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Co-amplified genes at 8p12 and 11q13 in breast tumors cooperate with two major pathways in oncogenesis

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Abstract

Co-amplification at chromosomes 8p11-8p12 and 11q12-11q14 occurs often in breast tumors, suggesting possible cooperation between genes in these regions in oncogenesis. We used high resolution array comparative genomic hybridization (array CGH) to map the minimal amplified regions. The 8p and 11q amplicons are complex and consist of at least four amplicon cores at each site. Candidate oncogenes mapping to these regions were identified by combining copy number and RNA and protein expression analyses. These studies also suggested that *CCND1* at 11q13 induced expression of *ZNF703* mapping at 8p12, which was subsequently shown to be mediated via the Rb/E2F pathway. Nine candidate oncogenes from 8p12 and four from 11q13 were further evaluated for oncogenic function. None of the genes individually promoted colony formation in soft agar or collaborated with each other functionally. On the other hand, *FGFR1* and *DDHD2* at 8p12 cooperated functionally with *MYC*, while *CCND1* and *ZNF703* cooperated with a dominant negative form of *TP53*. These observations highlight the complexity and functional consequences of the genomic rearrangements that occur in these breast cancer amplicons, including

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transcriptional cross-talk between genes in the 8p and 11q amplicons, as well as their cooperation with major pathways of tumorigenesis.

Introduction

Amplifications involving chromosomes 8p (*RAB11FIP1*, *FGFR1*), 11q (*CCND1*) and 17q (*ERBB2*) are among the most common high level copy number aberrations in breast tumors, occurring, for example, in one study, in 22.8%, 19.6% and 9.9% of tumors, respectively (Letessier et al., 2006). Amplification of 8p and 11q are most often observed in estrogen receptor positive tumors, while amplification of 17q (*ERBB2*) occurs in both estrogen receptor positive and negative tumors (Fridlyand et al., 2006; Letessier et al., 2006; Loo et al., 2004). Poor prognosis is associated with the presence of these amplicons in breast cancer. Thus, overexpressed genes within amplicons are attractive targets for therapy, as exemplified by the targeted use of herceptin to treat patients with tumors with *ERBB2* amplification.

Frequently, two or more of these amplicons are present in a given tumor, suggesting that genes in the two amplified regions may collaborate in formation of the transformed phenotype (Yang, Moffa, Haddad, Streicher and Ethier, 2007). Of interest in this regard, amplification of *FGFR1* at 8p12 and *CCND1* at 11q13 occurs preferentially in breast cancers, with amplification of 8p12 having been reported in 30–40% of tumors with *CCND1* amplification. Co-amplification of these genes is associated with significantly reduced patient survival (Cuny et al., 2000). In some cases, cytogenetic findings are consistent with physical co-amplification of the regions (Bautista and Theillet, 1998; Paterson et al., 2007), but selection for elevated copy number of a translocation-fusion gene is not thought to drive amplification (Paterson et al., 2007). Amplification and overexpression of a number of candidate oncogenes mapping at 8p12 and 11q13 are associated with the luminal breast cancer subtype (Adelaide et al., 2007). These observations raise the question as to whether collaboration between oncogenes within the two amplified regions from each chromosome in formation of the transformed phenotype provides a selective advantage for co-amplification. Here, we investigated this question by comprehensively profiling the 8p12 and 11q13 amplicons to define the recurrent regions of amplification and to identify candidate oncogenes. Subsequently, we evaluated these genes for their ability to (a) transform MCF10A cells by themselves, (b) in combination with genes from the other chromosome and (c) with genes involved in major pathways of cancer.

Results

Copy number profiling of tumors with amplicons at 8p12 and 11q13

We assembled a microarray with 64 BACs providing near tiling path coverage of the 9 Mb region of chromosome 8p11-p12 from *RNF122* to the centromere and 101 BACs at 11q12-q14. The 8p12 or 11q13 amplicons identified in tumors and cell lines using our standard genome scanning arrays of 2464 BACs (Snijders et al., 2001) were then fine mapped on this 8p11q array. The analysis of six tumors with 8p amplification from our previous study (Fridlyand et al., 2006) allowed us to define four amplicon cores in the 8p11-p12 region

(Figure 1A, Supplementary Table 1). Further analysis of a second set of breast tumors (Climent et al., 2007) in which 8p amplification was present in nine tumors yielded a similar result (Supplementary Table 1). The minimal amplified regions are summarized in Table 1. Amplicon cores A1 and A2 occurred most frequently, each being present in 7% of tumors (n=360) from three breast cancer array CGH datasets (Chin et al., 2006; Climent et al., 2007; Fridlyand et al., 2006). The minimal amplicon core A1 spanned 0.4 Mb as defined by tumor S0131 and cell line SUM225 (Supplementary Table 1). Amplification of the two more centromere proximal amplicon cores, A3 and A4 occurred less frequently (5% of tumors, n=360).

Eighteen tumors with amplification at 11q13 were analyzed on the 8p11q array and the 11q13-q14 copy number profiles generally showed four regions of amplification (Table 1) extending distally from the region encompassing *CCND1* (Figure 1B). An amplicon core mapping more proximally to *CCND1* was also present. Variably positioned amplicons between A2 and A3 were also observed. The A3 amplicon was further subdivided into two sub amplicon cores, A3.1 and A3.2. Three tumors (J3891, S0065 and S0081, Supplementary Table 1, Supplementary Figure 1) defined a minimal amplified region bracketed by *UVRAG* and *LRRC32* including *C11orf30*, whereas a minimal amplified region bracketed by *C11orf30* and *PHCA*, including *LRRC32* was present in two other tumors (J1144 and J5683, Supplementary Table 1, Supplementary Figure 1). Amplification at 11q13 varied in the number of cores which were present (Supplementary Figure 2). For example, in five tumors, J3981, J1901, S0132, S1534 and S1598 all four cores were present, whereas only A1 and A2 were amplified in J1333. In two tumors (J363 and J665), high level copy number was restricted to only A2 (Supplementary Table 1 and Supplementary Figures 2 and 3). Thus, the amplicon structure at 11q13 is highly complex; amplification of the four cores can occur independently, suggesting the presence of several driver genes for amplification.

Selection of candidate driver genes for amplification

We subjected genes in the amplicons to four tests for candidacy as driver oncogenes with the rationale that candidate oncogenes should be overexpressed when amplified, although overexpression may also occur by other means. Thus, we evaluated genes for copy number and expression by first accessioning a dataset of 90 breast tumors for which both DNA copy number and expression measurements were available (Chin et al., 2006). We then asked if expression levels of genes showed significant correlation with amplification at 8p12 or 11q13 (Supplementary Table 2). In addition, because co-amplification of 8p12 and 11q13 is frequent, we asked whether expression levels of genes mapping on 8p12 or 11q13 were responsive to copy number of the other chromosome by assessing correlation of expression of genes on the one chromosome with copy number of the other. In the second test, we carried out the same analysis on breast cancer cell lines previously profiled for transcript levels and DNA copy number (Neve et al., 2006). In the third and fourth assays, we compared transcript and protein levels in breast cancer cell lines with amplification at 8p12 alone, 11q13 alone or 8p12 and 11q13 to MCF10A (Figures 2 and 3 and Supplementary Table 3). The cell lines were profiled on the 8p11q array and included BT549 with a gain of 8p, SUM225 with amplification of 8p12 alone (Willmarth, Albertson and Ethier, 2004; Yang, Albertson and Ethier, 2004), 600MPE and MDA-MB-175 with amplification of

11q13 (Garcia et al., 2005; Snijders et al., 2001), SUM52, SUM44 and MDA-MB-134 with amplification of 8p12 and 11q13 (Garcia *et al.*, 2005; Yang, Albertson and Ethier, 2004) (Supplementary Table 1).

The cell line, MCF10A is immortal, but not transformed and does not form colonies in soft agar. We considered genes to be overexpressed at the transcript level if levels were at least five times that in MCF10A. This cutoff was selected because cell lines typically showed amplification levels greater than four-fold (e.g. SUM225 and MDA-MB-134 are amplified eight-fold on 8p relative to the median copy number for the cell line, Figure 1A). Similarly, we expected protein expression levels of candidate driver genes to be higher in breast cancer cell lines compared to MCF10A and also to be expressed at low levels in MCF10A. The outcome of these analyses is summarized in Figure 4. In addition, three genes mapping at 8p12 (*ADAM9*, *ADAM2* and *ANK1*) showed significant correlation of expression with copy number at 11q13, but no association of chromosome 11q gene expression with copy number at 8p was found (Supplementary Table 2).

In the 8p amplicon, 10 genes met the criteria to be classified as candidate oncogenes for all assays for which they were tested, although the number of tests to which they were subjected varied from all four to only one. Thus, *ZNF703*, *ERLIN2* (*C8orf2*), *PROSC*, *RAB11FIP1*, *ASH2L*, *LSM1*, *BAG4*, *DDHD2*, *PPAPDC1B* and *WHSC1L1* were considered good candidates. We consider *FGFR1* also to be a reasonably strong candidate, because it met the criteria for three of the four tests. Eight of these genes mapped within the minimal amplicon cores A1 and A2, providing further support for their candidacy as driver genes for these amplicons. Additional possible candidates include *TAAC1*, *TM2D2* (*BLP1*) and *ADAM18*, because they variably showed some increase in expression in cell lines by quantitative RT-PCR and/or expression levels were correlated with copy number in breast tumors or cell lines. We ruled out *TM2D2* (*BLP1*), because it maps outside the minimal amplicon cores, but *TAAC1* and *ADAM18* map at the distal edge of the A2 and A3 amplicon cores, respectively, and so remain possible drivers for amplification. We ruled out two additional genes, *GPR124* and *BRF2*, because of high protein levels in MCF10A. A similar analysis of the 11q13 amplicon revealed that four genes, which were subjected to all four tests met the criteria for three of them. They included *CCND1*, *FADD*, *PPFIA1* and *CTTN*.

Transcriptional crosstalk between chromosome 8p12 and 11q13 amplicons

Amplification of 8p12 and 11q13 frequently occur together suggesting possible interactions between the genes in these two amplicons. Indeed, it has been reported previously that *FGFR1* (at 8p12) is up-regulated by increased expression of *CCND1* (at 11q13) in fibroblasts, and occurs via *CCND1* mediated activation of the pRB/E2F pathway (Tashiro et al., 2003). In our evaluation of expression of genes in the 8p amplicons in cell lines, we observed increased expression of *ZNF703* and *RAB11FIP1* at the RNA level and *BAG4*, *RAB11FIP1* and *WHSC1L1* at the protein level in cell lines with amplification at chromosome 11q12-11q14, even when these genes, which map to 8p11-8p12 were not amplified (Figure 2). In order to determine if any of these genes could be regulated by *CCND1*, we assessed expression of the genes when *CCND1* was knocked down in SUM44 cells, which harbor both the 8p12 and 11q13 amplicons and also following forced

overexpression of *CCND1* in MCF10A cells. When two different shRNAs were used to knock down the transcript and protein expression levels of *CCND1* by 60% and 70%, respectively in SUM44 cells, only expression of *ZNF703* decreased by 40%, while there was no change in expression of *BAG4*, *FGFR1*, *RAB11FIP1*, and *WHSC1L1* (Figures 5A and B). This reduction in *ZNF703* expression was observed at 72 and 96 hours after infection with shRNA. Nevertheless, it was not maintained after one week in cells selected for stable expression of the shRNA, even though *CCND1* levels remained low compared to control infected cells (results not shown). By contrast, stable infection of MCF10A cells with a *CCND1* lentivirus resulted in a maximal four-fold increase in expression of *ZNF703* and 1.5-fold expression of *BAG4*, *FGFR1*, *RAB11FIP1*, and *WHSC1L* (Figure 5C). Since the reported up-regulation of *FGFR1* by *CCND1* occurs via activation of the pRB/E2F pathway (Tashiro et al., 2003), we investigated whether the up-regulation of *ZNF703* is similarly mediated by *E2F1*. Overexpression of *E2F1* in MCF10A consistently resulted in up-regulation of all genes tested, but much greater up-regulation of *ZNF703* and *FGFR1*. These observations suggest that expression of at least two genes in the 8p12 amplicon, *FGFR1* and *ZNF703*, are regulated by *E2F1* and *CCND1*. Nevertheless, examination of a breast cancer expression array dataset comprised of all breast cancer subtypes (Chin et al., 2006) revealed no significant correlation of *FGFR1* expression with either *E2F1* or *CCND1* expression levels (Spearman correlation < 0.25). On the other hand, when considering only luminal tumors, all four *FGFR1* probesets showed some association with one or both *E2F1* probesets (Spearman correlation > 0.3), and the correlation was considered significant for two of the *FGFR1* probesets (Spearman correlation ≥ 0.4, FDR < 0.05), suggesting some association.

Transforming activity of overexpressed genes in 8p12 and 11q13 amplicons

To evaluate possible oncogenic functions of genes in the amplicons, they were overexpressed in MCF10A and assessed for the capability to promote growth in soft agar. We selected six overexpressed genes from the A1 and A2 cores of the 8p12 amplicon (*ZNF703*, *ERLIN2*, *RAB11FIP1*, *DDHD2*, *WHSC1L1* and *FGFR1*), three additional overexpressed genes at the boundary of A2 (*ASH2L*, *LSM1* and *BAG4*), and four genes from the 11q13 amplicon (*CCND1*, *FADD*, *PPFIA1* and *CTTN*) for study. The genes were expressed by themselves in MCF10A, and we also asked whether they collaborate with *MYC* or *TP53*, genes deregulated in breast cancer. Amplification and/or overexpression of *MYC* occurs in 30–50% of breast cancers and has been associated with poor prognosis and resistance to anti-estrogen therapy (Agrawal, Yang, Murphy and Agrawal, 2006). TP53 is altered in ~18% of breast cancers and can be further inactivated by deregulation of interacting genes such as MDM2 (*i.e.* alterations in the *CDKN2A – MDM2 – TP53* pathway have been reported in 25% of breast cancers) (Ho et al., 2001). Expression of the genes individually did not promote colony formation in soft agar; however greater numbers of colonies were observed in cells co-expressing *MYC* and *FGFR1* or *DDHD2* (Figure 6A, Supplementary Figure 3). Expression of GSE56, a dominant negative mutant of *TP53* together with *CCND1* or *ZNF703* also resulted in more than two-fold increase in numbers of colonies, while expression with *CTTN* enhanced colony formation, but less than two-fold compared to MCF10A cells expressing GSE56 alone (Figure 6B, Supplementary Figure 4). Because 8p12 and 11q13 are often co-amplified in breast tumors, we also assessed whether

selected genes from these two amplicons cooperated with each other. Genes from 8p (*ZNF703*, *BAG4*, *WHSC1L1* or *FGFR1*) were assayed in combination with (*CCND1*, *FADD*, *CTTN* or *PPFIA1*); however, no increase in the number of colonies was observed in any of the combinations (data not shown).

Discussion

The 8p12 amplicon has been the subject of a number of studies using high resolution mapping by tiling path BAC array CGH and FISH. Three studies (Garcia et al., 2005; Prentice et al., 2005) concluded that there was a single minimal amplicon encompassing *ZNF703* (FLJ14299), *ERLIN2* (*SPFH2*, *C8orf2*), *BRF2* and *RAB11FIP1* and corresponding to the A1 core defined here. On the other hand, Gelsi-Boyer and colleagues (Gelsi-Boyer et al., 2005) defined four overlapping amplicon cores at 8p11-p12, with their most telomeric amplicon cores, A1 and A2 corresponding to the A1 and A2 cores defined here. There is less agreement on the definition of the minimal amplicon cores A3 and A4 in the two studies, as we considered them to map more distally (Table 1). Amplification of cores largely similar to A1 and A2 have also been observed in familial breast cancer (Melchor et al., 2007). A partial explanation for the failure of Garcia et al. (Garcia et al., 2005) and Pole et al. (Garcia et al., 2005) to find multiple amplicons can in part be due to the use of an array in these two studies that spanned the region from *WRN* to *ZMAT4* and so excluded the A4 core. On the other hand, Prentice et al. (Prentice et al., 2005) studied only five samples on a whole genome tiling path array and may have observed only the A1 amplicon core due to the small sample size. By contrast, Haverty and colleagues (Haverty et al., 2008) recently reported that their data on ~50 samples did not support the existence of multiple cores, as only the more frequently amplified A1 amplicon core was identified using the GISTIC algorithm (Beroukhim et al., 2007). Several lines of evidence argue against there being only a single 8p11-p12 amplicon core of importance in breast cancer, including (a) the consistency with which multiple 8p amplicon cores have been observed [this report and (Gelsi-Boyer et al., 2005; Melchor et al., 2007)], (b) the fact that the cores can be amplified independently [Figure 1A and Supplementary Table 1 and (Garcia et al., 2005; Gelsi-Boyer et al., 2005; Prentice et al., 2005; Ray et al., 2004)], (c) the association of particular amplicon cores with different breast tumor types (Reis-Filho et al., 2006), and (d) prognostic significance associated with individual cores (Gelsi-Boyer et al., 2005). Thus, it appears that aggregation of copy number data using the GISTIC procedure is insensitive to the complexity of amplified regions such as those present in breast tumors.

Amplification of 11q13 is also frequently complex in breast and other tumor types. Four to five amplicon cores have been identified in breast cancers by Southern blotting, FISH and chromosome CGH (Bekri et al., 1997; Janssen et al., 2002; Karlseder et al., 1994; Rodriguez et al., 2004), and here by high resolution array CGH. The amplicon cores may be amplified independently and their presence has been associated with different clinical features (Bekri et al., 1997; Cuny et al., 2000; Janssen et al., 2002; Karlseder et al., 1994; Rodriguez et al., 2004; Rots et al., 1999). These observations suggest that multiple driver oncogenes map to 11q13 and further that they may define different breast tumor subtypes.

Generally, studies of amplicons have identified a number of likely candidate oncogenes based on correlation of overexpression with amplification. Subsequently, a variety of assays can be used to provide evidence for oncogenic function; however the conclusions appear to be highly dependent on the assay used. For example, *LSM1* and *BAG4*, but not *PPAPDC1B* were reported to be transforming by one group (Yang, Streicher, Ray, Abrams and Ethier, 2006), while another group came to the opposite conclusion and attributed the difference to the assays used by the two groups (Bernard- Pierrot et al., 2008). In our studies, none of the tested genes, including *LSM1* and *BAG4* enhanced colony formation in soft agar when expressed in MCF10A. In addition, when assayed in combination with other genes from 8p12 or 11q13, no evidence of cooperation was found that might have provided selective advantage for the frequent co-amplification of 8p and 11q. Similarly, we found no genes for which the expression of a gene on 8p or 11q was correlated with amplification of the other chromosome. We did find, however, that transcriptional cross-talk occurred between the two regions, as expression of *ZNF703* on chromosome 8p12 was induced by expression of *CCND1* on chromosome 11q13 via the Rb/E2F pathway. On the other hand, functional cooperation of genes on 8p with *MYC* (*FGFR1* and *DDHD2*) and loss of *TP53* (*ZNF703*) supports their candidacy as driver oncogenes for the 8p12 amplicons.

Little is known about *ZNF703* and *DDHD2*, aside from the presence of conserved domains including a zinc finger domain in *ZNF703*, and in *DDHD2*, a sterile alpha motif found in signaling and nuclear proteins, as well as a DDHD domain that may be involved in metal binding. In a comparison of basal and luminal breast cancer expression profiles, *ZNF703* emerged as the candidate oncogene most significantly associated with the luminal subtype (Adelaide et al., 2007). Further investigation will be required to understand the possible oncogenic functions of these genes. Similarly, although expression of *FGFR1* in mammary epithelial cells induces a number of transformed properties (Xian, Schwertfeger, Vargo-Gogola and Rosen, 2005), a mechanistic understanding of the cooperation between *FGFR1* and *MYC* in promoting a transformed phenotype is currently lacking. Cooperation between *CCND1* and *TP53* loss in promoting growth in soft agar also requires further investigation. Although not observed in breast cancer (Fridlyand et al., 2006), a positive correlation of *TP53* mutation with *CCND1* amplification is observed in oral squamous cell carcinoma (Mineta, Borg, Dictor, Wahlberg and Wennerberg, 1997; Snijders *et al.*, 2005). Possibly, the cooperation between *CCND1* and *TP53* dysfunction results from suppression of growth control mechanisms similar to the p53-dependent cell cycle arrest pathway reported to function independently of *RB1* in cells with *CCND1* overexpression (Kan, Patton, Stark and Jackson, 2007).

The heterogeneity of breast cancer is highlighted by the discrimination of tumor subtypes by genome-wide copy number and expression profiling. Genomic copy number changes are expected to reflect cancer pathways active in the different tumor subtypes. Identification of the driver oncogenes and the functional consequences of their overexpression, together with the knowledge of their gene-gene interactions will enable tailoring of therapeutics for each tumor subtype. Our studies of the 8p12 and 11q13 amplicons have contributed to such an effort by further refining the mapping of the minimal amplicons and identifying *ZNF703*,

DDHD2 and *FGFR1*, genes amplified and overexpressed at 8p12 and *CCND1* at 11q13 as genes that functionally cooperate with major pathways of oncogenesis.

Materials and Methods

Cell lines and tumors

Cell lines obtained from the American Type Culture Collection (ATCC) included MCF10A, BT474, BT549, 600MPE, MDA-MB-175, and MDA-MB-134. MCF10A cells were grown in ATCC growth medium with 2% rather than 5% horse serum, since growth and colony formation in soft agar was inhibited in the higher serum concentration (Supplementary Figure 5). Moreover, propagation of cells in 5% serum promoted selection in later passages for cells with improved growth and higher frequency of spontaneous colony formation in soft agar. MDA-MB-134 cells were grown in 10% instead of 20% fetal bovine serum as we found that decreasing the serum did not affect their growth. SUM52, SUM225 and SUM44 cell lines were the kind gift of Stephen P. Ethier (Ethier, 1996; Ethier, Mahacek, Gullick, Frank and Weber, 1993; Forozan, Karhu, Kononen, Kallioniemi and Kallioniemi, 1997).

Breast tumors with amplification at 8p12 and/or 11q13 were accessioned from two sets of breast tumors previously profiled by array CGH (Climent et al., 2007; Fridlyand et al., 2006). Data from a third set of 90 tumors (Chin et al., 2006), profiled for both DNA copy number by array CGH and expression using the Affymetrix HTA system, were used for the analysis of gene expression with respect to DNA copy number.

Array CGH

The 8p11q array, providing near tiling path coverage of 8p and 11q13, was assembled with 64 BACs spanning the 8p11-p12 region, 101 BACs at 11q12-q14 and a set of 192 BACs distributed across the genome (Snijders et al., 2003). Clones covering the 8p and 11q regions were selected by reference to the genome assembly using the UCSC genome browser (July 2003 assembly). The DNA spotting solutions for the arrays were prepared using ligation mediated PCR as described previously (Snijders et al., 2001) after isolation of BAC DNA using the PhasePrep™ BAC DNA Kit (Sigma-Aldrich). Array CGH, imaging and data analysis were carried out as described previously (Snijders et al., 2001). The array CGH data for the 8p11q array were deposited in the NCBI Gene Expression Omnibus database, accession number GSE12761.

Statistical analysis of gene expression and amplification

All analyses were performed using the freely available R language. Copy number and expression data were obtained for breast tumors (Chin et al., 2006) and cell lines (Neve et al., 2006). To study the main effects of chromosome 8 and 11 amplification (as previously defined (Fridlyand et al., 2006) when multiple adjacent clones were included in the region or as log2ratio = 0.75 for single clones) on gene expression, an additive linear model was fit to each clone in the regions of amplification on chromosomes 8 and 11 with the two indicator variables as independent variables and gene expression as response. Clone-wise test of the main effect was done using moderated t-statistics (Smyth, 2004), based on empirical Bayes method of shrinkage of standard errors towards a common value, using smoothed data and

adjusting for multiple testing by controlling for false discovery rate (FDR) (Hochberg, 1995). An FDR adjusted p-value less than 0.05 was used to declare a clone significant. Further, because of the higher variability in gene expression in the samples with amplifications, a robust linear model applying iterated re-weighted least squares (IWLS) method (Smyth, 2004) was applied.

To study the correlation between copy number and gene expression for the 8p12 and 11q13 regions, the gene expression probesets were mapped to the nearest BAC clone from the 8p12 and 11q13 regions. Correlation was computed for each probeset and a Spearman correlation coefficient of at least 0.4 and FDR adjusted p-value cut-off of 0.05 were used to identify clones having positive correlation between copy number and gene expression. In order to avoid spurious correlations in the absence of real copy number changes, correlations were computed for the gene transcripts whose absolute assigned copy number exceeded 0.25 in at least five samples

Expression of candidate oncogenes in MCF10A

The source of cDNA clones is given in Supplementary Table 6. Clones in pOTB7 were recombined into Gateway entry vector, pDONR221, generating the genes in pENTR221 vector (pDONR221 is renamed pENTR221 when the genes are recombined into it and the *ccdB* gene is recombined out). The pENTR clones were then recombined with Gateway competent pLenti-V6-dest lentiviral vector with blasticidine resistance to generate genes in the pLenti-V6-dest blasticidine vector. The LPCX vector has been converted into a Gateway entry vector by insertion of sequences for recombination and was also used to recombine with the pENTR clones to generate genes in LPCX. All recombination experiments and Gateway conversion were carried out as described in the Gateway Technology protocol (Invitrogen, Inc.). Expression of all genes was checked with western blotting when antibodies were available and/or by real-time PCR.

CCND1 shRNA

Five shRNAs against *CCND1* in pLKO.1 lentiviral puro vector (Sigma-Aldrich Co. cat# TRCN0000040038, TRCN0000040039, TRCN0000040040, TRCN0000040041, TRCN0000040042) were evaluated for knockdown of CCND1 protein levels by western blotting. The two shRNAs (TRCN0000040038 and TRCN0000040041) that gave the greatest knockdown were used for further assays.

Soft Agar Assay

We observed that late passages of the MCF10A cell line developed the ability to grow in soft agar. Therefore, MCF10A cells lacking soft agar growth capability were identified by dilution-cloning. The clonal MCF10A cells were infected with retroviral or lentiviral vectors alone or with the vectors expressing the cDNA of the gene of interest. The infected cells were exposed to the appropriate antibiotics for the selectable markers present in the vectors until uninfected cells were completely dead. For doubly infected cells, the infections and selections were carried out sequentially. Following selection with the appropriate antibiotics for the selectable marker in the first construct, antibiotics were removed from the media, and the cells were infected with the second construct containing a different selectable marker

and selected for infection accordingly. At least two independent infections for each construct were carried out and assayed in soft agar as described by David Bowtell (http://www.bio.com/protocolstools/protocol) with the following modifications: The bottom and top agar layers were 1.4% and 0.8% Bacto Agar (Difco), respectively. For each cell line, 2×10^4 cells were plated in each of three wells of a six well plate; however the number of cells was reduced to 1×10^4 cells per well if the cells were previously transduced with GSE56. *HRAS* transduced MCF10A was plated with every assay as a positive control for growth in soft agar (Wang, Soule and Miller, 1997). Culture medium on top of the agar was changed weekly to prevent the agar from drying out. Colonies, which were usually visible between two to three weeks after incubation, were fixed and stained with 0.005% crystal violet in 50% methanol/50% PBS. Photomicrographs of each well were obtained using a dissecting microscope and the number of colonies was counted using ImageJ (http://rsbweb.nih.gov/ij/).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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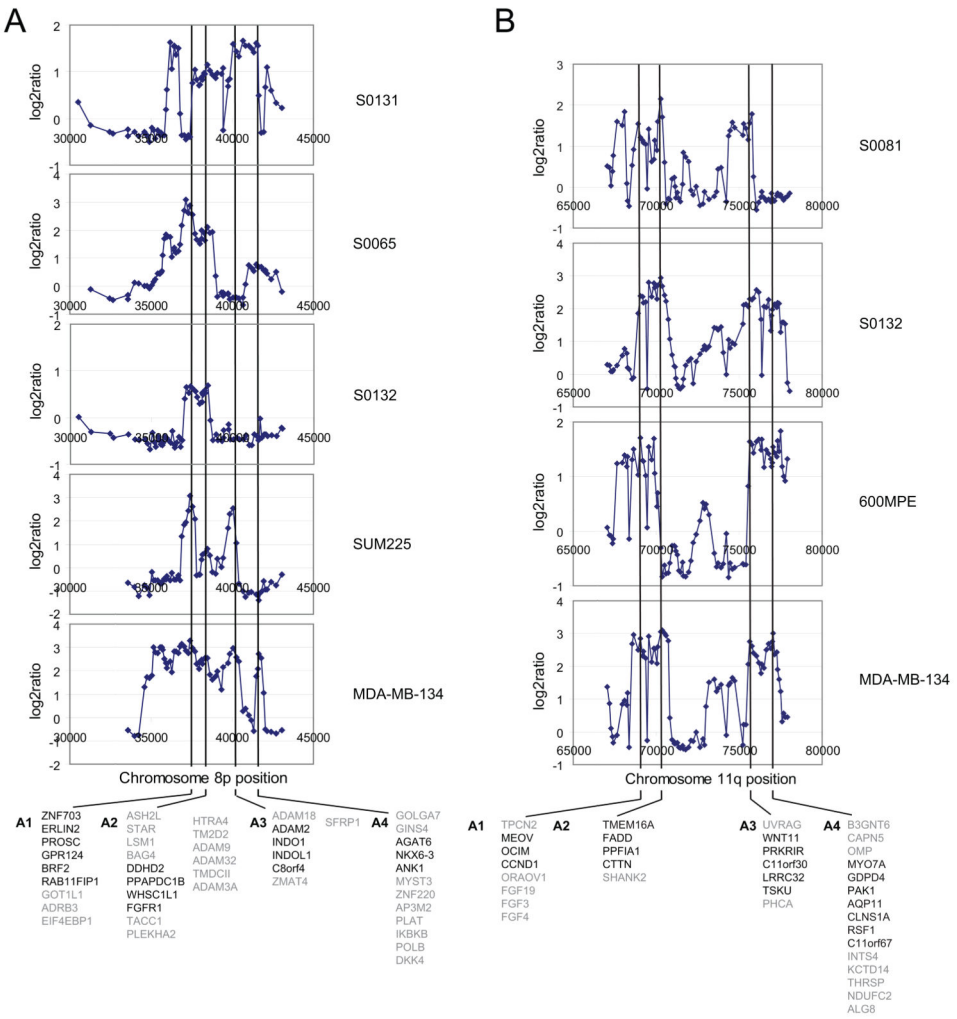


Figure 1.

Copy number profiles of 8p and 11q amplicons. Copy number profiles of selected tumors and cell lines are shown as determined by array CGH on the 8p11q BAC array. The centers of amplicon cores are indicated together with genes in each core region. (A) Amplicons at 8p11-p12. (B) Amplicons at 11q13-q14.

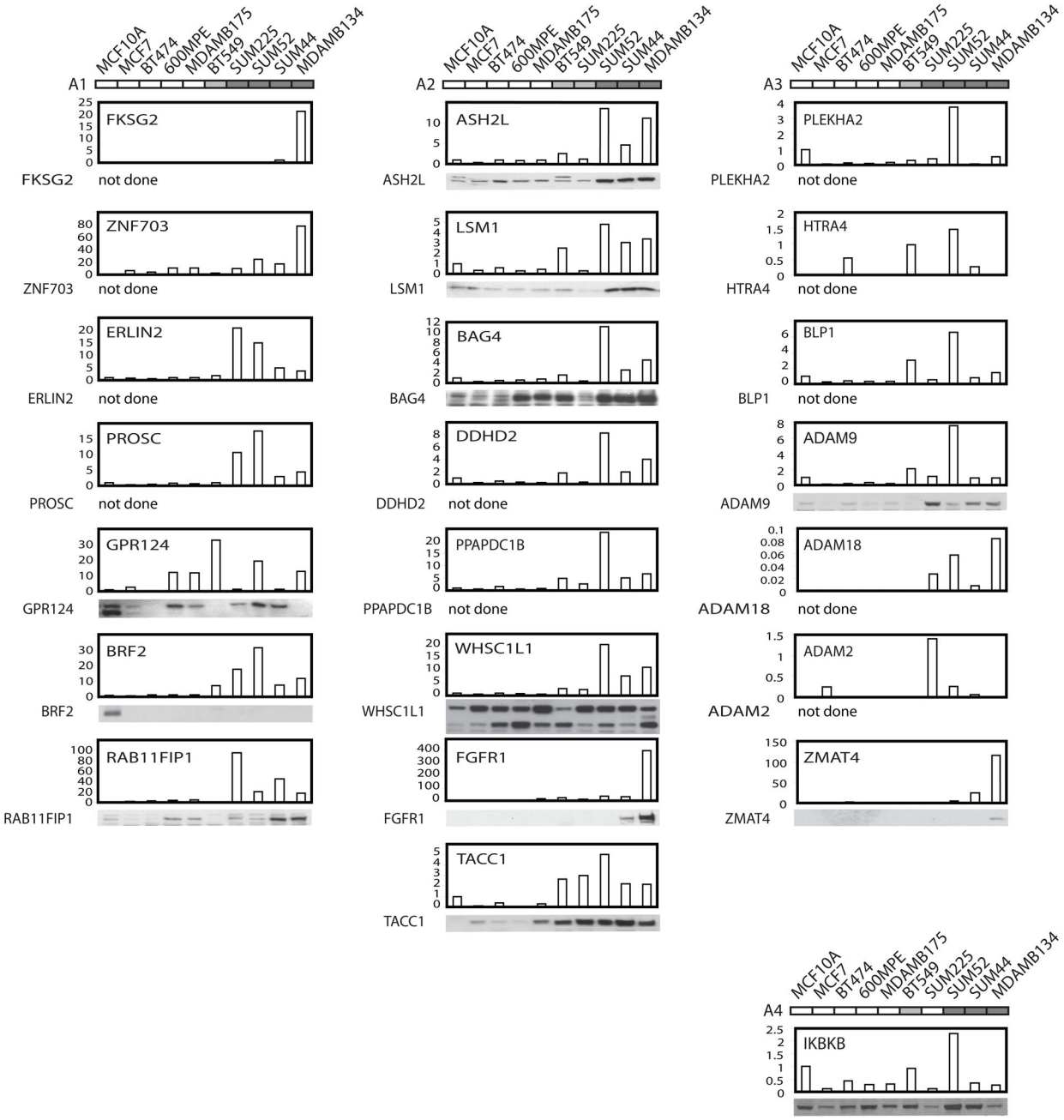


Figure 2.

Expression of selected genes in the 8p12 amplicon. Transcript levels for each gene were determined by quantitative RT-PCR and normalized to *GUSB*. The PCR reaction conditions and primers and probes for each gene are given in Supplementary Table 4. The data are displayed as expression levels relative to MCF10A (histograms) or as expression relative to *GUSB* if no expression was detected in MCF10A (*FKSG2*, *PLEKHA2*, *TM2D2*, *ADAM18*, *ADAM2*, *ZMAT4*). No expression of *HTRA4* was detected. Protein expression levels were determined if suitable antibodies were available (Supplementary Table 5). Western blots are shown below the RT-PCR histograms. Equal loading of lanes was confirmed by expression

of β-actin (data not shown). Genes are displayed according to inclusion in 8p amplicons A1-A4 and the copy number of the locus in each cell line is shown below the name of the cell line (white = average copy number, light gray = gain, and dark gray = amplification).

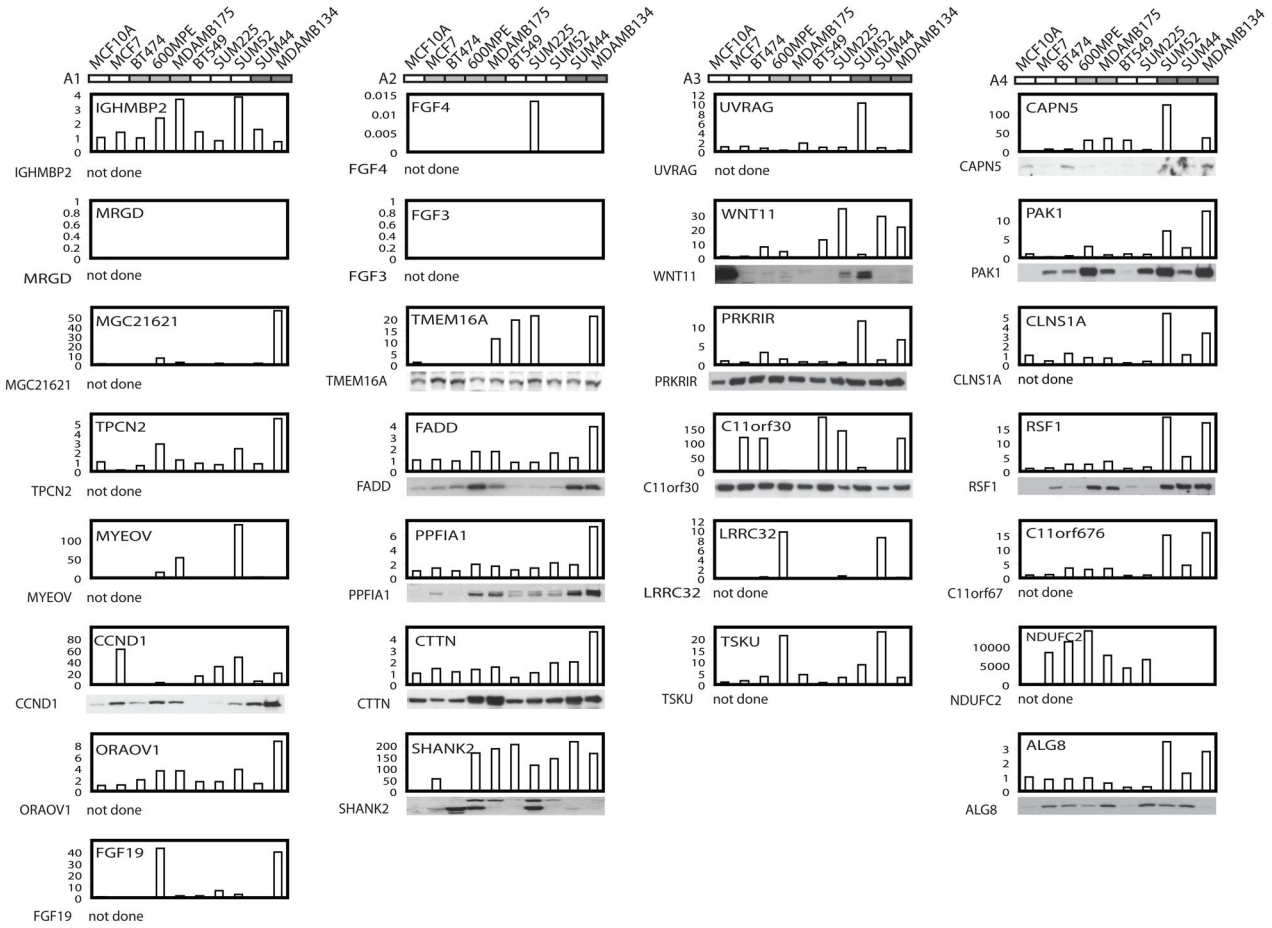
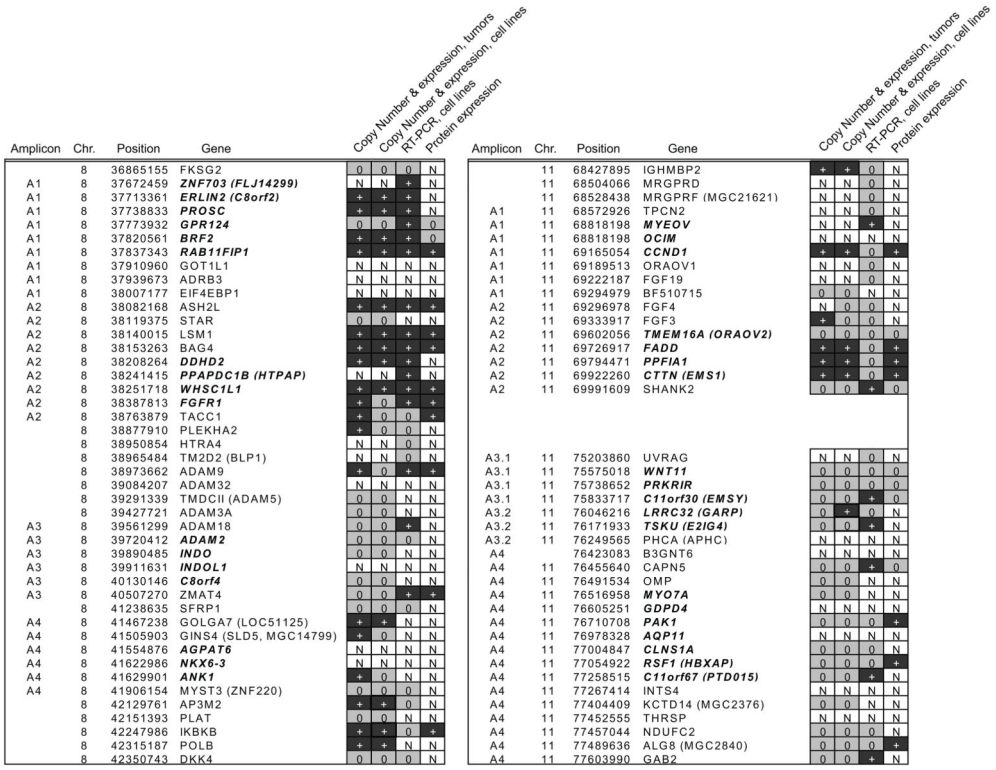


Figure 3.

Expression of selected genes in the 11q13 amplicon. Transcript levels for each gene were determined by quantitative RT-PCR and normalized to *GUSB*. The PCR reaction conditions and primers and probes for each gene are given in Supplementary Table 4. The data are displayed as expression levels relative to MCF10A (histograms) or as expression relative to *GUSB* if no expression was detected in MCF10A (*FGF4* and *LRRC32*). No expression of *MRGD* or *FGF3* was detected in any cell line. Protein expression levels were determined if suitable antibodies were available (Supplementary Table 5). Western blots are shown below the RT-PCR histograms. Equal loading of lanes was confirmed by expression of β-actin (data not shown). Genes are displayed according to inclusion in 11q13 amplicons A1–A4 and the copy number of the locus in each cell line is shown below the name of the cell line (white = average copy number, light gray = gain, and dark gray = amplification).



Amplicon	Chr.	Position	Gene	Copy Number & expression, tumors	Copy Number & expression, cell lines	RT-PCR, cell lines	Protein expression
	8	36865155	FKSG2	0	0	0	N
A1	8	37672459	***ZNF703 (FLJ14299)***	N	N	+	N
A1	8	37713361	***ERLIN2 (C8orf2)***	+	+	+	N
A1	8	37738833	***PROSC***	+	+	+	N
A1	8	37773932	***GPR124***	0	0	+	0
A1	8	37820561	***BRF2***	+	+	+	0
A1	8	37837343	***RAB11FIP1***	+	+	+	+
A1	8	37910960	GOT1L1	N	N	N	N
A1	8	37939673	ADRB3	N	N	N	N
A1	8	38007177	EIF4EBP1	N	N	N	N
A2	8	38082168	ASH2L	+	+	+	+
A2	8	38119375	STAR	0	0	N	N
A2	8	38140015	LSM1	+	+	+	+
A2	8	38153263	BAG4	+	+	+	+
A2	8	38208264	***DDHD2***	+	+	+	N
A2	8	38241415	***PPAPDC1B (HTPAP)***	N	N	+	N
A2	8	38251718	***WHSC1L1***	+	+	+	+
A2	8	38387813	***FGFR1***	+	0	+	+
A2	8	38763879	TACC1	+	0	0	+
	8	38877910	PLEKHA2	+	0	0	N
	8	38950854	HTRA4	N	N	0	N
	8	38965484	TM2D2 (BLP1)	N	N	0	N
	8	38973662	ADAM9	+	0	+	+
	8	39084207	ADAM32	N	N	N	N
	8	39291339	TMDCII (ADAM5)	0	0	N	N
	8	39427721	ADAM3A	0	0	N	N
A3	8	39561299	ADAM18	0	0	+	N
A3	8	39720412	***ADAM2***	0	0	0	N
A3	8	39890485	***INDO***	0	0	N	N
A3	8	39911631	***INDOL1***	N	N	N	N
A3	8	40130146	***C8orf4***	0	0	N	N
A3	8	40507270	ZMAT4	0	0	+	+
	8	41238635	SFRP1	0	0	0	N
A4	8	41467238	GOLGA7 (LOC51125)	+	+	N	N
A4	8	41505903	GINS4 (SLD5, MGC14799)	+	0	N	N
A4	8	41554876	***AGPAT6***	N	N	N	N
A4	8	41622986	***NKX6-3***	N	N	N	N
A4	8	41629901	***ANK1***	+	0	N	N
A4	8	41906154	MYST3 (ZNF220)	0	0	0	N
	8	42129761	AP3M2	+	+	0	N
	8	42151393	PLAT	0	0	N	N
	8	42247986	IKBKB	+	+	0	+
	8	42315187	POLB	+	+	N	N
	8	42350743	DKK4	0	0	0	N

Amplicon	Chr.	Position	Gene	Copy Number & expression, tumors	Copy Number & expression, cell lines	RT-PCR, cell lines	Protein expression
	11	68427895	IGHMBP2	+	+	0	N
	11	68504066	MRGPRD	N	N	0	N
	11	68528438	MRGPRF (MGC21621)	N	N	0	N
A1	11	68572926	TPCN2	N	N	0	N
A1	11	68818198	***MYEOV***	N	N	+	N
A1	11	68818198	***OCIM***	N	N	N	N
A1	11	69165054	***CCND1***	+	+	0	+
A1	11	69189513	ORAOV1	N	N	0	N
A1	11	69222187	FGF19	N	N	0	N
A1	11	69294979	BF510715	0	0	N	N
A2	11	69296978	FGF4	N	0	0	N
A2	11	69333917	FGF3	+	0	0	N
A2	11	69602056	***TMEM16A (ORAOV2)***	0	0	0	0
A2	11	69726917	***FADD***	+	+	0	+
A2	11	69794471	***PPFIA1***	+	+	0	+
A2	11	69922260	***CTTN (EMS1)***	+	+	0	+
A2	11	69991609	SHANK2	0	0	+	0
A3.1	11	75203860	UVRAG	N	N	0	N
A3.1	11	75575018	***WNT11***	0	0	0	0
A3.1	11	75738652	***PRKRIR***	0	0	0	0
A3.1	11	75833717	***C11orf30 (EMSY)***	0	0	+	0
A3.2	11	76046216	***LRRC32 (GARP)***	0	+	0	N
A3.2	11	76171933	***TSKU (E2IG4)***	0	0	+	N
A3.2	11	76249565	PHCA (APHC)	N	N	N	N
A4		76423083	B3GNT6	N	N	N	N
A4	11	76455640	CAPN5	0	0	+	0
A4	11	76491534	OMP	0	0	N	N
A4	11	76516958	***MYO7A***	0	0	N	N
A4	11	76605251	***GDPD4***	N	N	N	N
A4	11	76710708	***PAK1***	0	0	0	+
A4	11	76978328	***AQP11***	N	N	N	N
A4	11	77004847	***CLNS1A***	0	0	0	N
A4	11	77054922	***RSF1 (HBXAP)***	0	0	0	+
A4	11	77258515	***C11orf67 (PTD015)***	0	0	+	N
A4	11	77267414	INTS4	N	N	N	N
A4	11	77404409	KCTD14 (MGC2376)	0	0	N	N
A4	11	77452555	THRSP	N	N	N	N
A4	11	77457044	NDUFC2	0	0	0	N
A4	11	77489636	ALG8 (MGC2840)	0	0	0	+
A4	11	77603990	GAB2	0	0	+	N

Figure 4.

Expression of candidate oncogenes in 8p12 and 11q13 amplicons. Genes in the amplicons are shown in genome order. The results of the expression analyses described in the text are shown from left right, correlation of copy number with expression in the breast tumor dataset, correlation of copy number with expression in the cell line dataset, transcript and protein levels in cell lines. + = significant positive correlation, transcript overexpression or protein overexpression, 0 = no correlation or overexpression, and N = not done.

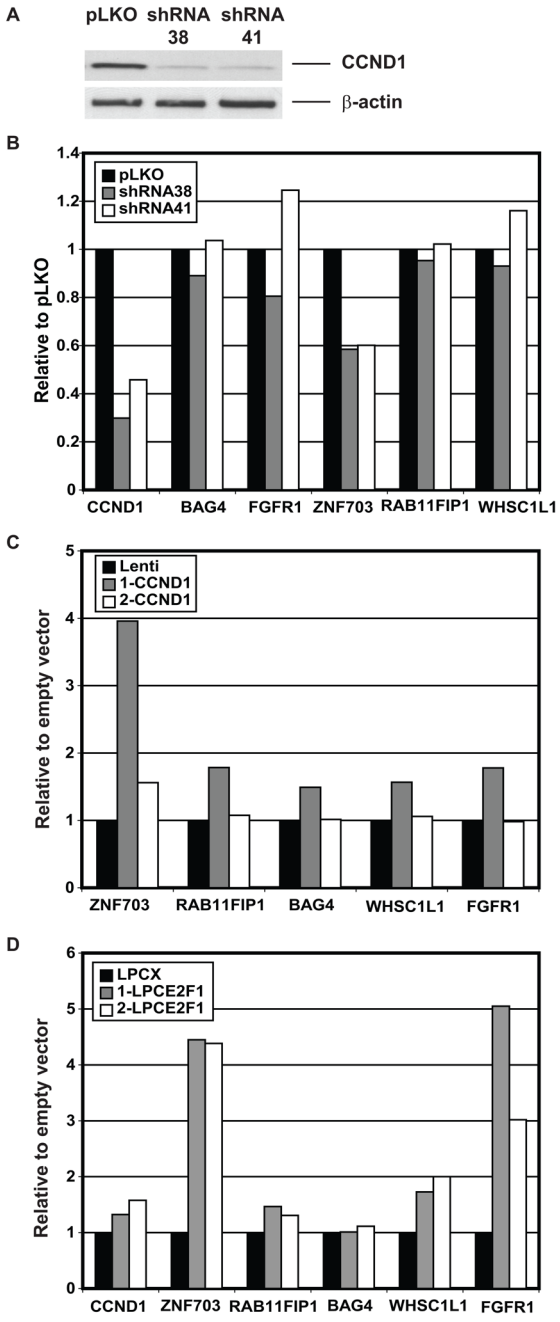


Figure 5.

Interaction between genes at 8p12 and 11q13.

A. Knockdown of *CCND1* by two shRNAs. Western blot showing decreased expression of CCND1 protein in SUM44 cells stably infected with either of two different shRNA lentiviruses 38 and 41 compared to a control shRNA pLKO (upper panel) and levels of β-actin on the same membrane (lower panel).

B. Transcript levels of genes normalized to *GUSB* in SUM44 cells expressing *CCND1* shRNAs compared to SUM44 cells infected with empty vector (pLKO).

C. Transcript levels of genes normalized to *GUSB* in MCF10A cells overexpressing *CCND1* compared to control MCF10A cells infected with an empty vector (Lenti). The results of two separate infections of MCF10A cells (1-CCND1 and 2-CCND1) are shown. Note that expression of *CCND1* in 1-CCND1 was 2.7 times the level achieved in 2- CCND1. In both experiments, expression of *ZNF703* was reproducibly induced to 5% of the level of expression of *CCND1*.

D. Transcript levels of genes normalized to *GUSB* in SUM44 cells infected with *E2F1* expressing retrovirus compared to control cells infected with empty vector (LPCX). The results of two separate infections of SUM44 cells (1-LPCE2F1 and 2-LPCE2F1) are shown.

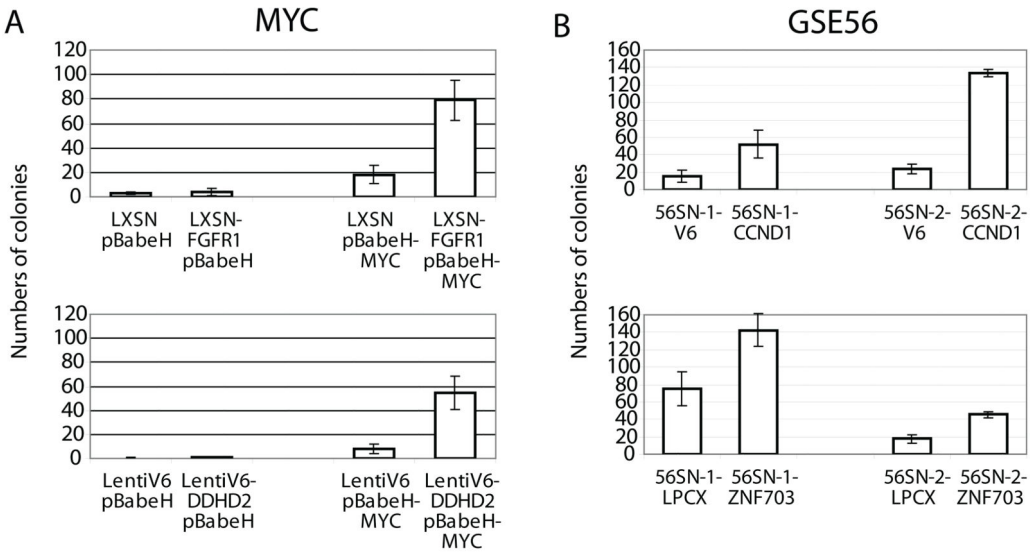


Figure 6.

Soft agar assays to evaluate the oncogenic function of genes on 8p12 and 11q13.

A. Expression of genes individually and in combination with *MYC*. Expression of *MYC* alone in MCF10A gave a few to ~40 colonies per well of a six-well plate. To control for the variability in the number of colonies in *MYC* expressing cells, all assays with were carried out with *MYC* alone control. The number of colonies from *MYC* expressing cells varied little within any one assay. Assays were carried out in triplicate with two different cell lines expressing *MYC* and each gene generated from two independent infections. Shown are the numbers of colonies per six-well plate (mean and standard deviation) for MCF10A cells stably infected with vector control (empty vector LentiV6 or LPCX) or vectors expressing individual genes, and MCF10A cells stably co-infected with control pBabeH (empty vector) plus appropriate corresponding empty vector for a candidate gene or co-expressing pBabeH-MYC and the selected genes.

B. Expression of genes in combination with GSE56. Expression of GSE56 alone in MCF10A cells produced colonies in soft agar that differed greatly in size, which by western-blotting of clonal cell lines derived from the GSE56-infected MCF10A appeared to result from variable expression levels of GSE56. Therefore, candidate genes were expressed in two different clonal cell lines, one expressing a high level of GSE56 in LXSN (56SN-1) and the other a moderate level (56SN-2). Shown are the numbers of colonies per six-well plate (mean and standard deviation) for MCF10A cells stably infected with vector control (56SN-1 or 56SN-2 and either empty vectors LPCX or LentiV6, V6) or vectors expressing individual genes together with either of the two GSE56-expressing MCF10A clones (56SN-1 or 56SN-2). Cells expressing CCND1 or ZNF703 alone do not form colonies in soft agar (Supplementary Figure 2).

Table 1

Minimal amplicon cores at 8p12 and 11q13

Amplicon	Flanking clone	Genes in (or partially in) flanking clone or amplicon core
8p A1	RP11-101H15	no genes
		ZNF703, ERLIN2, PROSC, GPR124, BRF2, RAP11FIP1
	CTC-497A2	*GOT1L1, ADRB3, EIF4EBP1*
8p A2	RP11-90P5	*ASH2L, STAR, LSM1, BAG4, (DDHD2)*
		DDHD2, PPAPDC1B, WHSC1L1, FGFR1
	RP11-647P1	*TACC1, (PLEKHA2)*
8p A3	RP11769N8	*ADAM18*
		ADAM2, INDO1, INDOL1, C8orf4
	RP11-51K12	*ZMAT4*
8p A4	RP11-407N24	*GOLGA7, GINS4, (AGAT6)*
		AGAT6, NKX6-3, ANK1
	RP11-109A10	*MYST3*
11q A1	RP11-40B5	*TPCN2*
		MEOV, OCIM, CCND1
	CTD-3190C8	*ORAOV1, FGF19, BF510715, FGF4, FGF3*
11q A2	RP11-368I20	*no genes*
		TMEM16A, FADD, PPFIA1, CTTN
	RP11-916J3	*SHANK2*
11q A3.1	RP11-133F12	*UVRAG*
		WNT11, PRKRIR, C11orf30
	RP11-115O9	*LRRC32*
11q A3.2	CTD-2501F13	*C11orf30*
		LRRC32, TSKU
	RP11-259H21	*PHCA*
11q A4	CTD-2504L8	*PHCA, B3GNT6, OMP, CAPN5*
		MYO7A, GDPD4, PAK1, AQP11, CLNS1A, RSF1, C11orf67
	RP11-7I15	*INTS4, KCTD14, THRSP, NDUFC2*