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Mutant p53 drives invasion in breast tumors through up-regulation of miR-155

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Abstract

Loss of p53 function is a critical event during tumorigenesis, with half of all cancers harbouring mutations within the *TP53* gene. Such events frequently result in the expression of a mutated p53 protein with gain-of-function properties that drive invasion and metastasis. Here, we show that the expression of miR-155 was up-regulated by mutant p53 to drive invasion. The miR-155 host gene was directly repressed by p63, providing the molecular basis for mutant p53 to drive miR-155 expression. Significant overlap was observed between miR-155 targets and the molecular profile of mutant p53-expressing breast tumors *in vivo*. A search for cancer-related target genes of miR-155 revealed ZNF652, a novel zinc finger transcriptional repressor. ZNF652 directly repressed key drivers of invasion and metastasis, such as *TGFB1*, *TGFB2, TGFBR2, EGFR, SMAD2* and *VIM.* Furthermore, silencing of ZNF652 in epithelial cancer cell lines promoted invasion into matrigel. Importantly, loss of ZNF652 expression in primary breast tumors was significantly correlated with increased local invasion and defined a population of breast cancer patients with metastatic tumors. Collectively, these findings suggest that miR-155 targeted therapies may provide an attractive approach to treat mutant p53-expressing tumors.

Introduction

The p53 tumor suppressor is a transcription factor that plays a pivotal role in the prevention of neoplastic transformation. Disruption of the p53 pathway is arguably a universal requirement for the development of cancer and is ultimately achieved through either direct mutation of the *TP53* gene or indirect inactivation of downstream p53 signalling pathways. However, inactivation of the tumor suppressor function of p53 is not the only outcome of a *TP53* mutation. Increasing evidence suggests that the mutated p53 protein exhibits traits consistent with the acquisition of an oncogenic gain-of-function (1, 2). For example, mutations of *TP53* gene are frequently missense, involve similar codons within the p53 DNA binding domain and are expressed at remarkably high levels in tumor cells (1). Somatic *TP53* mutations occur in approximately 20% of breast tumors, which in turn define a population of particularly aggressive breast lesions associated with a poor prognosis (3, 4).

It is postulated that mutant p53 acquires new oncogenic properties as a result of local or global distortion of the protein structure, exposing previously hidden binding interfaces to facilitate gain-of-function interactions with novel binding partners. In particular, mutant p53 has been shown to acquire the ability to sequester p63 and p73, and this is thought to promote tumor invasion and metastasis (5, 6). So what are the key downstream target genes of p63 and p73 that are deregulated to promote tumor progression in cancer cells expressing mutant p53? Recent studies have identified the metastasis suppressors SHARP1 and cyclin G2 as critical mutant p53 targets that are deregulated through loss of p63 signalling (6). The expression of these two genes alone defined a subset of particularly aggressive breast tumors associated with poor patient outcome (6). Such examples demonstrate the potential utility of targets of mutant p53 as robust clinical prognostic tools and therapeutic targets in mutant p53 expressing tumors, however a complete understanding of the

mechanisms and pathways exploited by mutant p53 to drive tumorigenesis remain poorly understood. A major aspect of the anti-metastatic activity of p63 resides in its ability to regulate non-coding RNAs (7) and microRNA processing complexes (8), yet the ability of mutant p53 to dysregulate these pathways remains unexplored. We report here that mutant p53 inactivates the p63 signalling pathway to up-regulate the expression of an oncogenic microRNA, miR-155. In turn, elevated miR-155 levels promote cellular transformation and invasion through the targeting of key suppressors of metastasis.

Results

miR-155 promotes migration, invasion and amoeboid transformation

Several microRNAs have been recently implicated in the promotion of tumorigenesis and have emerged as potential therapeutic targets. In particular, miR-155 is considered as an oncomiR as it is up-regulated in breast tumors (9) and this increased expression is associated with the promotion of tumorigenesis (10). To gain a wider appreciation for the role of miR-155 during breast tumorigenesis, we measured miR-155 levels in established breast cancer cell lines of epithelial (non-invasive) and mesenchymal (invasive) origin (11). Indeed, miR-155 was almost absent in epithelial lines, but was highly expressed in 4 out of 5 of mesenchymal lines (Supplementary Figure 1), suggesting an upregulation of miR-155 during epithelial to mesenchymal transition (EMT) of breast cancers. Importantly, this observation was not restricted to breast cancer cell lines, as miR-155 levels were absent in the majority of epithelial lines from the NCI-60 panel of cancer cell lines whilst showing significantly higher expression in the mesenchymal lines (Figure 1A). To assess whether miR-155 can drive EMT in breast tumors, we stably expressed miR-155 in the ZR-75-1 epithelial breast cancer cell line. Although present at only modest levels as compared to some mesenchymal breast cancer lines (Supplementary Figure 2), exogenous miR-155 dramatically enhanced the ability of ZR-75-1 cells to migrate in a scratch-wound assay (Figure 1B). Expression of miR-155 also endowed ZR-75-1 cells with a newly acquired ability to invade through matrigel (Figure 1C). Interestingly, ZR-75-1 cells expression induced an amoeboid transformation of this epithelial cell line, as characterised by the enlarged filamentous cytoplasm with frequent protrusion of lamellipodia from these ZR-75-1 cells expressing miR-155 (Figure 1D).

To ensure that these observations were not restricted to the ZR-75-1 cell line, miR-155 was also stably expressed in the non-malignant breast epithelial cell line, MCF-10A (Supplementary

Figure 2B). Exogenous miR-155 enhanced the ability of MCF-10A cells to migrate (Figure 1B) or invade through matrigel (Figure 1C) to similar extents of that observed in ZR-75-1 cells. Although miR-155 was able to enhance the frequency of lamellipodia protrusions from MCF-10A cells (Figure 1D), it did not alter the size of the MCF-10A cells (data not shown). These findings indicate that miR-155 has general pro-migratory and pro-invasive characteristics in epithelial breast cell lines, however the mode of transformation (i.e. amoeboid) may be specific to cell lines.

To examine the altered transcriptional events associated with the miR-155-mediated amoeboid transformation and accompanying pro-migratory and invasive characteristics, the expression levels of 84 EMT-related genes (see Materials and Methods) were assessed by real-time PCR (Figure 1E). Indeed, expression of miR-155 in ZR-75-1 cells was associated with a significant up-regulation of numerous mesenchymal markers, with the major transcriptional reprogramming by miR-155 converging upon the TGF-β signalling pathway. Therefore, we proposed the miR-155 may be aberrantly driving the TGF-β signalling pathway in the absence of an exogenous TGF-β ligand. Indeed, the addition of exogenous TGF-β to ZR-75-1 cells phenocopied the effects of miR-155 expression, as characterised by an increased invasive capacity and an amoeboid transformation involving enlarged cytoplasm and increased frequency of lamellipodia (Supplementary Figure 3). Thus, our findings demonstrate that miR-155 aberrantly stimulates the TGF-β pathway in the absence of exogenous TGF-β.

miR-155 is a target microRNA of mutant p53

Mutant p53 and miR-155 expression are both associated with invasive properties *in vivo* (2, 10), which prompted us to investigate whether they promote malignant transformation through a similar oncogenic axis. We initially determined if miR-155 can phenocopy mutant p53 in its ability to drive invasion and mensenchymal transformation when expressed in the same genetic background.

Expression of miR-155 in H1299 cells significantly enhanced their invasive capabilities and drove a mesenchymal phenotype with close resemblance to that observed upon induced mutant p53 expression (Supplementary Figure 4). These observations indicate that miR-155 and mutant p53 may drive overlapping oncogenic pathways. Since mutation of *TP53* is considered an early event during breast tumorigenesis (12, 13), we speculated that miR-155 may be a down-stream target of mutant p53 that reprograms the cancer transcriptome to drive invasion and metastasis. Indeed, induced expression of either the p53 mutant R248Q or R282W in the p53-null H1299 background was associated with a dose-dependent increase in MIR155HG (the precursor transcript for miR-155) expression or mature miR-155 levels (Figure 2A). We next examined if endogenous mutant p53 can constitutively regulate the expression of miR-155 levels. The mesenchymal breast cancer cell line BT549 possesses the highest miR-155 levels of those tested (Supplementary Figure 1) and also expresses an endogenous p53 mutant, R249S. Knockdown of this endogenous p53 mutant in BT549 cells resulted in a significantly reduced level of miR-155 (Figure 2B), confirming a role for mutant p53 in the aberrant activation of miR-155.

We next examined if miR-155 was up-regulated by mutant p53 *in vivo*, which was achieved through the analysis of a publically available cohort of 100 breast tumors in which both the global microRNA expression levels had been profiled and the *TP53* gene sequenced. Indeed, miR-155 expression levels were significantly higher in breast tumors with p53 mutations as compared to those breast tumors that had retained wild-type p53 (Figure 2C). Furthermore, miR-155 expression was most elevated in the basal breast tumors, the most aggressive breast cancer subtype frequently associated with p53 mutations (Figure 2D). Collectively, these observations support the notion of miR-155 as a mutant p53 target gene in breast tumors.

miR-155 is directly repressed by p63

Mutant p53 drives invasion and metastasis through inactivation of targets such as p63 (5, 6), ablating the p63 signalling pathways responsible for the suppression of invasion and metastasis. Therefore, we examined a role for p63 in the transcriptional control of *MIR155HG*. *In silico* analysis using the p63Scan software (14) identified a putative p63 response element (RE) in the 3rd exon of the *MIR155HG* gene. Chromatin immunoprecipitation (ChIP) experiments were used to subsequently demonstrate that endogenous p63 was directly recruited to this consensus p63-RE in MCF10A, a non-malignant breast epithelial cell line (Figure 2E). This association of p63 with the *MIR155HG* p63-RE was significantly reduced upon silencing of endogenous p63 in MCF10A cells. Knockdown of p63 also resulted in a significantly increased expression level of both the MIR155HG transcript and the mature miR-155 (Figure 2F). Collectively, these findings indicate that p63 functions as a direct transcriptional repressor of miR-155. Previous observations have also shown that miR-155 was repressed by wild-type p53, albeit through an unknown mechanism (15). Indeed, induction of wild-type p53 in H1299 cells repressed MIR155HG expression (Supplementary Figure 5). Our studies provide mechanistic insight into this repression, as ChIP experiments demonstrate that this induced wild-type p53 protein is also recruited to this newly identified p63-RE located within the *MIR155HG* gene (Figure 2E). These findings suggest a redundancy of function between wild-type p53 and p63 in the control of miR-155 expression via transcriptional repression of the precursor MIR155HG transcript.

Identification of downstream targets of the miR-155 • mutant p53 axis

Having established miR-155 as a downstream oncomiR of mutant p53, we sought to identify the specific targets of the miR-155 • mutant p53 axis that drive invasion in breast tumors. To obtain an understanding of the landscape of miR-155 targets, a comprehensive list of *bone fide* miR-155

targets was collated from the literature (Supplementary Table 1). Selection criteria for these published miR-155 target genes included both the demonstration of endogenous transcript regulation by miR-155 and validation of the miR-155 seed sequence through a reporter assay. This list contains 140 genes and is enriched with known tumor suppressors. Therefore, we speculated that mutant p53 may indirectly down-regulate the expression of these genes through aberrant up-regulation of miR-155. A relationship between the defined miR-155 targets and mutant p53 in breast cancer was examined through analysis of publically available transcript profiles of 251 p53-sequenced primary breast tumors (4). Remarkably, 42 of these 140 miR-155 targets (30%) showed a significantly lower expression in breast tumors with mutant p53 compared with tumors with wild type p53 (Figure 3A; Supplementary Table 2). These 42 genes can be considered as the key downstream targets of the mutant p53 • miR-155 axis and are therefore likely to play critical roles in the suppression of breast cancer invasion and metastasis.

The potential of the expression of these 42 genes to predict breast cancer metastasis was evaluated through the analysis of a publically available dataset of expression profiling in 78 breast tumors (16). Low expression of four genes (*ZNF652*, *PDCD4*, *TCF12* or *IL17RB*) was associated with a significantly higher frequency of metastasis-related poor outcomes (Figure 3B). This suggests that these four miR-155 • mutant p53 targets drive pathways that prevent breast cancer invasion and metastasis. ZNF652 was considered an excellent candidate for further investigation as this gene encodes a classical zinc-finger DNA binding transcription factor that functions as a transcriptional repressor (17, 18), potentially orchestrating downstream pathways of the miR-155 • mutant p53 axis.

ZNF652 is an epithelial marker and suppresses tumor cell invasion

Initially we measured ZNF652 transcript and protein levels in breast cancer cell lines, and found they are highly expressed in some epithelial cell lines, but are significantly down-regulated in mesenchymal cell lines (Figure 4A and B). The expression profile of ZNF652 in these cell lines resembles an inverse reflection of their miR-155 expression profiles, which is consistent with previous reports demonstrating that miR-155 targets the ZNF652 transcript through a specific seed sequence in the 3'UTR (19, 20). Indeed, over-expression of miR-155 markedly reduced endogenous ZNF652 protein levels in the H1299 epithelial lung carcinoma cell line (Figure 4C), further demonstrating ZNF652 as a miR-155 target. In a wider survey of ZNF652 expression in breast cell lines using gene expression microarray data of 51 breast cancer cell lines (21) classified into luminal (epithelial) and basal B (mesenchymal) (11), the expression of ZNF652 is highly negatively correlated with vimentin expression (r^2=0.33, p<0.0001) (Supplementary Figure 6). The luminal (epithelial) cell lines have a significantly higher expression of ZNF652 compared with the basal B (mesenchymal) cell lines (p<0.0001), while high expression of vimentin is a well known marker of mesenchymal cells. These data define ZNF652 as a novel marker for breast epithelial cells and indicate that ZNF652 expression is down-regulated during mesenchymal transformation, presumably in response to up-regulation of miR-155. We further examined if the loss of ZNF652 alone is sufficient to drive invasion. ZNF652 expression was silenced using a mixture of two specific siRNAs in H1299 cells, and their subsequent capacity to invade through matrigel assessed (Figure 4D). Ablation of ZNF652 expression resulted in a significant increase in the invasive potential of H1299 cells. These data indicate that ZNF652 is a key downstream target of the miR-155 • mutant p53 axis that regulates the invasive properties of tumor cells.

ZNF652 is a master regulator of the EMT gene network

ZNF652 is a repressor of gene transcription (17), and we hypothesised that this transcription factor may suppress invasion and metastasis through constitutive repression of key drivers of mesenchymal transformation. Identification of the key ZNF6562 targets will further elucidate the downstream regulatory network responsible for metastasis in mutant p53-miR-155 expressing breast tumors. We identified putative consensus ZNF652 DNA binding sequences (AnGGGTTAA) (18) in the classical promoter region, first intron or 3' UTR of *TGFB1*, *TGFB2, TGFBR2, EGFR, SMAD2* and *VIM* (Figure 4E). Direct recruitment of endogenous ZNF652 to these gene regulatory elements within *TGFB1*, *TGFB2, TGFBR2, EGFR, SMAD2* and *VIM* was demonstrated through ChIP analysis in ZR-75-1 cells (Figure 4E), confirming these genes as direct targets of ZNF652. Silencing of ZNF652 expression in the ZR-75-1 cell line also resulted in a de-repression of several of these target genes, including *TGFB1*, *TGFBR2, EGFR* and *VIM* (Supplementary Figure 7). EMT gene expression profiling (as described in Figure 1E) was subsequently performed to assess the global influence of loss of ZNF652 expression on ZR-75-1 cells. There was considerable overlap between the EMT gene expression profiles in response to loss of ZNF652 expression or over-expression of miR-155 (Supplementary Figure 8), suggesting that a large proportion of EMT gene regulation by miR-155 may be facilitated through its ability to target ZNF652. The identified ZNF652 targets converge upon the TGF-β signalling pathway, as was observed for miR-155. Thus, we hypothesize that ZNF652 may suppress invasion through repression of the TGF-β signalling pathway. Indeed, forced expression of ZNF652 ablated the ability of exogenous TGF-β to activate SMAD proteins, as demonstrated using a SMAD reporter assay (Supplementary Figure 9). Collectively, the data suggest that ZNF652 is a master repressor of the EMT gene network.

ZNF652 suppresses invasion *in vivo*

The potential of ZNF652 to suppress invasion *in vivo* was evaluated in human breast tumors by staining tumor microarrays of paraffin embedded formalin fixed tissues with our affinity purified polyclonal anti-ZNF652 antibody (Figure 5A). ZNF652 protein levels were assessed in 112 breast tumors of known invasive potential, as assessed by the TNM scoring available for each tumor. There is a highly significant association of low ZNF652 levels and increased propensity for tumor invasion in distant organs or structures ($p < 0.001$; Fisher exact test), thus demonstrating that ZNF652 is a *bone fide* suppressor of tumor cell dissemination and invasion.

Based on our evidence, we propose that epithelial cancers expressing mutant p53 acquire enhanced invasive and metastatic potential through up-regulation of miR-155. This oncomiR drives invasion by directly repressing the target transcript ZNF652, which as a consequence causes the de-repression of a network of EMT genes and acquisition of an invasive cell phenotype (summarised diagrammatically in Figure 5B). Collectively, this study provides evidence that miR-155 targeted therapies may provide an attractive approach to treat mutant p53 expressing breast tumors. Furthermore, histological assessment of the key targets of the mutant p53 • miR-155 axis may have prognostic implications in the clinical management of breast cancer patients.

Discussion

miR-155 drives breast cancer cell transformation and invasion

This study implicates a role for miR-155 in the transformation of the ZR-75-1 and MCF-10A epithelial breast cells (Figure 1). It is of particular interest that miR-155 could impart pro-migratory and pro-invasive properties to the non-malignant MCF-10A cell line, as these findings potentially indicate that miR-155 possesses transforming capabilities in the breast epithelium. In addition, miR-155 drove an amoeboid transformation of ZR-75-1 cells. The amoeboid transition of epithelial tumor cells represents a major mode of migration and invasion (22). This pro-invasive amoeboid transformation was associated with the formation of lamellipodia, which are typically restricted to highly mobile and invasive cells (23), properties not frequently associated with the epithelial ZR-75-1 cell line. Our findings are consistent with the pre-existing notion of miR-155 as a breast cancer oncomiR. Previous studies have shown that ectopic expression of miR-155 mimics in the MCF-7 or BT-474 breast cancer cell lines was associated with increased proliferation rates (10, 24). Furthermore, the ectopic expression of miR-155 mimics in the MDA-MB-231 breast cancer cell line accelerated the growth of mammary fat pad tumor xenograft tumors *in vivo* (10). Our findings suggest that these previously observed oncogenic activities of miR-155 in breast cancer cell lines result from its ability to rearrange the actin cytoskeleton to reconfigure the cell with a pro-invasive morphology (Figure 1).

In contrast to our findings, a previous report suggests that ectopic expression of miR-155 did not drive a mesenchymal phenotype in the NMuMG murine mammary epithelial cell line (25). Furthermore, ectopic expression of miR-155 had no influence on the proliferation or primary tumor growth of 4T1 murine mammary cell lines *(26)*. In fact, findings from Xiang et al suggest that miR-155

suppresses the ability of 4T1 cells to undergo EMT. Indeed, it appears that the biological activity of miR-155 in human cell lines (10, 24) is vastly different to that observed upon miR-155 expression in mammary cell lines that originate from mice (25, 26), suggesting a lack of conservation of miR-155 seed sequences in its target 3'UTRs across these two species.

The complex regulation of miR-155 expression

Herein, we demonstrated that either p53 or p63 can directly repress miR-155 levels through direct association with the promoter of *MIR155HG*. As such, a gain-of-function mutation in the *TP53* gene would act as a double-edged sword to activate miR-155 levels through both a loss of wild-type p53-mediated repression and also through the inactivation of p63-mediated repression as a result of the oncogenic activities of mutant p53 (5, 6). Findings from Adorno *et al* suggest that the presence of TGF-β is a critical factor for mutant p53 to sequester p63 from its target genes. Therefore, it is tempting to speculate a role for TGF-β in the regulation of miR-155 expression. This is indeed the case, as miR-155 has been previously shown to be upregulated upon TGF-β treatment (25). The nature of the relationship between miR-155 and TGF-β is quite complex, as we demonstrated that the major transcriptional reprogramming by miR-155 converged upon the TGF-β pathway and ectopic expression of miR-155 aberrantly sensitised cells to exogenous TGF-β (Figure 1 and Supplementary Figure 3). Collectively, these observations suggest that miR-155 and TGF-β exist in a positive feedback loop.

Targets of the mutant p53 • miR-155 axis

We suggest that *ZNF652*, *PDCD4*, *TCF12* and *IL17RB* are critical targets of the mutant p53 • miR-155 axis in breast cancer (Figure 3), thus implicating them as suppressors of breast cancer invasion and

metastasis. Consistent with this hypothesis, PDCD4 has been previously shown to inhibit motility and invasion of breast cancer cell lines *in vitro* (27, 28) and is down-regulated in invasive breast carcinomas *in vivo* (29). Likewise, loss of IL17RB expression is a strong biomarker for clinical outcome in breast cancer patients receiving tamoxifen monotherapy (30). Our findings are consistent with a similar role for ZNF652 as a suppressor of breast cancer cell invasion. We demonstrate that ZNF652 directly represses *TGFB1*, *TGFB2, TGFBR2, EGFR, SMAD2* and *VIM*; genes that have all been previously implicated in the promotion of mesenchymal transformation and invasion (6, 31-34). As such, we have defined a new role for ZNF652 as a master regulator of the EMT gene network.

In conclusion, we have identified miR-155 as a novel target of mutant p53 • p63 axis. Elevated miR-155 levels promote cellular transformation and invasion through the targeting of key suppressors of metastasis, such as ZNF652. Collectively, these findings suggest that miR-155 targeted therapies may provide an attractive approach to treat mutant p53-expressing tumors.

Materials and Methods

Cell culture, plasmids and treatments

H1299 cells were maintained in DMEM supplemented with 10% FBS. ZR-75-1 and BT549 cells were maintained in RPMI supplemented with 10% FBS and 1mM sodium pyruvate. H1299 cells with inducible expression of wild-type or mutant p53 are as described previously (35). H1299 and ZR-75-1 were engineered to express either miR-155 or a non-targeting SCR control through retroviral mediated transduction with viruses generated by the pMSCV-Puro-GFP-miR-155 or pGIPZ-non-targeting (Open Biosystems) vectors, followed by selection in 500ng/mL puromycin (Sigma Aldrich, Castle Hill, NSW). To silence gene expression in cell lines, cells were transfected with 10 µM of various specific siRNA molecules (Qiagen, Doncaster, VIC; listed in Supplementary Table 3) and transfected using lipitoid transfection reagent following manufacturer's protocol (36). For constitutive knockdown, the pGIPZ lentiviral shRNAmir system expressing two independent ZNF652-specific shRNA oligonucleotides (V3LHS_368392 or V2LHS_229362) or a p63-specific shRNA oligonucleotides (V2LHS_24250) was used in accordance with the manufacturer's protocol (Open Biosystems, Huntsville, AL).

Immunofluorescence

Cells were plated at 10% confluence on glass cover slips and grown for 12 days. Where indicated, cells were treated with 2ng/mL TGF-β. Media was refreshed every 3 days. For actin staining, media was removed from cells followed by 1 × wash with warmed PBS. Cells were fixed with warmed 4% PFA for 15 minutes and blocked in 1% BSA/PBS for 20 minutes. Cells were washed in PBS and permeabilised for 5 minutes with 0.1% Triton-X/PBS. Phalloidin antibody (diluted 1:500 in 0.1% Triton-X/PBS) was added and incubated for 1 hour at room temperature in the dark. Cells were

washed 2 × with PBS and mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Cells were imaged using Olympus IX70 inverted microscope.

To score cell size, the major and minor axis of the cells was measured. To score lamellopodia, cells with lamellopodia (visualised with actin stain) were counted. Total cell numbers were determined by DAPI stain. Analysis was performed using ImageJ software over ≥4 fields of view. A minimum of 100 cells were counted per condition.

Isolation of RNA, RT-PCR and microRNA analysis

Briefly, total RNA was extracted from cells using RNeasy mini kit (Qiagen), with quantitative real-time PCR performed as previously described (37). Specific primers for real-time PCR are listed in Supplementary Table 3. Low density PCR arrays of 84 EMT-related genes were performed using the Human Epithelial to Mesenchymal Transition RT^2Profiler PCR Array System (SABiosciences, Frederick, MD) as per manufacturer's protocol. MiR-155 analysis was performed using the ABI Taqman miRNA assay for miR-155, normalising levels to U6, U48 and miR-16 according to the standard protocol.

Western blot analysis and Chromatin Immunoprecipitation (ChIP)

Western blot and ChIP analysis are described Supplementary Materials and Methods.

Invasion/Migration Assays

Real-time invasion assays were performed using the xCelligence Real-Time Cell Analyzer (RTCA) DP (Roche, Castle Hill, NSW), as per the manufacturer's protocol. Briefly, sub-confluent cell cultures were collected in serum free media and plated at 2×10^4 cells per well in the top chamber of a CIM-

Plate 16 pre-coated with 5% matrigel (BD Biosciences). DMEM containing 10% FCS was used as a chemo-attractant. Real-time migration assays were performed using Incucyte (Essen, Michigan, USA). Phase images were taken every 15 minutes and wound closure and cell confluence calculated using specific Incucyte software.

Breast tissue microarrays and immunohistochemistry

Breast tissue microarrays BR951, BR961 and BR963 were sourced from US Biomax Inc. Immunohistochemistry was performed to determine ZNF652 expression. Briefly, slides were heated at 50˚C for 2 hours followed by dewaxing 3 × 5 min with xylene, rehydration 3 × 5 min with 100% ethanol and 2 × 3 min with PBS. Slides were subsequently treated with 1:100 dilution H_2O_2 in PBS for 5 min at RT followed by 2 × 3 min wash in PBS. Slides were immersed in citrate buffer and microwaved on high for 3 min (until boiling) followed by 15 min on low. Cooled slides were washed 2 × 3 min in PBS. Slides were blocked in 5% rabbit serum in PBS at RT for 30 min and incubated with an affinity purified rabbit polyclonal antibody against ZNF652 (17) overnight at 4˚C. Slides were washed 2 × 3 min in PBS followed by addition of biotinylated rabbit specific secondary antibody (Dako Australia, Cambellfield, VIC) at 1:400 in blocking solution and incubated for 1 hr at RT in a humid chamber. Streptavidin (1:500 dilution in PBS) was added and incubated for 1 hr at RT in a humid chamber. DAB and H_2O_2 solutions were added and incubated at RT for 6 min. Sections were counterstained with Lillie-Mayer haematoxylin for 15-30 sec, washed once with water and dehydrated through 3 × 5 min washes 100% ethanol and 3 × 5 min washes with xylene. Slides were subsequently mounted in DPX.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. miR-155 expression drives invasion in ZR-75-1 and MCF-10A cells

A. The relative expression levels of miR-155 were determined in the NCI-60 panel. Cell lines were classified as epithelial and mesenchymal based on the E-cadherin / vimentin protein ratio (38).

B. The ability of ZR-75-1 cells (control or miR-155) or MCF-10A cells (control or miR-155) to migrate was determined by a scratch-wound assay using Incucyte (Essen). Phase images were taken every 120 minutes and relative wound width was calculated in real-time using Incucyte software.

C. ZR-75-1 cells (control or miR-155) or MCF-10A cells (control or miR-155) were plated in the upper chamber of a CIM-16 plate coated with 5% matrigel. Invasion was measured in real-time using an xCelligence RTCA DP analyser.

D. ZR-75-1 cells (control or miR-155) or MCF-10A cells (control or miR-155) were seeded at 10% confluence and grown for 12 days. Cells were stained for F-actin using a phalloidin antibody, with lamellopodia and cell size scored as described in Materials and Methods. Representative images of ZR-75-1 cells (control or miR-155) are shown.

E. The expression of EMT related genes in ZR-75-1 cells expressing miR-155 was determined using a low density PCR array. Data are presented as a fold change in expression relative to ZR-75-1 expressing a scrambled RNA sequence (control). Data presented is an average of three independent biological replicates.

Figure 2. miR-155 expression is regulated by p63

A. H1299 inducible p53-R248Q or p53-R282W cells were treated with 2.5 µg/mL PonA for 0, 24, 48 or 72 hours to induce either p53 R248Q or p53 R282W protein expression. Relative *MIR155HG* expression levels or mature miR-155 levels were determined by specific RT-PCR. Induction of p53 R248Q and p53 R282W proteins were determined by Western blot analysis.

B. Endogenous mutant p53 (R249S) expression was silenced by transient transfection of a specific siRNA targeting p53 (see Materials and Methods). Relative miR-155 expression was determined by an ABI Taqman miRNA assay. Knockdown of endogenous p53 R249S was confirmed by Western blot analysis.

C. The expression levels of miR-155 and p53 status of a cohort of 100 breast tumors were derived from a publically available dataset (GSE19536).

D. Publically available gene expression profiling of the cohort of 100 breast tumors from (C) was used to segregate the cases into breast cancer subtypes. The expression levels of miR-155 was significantly different across the subtypes, as determined using a 1-way ANOVA.

E. DNA-p63 complexes were immunoprecipitated from MCF-10A cell lysates using a p63 antibody (or IgG control) and normalised against two unrelated genomic regions (see Materials and Methods). Fold enrichment of a putative p63-RE within the *MIR155HG* gene (as compared to IgG control) was determined. The anti-p63 antibody (H-129) detects both the TAp63 and ΔNp63 isoforms. The ability of p53 to bind this response element was determined in H1299 cells upon induction of wild-type p53 protein. Fold enrichment of p53 within this region of the *MIR155HG* gene was determined by ChIP analysis following induction of p53-WT in EI H1299 cells for 24 hours.

F. Endogenous p63 was silenced in MCF-10A cells through constitutive lentiviral-mediated expression of a specific shRNA targeting the p63 transcript (left panel). Relative expression levels of *MIR155HG* or mature miR-155 were determined by specific RT-PCR in MCF10A cells expressing p63-specific shRNA (which targets both the TAp63 and ΔNp63 isoforms) or a non-targeting control shRNA (as above). Knockdown of p63 expression was confirmed by real-time PCR (using primers that detect both the TAp63 and ΔNp63 isoforms) or through Western blot analysis (using the H-129 antibody that detects both TAp63 and ΔNp63 isoforms).

Figure 3. Four genes commonly regulated by the mutant p53•miR-155 axis are associated with poor metastasis-free survival

A. Of the 140 published miR-155 target genes (see Supplementary Table 1), the expression of 42 of these genes was significantly correlated with mutant p53 status *in vivo* (4). Loss of expression of 4 of these 42 genes predict metastasis *in vivo*, as determined from a publicly available dataset of 78 breast tumors (16).

B. Kaplan-Meier plots derived from publically available survival data associated with a cohort of 78 breast tumors with metastasis-free survival data (16). Expression of ZNF652, PCDC4, TCF12 and IL7RB was sourced from expression microarray analyses. Tumors were ranked for gene expression, with those tumors below the median expression level of the cohort defined as 'Low Expression' and those tumors above the median expression level of the cohort defined as 'High Expression'.

Figure 4. ZNF652 is an epithelial cell marker which is down-regulated in EMT by miR-155

A. Nuclear extracts from a panel of epithelial and mesenchymal breast cancer cell lines were screened for ZNF652 protein expression with a specific polyclonal anti-ZNF652 antibody (Kumar, 2006). Equal loading was determined by Lamin A/C.

B. Relative mRNA expression of ZNF652 was determined in a panel of cell lines by specific RT-PCR.

C. ZNF652 protein levels were determined in H1299 cells following constitutive expression of miR-155 (described and characterised in Supplementary Figure 4).

D. Knockdown of ZNF652 expression using a 50:50 mixture of two specific siRNA oligonucleotides in H1299 resulted in an enhanced ability of H1299 cells to invade into matrigel, as determined in real-time using an xCelligence RTCA DP analyser. ZNF652 transcript levels were determined by real-time PCR following siRNA-mediated knockdown for 48 hours.

E. *In silico* screening of the classical promoter regions (10kB upstream), first introns and 3'UTRs of *TGFB1*, *TGFB2, TGFBR2, EGFR, SMAD2* and *VIM* identified multiple putative ZNF652 binding sites (BS), as illustrated in schematic diagram. ChIP analysis was performed in ZR-75-1 cells using a specific ZNF652 antibody, with the fold-enrichment of ZNF652 bound at each putative ZNF652 binding site determined relative to an IgG control and normalised against two unrelated genomic regions (see Materials and Methods).

Figure 5. Loss of ZNF652 expression is associated with increased tumor invasion *in vivo*

A. Immunohistochemistry was performed on breast tissue microarrays to determine ZNF652 expression *in vivo*. ZNF652 expression was significantly associated (* indicates $p<0.001$, Fisher exact test) with tissue invasion as assessed by the TNM staging (where 'low', 'medium' or 'high' indicates T2, T3 or T4 classifications, respectively) as defined by the American Joint Committee on Cancer. Tissues were classified as low ZNF652 expression when there was negligible staining of 95% of the breast tumor nuclei.

B. Schematic diagram representing the proposed mechanism of mutant p53 driving EMT and tumor cell invasion through a pathway involving miR-155 and ZNF652.