Cellular Response to ATM Deletion: A Multi-Omics Approach

Application Note

Authors

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Abstract

The GeneSpring family of software products has been providing comprehensive analytical and visualization tools for multiple data types including microarray experiments and mass spectrometry runs. GeneSpring enhances the researcher's ability to leverage concordances among multiple data types by supporting the integrated analysis of multiple experiment types in a single application. This utility is further advanced by adding support for the import of sequencing experiments analyzed in Strand NGS. Scientists can perform multi-omics analysis combining data derived from microarray experiments, mass spectrometry, and high-throughput sequencing studies. This Application Note highlights the value of integrated analysis of gene expression microarrays with small RNA next-generation sequencing. The messenger RNA (mRNA) and microRNA (miRNA) interactions elucidated using the combined correlation and pathway workflows demonstrate the potential of combinatorial approach towards analyzing biological data. In this study, we examined interdependency of mRNA and miRNA in wild-type and ATM-deficient normal (noncancerous) human epithelial cells (HME-CC).

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Introduction

Ataxia Telangiectasia Mutated (ATM) is a serine/threonine protein kinase that phosphorylates key tumor-suppressor proteins that, in turn, initiate the activation of a DNA damage checkpoint leading to cell-cycle arrest, DNA repair, or apoptosis. Mutations in the ATM gene are known to cause a disorder called Ataxia Telangiectasia, from which it derives its name.

ATM has been widely studied as a tumor suppressor gene[1,2]. Copy number changes and epigenetic silencing of ATM through methylation have been implicated in susceptibility to multiple cancer types, including breast cancer[3]. Hesse *et al*[4]. have investigated the effects of ATM on mRNA and miRNA expression levels in normal noncancerous human mammary epithelial cells (HME-CCs). In this study, we used multi-omics analysis (MOA) in GeneSpring to jointly explore these changes. The study allowed us to generate a testable hypothesis on mechanisms by which the noncancerous cells might protect themselves upon loss of a key tumor suppressor, such as ATM.

Materials and Methods

Expression arrays: sample import and analysis

Microarray data by Hesse *et al*[4]. (accession number GSE36267) was downloaded from the Gene Expression Omnibus (GEO). These data were acquired using an Agilent-014850 Whole Human Genome Microarray 4x44K G4112F platform. Probe level information was imported into GeneSpring 13.0 (http://www.genespring-support.com). The probes were summarized using Entrez ID to create a gene level experiment. Differentially regulated mRNAs between wild type and ATM-deficient epithelial cells, exhibiting expression differences with a fold change ≥ 1.2 were identified using a moderated t-test (p-value ≤ 0.05 with Storey-Bootstrapping correction).

SmallRNA-seq: sample import and data analysis

All NGS-related analysis for smallRNA-seq data, including read alignment, experiment creation, quantification, and identification of miRNA targets were carried out using Strand NGS 2.1 (http://www.strand-ngs.com/). Fastq files available for smallRNA sequencing of wild type and ATM-deficient human epithelial cells by Hesse *et al*[4]. were downloaded from GEO (Accession number GSE36267) and aligned to the human genome build hg19 using COBWeB[5], the built-in aligner in Strand NGS. Reads with alignment length less than 10 base pairs were ignored, no gaps were allowed, and a maximum of one mismatch was allowed.

Bam files generated were used to create the smallRNA-seq experiment. Reads mapping to miRNA regions were filtered for uniquely mapped reads having a read quality > 20. Quantification and normalization was performed using DESeq[6]. Differentially regulated miRNAs between wild type and ATM-deficient epithelial cells were identified using a t-test with Storey-Bootstrapping correction (q-value ≤ 0.05) and fold change ≥ 1.5. Target mRNAs were found using target prediction databases downloaded in Strand NGS through the annotation manager, namely, miRBase v20, PicTar, PITA Catalog v6, Tarbase v5, and TargetScan v6.2.

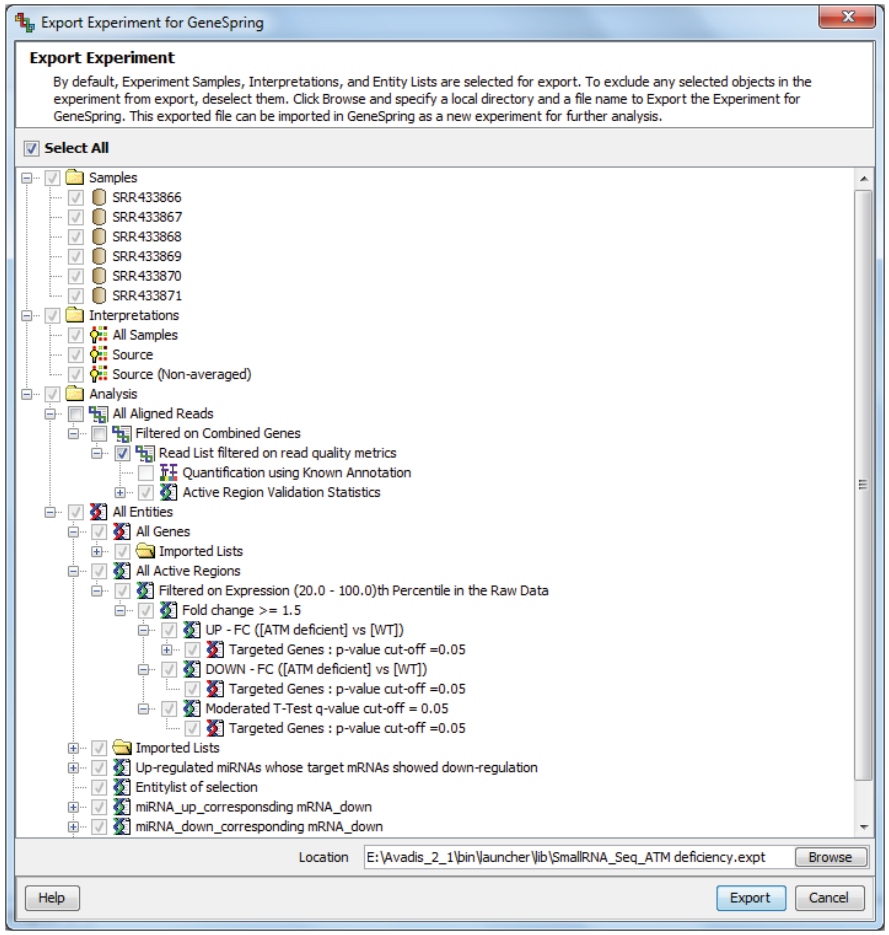


Figure 1. Export of sequencing experiment from Strand NGS 2.1 for import into GeneSpring.

Export of a SmallRNA-seq experiment from Strand NGS 2.1 into GeneSpring 13.0 and creation of a multi-omics experiment

All smallRNA-seq analysis was performed in Strand NGS 2.1. For comparative and multi-omic analysis, the small RNA sequencing experiment was exported from Strand NGS as an .expt file and imported into GeneSpring, as shown in Figure 1. All entity lists and active regions are exported by default. In addition, we selected the filtered read list used for quantification to be exported out to GeneSpring. This enabled a direct comparison of the mRNA data generated using microarrays with the miRNA data generated using NGS through the Multi-Omics Analysis.

An MOA experiment was created using the microarray and the imported Strand NGS smallRNA-Seq experiments (see many how to videos at http://www.genomics.agilent.com/article.jsp?pageId=1500006, including how to add an experiment to an existing project). Both experiments had the interpretation containing the two experimental groups of wild type and ATM-deficient cells.

Additional analysis

Pathway analysis

Multi-omics Pathway Analysis was performed in GeneSpring 13.0 using the KEGG database[7,8], Release 71.

Correlation analysis

Correlation framework in GeneSpring supports correlation between molecules studied by a single-omics platform, or between two different omics platforms. In this study, entity-level correlation analysis was performed on the miRNA and the mRNA identified to be differentially expressed by smallRNA-seq and microarray analysis, respectively. Correlation coefficients were calculated using Pearson's similarity metrics. Clustering on the correlation coefficients was done using Pearson's centered distance metric.

Results and Discussion

Changes in expression profiles of miRNAs after depletion of ATM

In our analysis, we found a total of 125 miRNAs to be differentially expressed (using previously described criteria), of which 62 were up-regulated in ATM-deficient cells, while 63 were down-regulated. Consistent with studies done by Hesse *et al*[4]., we found several (six) tumor suppressors among the down-regulated miRNAs. Among the up-regulated miRNAs were four onco-miRs and two miRNAs associated with chemo-sensitivity of tumor cells (http://202.38.126.151/hmdd/tools/tam.html/). Figures 2 and 3 respectively show the down-regulation of a representative tumor suppressor miRNA, hsa-mir-96, and the up-regulation of an onco-mir, hsa-mir-221, as previously reported (Hesse *et al.*)[4].

We also found the let-7 family, implicated in cell death, cell fate determination, and preventing unchecked proliferation to be up-regulated. The let-7 family of miRNAs are largely tumor suppressors, but oncogenic properties have been attributed to let-7a[9]. In our analysis, hsa-let-7a was up-regulated with let-7a-3 showing an 8 fold increase in expression levels, and let-7a-2 showing 3.4 fold up-regulation in ATM-deficient cells (Figure 4). Other members of the family also show varying levels of increased expression in ATM-deficient cells, as compared to WT.

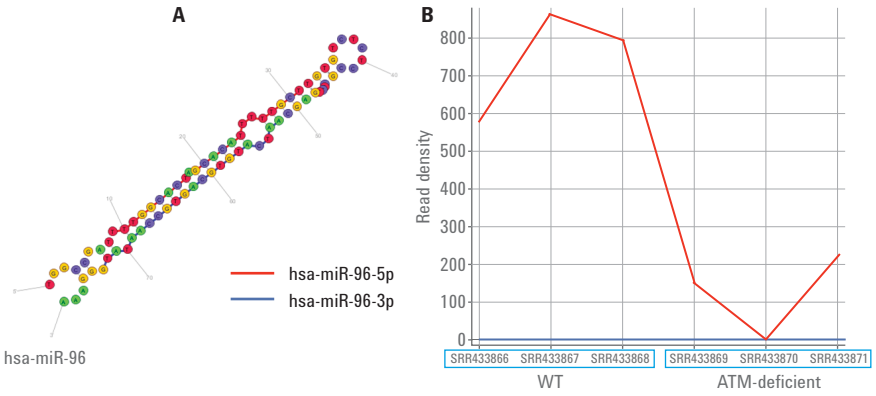


Figure 2. Gene view from Strand NGS 2.1 showing the (A) structure and (B) the down-regulation of hsa-miR-96 in ATM-deficient cells.

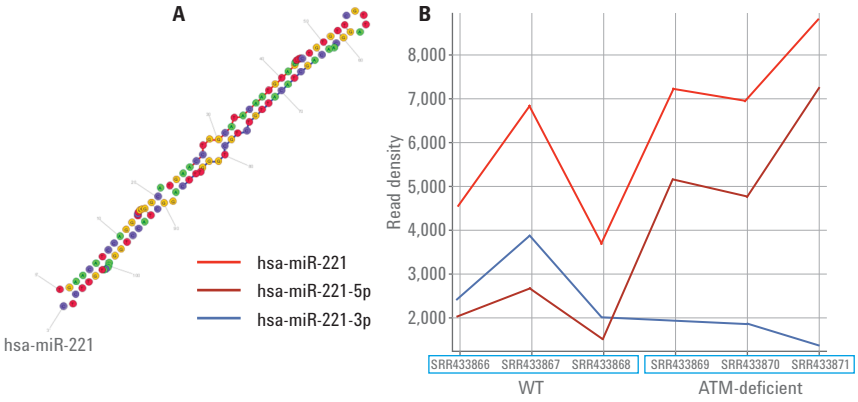


Figure 3. Gene view from Strand NGS 2.1 showing the (A) structure and (B) the up-regulation of hsa-miR-221 in ATM-deficient cells.

The up-regulated and down-regulated miRNAs were used individually with target prediction databases PITA, TargetScan, TarBase, PicTar, and microRNAorg to identify the possible targets. Targets identified by a minimum of one database were used for further downstream analysis.

Changes in gene expression profiles due to changes in regulatory miRNA profiles

Using a moderated t-test (with Storey-Bootstrapping as the false discovery rate method) with q-value threshold at 0.05, we found several genes to be differentially expressed at a fold change value of ≥ 1.2, representing potential targets of miRNA regulation. However, not all of the differential expression could be due to miRNA regulation. Conversely, due to the multiple ways by which miRNA-mediated gene silencing mechanisms operate, not all the possible targets of the differentially regulated miRNAs might be found to be differentially expressed[10]. To establish the genes possibly regulated by miRNAs at the transcript level in the current scenario, the predicted gene targets of up-regulated miRNAs were exported from Strand NGS and imported into GeneSpring. A Venn diagram between these targets and the down-regulated genes from the expression arrays identified 567 entities as probable targets of miRNA regulation, as shown in Figure 5A. A total of 83 of the 567 entities were protein coding cancer genes, as assessed by comparison with a manually curated list of 2,000 protein coding cancer genes from NCG 4.0, the Network of Cancer Genes (http://ncg.kcl.ac.uk/)[11].

To identify the subset of genes whose higher expression levels in the absence of ATM is a consequence of miRNA regulation, a Venn diagram was drawn between possible targets of down-regulated miRNAs (the entity list imported from Strand NGS) and genes found to be up-regulated, as shown in Figure 5B.

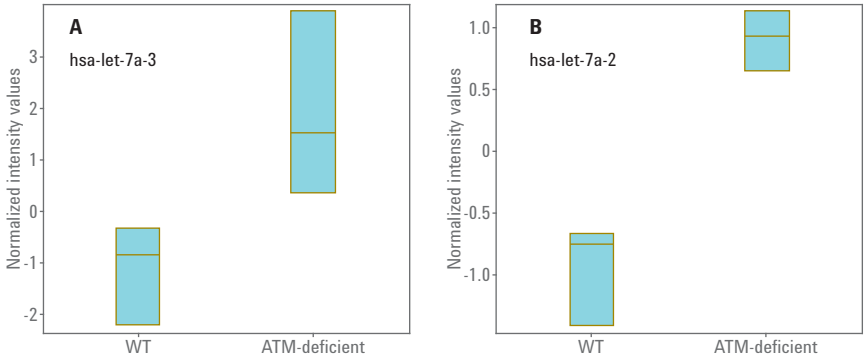


Figure 4. Box-whisker plots showing the up-regulation of (A) hsa-let-7a-3 and (B) hsa-let-7a-2 in ATM-deficient cells.

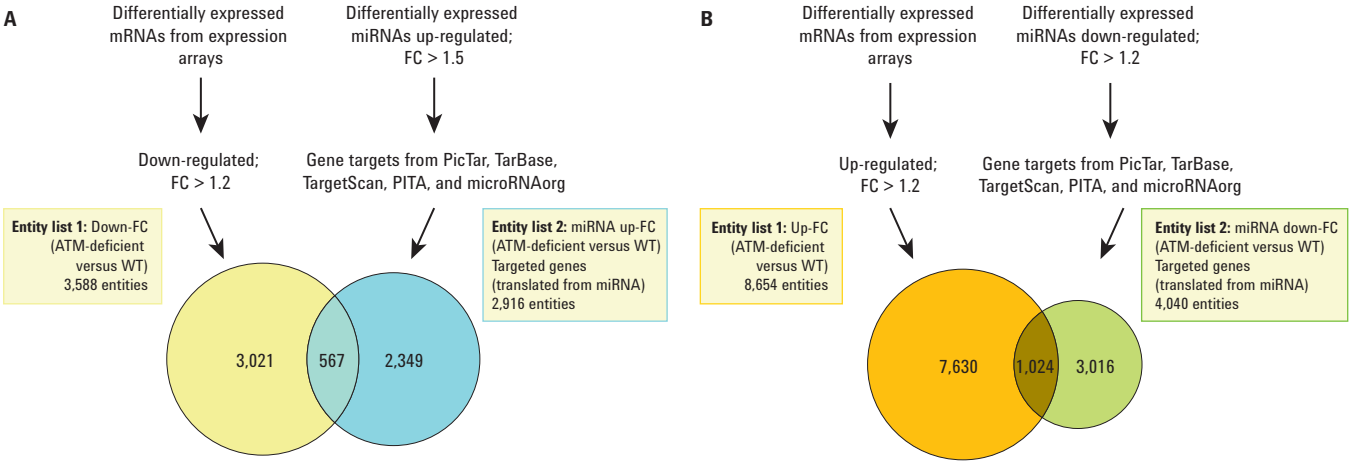


Figure 5. Probable targets of miRNA regulation A) Overlap between down-regulated mRNAs and targets of up-regulated miRNAs identify a common set of 567 genes. B) Overlap between up-regulated mRNAs and targets of down-regulated miRNAs identify a common set of 1,024 genes.

Having identified the putative targets, we next sought to establish the subset of differentially expressed miRNAs that directly regulated their target mRNAs in the current scenario. A differentially expressed gene list was exported from GeneSpring, and imported into Strand NGS to find targeting miRNAs from the PITA, PicTar, TargetScan, microRNAorg, and TarBase databases. Similar to the approach used for identifying the probable target genes, we looked at the overlap of the up-regulated miRNAs with the predicted miRNA regulators of repressed or down-regulated genes. An identical approach was used for the miRNAs undergoing a decrease in expression upon ATM deficiency, as shown in Figure 6.

As may be noted from Figure 6A, only five out of the 63 down-regulated miRNAs seem to have resulted in the elevated expression levels of their gene targets. The large overlap in Figure 6B, and the contrastingly low overlap in Figure 6A are noteworthy, and probably are due to the fact that down-regulated miRNAs are known to impact their targets more at a translational level[12]. The up-regulated miRNAs however, seem to be showing a more direct effect by repressing their targets. We, therefore, directed our attention to the miRNAs whose expression levels had increased upon ATM deletion.

ATM has been linked with breast cancer susceptibility[13]. Up-regulation of several onco-mirs and down-regulation of tumor suppressor miRNAs observed upon ATM deletion point to the critical role played by ATM in breast cancer predisposition. However, as noted in Figure 4, the let-7 family of tumor suppressors also showed high expression levels in ATM-deficient cells as compared to wild type cells. This indicates that the let-7 family either functions in an oncogenic role in breast cancer, being involved in tumorigenic transformation of mammary cells upon ATM depletion, or the increased expression of let-7 family members serves an as yet unknown function. To investigate either possibility, we carried out multi-omics pathway analysis with the 83 down-regulated protein coding cancer genes and the 57 up-regulated miRNAs. We identified the KEGG pathway "hsa05206-MicroRNAs in Cancer" as being significantly enriched across both the expression and the smallRNA-seq experiments.

As seen in this pathway, the let-7 family of miRNAs negatively regulate NRAS and HMGA2 in normal mammary cells, but not upon cancer initiation[14]. The overlay of the data indicates that this negative regulation probably becomes more pronounced upon ATM deletion, with levels of NRAS and HMGA2 decreasing while levels of let-7 increase.

We next computed correlation coefficients between the 83 mRNAs and the 57 miRNAs, and clustered the entities based upon the correlation coefficients. Keeping in mind that correlation does not indicate causation, it is noteworthy that NRAS and HMGA2 are among the mRNAs which are negatively correlated with the let-7 family members. Pair-wise correlation coefficient values indicate that NRAS has a stronger negative correlation with let-7 compared to HMGA2.

Results of the multi-omics pathway and correlation analysis suggest that upon deletion of ATM, levels of let-7 increase. The let-7 family of miRNAs are known to function as tumor suppressors[9], in gastric[15] and colon cancer[16, 17], and reduce proliferation of self-renewing tumor initiating cells in breast cancer[18]. High levels of let-7 have been shown to repress NRAS and HMGA2[19,20]. Thus, the decreased expression levels of NRAS and HMGA2 observed here is likely a direct outcome of a let-7 increase, rather than of ATM deletion. HMGA2 and NRAS are potent oncogenes, and are seen in a variety of different tumors. They are repressed through let-7 binding to their 3'UTR[17, 20]. Apart from let-7 repression, HMGA2 has been speculated to be under control of miRNAs other than let-7[18] as well, which could explain the low correlation values seen.

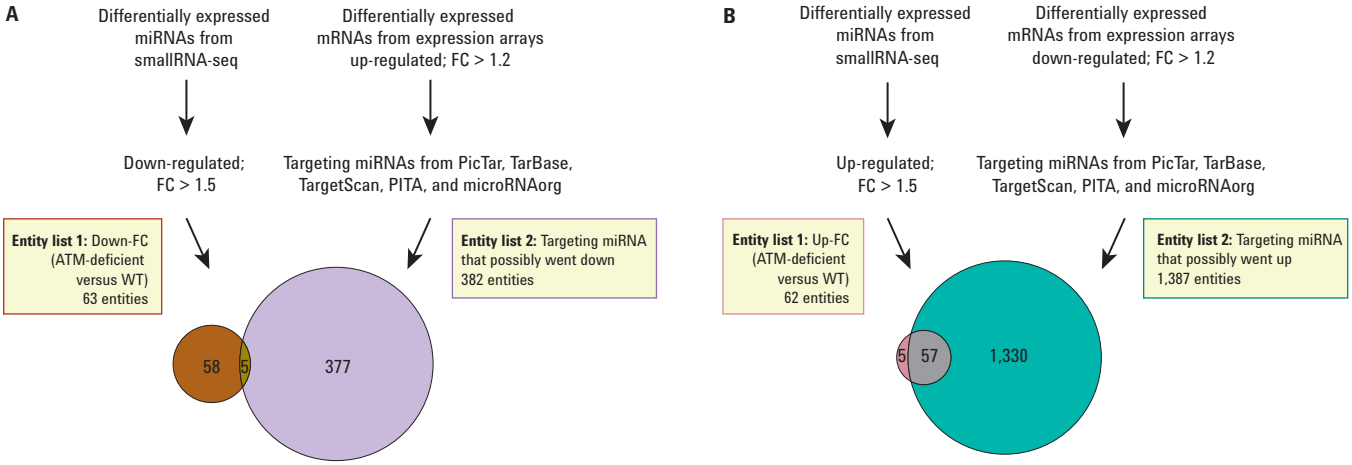


Figure 6. Probable list of regulatory miRNAs A) Overlap between down-regulated miRNAs and known regulators of up-regulated mRNAs identify five miRNAs. B) Overlap between up-regulated miRNAs and known regulators of down-regulated mRNAs identify 57 miRNAs.

What does this mean with respect to biological changes occurring in the mammary cells in response to ATM depletion? Since ATM is a tumor repressor, its loss would make the cells vulnerable to DNA damage and expose them to neo-plastic transformation. As a protective mechanism, cells elevate the levels of another tumor suppressor, the let-7 miRNA. Increase in let-7 levels with concurrent decrease in levels of HMGA2 and NRAS could be one of the compensatory and protective mechanisms employed by the cells to prevent oncogenesis. While HMGA2 over-expression is known to lead to poor prognosis and increased metastasis for breast cancer patients, elevated levels in normal mammary epithelial cells also induces transformation with cells showing lack of proliferation suppression, disruption of cell-cell adhesion, and colony formation on soft agar. HGMA2 levels are low in normal adult tissues[19,21], and it is possible that using this method, the cells can keep the levels of onco-genes lower than normal.

Conclusions

The ability to import relevant information from Strand NGS to GeneSpring allowed us to perform a multi-omics analysis of gene expression combining mRNA and miRNA datasets collected using different measurement platforms. This enabled formulation of a testable hypothesis regarding cellular response to loss of a tumor suppressor.

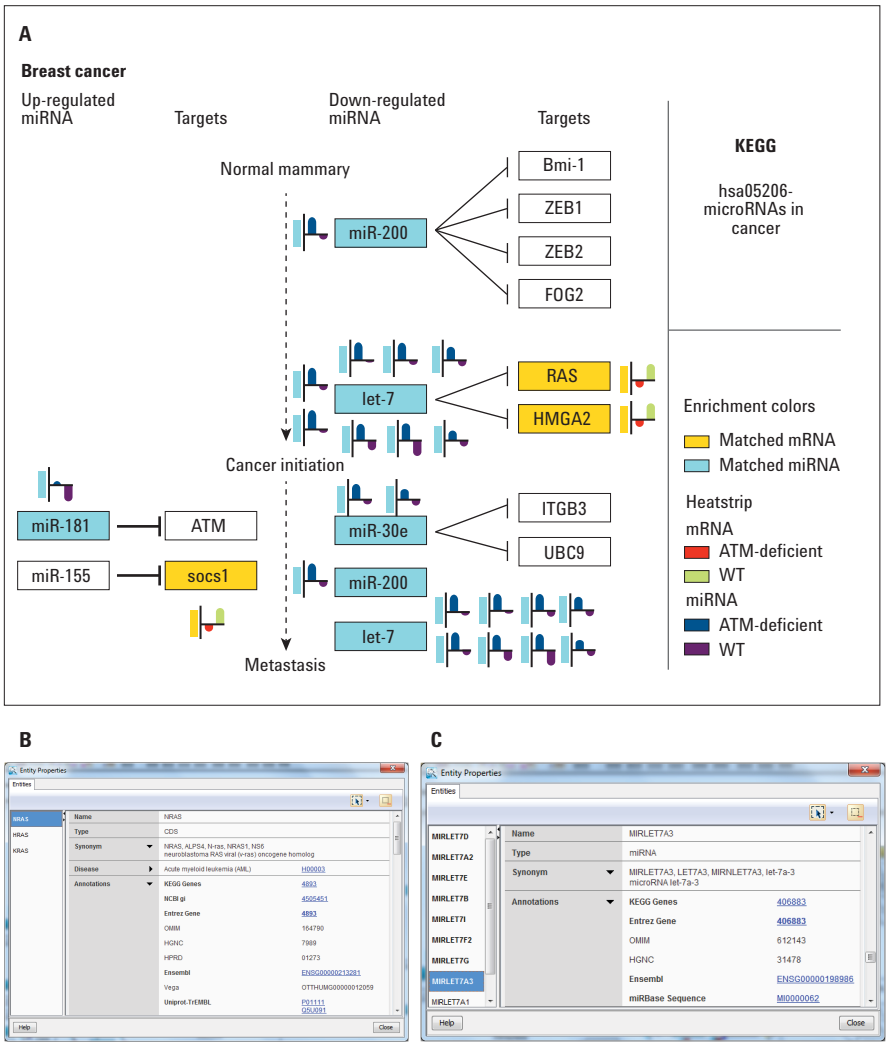
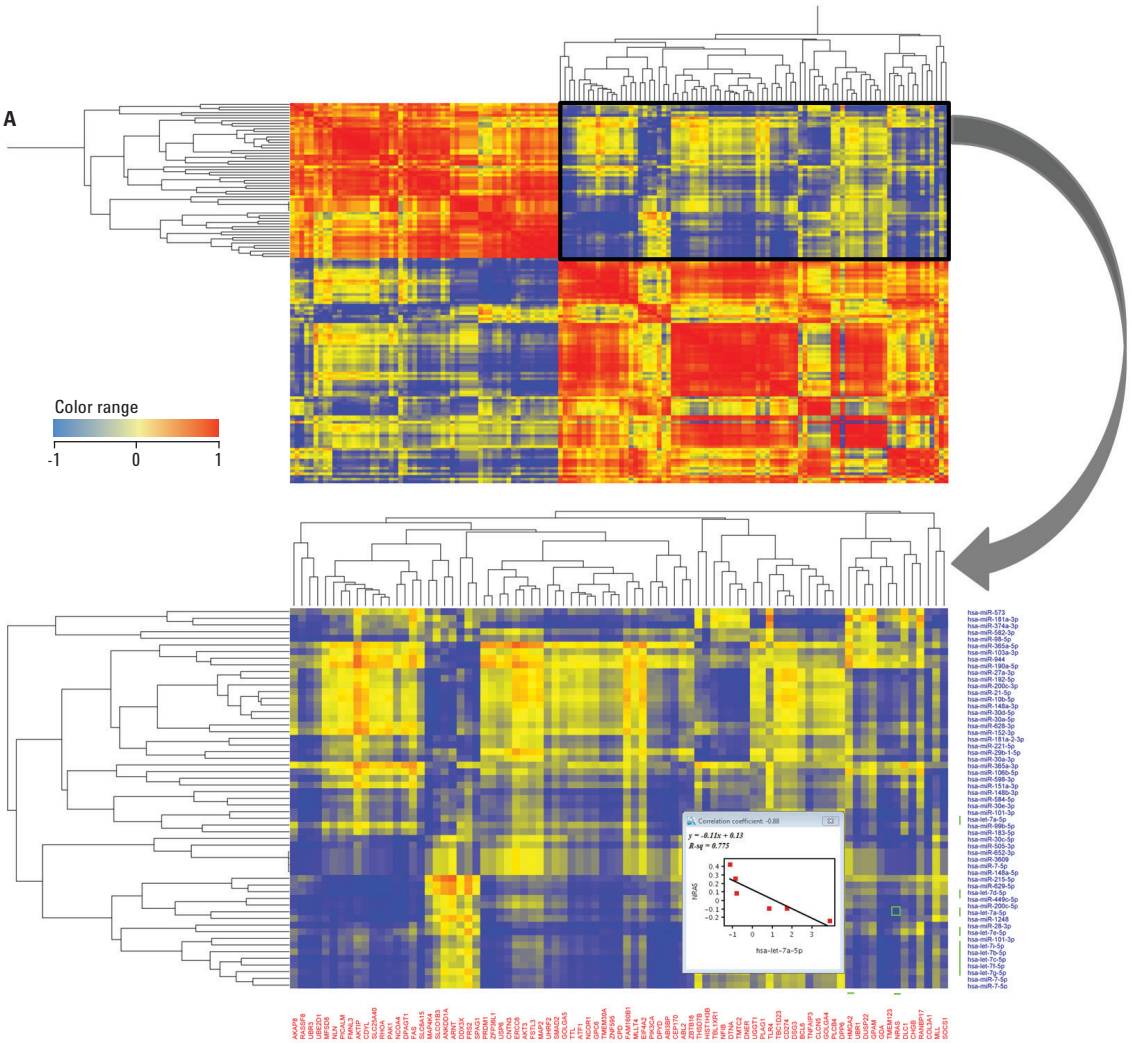


Figure 7. KEGG pathway- MicroRNAs in cancer. A) Pathway view from GeneSpring showing matched entities from down-regulated mRNAs and up-regulated miRNAs. B) Dialog showing properties of matched mRNA, NRAS. C) Dialog showing properties of matched miRNAs, let-7 family.



B

miRNA	NRAS	HMGA2
hsa-let-7a-5p	–0.880	–0.615
hsa-let-7c-5p	–0.877	–0.597
hsa-let-7i-5p	–0.874	–0.625
hsa-let-7a-5p	–0.833	–0.571
hsa-let-7b-5p	–0.826	–0.772
hsa-let-7f-5p	–0.807	–0.597
hsa-let-7g-5p	–0.732	–0.673
hsa-let-7d-5p	–0.616	–0.472
hsa-let-7e-5p	–0.508	–0.504

Figure 8. Correlation of up-regulated miRNAs and down-regulated mRNAs. A) Correlation heat-map highlighting HMGA2, NRAS and the let-7 family. The summary view with four quadrants is shown on the top, while the detailed view is on the bottom. B) Correlation coefficients between NRAS, HMGA2 and the let-7 family.

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