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Mutant GATA3 Actively Promotes the Growth of Normal and Malignant Mammary Cells

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Abstract

Background/Aim—GATA3, a transcription factor expressed in luminal breast epithelial cells, is required for mammary gland development. Heterozygous GATA3 mutations occur in up to 15% of estrogen receptor (ER)-positive breast tumors and have been proposed to be null alleles resulting in haploinsufficiency; however, the mutation spectrum of GATA3 in breast cancer is in sharp contrast to that found in HDR syndrome, a true GATA3 haploinsufficiency disease. Materials and

Methods—Transgenic mice, 3D cultures and xenografts were used to examine the effect of mutant GATA3 expression on mammary cell proliferation.

Results—Mutant GATA3 accelerated tumor growth of ZR751 cell xenografts and promoted precocious lobuloalveolar development in transgenic mouse mammary glands.

Conclusion—These data indicate that the GATA3 mutations, recently observed in breast cancer, encode active transcription factors, which elicit proliferative phenotypes in normal mammary epithelium and promote the growth of ER-positive breast cancer cell lines.

Keywords

GATA3; breast cancer; transcription factor; mammary gland biology

GATA3 is expressed in normal luminal mammary epithelial cells and is a key transcription factor in mammary gland development (1). Mice with mammary-specific deletion of GATA3 have significant defects in mammary terminal end bud formation, branching morphogenesis, and lactation (2, 3). Concerning human breast cancer, GATA3 is highly expressed in the majority of estrogen receptor-positive (ER+) tumors (4, 5), where GATA3 and ERα are involved in a positive cross-regulatory feedback loop (6). Somatic *GATA3* mutations have

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recently been identified in breast cancer, primarily in Luminal A and Luminal B ER+ tumors (7–9). In the largest of these studies (8), *GATA3* mutations were detected in 14% of Luminal A and 15% of Luminal B tumors, making it the second most frequently mutated gene in Luminal A tumors (after *PIK3CA*) and the third most frequently mutated gene in Luminal B tumors (after *PIK3CA* and *TP53*). The vast majority of mutations are frameshifts. Approximately 40% of the mutations identified in the TCGA study lack the second zinc finger, which is responsible for binding the canonical GATA motif (10, 11), while the remainder encode longer proteins containing both of the GATA3 zinc fingers.

Although the precise nature of the contribution of GATA3 mutation to tumorigenesis is unclear, its high mutation frequency indicates that this alteration plays an important role in breast tumor pathogenesis. Prior studies have found that *GATA3* mutations in breast cancer are clustered in exons 4, 5 and 6, which encode the zinc finger domains necessary for DNA binding. In addition, the wild-type *GATA3* allele tends to be retained and both alleles are expressed in these tumors (9, 12). The initial interpretation in the literature suggests that *GATA3* is a haploinsufficient tumor suppressor gene in breast cancer and that the mutant alleles encode non-functional proteins (9); however, this model has not been rigorously tested. Previous studies (12–14) have noted that comparison of the mutation spectrum of *GATA3* in breast cancer with that found in hypoparathyroidism, deafness and renal dysplasia (HDR) syndrome, a genuine human *GATA3* haploinsufficiency disease, casts doubt on this interpretation. HDR syndrome is generally associated with *GATA3* mutations throughout the open reading frame or whole gene deletions (15). However, the mutations found in breast cancer specimens cluster predominantly at the C-terminus of the protein. Data from HDR cases have clearly shown that GATA3 function can effectively be disrupted by mutations throughout the coding sequence, but these mutations are not selected for in breast cancer. Instead, there appears to be a selective pressure to retain and express a substantial proportion of the *GATA3* open reading frame, which suggests that the encoded protein may have a residual or novel function rather than simply being a null allele. Consistent with this interpretation, a recent study has shown that CRISPR-mediated deletion of mutant *GATA3* attenuates tumorigenesis in MCF7 cell line xenografts and that overexpression of mutant GATA3 increased tumor growth rate in CAMA-1 cell line xenografts (16).

Previous animal studies have relied on xenografts in immunocompromised mice to functionally characterize GATA3 mutant proteins. In this study, we developed a transgenic mouse with mammary-specific expression of human mutant GATA3 to more rigorously determine whether the truncating GATA3 alleles are truly null alleles or if the encoding proteins may retain residual or novel functions relevant to tumorigenesis. We showed that mutant GATA3 elicits hyperproliferative phenotypes during mammary gland development. Consistent with this phenotype, studies with two GATA3 hotspot mutations showed that ectopic expression of these proteins increased the growth rate of ZR751 cell line both *in vitro* and in xenografts. These findings suggest a model in which the studied recurrent breast cancer mutations do not result in haploinsufficiency or simple loss-of-function, but encode functional proteins, which actively contribute to tumor growth.

Materials and Methods

Generation of MMTV-GATA3^{335fs} mice

The *GATA3^{335fs}* coding sequence was subcloned under the control of the steroid hormone-responsive mouse mammary tumor virus (MMTV) promoter into the MMTV-SV40-BSSK plasmid (kindly provided by Philip Leder, plasmid #1824, Addgene) (17). Constructs were linearized and injected into FVB pronuclei and implanted into surrogate FVB mice. Germline transmission was confirmed by PCR of tail DNA (5′-ACTCCAGCCACATGCTGAC and 5′-TCACACCACAGAAGTAAGGTTCC). Whole mount analysis of inguinal mammary glands from estrus-matched (18) 9-week old non-parous mice was performed as previously described (19). The extent of mammary gland development was quantified on a 0–3 scale as has previously been described (20).

Cell culture

ZR751 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and identity was verified by short tandem repeat (STR) profiling at our institutional genomics core facility using the Genemarker 10 kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. ZR751 cells stably expressing FLAG-tagged GATA3^{308*}, GATA3^{335fs}, or the empty vector control were generated using pBM-IRES-Puro retroviral vector (21). 3D culture was performed as previously described (22).

Xenografts

Xenografts were performed in athymic mice (National Cancer Institute) and were approved by the Institutional Animal Use and Care Committee. Thirty nulliparous 5-week old female mice were implanted subcutaneously with 60-day slow-release pellets containing 0.72 mg of 17β-estradiol (Innovative Research, Sarasota, FL, USA). Two million ZR751 cells in 100 μl of a 1:1 mixture of RPMI 1640 (Thermo Fisher, Waltham, MA, USA) and Matrigel (BD Biosciences, San Jose, CA, USA) were injected orthotopically into the right and left 4th inguinal mammary fat pads using a 21½ gauge needle. Animals were checked daily for tumor formation by palpation. Tumor size was measured thrice per week using a caliper and was calculated using the formula ½ x length x width x height

Western Blot

Mammary glands from 9 week, 15.5 day pregnant, and lactating wildtype and transgenic mice were minced, resuspended in lysis buffer (25 mM Tris-HCl pH 7.6, 200 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS) and homogenized using a tissue grinder. Samples were resolved by SDS-PAGE on 4–20% gradient gels (Biorad Laboratories, Hercules, CA) and transferred to PVDF membranes. Antibodies used were against GATA3 (BD Biosciences, San Jose, CA, USA) and E-Cadherin (Cell Signaling Technology, Danvers, MA, USA) and were incubated overnight prior to washing and detection with HRP-conjugated secondary antibodies and the Super Signal Chemilumescent Substrate (Pierce, Waltham, MA, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism (Graphpad Software, La Jolla, CA, USA). The frequencies of categories of mammary phenotypes in transgenic and matched control mice were compared using a Chi-squared test. Differences between median colony sizes in 3D culture were evaluated using the Mann-Whitney test. For xenograft studies, Kaplan-Meier analysis with log-rank test was used to compare time to tumor formation between groups, and differences between experimental and control xenograft volumes at each time point shown were evaluated using a t-test. Cell culture experiments were repeated twice, and animal experiments included the numbers of replicate animal indicated in each figure legend. $P < 0.05$ was the threshold for statistical significance in all cases.

Results

Two common *GATA3* mutations were chosen for further analysis; a splice acceptor site deletion affecting codon 308, modeled as a stop codon ($GATA3^{308*}$), and a frameshift at codon 335 ($GATA3^{335fs}$), which, collectively, are reported in more than 100 breast cancer cases in cBioportal (7–9, 23, 24). Codon numbering was according to NCBI reference sequence NM_002051.2. The $GATA3^{335fs}$ mutant was evaluated in a transgenic model, and both mutants in cancer cell line xenografts.

$GATA3^{335fs}$ leads to precocious lobuloalveolar development in the mammary gland

To determine whether expression of the $GATA3^{335fs}$ protein might confer a competitive proliferative advantage on mammary epithelial cells in a physiologic setting, transgenic mice expressing this allele under the control of the MMTV promoter were generated (Figure 1A). This strategy also allowed the comparison of the present $GATA3^{335fs}$-induced phenotype to prior reports of GATA3 manipulation in the mammary gland. Mammary-specific *GATA3* deletion has been reported to cause a failure in mammary gland development (2, 3); a finding expected to be phenocopied if the mutant *GATA3* allele encodes a dominant negative protein. In contrast, the overexpression of wild-type GATA3 in mice using the MMTV promoter resulted in precocious lobuloalveolar development reminiscent of early pregnancy in the mammary gland (25).

Western blot analysis indicated that the mutant protein was expressed in the non-parous gland and that expression is increased during pregnancy and lactation (Figure 1B), consistent with control by the hormonally-regulated MMTV promoter (26). Lysate from MCF7 cells which endogenously express this mutant protein was used as a positive control. Transgenic female mice were indistinguishable from wild-type littermates in general health and body weight and were able to suckle pups normally. Whole mount analysis was performed on mammary glands from non-parous 9-week old mice, all of which were in metestrus. Representative examples showed significantly increased branching and precocious lobuloalveolar development in $GATA3^{335fs}$ glands compared to wild type glands (Figure 1C). Mammary glands were scored by the extent of their morphological development using a scale from 0 to 3 as described by Fata and colleagues (20). Glands were observed across the full range of developmental stages from Grade 0 (consisting mostly of primary ducts

with a limited number of secondary ducts) to Grade 3 (fully branched with extensive alveolar development). Samples were scored blindly and independently by two individuals. A significant shift to a precocious lobuloalveolar phenotype was observed in the mammary glands expressing GATA3^{335fs}. This precocious development of the mammary gland more closely resembled the phenotype observed in MMTV-*GATA3*^{WT} mice (25) and was in stark contrast to the failure of mammary gland development in *GATA3* conditional deletion mice (2, 3), suggesting that this mutant does not act in a dominant negative fashion, and is able to induce a proliferative phenotype.

Characterization of two GATA3 mutant proteins in human breast cancer cells

To further address the potential functions of these mutant proteins, stable cell lines were generated in the ZR751 cell line, an ER+ luminal breast cancer cell line that expresses endogenous wild-type GATA3. Specifically, ZR751 cells were transfected with either the empty vector, or with plasmids encoding FLAG-tagged GATA^{308*} and GATA3^{335fs}. This cell line was chosen since it more closely resembles the ER+ luminal tumors in which *GATA3* mutations are observed in the clinic than the ER-negative cell lines typically used in prior studies. Accordingly, it may be a more physiologically relevant model for analyzing the role of GATA3 and its mutants in tumorigenesis.

In 3D culture, the size of colonies formed by the ZR751 cell line was estrogen-dependent, with the control cells having an 88% higher median colony size when cultured in the presence of 100 nM estradiol. Interestingly, ectopic expression of both mutant GATA3 proteins in the ZR751 cell line led to a larger colony size in the absence of estradiol (gray boxes, Figure 2). The magnitude of the proliferative response to estrogen was not as large in the GATA3^{308*} and GATA3^{335fs} expressing cells; 29.5 % larger (p<0.0001) and 22 % larger (p<0.0001), respectively. These data demonstrated that the expression of the single zinc finger mutants in ZR751 cells caused increased colony size in the 3D culture in the absence of estrogen. This outcome approached the effect size observed when the control cells were treated with estradiol.

To determine whether the GATA3 mutants conferred a similar growth advantage *in vivo*, these cell lines were xenografted into the fat pads of athymic nude mice implanted with slow-release estrogen pellets (n=20 xenografts per cell line). Mice were followed up until the larger tumors reached a volume of 600 mm^3 (48 days). The time required to reach 100 mm^3 volume was measured and it was found that the GATA3^{308*} and GATA3^{335fs} expressing tumors reached the target volume significantly more quickly than the controls (Figure 3A). The GATA3^{308*} and GATA3^{335fs} expressing tumors were all significantly larger compared to the control tumors at the indicated time-points (Figure 3B).

Discussion

In this study, we challenged the existing paradigm that haploinsufficiency is the main outcome for breast cancer-specific *GATA3* mutations. We show that expression of mutant GATA3 leads to precocious lobuloalveolar development in non-parous mice in the presence of two wild-type *GATA3* alleles, and increased xenograft growth of a luminal breast cancer cell line *in vivo*.

We considered the possibility that the mutant protein might somehow antagonize the transcriptional activity of the wild-type protein in a dominant-negative fashion; however, we did not see any dominant negative effects of the mutants on wildtype GATA3 in luciferase assays (data not shown). Other studies that have recently tested three different GATA3 mutants in a similar reporter assay also did not show any dominant-negative effect with truncated mutants (G314fs and R330fs) that lack the second zinc finger, but detected a modest dominant-negative effect in two other mutants (R330del and P408fs) that retained both zinc fingers, albeit only in one of the two cell lines they studied (12). A more rigorous test of dominant-negative activity than these artificial luciferase reporter constructs is to test whether expression of the mutant protein in the mouse mammary gland can phenocopy the defects in mammary outgrowth described in the mammary-specific *GATA3* knockout models (2, 3). Unlike the substantial reduction in mammary epithelial tree growth that was reported in those mice (2, 3), mice expressing the $GATA3^{335fs}$ mutant transgene developed full mammary glands with a precocious lobuloalveolar development phenotype. These data indicate that – even in the presence of the two endogenous wild-type *GATA3* alleles – expression of this truncated mutant is sufficient to confer a proliferative phenotype. This mammary phenotype strongly resembles that of MMTV-$GATA3^{WT}$ mice (25). In our cell line model, the expression of mutant GATA3 was sufficient to accelerate tumor growth. These findings argue against both the simple haploinsufficiency model and the dominant-negative model for the role of mutant GATA3 in breast cancer and are more consistent with the mutant protein having a residual or novel function.

Genes mutated in cancer can be considered to fall into one of four categories – passenger mutations, classical two-hit tumor suppressor genes, haploinsufficient tumor suppressor genes or proto-oncogenes. The prevalence of *GATA3* mutations in ER+ breast cancer and their restriction to this breast cancer subtype argues strongly against a passenger mutation hypothesis. It is also clear from earlier work (9) that wild-type GATA3 and mutant GATA3 are co-expressed, indicating that *GATA3* does not follow the classical two-hit tumor suppressor gene model. The haploinsufficiency model has received most attention (7, 9) so far, and while our data do not fully exclude a potential contribution for haploinsufficiency, one might expect a far greater prevalence of single allele GATA3 deletion and/or mutations throughout the open reading frame if this was the primary mechanism, and we have demonstrated in this study that the mutants are not null alleles.

Instead, given the facts that (i) the mutant proteins are expressed in $GATA3^{mut}$ tumors, (ii) the selective pressure in tumor evolution appears to favor truncating mutations rather than gene deletions, and (iii) the mutant proteins exert proliferative phenotypes on both normal and malignant mammary epithelial cells, we believe that the data are most consistent with *GATA3* being a mammary proto-oncogene. This contention would be strengthened by demonstrating mammary tumorigenesis in the MMTV-$GATA3^{335fs}$ mice. We observed no tumors in a cohort of 13 non-parous females that we followed (median age 553 days, range 371–685 days), although in several other MMTV-driven mammary tumor models tumor incidence increases with parity, an interaction we have not addressed in our study. Tests to determine whether mammary tumorigenesis is accelerated in other models by crossing with MMTV-$GATA3^{335fs}$ mice would also provide a rigorous test for the proto-oncogene hypothesis.

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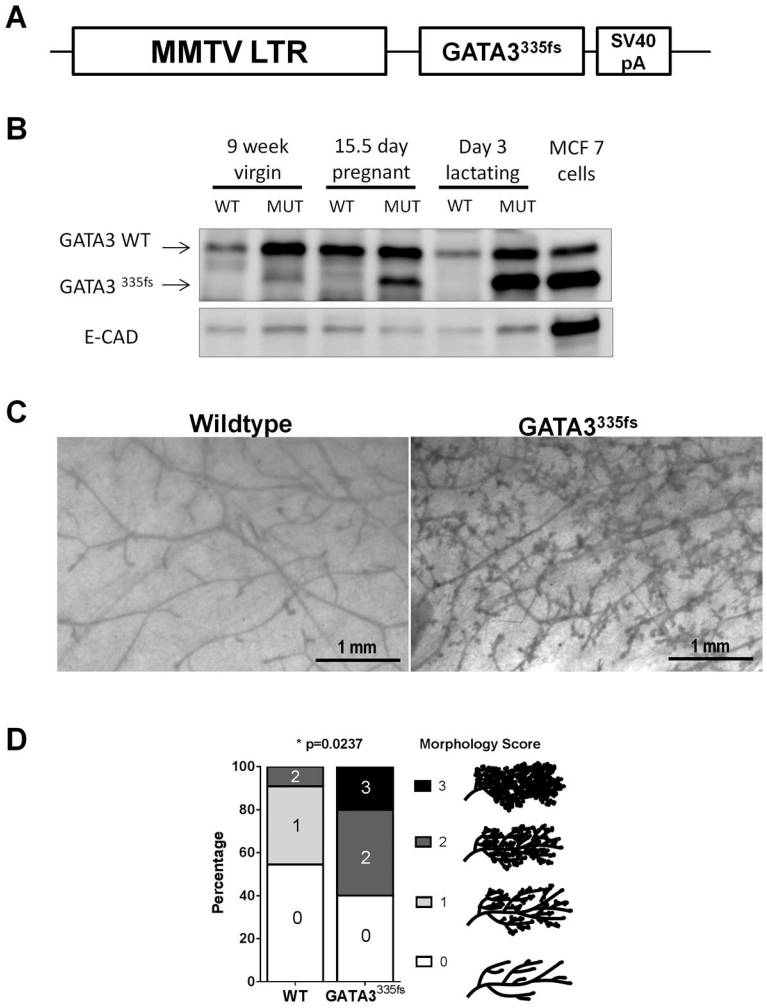

Figure 1.

Mammary specific expression of GATA3^{335fs} in transgenic mice leads to precocious lobuloalveolar development. A. MMTV-GATA3^{335fs} construct with GATA3^{335fs} cloned downstream of the luminal cell-specific MMTV-LTR promoter. B. Expression of GATA3^{335fs} transgenic protein in virgin, pregnant, and lactating mouse mammary glands analyzed by western blot. MCF7 cells, which express this mutant, were used as a positive control. E-cadherin, an epithelial cell marker, was used as a loading control. C. Representative carmine-stained mammary gland whole mounts in metestrus-matched 9 week MMTV-GATA3^{335fs} and littermate wildtype controls. D. The extent of mammary gland development was quantified in 9 week old MMTV-GATA3^{335fs} (n= 10) and wild-type (n=11) mice ($p<0.0237$, Fisher's Exact Test).

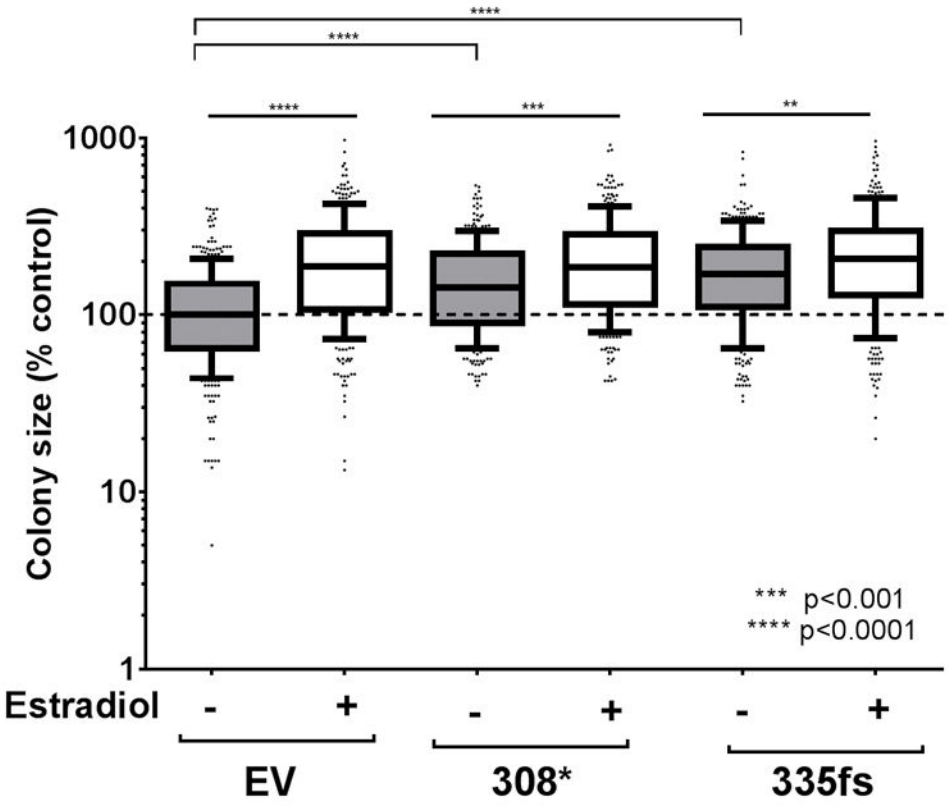

Figure 2.

In vitro growth of ZR751 cells expressing ectopic mutant GATA3.

3D culture colony growth of the ZR751 stable cell lines in the absence or presence of 100 nM estradiol. The graph represents quantification of two independent experiments, each normalized to the median colony size in the control cultures at the time of quantification (**p<0.01, ***p<0.001 and ****p<0.0001, Mann-Whitney test). EV, ZR751 cells transfected with empty vector control; 308*, ZR751 cells expressing FLAG-tagged GATA3^{308*}; 335fs, ZR751 cells expressing FLAG-tagged GATA3^{335fs}

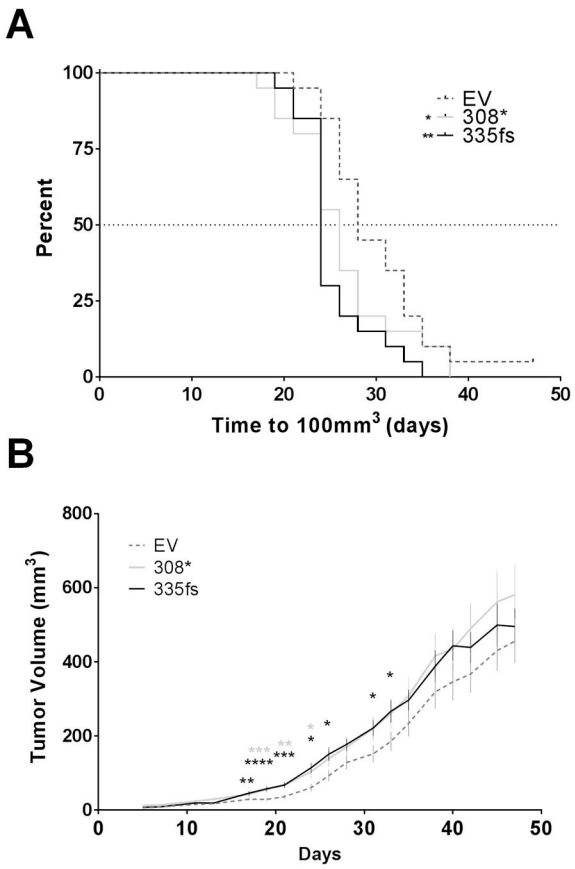

Figure 3.

In vivo growth of ZR751 cells expressing ectopic mutant GATA3. A. Kaplan-Meier curve showing the time to 100 mm^3 xenograft tumor volume (*p<0.05, **p<0.01, Log-rank test). B. Comparison of growth rates of ZR751 xenograft tumors. Graph shows tumor volumes and standard error of the mean (n=20 per group, (*p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, Student's t-test). EV, ZR751 cells transfected with empty vector control; 308*, ZR751 cells expressing FLAG-tagged $GATA3^{308*}$; 335fs, ZR751 cells expressing FLAG-tagged $GATA3^{335fs}$