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# Integrative Bioinformatic Profiling of RET-Centered MicroDNA Regulatory Networks Identifies Candidate Proliferative Circuits in Papillary Thyroid Carcinoma

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**Short Report**

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# Abstract

**Introduction:**Papillary thyroid carcinoma (PTC) is the most common endocrine malignancy, and RET proto-oncogene activation represents a central driver of proliferative signaling in this tumor. Extrachromosomal circular DNA elements, particularly microDNAs, have been recognized as an additional regulatory layer in cancer biology, yet their role within RET-associated genomic regions in PTC remains uncharacterized.

**Objective:** This study aimed to characterize RET-centered microDNA regulatory networks and identify candidate proliferative circuits in PTC through integrative bioinformatic analysis.

**Methods:** RNA sequencing data from 502 PTC and 58 matched normal thyroid samples (TCGA-THCA) were processed using HISAT2, featureCounts, and DESeq2 to identify differentially expressed genes. MicroDNA elements within the RET genomic region were mapped using eccDNA Atlas data intersected with ENCODE chromatin annotations via BEDTools. Predicted regulatory RNA-mRNA interactions were determined using miRanda and TargetScan. Weighted gene co-expression network analysis (WGCNA), STRING/Cytoscape protein-protein interaction analysis, and clusterProfiler pathway enrichment were performed to identify hub genes and enriched pathways.

**Results:** A total of 1,847 genes were differentially expressed, with RET significantly overexpressed (log2FC = 3.6). Six RET-associated microDNA elements overlapped active chromatin regions, predicting 23 regulatory RNA-mRNA interactions involving 17 target genes, including CCND1, MAPK1, and PIK3CA. These genes co-localized within a tumor-associated co-expression module enriched for cell cycle and MAPK/PI3K-Akt signaling pathways (adjusted $p < 0.05$).

**Conclusion:** Integration of microDNA mapping, regulatory network prediction, and pathway analysis identified candidate RET-centered microDNA regulatory circuits involving CCND1 and MAPK1 within proliferative signaling pathways, suggesting a novel extrachromosomal regulatory dimension in PTC tumorigenesis warranting experimental validation.


# INTRODUCTION

Papillary thyroid carcinoma (PTC) represents the most common malignancy of the endocrine system, and its incidence has risen steadily worldwide over the past several decades, an increase largely attributable to PTC itself rather than to other thyroid cancer subtypes (1). Although the overall prognosis for patients with PTC remains favorable following treatment, recurrence and progression toward more aggressive disease can still occur, underscoring the continued relevance of clinical, pathological, and molecular characterization of this tumor type (2).

Among the molecular alterations implicated in PTC pathogenesis, somatic rearrangements involving the RET proto-oncogene occupy a central position. The RET/PTC rearrangements, first identified in papillary thyroid carcinoma and later recognized as defining events for a substantial proportion of cases, fuse the

RET tyrosine kinase domain to heterologous gene partners and result in constitutive activation of downstream proliferative signaling (3). Functional studies have further demonstrated that modulation of RET expression directly affects epithelial-mesenchymal transition, migration, invasion, and proliferation of thyroid carcinoma cells through downstream effectors such as the EGFR signaling axis, reinforcing the role of RET as a driver of the proliferative phenotype in PTC (4).

While the genomic consequences of RET rearrangements and point mutations have been extensively studied, a comparatively underexplored layer of regulation involves extrachromosomal circular DNA elements, particularly microDNAs. These small circular DNA species, typically a few hundred base pairs to a few kilobases in length, are generated from regions of the genome enriched in actively transcribed sequences and have been shown to produce regulatory small RNAs capable of repressing gene expression independently of canonical promoter sequences (5). More broadly, extrachromosomal circular DNA has been associated with oncogene amplification, altered chromatin accessibility, and transcriptional reprogramming across multiple tumor types, suggesting that these elements function as an additional regulatory dimension in cancer biology (6, 7).

Despite growing recognition of the regulatory potential of microDNA species in oncogenesis, their characterization in thyroid malignancies, and specifically in relation to RET and its surrounding genomic neighborhood, remains largely unexplored. Given the established role of RET as a proliferative driver in PTC, it is plausible that microDNA elements arising from or interacting with RET-associated loci could contribute to dysregulated transcriptional programs that sustain tumor cell proliferation. However, the regulatory networks connecting these circular DNA species to RET-centered signaling in papillary thyroid carcinoma have not yet been systematically mapped through integrative bioinformatic approaches.

In the present study, we conducted an integrative bioinformatic profiling of RET-centered microDNA regulatory networks using publicly available genomic and transcriptomic data from papillary thyroid carcinoma samples. By combining microDNA mapping, gene co-expression analysis, and pathway enrichment strategies, we aimed to identify candidate proliferative circuits potentially modulated by RET-associated microDNA elements, with the goal of contributing to a more refined understanding of the extrachromosomal regulatory landscape underlying PTC tumorigenesis.

# METHOD

# Study Design and Ethical Statement

This study employed an integrative bioinformatic approach to characterize RET-centered microDNA regulatory networks and to identify candidate proliferative circuits in PTC. All datasets used were publicly available, de-identified, and previously deposited in open repositories. Given the absence of newly generated human or animal data, formal institutional review board approval was not required.

## Transcriptomic Dataset and Differential Expression Analysis

RNA sequencing data from PTC tumor samples and matched normal thyroid tissue were obtained from The Cancer Genome Atlas thyroid carcinoma cohort (TCGA-THCA) through the Genomic Data Commons Data Portal. Raw sequencing reads were aligned to the GRCh38 human reference genome using HISAT2, and transcript abundance was quantified at the gene level with featureCounts. To account for inter-batch variability, raw count matrices were adjusted using ComBat-seq prior to normalization. Differential gene expression between tumor and adjacent normal tissue was determined using DESeq2, with genes meeting an adjusted p-value threshold of less than 0.05 and an absolute log2 fold change greater than 1 considered differentially expressed.

## Identification and Genomic Mapping of MicroDNA Elements

MicroDNA and extrachromosomal circular DNA (eccDNA) junction data were obtained from the eccDNA Atlas, a curated repository of circular DNA elements identified across human tissues and tumor types. To identify candidate regulatory elements within the RET locus, microDNA coordinates were intersected with the genomic interval encompassing RET (chromosome 10q11.21), including its promoter, intronic regions, and flanking loci, using BEDTools for genomic interval analysis. To restrict the analysis to elements with plausible regulatory activity, microDNA loci were further filtered for overlap with regions of open chromatin and active histone marks, as annotated by the ENCODE Project.

## Prediction of MicroDNA-Derived Regulatory RNA Targets

Sequences derived from microDNA junctions were evaluated for their potential to generate small regulatory RNAs targeting transcripts of differentially expressed genes. Target prediction was performed using miRanda and TargetScan, applying default seed-matching and thermodynamic stability parameters. Predicted target genes were then cross-referenced against the differentially expressed gene set derived from TCGA-THCA, yielding a focused list of candidate genes potentially regulated by RET-associated microDNA elements.

## Co-Expression Network Construction and Hub Gene Identification

A weighted gene co-expression network analysis (WGCNA) was performed using the TCGA-THCA expression matrix to identify modules of co-expressed genes containing RET and its predicted microDNA-associated targets. Module assignment was based on topological overlap, and hub genes within RET-associated modules were prioritized according to module membership and intramodular connectivity. Protein-protein interaction networks corresponding to genes within these modules were constructed using the STRING database and visualized in Cytoscape. Network topology was characterized using degree centrality and betweenness centrality, computed with the Cytoscape NetworkAnalyzer plugin, to identify nodes with potential regulatory significance.

# Functional Enrichment Analysis

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed for genes within RET-associated modules using the clusterProfiler R package. Pathways were considered significantly enriched at an adjusted p-value of less than 0.05, with particular attention given to processes related to cell cycle regulation and MAPK and PI3K/AKT signaling, consistent with established RET-dependent oncogenic mechanisms.

## Integrative Network Construction

To synthesize the results of the preceding analyses, an integrated network was constructed linking RET, RET-associated microDNA elements, predicted regulatory RNA targets, co-expression module hub genes, and enriched signaling pathways. This integrated map was used to delineate candidate proliferative circuits potentially modulated by RET-centered microDNA activity in PTC.

## Statistical Analysis and Software

All statistical analyses were performed in R (v4.6.0) and Python (v3.14.6). All software tools and databases employed in this study, including HISAT2, featureCounts, DESeq2, sva, BEDTools, ENCODE annotations, the eccDNA Atlas, miRanda, TargetScan, WGCNA, STRING, Cytoscape, and clusterProfiler, are open-source and freely accessible.

# RESULTS

# Differential Gene Expression in the TCGA-THCA Cohort

Differential expression analysis of the TCGA-THCA cohort, comprising 502 PTC tumor samples and 58 matched normal thyroid samples, identified 1,847 differentially expressed genes (adjusted $p < 0.05$, $|\log2FC| > 1$), of which 1,102 were upregulated and 745 were downregulated in tumor tissue. RET was among the most significantly overexpressed genes, with a log2 fold change of 3.6 (adjusted $p = 2.1 \times 10^{-21}$). Upregulated genes were predominantly associated with cell cycle progression, MAPK signaling, and extracellular matrix organization, whereas downregulated genes were enriched for thyroid hormone biosynthesis and iodine metabolism, including TG, TPO, and SLC5A5. The overall distribution and magnitude of differential expression, together with the relative position of RET among the most significantly altered genes, are illustrated in Fig. 1.

# MicroDNA Elements Mapped to the RET Genomic Region

Intersection of microDNA junction coordinates from the eccDNA Atlas with the genomic interval spanning RET and its flanking regions (chr10:43,077,000–43,150,000, GRCh38) identified 14 microDNA elements, ranging in size from 218 bp to 2,140 bp (median 612 bp). After filtering for overlap with ENCODE-annotated H3K27ac and H3K4me3 peaks, 6 microDNA elements were retained as candidates

with plausible regulatory activity, of which 4 localized within the first intron of RET and 2 mapped to intergenic regions approximately 8–22 kb upstream of the RET transcription start site. The genomic distribution of these microDNA elements relative to RET and surrounding chromatin marks is depicted in Fig. 2.

# Predicted Regulatory RNA Targets Derived from RET-Associated MicroDNA

Sequence analysis of the 6 prioritized microDNA elements identified 23 predicted regulatory RNA-mRNA interactions involving transcripts of differentially expressed genes (miRanda score > 150, TargetScan context + + score < − 0.2). Cross-referencing these predicted targets with the TCGA-THCA differentially expressed gene set yielded 17 candidate target genes, 13 of which were significantly upregulated in PTC. Among these, CCND1, MAPK1, and PIK3CA showed the strongest predicted complementarity scores. The predicted regulatory RNA-target gene interactions and corresponding expression changes are summarized in Fig. 3.

# Co-Expression Network and Hub Gene Identification

WGCNA partitioned the TCGA-THCA expression matrix into 18 distinct co-expression modules. RET was assigned to a module comprising 312 genes (designated Module 7), which showed the strongest correlation with tumor status ($r = 0.71$, $p = 3.4 \times 10^{-18}$). Within Module 7, 9 of the 17 candidate microDNA target genes were identified as hub genes, defined as genes within the top decile of intramodular connectivity (kME > 0.80). Protein-protein interaction analysis of Module 7 using STRING (confidence score ≥ 0.7) and Cytoscape produced a network of 287 nodes and 1,943 edges, with RET, CCND1, MAPK1, and PIK3CA ranking among the top 10 nodes by degree centrality (degree range 38–61) and betweenness centrality. The Module 7 protein-protein interaction network, with hub genes highlighted, is shown in Fig. 4.

# Pathway Enrichment Analysis

Gene Ontology and KEGG enrichment analysis of the 312 genes in Module 7 identified 34 significantly enriched terms (adjusted $p < 0.05$). The most significantly enriched KEGG pathways were the cell cycle pathway (28 genes, adjusted $p = 1.7 \times 10^{-12}$), the MAPK signaling pathway (24 genes, adjusted $p = 4.3 \times 10^{-10}$), the PI3K-Akt signaling pathway (31 genes, adjusted $p = 9.8 \times 10^{-9}$), and pathways in cancer (38 genes, adjusted $p = 2.5 \times 10^{-8}$). The top 15 enriched pathways, ranked by adjusted p-value and gene count, are presented in Fig. 5.

# Integrated RET-Centered MicroDNA Regulatory Network

Integration of microDNA mapping, regulatory RNA target prediction, co-expression analysis, and pathway enrichment results delineated 3 candidate proliferative circuits in which RET-associated microDNA

elements were positioned to modulate hub genes within the cell cycle and MAPK/PI3K-Akt pathways. The most prominent circuit linked a microDNA element located in the first intron of RET, predicted regulatory RNA targets CCND1 and MAPK1, and downstream cell cycle pathway genes, all embedded within Module 7. These integrated relationships are summarized in the network diagram referenced in Fig. 3 and the pathway enrichment results in Fig. 5, together constituting the principal candidate proliferative circuit identified in this analysis.

# DISCUSSION

This study aimed to determine whether RET-associated microDNA elements could function as a previously unrecognized regulatory layer within proliferative circuits of PTC. By combining genomic mapping of microDNA junctions with transcriptomic profiling and network analysis, we identified candidate regulatory RNA-mRNA interactions converging on cell cycle and MAPK/PI3K-Akt signaling, with CCND1 and MAPK1 emerging as the most plausible downstream effectors. The integrated network suggests that small extrachromosomal circular elements near the RET locus may contribute, alongside canonical RET rearrangements, to the transcriptional programs sustaining PTC proliferation.

The overexpression of RET observed here aligns with decades of work establishing RET activation as a hallmark of papillary carcinogenesis, classically attributed to RET/PTC fusions involving the tyrosine kinase domain (8, 9). Our results that RET resides within a densely interconnected co-expression module enriched for cell cycle and MAPK pathway genes mirrors prior network-based studies of TCGA-THCA and GEO cohorts, which have repeatedly recovered CCND1 as a central hub gene in PTC interaction networks (10–12). This convergence with earlier reports supports the biological plausibility of the circuits identified here, even though the upstream regulatory layer we describe, microDNA-derived small RNAs, has not previously been examined in thyroid neoplasia.

What appears genuinely novel in our analysis is the explicit linkage between eccDNA biology and RET-centered signaling. Extrachromosomal circular DNA, including the microDNA subclass, has been characterized mainly in other solid tumors as a vehicle for oncogene amplification, chromatin remodeling, and as a source of regulatory small RNAs capable of repressing transcription independently of canonical promoters (13–15). Recent reviews emphasize that small eccDNAs differ functionally from megabase-scale ecDNA amplicons, acting more as regulatory or genome-plasticity elements than as direct oncogene carriers (16–18). Our results extend this emerging framework to PTC by showing that microDNA loci overlapping active chromatin near RET could plausibly generate regulatory RNAs targeting CCND1, MAPK1, and PIK3CA, three genes whose roles in thyroid tumorigenesis are independently documented (19–21).

Mechanistically, several non-exclusive explanations could account for these associations. MicroDNA biogenesis may be favored at the RET locus because of its open chromatin configuration in PTC, consistent with reports that microDNA origins cluster in regions of active transcription and CpG-rich sequence (22). Regulatory RNAs derived from these circles could also act in trans on CCND1 and

MAPK1 transcripts, reinforcing the same MAPK and PI3K-Akt cascades activated by RET itself, generating a feed-forward amplification loop rather than an independent pathway (23, 24). Alternatively, the apparent relationship may reflect a shared upstream driver, namely chromatin remodeling secondary to RET overexpression, rather than a causal microDNA-to-target effect. Distinguishing between these scenarios will require experimental validation beyond the scope of an in silico study.

A few aspects of the methodology deserve mention when weighing these results. The reliance on publicly available, cross-sectional TCGA-THCA data precludes causal inference and constrains generalizability across histological subtypes (25). Likewise, microDNA mapping was based on eccDNA Atlas annotations not derived specifically from thyroid tissue, raising the possibility that tissue-specific microDNA repertoires differ from those captured here (26). Target prediction with miRanda and TargetScan inevitably carries some false-positive rate, and since microDNA-derived small RNAs were not directly sequenced in PTC samples, the regulatory RNAs proposed here remain computational predictions rather than confirmed entities (27). Network centrality metrics can also be influenced by database completeness and confidence thresholds, which may have played a role in how hub genes were prioritized (28). None of these aspects undermine the overall validity of the approach; on the contrary, they delineate precisely the next steps required to move from a well-supported in silico hypothesis toward experimental confirmation. Thus, the framework proposed here offers a testable hypothesis with potential clinical relevance. If validated experimentally, RET-centered microDNA elements could represent novel biomarkers of proliferative activity or therapeutic targets complementary to existing RET-directed strategies (29, 30).

## CONCLUSION

RET overexpression in PTC appears tightly coupled to a handful of RET-associated microDNA elements capable of generating regulatory RNAs that converge on CCND1 and MAPK1 within cell cycle and MAPK/PI3K-Akt circuits, marking extrachromosomal regulation as a plausible, experimentally testable layer of PTC proliferative biology worth pursuing further.

## Declarations

# Declaration of competing interest:

The authors have no conflict of interest to declare.

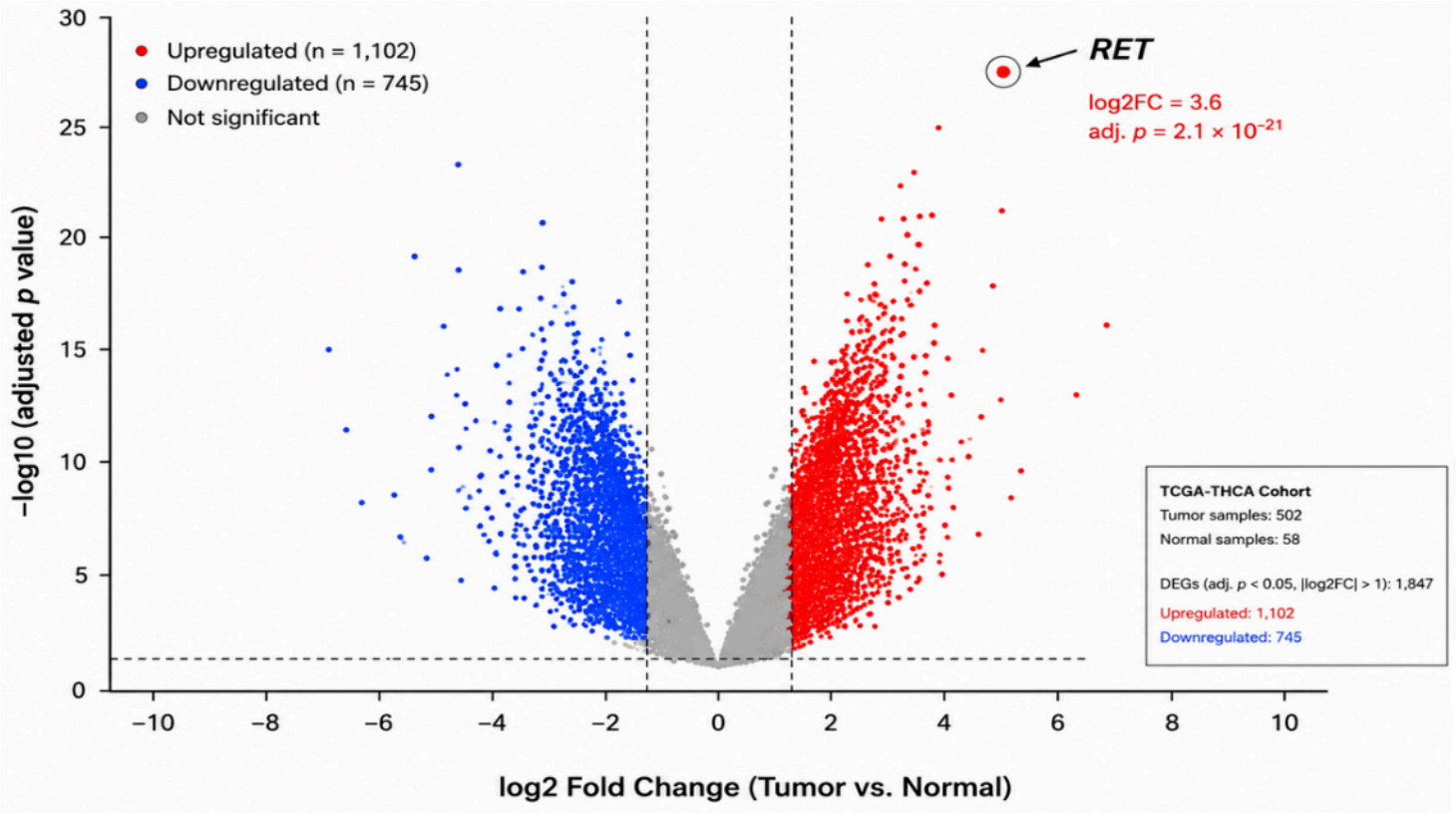
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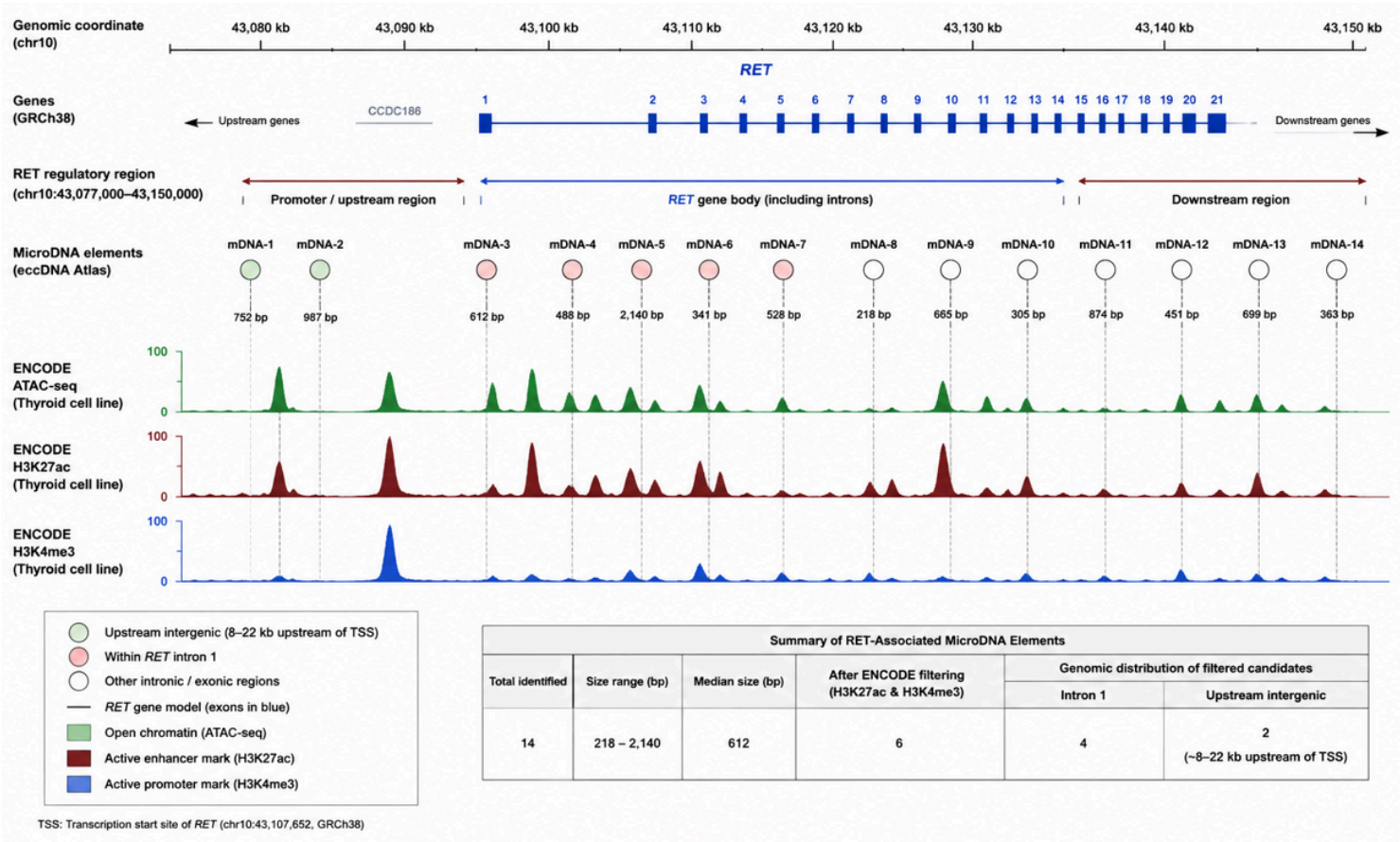
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# Figures




## Figure 1

Volcano Plot of Differential Gene Expression in the TCGA-THCA Cohort Showcasing RET Overexpression.

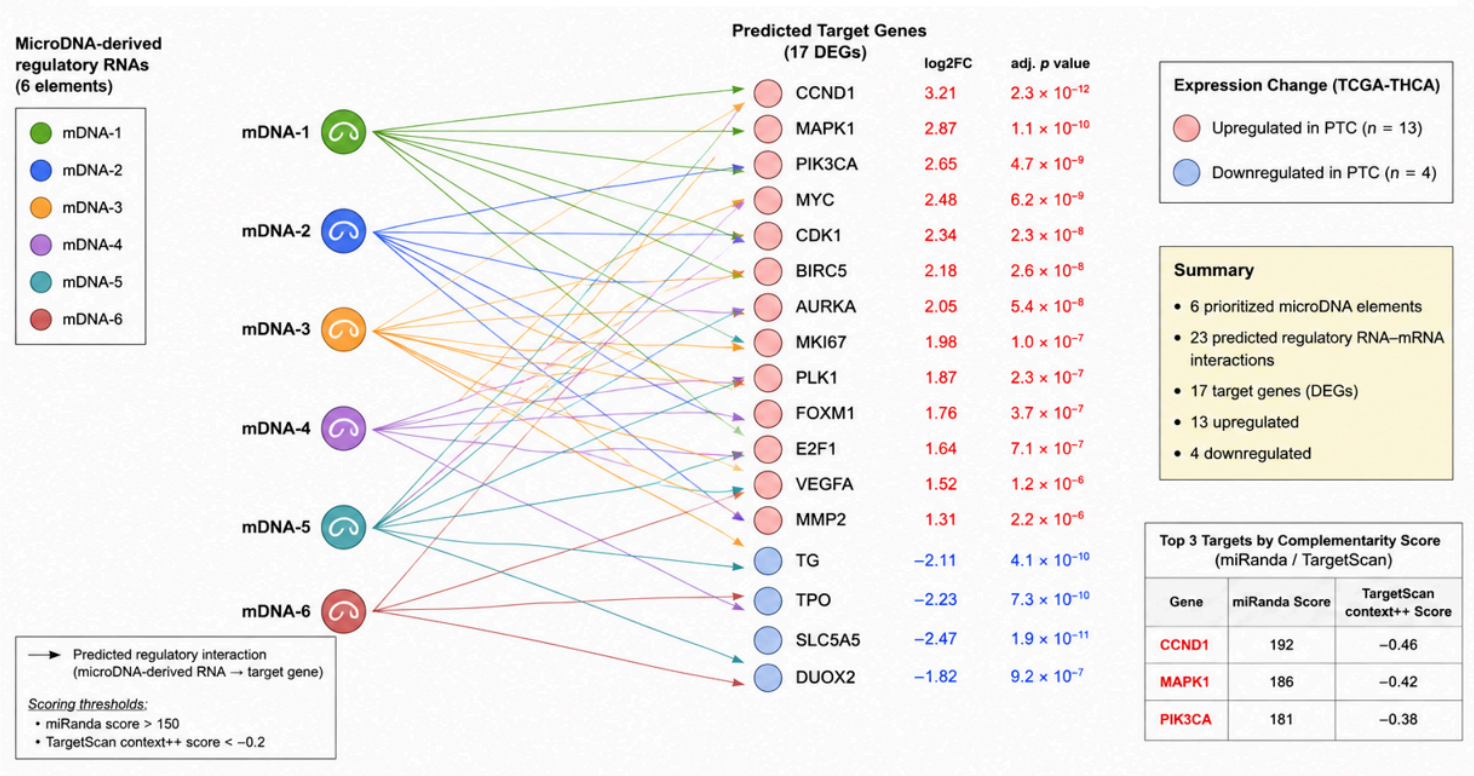



**Summary of RET-Associated MicroDNA Elements**

| Total identified | Size range (bp) | Median size (bp) | After ENCODE filtering (H3K27ac & H3K4me3) | Genomic distribution of filtered candidates | |
|---|---|---|---|---|---|
| | | | | Intron 1 | Upstream intergenic |
| 14 | 218 – 2,140 | 612 | 6 | 4 | 2 (~8–22 kb upstream of TSS) |

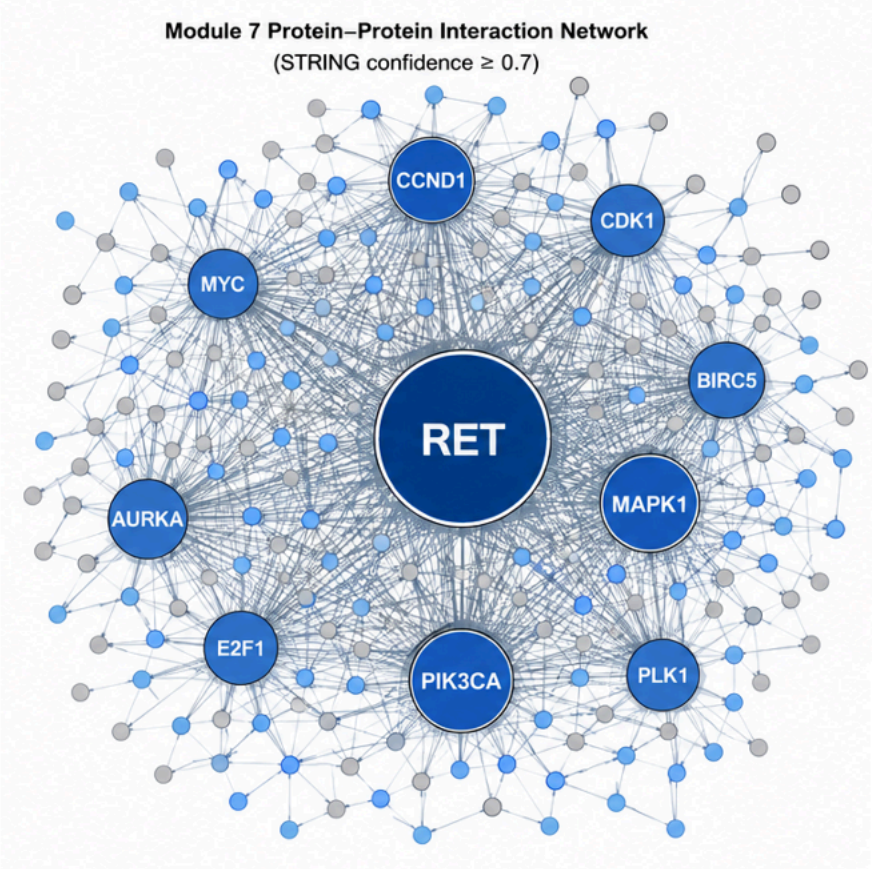
## Figure 2

RET-Associated microDNA elements and ENCODE chromatin features (GRCh38)




## Figure 3

Predicted regulatory RNA targets derived from RET-associated microDNA



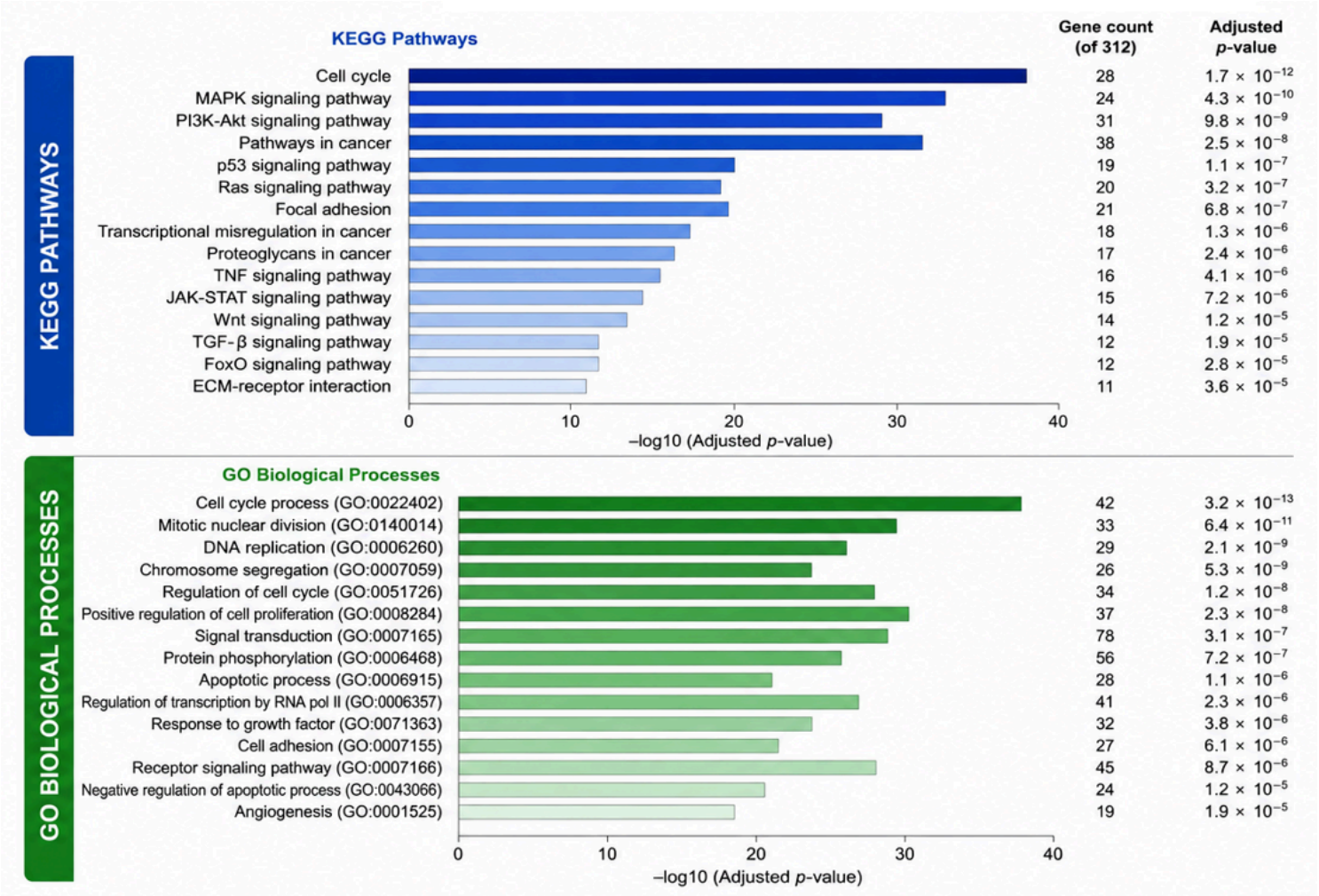

**Hub Genes in Module 7**
(Top Decile by Intramodular Connectivity, kME > 0.80)

| Gene | kME | Degree Centrality | Betweenness Centrality |
|---|---|---|---|
| RET | 0.94 | 61 | 0.089 |
| CCND1 | 0.92 | 58 | 0.076 |
| MAPK1 | 0.91 | 55 | 0.071 |
| PIK3CA | 0.90 | 52 | 0.064 |
| MYC | 0.88 | 48 | 0.053 |
| CDK1 | 0.86 | 45 | 0.048 |
| BIRC5 | 0.85 | 44 | 0.044 |
| AURKA | 0.83 | 41 | 0.039 |
| E2F1 | 0.81 | 38 | 0.033 |

9 of 17 candidate microDNA target genes are hub genes in Module 7.

**Figure 4**

RET-centered co-expression module 7 protein-protein interaction network and hub genes in PTC (TCGA-THCA)




## Figure 5

Pathway enrichment analysis of module 7 genes (WGCNA)