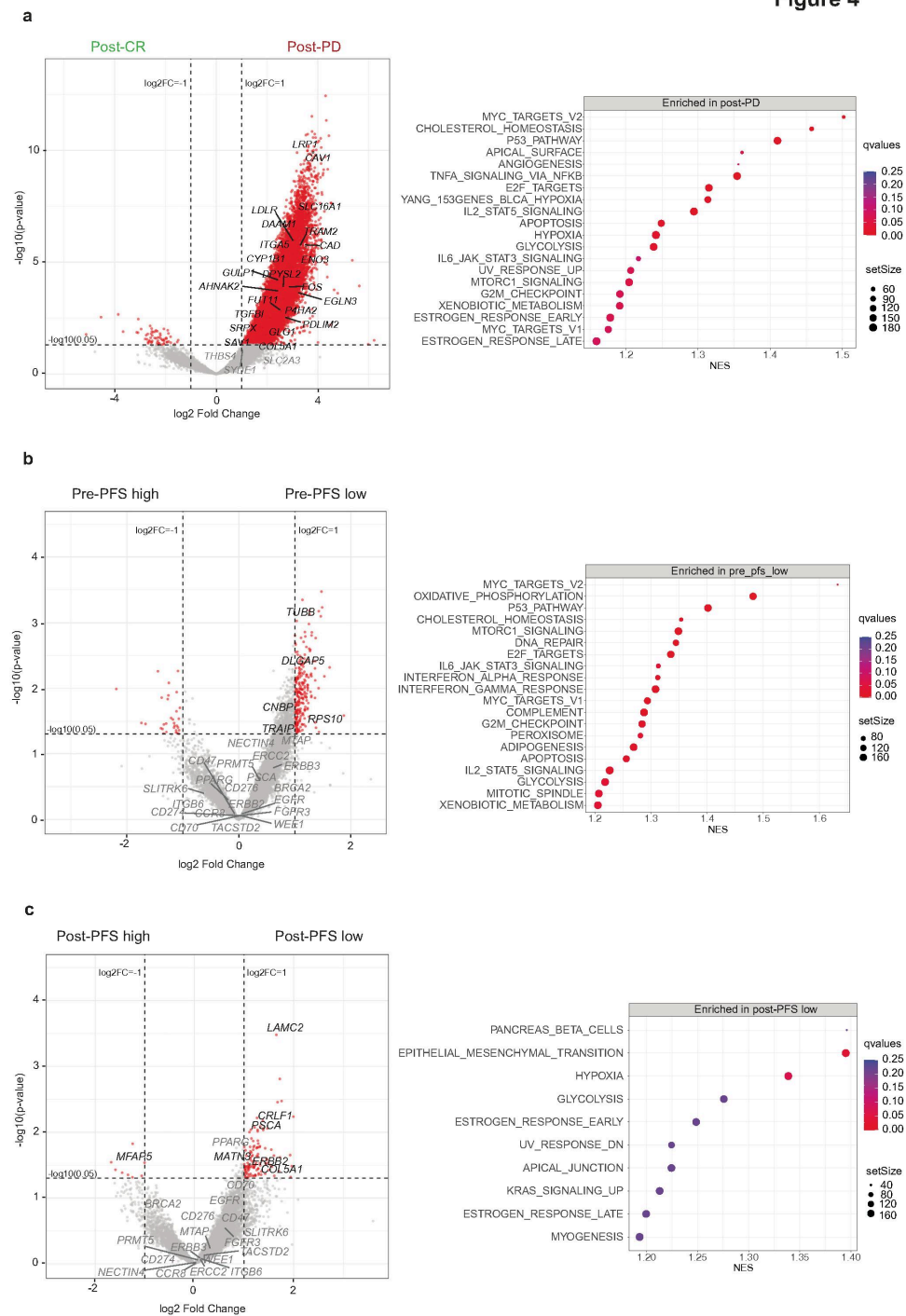


Figure 4

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