Amplification of Zinc Finger Gene 217 (*ZNF217*) and Cancer: When Good Fingers Go Bad

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SUMMARY

Chromosome 20q13 is highly amplified in human cancers, including 20-30% of early stage human breast cancers. The amplification correlates with poor prognosis. Over-expression of the zinc-finger protein 217 (ZNF217), a candidate oncogene on 20q13.2, in cultured human mammary and ovarian epithelial cells can lead to their immortalization, indicating that selection for ZNF217 expression may drive 20q13 amplification during critical early stages of cancer progression. ZNF217 can also attenuate apoptotic signals resulting from exposure to doxorubicin, suggesting that ZNF217 expression may also be involved in resistance to chemotherapy. Recent findings indicate that ZNF217 binds specific DNA sequences, recruits the co-repressor C-terminal binding protein (CtBP), and represses the transcription of a variety of genes. Inappropriate expression of ZNF217 may lead to aberrant down-regulation of genes involved in limiting the proliferation, survival, and/or invasiveness of cancer cells. Better understanding of ZNF217 and its associated pathways may provide new targets for therapeutic intervention in human cancers.

INTRODUCTION

Zinc finger (ZNF) containing proteins are extremely abundant in higher eukaryotes and can function as sequence-specific DNA-binding factors. Following the sequencing of the human genome, genes encoding hundreds of zinc finger proteins have been uncovered, but the functional significance of most is partially or completely unknown. One zinc finger protein that has received recent interest as a potential oncogene is ZNF217. This review will focus on the evidence that selection for *ZNF217* expression drives 20q13 amplification in a variety of cancers, and on new molecular studies that have begun to dissect the mechanisms by which *ZNF217* amplification may provide selective advantages to cancer cells.

Chromosome 20q13 amplifications in human cancers

DNA amplifications are common in human cancers, can result in increases in gene expression, and are often sites of oncogenes that confer proliferative and/or survival advantages to host cells. Amplifications involving human chromosome 20q13 are observed in a number of different cancer types, including breast [1-3], ovarian [4, 5], and squamous cell cancers [6, 7]. 20q13 amplifications have been shown to be associated with reduced disease-free survival [8, 9], reduced patient survival [10], increased mean cell proliferation activity [11], increased tumour grade/aggressiveness [9, 12], and to correlate with poor prognosis. 20q13 amplifications are thought to occur early in the course of breast cancer development [13]. The finding that the 20q13 region is selectively targeted for amplification during cancer progression suggests that it contains one or more genes that confer selective advantages to cancer cells.

In order to understand the contribution of 20q13 amplification to tumorigenesis, research has focused on defining the minimal common amplified region. In 1994, a 1.5 Mb region was identified in breast cancer cell lines, but at the time no obvious candidate oncogenes within this region were known [14]. However, as information about the human genome has accumulated, a number of genes found on chromosome 20q13 have been identified as possible oncogenic targets of amplification. These include *STK15/BTAK/Aurora2* [15-17], *CAS* [18-20], *BCAS1/NABC1* [21], *PFDN4* [22], *EEF1A2* [23], *CYP24* [24] and *ZNF217* [25][24]. It is likelypossible that there is more than one oncogene in the 20q13.2 amplicon as a case can be madethere is evidence that manyseveral of these genes may contribute towards tumour development. For example, *EEF1A2,* which encodes a protein elongation factor, has been shown to transform NIH-3T3 fibroblasts and injection of these transformed cells into mice was sufficient tothese transformed cells induced tumours in mice [23, 26]. Similarly, the *STK15* gene which encodes the centromere associated kinase Aurora2 has been shown to transform NIH-3T3 fibroblasts and MCF7 breast carcinoma cells [27]. In this review we will focus on the evidence that *ZNF217* is an the oncogene *ZNF217.*

ZNF217 is a candidate oncogene on 20q13.2

In order for a particular gene in an amplicon to be driving the selection of cells with the amplification, this gene must be located at the core of the amplified region and amplification must lead to over-expression of the gene. In 1998, Collins and colleagues [25][24] cloned a 1 Mb region of chromosome 20q13. They defined a ~260 kb region of maximal amplification that was common to a series of breast tumours and described a new gene, *ZNF217*, which was found within this 260 kb region. *ZNF217* was shown to be more highly expressed in all ten breast cancer cell

lines carrying the 20q13 amplification than in lines with normal 20q13 copy number. This study provided evidence that ZNF217 fits the criteria for a driver gene in the 20q13 amplicon.

A number of subsequent studies have provided additional evidence that *ZNF217* is a candidate target gene driving 20q13 amplification in a significant proportion of tumours and cell lines from a range of human cancer types, including cancers of the breast, colon, stomach, ovary and pancreas (Table 1). In addition, *ZNF217* was found in separate studies to be within a narrow region of high copy number in breast tumours [28][25] and gastric adenocarcinomas [29][26]. Increased copy number of *ZNF217* has been shown to correlate with increased expression in a gene dosage dependent manner [30][27]. *ZNF217* amplification has also been shown to correlate with shorter patient survival in ovarian cancer [19, 31][19, 28] and colon cancer [32][29]. The copy number of the *ZNF217* genes has been found to be higher in metastatic melanoma than in primary melanoma [33][30]. The frequency of increased *ZNF217* copy number has also been shown to be higher in liver metastatic lesions from colorectal cancer than in the primary colorectal cancers that gave rise to the metastases. None of the colorectal cancers which did not give rise to liver metastasis showed *ZNF217* copy number amplifications [34][31]. Finally, *ZNF217* amplification has been shown to correlate with tumour size in gastric cancer [35][32].

Compelling support for the hypothesis that *ZNF217* can contribute to cancer came from a study which demonstrated that transduction of finite life-span human mammary epithelial cells with *ZNF217* gave rise to immortalized cells [36][33]. This provided the first direct evidence for a potentially oncogenic function of *ZNF217*.

The results suggested that the selective amplification of *ZNF217* may allow cancer cells to overcome senescence and become immortal, thought to be a crucial event in the development of many cancers [37][34]. *ZNF217*-immortalized cell lines were shown to have increased telomerase activity and stable telomere lengths, suggesting that ZNF217 may function, in part, by allowing pre-malignant cells to de-repress telomerase and overcome senescence due to telomere dysfunction [36] [33].

In support of this original study, *ZNF217* has recently been shown to immortalize ovarian cells [38][35]. *ZNF217* transduction into ovarian surface epithelial cells with extended but finite life-spans, combined with EGF treatment, led to cellular immortalization. Although EGF treatment was necessary during the immortalization process, the permanent lines that were established were able to grow without EGF. The siRNA mediated knock-down of *ZNF217* in these permanent lines led to growth arrest, demonstrating that the continued proliferation was dependent on *ZNF217*. *ZNF217* transduction also allowed the acquisition of three other malignancy associated characteristics in ovarian surface epithelial cells: reduced serum dependence, anchorage independence and increased telomerase activity.

In addition to the ability to proliferate indefinitely, cancer cells acquire the ability to resist apoptotic signals. Huang and colleagues [39][36] investigated the effects of ZNF217 on cellular survival in a cell culture model. They demonstrated that cells over-expressing ZNF217 were more resistant than control cells to spontaneous cell death and cell death caused by the chemotherapeutic agent doxorubicin. Cells over-expressing ZNF217 were also more resistant to cell death induced by disruptions to telomere integrity, a potential link with the increased ability of

ZNF217-expressing cells to overcome telomere dysfunction. Knock-down of *ZNF217* expression by siRNA in various cancer cell lines resulted in increased cell death and increased susceptibility to doxorubicin. To dissect potential targets of ZNF217 promoting cell survival, the effect of ZNF217 levels on Akt phosphorylation was examined. Akt becomes active when phosphorylated and Akt activation is a survival signal associated with cells which are resistant to apoptosis [40][37]. Over-expression of ZNF217 led to increased Akt phosphorylation and siRNA knock-down led to decreased Akt phosphorylation. These results suggest that *ZNF217* amplification can attenuate apoptotic signals.

ZNF217 is a transcription factor

The original paper by Collins and colleagues which identified the *ZNF217* gene within the 20q13.2 amplified region noted that ZNF217 contains 8 zinc fingers (Figure 1) and proposed that it functions as a sequence-specific DNA-binding transcription factor [25][24]. GFP-ZNF217 was later shown to localize to the nuclei in HeLa cells [41][38]. New research has provided experimental evidence that ZNF217 is indeed a transcription factor, and suggests that *ZNF217* amplification may promote cancer through alterations in the regulation of key target genes.

Supporting evidence for the role of ZNF217 as a transcription factor came first from co-immunoprecipitation experiments (Figure 2). ZNF217 was found in complexes immunoprecipitated by antibodies to the transcriptional co-repressor CoREST [42][39], the lysine specific histone demethylase LSD-1 (also known as BHC110) [43-46][40-43] and the transcriptional co-repressor CtBP1 [46][43]. Mass spectroscopy indicated that each of these immunoprecipitated complexes contained ZNF217,

CoREST, histone deacetylases HDAC1 and 2, and LSD-1. Three of the complexes also contained CtBP proteins (Figure 2). ZNF217 has since been shown to be a direct binding partner of CtBP, and the sites through which interaction is achieved have been identified [47][44]. Detailed mutational and structural analysis revealed that ZNF217 binds CtBP through both a classical PXDLS motif and a newly identified RRT motif. Interestingly, this new CtBP-interacting motif has been noted in other zinc-finger proteins such as ZNF516 and RIZ1, suggesting that ZNF217, ZNF516 and RIZ may represent a new class of CtBP partners (Figure 1) [47][44].

ZNF217 has been shown to bind and participate in the repression of several promoters, including one of a known CtBP target gene, *E-cadherin* [47][44]. This repression activity is partially dependent on the ability of ZNF217 to bind CtBP. A ZNF217 mutant that could not bind to CtBP had reduced, but still significant, ability to repress transcription. This observation indicates that ZNF217 retains some transcriptional repression activity in the absence of CtBP (Figure 3).

Using a cell-free PCR-based enrichment assay, Cowger and colleagues [48][45] recently demonstrated a DNA sequence preference for ZNF217 binding. Using a pool of random oligonucleotides incubated with a GST-ZNF217 fusion protein encompassing the sixth and seventh zinc fingers (Figure 1), they identified a consensus sequence preferentially bound by ZNF217. The core consensus identified, CAGAAY (where Y represents a C or T), was found to be present in the human *E-cadherin* proximal promoter. This group went on to demonstrate using ChIP assays that ZNF217 and the other components of an associated repression complex are present at the *E-cadherin* promoter in the breast cancer cell line MCF7. ZNF217 protein levels were shown to inversely correlate with expression from an *E-*

cadherin promoter linked to a reporter gene - siRNA knockdown of *ZNF217* led to increased expression from the *E-cadherin* reporter and over-expression of ZNF217 led to decreased expression.

More recently, Krig and colleagues have employed genome-wide ChIP-CHIP assays to identify a large number of target genes bound by ZNF217 complexes [49] ~~[46]~~. Three tumour cell lines (MCF7, the colon cancer line SW480, and the teratocarcinoma line Ntera2) were used for these analyses. Sets of promoters physically associated with ZNF217 in the cell lines were subjected to bioinformatic analysis. This analysis led to the identification of an 8 base pair consensus sequence **ATTCCNAC** (reverse complement strand **GTNGGAAT**) with a five base pair core consensus **ATTCC** (reverse complement strand **GGAAT**) for genes regulated by ZNF217 in all three cell lines. However, when the authors analyzed the three cell lines individually, they found an AP-1 like binding motif **ANGAGTCA** in MCF-7 cells, a **CATTCC** binding motif in SW480 cells, and no statistically significant consensus binding motif in Ntera2 cells [49]~~[46]~~.

The ZNF217 consensus binding motifs identified by ~~ChiP~~ChIP-CHIP differed significantly from that identified in the cell-free site selection experiments [48]~~[45]~~. There are a number of possible explanations for this discrepancy. The site selection experiments used only a fragment of ZNF217 containing zinc fingers 6 and 7. Typically three zinc fingers, each recognizing three nucleotides, are used for specific binding to DNA [50]~~[47]~~. Thus additional ZNF217 domains may influence its binding in vivo. Depending on what additional proteins it is bound to, the full-length ZNF217 protein may display additional or ~~different~~ altered binding preferences. Additionally, the site selection assay only detects sites to which ZNF217 binds

directly, whereas the ChIP-CHIP assays are also expected to detect sites to which ZNF217 binds indirectly through interactions with other DNA binding proteins.

The mechanisms through which ZNF217 functionally represses gene expression also appear complicated. It is likely that there are multiple mechanisms [49][46] (Figure 3). First, ZNF217 may bind directly to promoters via zinc fingers 6 and 7 and recruit the CtBP co-repressor complex through direct protein-protein interactions (Model 1). Alternatively, other DNA binding factors may mediate contact between promoters and the ZNF217/CtBP repressor complex (Model 2). It is also possible that ZNF217 and other transcription factors are recruited to the same promoters and synergize with CtBP to promote gene silencing (Model 3). Finally, ZNF217 may repress transcription on its own by an unknown mechanism (Model 4). One unexplored possibility is that ZNF217 itself contains enzymatic activity similar to the demonstrated histone methyltransferase activity of the related repressor RIZ [51] [48] (Figure 1). These four models are non-exclusive and their relative contributions could depend on cell and promoter context.

How might ZNF217 influence tumorigenesis?

From the set of promoters that were identified to be bound by ZNF217 in ChIP-CHIP experiments, Krig and colleagues selectedidentified the top 1000 target promoters from the two sets ofbound by ZNF217 in Ntera2 cellls promoter array data (1276 promoters in total) and examined the RNA expression levels of the 1077 of these targets which were represented onusing an expression array [52]. These expression arrays were used to The identificationy of sets of genes associated with expression levels which correlate with ZNF217 expression in vivo allowed an analysis of the processes that ZNF217 is likely to regulate [49][46]. The majority of

th ~~The majority of th~~e top-ranked target genes had low levels of RNA expression, supporting the hypothesis that ZNF217 is a repressor of transcription and is silencing the promoters to which it binds. However, a small subset of the top-ranked target genes had high levels of RNA expression, indicating that ZNF217, like many transcription factors may also act as ~~both~~ an activator ~~and repressor~~ of transcription at some promoters.~~–~~ The promoters that ZNF217 was shown to occupy were categorized and were shown to be associated with proteins involved in processes such as transcriptional regulation, organ development, cell differentiation, cell adhesion, and embryonic development. In further ChIP-CHIP assays, the promoters bound by ZNF217 and CtBP were compared. The majority of ZNF217 targets were also bound by CtBP in both Ntera2 and MCF7 cells, suggesting that these two proteins often co-operate in gene repression.

Despite the gains in understanding of ZNF217 interactions and binding, there remains a large gap in understanding how these biochemical features contribute mechanistically to malignancy in cells that aberrantly express this protein. The demonstration that ZNF217 can act as a repressor of transcription and that the tumour suppressor *E-cadherin* may be a ZNF217 target gene is one connection that is being pursued. The findings that ZNF217 binds directly to DNA sequences in the *E-cadherin* promoter and that it must be present for other components of the associated complex to repress expression from the *E-cadherin* promoter [48]~~[45]~~ suggest that ZNF217 may serve as an important targeting factor for *E-cadherin* gene repression. Decreased expression of E-cadherin occurs in many human cancers and often correlates with epithelial to mesenchymal transition (EMT). EMT in tumours of epithelial origin allows cancer cells to leave the tumour and metastasize to other organs [53]~~[49]~~.

Additional cancer-related targets of ZNF217-mediated repression are suggested by the ChIP-CHIP and expression profiling experiments. The increased repression of genes involved in such processes as development, cell adhesion, and differentiation could promote proliferative and invasive behavior ordinarily associated with stem or progenitor cells. The increases in Akt phosphorylation observed in cells over-expressing ZNF217 [39][36] remain to be explained, and are likely to be due to altered expression of genes encoding proteins that regulate this phosphorylation.

CONCLUSION

Clinical data suggest that *ZNF217* amplification is associated with poor patient survival and increased metastatic potential of cancers. The finding that ZNF217 is a DNA-binding protein that can repress transcription by directly recruiting the co-repressor CtBP, together with the characterization of ZNF217 target genes and induced cellular phenotypes, are important steps in understanding the biological effects associated with ZNF217 over-expression. The mis-regulation of E-cadherin and as yet ~~uncharacterised~~ uncharacterized ZNF217 target genes (from the lists generated in Krig *et al*, 2007) could account for the alterations in cellular immortalization, apoptosis resistance, resistance to chemotherapeutic agents, and Akt phosphorylation, that are seen in cells in which *ZNF217* is highly expressed. Although hundreds of genes are potential targets of ZNF217 and consensus binding sites have been proposed, further analysis will be required to understand how precisely ZNF217 is localized to these genes, the mechanism(s) through which it alters their expression, and to define the subset of targets that are most important in cancer formation and progression.

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FIGURE LEGENDS

Figure 1. Schematic representation of the zinc finger proteins ZNF217, RIZ and ZNF516 with their different functional domains.

hZNFnf217, hRIZ1 and hZNF516 possess both PXDLS motifs and RRT motifs. PXDLS-like motifs (for example PLDLS, PLNLS, VLDLS) which are found in many CtBP interacting proteins have been shown to be directly responsible for the interaction with CtBP and are indicated by white rectangles. RRT-like motifs (for example RRTGCPPAL, RRTSSPPSS, GRTGPPPAL) are a recently identified second type of CtBP interaction motif which haves been found in a subset of CtBP interacting proteins. andThese are indicated in this diagram by grey rectangles. Predicted zinc-fingers are shown as arches and numbered, PXDLS motifs are indicated by white rectangles, RRT motifs are indicated by grey rectangles. . The main functional domains of ZNF217 and RIZ1 are indicated (ZNF217: Tthe CoREST and the DNA binding regions according to (Cowger et al., 2006,) and the proline- rich region that is often found in transcriptional activators, according to (Collins et al., 1998); RIZ1: tThe PR/SET domain with histone methyl-transferase activity).

The PR/SET domain in the RIZ protein is indicated by wide grey rectangles. Putative functional domains are underlined.

Figure 2. The ZNF217-containing transcriptional repressor complexes.

A scheme is presented of ZNF217-containing transcriptional repressor complexes that have been identified through co-immunoprecipitation experiments. Components common to all four complexes are highlighted in grey.

Figure 3. Models for ZNF217 recruitment and function as a component of transcriptional repressor complexes.

(1) ZNF217 binds DNA directly and orients an associated repressor complex. **(2)** Another transcription factor recruits ZNF217 and its partners to the DNA. **(3)** ZNF217 cooperates with other transcription factors in binding and recruitment of co-repressors. **(4)** ZNF217 uses intrinsic enzymatic activity to repress transcription.