Blood Cancer Journal www.nature.com/bcj

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Sex-specific dysregulation of exosomal non-coding RNAs drives multiple myeloma progression

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Multiple myeloma (MM) is characterized by the clonal proliferation of plasma cells in the bone marrow. Although the precise molecular mechanisms differentiating men and women in MM are not fully understood, uncovering these differences is crucial for improving personalized therapeutic approaches. Here, we show sex-specific dysregulation of exosomal non-coding RNAs (ncRNAs) in MM. We conducted an in-depth analysis of dysregulated ncRNAs in male and female patients, as well as MM cell lines, revealing distinct expression signatures across multiple clinical contexts, including newly diagnosed, relapse, progression, Hyperdiploid, non-Hyperdiploid, and treatment exposure. Our findings highlight the pivotal roles of lncRNAs and miRNAs in MM pathogenesis, detecting alterations in enriched pathways that influence key biological processes such as cellular proliferation, apoptosis, and gene regulation. We established a panel of ncRNAs with distinct sex-specific expression patterns, significant effects on mRNA regulation, and involvement in MM-associated biological pathways. Our results demonstrate that exosomes provide enhanced analytical resolution for detecting non-coding RNAs, enabling more sensitive and precise identification of transcriptomic alterations. These results suggest that sex-specific dysregulation of ncRNAs may contribute to differences in MM progression and therapy response. Ultimately, this study underscores the importance of exosomal ncRNA profiling in designing sex-tailored therapeutic strategies targeting dysregulated ncRNAs, paving the way for personalized medicine in MM.

Blood Cancer Journal (2025)15:162 ; https://doi.org/10.1038/s41408-025-01362-1

INTRODUCTION

Multiple myeloma (MM) is characterized by uncontrolled proliferation and accumulation of clonal plasma cells within the bone marrow [1]. Chromosomal translocations, aneuploidy, genetic mutations, and epigenetic aberrations are essential in disease initiation and progression [2]. In addition, men are generally more likely than women to develop MM. While the reasons for the higher incidence of MM in males (M) compared to females (F) are not well understood, numerous studies have been conducted to uncover sex-dependent genetic events, causes, prevalence, and survival differences of this cancer [3]. Sex-based differences in the incidence and malignancy of cancers, including MM, are particularly relevant since certain tumors are initiated via abnormalities in sex chromosomes or are influenced by sex hormones [4, 5]. The significant differences in the prevalence, incidence, and progression of MM in male and female patients have been estimated across different populations [6, 7]. Furthermore, sex-dependent differences in the primary genetic events of MM, such as immunoglobulin heavy chain gene translocations, which are more common in women, and hyperdiploidy, which is more frequent in men, suggest a complex interplay of biological factors [8]. While sexual classification did not significantly influence patient mortality, some retrospective studies have shown that mortality and incidence rates of MM are generally higher in men than in women, particularly in cases of the rare and aggressive subtype, extramedullary MM [9, 10]. In addition, some clinical trials suggest that genetic lesions involved in the initiation and progression of MM may differ between the sexes, and in female patients, the trial treatments may have mitigated some of the adverse effects of high-risk cytogenetic lesions [11]. These findings underscore the importance of sex-specific research in understanding MM biology and improving therapeutic approaches.

Non-coding RNAs (ncRNAs) are functional RNA molecules that do not encode proteins but play essential regulatory roles in cellular processes. They are involved in various mechanisms such as transcriptional and post-transcriptional regulation, chromatin remodeling, and cell signaling pathways [12]. The primary classes of ncRNAs examined in this study are microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). miRNAs are short RNA molecules (~22 nucleotides) that negatively regulate gene expression by promoting mRNA degradation or inhibiting translation [13]. In contrast, lncRNAs are typically longer than 200 nucleotides and participate in chromatin modification, transcriptional regulation, and the modulation of key signaling networks [14]. Recent research has demonstrated that the dysregulation of ncRNAs contributes significantly to the pathogenesis and progression of MM [15]. These molecules influence the tumor microenvironment and have emerged as promising biomarkers and therapeutic

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Received: 19 January 2025 Revised: 5 August 2025 Accepted: 2 September 2025
Published online: 16 October 2025

targets in MM. Understanding the functional roles of ncRNAs in MM will pave the way for the development of novel, personalized therapies that are more precise, effective, and tailored to the molecular profile of individual patients.

In this regard, exosomes are gaining prominence as critical mediators of MM pathogenesis and progression [16]. These nanosized extracellular vesicles (<150 nm), secreted by various cells, including malignant plasma cells, play a significant role in shaping the tumor-supportive bone marrow (BM) microenvironment. Exosomes primarily carry non-coding RNAs (ncRNAs) along with a diverse array of bioactive cargoes, including proteins, lipids, and nucleic acids, which they deliver to recipient cells, thereby modulating various malignant processes. In MM, exosomes have been implicated in promoting bone disease through osteoclast activation and osteoblast inhibition, enhancing angiogenesis by transferring pro-angiogenic factors like VEGF, and fostering immune suppression by modulating T cells and myeloid-derived suppressor cells [16–18]. Additionally, exosomes contribute to drug resistance by transferring survival-promoting signals and molecules that counteract therapeutic agents [19, 20].

Here, we studied exosomal miRNAs and lncRNAs in BM aspirate plasma samples from MM patients using RNA sequencing, with analysis conducted in both male and female groups. Additionally, we incorporated mRNA expression data from BM samples of male and female MM patients obtained from the Genomic Data Commons (GDC) database (MMRF-COMMPASS cohort). We developed a multi-step filtering process and utilized validated databases to examine interactions between non-coding and coding RNAs, aiming to identify key ncRNAs that are differentially expressed during MM progression, significantly influence gene regulation, and exert measurable effects on MM pathways. Ultimately, we established sex-specific panels of miRNAs and lncRNAs that play key functional roles in MM pathogenesis across sexes.

METHODS

Ethics approval and consent to participate

All methods were carried out in accordance with relevant guidelines and regulations, including the Declaration of Helsinki. Approval for the collection and research use of patient samples was obtained from the Mayo Clinic Institutional Review Board (IRBs 919-04, 15–009436, 18–003198, 2207-02). Informed consent was obtained in writing from all participants prior to sample collection.

Cell lines and patient samples

MM cell lines including LP1, NCI-H929, JJN-3, U-266, and KMS-11 were obtained from Dr. Walker's lab at Indiana University. The ANBL-6 cell line was obtained from Dr. Jelinek's lab at Mayo Clinic. All cell lines were cultured in RPMI media (Cytvia) with 10% exosome-depleted fetal bovine serum (Gibco), 1% penicillin-streptomycin (Cytiva), and 1x GlutaMAX (Gibco). The ANBL-6 cell line was dependent on 1 ng/ml IL-6. H929 cells required 50 µM 2-Mercaptoethanol (Thermo Scientific).

MM patient samples were obtained from bone marrow aspirate supernatant following centrifugation. Primary human MM cells were recovered from bone marrow aspirates collected from all Mayo Clinic sites.

Exosome isolation and characterization

The conditioned media were collected for exosome isolation. The media was first centrifuged at 1500 × *g* for 10 min to remove cells and large particles. It was then concentrated using Pierce™ Protein Concentrators PES, 100 K MWCO (Thermo Scientific). Specifically, 1 L of media was concentrated down to 5 mL and applied to size exclusion chromatography columns (SEC) (IZON Science). An automatic fraction collector was used to gather the exosome-rich fractions, which were then combined.

Supernatant from patient bone marrow aspirates and normal human plasma samples were centrifuged at 1500 × *g* for 10 min to remove large particles. Plasma was not concentrated and was applied directly to SEC columns for exosome isolation. Finally, the exosomes were filtered through a 0.2 µm filter (MilliporeSigma) and characterized for size and concentration using the NanoSight NS300 Nanoparticle Tracking Analyzer (Malvern Panalytical). The exosomes were aliquoted and stored at −80 °C. RNA content, including ncRNAs, was characterized for exosomes derived from both MM cell lines and patient samples, enabling the study of their potential role in MM pathogenesis.

RNA isolation, library preparation, and sequencing

RNAs were isolated from exosomes using the MagMAX™ mirVana™ Total RNA Isolation Kit (Thermo Scientific), following the manufacturer's protocol. After isolation, the RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific), and RNA integrity was assessed with an Agilent Bioanalyzer (Agilent Technologies). For library preparation, the isolated RNA was processed using the NEXTFLEX® Small RNA-Seq Kit v4 with UDIs (Revvity) according to the manufacturer's instructions. This protocol involved 3′ and 5′ adapter ligation, reverse transcription, PCR amplification, and size selection. Libraries were quantified using Qubit dsDNA HS Assay (Thermo Scientific) and validated for size distribution using a Bioanalyzer (Agilent Technologies). Sequencing was performed on an Illumina NovaSeq X Plus platform (The Center for Medical Genomics at Indiana University School of Medicine), following the standard procedures for small RNA sequencing.

Detection of lncRNA, miRNA, and mRNA

Comprehensive RNA identification was conducted using Partek® Flow® software version 11.0.23.1204 (Illumina). First, the quality control of raw FASTQ files was assessed for each sample. Adaptors were trimmed using CUTADAPT v4.2 to conform to a quality trim with a quality cutoff of 20. Trimming was performed on first and second mate adapters from both the 3′ and 5′ ends and read lengths shorter than 16nt were discarded. Next, we aligned the lncRNA data to the GRCh38 (hg38) reference genome using STAR v2.7.8a with a max mismatches value of 3 and all other input parameters set to their default values. Furthermore, the miRNA data was aligned using Bowtie2 v2.2.5 with a seed length of 10 and seed mismatches of 1, while keeping all other input settings at their default values.

Afterward, the transcript abundance of reads aligned to the genome annotation model by Partek E/M was quantified when the filter features were set to 1 and the other input settings were left unchanged. Finally, we applied the "Total miRNA" and "Transcript count" outputs to obtain the final annotated read count for miRNAs and lncRNAs, respectively.

Furthermore, raw count data for mRNA expression from BM samples of male and female MM patients were obtained from the MMRF-COMMPASS cohort as a part of the GDC database via TCGAbiolinks R package v2.32.

Statistical analysis

Data on coding and ncRNA expression were obtained from annotated read count related to healthy control samples, MM patient samples and MM-related cell lines. The obtained read count of expression was analyzed using DESeq2 v.1.44.0 [21] for differential expression analysis. A cutoff of 0.01 was selected for the adjusted *P*-value based on the Benjamini–Hochberg algorithm [22] to distinguish significant differential genes. Figures were generated using the R graphics packages ggplot2 v3.5.1, ggalluvial v0.12.5, and venn v1.12. Furthermore, the networks were built using Cytoscape v3.10.2.

Data filtering via computational and biological approaches

We established a multi-step filter aimed at recognizing significant ncRNAs which were highly dysregulated across several comparisons and potentially influenced coding RNA functionality. The initial filter was defined as the dysregulation of each RNA, such as mRNA, miRNA, and lncRNA, was considered acceptable if the adjusted *P*-value was below 0.01 and the absolute magnitude of log2 (fold change) exceeded 2. In the second filtering step, differentially upregulated RNAs in patients were considered significant if they met the following criteria: (i) an adjusted *P*-value < 0.01, (ii) a $\log_2$(fold change) >2, and (iii) an overall expression level in the corresponding cell line ≥6. Similarly, differentially downregulated RNAs were retained if they satisfied: (i) an adjusted *P*-value < 0.01, (ii) a $\log_2$(fold change) < −2, and (iii) an overall expression level in the corresponding cell line <6.

The third filter distinguished simultaneous dysregulation of coding and ncRNAs while checking the interactions according to databases. The LncSEAv2 database, which contains 2,878,179 verified lncRNA-mRNA interactions at three levels: chromatin, lncRNA-protein pairings, and

RNA-target [23] was employed in the third filter. Also, the miRTarBase database, which includes 938,073 verified miRNA-mRNA interactions [24], was used to utilize specific interactions. Hence, the third filter exclusively identified the coding and ncRNAs that were both dysregulated at the same sex and their interactions were documented in the relevant database. In the fourth filter, we isolated the dysregulated non-coding or coding RNA data detected exclusively in either males or females and eliminated the shared data. The objective of characterizing these filters is to identify the key ncRNAs that affect the progression of MM in the two sexes and have a significant impact on the regulation of mRNA expression.

Comparative investigation of pathways enrichment

Pathway enrichment analysis was performed using the KEGG PATHWAY database (last updated September 1, 2024) and the Molecular Signatures Database (MSigDB Hallmark). The analysis was conducted with the *enrichR* R package v3.4, employing a hypergeometric test with Homo sapiens genes used as the background set. *P*-values were adjusted for multiple comparisons using the Benjamini–Hochberg method.

RESULTS

In order to elucidate the role of exosomal ncRNA in sex-specific occurrence of the main genetic events and development of MM, our study focused on the expression pattern of ncRNAs and their interaction with mRNA. We isolated exosomes from six MM cell lines (LP1, NCI-H929, JJN-3, U-266, KMS-11, and ANBL-6) and 24 patient bone marrow aspirates, consisting of 8 females and 16 males, as well as from 7 healthy control samples. The exosomes were characterized for size and concentration using NanoSight NS300, confirming uniform size distribution across the samples (Supplementary Fig. S1). Their RNA cargo was isolated and analyzed for RNA content, including lncRNAs, miRNAs and mRNAs, to investigate their regulatory effects in MM. All exosomes showed a uniform size distribution in their category (Supplementary Fig. S1). We isolated ncRNA from exosomes and sequenced by Illumina sequencing. The annotated FASTQ data pertaining to patients and cell lines were utilized to analyze based on the main workflow. Moreover, a total of 451 cancerous and 170 normal BM samples from male patients, and 313 cancerous and 154 normal BM samples from female patients were obtained from the MMRF-COMMPASS cohort, available through the GDC database. The data underwent filtration in four stages, and the expressions of the final dysregulated coding and non-coding RNAs were applied to pathways enrichment analysis. The results demonstrated that exosomes offer higher resolution for the discovery of non-coding RNAs, enabling more sensitive and specific identification of transcriptomic alterations. Furthermore, the shared pathway perturbations observed across sexes in response to multiple myeloma appear to be mediated by distinct mRNA–non-coding RNA interaction networks (Fig. 1). Samples were studied from different aspects including male or female, cytogenetic abnormalities, disease status, and treatment exposure.

Since the primary aim of this study was to detect significant ncRNAs across sex groups in MM patients, 'sex' was the first and the most important demographic feature in this study. Statistical analysis was conducted on the RNA expression data to determine the influence of sex on the development of MM. The expression data for six different cell lines is shown in Supplementary Table S1. We categorized the patients with cytogenetic abnormalities such as TP53 deletion, t(11;14), and monosomy 13 as non-Hyperdiploid (non-Hype) group. In terms of disease status, we categorized the patients into newly diagnosed (ND), and progression and relapse (PRE) groups. In terms of treatment exposure (TX), we separated the individuals who took the medicine (TX) to those who did not (0), which were corresponding to the ND versus PRE groups. In terms of plasma cell percentage in BM aspirate sample (PC.As), we split samples to more than 50% (up) and less than 50% (down). In terms of patient status, we compared Deceased patient vs Alive ones (DE). The clinical characteristics of the patients are presented in Table 1.

We compared the deceased versus alive, Hype/non-Hype versus healthy, ND/PRE vs healthy, and TX versus no-TX patients on female and male samples individually. All the patients diagnosed with progressive or relapsed disease received individualized combinations of therapeutic agents outlined in the Appendix. Combinations were selected from various categories based on specific clinical indications and disease characteristics. It is important to note that such categorical classifications were not available for the mRNA data. Therefore, in the downstream analysis, all dysregulated mRNAs with validated interactions, were included for network construction.

Out of the 14,103 lncRNA transcripts associated with 8930 distinct lncRNAs, 1891 lncRNAs showed altered expression across various comparison groups and 1516 lncRNAs met the filtering criteria for further analysis. Moreover, from the analysis of 667 input miRNAs derived from FASTQ data, we identified 420 dysregulated miRNAs across multiple comparison groups. After integrating the data with ncRNA–mRNA interaction information obtained from the LncSEA v2 and miRTarBase databases and applying defined filtering criteria, a total of 19,886 and 32,791 significant ncRNA–mRNA interactions associated with different categories were identified in female and male samples, respectively. A total of 104 effector miRNAs and 305 effector lncRNAs passed all filtering criteria across both males and females and were included in the downstream analysis. The interactions between coding and non-coding RNAs, which exhibited differential expression in both male and female, are shown in Supplementary Table S2.

Notably, among the ncRNAs and mRNAs identified in the PCAs_up vs down comparison, no lncRNA-mRNA or miRNA-mRNA interactions met the third filtering criterion within female samples.

Exosomes offer enhanced sensitivity and resolution in detecting non-coding RNAs compared to direct sampling from tissue

Given our specific interest in dysregulated lncRNAs and miRNAs that interact with dysregulated mRNAs, we hereafter refer to these ncRNAs as "effector lncRNAs" and "effector miRNAs."

Comparison of effector ncRNA profiles between exosome samples and bone marrow (BM) aspirate across sex-specific MM groups revealed that the majority of prominently dysregulated ncRNAs identified in exosomes were not significantly altered in whole BM samples. Considering the established role of exosomes in transferring ncRNAs and facilitating ncRNA-mRNA interactions, these identified exosomal ncRNAs are likely critical to biological functions. Notably, the majority of exosomal lncRNAs showed insignificant changes in BM tissue samples, with females displaying a higher proportion of unchanged expression patterns compared to males. For subsequent analyses, lncRNAs were categorized based on their dysregulation status: "No Dysregulation in GDC", "Same Expression Pattern in Exosome and GDC", and "Upregulated in Exosome and Downregulated in GDC". LncRNAs downregulated in exosomes but upregulated in the GDC database were excluded (Fig. 2A). Among the effector lncRNAs shared between sexes, CAHM was notable for regulating ~2500 mRNAs in each sex group despite showing no significant dysregulation in female BM samples (Fig. 2B). Additionally, sex-specific effector lncRNAs with the highest number of regulated mRNAs are highlighted individually for females and males in Fig. 2C, D, respectively.

All 29 dysregulated miRNAs common among exosome samples were not significantly altered in BM samples across both sexes. Among these shared miRNAs, hsa-miR-335-5p regulated the highest number of mRNAs in both sex groups (Fig. 2E). Female-specific dysregulated miRNAs detected exclusively in exosomes, without corresponding dysregulation in the GDC database, and

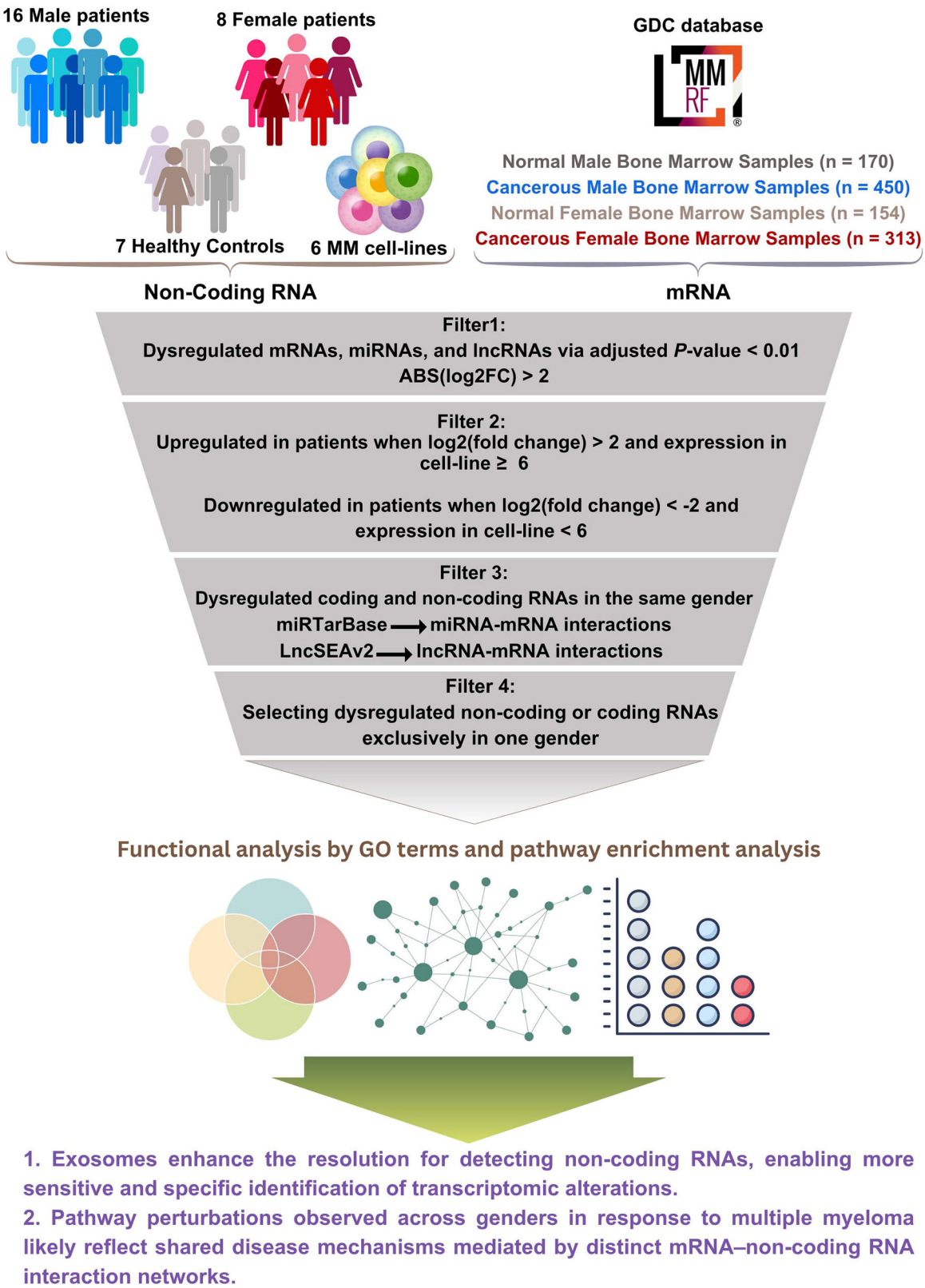


Fig. 1 The main workflow for data analysis. The annotated FASTQ data from six cell lines, 24 MM patient samples, and seven healthy controls were analyzed to achieve non-coding RNA data in males and females. In addition, Bone Marrow samples including cancerous and normal associated with males and females extracted from Genomic Data Commons (GDC) database (MMRF-COMMPASS cohort), were utilized to obtain mRNA data following the main workflow. After a four-stage filtration process, the expressions of dysregulated coding and non-coding RNAs were subjected to pathway enrichment analysis.

Table 1. The clinical characteristics of the patients.

Clinical character	Levels	Male	Female
FISH	1q gain/ amplification	7	4
	Hyperdiploid	5	5
	t(4;14)	1	0
	t(11;14)	6	2
	t(14;16)	1	0
	monosomy 13	7	4
	monosomy 17	1	0
	monosomies 1,8,13, and 14	1	0
	trisomies 3, 7, 9 and 15	0	1
	trisomies 3, 8, 9 and 11	1	0
	trisomies 7 and 11	1	0
	trisomy 8	1	0
Patient status	Deceased	5	6
	Alive	11	2
Disease status at collection	Newly diagnosed	7	3
	Progression	7	2
	Relapse	2	2 (1 unknown)
Medication	0	7	3
	TX	9	5
PC.As	up	8	4
	down	8	4

those regulating the highest number of mRNAs are presented in Fig. 2F. Male-specific dysregulated miRNAs identified solely in exosomes are shown in Fig. 2G, with the exception of hsa-let-7i-5p, which exhibited opposite regulation (upregulated in exosomes and downregulated in the GDC database).

Overall, the utilization of exosome samples allows for more precise characterization of ncRNA expression patterns compared to tissue-based sampling.

Sex-specific expression profiles of regulated effector lncRNAs, and miRNAs

The obtained data on lncRNA-mRNA interactions and miRNA-mRNA interactions that met the filters were used to display the numbers of effector lncRNAs, effector miRNAs, and regulated mRNAs using male and female data (Fig. 3A). Out of 3458 regulated mRNAs, 530 mRNAs were potentially controlled by all four categories of ncRNA. The number of mRNAs which were particularly regulated via male-specific miRNAs, female-specific miRNAs, male-specific lncRNAs and female-specific lncRNAs were 38, 48, 126 and 137, respectively.

The effector lncRNAs that were dysregulated in more than two comparison groups are shown in Fig. 3B. Also, all effector miRNAs across several comparisons across males and females which passed the filters are displayed in Fig. 2C. Different lncRNAs expression signatures were indicated across the sex groups. Most of the dysregulations were detected in male samples. The corresponding mRNAs that passed the filtering criteria and were regulated by the most ncRNAs, demonstrating regulation in both female and male groups, are depicted in Supplementary Fig. S2A.

Furthermore, variations in the expression profiles of dysregulated effector lncRNAs were observed between males and females and also in those dysregulated exclusively in either males or females (Supplementary Fig. S2B). The effector ncRNAs showed greater variability in expression across male samples, whereas expression levels in female samples were more consistent and concentrated. Furthermore, the number of dysregulated ncRNAs that were identified only in the male group was greater than the female group.

Chromosomal distribution of the effector non-coding RNAs in male and female patients

To further elucidate sex-specific chromosomal patterns, we analyzed the distribution of ncRNAs on the sex chromosomes (Fig. 3D). EIF1AX-AS1 was identified as the sole common effector lncRNA located on chromosome X shared between both sexes. Moreover, three miRNAs (hsa-miR-221-5p, hsa-miR-222-3p, and hsa-miR-223-5p) and six lncRNAs (FTX, LINC01281, SLC25A5-AS1, TMSB15B-AS1, UXT-AS1, and ZNF674-AS1) were specifically detected on chromosome X in male patients. Additionally, hsa-miR-222-5p was uniquely expressed on chromosome X in female patients, whereas TTTY14 was exclusively found on chromosome Y in male patients. Circos plots were generated to illustrate the chromosomal distribution of differentially expressed non-coding RNAs (ncRNAs) (Fig. 4A, B).

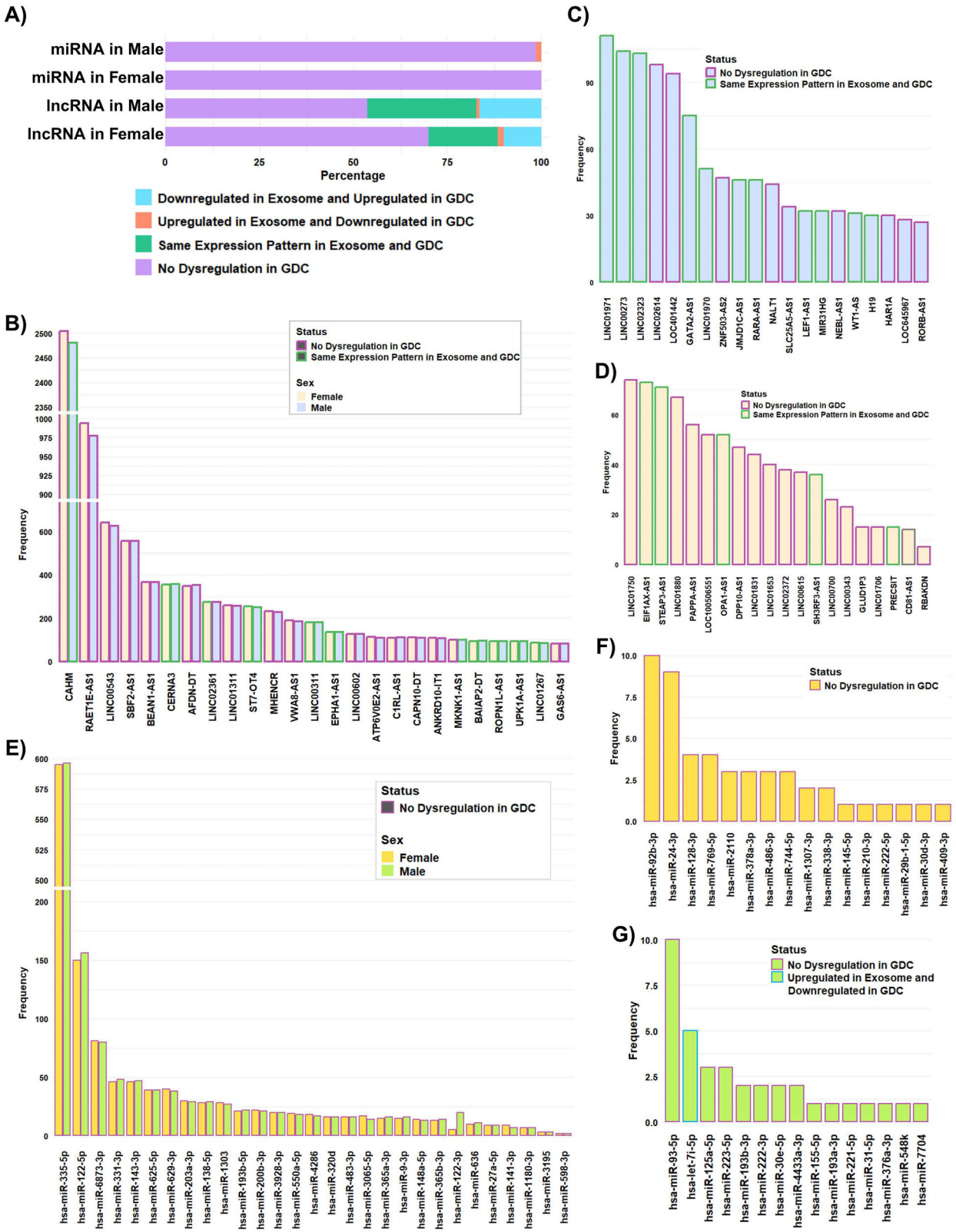
Distinct disruption observed in expression patterns for male compared to females in cytogenetic comparisons

Pathway enrichment analysis was systematically performed using sex-specific regulated mRNAs identified through cytogenetic comparisons. These comparisons involved two distinct conditions: “Hype vs. Healthy” and “non-Hype vs. Healthy”, each analyzed independently within male and female datasets. Specifically, regulated mRNAs from these sex-specific cytogenetic contrasts served as inputs for subsequent enrichment analyses.

It is noteworthy that the mRNA expression data utilized in this study were extracted from the MMRF-COMMPASS cohort, available through the GDC database, and contained no additional cytogenetic, demographic, or pharmacological metadata aside from sex information. Consequently, within each cytogenetic comparison, the constructed ncRNA-mRNA networks were based exclusively on dysregulated ncRNAs interacting with dysregulated mRNAs identified in the same sex-specific cohort. The validity of these interactions was corroborated via corresponding reference databases. The resultant mRNAs were subsequently employed for pathway enrichment analysis, aiming to elucidate the biological perturbations specific to each sex-related comparison.

The “Hype vs. Healthy” comparison in male samples, involving three downregulated mRNAs (GATA3, CEBPB, and HNF4A), as well as the “non-Hype vs. Healthy” comparison in female samples, encompassing two upregulated mRNAs (MYC and JUN), did not yield significant enrichment in MSigDB Hallmark pathways. Notably, the three male-specific mRNAs identified were modulated by thirteen lncRNAs and one miRNA, all of which exhibited differential expression exclusively in exosome-derived datasets, with no significant corresponding changes in bone marrow (BM) samples. Specifically, these mRNAs were regulated by downregulated lncRNAs, including EIF1AX-AS1, exhibiting expression patterns that aligned distinctly with exosome datasets (Fig. 4C). In the female samples categorized as non-Hype, MYC and JUN mRNAs were upregulated, regulated by seven downregulated ncRNAs similarly demonstrating significant expression solely within exosome-derived datasets, absent parallel changes in BM samples (Fig. 4D). The full table of enriched MSigDB Hallmark pathways is illustrated in Supplementary Table S3.

Further examination of MSigDB Hallmark pathway enrichment across the remaining datasets revealed six common pathways between sexes, namely “TNF-α Signaling via NF-κB”, “Heme Metabolism”, “Hedgehog Signaling”, “Hypoxia”, “Allograft Rejection”, and “Apoptosis”. Among these enriched pathways, MYC and



NFAT5 emerged as female-specific dysregulated mRNAs, whereas BCL3, FOSL2, and GAPVD1 were identified as male-specific. Additionally, twenty-two dysregulated mRNAs were found consistently altered across both male and female (Fig. 4E). Collectively, these findings underscore pronounced sex-specific regulatory dynamics in cytogenetic expression profiles, suggesting potential implications for sex-tailored therapeutic approaches in future clinical applications.

Fig. 2 Comparison of non-coding RNA profiles in exosomes and bone marrow tissue across male and female groups in MM. A Stacked bar plots showing the detection percentage of non-coding RNAs in exosomes compared to bone marrow tissue samples. **B** Common effector lncRNAs between males and females with the highest number of regulated mRNAs. Novel lncRNAs detected exclusively in exosomes (not dysregulated in the GDC database) are marked with magenta borders, while those exhibiting the same expression pattern in both exosomes and the GDC database are outlined in green. **C** Male-specific dysregulated lncRNAs associated with the highest number of regulated mRNAs. Distinct border colors represent different expression statuses in the GDC database. **D** Female-specific dysregulated lncRNAs with the highest number of regulated mRNAs. Border colors indicate differing expression patterns relative to the GDC database. **E** Common effector miRNAs between males and females were detected exclusively in exosomes and not found to be dysregulated in the GDC database. **F** Female-specific dysregulated miRNAs with the highest number of regulated mRNAs detected only in exosomes, with no corresponding dysregulation identified in the GDC database. **G** Male-specific dysregulated miRNAs with the highest number of regulated mRNAs detected only in exosomes, except for hsa-let-7i-5p, which was upregulated in exosomes and downregulated in the GDC database.

MSigDB hallmark enriched pathways in additional comparative groups

Building upon the analysis of pathway dysregulation across cytogenetic subgroups in multiple myeloma (MM) patients, we next examined differentially expressed mRNAs identified through additional pairwise comparisons. In the comparative analysis of deceased versus alive patients, the hallmark pathways "Notch Signaling" and "TNF-α Signaling via NF-κB" were specifically enriched in females and males, respectively. Notably, no pathways reached significance in the female-specific comparisons of "ND versus Healthy" and "PC up versus down", whereas dysregulated mRNAs in the comparison "PRE versus Healthy" revealed the greatest number of enriched pathways for both sex levels. Sex-specific mRNAs identified through the "TX versus 0" comparison, examining the drug exposure effects, yielded distinctive patterns: "TNF-α Signaling via NF-κB" was universally enriched across both sex levels, whereas "IL2/STAT5 Signaling" and "KRAS Signaling UP" were specifically enriched in females, and "Heme Metabolism" and the "p53 Pathway" emerged distinctly enriched in males (Fig. 5A).

A sex-specific ncRNA-mRNA regulatory network for the 'Heme Metabolism' pathway was constructed in male patients across multiple comparisons. To enhance interpretability, lncRNAs exhibiting identical expression patterns in bone marrow (BM) samples were consolidated into single nodes, outlined in green. The network revealed consistent downregulation of key mRNAs, including LMO2, E2F2, C3, SYNJ1, and GAPVD1. Notably, SYNJ1 and GAPVD1 were targeted by the greatest number of ncRNAs uniquely dysregulated in exosome-derived profiles. Furthermore, the lncRNA LOC1005065551, denoted as LOC1005065551.d to indicate its downregulation, was identified as a key regulator of LMO2 and displayed differential expression patterns across multiple comparisons, as illustrated by green directional arrows (Fig. 5B).

Given that 'TNF-α Signaling via NF-κB' consistently emerged as the most recurrently enriched pathway across multiple comparisons, sex-specific ncRNA–mRNA interaction networks were constructed to delineate regulatory relationships underlying this pathway (Fig. 6A, B). In the male-specific dataset, the TNF-α signaling network revealed downregulation of LIF, REL, RNF19B, IFIT2, F2RL1, and BCL3, alongside upregulation of NR4A1. Among these, BCL3 exhibited the highest number of interactions with ncRNAs uniquely dysregulated in exosome-derived datasets, suggesting a potential exosome-specific regulatory mechanism. Consistent with previous network representations, lncRNAs exhibiting similar expression patterns in BM samples were consolidated into single nodes outlined in green. Notably, RAET1E-AS1 was found to regulate five distinct mRNAs, while LOC1005065551 modulated BCL3 and displayed a unique differential expression trajectory across multiple comparisons, as denoted by green directional arrows (Fig. 6A).

In female-specific datasets, the 'TNF-α Signaling via NF-κB' pathway included the downregulated genes MSC and GFPT2, alongside the upregulated MYC, which was regulated by the greatest number of ncRNAs uniquely dysregulated in exosome-derived profiles. To streamline network visualization, lncRNAs exhibiting identical expression patterns in BM samples were consolidated into a single node, outlined with a green border. Notably, the upregulated hsa-miR-92b-3p was predicted to target the downregulated GFPT2, whereas a downregulated miRNA interacted with the upregulated MYC. The effector lncRNA CAHM, previously identified as one of the most recurrent regulatory elements, modulated all three mRNAs within this network. Additionally, CD81-AS1 which targets MYC, exhibited an inverse expression pattern across sample types, being upregulated in exosome-derived samples and downregulated in BM. Furthermore, the effector lncRNA LOC1005065551 specifically regulated MYC in a repressive manner, uniquely within the female dataset (Fig. 6B).

KEGG-enriched pathway reconstruction highlights differential RNA expression patterns between male and female patients

A total of 89 enriched pathways were identified across multiple comparisons in both sexes (Supplementary Table S4). Among these, pathways recurrently enriched in at least three comparative analyses are highlighted in Fig. 7A. Notably, a significant proportion of these pathways were cancer-related, suggesting that the dysregulated mRNAs may function as upstream drivers of tumorigenesis.

To further elucidate sex-specific molecular differences, three KEGG pathways were analyzed in detail. The "Th17 Cell Differentiation" pathway, implicated in multiple myeloma (MM) progression, was first examined. After excluding shared ncRNA–mRNA interactions, the remaining network featured only male-specific interactions. These included six upregulated miRNAs, such as hsa-miR-335-5p, which collectively targeted and suppressed MAPK1 (Fig. 7B).

The second network, "Pathways in Cancer", revealed distinct sex-based regulatory patterns after removal of overlapping interactions. In males, the network included a single downregulated mRNA, SOS2, regulated by 24 downregulated lncRNAs and one upregulated miRNA, hsa-miR-335-5p (Fig. 7C). For visualization clarity, lncRNAs exhibiting identical expression profiles in BM samples were grouped into single nodes with green borders.

In contrast, the female-specific cancer pathway network comprised three downregulated mRNAs (LAMC3, GLI2, and TCF7) and one upregulated mRNA (MYC), each regulated by multiple ncRNAs uniquely dysregulated in exosome-derived data. Among them, the effector lncRNA CAHM regulated all four mRNAs, while CD81-AS1 displayed opposing expression trends between exosomes and BM samples. Additionally, LOC1005065551 specifically regulated MYC in a female-specific, downregulated manner (Fig. 7D).

Finally, the "Wnt Signaling Pathway" network, after excluding shared interactions, retained only female-specific regulatory events involving MYC and TCF7. These were modulated by several ncRNAs including CAHM, CD81-AS1, and LOC1005065551 (Supplementary Fig. S3A). Collectively, these findings reinforce earlier MSigDB-based observations, underscoring the presence of sex-dependent transcriptomic divergence across KEGG-enriched pathways.

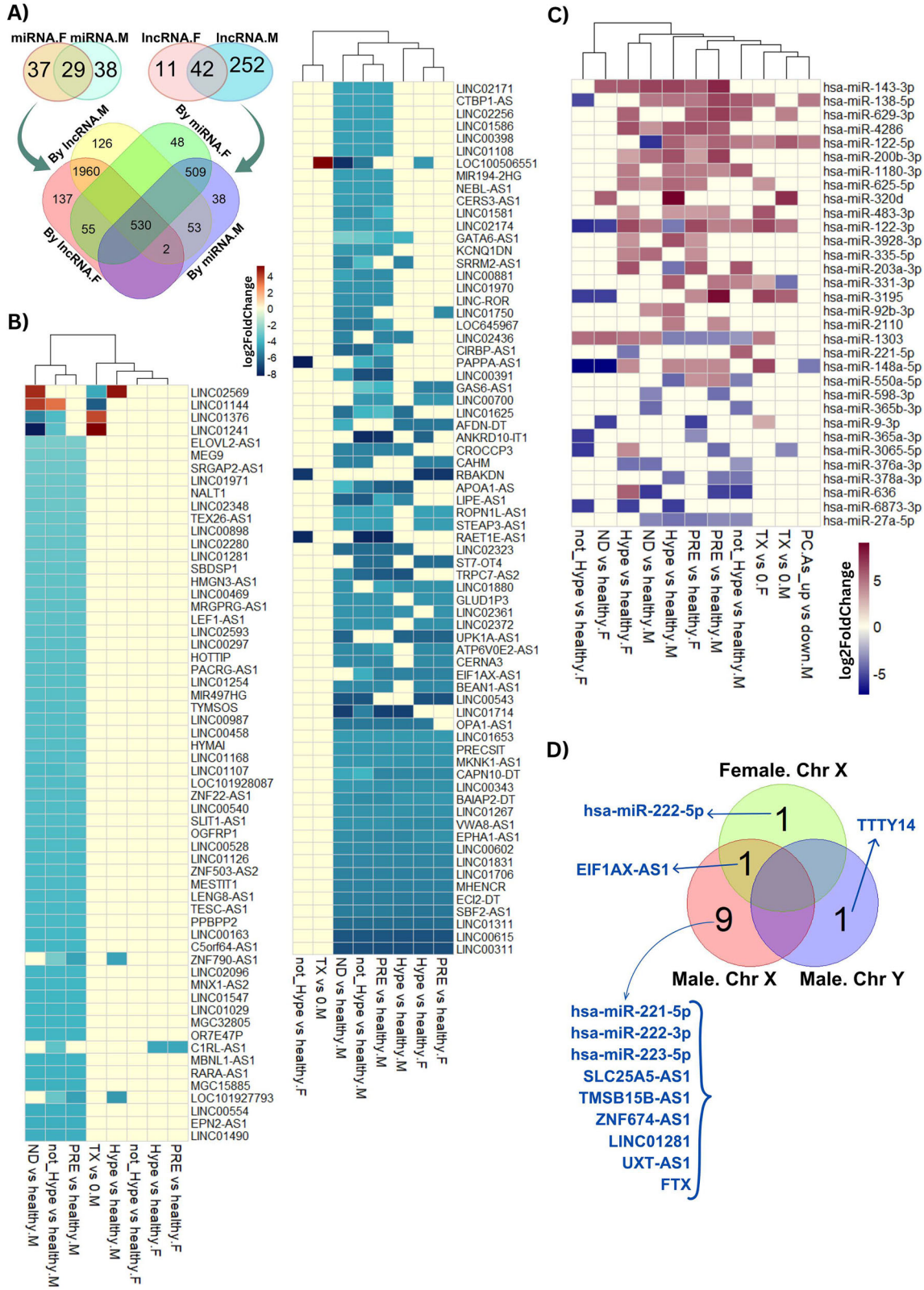


Fig. 3 Overview of non-coding and coding RNA expression data. A Venn diagrams showing significantly expressed lncRNAs and miRNAs in males (M) and females (F). along with the mRNAs regulated by each of these four groups. **B** The lncRNAs present in at least three comparison groups. **C** Significantly expressed miRNAs across various comparisons. **D** The distribution of differentially expressed lncRNAs and miRNAs originating from sex chromosomes (X and Y) in male and female samples.

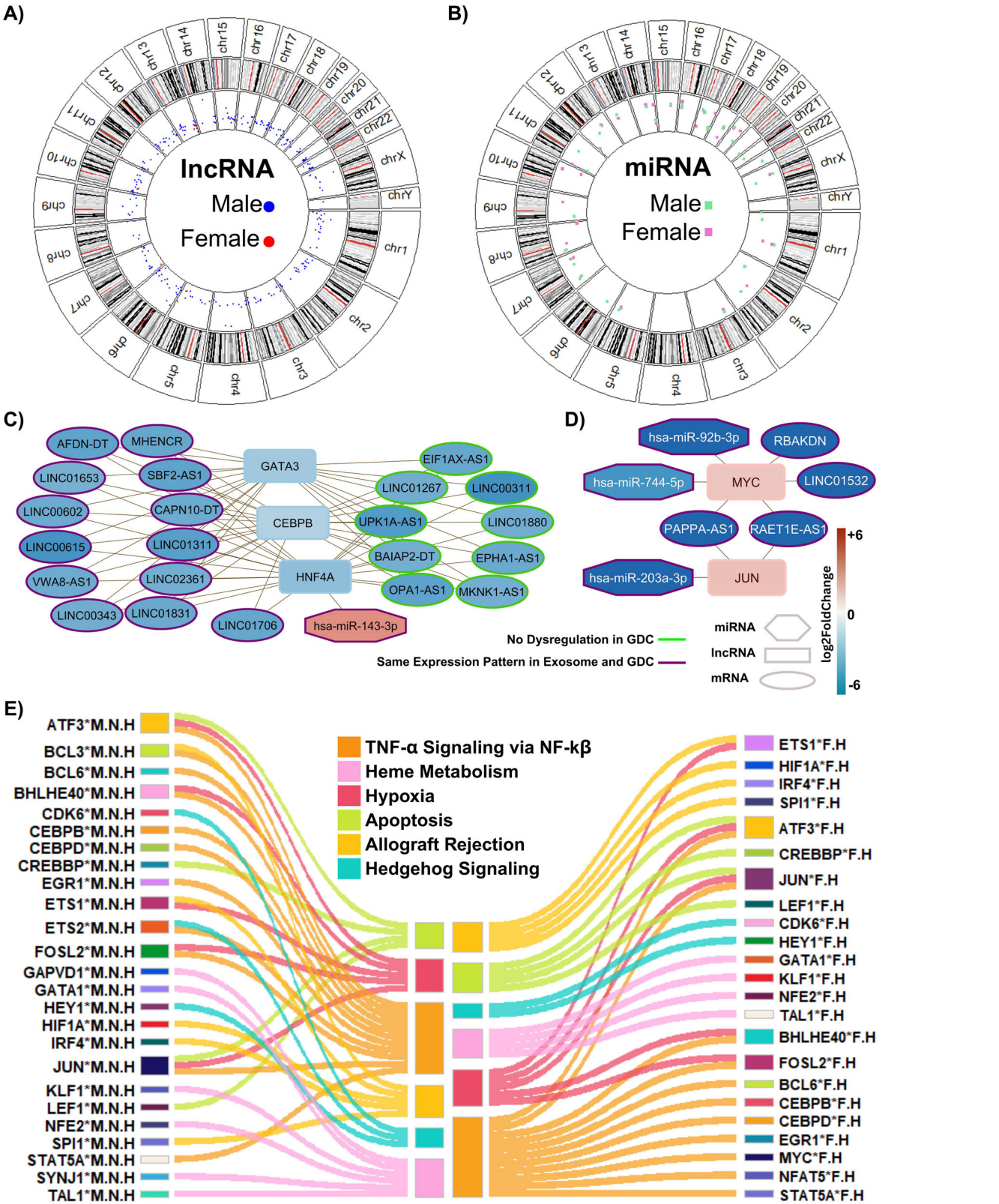


Fig. 4 Chromosomal distribution and cytogenetic network analysis of ncRNAs in MM patients. A Chromosomal distribution of lncRNAs across male (M) and female (F) samples. **B** Chromosomal distribution of miRNAs across male (M) and female (F) samples. **C** Sex-specific ncRNA-mRNA interaction network identified in the "Hyperdiploid (Hype) vs. Healthy" comparison within male samples. **D** ncRNA-mRNA network constructed for the "Non-Hyperdiploid (non-Hype) vs. Healthy" comparison in female samples. **E** Shared MSigDB hallmark pathways enriched based on dysregulated mRNAs identified in cytogenetic abnormality comparisons: "non-Hype vs. Healthy" in males (left) and "Hype vs. Healthy" in females (right).

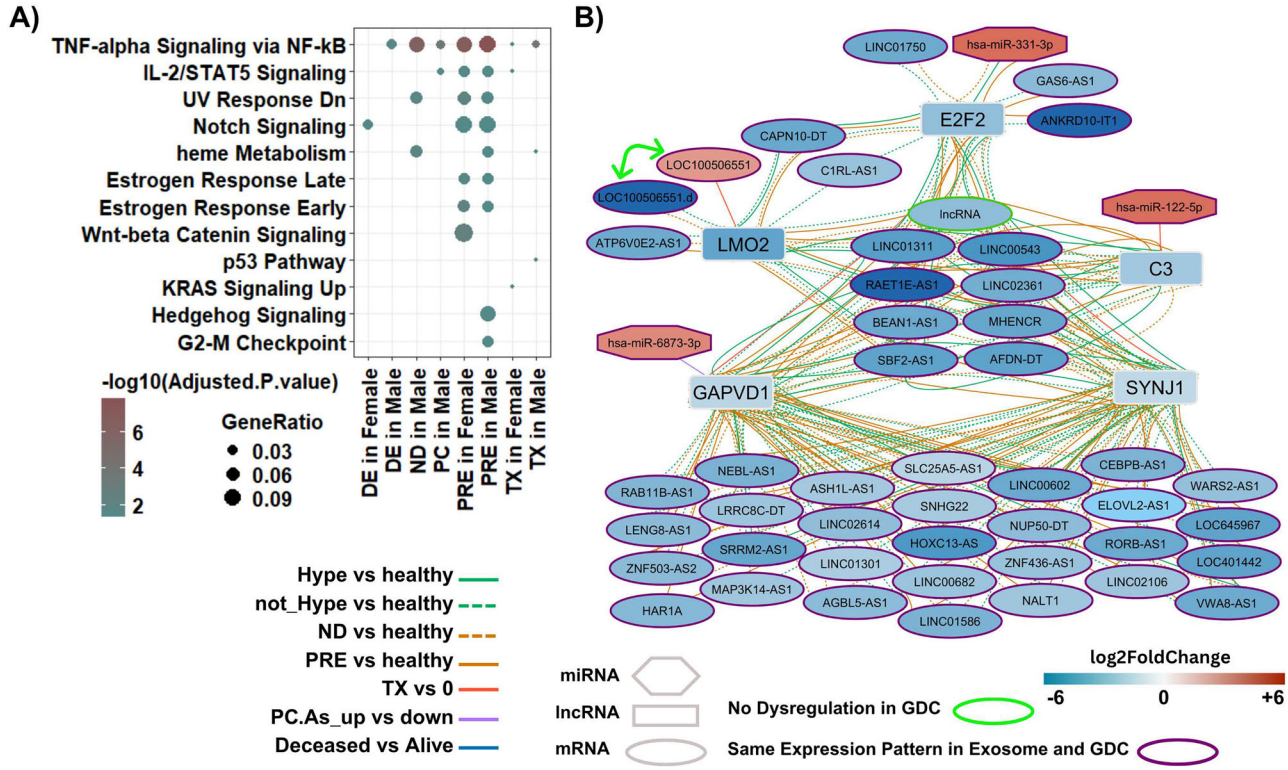


Fig. 5 Enriched MSigDB hallmark pathways beyond cytogenetic comparisons and the Heme metabolism network in males. A Significant MSigDB hallmark pathways enriched in comparisons beyond cytogenetic abnormalities, displayed separately for male (M) and female (F) datasets. **B** ncRNA-mRNA regulatory network associated with the "Heme Metabolism" pathway reconstructed from male-specific datasets across multiple comparisons.

Non-coding RNAs displaying distinct expression patterns across comparative groups

In the final analytical step, ncRNAs exhibiting differential expression patterns across various comparisons between male and female patients were investigated. Thirteen ncRNAs, including four miRNAs and nine lncRNAs, demonstrated distinct sex-specific expression profiles. The comparison involving medication usage accounted for the majority of these expression differences, revealing an inverse expression trend across most identified ncRNAs.

Notably, three effector miRNAs, hsa-miR-2110, hsa-miR-122-5p, and hsa-miR-92b-3p, showed upregulation in patients receiving medication compared to those without drug treatment, whereas they were consistently downregulated in other comparative analyses (Supplementary Fig. S3B).

Shared and sex-specific exosomal non-coding RNA dysregulation reveals distinct regulatory networks in multiple myeloma pathogenesis

Our results revealed shared and sex-specific dysregulation patterns of exosomal ncRNAs in MM (Fig. 8). Utilizing exosomes significantly improves the resolution for non-coding RNA profiling, thus enabling greater sensitivity and specificity in uncovering transcriptomic dysregulation. Across both sex levels, the lncRNA CAHM (absent in female tissue samples), RAET1-AS1, and LINC00543 emerged as the most influential, regulating numerous mRNAs. Similarly, miRNAs including hsa-miR-335-5p, hsa-miR-122-5p, and exosome-specific hsa-miR-6873-3p were identified as key regulators. In hyperdiploid MM comparisons, EIF1AX-AS1 from chromosome X displayed distinct expression patterns in exosomes of both male and female, affecting pathways such as TNF-α Signaling via NF-κB, Heme Metabolism, Hedgehog Signaling, Hypoxia, Allograft Rejection, and Apoptosis. Additionally, miRNAs hsa-miR-2110, hsa-miR-122-5p, and hsa-miR-92b-3p showed upregulation linked to medication status.

Sex-specific analyses highlighted notable differences. Male-specific dysregulation involved LINC01971, miRNAs hsa-miR-93-5p (exosome-exclusive) and hsa-let-7i-5p, and several mRNAs including GATA3, BCL3, and GAPVD1. Pathways such as TNF-α Signaling via NF-κB exhibited downregulation of key genes (LIF, REL, RNF19B) and upregulation of NR4A1, with RAET1E-AS1 regulating multiple mRNAs. Additionally, Th17 Cell Differentiation involved hsa-miR-335-5p targeting MAPK1.

In females, dysregulation was distinct in LINC01750, miRNAs hsa-miR-92b-3p and hsa-miR-24-3p, and genes like MYC and JUN. CD81-AS1 regulated MYC inversely across exosomes and bone marrow samples. Moreover, female-specific "Pathways in Cancer" and Wnt Signaling Pathways demonstrated extensive regulation by ncRNAs such as CAHM, CD81-AS1, and LOC1005065551.

DISCUSSION

Our study investigated the expression patterns of ncRNAs that interact with regulatory genes to uncover sex-specific differences in the prevalence and progression of MM. By analyzing exosomes isolated from MM patient samples, we profiled ncRNA expression, offering novel insights into the molecular mechanisms driving disease pathogenesis. Among the ncRNAs that met our stringent filtering criteria, effector miRNAs displayed distinct characteristics compared to effector lncRNAs. Notably, the number of identified effector miRNAs ($n = 104$) was substantially lower than that of effector lncRNAs ($n = 305$), highlighting potential differences in their regulatory roles and expression dynamics in MM. Moreover, a significantly higher proportion of effector miRNAs exhibited upregulated expression relative to effector lncRNAs, suggesting

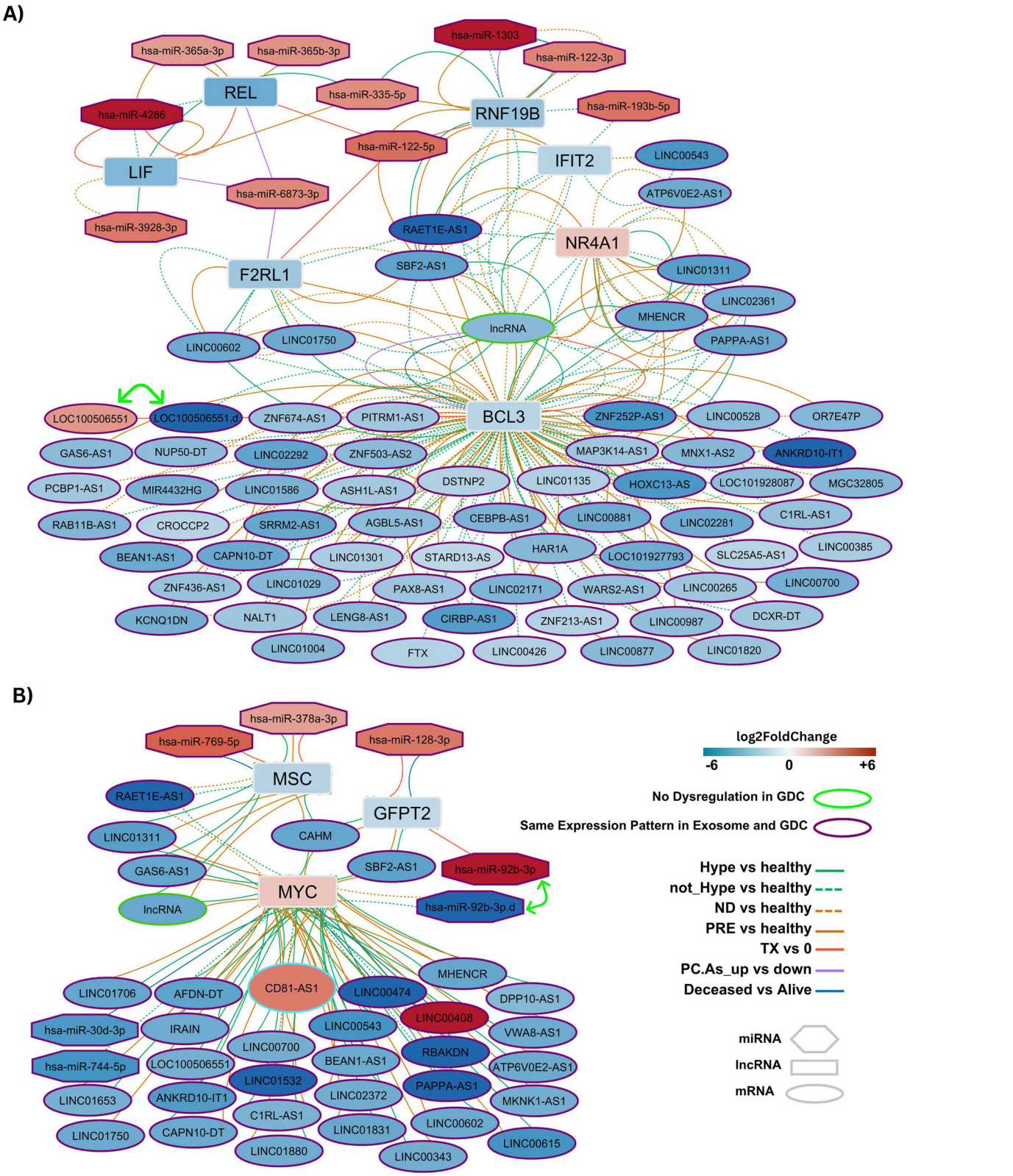


Fig. 6 Sex-specific regulatory networks of the enriched "TNF-α Signaling via NF-κB" pathway. A ncRNA-mRNA interaction network underlying the "TNF-α Signaling via NF-κB" pathway in male samples. **B** ncRNA-mRNA interaction network associated with the same pathway in female samples.

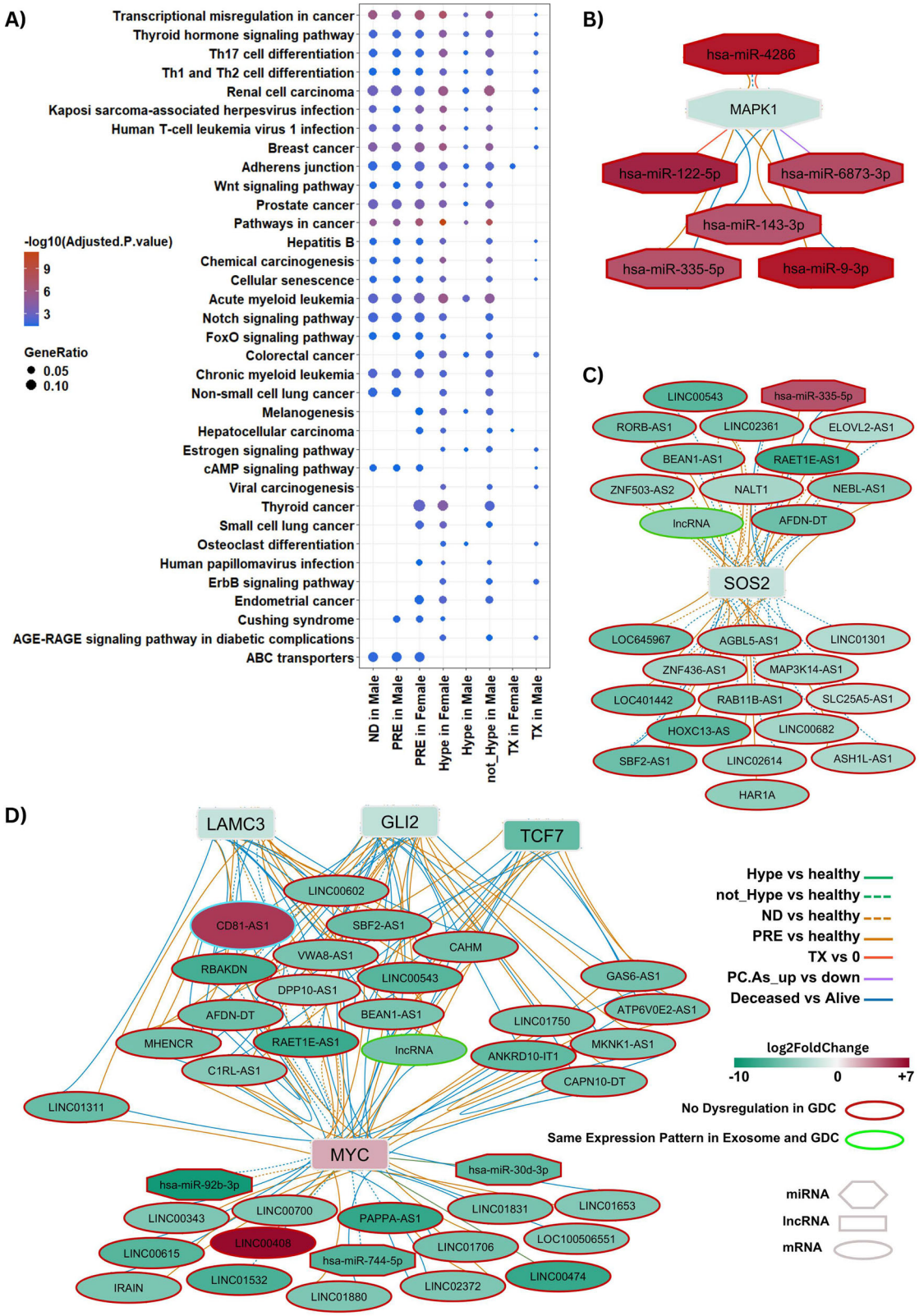


Fig. 7 Sex-specific enrichment and regulatory networks in KEGG pathways. A Enriched KEGG pathways derived from dysregulated mRNAs in disease status comparisons for male (M) and female (F) samples. **B** Male-specific ncRNA-mRNA interaction network associated with the "Th17 Cell Differentiation" pathway. **C** Male-specific regulatory network within the "Pathways in Cancer" KEGG pathway. **D** Female-specific ncRNA-mRNA interaction network identified in the "Pathways in Cancer" KEGG pathway.

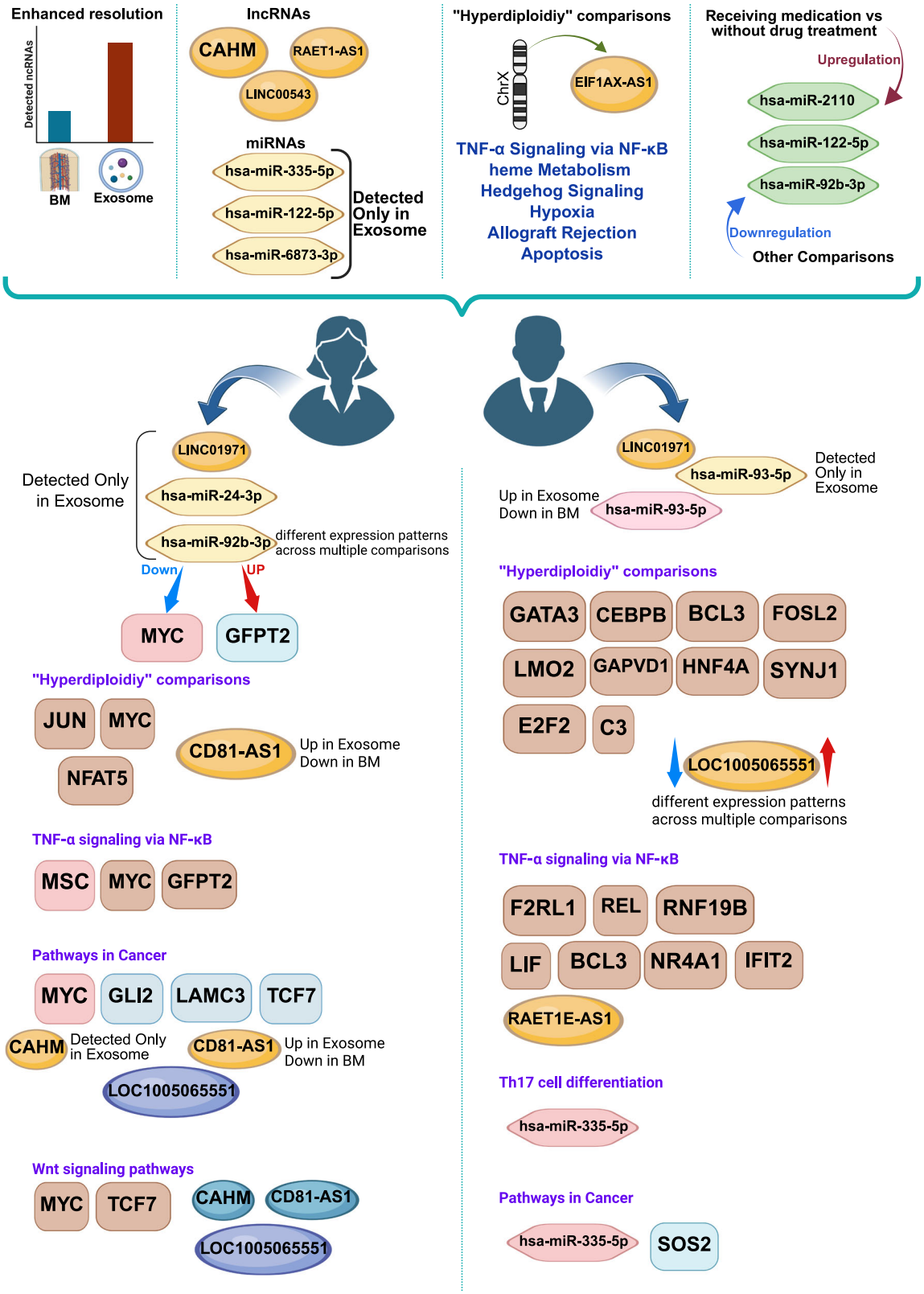


Fig. 8 Summary of key findings. Schematic illustration of shared and sex-specific ncRNA–mRNA interactions regulating critical pathways in multiple myeloma pathogenesis.

a more consistent activation pattern among miRNAs in MM pathogenesis.

Our study underscores the enhanced sensitivity of exosome-derived profiles in detecting functionally relevant ncRNAs in MM. Effector lncRNAs and miRNAs identified in exosomes exhibited limited overlap with BM tissue profiles, emphasizing the exosome's distinct regulatory landscape. The lack of significant dysregulation in BM samples, particularly among female subjects,

further supports the unique expression dynamics captured in exosomes. Notably, sex-specific and shared effectors such as CAHM and hsa-miR-335-5p regulated extensive mRNA networks. Given the capacity of exosomes to circulate systemically, their cargo of ncRNAs likely plays a critical role in eliciting broad biological responses.

By leveraging exosome-derived ncRNA data, our study reveals distinct regulatory roles and expression patterns of ncRNAs in MM pathogenesis, providing valuable insights into sex-specific molecular mechanisms underlying the disease. To further explore disease heterogeneity, samples were stratified based on additional clinical traits, including cytogenetic subtypes. Given that plasma cell drivers differ between Hype and non-Hype subtypes due to distinct cytogenetic aberrations, this feature was prioritized as the first stratification parameter in our analysis.

EIF1AX-AS1 has been reported to inhibit cancer cell proliferation and promote apoptosis [25]. Therefore, its downregulation in Hyperdiploid male patients compared to healthy individuals suggests a potential role in tumor progression, supporting a poorer prognosis and enhanced proliferative capacity of malignant cells. Moreover, MYC and JUN were upregulated in non-Hyperdiploid female patients compared to healthy individuals. MYC is a proto-oncogene that plays a critical role in cell growth, proliferation, and metabolism. In MM, elevated MYC and JUN expression contribute to disease progression and drug resistance. Targeting these transcription factors, either individually or in combination, is a promising area of research for developing new MM therapies [26, 27]. "TNF-α Signaling via NF-κB" enriched in most of the comparisons, particularly those involving cytogenetic subgroups, plays a critical role in multiple myeloma (MM) progression and the dynamic interplay between myeloma cells and the bone marrow microenvironment. TNF-α, a key pro-inflammatory cytokine, activates the NF-κB pathway, thereby promoting cellular processes such as proliferation, drug resistance, and immune evasion within the marrow niche [28, 29]. Additionally, TNF-α can induce epithelial–mesenchymal transition (EMT), enhancing cancer cell migration and invasion [30]. NF-κB activation in myeloid cells, often mediated by TNF-α, also stimulates the release of secondary pro-inflammatory cytokines, creating a self-reinforcing inflammatory loop that supports tumor progression [29].

NFAT5 emerged as female-specific dysregulated mRNA. While primarily recognized as a transcription factor involved in cellular adaptation to osmotic stress, NFAT5 also contributes to inflammatory and immune responses. Notably, it has been found to be highly expressed in exhausted $CD8^+$ T cells within the tumor microenvironment of several cancers, including melanoma, suggesting a role in immune dysfunction and tumor immune evasion [31].

In contrast, the "Heme Metabolism" pathway was uniquely enriched in male samples across multiple comparisons. Beyond its classical role in oxygen transport, heme is essential for mitochondrial respiration and B-cell differentiation, both of which are critical for plasma cell development. As MM originates from plasma cells, malignant cells retain and reprogram these metabolic pathways. Importantly, recent studies have shown that elevated heme levels promote resistance to venetoclax, a BCL-2 inhibitor used in MM treatment. Inhibiting heme biosynthesis, e.g., by targeting ferrochelatase, has been shown to enhance venetoclax sensitivity in both MM cell lines and primary patient samples [32].

Regarding non-coding RNAs, miR-92b-3p demonstrated context-dependent behavior in our study. It was downregulated in newly diagnosed MM patients compared to healthy individuals, yet upregulated in medicated MM patients relative to those newly diagnosed. Previous studies have linked miR-92b-3p to MM pathogenesis and relapse, supporting its role as a potential oncogenic miRNA. In other cancers, elevated miR-92b-3p levels have been associated with poor prognosis and reduced overall survival, suggesting a similar oncogenic role in MM progression [33].

Our study revealed that hsa-miR-335-5p, which was upregulated in exosomes, suppressed the highest number of mRNAs across both male and female groups. This miRNA has been previously identified as a potential diagnostic biomarker for bone disease in newly diagnosed multiple myeloma patients [34]. Elevated expression of miR-335 has been reported in various hematologic malignancies, including MM, suggesting its possible oncogenic role in disease progression [35]. These findings support the notion that, in certain contexts, miR-335-5p may be upregulated and contribute to MM development by regulating critical target mRNAs involved in tumor-associated processes. Besides, CAHM lncRNA emerged as the most critical, regulating more than 80% of mRNAs in both male and female groups. CAHM's function has been extensively demonstrated in multiple human malignancies [36].

Among the KEGG-enriched pathways, "Th17 Cell Differentiation" emerged as particularly relevant due to its established role in multiple myeloma (MM) pathogenesis. Th17 cell levels have been reported to fluctuate throughout disease progression, elevated during partial remission, normalized in complete remission, and rising again at recurrence [37]. After excluding shared ncRNA-mRNA interactions between sexes within this pathway, a male-specific regulatory module remained in which six upregulated miRNAs, including hsa-miR-335-5p, were predicted to suppress MAPK1 across multiple comparisons. Inhibition of MAPK1 within the Th17 differentiation axis may disrupt the balance of T cell subsets, potentially skewing differentiation toward immunosuppressive regulatory T cells (Tregs), thus contributing to the immunosuppressive tumor microenvironment that facilitates MM progression [38, 39].

Similarly, sex-specific differences were identified in the "Pathways in Cancer" network. Upon removal of overlapping interactions, downregulated SOS2 persisted exclusively in males, while LAMC3, GLI2, and TCF7 were downregulated in females. Suppression of SOS2 has implications for MM progression, primarily via disruption of the PI3K/AKT pathway and attenuation of therapeutic resistance, particularly in KRAS-driven malignancies and in the context of resistance to EGFR-TKIs [40]. While SOS1 is typically the dominant RAS activator, SOS2 can act as a compensatory effector, especially in KRAS-mutant tumors [41]. These findings highlight the potential of certain ncRNAs identified in this study to serve as therapeutic targets in MM.

The downregulation of LAMC3 is associated with malignant progression and poor prognosis in various cancers. It may function in modulating cell adhesion and signaling pathways critical for tumor growth and dissemination [42]. Likewise, GLI2, a central transcription factor in the Hedgehog signaling pathway, is implicated in MM cell proliferation, survival, and drug resistance. Inhibition of GLI2 has been shown to suppress MM cell viability and enhance sensitivity to agents such as bortezomib, suggesting its value as a therapeutic target [43].

Further supporting the involvement of oncogenic signaling cascades, the Wnt Signaling Pathway was also highlighted. It is well-recognized as a key driver of MM pathogenesis and is frequently aberrantly activated, even in the absence of exogenous Wnt ligands, indicating possible autocrine or paracrine activation mechanisms [44, 45]. The bone marrow microenvironment, particularly its stromal cell components, plays a pivotal role by secreting Wnt ligands that activate this pathway in MM cells [46].

In the final stage of our analysis, ncRNAs exhibiting divergent expression patterns across multiple comparisons were examined. Among them, hsa-miR-122-5p presented particularly noteworthy findings. This miRNA has been characterized as a tumor suppressor in hepatocellular carcinoma (HCC), where ectopic expression impairs tumorigenicity and enhances chemosensitivity. Conversely, its downregulation has been linked to doxorubicin resistance in HCC, partially through its regulation of BCL9, a co-

activator in the Wnt/β-catenin pathway aberrantly expressed in several malignancies. Additionally, elevated serum levels of miR-122-5p have been identified as an independent prognostic marker of hepatotoxicity in patients undergoing autologous stem cell transplantation (ASCT), including those with MM [47].

Furthermore, the male-specific and female-specific ncRNAs listed in Supplementary Table S2 have been previously identified as significant regulators in the development of various cancers. However, this is the first time to reveal this panel of ncRNAs as sex-specific regulators in MM.

The distinct expression patterns of both coding and non-coding RNAs across male and female patients, along with other clinical traits such as disease stage (newly diagnosed, relapse, progression), cytogenetic profiles (hyperdiploid vs. non-Hyperdiploid), and treatment status, underscore the critical need for sex-specific therapeutic strategies in MM. These findings emphasize the pivotal roles of exosomal ncRNAs, particularly lncRNAs and miRNAs, as regulators of gene expression in MM. Given their involvement in oncogenic processes, targeting dysregulated ncRNAs in a sex-specific manner could enhance treatment efficacy and pave the way for personalized therapeutic approaches. Notably, the greater variability observed in males highlights the potential for novel, precise interventions to improve clinical outcomes for both male and female MM patients.

DATA AVAILABILITY

All sequencing data generated in this study are deposited in the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE294154.

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ACKNOWLEDGEMENTS

This work was primarily supported by funds to RS from the Indiana University, Hematology/Oncology Division, Brown Center for Immunotherapy, Indiana University Simon Comprehensive Cancer Center, Showalter Trust Award, American Society of Gene and Cell Therapy and Cystic Fibrosis Foundation, The 100 Voices of Hope Award, Cooperative Center of Excellence in Hematology (CCEH) pilot feasibility grant. Research reported in this publication was supported by the National Institute of Diabetes And Digestive And Kidney Diseases of the National Institutes of Health under Award Number U24DK126127. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We especially thank Dr. Brian Walker for providing MM cell lines.

AUTHOR CONTRIBUTIONS

RS conceived and designed the study and provided funding support. SRB performed the experiments. S.M. analyzed data, and generated figures. SM and RS reviewed and interpreted data. GA, RF, and DFJ provided patient samples and reviewed the paper. All authors reviewed and edited the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41408-025-01362-1.

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