Published in final edited form as:

Ann Surg Oncol. 2013 July ; 20(7): 2204–2212. doi:10.1245/s10434-012-2570-5.

Expression of the *RET* Proto-oncogene Is Regulated by TFAP2C in Breast Cancer Independent of the Estrogen Receptor

Philip M. Spanheimer, MD, George W. Woodfield, MS, Anthony R. Cyr, BS, Mikhail V. Kulak, PhD, Lola S. White-Baer, Thomas B. Bair, PhD, and Ronald J. Weigel, MD, PhD

Department of Surgery, University of Iowa, Iowa City, IA

Abstract

Background—The *RET* proto-oncogene is expressed as part of the estrogen receptor (ER) cluster in breast cancer. We sought to determine if TFAP2C regulates Ret expression directly or indirectly through ER.

Methods—Chromatin immunoprecipitation sequencing (ChIP-Seq) and gel-shift assay were used to identify TFAP2C binding sites in the *RET* promoter in four breast cancer cell lines. Ret mRNA and protein levels were evaluated in ER-positive and ER-negative breast cancer cell lines after knockdown of TFAP2C. Luciferase expression assay was performed to assess expression from two of the identified sites.

Results—ChIP-Seq identified five main binding peaks for TFAP2C in the *RET* promoter at –101.5 kb, –50.7 kb, –32.5 kb, +5.0 kb, and +33.6 from the *RET* transcriptional start site. Binding at three of the AP-2 sites was conserved across all four cell lines, whereas the *RET*–101.5 and *RET*+33.6 sites were each found to be unbound by TFAP2C in one cell line. A TFAP2C consensus element was confirmed for all five sites. Knockdown of TFAP2C by siRNA in ER-positive MCF-7 cells resulted in significant down regulation of Ret mRNA compared to nontargeting (NT) siRNA (0.09 vs. 1.0, $P < 0.001$). Knockdown of TFAP2C in ER-negative MDA-MB-453 cells also led to a significant reduction in Ret mRNA compared to NT siRNA (0.16 vs. 1.0, $P < 0.001$). In MCF-7 cells, knockdown of TFAP2C abrogated Ret protein expression (0.02 vs. 1.0, $P < 0.001$) before reduction in ER.

Conclusions—TFAP2C regulates expression of the *RET* proto-oncogene through five AP-2 regulatory sites in the *RET* promoter. Regulation of Ret by TFAP2C occurs independently of ER expression in breast carcinoma.

Mutations of the Ret receptor tyrosine kinase are well described in the multiple endocrine neoplasia (MEN) 2 syndromes, familial medullary thyroid cancer, and Hirschsprung disease. Recently, expression of the *RET* gene has been identified in breast cancer, with a strong association with expression of the estrogen receptor (ER) alpha.¹ Activation of the Ret receptor by its ligand, glial cell–line–derived neurotrophic factor (GDNF), leads to phosphorylation of Erk1/2, Akt, and results in proliferation and growth in breast cancer cells.^{2,3} Ret is postulated to have an important role in breast cancer, and although mutations are rarely identified, expression and over-expression are common in breast carcinoma.^{4,5} Ret

expression is additionally associated with a poor prognosis in ER-positive breast cancers and may be involved in hormone resistance.^{4,6}

The ER pathway is an important regulator involved in the development and progression of breast cancer.⁷ Most effects of estrogen in breast cancer are mediated through transcriptional activation of ER. Along with observed coexpression in primary breast cancer and breast cancer cell lines, a role for ER in the regulation of *RET* has been identified. Multiple experiments have demonstrated increased activity of the *RET* promoter with activation of ER by estrogen and ER regulates transcription of the *RET* promoter through multiple estrogen response elements (EREs) binding sites.^{7,8} Furthermore, *RET* is coexpressed with the ER-cluster genes in breast cancer cell lines and primary breast tumors.⁵

TFAP2C is a member of the AP-2 family of transcription factor regulators. AP-2 factors bind to a consensus sequence originally described as GCCNNNGGC, which is now recognized to have some variability in the end triad as well as preference for the internal triad.^{9,10} TFAP2C has been shown to regulate expression of ER, as well as other genes in the ER-associated expression cluster both directly and indirectly through regulation of ER.¹¹ Because the *RET* gene is expressed in association with ER and TFAP2C is a global regulator of genes expressed in the ER cluster, we investigated regulation of *RET* by TFAP2C. We sought to characterize TFAP2C regulation of *RET* in the presence and absence of ER to determine whether TFAP2C effects are direct or mediated through regulation of ER expression. Because *RET* is also expressed in a subset of ER-negative tumors, we further examined the role of TFAP2C in regulating *RET* expression in ER-negative breast cancer cells.

METHODS

Cell Lines

The MCF-7, MDA-MB-453, SKBR-3 and BT-474 cell lines were obtained from the American Type Culture Collection (ATCC). MCF-7, SKBR-3 and BT-474 cells were grown using Dulbecco modified Eagle medium (DMEM) medium with 10 % fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C and 5 % CO₂. MDA-MB-453 cells were cultured using DMEM/F12 with 20 % FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C and room air CO₂.

Chromatin Immunoprecipitation Sequencing

Chromatin immunoprecipitation with direct sequencing (ChIP-Seq) was performed as previously described using TFAP2C monoclonal antibody SC-12762X (Santa Cruz Biotechnology, Santa Cruz, CA).¹² Sequence reads were aligned to the human HG18 genome using the program ELAND. Peaks were defined as previously described.¹¹ TFAP2C binding to the *RET* promoter was assessed by searching the University of California–Santa Cruz, genome browser (<http://genome.ucsc.edu/cgi-bin/hgTracks?org=human>) for *RET* with our ChIP-Seq tracks overlaid. Graphical representation of the ChIP-Seq data is presented in Figs. 1 and 2.

Gel-shift Assay

Gel-shift assay was performed using the Gel Shift Assay Core System E3050 (Promega, Madison, WI). A 150 bp oligonucleotide probe was created from the sequence underlying each peak on ChIP-Seq. Probes were labeled with ³²P according to manufacture protocol. TFAP2C protein was synthesized from pcDNA3.1-AP2C using TNT T7 Quick Coupled Transcription/Translation System 1170 (Promega).¹⁰ Probe and competitor design is

graphically represented in Fig. 2, and sequences are provided in the Supplemental Data. Supershift was performed with TFAP2C antibody SC-12762 (Santa Cruz Biotechnology).

Small Interfering RNA Transfections

Small interfering RNAs were obtained for *RET* (sc-156121, Santa Cruz Biotechnologies, Santa Cruz, CA), TFAP2C (D-005238-01, Dharmacon, Lafayette, CO), and nontargeting (NT) (D-001210-01-05, Dharmacon). Transfection was performed according to the manufacturer protocol using Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA).

Real-time Polymerase Chain Reaction

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) 48 h after siRNA transfection. Total RNA was converted to cDNA using the Superscript III kit (Invitrogen) using random hexamer primers. Quantitative PCR was performed according to standard TaqMan Fast protocol (Applied Biosystems, Carlsbad, CA). TaqMan primer/probe combinations for specific genes used were *RETHs01120030* and TFAP2C Hs00231476. Human beta-2 microglobulin (Hs00984230) was used for endogenous control (Applied Biosystems). RT-PCR was performed in triplicate and averages, standard deviations, and statistical analysis were determined with three biologic replicates.

Western Blot Testing

Total protein was isolated 72 and 120 h after siRNA transfection using RIPA buffer with Halt protease inhibitor cocktail (Thermo Scientific, Rockford, IL). The antibodies used for Western blot testing were: Ret (Cell Signaling Technologies, Danvers, MA), TFAP2C (Santa Cruz Biotechnologies), ESR1 (Santa Cruz Biotechnologies), and GAPDH (Santa Cruz Biotechnologies).

Luciferase Reporter Assay

Luciferase reporter constructs containing the TFAP2C binding site and AP-2 mutations at *RET*-50.7 and *RET*+33.6 were obtained as a generous gift from Edwin Cheung.¹³ The pGL3 vector (Promega) was used in control transfections. MCF-7 and MDA-MB-453 cells were transfected with reporter constructs using Lipofectamine LTX (Invitrogen) as recommended by the manufacturer. Twenty-four hours after transfection cells were washed with PBS, treated with passive lysis buffer, and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer protocol on an Infinite 200 Pro plate reader (Tecan, Switzerland). Samples were averaged over three technical and three biologic replicates.

Statistical Analysis

Statistical analysis was performed by unpaired two-sided Student's *t* test. Statistical significance was defined as $P < 0.05$.

RESULTS

Identification of TFAP2C Binding Sites in the RET Gene Regulatory Region

Previous analysis examining the regulation of Ret expression by ER identified 10 major EREs extending from approximately 94.4 kbp upstream to 32.8 kbp downstream of the *RET* gene transcriptional start site.⁸ We compared ChIP-Seq data for genomic binding of TFAP2C in the same genomic region in four breast carcinoma cell lines, MCF-7, BT-474, MDA-MB-453 and SKBR-3. As seen in Fig. 1, there are five major TFAP2C genomic binding peaks at *RET*-101.5, *RET*-50.7, *RET*-32.5, *RET*+5.0 and *RET*+33.6, relative to the *RET* transcriptional start site (TSS). Each of these TFAP2C binding peaks closely

corresponds to genomic regions previously described for the location of ERE sites. Furthermore, the pattern of genomic binding of TFAP2C was similar in all four cell lines with two notable exceptions: the TFAP2C binding peak at *RET*-101.5 was absent in the BT-474 cell line and the TFAP2C peak at +33.6 was absent in MDA-MB-453 cells. To confirm that TFAP2C bound directly to the DNA in these genomic regions and to define the AP-2 regulatory site precisely, gel-shift assay was performed with probes derived from these five genomic TFAP2C-binding regions of the *RET* gene (Fig. 2). Gel-shift assay confirmed that cloned TFAP2C protein bound to each genomic region and competitors were used to identify precisely the AP-2 consensus element for each major TFAP2C binding site. These data confirm that TFAP2C binds directly to AP-2 consensus elements at the genomic regions identified by ChIP-Seq.

Knockdown of TFAP2C Represses RET Expression

In order to demonstrate that TFAP2C regulates Ret expression, siRNA was used to knock down expression of TFAP2C or Ret (as a control) in breast cancer cell lines. In ER-positive MCF-7 cells, knockdown of Ret by siRNA led to a significantly decreased level of Ret mRNA compared to NT transfection (0.35 vs. 1.0, $P < 0.001$). Knockdown of Ret did not lead to a change in TFAP2C mRNA levels compared to NT siRNA transfection (0.94 vs. 1.0, $P = 0.58$). Knockdown of TFAP2C by siRNA resulted in a significant reduction in TFAP2C mRNA (0.13 vs. 1.0, $P < 0.001$) and Ret mRNA (0.09 vs. 1.0, $P < 0.001$) compared to NT siRNA transfection (Fig. 3). In the ER-negative MDA-MB-453 breast cancer cell line, knockdown of Ret by siRNA also resulted in a significantly decreased level of Ret mRNA compared to NT transfection (0.38 vs. 1.0, $P < 0.001$). Knockdown of Ret did not cause a significant change in levels of TFAP2C mRNA levels compared to NT transfection (0.84 vs. 1.0, $P = 0.36$). Similar to results in MCF-7 cells, transfection with TFAP2C siRNA led to a significant reduction in TFAP2C mRNA (0.12 vs. 1.0, $P < 0.001$) and Ret mRNA (0.16 vs. 1.0, $P < 0.001$) compared to NT siRNA transfection (Fig. 3).

Regulation of Ret Expression by TFAP2C Is Independent of ER

Demonstrating similar regulation of Ret expression by TFAP2C in ER-positive and ER-negative breast carcinoma cell lines indicates that TFAP2C is able to regulate expression of Ret independently of ER. However, previous findings have shown that knock down of TFAP2C can result in repression of ER expression in ER-positive cell lines.¹⁴ Hence, it is still possible that the effect on Ret expression with knock down of TFAP2C in MCF-7 cells may be mediated through secondary effects of ER expression. To more clearly demonstrate the effects of TFAP2C on *RET* expression independently of ER, knock down of TFAP2C was performed in MCF-7 cells at an early time, before ER expression was lost, and late, after repression of ER occurred. As seen in Fig. 4, Western blot test confirmed that transfection with siRNA to Ret caused a significant reduction in Ret protein compared to NT transfection at 72 h after transfection (0.08 vs. 1.0, $P < 0.001$) and 120 h after transfection (0.02 vs. 1.0, $P < 0.001$). Transfection with TFAP2C siRNA led to a significant reduction in TFAP2C protein at 72 (0.02 vs. 1.0, $P < 0.001$) and 120 h (0.03 vs. 1.0, $P < 0.001$). Transfection with TFAP2C siRNA similarly resulted in a significant reduction in Ret protein at 72 h (0.02 vs. 1.0, $P < 0.001$) and 120 h after transfection (0.02 vs. 1.0, $P < 0.001$). However, the level of the ER protein was not significantly altered at 72 h after transfection (0.95 vs. 1.0, $P = 0.34$), but was significantly reduced by 120 h (0.27 vs. 1.0, $P < 0.001$) after transfection with TFAP2C siRNA. Hence, the effects of knock down of TFAP2C on *RET* expression was found to occur early in MCF-7 cells, before effects on ER expression. As an additional control, transfection with Ret siRNA did not affect ER protein levels at 72 h (0.96 vs. 1.0, $P = 0.21$) or at 120 h after transfection (1.03 vs. 1.0, $P = 0.61$) (Fig. 4).

TFAP2C Binding Induces RET Transcription

Luciferase reporter constructs containing the *RET*-50.7 and the *RET*+33.6 promoter region cloned into the minimal promoter, TATA-LUC, were assayed in MCF-7 and MDA-MB-453 cells. In both cell lines, the AP-2 regulatory region at *RET*-50.7 was able to significantly induce reporter expression compared to empty vector and mutation of the AP-2 consensus binding sequence led to a significant reduction in reporter expression, indicating that the site at *RET*-50.7 was important to TFAP2C regulation of RET expression in both cell lines (Fig. 5). In MCF-7 cells, the site at *RET*+33.7 demonstrated comparable expression compared to the *RET*-50.7 construct, and mutation of the AP-2 site significantly reduced expression. However, in MDA-MB-453 cells, the *RET*+33.6 region demonstrated minimal activity when compared to the effect of the AP-2 regulatory region located at *RET*+50.7. In addition, mutation of the AP-2 consensus element in the *RET*+33.7 construct had an insignificant effect on expression in MDA-MB-453 cells. These data corroborate the ChIP-Seq data in which the TFAP2C peak in MDA-MB-453 at *RET*+33.6 is absent and indicates that the AP-2 binding site at *RET*+33.6 is not likely to play an important role in expression of *RET* in MDA-MB-453 cells.

DISCUSSION

Characteristic patterns of gene expression have been described for different breast cancer phenotypes.⁵ Expression of the Ret receptor tyrosine kinase has been observed in primary breast cancers and is associated with the ER-positive breast cancer phenotype.⁵ Studies investigating regulation of the *RET* gene in breast cancer have identified EREs in the *RET* promoter and have shown that activation of ER in the presence of estrogen leads to up-regulation of the *RET* gene.⁸ Previously, we have shown that TFAP2C is an important regulator of a large number of genes in the ER-associated gene expression cluster, including direct regulation of ER.¹¹ Our findings establish that TFAP2C is able to directly regulate Ret expression independently of ER expression. These results have important implications for Ret expression in the subset of Ret-expressing, ER-negative breast cancers, as well as for Ret expression in hormone-resistant ER-positive breast cancers, where regulation may not be responsive to estrogen. The results further indicate that although ER may be capable of inducing *Ret* expression, loss of TFAP2C abrogates activation of Ret by ER. It is also possible that in some cells loss of TFAP2C may exert its effects on *Ret* expression either directly or through regulation of ER expression.

All five of the TFAP2C interaction sites identified in the current study correspond spatially to previously described EREs in the *RET* promoter.⁸ Specifically, we identified TFAP2C binding sites at *RET*-101.5, *RET*-50.7, *RET*-32.5, *RET*+5.0 and two sites separated by 100 bp at *RET*+33.6 and +33.7 (see Supplemental Table 1 for details). These sites correspond to EREs previously reported at *RET*-94.4, *RET*-49.8, *RET*-31.7, *RET*+4.8 and *RET*+32.8; hence, each of the TFAP2C sites is within 200 to 700 bp of an ERE. Furthermore, regulation of Ret expression by ER may also involve FOXA1 acting through the regulatory region at *RET*-49.8, in close proximity to the AP-2 site at *RET*-50.7, and potentially the other regulatory regions identified. In fact, we previously reported that FOXA1 is a TFAP2C target gene.¹¹ Because TFAP2C regulates the expression of both ER and FOXA1, regulation of gene expression by TFAP2C provides a transcriptional mechanism that coordinately induces expression of ER and FOXA1 in luminal breast cancer. Recent studies have confirmed our previous report of TFAP2C binding specificity determined by ChIP-Seq and the likelihood that TFAP2C interacts as a complex involving ER, FOXA1 and TFAP2C in the regulation of genes in the luminal breast cancer expression cluster.^{11,13} Further work on understanding the role of TFAP2C in regulation of gene expression profiles in luminal breast cancer is necessary and *RET* is an excellent candidate gene to elucidate mechanisms of gene regulation by TFAP2C. Additional sites may also be

important to regulation of the *RET* promoter in certain cells. For example, an additional peak is found at approximately +12 *RET*, which is most prominent in BT-474 cells, and the role of such sites will require additional studies to evaluate the role of TFAP2C binding to certain locations. Furthermore, our reporter assay data indicate that the *RET*−50.7 site is controlled by TFAP2C in the same manner in ER-positive and ER-negative cells, whereas the *RET*+33.6 site does not appear to be necessary for *RET* regulation in the ER-negative cell line, MDA-MB-453. TFAP2C induction of transcription via *RET*+33.6 in MCF-7 but not MDA-MB-453 could indicate a different regulatory mechanism at these two sites. One explanation is that ER, or another TFAP2C cofactor such as p53 or FOXA1, is needed for TFAP2C binding at *RET*+33.6 but not at *RET*−50.7. Previous study has shown the regulatory region at *RET*+32.8, adjacent to the AP-2 binding site, is estrogen-responsive.⁸ Identifying this model provides an important opportunity to further characterize mechanisms of TFAP2C DNA binding and induction of transcription, allowing the elucidation of differences in RET regulation in different cell types.

Our results in the ER-negative MDA-MB-453 cell line are particularly interesting with regard to *RET* gene regulation in ER-negative breast cancer cells. Previous studies reported that Ret expression was not affected by estrogen in this cell line, which is consistent with the cells not expressing ER.⁶ However, our data demonstrated that TFAP2C regulates *RET* gene expression in MDA-MB-453, providing further evidence for the ability of TFAP2C to act independently of ER. Because Ret is known to regulate proliferative response and invasion via Erk1/2 activation, the findings suggest an important role for TFAP2C regulation of Ret in at least a subset of ER-negative breast cancers. Our data show that TFAP2C is able to induce Ret expression independent of ER and hormone responsive mechanisms. The data with regard to MDA-MB-453 is further evidence that TFAP2C is likely to associate with a different set of coactivators in breast cancer cells of different phenotypes.

The functional implications of Ret expression in breast cancer are not yet fully understood. Expression of Ret has been linked to poor prognosis in ER-positive breast cancers and may be involved in mechanisms of hormone resistance.^{4,6,8} Activation of the Ret receptor leads to activation of intracellular mediators of proliferation and growth including Erk1/2. Blocking Ret pathway signaling has been shown to increase sensitivity to Tamoxifen in ER-positive breast cancer. Additionally, Ret pathway activation has also been implicated in the development of resistance to hormonal therapy in ER-positive breast cancer.⁵ On the basis of these findings, modulation of Ret activation could provide an important target for slowing the growth pattern and preventing resistance to hormone therapy in breast cancers. Regulation by TFAP2C is an additional target for modulation of Ret expression, as well as a target for concurrent activity of other ER-associated genes including ER itself. Further study is needed to investigate the potential of targeting TFAP2C as a single therapy for intervention in multiple cell proliferation pathways including Ret and ER in breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported in part by the National Institutes of Health grants R01CA109294 (PI: R.J. Weigel), T32CA 148062 (PI: R. J. Weigel) and by a generous gift from the Kristen Olewine Milke Breast Cancer Research Fund. P.M.S. was supported by the NIH grant T32CA148062.

REFERENCES

1. Tozlu S, Girault I, Vacher S, et al. Identification of novel genes that co-cluster with estrogen receptor alpha in breast tumor biopsy specimens, using a large-scale real-time reverse transcription–PCR approach. *Endocr Relat Cancer*. 2006; 13:1109–1120. [PubMed: 17158757]
2. Essegir S, Todd SK, Hunt T, et al. A role for glial cell derived neurotrophic factor induced expression by inflammatory cytokines and RET/GFR alpha 1 receptor up-regulation in breast cancer. *Cancer Res*. 2007; 67:11732–11741. [PubMed: 18089803]
3. Boulay A, Breuleux M, Stephan C, et al. The Ret receptor tyrosine kinase pathway functionally interacts with the ERalpha pathway in breast cancer. *Cancer Res*. 2008; 68:3743–3751. [PubMed: 18483257]
4. Plaza-Menacho I, Morandi A, Robertson D, et al. Targeting the receptor tyrosine kinase RET sensitizes breast cancer cells to tamoxifen treatment and reveals a role for RET in endocrine resistance. *Oncogene*. 2010; 29:4648–4657. [PubMed: 20531297]
5. Kao J, Salari K, Bocanegra M, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS One*. 2009; 4:e6146. [PubMed: 19582160]
6. Wang C, Mayer JA, Mazumdar A, Brown PH. The rearranged during transfection/papillary thyroid carcinoma tyrosine kinase is an estrogen-dependent gene required for the growth of estrogen receptor positive breast cancer cells. *Breast Cancer Res Treat*. 2012; 133:487–500. [PubMed: 21947652]
7. Sommer S, Fuqua SA. Estrogen receptor and breast cancer. *Semin Cancer Biol*. 2001; 11:339–352. [PubMed: 11562176]
8. Stine ZE, McGaughey DM, Bessling SL, et al. Steroid hormone modulation of RET through two estrogen responsive enhancers in breast cancer. *Hum Mol Genet*. 2011; 20:3746–3756. [PubMed: 21737465]
9. Williams T, Tjian R. Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes Dev*. 1991; 5:670–682. [PubMed: 2010091]
10. McPherson LA, Weigel RJ. AP2alpha and AP2gamma: a comparison of binding site specificity and trans-activation of the estrogen receptor promoter and single site promoter constructs. *Nucleic Acids Res*. 1999; 27:4040–4049. [PubMed: 10497269]
11. Woodfield GW, Chen Y, Bair TB, et al. Identification of primary gene targets of TFAP2C in hormone responsive breast carcinoma cells. *Genes Chromosomes Cancer*. 2010; 49:948–962. [PubMed: 20629094]
12. Woodfield GW, Hitchler MJ, Chen Y, et al. Interaction of TFAP2C with the estrogen receptor-alpha promoter is controlled by chromatin structure. *Clin Cancer Res*. 2009; 15:3672–3679. [PubMed: 19458056]
13. Tan SK, Lin ZH, Chang CW, et al. AP-2gamma regulates oestrogen receptor-mediated long-range chromatin interaction and gene transcription. *EMBO J*. 2011; 30:2569–2581. [PubMed: 21572391]
14. Woodfield GW, Horan AD, Chen Y, Weigel RJ. TFAP2C controls hormone response in breast cancer cells through multiple pathways of estrogen signaling. *Cancer Res*. 2007; 67:8439–8443. [PubMed: 17875680]

FIG. 1.

Genomic binding of TFAP2C in the *RET* locus. ChIP-Seq data graphical representation from the University of California–Santa Cruz, genome browser demonstrating locations of TFAP2C binding in the *RET* promoter. Five binding peaks are identified in MCF-7, BT-474, MDA-MB-453, and SKBR-3 at locations -101.5 kb, -50.7 kb, -32.5 kb, $+5.0$ kb, and $+33.6$ kb from the *RET* transcriptional start site. *Bottom*: Higher-resolution images around each peak. All peaks are conserved across the cell lines, except the *RET* -101.5 peak is absent in the BT-474 and the *RET* $+33.6$ peak is absent in MDA-MB-453

FIG. 2.

Localization of AP-2 sites corresponds to ChIP-Seq peaks. Gel-shift assay confirming binding of synthesized TFAP2C protein at each binding site identified by ChIP-Seq. *Upper left:* ChIP-Seq data from MCF-7 demonstrating the location of the gel-shift probe with respect to the ChIP-Seq peak. Shift of the probe is demonstrated at all sites confirming TFAP2C binding in that region, and supershift was performed with TFAP2C antibody to confirm specificity of the gel-shift complex. Beneath each gel-shift image is a graphical representation of the probe and competition oligonucleotides. For oligonucleotides competing for binding of synthesized TFAP2C protein the sequence is provided, with the consensus AP-2 family binding sequence underlined. For the *RET*-32.5 peak the AP-2 family site was split between competitor 3 and competitor 4, with competition for binding demonstrated by joining 3 and 4 as well as creating an oligonucleotide of the end of 3 and beginning of 4. The binding sequence at the *RET*+5.0 site was confirmed when mutation of 2 base pairs in the AP-2 binding sequence (boxed 4*) failed to compete for TFAP2C

binding. Partial competition occurred with competitors 1 and 5 at the *RET*+33.6 kb site; complete competition occurred with a competitor composed of 1 + 5 linked and is indicative of deviation of these 2 sites from an optimal AP-2 consensus element

FIG. 3.

Knockdown of TFAP2C abrogates *RET* expression. RT-PCR demonstrated the effects of siRNA knockdown of Ret and TFAP2C on Ret and TFAP2C expression in ER-positive MCF-7 and ER-negative MDA-MB-453 breast cancer cell lines. mRNA levels were normalized to human beta-2-microglobulin and to nontargeting (NT) transfection. RT-PCR was performed in triplicate and averages and standard deviations were calculated from 3 biologic replicates. Asterisks indicate $P < 0.001$ compared to NT transfection

FIG. 5.

Expression of RET is induced by TFAP2C at *RET*-50.7 and *RET*+33.6. Luciferase reporter constructs with AP-2 regulatory regions cloned into TATA-LUC minimal promoter were assayed in MCF-7 and MDA-MB-453 cells. Empty vector (EV) was performed with transfection of pGL3. Construct with *RET*-50.7 region was active in both cell lines and expression was abolished by mutation of the AP-2 site (mutAP2). Relative expression in MCF-7 cells demonstrated expression of the construct with the AP-2 regulatory region corresponding to the *RET*+33.6 site and significant reduction of expression by mutation of the AP-2 site. In MDA-MB-453, relative expression from the *RET*+33.6 site was significantly reduced with minimal effect noted with mutation of the AP-2 site