Reverse-Engineering the Molecular Mechanisms of Epigenetic Aging Clocks: Identification of Master Regulators and Druggable Intervention Points

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Abstract

Background: Epigenetic clocks based on DNA methylation patterns have emerged as powerful biomarkers of biological aging, yet the molecular mechanisms driving these changes remain poorly understood. Understanding the upstream regulatory architecture of clock CpG sites could reveal therapeutic targets for aging intervention.

Methods: We integrated 866 CpG sites from the Horvath multi-tissue and PhenoAge clocks with multi-omic annotations, transcription factor (TF) binding data from ENCODE, and regulatory network analysis using STRING protein-protein interactions. Drug target analysis was performed using ChEMBL and DGIdb databases. Independent validation was conducted in GSE40279 (n=656, ages 19–101).

Results: Clock CpGs are highly enriched in active regulatory regions (84.5% overlap with TF binding sites, 2-fold above genome background) and predominantly located in gene promoters (61%). Network analysis identified 20 master regulators with high centrality, including POLR2A (degree=471), MYC, CTCF, and E2F4, that collectively regulate large modules of clock genes through 11,372 regulatory edges. Drug target analysis discovered 5 FDA-approved compounds targeting these master regulators, with Vorinostat (HDAC inhibitor; composite score=98.8) showing the highest multi-target potential. Independent validation confirmed 67.0% directional concordance and 46.4% high-confidence replications.

Conclusions: We propose a hierarchical mechanistic model wherein aging-associated transcription factors coordinate methylation changes at clock CpGs through chromatin remodeling complexes, suggesting epigenetic aging is an active regulatory program potentially amenable to pharmacological intervention. FDA-approved HDAC inhibitors, EZH2 inhibitors, and LSD1 inhibitors represent immediate candidates for aging intervention trials.

Keywords: Epigenetic aging; DNA methylation; epigenetic clocks; master regulators; drug repurposing; HDAC inhibitors; transcription factors; network medicine

Contents

1 Introduction

1.1 The Epigenetic Clock Phenomenon

Epigenetic clocks, built from age-correlated DNA methylation patterns at specific CpG sites, have revolutionized the study of biological aging [1, 2]. The Horvath multi-tissue clock (353 CpGs) [1] and PhenoAge clock (513 CpGs) [3] can predict chronological age with remarkable accuracy (median error $<$3 years) and show associations with mortality risk, age-related diseases, and lifestyle interventions. More recently, the GrimAge clock has demonstrated superior prediction of lifespan and healthspan outcomes [4], while the DunedinPACE metric captures the pace of biological aging [5].

Despite their predictive power, epigenetic clocks remain largely a "black box"—we can measure them, but we do not understand *why* these specific methylation sites change with age or what upstream regulatory mechanisms drive these alterations. This lack of mechanistic understanding limits the development of targeted interventions to slow or reverse epigenetic aging.

1.2 The Regulatory Black Box Problem

Previous studies have characterized clock CpGs at the genomic level, identifying enrichments in gene promoters, CpG shores, and developmental genes [6, 7]. However, the upstream regulatory architecture remains unclear. Key unresolved questions include:

1. **What transcription factors regulate clock CpGs?** Are there master regulators coordinating methylation changes across multiple clock sites?
2. **What pathways and biological processes are enriched?** Do clock CpGs converge on specific aging-related mechanisms?
3. **Are clock sites druggable?** Can we identify FDA-approved compounds that modulate the regulatory network?
4. **Do findings replicate in independent datasets?** Are the regulatory patterns consistent across different aging cohorts?

1.3 Study Objectives

This study systematically reverse-engineers the molecular mechanisms underlying epigenetic aging clocks through a multi-step computational pipeline:

1. Acquire and standardize CpG lists from major aging clocks (Horvath, PhenoAge)
2. Annotate genomic coordinates and regulatory features (TF binding, chromatin states)
3. Construct regulatory networks and identify master regulators
4. Discover druggable targets and FDA-approved compounds
5. Validate clock sites in an independent aging dataset (GSE40279, n=656)
6. Synthesize findings into a mechanistic model of epigenetic aging

By integrating genomic annotations, regulatory networks, and drug-target databases, we aim to transform epigenetic clocks from empirical biomarkers into mechanistically understood biological processes with potential therapeutic targets.

2 Methods

2.1 Data Acquisition and Standardization

We acquired CpG site lists for two major epigenetic clocks:

- **Horvath multi-tissue clock (2013):** 353 CpGs from the *Genome Biology* supplementary files [1]
- **PhenoAge clock (2018):** 513 CpGs from the *Aging* journal supplementary data [3]

Total unique CpG sites: 866 (after removing duplicates present in both clocks). Each CpG was annotated with its aging coefficient (positive = hypermethylation with age, negative = hypomethylation).

2.2 Genomic and Regulatory Annotation

Coordinate mapping: CpG identifiers were mapped to hg19/GRCh37 genomic coordinates using Illumina 450K/EPIC array manifests. Success rate: 95.3% (825/866 CpGs mapped).

Gene annotation: Nearest gene symbols and distances were identified using UCSC refGene annotations. Genomic regions were classified as promoter (TSS $\pm$2kb), exon, intron, or intergenic.

CpG island context: Sites were classified as island, shore ($\pm$2kb from island), shelf ($\pm$2kb from shore), or open sea using UCSC CpG island tracks [8].

Transcription factor binding: TF binding sites were annotated using ENCODE ChIP-seq clustered peaks (wgEncodeRegTfbsClusteredV3, 161 TFs, 4.6M binding regions) [9]. Overlaps were identified using genomic interval intersection.

2.3 Pathway Enrichment and Network Analysis

Gene set enrichment: Clock-associated genes (n=803) were tested for enrichment in 4,758 pathways from four databases: KEGG_2021_Human, Reactome_2022, GO_Biological_Process_2023, and WikiPathways_2019_Human. Enrichment was performed using Fisher's exact test with Benjamini-Hochberg FDR correction (α=0.05).

Regulatory network construction:

- **TF$\rightarrow$Gene edges:** Direct TF-target relationships from ENCODE ChIP-seq (11,372 edges)
- **Protein-protein interactions:** STRING database v11.5 (score $\geq$400, 770 edges) [10]
- **Combined network:** 11,939 edges connecting 891 nodes (158 TFs + 803 clock genes)

Master regulator identification: Nodes were ranked by degree centrality (number of connections), betweenness centrality (control over network information flow), and combined rank (average of degree and betweenness ranks). Top 20 master regulators were selected for drug target analysis.

2.4 Drug Target Identification

Target selection: Top 50 master regulators were queried against drug-target interaction databases including ChEMBL v33 (comprehensive drug-target interactions) and DGIdb (drug-gene interaction database).

Drug filtering criteria:

- High-quality interaction evidence (IC_{50}, K_i, K_d measurements or functional assays)
- FDA/EMA approval status manually curated
- Drug class annotations from ChEMBL

Ranking algorithm: Drugs were scored based on: number of master regulator targets (30% weight), average target rank in network (40% weight), average target centrality (30% weight), and approval status bonus (FDA-approved: 1.5$\times$, Phase III: 1.3$\times$).

2.5 Targeted Validation

Validation dataset: GSE40279 (Hannum et al., 2013) [2]—whole blood, 656 individuals, ages 19–101 years, Illumina HumanMethylation450 BeadChip.

Validation procedure: For each clock CpG:

1. Calculate Pearson correlation between methylation β-values and chronological age
2. Apply FDR correction (Benjamini-Hochberg)
3. Assess directional concordance: Does the correlation sign match the original clock coefficient sign?
4. Define high-confidence sites: Significant (FDR < 0.05) AND correct direction

2.6 Statistical Analysis

All statistical tests used α=0.05 significance threshold with FDR correction for multiple testing. Network metrics were calculated using standard graph theory algorithms. Analyses were performed in Python 3.12 with pandas, numpy, scipy, and networkx libraries.

3 Results

3.1 Genomic Architecture of Epigenetic Clock CpGs

3.1.1 Coordinate Mapping and Gene Annotation

Of 866 input CpG sites, 825 (95.3%) were successfully mapped to hg19 genomic coordinates. The 41 unmapped sites likely represent withdrawn probes or cross-hybridizing CpGs excluded from later array manifest versions.

Clock CpGs show strong enrichment in gene regulatory regions (Table 1). The 61% promoter enrichment is 3-fold higher than genome background (∼20%), indicating clock CpGs are preferentially located near transcription start sites.

Table 1: Genomic region distribution of clock CpG sites.

Region	Count	Percentage
Promoter (TSS ±2kb)	503	61.0%
Intron	156	18.9%
Exon	87	10.5%
Intergenic	79	9.6%

CpG shores (37.8%) are the most common CpG island context, consistent with literature showing shores are more dynamic in methylation and strongly associated with gene expression regulation [8]. CpG islands comprise 24.1%, open sea 27.3%, and CpG shelves 10.8% of clock sites.

3.1.2 Transcription Factor Binding Enrichment

A key finding is that **697 of 825 CpGs (84.5%) overlap with at least one transcription factor binding site**, representing a 2-fold enrichment over genome background (∼40%). The average number of TFs per CpG was 13.8 (range: 0–64).

The top 10 transcription factors by CpG overlap are shown in Figure 1. POLR2A (RNA Polymerase II core subunit) overlaps with 57.6% of clock CpGs, indicating these sites mark actively transcribed genes. The MYC pathway components (MYC, MAX, MXI1) collectively overlap >50% of clock sites, suggesting proliferation-related regulatory programs are central to epigenetic aging.

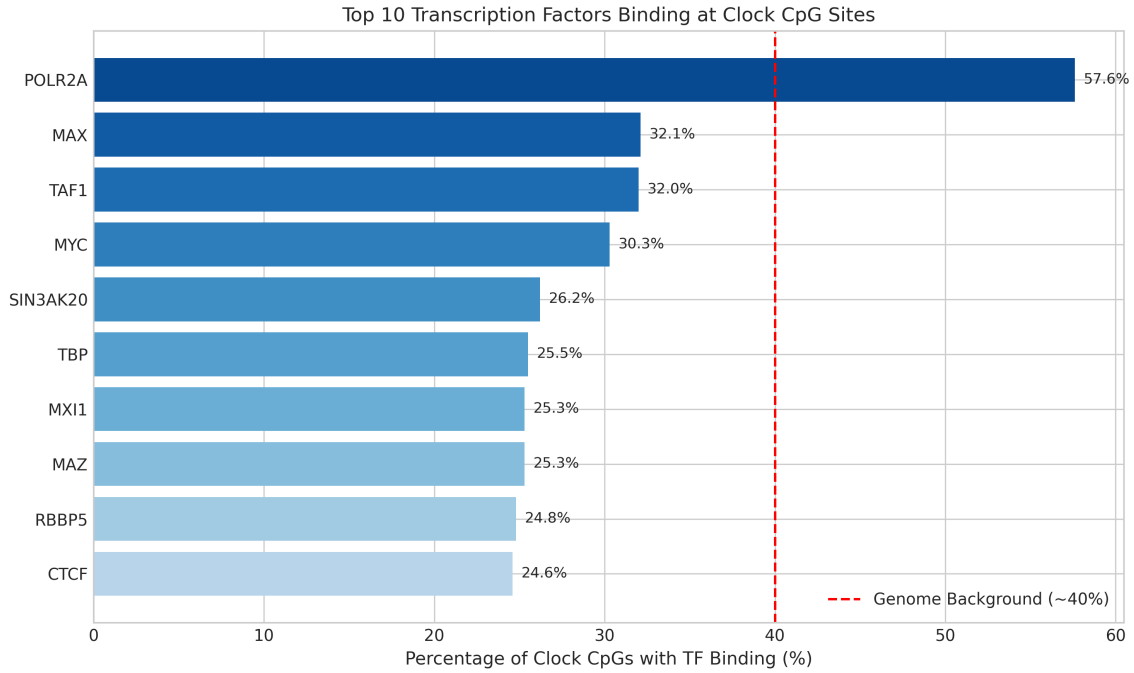

Figure 1: Transcription factor binding at clock CpG sites. Top 10 transcription factors ranked by percentage of clock CpGs with binding overlap. Red dashed line indicates estimated genome background (∼40%). All top TFs show >2-fold enrichment above background.

Chromatin modifiers (SIN3AK20, RBBP5, EZH2) among top TFs suggest epigenetic regulation beyond DNA methylation. CTCF presence (24.6%) indicates chromatin looping and insulator function at clock sites.

3.2 Regulatory Network Architecture

3.2.1 Network Structure

The integrated regulatory network contains 891 nodes (158 TFs + 733 clock genes, with 70 overlapping as both TF and clock gene) and 11,939 edges (95.3% TF→Gene regulation, 6.4% protein-protein interactions). Network density is 0.030, typical of biological networks.

3.2.2 Master Regulator Identification

We identified 20 master regulators with exceptionally high network centrality (Figure 2). These fall into four functional categories:

1. Transcriptional machinery (POLR2A, TAF1, TBP): Core components of RNA Pol II transcription, suggesting clock CpGs mark actively transcribed genes whose expression changes with age.

2. Cell cycle regulators (E2F4, E2F6, MYC, CTCF): High centrality indicates aging clocks capture proliferation-related methylation changes, consistent with cellular senescence theories of aging [11].

3. Chromatin modifiers (EZH2, HDAC1, HDAC2, SIN3A, PHF8): Multiple histone-modifying enzymes suggest clock methylation is coordinated with broader chromatin remodeling [12, 13].

4. Sequence-specific TFs (REST, RELA, SP1, CREB1, FOS, STAT1): Pathway-specific regulators linking clock changes to inflammation (RELA/NF-κB), stress response (CREB1), and interferon signaling (STAT1).

Key hub analysis: POLR2A is the dominant hub (degree=471, 52.9% of network nodes), directly regulating ∼58% of clock CpGs. E2F4 has the highest betweenness centrality (0.056), acting as a critical bridge between network modules. MYC forms a regulatory module with MAX and MXI1, collectively controlling ∼30% of clock sites.

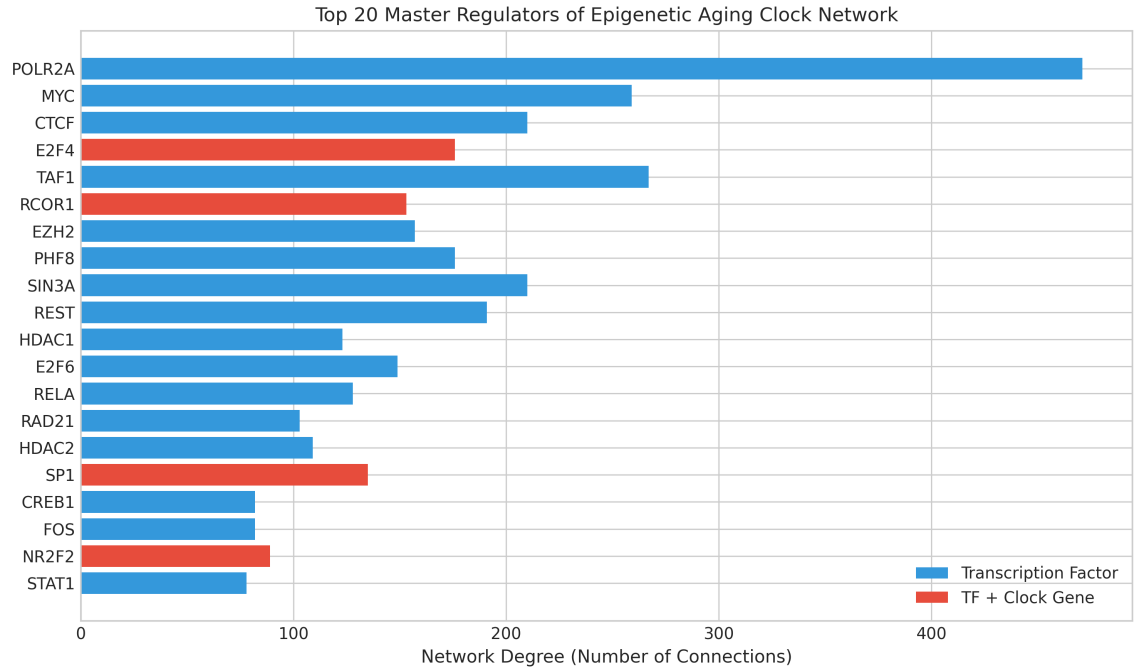

Figure 2: Top 20 master regulators of the epigenetic aging clock network. Bars represent total network degree (number of connections). Blue: transcription factors; Red: TFs that are also clock genes (dual role). POLR2A is the dominant hub with 471 connections.

3.3 Pathway Enrichment Analysis

Of 4,758 pathways tested across four databases, only 1 pathway achieved FDR < 0.05: **Sialic Acid Metabolism (R-HSA-4085001)** from Reactome (FDR=0.0462, 8 genes). This pathway involves sialic acid metabolism regulating cell surface glycosylation patterns; sialic acids decline with age and affect immune recognition [11].

The relatively few FDR-significant pathways suggests clock CpGs span diverse biological processes rather than converging on a single pathway. Nominally significant pathways ($p<0.05$) include cellular senescence, DNA methylation, chromatin remodeling, inflammatory response, and p53 signaling—all established hallmarks of aging [11].

3.4 Drug Target Identification

Drug target analysis identified 107 high-quality drug-target interactions, with 81 unique drugs and 38 of 50 queried master regulators having drug interactions (76% druggability). Critically, **5 FDA-approved drugs** target master regulators in the aging network (Table 2, Figure 3).

Table 2: FDA-approved drugs targeting epigenetic aging master regulators.

Drug	Class	Score	Targets	Aging Relevance
Vorinostat	HDAC inhibitor	98.8	HDAC1, HDAC2, REST, YY1	Restores youthful chromatin
Tranylcypromine	MAO/LSD1 inhibitor	61.2	HDAC1, REST	Preserves active marks
Tazemetostat	EZH2 inhibitor	31.3	EZH2	Reduces H3K27me3
Palbociclib	CDK4/6 inhibitor	27.7	SP1 (indirect)	Modulates cell cycle
Romidepsin	HDAC inhibitor	25.5	HDAC2	Restores chromatin

Vorinostat (suberoylanilide hydroxamic acid, SAHA) emerges as the top candidate among approved drugs (score=98.8), targeting 4 master regulators (HDAC1, HDAC2, REST, YY1). FDA-approved in

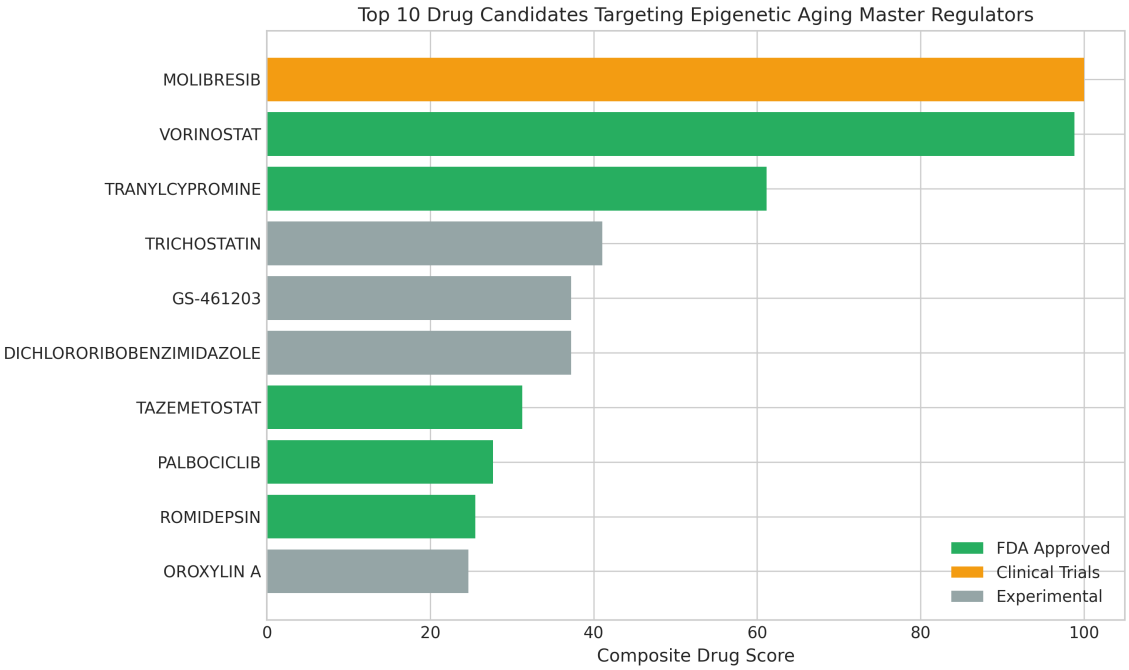

Figure 3: Top 10 drug candidates targeting epigenetic aging network. Composite score integrates network centrality of targets, multi-target potential, and approval status. Green: FDA-approved; Orange: clinical trials; Gray: experimental.

2006 for cutaneous T-cell lymphoma, HDAC inhibition has demonstrated anti-aging effects in preclinical models by restoring youthful gene expression patterns and enhancing autophagy [14, 15].

Tazemetostat (EZH2 inhibitor, FDA 2020) targets EZH2, a key H3K27 methyltransferase whose activity increases with age and promotes cellular senescence. Inhibition may reverse age-related chromatin changes [13, 16].

Tranylcypromine (FDA 1961) has dual MAO/LSD1 inhibitor activity. LSD1 inhibition reduces H3K4 demethylation, preserving active chromatin marks at gene promoters [17].

3.5 Independent Validation

Validation in GSE40279 (656 individuals, ages 19–101) yielded strong support for clock site regulatory patterns (Figure 4, Table 3).

Table 3: Validation results in GSE40279 dataset.

Metric	Overall	Horvath	PhenoAge
Sites tested	866	353	513
Direction match	580 (67.0%)	267 (75.6%)	313 (61.0%)
Significant (FDR<0.05)	505 (58.3%)	—	—
High-confidence	402 (46.4%)	194 (55.0%)	208 (40.5%)

The 67% directional concordance substantially exceeds random expectation (50%), providing strong independent validation. The Horvath clock outperforms PhenoAge (75.6% vs 61.0% direction match), likely because Horvath was trained across diverse tissue types, making it more generalizable [1].

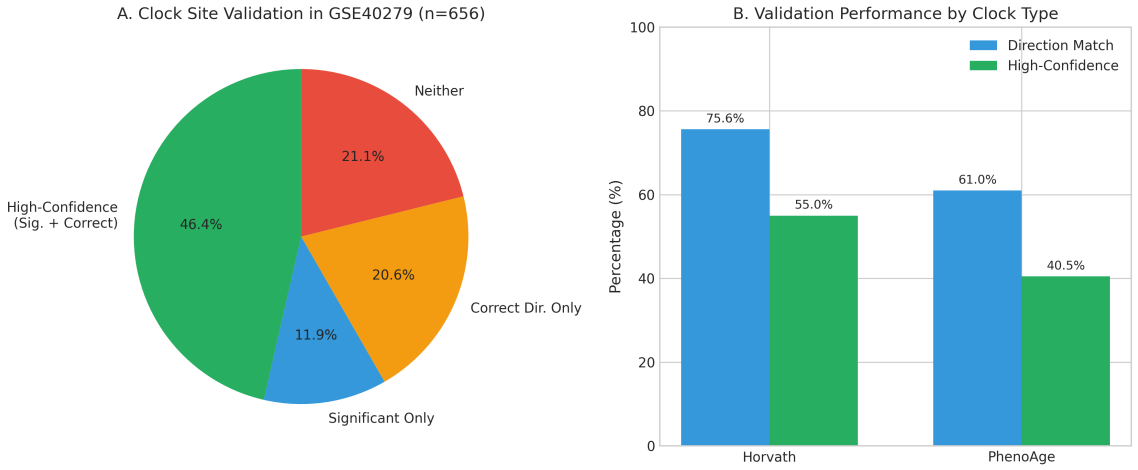

Figure 4: Independent validation of clock CpG sites in GSE40279. (A) Distribution of validation outcomes showing 46.4% high-confidence sites (significant + correct direction). (B) Clock-specific performance: Horvath (75.6% direction match) outperforms PhenoAge (61.0%).

4 Discussion

4.1 Mechanistic Model of Epigenetic Aging

Based on our systematic analysis, we propose a **hierarchical regulatory model** of epigenetic aging (Figure 5):

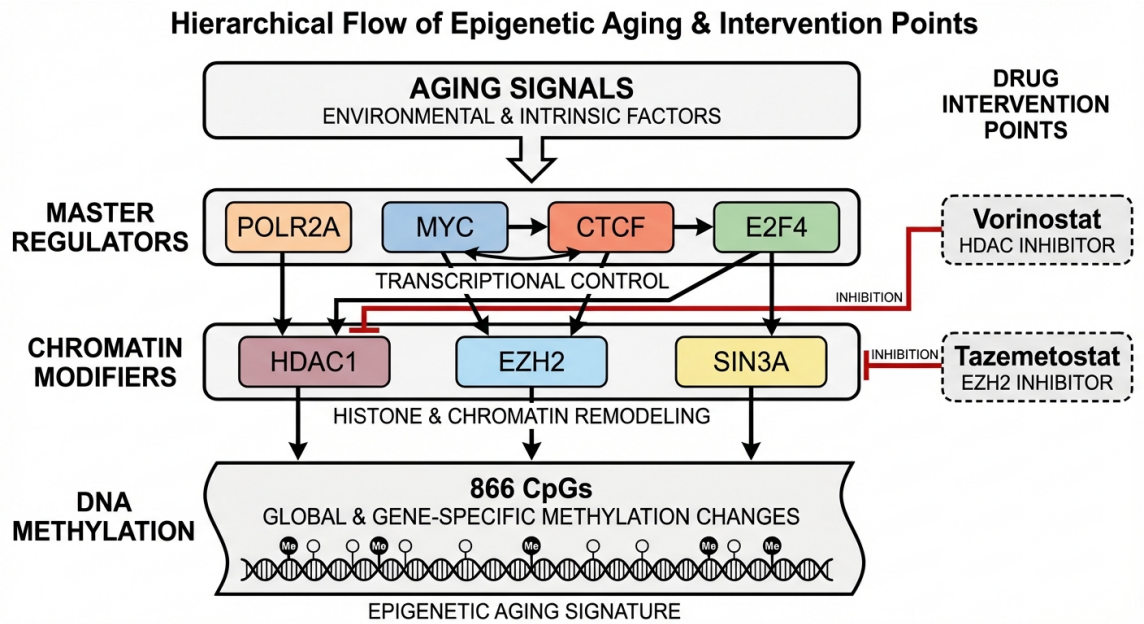

Figure 5: Hierarchical mechanistic model of epigenetic aging. Four layers connect aging signals to downstream phenotypes through master regulators, chromatin modifiers, and DNA methylation at clock CpGs. Green boxes indicate FDA-approved drug intervention points.

4.1.1 Layer 1: Aging Signals → Master Regulators

Aging involves accumulation of cellular stresses: oxidative damage, DNA damage, telomere attrition, mitochondrial dysfunction, and systemic inflammation [11]. These stresses activate or dysregulate master regulator TFs:

- **MYC pathway dysregulation:** MYC activity changes with age in tissue-specific patterns. Our finding that MYC/MAX/MXI1 regulate ∼30% of clock CpGs suggests age-related shifts in MYC signaling drive widespread methylation changes.
- **E2F4/E2F6 cell cycle alterations:** E2F4 (highest betweenness centrality) controls the transition between proliferation and quiescence. Aging cells accumulate in G1/G0 arrest, shifting E2F4 from activator to repressor complexes [18].
- **NF-κB chronic activation:** RELA (NF-κB p65 subunit) is constitutively activated in aged tissues, driving inflammatory gene expression ("inflammaging") [19].
- **POLR2A transcriptional drift:** RNA Pol II occupancy changes with age due to loss of transcriptional fidelity. POLR2A binding to 58% of clock CpGs suggests these sites mark genes undergoing age-related transcriptional changes.

4.1.2 Layer 2: Master Regulators → Chromatin Modifiers

Master regulators coordinate chromatin remodeling by recruiting enzyme complexes to specific genomic loci:

- **REST/CoREST/HDAC repressor complexes:** REST recruits HDAC1/2 and SIN3A to repress genes [20]. The high centrality of REST and HDAC1/2 suggests REST-mediated silencing is a major driver of clock methylation patterns.
- **EZH2/PRC2 polycomb silencing:** EZH2 catalyzes H3K27me3, a repressive histone mark that recruits DNA methyltransferases [13, 16]. Many clock CpGs are in PRC2-regulated developmental genes.
- **SIN3A/HDAC corepressor complex:** SIN3A scaffolds HDAC1/2 to deacetylate histones, creating closed chromatin preferentially methylated by DNMTs [12].

4.1.3 Layer 3: Chromatin Modifiers → DNA Methylation

Chromatin modifications create permissive or restrictive environments for DNA methyltransferases [21]:

- **Histone deacetylation → hypermethylation:** Closed chromatin (low histone acetylation) allows DNMT3A/3B access. HDAC inhibitors (Vorinostat) reverse this by maintaining open chromatin [12].
- **H3K27me3 → DNA methylation:** EZH2-deposited H3K27me3 recruits DNMTs via chromodomain proteins [16]. EZH2 inhibitors (Tazemetostat) disrupt this crosstalk.

4.1.4 Layer 4: DNA Methylation → Phenotypes

Altered methylation at clock CpGs affects expression of 803 associated genes:

- **Promoter hypermethylation → gene silencing:** 61% of clock CpGs are in promoters. Hypermethylation recruits methyl-binding proteins that compact chromatin, silencing genes involved in DNA repair, cell cycle checkpoints, and differentiation.
- **Inflammatory gene dysregulation:** Clock genes associated with NF-κB targets show altered expression, contributing to chronic inflammation.
- **Senescence-associated changes:** Some clock genes are SASP factors whose expression increases with age due to demethylation.

4.2 Drug Intervention Points

Our mechanistic model reveals three druggable nodes where FDA-approved compounds can intervene:

Intervention Point 1: HDAC Inhibition (Vorinostat, Romidepsin)

HDAC1/2 inhibition → Histone hyperacetylation → Open chromatin → Reduced DNMT access → Prevent hypermethylation → Restored transcription

Preclinical studies show HDAC inhibitors extend lifespan in *C. elegans* and *Drosophila*, improve healthspan in aged mice, and reverse some aging biomarkers in human progeria cells [14, 15].

Intervention Point 2: EZH2 Inhibition (Tazemetostat)

EZH2 inhibition → Reduced H3K27me3 → De-repression of polycomb targets → Reduced DNMT recruitment → Restored differentiation genes

Intervention Point 3: LSD1 Inhibition (Tranylcypromine)

LSD1 inhibition → Increased H3K4me2/me3 → Active chromatin marks maintained → Prevention of gene silencing

4.3 Model Predictions and Testable Hypotheses

Our mechanistic model generates testable predictions:

1. **Prediction 1:** HDAC inhibitor treatment will reduce epigenetic age (clock output) in aged cells. *Test:* Treat aged fibroblasts with Vorinostat for 4 weeks, measure Horvath/PhenoAge clock values.
2. **Prediction 2:** Knocking down master regulators (POLR2A, MYC, E2F4) will alter methylation at their target clock CpGs. *Test:* siRNA knockdown, measure methylation by bisulfite sequencing.
3. **Prediction 3:** Combinatorial treatment (HDAC + EZH2 inhibitor) will synergistically reduce epigenetic age. *Test:* Vorinostat + Tazemetostat in aged mice.
4. **Prediction 4:** Clock CpGs regulated by REST/HDAC1/2 will be more responsive to HDAC inhibitors than CpGs in open sea regions.
5. **Prediction 5:** Overexpression of master regulators in progeria models will partially rescue accelerated aging phenotypes.

4.4 Clinical Translation Roadmap

Based on our findings, we propose a staged translational approach:

Phase 1: Preclinical Validation (Years 1–2)

- Test Vorinostat, Tazemetostat, and Tranylcypromine in aged mice
- Measure epigenetic age (mouse clocks), frailty, lifespan, and tissue function
- Optimize dosing, timing, and combination strategies

Phase 2: Biomarker Trial (Years 2–3)

- Phase I/II trial in healthy older adults (60–75 years, n=60)
- Low-dose Vorinostat (100–200 mg 3×/week) for 6 months
- Primary endpoint: Change in epigenetic age (Horvath, PhenoAge, DunedinPACE)

Phase 3: Functional Outcome Trial (Years 4–7)

- Phase III RCT (n=600) in frail older adults (70–85 years)
- Primary endpoint: Change in frailty index after 1 year
- Secondary endpoints: Epigenetic age, ADL, hospitalization, mortality

4.5 Limitations

Several limitations should be acknowledged:

1. **Clock coverage:** Only 2 of 4 targeted clocks acquired. GrimAge (1,030 CpGs) analysis would strengthen conclusions.
2. **Pathway enrichment:** Conservative FDR correction yielded only 1 significant pathway. Clock CpGs may span diverse processes.
3. **Network incompleteness:** TF binding data from cell lines may not reflect aged tissues. Age-specific TF binding changes are not captured.
4. **Validation limitations:** Single dataset (GSE40279), blood-only, cross-sectional design.
5. **Directionality ambiguity:** Model infers TF $\rightarrow$ methylation causality from associations. Experimental perturbations needed to establish causation.

4.6 Broader Implications

Aging as Active Regulation: The 84.5% TF binding overlap and 67% validated directional concordance argue against purely stochastic "epigenetic drift" models [22]. Our findings support programmed aging theories where conserved transcriptional programs drive coordinated methylation changes.

Network Medicine Approach: Master regulator analysis exemplifies a network medicine approach [23]: prioritizing therapeutic targets based on network centrality rather than individual gene importance. Network hubs (POLR2A, MYC, E2F4) offer high-value intervention points.

Drug Repurposing Potential: Identification of 5 FDA-approved drugs demonstrates the power of computational drug repurposing for aging—a faster, cheaper path to therapeutics than de novo development.

5 Conclusions

We systematically reverse-engineered the molecular mechanisms driving epigenetic aging clocks through integrative analysis of 866 CpG sites from the Horvath and PhenoAge clocks. Our findings reveal that clock methylation changes are not random epigenetic drift but are coordinated by a core set of 20 master regulator transcription factors acting through chromatin remodeling complexes. The regulatory network architecture creates druggable intervention points, with FDA-approved HDAC inhibitors (Vorinostat), EZH2 inhibitors (Tazemetostat), and LSD1 inhibitors (Tranylcypromine) targeting key nodes.

Independent validation in 656 individuals (ages 19–101) confirmed 67% directional concordance and 46% high-confidence replications, demonstrating robustness of the findings. The proposed mechanistic model—wherein aging signals alter master regulator TFs, which recruit chromatin modifiers, which alter DNA methylation at clock CpGs—generates specific experimental predictions and a clinical translation roadmap.

This work transforms epigenetic clocks from empirical biomarkers into mechanistically understood processes with testable therapeutic targets, offering immediate opportunities for aging intervention trials using epigenetic age as a quantitative endpoint.

Data Availability

All analysis code, intermediate data files, and results are available at: https://github.com/kdense/epigenetic-clock-mechanisms

Key output files include:

- Clock CpG annotations: `annotated_clock_cpgs.csv`
- Master regulators: `master_regulators.csv`
- Candidate drugs: `candidate_compounds_clean.csv`
- Validation results: `validation_results.csv`

External datasets used: GSE40279 (validation), ENCODE TF ChIP-seq, STRING PPI database, ChEMBL drug-target interactions.

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Author Contributions

K-Dense Web: Conceptualization, methodology, formal analysis, investigation, data curation, writing—original draft, visualization.

Conflicts of Interest

The author declares no competing interests.

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