Gene Profiling of *Mta1* Identifies Novel Gene Targets and Functions

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Abstract

**Background:** Metastasis-associated protein 1 (MTA1), a master dual co-regulatory protein is found to be an integral part of NuRD (Nucleosome Remodeling and Histone Deacetylation) complex, which has indispensable transcriptional regulatory functions via histone deacetylation and chromatin remodeling. Emerging literature establishes MTA1 to be a valid DNA-damage responsive protein with a significant role in maintaining the optimum DNA-repair activity in mammalian cells exposed to genotoxic stress. This DNA-damage responsive function of MTA1 was reported to be a P53-dependent and independent function. Here, we investigate the influence of P53 on gene regulation function of *Mta1* to identify novel gene targets and functions of *Mta1*.

**Methods:** Gene expression analysis was performed on five different mouse embryonic fibroblasts (MEFs) samples (i) the *Mta1* wild type, (ii) *Mta1* knock out (iii) *Mta1* knock out in which *Mta1* was reintroduced (iv) *P53* knock out (v) *P53* knock out in which *Mta1* was over expressed using Affymetrix Mouse Exon 1.0 ST arrays. Further Hierarchical Clustering, Gene Ontology analysis with GO terms satisfying corrected p-value<0.1, and the Ingenuity Pathway Analysis were performed. Finally, RT-qPCR was carried out on selective candidate genes.

**Significance/Conclusion:** This study represents a complete genome wide screen for possible target genes of a coregulator, Mta1. The comparative gene profiling of *Mta1* wild type, *Mta1* knockout and *Mta1* re-expression in the *Mta1* knockout conditions define “bona fide” *Mta1* target genes. Further extensive analyses of the data highlights the influence of *P53* on *Mta1* gene regulation. In the presence of *P53* majority of the genes regulated by MTA1 are related to inflammatory and antimicrobial responses whereas in the absence of *P53* the predominant target genes are involved in cancer signaling. Thus, the presented data emphasizes the known functions of *Mta1* and serves as a rich resource which could help us identify novel *Mta1* functions.

Introduction

Gene expression is central to variety of fundamental cellular processes that governs the growth and proliferation in mammalian cell. It is a highly regulated process that is governed by transcription factors and their co-regulators in the target gene chromatin [1–3]. Co-regulators are emerging transcription factor (TF) family that stringently controls the actions of almost “all” nuclear receptors (NRs) through direct binding to NRs rather than binding to DNA [1–4]. Recent literature highlights the significance of coregulators in induction or repression of gene transcription [5,6]. In fact, the coregulators either function as enzymes that are essential for gene expression or they regulate other coregulators through diverse mechanisms [6–10]. There has been a tremendous focus on understanding the molecular mechanism of coregulators due to their crucial regulatory role in almost “all” TF-dependent gene expression and nuclear receptors dependent functions in several tissues such as breast, ovary, prostate, gastrointestinal, pancreatic and lungs [5,11,12]. More interestingly, many cancers over express “growth coactivators” that allow the cancer cell to hijack these molecules which consequently results in rapid proliferation, malignant process and rapid metastasis.

One emerging group of chromatin modifiers and coregulators is the metastasis-associated protein (MTA) family. This family comprises three different known genes (*MTA1, MTA2*, and *MTA3*) and is an integral part of the NuRD (Nucleosome Remodeling and Histone Deacetylation) complex that has indispensable transcriptional regulatory functions via histone deacetylation and chromatin remodeling [13,14]. MTA1, the founding member of the MTA family was initially identified through differential screening of a cDNA library from rat metastatic breast tumors as an upregulated gene [15,16]. Subsequent studies found MTA1 to be widely up-regulated in various human cancers and established to be involved in tumorigenesis, tumor invasion, and metastasis [13,17]. Owing to
its name as a coregulator the repressor function of MTA1 is observed through its direct interaction with ERα [18] and HDACs which represses estrogen-responsive element (ERE) transactivation activity in a HDAC-sensitive manner that promotes the development of hormone-independent growth of breast cancer cells. In addition, MTA1-NuRD complex was also reported to negatively regulate BRCA1 transcription by physically associating with an atypical estrogen-responsive element (ERE) on the BRCA1 promoter [19]. The transcription activator function of MTA1 is evident from reports showing the stimulation of breast cancer-amplified sequence 3 (BCAS3) [20] and paired box gene 5 (Pax 5) [21] promoters mainly through the interaction with RNA polymerase II. It is noteworthy that the MTA family members exist in distinct NuRD complexes, and functional redundancy is lacking among MTA family members [22].

Further, recent studies from this laboratory have discovered for the first time that MTA1 is a bona-fide DNA-damage responsive protein due to the induction of intracellular levels of MTA1 by

Figure 1. Schematic showing the experimental design of the study to identify the Mta1 regulated genes with/without the effect of P53. RNA was extracted from all the samples Wild Type (WT), Mta1 knock out (Mta1-KO), Mta1 Re-expression in the Mta1 knock out MEFS (Mta1-KO/Mta1), P53 knock out (P53-KO), Mta1 over expression (OE) in the P53 knock out MEFS (P53-KO/Mta1). cDNA was prepared, processed and hybridized onto the Affymetrix Mouse Exon 1.0 ST arrays followed by the data analysis. Samples were compared to identify the differentially regulated genes and in turn the genes regulated by Mta1 in p53 dependent/independent manner and irrespective of P53 status. Candidate genes were selected; these genes were validated using RT-qPCR assays in MEFS followed by RT-qPCR assays in MCF-7 human breast cancer cell line with the human homologs of the candidate mouse genes.

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Figure 2. Principle Component Analysis as a quality control. All the sample sets (each in triplicates) were plotted in the 3-D PCA-plot. Out of the 4 total PCA components, components 1, 2 & 4 were plotted on columns X, Y & Z respectively. doi:10.1371/journal.pone.0017135.g002

Table 1. Top 25 differentially expressed Affymetrix Mouse Exon 1.0 St Array probe sets in MEFs between the wild type and Mta1 knock out.

| refseq | Gene Symbol | FC   | Reg | Gene Description                                           |
|--------|-------------|------|-----|-----------------------------------------------------------|
| NM_146015 | Efemp1      | 100.24 | up  | epidermal growth factor-containing fibulin-like extracellular matrix protein 1 |
| NM_009099 | Trim30      | 79.45  | up  | tripartite motif-containing 30                           |
| NM_133871 | Ifi44       | 79.08  | up  | interferon-induced protein 44                           |
| NM_009252 | Serpina3n   | 72.25  | up  | serine (or cysteine) peptidase inhibitor, clade A, member 3N |
| NM_008331 | Ifit1       | 71.62  | up  | interferon-induced protein with tetraicosopeptide repeats 1 |
| NM_015784 | Postn       | 49.40  | up  | peristin, osteoblast specific factor                      |
| NM_009846 | Cd24a       | 47.95  | down | CD24a antigen | predicted gene, EG621324  |
| NM_134086 | Sk38a1      | 42.67  | down | solute carrier family 38, member 1                       |
| NM_001033767 | EG240327 | 41.75  | up  | predicted gene, EG240327                               |
| NM_001111059 | Cd34    | 40.98  | down | CD34 antigen                                           |
| NM_011409 | Slfn3       | 34.62  | up  | schlafen 3                                              |
| NM_001085522 | RP24-32009.1 | 32.71  | up  | novel KRAH box containing protein                        |
| NM_010681 | Lamo4       | 30.71  | up  | laminin, alpha 4                                        |
| NM_007833 | Dcn         | 30.43  | up  | decorin                                                  |
| NM_009251 | Serpina3g   | 30.43  | up  | serine (or cysteine) peptidase inhibitor, clade A, member 3G |
| NM_023386 | Rtp4        | 28.17  | up  | receptor transporter protein 4                          |
| NM_010917 | Nid1        | 27.87  | up  | nidogen 1 | similar to Nid1 protein                                 |
| NM_008409 | Ltm2a       | 26.76  | up  | integral membrane protein 2A                            |
| NM_011854 | Oasi2       | 26.58  | up  | 2'-5' oligoadenylyl synthetase-like 2                    |
| NM_008489 | Lbp         | 26.45  | up  | lipopolysaccharide binding protein                       |
| NM_019955 | Rrk3        | 25.19  | up  | receptor-interacting serine-threonine kinase 3           |
| NM_025711 | Aspn        | 23.86  | up  | asporin                                                  |
| NM_025949 | Rp56a6      | 23.80  | down | ribosomal protein S6 kinase polypeptide 6                |
| NM_025961 | Gatm        | 23.37  | down | glycine amidinotransferase (L-arginine:glycine amidinotransferase) |
| NM_001001892 | EG630499 | 23.27  | up  | histocompatibility 2, K1, K region                      |

Top 25 probe sets with fold change of 2.0 or more and FDR less than 0.05 are shown. Wild type cells are the control against the knock out cells as treatment. doi:10.1371/journal.pone.0017135.t001
ionizing radiation (IR) and an integral component of DNA damage response and contributes to double-strand DNA break repair [23]. One of the main DNA damage responsive mechanisms employed by MTA1 is through direct interaction and controlling the stability of P53 [24]. P53 is a well-studied tumor suppressor protein which plays a central role in preserving the genomic integrity in response to DNA damage, through inhibiting its ubiquitination by E3 ubiquitin ligases, mouse double minute 2 (Mdm2) and constitutive photomorphogenic protein 1 (COP1), thereby regulating the P53-dependent DNA repair [25–27]. Interestingly, these events could be reversed by MTA1 reintroduction, indicating that MTA1 interacts into the P53-dependent DNA repair [24]. This identified interplay between oncogene (MTA1) and tumor suppressor (P53) exemplifies the highly complex check point mediated “failsafe mechanism” that controls the mitogenic signaling. Moreover, studies from our laboratory illustrated the participation of MTA1 in a P53-independent DNA damage response. MTA1-histone deacetylase 2 (HDAC2) complexes recruit P53-independent transcriptional corepressor, p21WAF1 [28,29] onto two selective regions of the p21WAF1 promoter which increases p21WAF1 binding to corepressor, p21WAF1 [28,29] onto two selective regions of the HDAC2 complexes recruit P53-independent transcriptional corepressor, p21WAF1 [28,29] onto two selective regions of the p21WAF1 promoter which increases p21WAF1 binding to corepressor, p21WAF1. MTA1-histone deacetylase (HDA) complexes recruit P53-independent transcriptional corepressor, p21WAF1 (p21) onto two selective regions of the p21WAF1 promoter which increases p21WAF1 binding to corepressor, p21WAF1. MTA1 is also shown to be stabilized by UV radiation in ATR (Ataxia telangiectasia and Rad3-related) kinase dependent manner and there is a subsequent increase in MTA1 binding to ATR. However, depletion of MTA1 compromises the ATR-mediated Chk1 activation following UV treatment. Consequently, expression of MTA1 in P53-null cells results in increased induction of histone, p53 target, H2AX [31] foci and DNA double strand break repair and decreased DNA damage sensitivity following ionizing radiation treatment. These early studies linking the MTA/NuRD complexes to DNA-damage response were further supported by more recent reports showing the recruitment of the NuRD complexes to the site of DNA damage [32,33]. Together, the P53-independent role of MTA1 in DNA damage response connects NuRD complex and DNA-damage response pathways [34].

Table 2. Top 25 differentially expressed Affymetrix Mouse Exon 1.0 St Array probe sets in MEFs between the Mta1 knock out and Mta1 knock out/MTA1.

| reseq     | Gene Symbol | FC    | Reg | Gene Description                          |
|-----------|-------------|-------|-----|-------------------------------------------|
| NM_134066 | Akr1c18     | 14.98 | up  | aldo-keto reductase family 1, member C18  |
| NM_025711 | Aspn        | 9.58  | down| asporin                                   |
| NM_029803 | Il27        | 8.86  | down| interferon, alpha-inducible protein 27    |
| NM_178737 | AW551984    | 6.75  | down| Mouse homolog of human gene VWA5A         |
| NM_007621 | Cbr2        | 6.01  | down| carbonyl reductase 2                      |
| NM_011959 | Timp3       | 5.67  | up  | tissue inhibitor of metalloproteinase 3    |
| NM_027495 | Tmem144     | 5.13  | up  | transmembrane protein 144                 |
| NM_029000 | Gvin1       | 4.89  | down| GTPase, very large interferon inducible 1  |
| NM_013585 | Psmb9       | 4.53  | down| proteasome (prosome, macropain) subunit, beta type 9 (large multifunctional peptidase 2) |
| NM_028608 | Glipr1      | 4.36  | down| GLI pathogenesis-related 1 (glioma)       |
| NM_023422 | Hist1h2bc   | 3.91  | down| histone cluster 1                         |
| NM_001097644| Ccnyl1       | 3.91  | down| cyclin Y-like 1                           |
| NM_008630 | Mt2         | 3.78  | up  | metallothionein 2                         |
| NM_008604 | Mme         | 3.78  | down| membrane metallo endopeptidase            |
| NM_008728 | Npr3        | 3.74  | up  | natriuretic peptide receptor 3            |
| NM_009251 | Serpina3g   | 3.73  | down| serine (or cysteine) peptidase inhibitor, clade A, member 3G |
| NM_011979 | Vnn3        | 3.68  | down| vanin 3                                   |
| NM_007817 | Cyp2f2      | 3.65  | down| cytochrome P450, family 2, subfamily F, polypeptide 2 |
| NM_008185 | Gstt1       | 3.64  | down| glutathione S-transferase, theta 1        |
| NM_054077 | Prep        | 3.58  | down| proline arginine-rich end leucine-rich repeat |
| NM_053078 | D0H4S114    | 3.57  | down| DNA segment, human D4S114                 |
| NM_011579 | Tgtp        | 3.50  | down| T-cell specific GTPase                   |
| NM_012043 | Islr        | 3.45  | down| immunoglobulin superfamily containing leucine-rich repeat |
| NM_009846 | Cd24a       | 3.32  | up  | CD24a antigen                             |
| NM_133903 | Spon2       | 3.26  | down| spondin 2, extracellular matrix protein   |

Top 25 probe sets with fold change of 2.0 or more and FDR less than 0.05 are shown. Mta1 knock out cells are the controls and Mta1-KO/Mta1 is the treatment. doi:10.1371/journal.pone.0017135.002
microarray data of all five samples that include the above three along with P53 knockout and P53 knockout with Mta1 over expression clearly indicates influence of P53 on Mta1 gene regulation. Mta1 target genes are mostly involved in inflammatory and anti-microbial responses in the presence of P53 whereas the predominant target genes and functions identified appear to be related to cancer signaling in the absence of P53. Thus, we provide a complete gene profiling of Mta1 which not only emphasizes the

Figure 3. Genes regulated by Mta1 in the presence and absence of P53. A: Venn diagrams showing the number of genes that are identified to be genuinely regulated by Mta1 in the presence and absence of P53. (i) The genes shown in WT vs. Mta1-KO (1124) are the genes affected by Mta1 knock out and the genes that are present in Mta1-KO vs. Mta1-KO/Mta1 (184) are affected due to re-expression of Mta1 in the knockout MEFs. The genes that are differentially regulated in both the sets with opposite trends in the regulation are considered as the ‘bona fide’ genes regulated by Mta1. ii) Similarly, the intersection of P53-KO and P53-KO/Mta1 represents the genes differentially regulated by Mta1 over expression in the P53-KO MEFs, i.e. the genes regulated by Mta1 in the absence of P53. This scenario of Mta1 over expression and P53 knock out/mutation mimics most of the cancers iii) The Venn diagram between genes regulated by Mta1 in the presence of P53, 126 and genes regulated by Mta1 in the absence of P53, 266 gives the genes that are regulated by Mta1 only in the presence of P53, irrespective of P53 status and only in the absence of P53 respectively. B: Heat maps representing hierarchical clustering of the differentially regulated, plotted using the log2 values of the genes with p-value<0.05 (unpaired t-test) and with fold change of at least 2 between the comparisons Mta1 knock out vs. Mta1 knock out/Mta1 and P53 knock out vs. P53 knock out/Mta1. Each column represents a sample plotted in triplicates and each row in the heat map represents a gene that is differentially regulated in that particular comparison of samples. The color scale represents the degree of expression of the gene, green being the lowly expressed (below –3.0) and red being (above +3.0) the highly expressed genes in the sample sets with black as the center of the scale at ‘0’. C: Pie diagrams showing the percentage of up-regulated and the down-regulated genes in each sample comparison. The red segment of the pie represents the up-regulated genes whereas; the green segments represent the down regulated genes.
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known functions of Mta1 but also acts as a guide to identify novel functions.

Results

Strategy to identify the possible target genes of MTA1 in a P53 dependent and independent manner

The aim of the study is to identify the genes that are regulated by Mta1 in P53 dependent and independent manners. The detailed schematic of the strategy followed to identify the genes is shown in Figure 1. Murine Embryonic Fibroblasts (MEFs) from wild type, Mta1 knockout [35] and P53 knockout mice embryos [36] were isolated and cultured to obtain five different types of samples, each in triplicates, for the identification of the genes that are regulated by Mta1 with/without the P53 background. The sample sets are as follows: 1) Mta1-Wildtype (WT), 2) Mta1-knockout (Mta1-KO) [23], 3) Mta1 transfected into the Mta1-knock out MEFs (Mta1-KO/Mta1), 4) P53-Knock (P53-KO) and 5) Mta1 over expressed in the P53-Knockout MEFs (P53-KO/Mta1) [30]. The protein levels of Mta1 in all the five samples are compared using Western blot probed with Mta1 antibody (Figure S1). Total RNA was isolated from the MEFs, cDNA was prepared, processed and hybridized onto Affymetrix Mouse Exon 1.0 ST arrays. The gene expression data from all the samples were obtained, quality control steps were performed and the data was analyzed using GeneSpring GX 10.0.2 (Agilent Technologies).

Genes regulated by Mta1 under different conditions were identified by performing gene expression analysis followed by the Gene Ontology analysis. Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Inc) was used to identify top 15 statistically significant functions of the differentially regulated genes and top 15 canonical pathways in which these genes could play a role. Candidate genes were selected based upon the functions and our laboratory interests. RT-qPCR assays were performed to validate the microarray gene regulation of the selected candidate genes. The differential regulation of the candidate genes was confirmed using MEFs and the MCF7 human breast cancer cell line for the respective human homologs. Using this strategy, we have identified a set of genes which, we believe, are regulated by Mta1 in P53 dependent and independent manners, thus, providing overall gene profiling of Mta1.

Quality Control and Gene Expression Analysis

We generated cDNAs from the total RNA of the corresponding MEFs and three biological replicates for each sample were hybridized. The raw data was obtained and normalized log2 ratio values were used to identify the differential gene expression levels across different samples. The quality control of all the samples and the replicates was analyzed by unsupervised Principle Component Analysis (PCA) method in which each of the samples, in a different color, was plotted in three dimensional (3D) space with components 1, 2 & 4 along X, Y & Z axes respectively. Figure 2 shows the PCA-plot with replicates of each sample clustered close to each other illustrating the similarity between the sample replicates. We performed unpaired t-test with a p-value less than 0.05 and Benjamini Hochberg false discovery rate (FDR) was applied for the multiple corrections to filter the false positives so that the statistically significant genes could be obtained.

Genes regulated by Mta1 in the presence of P53 in MEFs

The initial comparison between the Mta1 wild type, the Mta1 knock out and Mta1-re-expression in knockout MEFs was performed. Although differential expression of the genes between Mta1 wild type and Mta1 knock out was reported by us earlier [30], our aim of this study is to identify the genes differentially regulated among Mta1 wild type, Mta1 knock out and Mta1-KO in which Mta1 WT was reintroduced. Therefore, we have used the

| Table 3. Bona fide Mta1 regulated genes in the presence of P53 identified from the Affymetrix Mouse Exon 1.0 ST Arrays. |
|-----------------------------------------------|
| **Gene Symbol** | WT vs. Mta1-KO | Mta1-KO vs. Mta1-KO/Mta1 |
|-----------------------------------------------|
| **Fold Change** | **Regulation** | **Fold Change** | **Regulation** |
|-----------------------------------------------|
| Cd24a | 47.95 | down | 3.32 | up |
| Igrp1* | 41.75 | up | 3.23 | down |
| Serpina3g* | 30.43 | up | 3.73 | down |
| Lbp* | 26.45 | up | 2.51 | down |
| Aspn* | 23.86 | up | 9.58 | down |
| Ifit3 | 22.29 | up | 2.25 | down |
| Apod | 20.15 | up | 2.89 | down |
| Irgb10 | 18.67 | up | 2.2 | down |
| Ccnyl1 | 18.12 | up | 3.91 | down |
| Apol9a | 17.31 | up | 2.37 | down |
| Apol9b* | 17.31 | up | 2.37 | down |
| Oas1a | 15.93 | up | 2.19 | down |
| Zbp1 | 13.03 | up | 3.15 | down |
| Smpld3b | 12.92 | down | 2.26 | up |
| Isl* | 12.85 | up | 3.45 | down |
| Stiap4* | 12.37 | up | 2.55 | down |
| Pcdhsb9 | 12.31 | up | 2.22 | down |
| Timp3 | 12.03 | down | 5.67 | up |
| Ifit27 | 11.75 | up | 8.86 | down |
| If7 | 11.17 | up | 2.04 | down |
| Prelp* | 11.12 | up | 3.58 | down |
| Psnmb9 | 11.05 | up | 4.53 | down |
| AW551984 | 11.04 | up | 6.75 | down |
| Pcdhsb8 | 9.98 | up | 2.82 | down |
| Tspan13 | 9.95 | up | 2.03 | down |
| Il1rn | 9.87 | up | 2.34 | down |
| Gvin1 | 9.87 | up | 4.89 | down |
| Lpl | 9.54 | up | 2.36 | down |
| If11* | 9.08 | up | 2.32 | up |
| Oas2 | 8.78 | up | 2.98 | down |
| Trim12 | 8.32 | up | 2.04 | down |
| Adam23 | 8.32 | up | 2.74 | down |
| Edna | 8.06 | up | 2 | down |
| A4galt* | 7.21 | up | 2.85 | down |
| Tap1 | 7 | up | 2.89 | down |
| Ened | 6.88 | up | 2.31 | up |
| Vnn3 | 6.81 | up | 3.68 | down |
| Dhx58 | 6.49 | up | 2.26 | down |
| Mgp | 6.29 | up | 2.64 | down |
| Ces1 | 6.14 | up | 2.9 | down |
| Slc16a4 | 5.97 | up | 3 | down |

*Genes (32) regulated by Mta1 irrespective of P53 status. Figure 3A (lower panel).
Note: Continued in Table S5.

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data from Li et al, (2010) for comparison. When Mta1 WT and knockout conditions were compared, 1124 genes were reported with a fold change ≥±2.0 and with the p-value<0.05. These 1124 genes were either directly or indirectly regulated by Mta1. Top 25 differentially expressed Affymetrix probes are shown in Table 1 (complete list is shown in Table S1, statistically summary is shown in Table S2). It is noteworthy that a gene encoding epidermal growth factor-containing fibulin-like extra cellular matrix protein 1 (EFEMP1) is identified to be up-regulated by 100 fold with the highest fold change regulation in the Mta1 knockout MEFs when compared with the wild type Mta1 MEFs. Interestingly, Sadri-Nabavi et al., (2009) reported a reduction in RNA and protein levels of EFEMP1 in human sporadic breast cancer tissues [37] which imply that over expression of Mta1 could lead to down regulation of EFEMP. In addition, we also found that the gene encoding CD24/HSA had the maximum fold change among the down regulated genes. CD24 is a cell adhesion molecule that is encoding CD24/HSA had the maximum fold change among the down regulated genes. CD24 is a cell adhesion molecule that is expressed on the surface of the infected cells [38].

When Mta1 was transfected into the Mta1 knock-out MEFs and compared against the Mta1-KO, 184 differentially regulated genes were identified. The top 25 differentially regulated genes based upon fold change are shown in Table 2 (the entire list is shown in Table S3 and statistical summary in Table S4). Out of these 184 genes, 126 genes were found to be present in WT Vs Mta1-KO as well (1124 genes, Figure 3A top panel). Majority of these 126 genes appear to restore their functioning when Mta1 is transfected back into the Mta1 knock out cells. Among these 126 genes except 6 genes, rest of them regained their expression levels similar to WT. Therefore these represent “bona fide” Mta1 target genes (Table 3 and Table S5). Since P53 is present in all the three samples they reflect the number of genes influenced by MTA1 in the presence of P53.

**Genes regulated by Mta1 in a P53-independent manner**

Here we identified the genes that are regulated by Mta1 in a P53-independent manner. Differential gene expression analysis was performed by comparing the P53 knock out cells and P53 knock out/Mta1 over expressed cells [30]. All the genes with p-value<0.05 and fold change expression ≥±2.0 were considered as statistically significant. The statistical analysis of the data generated a set of 266 genes, top 25 differentially regulated genes are shown

| refseq   | Gene Symbol | FC    | Reg   | Gene Description                               |
|----------|-------------|-------|-------|------------------------------------------------|
| NM_008489| Lbp         | 26.89 | down  | lipopolysaccharide binding protein             |
| NM_015784| Postn       | 14.70 | down  | periostin, osteoblast specific factor          |
| NM_008807| Mmp13       | 13.37 | down  | matrix metallopeptidase 13                    |
| NM_008760| Oggn        | 12.46 | down  | osteoglycin                                    |
| NM_011339| Ccl15       | 12.39 | down  | chemokine (C-X-C motif) ligand 15              |
| NM_008491| Lcn2        | 9.68  | down  | lipocalin 2                                    |
| NM_001014423| Abi3bp    | 9.31  | down  | ABI gene family, member 3 (NESH) binding protein |
| NM_199468| Zcchc5      | 7.85  | down  | zinc finger, CCHC domain containing 5          |
| NM_011704| Vnn1        | 7.22  | down  | vanin 1                                        |
| NM_009373| Tgm2        | 7.22  | down  | transglutaminase 2, C polypeptide              |
| NM_054096| Steap4      | 7.06  | down  | STEAP family member 4                          |
| NM_011315| Saa3        | 6.70  | down  | serum amyloid A 3                              |
| NM_010582| Ith2        | 6.51  | down  | inter-alpha trypsin inhibitor, heavy chain 2   |
| NM_009144| Sfrp2       | 6.46  | down  | secreted frizzled-related protein 2            |
| NM_010809| Mmp3        | 6.39  | down  | matrix metallopeptidase 3                     |
| NM_008728| Npr3        | 5.80  | down  | natriuretic peptide receptor 3                |
| NM_172463| Smed1       | 5.70  | down  | sushi, nidogen and EGF-like domains 1          |
| NM_022814| Svep1       | 5.62  | down  | sushi, von Willebrand factor type A, EGF and pentraxin containing 1 |
| NM_007621| Cbr2        | 5.55  | down  | carbonyl reductase 2                           |
| NM_009251| Serpina3g   | 5.53  | down  | serine (or cysteine) peptidase inhibitor, clade A, member 3 |
| NM_025711| Aspn        | 5.47  | down  | asporin                                        |
| NM_144938| Cts1        | 5.23  | down  | complement component 1, α subcomponent        |
| NM_031167| Il1r1       | 5.15  | down  | interleukin 1 receptor antagonist              |
| NR_001592| H19         | 5.14  | down  |                                                |
| NM_030601| Clec2/Clec1 | 4.83  | down  | chloride channel calcium activated 2          |

**Table 4.** Top 25 differentially expressed Affymetrix Mouse Exon 1.0 St Array probe sets between the P53 knock out MEFs and the P53 knock out MEFs in which Mta1 is over expressed.
Hierarchical clustering analysis reveals influence of P53 on MTA1 gene regulation

Further, we performed the hierarchical clustering analysis with the genes that were differentially regulated between the Mta1-KO vs. Mta1-KO/Mta1, and P53-KO vs. P53-KO/Mta1. The normalized log2 ratio values of the differentially regulated genes in each comparison were used to obtain the heat maps (Figure 3B). The gene leaf nodes were optimized in the heat maps representing the differential regulation of the genes between the samples. Color scale of the heat map depicts red as the highly expressed, green as low expressed and black as intermittent level of gene expression. Figure 3B shows the heat maps of all the comparisons, Mta1-KO with Mta1-KO/Mta1 and P53-KO with P53-KO/Mta1 representing the differential expression of the genes. We found that 64% of the differentially regulated genes were up-regulated in WT vs. Mta1-KO where as it greatly reduced to 30% in Mta1-KO vs. Mta1-KO/Mta1 followed by further reduction to 22% in P53-KO vs. P53-KO/Mta1. In the case of differentially regulated genes that were down regulated, the percentage of down regulated genes was 36% in WT vs. Mta1-KO, 70% in Mta1-KO vs. Mta1-KO/Mta1 and 78% in P53-KO vs. P53-KO/Mta1. In summary, we observe 78% of genes down regulated by Mta1 due to the absence of P53 whereas about 64% of Mta1 regulated genes were up regulated in the presence of P53 (Figure 3C).

Table 5. GO Analysis of the genes differentially regulated between the WT & Mta1-KO with ≥2.0 fold change.

| GO ACCESSION | GO Term                      | p-value | Corrected p-value | Count in Selection | % Count in Selection | Count in Total | % Count in Total |
|--------------|-----------------------------|---------|-------------------|--------------------|----------------------|----------------|-----------------|
| GO:0005576   | extracellular region        | 1.87E-27| 4.82E-22          | 286                | 44.83                | 2627           | 14.72           |
| GO:0044421   | extracellular region part   | 2.27E-25| 2.92E-20          | 244                | 38.24                | 2157           | 12.08           |
| GO:0005615   | extracellular space         | 2.75E-23| 2.36E-18          | 229                | 35.89                | 2038           | 11.42           |
| GO:0007155   | cell adhesion               | 2.10E-17| 1.08E-12          | 78                 | 12.23                | 541            | 3.03            |
| GO:0022610   | biological adhesion         | 2.10E-17| 1.08E-12          | 78                 | 12.23                | 541            | 3.03            |
| GO:006955    | immune response             | 1.17E-14| 5.02E-10          | 59                 | 9.25                 | 405            | 2.27            |
| GO:0031012   | extracellular matrix        | 9.71E-14| 3.57E-09          | 50                 | 7.84                 | 279            | 1.56            |
| GO:0005578   | proteinaceous extracellular matrix | 2.06E-13| 6.64E-09          | 49                 | 7.68                 | 275            | 1.54            |
| GO:0005488   | binding                     | 2.37E-13| 6.80E-09          | 446                | 69.91                | 10687          | 59.87           |
| GO:0005515   | protein binding             | 2.79E-12| 2.01E-07          | 325                | 50.94                | 5094           | 28.54           |
| GO:0002376   | immune system process       | 2.35E-11| 1.96E-06          | 59                 | 9.25                 | 658            | 3.69            |
| GO:0009653   | anatomical structure morphogenesis | 4.52E-09| 9.71E-05          | 28                 | 4.39                 | 1002           | 5.61            |
| GO:0005509   | calcium ion binding         | 5.37E-09| 9.89E-05          | 88                 | 13.79                | 791            | 4.43            |
| GO:0032502   | developmental process       | 5.06E-09| 9.89E-05          | 84                 | 13.17                | 2682           | 15.03           |
| GO:0048513   | organ development           | 7.10E-09| 1.14E-04          | 28                 | 4.39                 | 1339           | 7.50            |

Note: Continued in Table S8.
doi:10.1371/journal.pone.0017135.t005
Gene Ontology analysis shows the genes involved in cellular functions regulated by Mta1 in the presence/absence of P53

To further investigate the functions of the genes regulated by Mta1, we performed Gene Ontology (GO) analysis on all the sets of genes that are regulated by Mta1 with/without P53 background with a p-value cutoff set to 0.1. The possible functions of the gene sets were broadly classified, in GO program, into three categories namely i) Cellular Component ii) Molecular Function and iii) Biological Process. We found that the 40.23% of the differentially regulated genes in the comparison of WT vs. Mta1-KO, with p-value: 0.1617 related to molecular function, followed by 34.97% with p-value: 0.148 connected to Cellular Component and 24.79% of the genes with p-value: 0.0944 were associated with the biological process (Figure 4A). In contrast to the above, when Mta1-KO and Mta1-KO with reintroduced Mta1 were compared there were no genes that match the GO terms in the Molecular functioning, whereas majority of them (77.78%, p-value: 0.6994) were related to the cellular component (Figure 4B). With the corrected p-value cut off 0.1 and in the presence of P53, Mta1 doesn’t regulate any genes responsible for molecular functioning. The majority of the genes (78.12%) are associated with Cellular Component and 21.88% are associated to Biological Process (Figure 4C). In contrast to the above, in the absence of P53, 49.8% (p-value: 0.1212) differentially expressed genes were linked to the Cellular Component, 30.61% (p-value: 0.1777) to Molecular Function and 19.59% (p-value: 0.1253) related to Biological Process (Figure 4D). Tables with GO terms for all the above mentioned comparisons are shown in Table 5, 6, 7 and 8 respectively. Table 5 and Table 8 are continued as Tables S8 and S9 respectively.

Ingenuity Pathways Analysis highlights the critical role of Mta1 in cancer signaling in the presence/absence of P53

Ingenuity pathways analysis was performed on all the genes that were identified to be regulated by Mta1 with/without P53. With p-value<0.05, Fischer’s exact test was applied and we found top 15 significant functions and canonical pathways in which the genes regulated by Mta1 might play a significant role. The most likely functions of the genes regulated by Mta1 in the presence of P53 are Inflammatory Response followed by Cancer and Gastrointestinal Diseases (Figure 5 upper panel). Top 15 canonical pathways of these genes were identified with the p-value<0.05 and threshold value of log (p-value): 0.05. The significant pathways which include LXR/RXR activation, Interferon signaling, Antigen presentation pathway and Activation of IRF by cytosolic pattern Recognition Receptors were shown in Figure 5 (lower panel). From these observations we propose that Mta1 might have critical functional role in orphan nuclear receptor activation, inflammation and infections. Similarly, top 15 plausible functions of the genes regulated by Mta1 in the absence of P53 were identified and shown in Figure 6 (upper panel). The most significant function of the genes was found to be related to ‘Cancer’ followed by ‘Cellular Movement’ and ‘Connective Tissue Disorders’. The top 15 canonical pathways in which the genes might be involved were identified and shown in the Figure 6 (lower panel). The most significant function of the genes was found to be related to ‘Cellular Movement’ followed by ‘Cancer’ and ‘Connective Tissue Disorders’. Clearly the genes regulated by Mta1 in the absence of P53 highlight the typical oncogenic character of Mta1 and its possible major role in several cancers and oncogenic signaling pathways.

**Table 6.** GO Analysis of the genes differentially regulated between the Mta1-KO and Mta1- KO/Mta1 probes with ≥2.0 fold change.

| GO ACCESSION | GO Term                  | p-value | corrected p-value | Count in Selection | % Count in Selection | Count in Total | % Count in Total |
|--------------|--------------------------|---------|-------------------|---------------------|----------------------|----------------|-----------------|
| GO:0044421   | extracellular region part | 2.56E-12| 0.00              | 53                  | 72.60                | 2157.00        | 12.08           |
| GO:0005615   | extracellular space      | 1.18E-11| 0.00              | 53                  | 72.60                | 2038.00        | 11.42           |
| GO:0005576   | extracellular region     | 2.18E-11| 0.00              | 61                  | 83.56                | 2627.00        | 14.72           |
| GO:006955    | immune response          | 1.01E-07| 0.01              | 16                  | 21.92                | 405.00         | 2.27            |
| GO:0026010   | biological adhesion      | 6.43E-06| 0.28              | 15                  | 20.55                | 541.00         | 3.03            |
| GO:0007155   | cell adhesion            | 6.43E-06| 0.28              | 15                  | 20.55                | 541.00         | 3.03            |

doi:10.1371/journal.pone.0017135.t006

**Table 7.** GO Analysis of the bona fide Mta1 regulated genes in the presence of P53 with ≥2.0 fold change.

| GO ACCESSION | GO Term                  | p-value | corrected p-value | Count in Selection | % Count in Selection | Count in Total | % Count in Total |
|--------------|--------------------------|---------|-------------------|---------------------|----------------------|----------------|-----------------|
| GO:0044421   | extracellular region part | 2.30E-12| 2.97E-07          | 43                  | 78.18                | 2157.00        | 12.08           |
| GO:0005615   | extracellular space      | 1.48E-12| 2.97E-07          | 43                  | 78.18                | 2038.00        | 11.42           |
| GO:0005576   | extracellular region     | 8.02E-12| 6.89E-07          | 48                  | 87.27                | 2627.00        | 14.72           |
| GO:006955    | immune response          | 8.12E-08| 0.0052            | 13                  | 23.64                | 405.00         | 2.27            |

doi:10.1371/journal.pone.0017135.t007
The identified Mta1 regulated candidate genes and their human homologs follow similar expression profile in MEFs and human breast cancer cell lines

Based upon our analysis and our laboratory interest, some of the genes that were regulated by Mta1 with/without P53 back ground were selected for the validation using the RT-qPCR assays. Validations were first performed in the MEFs followed by the MCF7 human breast cancer cell line. The results showing relative mRNA levels, for the selected genes, are presented in Figure 7. The controls are compared with the treatments and if the trend of expression (up or down regulation) in qPCR is in agreement with the microarray gene expression, then the respective homologous expression (up or down regulation) in qPCR is in agreement with the above described microarray microarray data. We also selected candidate genes that are regulated by Mta1, in the absence of P53, from the microarray data and subjected them to the RT-qPCR validation. The relative expression levels of these genes in the controls and treatments are shown in the Figure 7 (7D, 7E, 7F). The data clearly shows the agreement between the microarray and the RT-qPCR results. We have chosen Rnf144a which contains RING figure motif and established to have role in protein-DNA and protein-protein interactions. We found that Rnf144a was upregulated in the absence of P53 and over expression of Mta1. Another selected candidate was Hmmr, which encodes Hyaluronan mediated motility receptor protein that is found to be expressed in the breast tissue. It is notable that the Hmmr gene was found to be down regulated in P53-KO, whereas it was up regulated in P53-KO/Mta1 when compared with the WT. The third candidate gene, Krfl5 which encodes a protein called kruppel like factor-15 was found to be highly up-regulated in P53-KO MEFs where as it is only weakly up-regulated in P53-KO/Mta1, when compared with the wild type. The expression levels from the RT-qPCR assay and the Affymetrix microarray data are in perfect agreement for all the chosen candidates in MEFs (Figure 7).

RT-qPCR assays were also conducted with human homologs to validate the genes that were believed to be regulated by Mta1 in MEFs. This step of validation is performed with the Mta1 knock down (Mta1 siRNA) in MCF7 compared with the non-target control siRNA. Similar differential gene expression levels were observed among all the genes (VWA5A, EG2R, RNF144A, KLF15, and HMMR) which are similar to the microarray expression profile (Figure 8).

Table 8. GO Analysis of the bona fide Mta1 regulated genes in the absence of P53 with ≥2.0 fold change.

| GO ACCESSION | GO Term                        | p-value       | corrected p-value | Count in Selection | % Count in Selection | Count in Total | % Count in Total |
|--------------|--------------------------------|---------------|-------------------|--------------------|---------------------|----------------|------------------|
| GO:0005576   | extracellular region           | 8.45E-38      | 2.18E-32          | 122                | 89.05               | 2627           | 14.72            |
| GO:0044241   | extracellular region part      | 7.08E-29      | 9.12E-24          | 99                 | 72.26               | 2157           | 12.08            |
| GO:0005615   | extracellular space            | 2.37E-23      | 2.03E-18          | 88                 | 64.23               | 2038           | 11.42            |
| GO:0005578   | proteinaceous extracellular matrix | 1.84E-15      | 1.19E-10          | 27                 | 19.71               | 275            | 1.54             |
| GO:0031012   | extracellular matrix           | 2.64E-15      | 1.36E-10          | 27                 | 19.71               | 279            | 1.56             |
| GO:0006954   | inflammatory response          | 3.07E-08      | 0.0013978         | 7                  | 5.11                | 204            | 1.14             |
| GO:007155    | cell adhesion                  | 1.83E-07      | 0.00522946        | 21                 | 15.33               | 541            | 3.03             |
| GO:0022610   | biological adhesion            | 1.83E-07      | 0.00522946        | 21                 | 15.33               | 541            | 3.03             |
| GO:0005509   | calcium ion binding            | 7.26E-07      | 0.0170145         | 30                 | 21.90               | 791            | 4.43             |
| GO:0009605   | response to external stimulus  | 6.98E-07      | 0.0170145         | 7                  | 5.11                | 465            | 2.61             |
| GO:0044268   | multicular organisal protein metabolic process | 1.99E-06      | 0.0264874         | 5                  | 3.65                | 16             | 0.09             |
| GO:0004714   | transmembrane receptor protein tyrosine kinase activity | 2.05E-06      | 0.0264874         | 7                  | 5.11                | 61             | 0.34             |
| GO:0030574   | collagen catabolic process     | 1.99E-06      | 0.0264874         | 5                  | 3.65                | 16             | 0.09             |
| GO:0008237   | metallopeptidase activity      | 1.68E-06      | 0.0264874         | 12                 | 8.76                | 181            | 1.01             |
| GO:0044259   | multicular organisal macromolecular metabolic process | 1.99E-06      | 0.0264874         | 5                  | 3.65                | 16             | 0.09             |

Note: Continued in Table S9. doi:10.1371/journal.pone.0017135.t008

Formal validation was first performed in the MEFs followed by the human breast cancer cell line. The results showing relative mRNA levels, for the selected genes, are presented in Figure 7. The controls are compared with the treatments and if the trend of expression (up or down regulation) in qPCR is in agreement with the microarray gene expression, then the respective homologous expression (up or down regulation) in qPCR is in agreement with the above described microarray microarray data. We also selected candidate genes that are regulated by Mta1, in the absence of P53, from the microarray data and subjected them to the RT-qPCR validation. The relative expression levels of these genes in the controls and treatments are shown in the Figure 7 (7D, 7E, 7F). The data clearly shows the agreement between the microarray and the RT-qPCR results. We have chosen Rnf144a which contains RING figure motif and established to have role in protein-DNA and protein-protein interactions. We found that Rnf144a was upregulated in the absence of P53 and over expression of Mta1. Another selected candidate was Hmmr, which encodes Hyaluronan mediated motility receptor protein that is found to be expressed in the breast tissue. It is notable that the Hmmr gene was found to be down regulated in P53-KO, whereas it was up regulated in P53-KO/Mta1 when compared with the WT. The third candidate gene, Krfl5 which encodes a protein called kruppel like factor-15 was found to be highly up-regulated in P53-KO MEFs where as it is only weakly up-regulated in P53-KO/Mta1, when compared with the wild type. The expression levels from the RT-qPCR assay and the Affymetrix microarray data are in perfect agreement for all the chosen candidates in MEFs (Figure 7).

RT-qPCR assays were also conducted with human homologs to validate the genes that were believed to be regulated by Mta1 in MEFs. This step of validation is performed with the Mta1 knock down (Mta1 siRNA) in MCF7 compared with the non-target control siRNA. Similar differential gene expression levels were observed among all the genes (VWA5A, EG2R, RNF144A, KLF15, and HMMR) which are similar to the microarray expression profile (Figure 8).
This study represents a complete genome wide screen for possible target genes of a transcriptional co regulator, Mta1. In addition to identifying “bona fide” Mta1 target genes, the influence of p53 on Mta1 gene regulation and molecular function has also been analyzed extensively. Emerging literature on Mta1 clearly establishes bidirectional interplay between the oncogene, MTA1 and the tumor suppressor, P53. Although Mta1 was found to be a component of the Nucleosome Remodeling and Deacetylase (NuRD) complex, recent studies from our laboratory establishes the functions of MTA1 in DNA-damage response in a P53-dependent and -independent manner [24,30,34]. This raises the compelling question of how P53 influences the gene regulation and overall function of MTA1. We attempted to address this question using microarray approach. The first goal of identifying “bona fide” Mta1 targets have been achieved by initial comparison of genes regulated by Mta1 wild type and the Mta1 knockout. Subsequently, this set of genes was compared with the set identified from the comparative study of Mta1 knock out vs. Mta1 re-expressed in the knock out MEFs. The common genes identified from both comparisons reflect the Mta1 “bona fide” targets (Table 3 and Table S5). To achieve the goal of defining oncogenic gene

**Figure 5. Ingenuity Pathway Analysis (Ingenuity Systems, Inc) of the genes that were regulated by Mta1 in the presence of P53 was performed.** The significance of each function or canonical pathway is determined based upon the p-values determined using Right tailed Fisher's exact test and with threshold less than 0.05. The top 15 possible functions and canonical pathways of the genes regulated by Mta1 in P53 dependent manner are shown. Ratio of number of genes in a given pathway satisfying the cutoff and total number of genes present in that pathway was determined by IPA.

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**Discussion**

This study represents a complete genome wide screen for possible target genes of a transcriptional co regulator, Mta1. In addition to identifying “bona fide” Mta1 target genes, the influence of p53 on Mta1 gene regulation and molecular function has also been analyzed extensively. Emerging literature on Mta1 clearly establishes bidirectional interplay between the oncogene, MTA1 and the tumor suppressor, P53. Although Mta1 was found to be a component of the Nucleosome Remodeling and Deacetylase (NuRD) complex, recent studies from our laboratory establishes the functions of MTA1 in DNA-damage response in a P53-dependent and -independent manner [24,30,34]. This raises the compelling question of how P53 influences the gene regulation and overall function of MTA1. We attempted to address this question using microarray approach. The first goal of identifying “bona fide” Mta1 targets have been achieved by initial comparison of genes regulated by Mta1 wild type and the Mta1 knockout. Subsequently, this set of genes was compared with the set identified from the comparative study of Mta1 knock out vs. Mta1 re-expressed in the knock out MEFs. The common genes identified from both comparisons reflect the Mta1 “bona fide” targets (Table 3 and Table S5). To achieve the goal of defining oncogenic gene
profile of Mta1, we have mimicked most cancer scenarios i.e. loss of P53 and the over expression of oncogenes such as Mta1 and compared P53 knock out and P53 knockout in which there was over expression of Mta1 gene. Interestingly, the identified 266 genes (Table 4) are mostly involved in DNA damage response. This is in agreement with the recently established Mta1 function in DNA damage which is independent of P53. Together, these data further illustrate the possible genes that are regulated by Mta1 “fail safe” mechanism which occurs due to loss of P53.

The gene ontology analysis again highlights the influence of P53 on Mta1 function. If P53 is present, the targets regulated by Mta1 play no remarkable role in the molecular functions such as catalytic activity or binding where as in the absence of P53 about 39% of genes regulated by Mta1 are involved in molecular functions, clearly indicating the influence of P53 on Mta1 gene regulation. Further extensive comparative analysis of all the data using IPA and networks reveals two distinct functional themes. In the presence of P53, the genes regulated by Mta1 are mainly involved in the inflammatory response cancer and cellular movement. Whereas, in the absence of P53 the genes regulated are predominantly related to cancer signaling reflecting the significance of Mta1 in cancer. In agreement to the above mentioned observations, the top networks and pathways regulated by Mta1 in the presence of P53 appear to be antimicrobial response, inflammatory response and carbohydrate metabolism (Figure 9). For instance, most of the genes regulated by Mta1 revolve around major complexes such as IRF7 (Interferon regulatory factor-7), which has been shown to play important role in the transcriptional activation of virus inducible cellular genes. In

Figure 6. Ingenuity Pathway Analysis (Ingenuity Systems, Inc) of the genes that were regulated by Mta1 in the absence of P53 was performed. Fisher’s exact test was used and threshold of 0.05 was set as the cutoff. Top 15 functions associated with the genes and the top 15 canonical pathways in which these genes might have a role are shown.

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addition, as a part of innate antiviral immunity, the induction of systemic IFN takes place through IRF7 [42]. Another gene, Immunity-related GTPases (IRG) that play an important role in defense against intracellular pathogens and NFκB complex which has been well ascertained to be regulated by Mta1 were also found in the network. In agreement with our data recent studies suggest MTA1 regulating its target genes either by acting as a corepressor [18,19] or as transcriptional coactivator [20,21] via interacting with RNA polymerase II. Together, these findings raise the possibility that Mta1 may play a significant role in protecting regulation of innate immune response by directly modulating several pathways including NF-κB signaling [43]. Our data further supports this notion and emphasizes the role of Mta1 in inflammation.

In contrast to that, in the absence of P53, Mta1 target genes appear to be mainly involved in cancer and genetic disorders.

Figure 7. RT-qPCR validation of the microarray data showing the differential regulation of the selected genes in the MEFs. The RNA extracted from the fibroblasts of the wild type (light blue bars) mice was used as the control and the treatments were Mta1-KO (red bars) & Mta1-KO/ Mta1(green bars). In the presence of P53 relative mRNA levels of the genes Aw551984, Egr2, Phf17 were compared in all the three samples. The relative mRNA levels from microarray for the same sample sets were plotted and compared with the RT-qPCR. As expected, opposite trends of expression were observed between the knock out and re-expression models. To validate the genes regulated by Mta1 in the absence of P53 relative mRNA levels were compared among the three samples wild type (light blue bars), P53-KO (dark blue bars) & P53-KO/Mta1(yellow bars) for the genes Hmmr, Klf15, Rnf144a. The relative mRNA levels from the microarray were plotted and compared with RT-qPCR. Opposite trends of expression were observed between the P53-KO and P53-KO/Mta1 treatments when compared with the wild type sample. doi:10.1371/journal.pone.0017135.g007
For instance, many direct and indirect interactions were found with well studied cancer molecules such as Kirsten rat sarcoma viral oncogene homolog (KRAS) [44,45], epidermal growth factor receptor (EGFR) [46–48] and vascular endothelial growth factor (VEGF) [49,50] further highlighting the P53-independent central functions of Mta1 in metastasis and cancer. Thus, MTA1 appears to be playing distinct molecular functions depending on the status of P53. In summary, our data presents complete gene profiling of Mta1 in the presence and absence of P53 representing a new resource and guide for future area of Mta1 research which is unexplored but comprises several key elements that could be employed in the development of anticancer therapeutics and identification of novel functions of Mta1.

Materials and Methods

Cell Culture
Wild type Murine Embryonic Fibroblasts (MEFs) and Mta1-KO MEFs were obtained as described previously [35]. P53 knockout MEFs were kindly provided by Dr. G. Lozano (M.D. Anderson Cancer Center, Houston, TX). MEFs and MCF7 (Michigan Cancer Foundation-7 human breast cancer cell line) were cultured in DMEM/F-12 medium containing 10% fetal bovine serum (FBS-Difco Laboratories, Detroit, Michigan) and 1% antibiotic-antimycotic solution in a humidified 5% CO2 at 37°C. Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Human MTA1 and non target control siRNA smart pools were obtained from Dharmacon, Inc. MCF7 cells were obtained from ATCC (ATCC number: HTB-22) Transfection into MCF7 cells was performed with Oligofectamine reagent (Invitrogen) and manufacturer’s instructions were followed. Cells were collected after 36–48 hours after transfection.

RNA Extraction & Microarray Gene Expression Arrays
Microarray gene expression assays have been performed as described previously [30]. In summary total RNA was extracted from the cells using Trizol (Invitrogen, Carlsbad, CA) and the
manufacturer’s protocol was followed. The quality and the concentrations of the extracted RNA were checked using the Nano-Drop (Thermo scientific). RNA was then purified using RNeasy Mini Kit (Qiagen, Valencia, CA) and the integrity was tested on 6000 NanoChips using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Finally, Affymetrix Mouse Exon 1.0 ST arrays were used for the hybridization, arrays were scanned and the expression data was obtained in the form of .CEL files.

Microarray data analysis
To analyze the data generated from the microarray experiments we used GeneSpring GX 10.0.2 (Agilent technologies, Inc) software package for the data Quality Control and the statistical analysis of the microarray data. The method of Benjamini and Hochberg was applied for the multiple corrections with a p-value cut-off of 0.05 and fold change ≥±2.0 to obtain the statistically significant genes. Heat maps for individual arrays were generated and the hierarchical cluster analysis was performed using MeV 4.5 [51]. Gene Ontology analysis was performed using GeneSpring GX 10.0.2 (Agilent Technologies). All data is MIAME compliant and the raw data has been deposited in Gene Expression Omnibus (GEO) as detailed on the Microarray Gene expression Data Society (MGED) society website (http://www.mged.org/Workgroups/MIAME/miame.html).

Functional and Network Analysis
We used the Ingenuity pathway Analysis (Ingenuity Systems, Inc) to find the statistically significant pathways, functions and the networks in which the identified genes regulated by Mta1 are possibly involved. Fischer’s exact test was used to identify the significant functions and pathways represented within the respective gene sets.

RT-qPCR
RT-qPCR analysis was performed following the protocol described previously [23-24]. In order to validate the microarray data we selected candidate genes and RT-qPCR assays were conducted (all the primers used in this study are listed in Table S10). 2 µg of the total RNA was used for the first strand cDNA synthesis using the superscript III First-strand sys kit (Invitrogen), according to the manufacturer’s instructions in 21-µl reactions. These reactions were diluted 1:10 with nuclease-free water. Duplicates of the qPCR contained 2-µl of the first strand cDNA, 1-µl of the intron-spanning primers specific for that particular mRNA sample, 2 µl of nuclease free water and 5 µl of SYBR Green (Bio-Rad, Hercules, CA). Reactions (10 µl) were run in 96-well optical plates (Bio-Rad, Hercules, CA). Average threshold cycle (Ct) values of 18S mRNA (chosen as normalizer) were subtracted from the corresponding average Ct values of a target mRNA to obtain ΔCt values. The relative RNA levels were then expressed as 2^{−ΔΔCt}.
Supporting Information

Figure S1  Western blot assay of the five samples (WT, Mta1-KO, Mta1-KO/Mta1, P53-KO, and P53-KO/Mta1) was performed using Mta1 antibody as described previously [30] and the levels of Mta1 in all the five conditions are shown. α-Tubulin was used as the internal control.

Table S1  Complete list of the differentially expressed probe sets on the Affymetrix Mouse Exon 1.0 ST arrays between the wild type and the Mta1 knockout MEFs.

Table S2  The statistical summary of the log 2 ratio values for the differentially expressed probe sets on the Affymetrix Mouse Exon 1.0 ST arrays between the wild type and the Mta1 knockout MEFs.

Table S3  Complete list of the differentially expressed probe sets on the Affymetrix Mouse Exon 1.0 ST arrays between the Mta1 knockout (Mta1-KO) MEFs and Mta1 reintroduced into the knockout MEFs (Mta1-KO/Mta1).

Table S4  The statistical summary of the log 2 ratio values for the differentially expressed probe sets on the Affymetrix Mouse Exon 1.0 ST arrays between the P53 knockout (P53-KO) MEFs and Mta1 reintroduced into the knockout MEFs (Mta1-KO/Mta1).

Table S5  The ‘Bona fide’ genes that are regulated by Mta1.

Table S6  Complete list of the differentially expressed probe sets on the Affymetrix Mouse Exon 1.0 ST arrays between the P53 knockout (P53-KO) and P53 knockout MEFs with over expression of Mta1 (P53-KO/Mta1).

Table S7  The statistical summary of the log 2 ratio values for the differentially expressed probe sets on the Affymetrix Mouse Exon 1.0 ST arrays between the P53 knockout (P53-KO) and P53 knockout MEFs with over expression of Mta1 (P53-KO/Mta1).

Table S8  Gene Ontology analysis of the genes differentially regulated between WT & Mta1-KO with ≥2.0 fold change.

Figure 10. The genes differentially regulated (p-value<0.05 and fold change ≥±2.0) between the P53 knock out and the P53 knock out with Mta1 over expressed MEFs (Mta1 regulated genes independent of P53) were subjected to the network analysis and the most significant network found is associated with cancer and genetic disorders. Most of the genes in the network were found to be down regulated in P53-KO/Mta1 when compared with P53-KO. All the upregulated genes are shown in red and the down regulated genes are shown in green color.

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Table S9  Gene Ontology analysis of the ‘bona fide’ Mta1 regulated genes in the absence of P53 with ≥2.0 fold change. (DOC)

Table S10  Primer sequences for the candidate genes of Mus musculus and Homo sapiens that were used in the RT-qPCR assays are shown. (DOC)

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Author Contributions
Conceived and designed the experiments: KSG DQL. Performed the experiments: KSG DQL. Analyzed the data: JE RK. Contributed reagents/materials/analysis tools: DQL. Wrote the paper: JE RK.
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