An intergenic enhancer deletion in 2q35 modulates breast cancer risk by deregulating IGFBP5 expression

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Abstract

Breast cancer is the most diagnosed malignancy and the second leading cause of cancer mortality in females (1). Previous association studies have identified variants on 2q35 associated with the risk of breast cancer (2-5). To identify functional susceptibility loci for breast cancer, we interrogated the 2q35 gene desert for chromatin architecture and functional variation correlated with gene expression. We report an intergenic enhancer copy number variation (enCNV; deletion) located approximately 400Kb upstream to *IGFBP5*, which overlaps an intergenic ERα-bound enhancer that loops to the *IGFBP5* promoter. The enCNV is correlated with modified ERα binding and monoallelic-repression of *IGFBP5* following estrogen treatment. We investigated the association of enCNV genotype with breast cancer in 1,182 cases and 1,362 controls, and replicate our findings in an independent set of 62,533 cases and 60,966 controls from 41 case control studies and 11 GWAS. We report a dose-dependent inverse association of 2q35 enCNV genotype (percopy OR=0.68 95%CI 0.55‑0.83, P=0.0002; replication OR=0.77 95%CI 0.73-0.82, $P=2.1x10^{-19}$) and identify 13 additional linked variants ($r^2>0.8$) in the 20Kb linkage block containing the enCNV ($P=3.2x10^{-15}$ - $5.6x10^{-17}$). These associations were independent of previously reported 2q35 variants, rs13387042 and rs16857609, and were stronger for ER-positive than ER-negative disease. Together, these results suggest that 2q35 breast cancer risk loci may be mediating their effect through *IGFBP5*.

Introduction

The 2q35 risk locus falls within a 400Kb gene desert bounded by genes *TNP1* (MIM: 190231) and *DIRC3* (MIM: 608262), nearby two members of the insulin growth factor binding protein family, *IGFBP5 (MIM: 146734)* and *IGFBP2 (MIM: 146731).* *IGFBP5* plays a critical role in mammary development (6, 7) and has been consistently implicated in tumorigenesis (6-10).The neighboring intergenic region contains the previously identified breast cancer (MIM: 114480) risk loci, rs13387042 (3) (Genbank: NC_000002 g.217041109A>G), rs16857609 (2)(Genbank: NC_000002 g.217431785C>T), and rs4442975(5) (Genbank: NC_000002 g.217920769G>T) as well as numerous intergenic enhancers, of which many whose function remains elusive.

We sought to identify intergenic variation that may affect the estrogen-mediated transcriptional regulation *IGFBP5* and to contribute to the understanding of functional chromatin architecture at the 2q35 risk locus.

Results

To evaluate the possibility that *IGFBP5* transcription is regulated by a distal enhancer within the 2q35 gene desert, we investigated the chromatin interaction profile across the 2q35 gene desert with the *IGFBP5* promoter using chromosome conformation capture (3C) (11) in the MCF7 breast cancer cell line. Results of this interaction analysis indicated strong physical proximity of the *IGFBP5* promoter with a region containing an estrogen receptor (ERα)-bound enhancer element approximately 400Kb telomeric to the *IGFBP5* promoter (**Figure 1**). Sequence analysis of this intergenic looping enhancer revealed a 1.3 Kb copy number variation (CNV; deletion) spanning the enhancer in MCF7 cells (**Figure S1**); however, the proximal estrogen response element (ERE) was not deleted (**Figure 2A**). We examined the implications of this enCNV on ERα binding activity using chromatin immunoprecipitation coupled with allele-specific qPCR (ChIP-qPCR). Our data revealed enhanced binding activity on the variant allele ($P<0.004$; **Figure 2B**), both before and after treatment with estrogen.

We hypothesized that differential allelic-binding of ERα at the 2q35 enCNV would affect allele-specific *IGFBP5* transcription in response to estrogen signaling. We investigated the effect of the polymorphic enhancer on *IGFBP5* expression by tracking a heterozygous *IGFBP5* intronic SNP (rs7565131; Genbank: NM_000599 c.338A>C) as a marker of allele specific expression (**Figure 2C**). Prior to estrogen treatment, MCF7 cells robustly express *IGFBP5*, although a majority (>95%) of expression is from the A-allele. Following treatment with low dose estrogen, the abundance of *IGFBP5* nuclear RNA (rs7565131-A) is markedly reduced at 1 hour, relative to vehicle treated cells ($P=0.027$).

This pattern of monoallelic repression is sustained at 24 hours of exposure to estrogen (P=0.014).

To resolve the question of ERα binding at this site being repressive of *IGFBP5-A* versus merely upregulating *IGFBP5-C*, we utilized a transactivator-fused nuclease-defective CRISPR system (13) to activate specific genomic sites in 2q35. We hypothesized that if ERα binding is repressive at this locus under normal conditions, when targeting a definitive transactivator molecule to this site, we would see the inverse transcriptional response (i.e. we expect to see an increase in *IGFBP5-A* relative to *IGFBP5-C*). Targeting of this construct to the IGFBP5 promoter showed no significant change in allelic balance (Figure S2; P=0.52 and 0.91). Targeting to the ERE at the 2q35 enCNV shows a significant increase in *IGFBP5-A* expression (Figure S2; P=0.004). Given that the activator *increases* expression of *IGFBP5-A* and, conversely, E2-bound ERα acting here as a repressor *decreases* expression of the same *IGFBP5-A* allele, we conclude that in MCF7s the 2q35 enCNV variant allele is in *cis* with *IGFBP5-A*. Additionally, these findings confirm our assertion that ERα binding at this distal enhancer is repressive of *IGFBP5* expression and suggests a functional mechanism for estrogen-induced regulation of *IGFBP5* transcription through this enhancer.

To investigate the hypothesis that variants, which influence *IGFBP5* expression, may be associated with breast cancer risk, we examined the relationship of the 2q35 enCNV with breast cancer in the Women's Circle of Health Study (WCHS) (**Table S1**). We identified 2,134 homozygous wildtype, 368 heterozygous, and 42 homozygous deleted (variant) individuals with an overall genotyping rate of 92%. We observed an inverse association between the 2q35 enCNV and breast cancer risk overall (per copy

OR=0.68 95%CI 0.55 - 0.83, P=0.0002; **Table S2**). The observed association was dose-dependent based on number of deleted alleles in both European American (EA) (P=0.03) and African American (AA) women (P=0.004), with homozygous deletion carriers having approximately 80% decreased breast cancer risk (OR=0.22 95%CI 0.09-0.52, P=0.0005; **Table S2**). The association was consistent in both pre and post-menopausal women combined, however a stronger effect was observed in pre-menopausal women (pre-menopausal per copy OR=0.60 95%CI 0.45-0.80, P=0.001; post-menopausal per copy OR=0.72 95%CI 0.53-0.97, P=0.03; **Table S2**). Among cases with available ER status (74.8%), the protective effect was confined to ER-positive tumors among all women combined (per copy OR=0.74 95%CI 0.58-0.96, P=0.02; **Table S3**).

To evaluate our association results in a larger, independent population, we replicated our findings in data from 46,785 cases and 42,892 controls from 41 case-control studies genotyped with a custom array, participating in the Breast Cancer Association Consortium (iCOGS; http://ccge.medschl.cam.ac.uk/research/consortia/icogs/)(2), together with data from 11 breast cancer GWAS, comprising 15,748 cases and 18,084 controls (2, 26) (http://gameon.dfci.harvard.edu/gameon/). All studies were of predominantly European origin and the 2q35 enCNV was not polymorphic in Asian populations in BCAC or 1000 genomes. The 2q35 enCNV was not genotyped on the iCOGS array or in any of the GWAS, but the variant is present in the 1000 genomes dataset (http://www.1000genomes.org/). We therefore derived imputed genotypes for all variants across a 1Mb interval (Chr 2: 217,731,785-218,796,508; hg19) that encompassed the 2q35 enCNV together with the flanking LD blocks containing the previously reported

2q35 susceptibility loci, rs13387042/rs4442975 and rs16857609. The 2q35 enCNV was reliably imputed in iCOGS (mean r^2=0.74) and in eight of the GWAS (r^2=0.54 to 0.73). The 2q35 enCNV was similarly associated with a reduced breast cancer risk (per copy OR=0.78 95%CI 0.74-0.84, P=6.9x10^{-16} in iCOGS; P=2.1x10^{-19}in iCOGS+GWAS combined). There was weak evidence for heterogeneity (I^2=29.29, *P*=0.04; **Figure S3**) largely driven by one study and the association remained highly significant after removing this study (OR=0.78 95%CI 0.73-0.83, P=4.1x10^{-16}). The OR for homozygous carriers of the deletion (OR=0.88 95%CI 0.56-1.38) did not differ significantly from that in heterozygous carriers (OR=0.77 95%CI 0.72-0.82), but a log-additive model could not be rejected. The association was stronger for ER-positive (OR =0.77 95%CI 0.71-0.82, P=3.1x10^{-13}) than ER-negative disease (OR=0.90 95%CI 0.80-1.01, P=0.09; P-diff=0.0079; **Table S4**), consistent with the effect observed in our initial study and previously for 2q35 loci.

The 2q35 enCNV lies in a linkage disequilibrium (LD) block of ~20Kb and strong sites of recombination separate it from the LD blocks containing the previously reported 2q35 risk loci, rs13387042/rs4442975, rs16857609; the 2q35 enCNV is uncorrelated with either locus (r^2<0.01) (**Figure 3**). In multiple regression analysis based on the iCOGS data, all three loci remain highly significantly associated with disease (**Table S5**). Only one SNP in the LD block containing the 2q35 enCNV, rs16856925 (Genbank: NC_000002 g.217096609A>G), was genotyped on the iCOGS array. This SNP was highly correlated with the 2q35 enCNV (r^2=0.90) and hence largely determined the imputed genotypes; rs16856925 was slightly more strongly associated with disease than the 2q35 enCNV (iCOGS *P*=3.7x10^{-16}; combined *P*=1.2x10^{-20}; **Figure S4 and**

Table S4). The most strongly associated variant in this block was rs34005590 (Genbank: NC_000002 g.217098337C>A; r^2=0.93; iCOGS *P*=5.6x10^{-17}; iCOGS+GWAS combined P=7.4x10^{-22}; **Figure 3, Table S4**). Fourteen variants in this block, including rs16856925 and 2q35 enCNV, were correlated with rs34005590 at r^2>0.8; however, none of these variants could be excluded as being causal at a likelihood ratio of 100:1(27). In conditional analyses, no additional SNPs were associated with disease after adjustment for rs34005590, 2q35 enCNV, or rs16856925; thus, the association results are consistent with a single causal variant within the 20Kb LD block containing the 2q35 enCNV.

Discussion

The understanding of factors affecting breast cancer risk has grown exponentially in recent years. *IGFBP5* and 2q35 have both been consistently implicated in cancer, though little was known about the nature of their interaction. Molecular studies of *IGFBP5* have revealed its essential role in normal mammary epithelial development (6, 7, 28, 29), contributing to the documented involvement of the IGF signaling axis in mammary density as a risk factor for breast cancer (30-32). A recent contemporaneous study describes a neighboring 2q35 breast cancer-associated variant nearby the locus we describe. Their intriguing and independent findings implicate an intergenic SNP in modifying expression of *IGFBP5*, however, their work focused on a narrow genomic region investigated in high resolution on the iCOGS array and excludes our reported risk locus (5). Here we shed light on the complexity of *IGFBP5* transcriptional control by estrogen and identify a polymorphic regulatory region ~400Kb upstream that

differentially regulates *IGFBP5* upon exposure to estrogen. Further, we utilize a transactivator-fused CRISPR system to evaluate 2q35 allele linkage in MCF7 cell line and confirm the repressive nature of ERα binding at the 2q35 enCNV. Targeting the wildtype sequence of the non-deleted enCNV allele results in no significant shift in the allelic balance. When considering the allelic preference of ERα binding at the 2q35 enCNV, these data suggest a model where the wildtype allele performs as a less efficient regulator of *IGFBP5* regulation, and the bulk of expression comes from the efficiently regulated *IGFBP5-A* allele. Our findings are consistent with the current understanding of chromatin architecture (33-35) and suggest that previously under-studied (36) larger CNVs, particularly in intergenic enhancers, may play a striking role in the etiology of disease.

Materials and Methods

Cell Culture and treatments

Cells were maintained according to manufacturer recommendation (ATCC). Briefly, MCF7 cells (passage 14-28) were maintained in complete DMEM (10%FBS, 5mg/mL insulin, 0.4% penicillin-streptomycin) at 37°C in humidified chamber with 5% CO_2. Cells were hormone starved prior to treatment for at least 48 hours in phenol red free media supplemented with 10% charcoal/dextran stripped FBS (Life Technologies, Carlsbad, CA). Cells were treated with vehicle (DMSO) or 17β-estradiol (10nM; Sigma-Aldrich, St. Louis, MO) for the indicated duration.

Chromatin Conformation Capture

Chromatin conformation capture was conducted as previously published with subtle modifications (11). Briefly, nuclei from 5 x 10^6 cells were isolated and crosslinked in 1% formaldehyde for 10 minutes at room temperature. Washed nuclei were resuspended in 1x restriction enzyme buffer and digested overnight with 400U of restriction enzyme (HindIII; New England Biolabs Inc., Ipswich, MA). Digested nuclei were disrupted and diluted to a final volume of 8 mL for ligation for 2-4 hours at 16°C. Ligated DNA was purified and resuspended in TE (Invitrogen Inc., Carlsbad, CA). Site-specific interactions with the "anchor" region (*IGFBP5* promoter) were assayed by realtime quantitative PCR with 100ng 3C DNA per reaction and normalized to a 3C positive control library prepared as previously described (11). All experiments were conducted in biological triplicates and qPCR reactions as technical duplicates. BACs (3096A13, 2565O2, 2505P8; Invitrogen Inc., Carlsbad, CA) were grown according to

manufacturer recommendations and purified (PureLink HiPure; Invitrogen Inc., Carlsbad, CA). Primer sequences are listed in the supplementary data.

Chromatin Immunoprecipitation

Experiments were performed as previously described according to manufacturer recommendation (Upstate Biotechnologies/EMD Millipore, Billerica, MA). Briefly, vehicle or estrogen (10nM in DMSO, 45 minutes) treated cells were crosslinked with 1% formaldehyde and washed. Cells were lysed and chromatin/protein complexes sheared by sonication. IgG or ERα (HC-20; Santa Cruz Biotechnology Inc., Dallas, TX) was immunoprecipitated overnight and complexes collected with protein A/G beads for one hour (Dynabeads; Invitrogen Inc., Carlsbad, CA). Eluted DNAs were decrosslinked and purified by ethanol precipitation. Experiments were conducted in biological triplicate and qPCR reactions in technical duplicate. Binding activity was calculated relative to input. Primer sequences are listed in the supplementary data.

Expression analysis

Nuclei from estrogen (10nM, DMSO) or vehicle (DMSO) treated cells were isolated (Nuclear extraction buffer: 100mM Tris, 100mM NaCl, 0.5% NP-40) and nuclear-enriched RNA was extracted with Trizol (Invitrogen Inc., Carlsbad, CA). Residual DNA contaminants were removed by DNAse treatment (Promega Inc., Madison, WI) and cDNA was synthesized per manufacturers recommendation (FirstStrand Synthesis Kit; Invitrogen Inc., Carlsbad, CA). Expression of total *IGFBP5* was quantified by RT-qPCR with primers targeting the 3' UTR and normalized to actin (Integrated DNA Technologies, Coralville, IA). Reactions performed at 95°C, 3min; and cycled 40x at 95°C, 15s; 61°C, 15s; 72°C, 15s, followed by melting curve analysis

(CFX96, Bio-Rad Laboratories, Hercules, CA). Allelic expression of IGFBP5 was determined by 20-cycle pre-amplification of a 700bp fragment surrounding heterozygous intronic rs7565131 A/C (95°C, 5min; cycled 20x 95°C, 30s; 61°C, 30s; 72°C, 30s, followed by a 10 min extension at 72°C). Amplified sequences were column purified (QIAamp PCR cleanup kit, Qiagen Inc., Valencia, CA) and detection was conducted using a modified RT-MAMA-qPCR with allele specific primers (12). All experiments were conducted in biological triplicates and qPCR reactions as technical duplicates. Primer sequences are listed in the supplementary data.

CRISPR-aided analysis of allele linkage

Briefly, MCF7 cells were grown in complete media and transfected with pAC154-dual-dCas9VP160-sgExpression (13) (Addgene, Cambridge, MA) containing appropriate guide RNAs by nucleofection, per manufacturer's recommendation (Nucleofector, Lonza Ltd, Basel, Switzerland). Constructs were validated by sequencing at our core facility. Guide RNAs targeted either *IGFBP5* promoter sites (Promoter site 1: CTACAAACTGGCTGGCAGCC; Promoter site 2: GTTTGTACTGCAAAGCTCCT), the ERE nearest the 2q35 enCNV (ERE: CTGAACTGTCCTCAAGTTCT), or the wildtype sequence within the deleted region (enCNV site 1: TAGATGGATCCCTCAGAAAT; enCNV site 2: CCATAGACAGGTCTTTTTTG). RNA was extracted for expression analysis as described above. Data represent technical and biological duplicates.

Women's Circle of Health Study

Study Population

The study was conducted using samples and data from the Women's Circle of Health Study (WCHS), a case-control study designed to examine risk factors for early/aggressive breast cancer among African American (AA) women compared to American women of European descent (EA). Details of the study design, inclusion criteria, and collection of survey data and biospecimens have been previously described (14, 15). Briefly, incident breast cancer cases were identified in four boroughs of metropolitan New York City using hospital-based case ascertainment, and in seven counties in New Jersey (NJ) using population-based case ascertainment through the NJ State Cancer Registry, a participant of the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program. Cases were women recently diagnosed with primary, histologically confirmed breast cancer with no previous history of cancer except for non-melanoma skin cancer who self-identified as AA or EA, 20-75 years of age, and were English speaking. Controls were frequency matched to cases by self-reported race and 5-year age groups and were recruited from the same target population as cases by using random digit dialing in the same residential area as cases. AA controls in NJ were supplemented by community recruitment efforts to assemble a control sample more representative of the general population (16). A total of 1,369 EAs (680 cases, 689 controls) and 1,403 AAs (628 cases, 775 controls) women were included in the study. The study was approved by the institutional review boards at Roswell Park Cancer Institute (RPCI), the Cancer Institute of New Jersey (CINJ), Mount Sinai School of Medicine (MSSM; now the Icahn School of Medicine at Mount Sinai), and all participating hospitals in New York.

Survey Data, DNA Collection, and Genotyping

Detailed survey data were collected by in-person interviews and included demographic and lifestyle information, family history of cancer, and medical history. Anthropometric measurements and biospecimen collections were obtained by trained interviewers. Pathology data were collected and abstracted by trained study staff from patient medical records and included information on tumor grade and stage, and ER status.

Genomic DNA for study participants was initially extracted from blood samples using the using the FlexiGene^{TM} DNA isolation kits (Qiagen Inc., Valencia, CA) and subsequently from Oragene^{TM} kits following the manufacturer's protocols, with the majority of DNA samples derived from saliva samples collected using Oragene^{TM} kits (DNA Genotek Inc., Kanata, Ontario, Canada). Genomic DNA was evaluated and quantitated by Nanodrop UV-spectrometer (Thermo Fisher Scientific Inc., Wilmington, DE) and PicoGreen-based fluorometric assay (Molecular Probes, Invitrogen Inc., Carlsbad, CA), and stored at -80°C until analysis.

Of 2,772 blinded samples initially included in the study, 228 samples could not be amplified leaving a total N=2,544 (EA: 613 cases, 630 controls; AA: 569 cases, 732 controls) in the study. Blinded samples were genotyped by a custom designed semi-automated multiplex fluorescent-coupled PCR in 96-well format followed by fragment length analysis. PCR reaction conditions were conducted per manufacturer recommendations (HotstarTaq Plus MasterMix, Qiagen Inc., Valencia, CA; 10ng DNA, initial activation of 95°C, 5min; and cycled 30x at 95°C, 30s; 57°C, 90s; 72°C, 30s, followed by a final 10min extension at 72°C). Amplified samples were diluted 4x and loaded for FLA by the Molecular Biology Core Facility at Dartmouth College. Genotypes

were assigned with a peak-calling algorithm in a 4bp window surrounding the expected amplicon size utilizing GeneMapper 4.0 software (Applied Biosystems). Briefly, calls were made by peak calling within 4bp bins centered on predicted sizes of 152 and 292bp. A threshold of 1,000 RFU was used to eliminate rare instances of signal bleed from neighboring overloaded wells (due to initial DNA concentration inconsistencies). Infrequent size calling software abnormalities were resolved manually using the same criteria as above. Quality control was conducted by secondary FLA of entire plates (N=4 x 96-well) and randomly selected individual samples (n=85).

To account for population admixture in the analysis, all samples were also genotyped at the Genomics Core Facility at Roswell Park Cancer Institute using the Illumina GoldenGate Assay (Illumina Inc., San Diego, CA) for a panel of 100 ancestry informative markers (AIMs) that were previously validated in the Black Women's Health Study Ruiz-Narváez, Rosenberg, Wise, Reich and Palmer (17). As a quality control measure, five percent duplicates and two sets of in-house trio samples were included across all plates. Proportions of European and African ancestry for each woman were computed using the Bayesian Markov Chain Monte Carlo clustering algorithm implemented in STRUCTURE (18). Since the sum of two ancestral proportions in each individual is always one, we used only the proportion of European Ancestry in all analyses.

Statistical Analysis

Continuous and categorical descriptive variables were compared between cases and controls using t-tests and chi-square tests for proportion, respectively. Odd ratios (OR) and 95% confidence intervals (CIs) for associations between 2q35 enCNV

genotype and breast cancer risk were estimated using unconditional logistic regression among all women, and stratified by self-reported race. Additional analyses were conducted to examine associations by menopausal and ER status. All analyses were adjusted for age, proportion of European ancestry, attained education, family history of breast cancer, smoking status, parity, use of hormone replacement therapy use, and study site (New York, New Jersey). Women with missing covariate data on smoking history (n=1), use of hormone replacement therapy (n=3), and family history of breast cancer (n=11), were considered to be non-smokers, non-users of hormone replacement therapy, and not to have a family history of breast cancer, respectively. For 4 women without ancestry data, race-specific median values for proportion of European ancestry were used. For analyses with pre- and post-menopausal women combined, menopausal status was also included in the model. For analyses combining EA and AA women together, self-reported race was also included in the model in addition to proportion of European ancestry estimates. Co-dominant models were analyzed and additive genotyping coding based on the number of rare alleles was used as an ordinal variable to determine P-values associated with each copy of the variant allele (p test for linear trend). Case-case unconditional logistic regression analysis was also performed to examine associations between 2q35 enCNV genotype and odds of being diagnosed with ER-negative versus ER-positive tumors. All analyses were conducted using SAS V9.3 (SAS Institute, Cary, CA). All tests were two-sided and considered statistically significant at P=0.05.

Breast Cancer Association Consortium

Genotype data for replication were derived from 11 breast cancer GWAS based on populations of European ancestry, together with 41 additional case-control studies

from populations of European ancestry participating in the Breast Cancer Association Consortium(2). The 11 GWAS were genotyped with using a variety of different platforms, while the 41 additional case-control studies were genotyped using a custom array (iCOGS). After quality control exclusions, data were available for 15,748 cases and 18,084 controls from the GWAS and 46,785 cases and 42,882 controls genotyped using the iCOGS array (after excluding samples overlapping with any GWAS; see Michailidou, 2013 for details). All studies were approved by the relevant local ethics review committee and subjects gave informed consent.

The GWAS genotype data were used to estimate genotypes for other common variants across the region in the study subjects by imputation, with IMPUTE v.2.2 (19) and the March 2012 release of the 1000 Genomes Project as reference panel, after prephasing using SHAPEIT (20) with the exception of three GWAS - BCFR, BPC3 and TNBCC - for which imputation was performed using MACH (21) and Minimac (22). Per-allele odds ratios (ORs) and standard errors for individual studies were generated using SNPTEST (23) and ProbABEL (24). For the iCOGS samples the imputation was performed in one step without pre-phasing using IMPUTE.v2 and the March 2012 release of the 1000 genomes as reference, analysis for the iCOGS samples was done using logistic regression in R. Estimated ORs for the combined analysis were generated using a fixed-effect meta-analysis adjusting for genomic control, using METAL (25). Data for SNPs with an imputation accuracy r2>0.3 in a given study were included in the combined analysis. For the combined analysis of the GWAS and iCOGS, we reanalyzed the iCOGS data to remove samples also included in a GWAS, to generate independent datasets. For the iCOGS data we adjusted for study and used nine principal components to adjust for

potential population stratification. GWAS were adjusted for differing sets of principal components as previously described (2). The iCOGS data were similarly used to estimate per-allele ORs separately for ER-positive and ER-negative disease (27,078 and 7,333 cases, respectively).

To evaluate the evidence for association between the 2q35 enCNV and other association SNPs on 2q35, we performed multiple logistic regression in the iCOGS dataset, including all SNPs together with study and principal component as covariates. The P value for each SNP, after adjustment for all other SNPs, was determined by a Wald test.

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Conflict of Interest Statement

AW is a founder and shareholder of Genextropy Inc.; the remaining authors declare no potential conflicts of interest.

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Legends to Figures

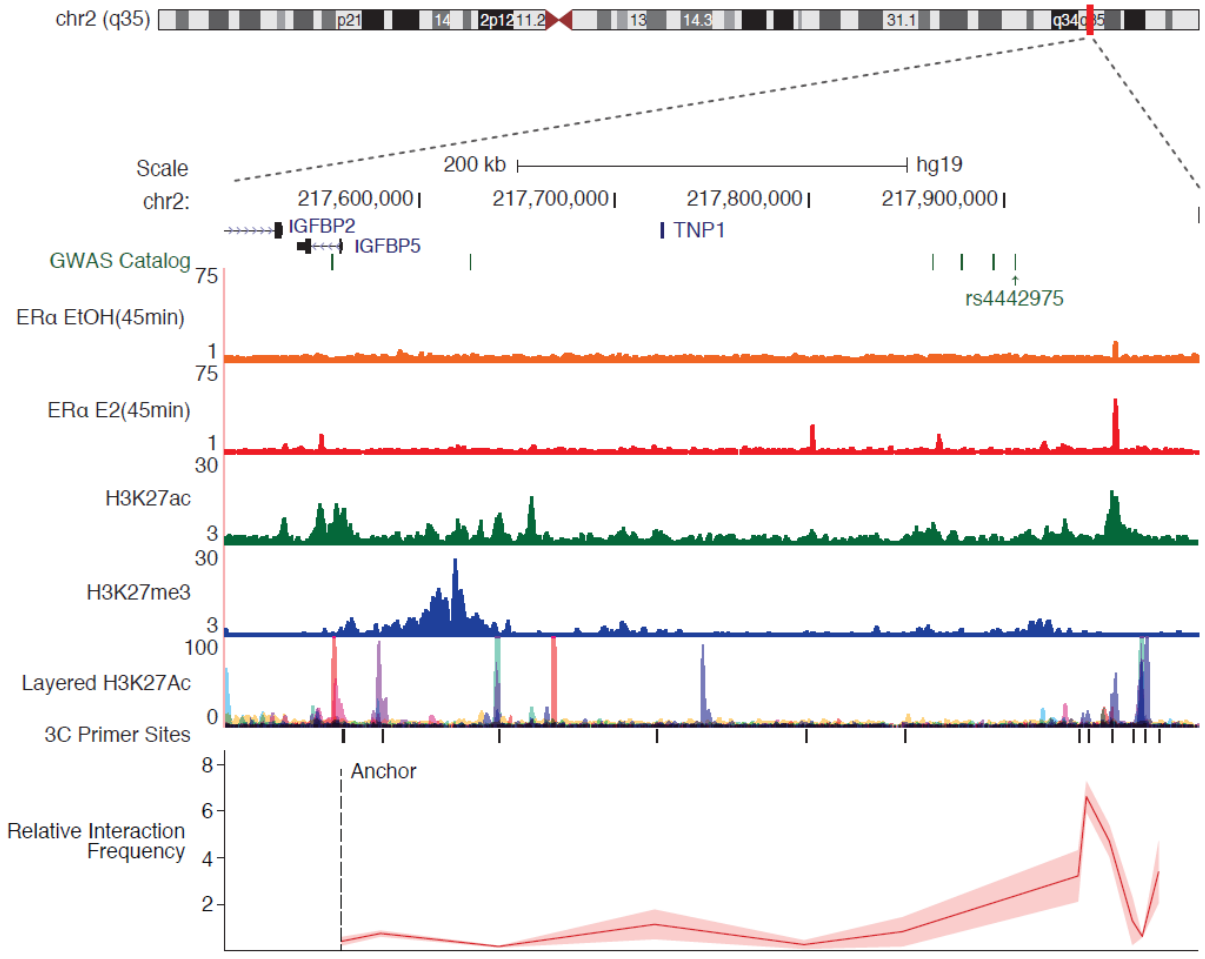
Figure 1: Epigenetic and chromatin interaction profiles of the 2q35 gene desert upstream of IGFBP5. ChIP-seq read density was plotted for estrogen receptor (ERα) (37), H3K27Ac, H3K27me3, and ENCODE layered H3K27Ac (33) for breast cancer cell line MCF7 (upper panels, as labeled). Relative interaction frequency was investigated with Chromosome Conformation Capture (3C) (11) for the *IGFBP5* promoter (Anchor) in breast cancer cell line MCF7 (lower panel). Primer locations for 3C are indicated, and average profile (red line) and standard deviation (shaded region) for biological triplicates are plotted. The browser graphic was modified from the UCSC genome browser (http://genome.ucsc.edu/index.html) (38).

Figure 2: Analysis of allelic binding and effects on allelic expression of IGFBP5. ChIP-seq read density for a 3Kb region overlapping the ERα-bound looping enhancer was plotted for ERα (37), H3K27Ac, H3K27me3, and ENCODE layered H3K27Ac (33) (panels as labeled). The blue bar indicates the location of the intergenic enhancer copy number variation. ERα binding activity at the ERE (orange bar) was assayed by Chromatin Immunoprecipitation (ChIP)-qPCR for the variant (red) and wildtype (blue) alleles, and a negative control region (in *ACTB*, purple), in heterozygous MCF7 cells with estrogen treatment (vehicle and estrogen indicated in light and dark shades for each site, respectively). Allelic detection primers were designed as indicated on inset map. Error bars represent SD of biological triplicates. *P<0.004; **P<0.002. Investigation of allele-specific expression of *IGFBP5* was conducted by allelic amplification of intronic marker SNP, rs7565131. Briefly, nuclear RNA from estrogen or vehicle treated cells was

isolated. Total *IGFBP5* nuclear RNA was determined by detection of 3'UTR sequence (total bar height; error bars represent SD of biological triplicates). Allelic expression was evaluated by detection of allele-specific products by a modified MAMA(12)-qPCR. Relative abundance (total signal %) indicated by color (rs7565131-A and C as red and blue, respectively). Error bars with hats represent SD of biological triplicates. ***P=0.027; ****P=0.014

Figure 3: Regional plots of the three independent 2q35 breast cancer risk loci in 41 case control studies and 11 GWAS (n=123,499). For imputed variation within a 500Kb region including the 2q35 enCNV, $-\log_{10}$ P-values are plotted against genomic position (human reference sequence, hg19). The most strongly associated SNP in the 20Kb linkage block containing the enCNV, rs34005590, is represented by a purple diamond. The 13 additional variants in high LD (r^2>0.8) cluster tightly around ~218,000,000 (**Table S4**). Previously identified independent loci, rs13387042/rs4442975 and rs16857609 lie in centromeric and telomeric peaks, respectively. Image drawn with LocusZoom (http://csg.sph.umich.edu/locuszoom/).

Figure 1


chr2 (q35)
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IGFBP2
IGFBP5
TNP1
GWAS Catalog
rs4442975
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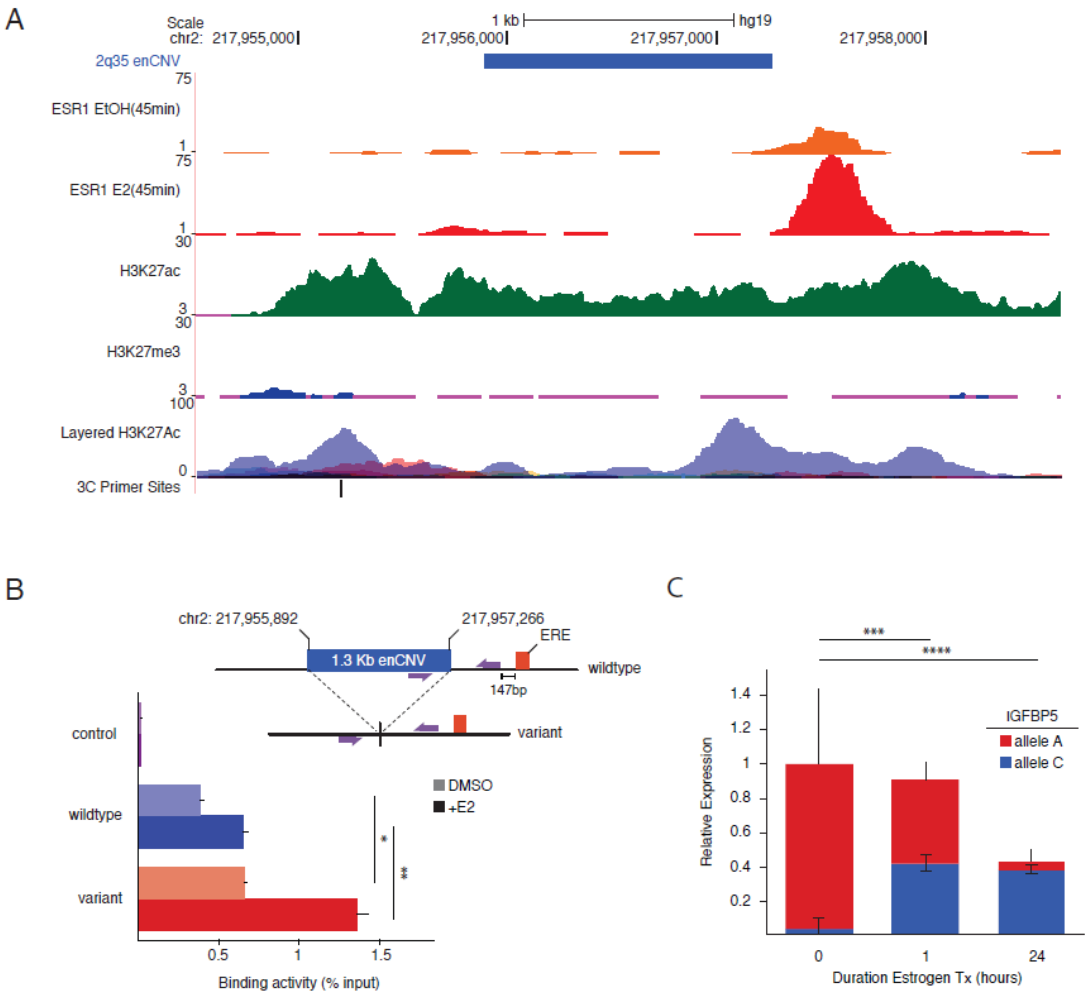
H3K27me3
Layered H3K27Ac
3C Primer Sites
Anchor
Relative Interaction Frequency

Figure 2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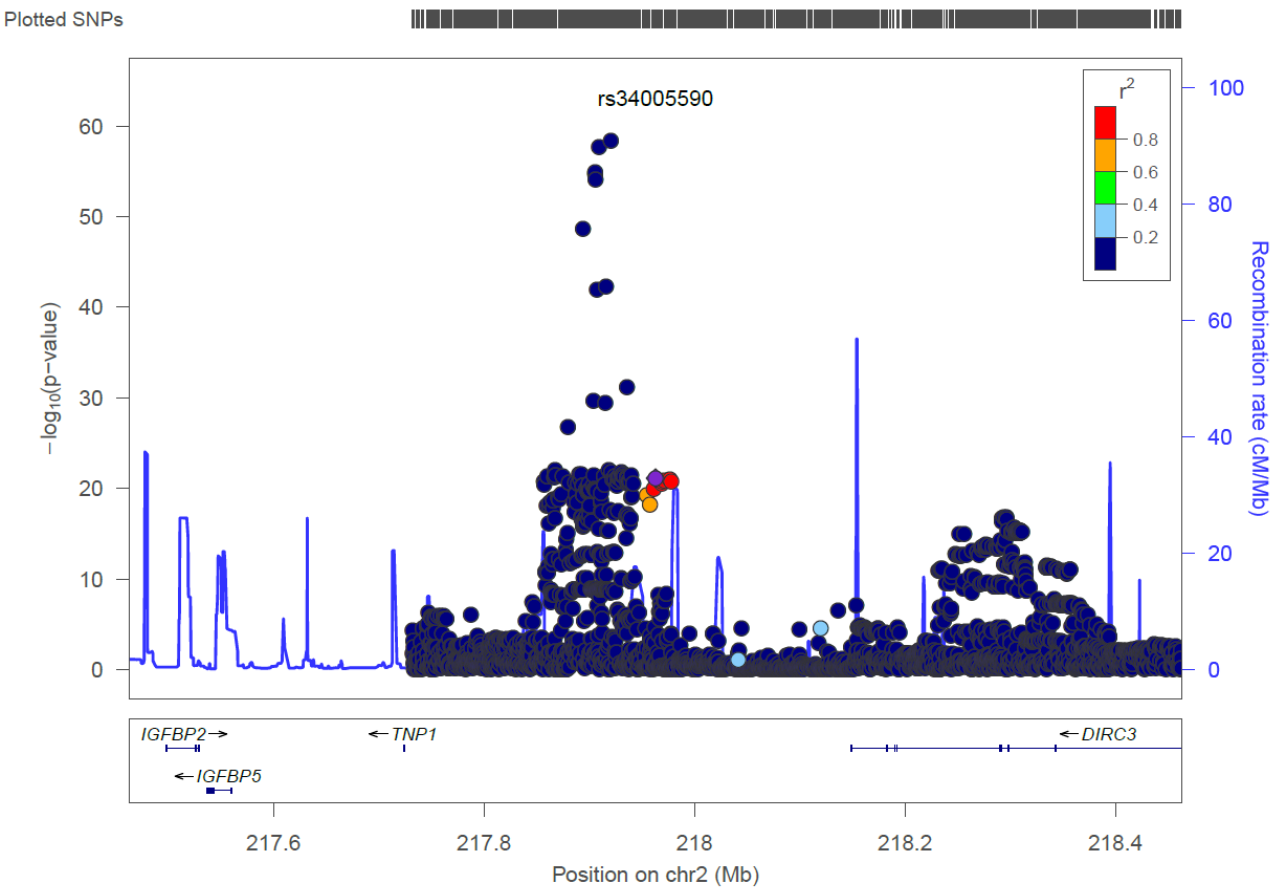
1 kb
hg19
217,956,000
217,957,000
217,958,000
2q35 enCNV
ESR1 EtOH(45min)
ESR1 E2(45min)
H3K27ac
H3K27me3
Layered H3K27Ac
3C Primer Sites
B
chr2: 217,955,892
217,957,266
ERE
1.3 Kb enCNV
wildtype
147bp
variant
control
wildtype
variant
DMSO
+E2
Binding activity (% input)
C
IGFBP5
allele A
allele C
Relative Expression
Duration Estrogen Tx (hours)

Figure 3


Plotted SNPs
rs34005590
r2
0.8
0.6
0.4
0.2
−log10(p−value)
Recombination rate (cM/Mb)
IGFBP2
TNP1
DIRC3
IGFBP5
Position on chr2 (Mb)