TISSUE SPECIFIC DELETION OF ZBP-89 IN THE MOUSE INTESTINE RESULTS IN EXPANSION OF GOBLET AND PANETH CELL LINEAGES

by

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To Dan, without your support and strength I could not have come this far

To my parents, Donald and Monica, for guiding me through life

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Chapter I

Introduction

Identifying the nuclear proteins essential to cell cycle control has far reaching effects in both health and disease, especially cancer. Due to the ability of ZBP-89 to form direct protein complexes with known tumor suppressor factors, eg. p53, p300, ataxia-telangiectasia mutated, the goal of this project has been to understand the role of this protein in vivo. A specific focus is on the function of ZBP-89 in the gastrointestinal tract given the known role for this factor and its interactors in cancers of this system.

The Krüppel-type transcription factor ZBP-89 was originally identified by screening a cDNA library prepared from a rat pituitary adenoma cell line (GH4) for proteins that bound to the epidermal growth factor (EGF) response element of the gastrin gene (61). ZBP-89 (also known as ZNF148, BERF-1, or BFCOL1) was originally named Zinc-finger Binding Protein-89 for its characteristic amino-terminal zinc finger region and predicted size of 89 kDa. This name stresses the size of the protein as opposed to its function since this ubiquitously expressed factor appears to play roles in cell growth arrest, gene regulation, and apoptosis (8, 72). Furthermore its bifunctional regulatory domains suggest it might function as both an activator and

repressor of transcription depending on the situation (34, 68). Overall this factor plays critical roles that remain relatively uncharted.

The focus of this dissertation is to study the effective targeting of the ZBP-89 gene and the resulting phenotype of mice that are conditionally null for ZBP-89 in the gastrointestinal tract. In Chapter 1 of this thesis I provide an overview of the current knowledge of ZBP-89 including its genomic localization, protein structure, interacting protein, roles in growth arrest and disease, and other in vivo models examining its function. In Chapter 2, I discuss the targeting strategy and generation of mice harboring a gene targeted ZBP-89 allele. Chapter 3 focuses on the deletion of ZBP-89 in the mouse intestine after breeding the targeted mice to a line which expresses Cre recombinase in the intestine. Finally, in Chapter 4, I summarize the contribution the conditionally null ZBP-89 mouse provides to furthering our understanding of gastrointestinal growth and transformation.

Locus and Species Characteristics of ZBP-89

ZBP-89 is located on human chromosome 3q21, which is also the site of breakpoints and translocations in some cases of acute myeloid leukemia (1, 69, 89). Specifically ZBP-89 is located between STS markers D3S1551 and D3S1765 (51). In humans the gene spans 130 kilobases (kb) of genomic DNA encompassing the 5'-untranslated region, 9 exons, 8 introns, and the 3'-untranslated region. The 5' untranslated region (UTR), including the promoter, is contained in the first three exons. The promoter contains high GC content, multiple transcription initiation

sites, and lacks canonical CAAT and TATA boxes (27). The start site of translation is in exon 4 and the stop codons reside in exon 9 (27). The exon sizes range from 82 base pairs (bp) to 5275 bp. Exon 9 is the largest exon; however, a vast majority of the sequence belongs to the 3'- UTR. Furthermore exon-intron boundaries follow the typical GT-AG rule for donor and acceptor splice sites. The smallest intron is between exons 8 and 9 and is 280 bp in length, while the largest is roughly 24 kb and is intron 7. This exon/intron organization correlates with unique domains within the protein whereby each exon encodes a distinct functional domain (Figure 1.1). These domains will be described in more detail in the following section. There are many forms of mRNA for this gene (major forms are 8.6, 7.6, and 4.2 kb) which vary by tissue with up to seven polyadenylation signals located in the 3'- UTR. ZBP-89 is ubiquitously expressed at low levels, although some tissues have an elevated level of expression. Observation of human expressed sequence tags (EST's) and genomic clones suggest that alternatively spliced transcripts of ZBP-89 exist and these lead to functionally diverse protein isoforms of various sizes (49).

Multiple promoter usage and alternative splicing are common occurrences at the pre-mRNA level which lead to increased genetic complexity (92). A splice isoform of ZBP-89 was identified that is normally expressed humans. This amino-terminally truncated isoform is generated by use of an alternate promoter and alternate exon 4 which are located in intron 4 of the gene. As a result, the truncated protein is missing the amino-terminal 127 amino acids and lacks the p300 interaction domain (49). This isoform is expressed at low levels in many normal and transformed human tissues (49). Comparative genomic analysis shows that this

isoform is also found in chimpanzees but not in mice, suggesting that this mechanism is restricted to hominids. While to date this is the only isoform that has been characterized, given what is known from EST's as well the presence of multiple ZBP-89 species observed on immunoblots it is highly likely that at least several isoforms exist in both humans and mice.

As expected there are many similarities between the human and mouse ZBP-89 genes. The chromosomal location is not conserved and therefore in mice ZBP-89 is found on chromosome 16. Here the entire gene covers a region of 110 kb, slightly smaller than the human counterpart. Overall there is 92.8% nucleotide and 97.2% amino acid similarity between the two species. Its genomic organization is similar to the homologous human gene including that it contains 9 exons, the first 3 of which are untranslated. Sequences of analogous exons range between 93 and 100% similar, with the sizes of each exon being conserved. The coding region starts at base 17 of exon 4 and terminates at base 1599 of exon 9. As in humans, this gene is ubiquitously expressed in mouse tissues with major mRNA transcripts of 7.6, 4.1, and 3.4 kb (68). While alternate splice forms are highly likely in the mouse, it was determined that this gene is present in a single copy in the genome and there are no other closely related genes in the mouse (27).

While the human and mouse ZBP-89 genes have been studied extensively there is little known about this gene in other species. However this gene is known to be phylogenetically conserved in mammals. In Zebrafish ZBP-89 shares a 61.8% amino acid identity and 74.1% DNA sequence similarity with human ZBP-89 (55). Perhaps the closest match to the human gene can be found in the chimpanzee (*Pan*

troglodytes) where there is 99.5% nucleotide identity and 99.6% amino acid similarity. Other species having 90% similarity or better when compared to humans include the dog and rat (Table 1.1). As the genomic sequences for more species are completed the genomic organization of ZBP-89 in those species can be examined.

Protein Structure of ZBP-89

ZBP-89 translates into 794 amino acid residues and the resulting protein has a predicted molecular mass of 89 kDa. Interestingly the first 454 amino acids are identical to the 49 kDa human DNA-binding protein ht-β, which binds T-cell receptor genes (61, 83). ht-β is generated by an in-frame stop codon at nucleotide 1753 as a result of two single base pair deletions compared to ZBP-89. Furthermore not a single immunochemical study of ZBP-89 has detected a band corresponding to ht-β. Therefore ht-β is possibly a truncated form of ZBP-89 found in T cells or perhaps the frameshifts resulted from somatic mutations of ZBP-89 in the Jurkat (acute T-cell leukemia) cell line used to isolate ht-β. However despite similarities with ht-β, ZBP-89 corresponds to the full-length protein and has distinct functions. ZBP-89 has a very distinctive domain structure setting it apart from other zinc-finger containing transcription factors. Proceeding from the amino terminus to the carboxy terminus of the protein the following domains are found: acidic, synergy control (SC), basic, zinc finger, two more basic surrounding a SC, serine-rich, and PEST domains.

The acidic domain is a region 46 amino acids long that is rich in glutamic acid and found in the amino terminus of the protein. This domain is encoded by exon 4 and is the site of an interaction with the co-activator p300 (6). Each SC motif is four amino acids in length and serves to limit synergistic transcription from multiple response elements (39). In particular these serve as sites for translational modification by members of the Small Ubiquitin-like Modifier (SUMO) family (19). A highly basic domain, rich in lysine, is encoded by exon 5. This domain is 25 amino acids in length and represents most of the sequence responsible for transcriptional repression (68). Inside this basic domain is a nuclear localization signal (NLS). The zinc finger domain consists of the four Cys_2-His_2-type (Krüppel-like) zinc fingers found near the center of the protein. This zinc finger region functions as a DNA binding domain and serves as a site for binding to many factors. These zinc fingers are encoded by exons 6-9 with roughly one zinc finger encoded per exon. In addition exon 9 encodes the carboxy terminus of the protein which includes two additional basic domains, a Synergy Control (SC) motif, as well as a Serine-rich/PEST domain. Similar to the upstream basic domain, the basic domain immediately downstream of the zinc fingers consists of 16 amino acids and also contains a NLS. Amino acids 522 to 639 of the protein contain not only a region rich in serine residues but a PEST domain (amino acids 569-596) as well. The serine-rich region is similar to that found in other factors that have been shown to form protein-protein interactions with Krüppel family members (75). The PEST domain is rich in the amino acids proline (P), glutamic acid (E), serine (S), and threonine (T) as its definition states. It is hypothesized that this PEST sequence acts as a signal

peptide for protein degradation as it is associated with proteins that have a short half-life, however there is currently no experimental data to confirm this hypothesis (73). The carboxy terminus of the protein also contains a transcriptional activation domain whose strength of activation depends upon the context in which it is bound (presence of other bound transcription factors) (34).

To date one other member of the ZBP-89 family of transcription factors has been identified. ZBP-99 was identified in a yeast one hybrid screen whereby a Jurkat human leukemia cDNA library was screened with a GC-rich ornithine decarboxylase (ODC) promoter element (48). This ubiquitously expressed 99 kDa protein also binds to a GC-rich region of the gastrin promoter. ZBP-99 also has a unique domain structure consisting of a glycine-rich, proline-rich, and histidine repeat regions, basic domains, and four closely spaced zinc fingers. Common features between the two family members include 91% sequence similarity within the zinc finger domains, and similarly located basic domains flanking the zinc finger domain. Recent attempts to identify other proteins that interact with ZBP-99 have not proven fruitful given the ability of this protein to function as an activator of all reporters used in a yeast two-hybrid screen (Adams-Romain and Merchant unpublished observations).

ZBP-89 Interacting Proteins

After ZBP-89 was identified, additional labs identified ZBP-89 as a factor binding to GC-rich elements used to screen expression libraries. The consensus

binding site for ZBP-89 is gccCCtxCxCC, where the lowercase letters are residues involved in but not essential for binding, the x's represent residues that have not been mutated, and uppercase C's represent cytidines essential for binding (52). Interestingly ZBP-89 competitively binds to the same GC-rich DNA element as the zinc-finger transcription factor Sp1. A dichotomy exists whereby in some instances ZBP-89 opposes the effect of Sp1 by repressing promoter activity and in other cases these factors synergize to regulate transcription (Figure 1.2). For example, ZBP-89 negatively regulates the gastrin promoter; however Sp1 has been shown to activate this promoter (61, 62). On the other hand, ZBP-89 binds directly to Sp1; forming a complex that then represses the intermediate filament vimentin promoter (84, 93). Aside from cooperating or competing with Sp1, ZBP-89 alone has the ability to either stimulate or repress transcriptional activity reliant on the promoter it binds to. In addition to those listed above ZBP-89 has been shown to bind to the promoters of the following genes: pro-α1/2 (I) collagen, cytochrome c oxidase (COX) Vb, epithelial neutrophil-Activating peptide-78 (ENA-78), L2 promoter of the growth hormone receptor, intestinal alkaline phosphatase, ornithine decarboxylase (ODC), lck, pre-TCR-α, β-enolase, $p21^{waf1}$, STAT1, and stromelysin (Table 1.2) (6, 7, 14, 15, 34, 42, 52, 59, 68, 71, 81, 88, 90). The transcriptional regulation of these promoters by ZBP-89 as well as Sp1 has been examined in vitro and depends greatly on the cell type, post-translational modification status, and cell state. In addition the ability to repress or stimulate can be due to positional effects of binding sites, alternative splicing, alternative transitional initiation, or competition/interference with other factors. Furthermore, ZBP-89 is known to possess these abilities.

Utilizing its unique domain structure, ZBP-89 is also capable of exerting effects through direct protein-protein interactions. While the zinc finger region typically serves as its DNA binding domain, proteins are also capable of binding to the zinc fingers or other portions of this factor. As an example, the zinc finger domain is capable of binding to the DNA binding and C-terminal domains of the transcription factor p53 (8). Pulse-chase analysis using adenovirus-infected cells demonstrated that as a result of this direct interaction p53 was stabilized by ZBP-89. In another example both the amino-terminus and the zinc finger domain are capable of interacting with ataxia-telangiectasia mutated (ATM); this complex then recruits the co-activator p300, which also binds to the amino-terminus of ZBP-89 during activation of the $p21^{waf1}$ promoter in response to sodium butyrate (4, 6). In addition to p300 and ATM, ZBP-89 is capable of binding to BMRF1 of the Epstein Barr Virus, Friend of Gata-1 (FOG-1), Gata-1, HDAC1, p53, Sp1, Stat3, Ubc9, and YY1 (6, 14, 36, 84-86). Given its ubiquitous expression the current list of ZBP-89 interacting proteins and promoters is likely to expand. Overall the interactions of ZBP-89 described above suggest roles for this factor in many pathways, however, its role in both p53 dependent and independent growth arrest pathways is perhaps the most studied.

Regulation of Growth Arrest by ZBP-89

Early analysis of ZBP-89 function in cells showed that it arrested growth through its interactions with both p53 and $p21^{waf1}$ suggesting key roles for ZBP-89 in cell cycle regulation. In eukaryotic cells, the cell cycle refers to the series of events

that lead to the replication of a given cell. The cell cycle consists of four distinct phases: G1 phase, S phase, G2 phase, and M phase. Activation of each phase is dependent on the proper progression and completion of the previous one and no phase is reversible. Regulation of the cell cycle involves detecting and repairing any acquired genetic damage so that after cell division the resulting cells do not malfunction. There are checkpoints used by the cell to monitor and regulate progression through the cycle. The checkpoint functions to verify completion of processes from the previous cycle phase and repair any DNA damage before allowing the next phase to begin (26). There are two main checkpoints: the G1/S checkpoint and the G2/M checkpoint. The G1/S checkpoint is a rate-limiting step in the cell cycle at the end of G1 phase and is known as a restriction point after which the cell is committed to entering S phase. Likewise after passing through the G2/M checkpoint the cell is committed to M phase where mitosis occurs(57). The tumor suppressor p53 plays an important role in triggering the control mechanisms at both checkpoints (56). Furthermore progression through the cell cycle phases and checkpoints is determined by two key classes of regulatory molecules known as the cyclins and cyclin-dependent kinases (CDKs). This network of tumor suppressors, cyclins, and CDKs control whether a cell is fit for progression through the cycle or whether the cell is unhealthy and needs repair or should proceed through an alternate pathway leading to cell growth arrest and cell death (57).

The tumor suppressor p53 mainly functions as a transcription factor by binding to specific DNA sequences and then transactivating or repressing its target genes (82). In response to cellular genotoxic stress the targets of p53 regulate the

DNA repair, cell-cycle arrest, and apoptosis pathways (29). Given its critical function the expression of p53 protein under normal physiological conditions must be tightly regulated due to the extreme outcomes in activating its target genes. Normally this constitutively expressed protein is found at low levels in most tissues (54). When p53 is upregulated it arrests the cell at both the G1/S and G2/M checkpoints (21, 25). Alterations in p53 can lead to extended proliferation in cells that would have otherwise undergone cell cycle arrest and apoptosis. Therefore, it is unsurprising that p53 mutations are found in over half of all human tumors (37, 54). However wild-type p53 has also been shown to be elevated in cancers (58). The exact mechanism for this has not been proven but likely results from the binding of other transcription factors followed by stabilization of the p53 protein. Understanding the levels of p53 as well as its mutants are important in the fight against cancer, as cancers with wild-type p53 appear more susceptible to chemotherapy (76).

When ZBP-89 is overexpressed, p53 protein expression is also increased (8). This overexpression of ZBP-89 leading to increased p53 occurs both when cells are forced to express exogenous ZBP-89 through an adenoviral vector as well as when the cells are serum starved leading to increased endogenous ZBP-89. Co-immunoprecipitation studies revealed a direct interaction between ZBP-89 and p53. Specifically the DNA-binding and carboxy-terminal domains of p53 were shown to bind the zinc-finger containing DNA binding domain of ZBP-89. ZBP-89 blocks the shuttling of p53 out of the nucleus and into the cytoplasm, where it is normally degraded by the proteosome (8). Thus the binding of ZBP-89 effectively prolongs the half-life of p53 thereby enhancing its transcriptional activity as well as stabilizing

the p53 protein and retarding its rapid degradation. Interestingly increased susceptibility to chemotherapy-induced cell death has been shown in both p53-null cells with elevated ZBP-89 levels and cells with an accumulation of p53 (wild-type) enhanced by ZBP-89 (64).

Given the connection between p53 mutation and cell cycle aberrations, the connection between ZBP-89 and these mutations has also been explored. Adenoviral overexpression of ZBP-89 in HT-29 cells did not show stabilization of the p53R273H mutant that is present in this cell line (8). However, GST pull-down assays showed ZBP-89 was still able to bind this mutant (8). Of the six additional p53 mutants examined none were stabilized by ZBP-89 except for the p53A161T mutation, which exhibited constitutive transcriptional activity (64). Therefore despite protein-protein interactions between the p53 mutants and ZBP-89, ZBP-89 is prevented from potentiating p53-dependent transcriptional activation. Furthermore mutations in the DNA binding domain of p53 have a dominant negative effect on ZBP-89, preventing ZBP-89 mediated p53-independent apoptosis. Together these findings further stress the importance of determining the expression levels of ZBP-89 as well as p53 wild-type and mutants in cancers.

The CDK inhibitor $p21^{waf1}$ controls progression through the cell cycle by binding to G_1 cyclin/CDK complexes (32, 33, 87). This control functions through both p53 dependent and independent mechanisms (94). In the situation where there is DNA damage in the cell this damage stimulates the transcription of $p21^{waf1}$ to trigger cell growth arrest in a manner that is dependent on p53 involvement (24). On the other hand transcription factor binding to the $p21^{waf1}$ promoter as well as

involvement of chemical agents can direct the cell cycle towards differentiation pathways in a manner that is p53 independent (28, 66, 70). Specifically GC rich sites in the $p21^{waf1}$ promoter bind members of the Sp family of transcription factors as well as ZBP-89 (6, 13, 45, 77).

The amino-terminal domain of ZBP-89 binds to the region of the $p21^{waf1}$ promoter from -245 to -215 in a manner that is dependent on the short chain fatty acid butyrate (6). When HT-29 cells are treated with sodium butyrate, ZBP-89 is recruited on to the $p21^{waf1}$ promoter within 30 minutes (46). Sodium butyrate provides an elevated level of histone acetyltransferase activity that is required for ZBP-89 to potentiate the transcription of $p21^{waf1}$. The transcriptional co-activator p300, known to mediate growth arrest, binds to ZBP-89 and further enhances the activation of the $p21^{waf1}$ promoter (6, 30). Thus increased histone acetyltransferase activity allows a complex of ZBP-89 and p300 to bind to the $p21^{waf1}$ promoter. Sp1 is then recruited to this complex and binds to both ZBP-89 and the $p21^{waf1}$ promoter. This mechanism functions to regulate $p21^{waf1}$ transcription promoting growth arrest and cellular differentiation.

ZBP-89 also binds other GC rich promoter elements to mediate programmed cell death in manners that are independent of p53. Extracellular signals utilize molecular mechanisms that are not clearly understood to trigger apoptotic pathways that do not require p53. These generally include pro-inflammatory cytokines and the withdrawal of growth factors leading to suppression of survival signals followed by destabilization of mitochondrial membranes and ultimately cell death. For example interferon-gamma (IFNγ) is a pro-inflammatory cytokine known to induce apoptosis

and sensitize cancer cells to death signals (3, 65, 74). IFNγ-induced phosphorylation of STAT1 triggers apoptosis. A reduction in ZBP-89 levels lead to decreased STAT1 promoter activity thus attenuating IFNγ-induced apoptosis (7). ZBP-89 binds to a G-rich element from +171 to +179 of STAT1 and is required for the constitutive expression of STAT1 (7).

Other pathways leading to apoptosis involve the Mitogen-Activated Protein (MAP) family of kinases which can work in both p53 dependent and independent manners. There are three MAP kinase subfamilies: Extracellular signal-Related Kinase (ERK) 1/2, c-Jun N-terminal Kinase (JNK) 1/2, and p38 MAP kinase. A time course of MAP kinase activation by ZBP-89 showed that while overall protein levels of all three MAP kinases remained unchanged the phosphorylation of these kinases increased. In vitro kinase assays were used to show the increases in this phosphorylation corresponded to an increase in kinase phosphorylation of specific substrates (9). Therefore ectopic ZBP-89 expression has been shown to induce activation of all three MAP kinase subfamilies. Blocking kinase activation through the use to specific inhibitors and dominant negative constructs showed ERK1/2 and p38 are dispensable for ZBP-89 induced apoptosis (9). However blockade of JNK using an inhibitor lead to a significant decrease in ZBP-89 induced apoptosis (9). p53 null cells infected with adenoviral ZBP-89 show activation of JNK (9). Therefore JNK is required for ZBP-89 induced apoptosis and this pathway is independent of p53.

ZBP-89 in Disease and Cancer

Perhaps the most fundamental characteristic of cells is their ability to proliferate. In healthy cells this proliferation is very tightly regulated and occurs in a systematic fashion as cells proceed through the cell cycle. When cells become damaged beyond repair they normally proceed down a pathway that ultimately leads to their death. However in some situations these cells are able to evade the body's programmed death response and the abnormal cells grow uncontrollably. The result is usually the development of a non-structured tumor. This uncontrolled growth of abnormal cells is no longer useful to the body and is often referred to as a cancer (38). Cancer can arise in almost every site in the body and its characteristics often differ from cancers originating in different organs. In order to generate better treatments to stop the spread of and/or eliminate cancers, we must first understand the environmental factors and genetic mutations that help the cancer develop. Due to our understanding of ZBP-89 function in growth arrest and apoptosis it should come as no surprise that it plays a role in the development of cancers in the gastrointestinal tract and other parts of the body.

Colorectal Cancer

Despite a decrease in incidence colorectal cancer is the third most common cancer in both men and women worldwide (40). Many risk factors associated with colorectal cancer are modifiable factors such as smoking, extreme alcohol consumption, obesity, and a diet short on fruits and vegetables. In addition inherited genetic mutations such as hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) have been shown to increase risk.

Chemotherapy in combination with surgery is the most common treatment and varies depending on the level of invasiveness of the cancer. It is estimated that 49,960 deaths will occur in 2008 due to colon and rectum cancer (40). These deaths are also on the decline in recent decades as word spreads on the importance of early detection and development of better treatments. It is expected that as scientists have an increased understanding of the roles genetic mutations and modifiable behaviors play the incidence and thus death rates for colorectal cancer will continue to decline.

In vitro studies using the colorectal cancer cell lines MKN-45, KatoIII, Hct 116, and Colo DM have shown an increase in the expression of ZBP-89 mRNA (80). In a study analyzing gene expression in 16 patients with Dukes' B colon cancer, ZBP-89 was shown to be down-regulated by both microarray analysis and real-time polymerase chain reaction (PCR) (11). The greatest amounts of downregulation were associated with relapse in these patients (11). This suggests that the lower the expression of ZBP-89 the more likely a patient diagnosed with and treated for colorectal cancer will need follow-up treatment for the disease and should be monitored closely for such relapse. In a separate study examining stromelysin-1 promoter mutations in patients with high microsatellite instability (MSI) colorectal tumors all of the mutations detected in this promoter were restricted to the region containing the ZBP-89 binding site (63). This suggests in high MSI colorectal tumors ZBP-89 promoter binding might be altered. However, this was not examined in any capacity in the study. It is important to keep in mind that both of the human studies described above had a very small sample size which could inaccurately reflect the

true role ZBP-89 plays in colorectal cancer. These contradictory findings between in vitro and patient data suggest a fine line exists whereby normal levels of ZBP-89 expression support homeostasis but altered expression levels, either an increase or a decrease, can lead to neoplastic changes.

Diet and Absorption in the Colon

In the colon, butyrate, a fiber derived fermentation product, is capable of maintaining epithelial cell differentiation (44). These effects are thought to be due to the suppression of histone deactylases (HDAC)s, since histone hyperacetylation is associated with butyrate induction of differentiation (60). While little is known concerning the gene specific effects of butyrate, the cyclin-dependent kinase inhibitor $p21^{Waf1}$ is a transcriptional target of butyrate (2). It is known that $p21^{Waf1}$ is regulated through binding of Sp1 and ZBP-89 to GC-rich elements in its promoter (6, 28, 35). In HT-29 (human colon adenocarcinoma) cells treated with butyrate the expression of $p21^{Waf1}$ was potentiated due to the recruitment of the coactivator p300 to the promoter by ZBP-89. This potentiation leads to growth arrest in a relatively short time period whereby expression of ZBP-89 is not affected. Furthermore elevated levels of ZBP-89 expression were seen when cells were exposed to butyrate for periods greater than 48 hours suggesting that factors other than $p21^{waf1}$ are involved the continued effects of butyrate (6). These studies suggest interplay between ZBP-89 and a high fiber diet in the regulation of differentiation and growth arrest in the colon leading to a protective effect in the fight to prevent cancer. Taken together is it possible ZBP-89 works through both genetic and modifiable factors to have an effect on cancer in the colon.

Gastric Cancer

Results suggest elevated levels of ZBP-89 in the stomach may be used as a marker of transformation. This is due in part to the human DNA Epstein-Barr Virus (EBV) which is present in 10% of gastric cancers. The EBV gene BMRF1 has been shown to activate the gastrin promoter and this is mediated through ZBP-89 binding, though the exact mechanism is unknown (36). While incidence is relatively low in the United States gastric cancer is the second most common cause of cancer death in the world (20). It is especially prevalent in Japan where gastric cancer is the most common cancer, and in Korea and China where this form ranks second most popular (53). Risk factors for this form of cancer include smoking, a diet short on fruits and vegetables, high consumption of smoked food, and infection with *Helicobacter pylori* (43, 47, 53, 91). Risk is also increased in those with a family history of gastric cancer, especially in those with a sibling or parent with the disease. When discovered at an early stage the disease is relatively treatable which accounts for a reduced mortality rate in the United States. However, given that many patients are asymptomatic until the disease has progressed to an advanced stage and screening availability is low in developing countries this attributes to the high mortality rate in Asia. While the exact treatment varies depending on the stage of the cancer, surgical techniques such as endoscopic mucosal resection and endoscopic submucosal dissection are often used in combination with chemotherapy (79). There is hope that in developing countries as screening availability increases, the sanitation practices improve, there is less reliance on smoking as a food preservation technique, and farming of fruits and vegetables becomes more popular

the rate of gastric cancer will decrease (41). Regardless of the exact rate due to modifiable factors there is still the need for a better understanding of how genetic factors influence this cancer.

The main physiological role of gastrin, a circulating peptide hormone expressed by G cells in the antrum of the stomach, is to regulate acid secretion by parietal cells as well as epithelial cell growth (23). Gastrin expression varies with changes in stomach environment such as pH and mucosal inflammation. However the expression is not confined to the stomach as it is known to be upregulated in a variety of pre-malignant conditions and established cancers. This tissue specific gastrin production could be the result of constitutive expression followed by secretion and ultimately leading to autocrine or paracrine activity. Conversely upregulated expression as a result of *H. pylori* infection or proton pump inhibitors can lead to systemic hypergastrinemia (31). Interestingly cases of both gastric and colorectal adenocarcinoma have been linked with systemic gastrin expression, with some of those attributed to *H. pylori* infection (10, 31). There is a GC-rich element in the human gastrin promoter that confers epidermal growth factor (EGF) responsiveness. This element may be involved in the upregulation of gastrin as a result of *H. pylori* infection, since colonization provokes inflammation leading to upregulation of heparin binding EGF-like growth factor (HB-EGF) (12). Furthermore, this element has been shown to be a site for binding of the transcription factor Sp1, whereby Sp1 stimulates gastrin expression (62). ZBP-89 also binds to this site and functions to negatively regulate Sp1 binding (61).

ZBP-89 binds specifically to the 5' half-site of this 16 base-pair region of the human gastrin promoter designated gastrin EGF response element (gERE). Co-expression studies show that when bound, ZBP-89 specifically inhibits EGF induction of the gastrin promoter. In order to address any role ZBP-89 might play in gastric cancer; in vitro studies examining ZBP-89 protein expression levels in gastric cell lines were performed. In three human gastric adenocarcinoma cell lines (AGS, MKN-45, and KatoIII) ZBP-89 protein was elevated (80). Additionally, freshly isolated gastric tumor tissue also showed elevated protein levels compared to normal tissues isolated from the same patient (80). This suggests the elevated expression levels are real and not merely an artifact of the extended life in culture of the cell lines. RT-PCR confirmed the additional protein was the result of an increase in gene expression (80). Collectively these results suggest that ZBP-89 is elevated in pre-malignant states and some gastric cancers and thus ZBP-89 may be used as a marker for transformation.

Other Cancers

While a role for ZBP-89 expression in cancers of the gastrointestinal tract has been examined this paradigm also exists in tumors found elsewhere in the body. For example, patients with primitive neuroectodermal tumors or ependymomas, both forms of brain tumors, typically display large amounts of a particular haplotype of platelet derived growth factor (PDGF). This haplotype, H2δ, contains a mutation that specifically disrupts the binding of ZBP-89 (22). In this case loss of ZBP-89 promotes tumor growth, speculating that in certain tissues ZBP-89 may function as a tumor suppressor. Similar to gastric tissues ZBP-89 protein was elevated in human

breast cancer cell lines (SKBR3, Hs578t) and a human melanoma cell line G361. However it is important to note that ZBP-89 levels were unchanged when primary carcinoma and epithelial cell lines from these tissues were examined.

In the case of recurrent human hepatocellular carcinoma (HCC) there is a longer interval between surgical resection and recurrence in patients with wild-type p53. Comparison of cancerous tissues and non-cancerous tissues in patients revealed that ZBP-89 protein was significantly higher in cancerous tissues regardless of p53 status (18). However, ZBP-89 expression in recurrent HCC was more likely to be higher in the nucleus when wild-type p53 was present and higher in the cytoplasm when p53 mutations were present (18). In patients with wild-type p53, 67% of these cases showed co-localization between ZBP-89 and p53 in the nucleus (18). These data suggest a role for ZBP-89 in the nuclear retention of p53 in a subset of recurrent HCC. As is true for other cancers, nuclear accumulation of p53 is associated with increased susceptibility to radiotherapy and chemotherapy induced apoptosis. Therefore, identifying recurrent HCC patients with nuclear co-localization of ZBP-89 and p53 may also identify patients better suited for chemotherapy treatment.

The role ZBP-89 plays in the progression towards cancer appears to vary depending on the tissue examined. Furthermore the exact mechanisms by which this occurs are likely to be quite complex and vary by tissue. Though a role for ZBP-89 in cancer is highly speculative, a consensus appears that in most tissues low levels of ZBP-89 gene expression correlate with homeostasis. Many of the studies described above were performed using cultured cell lines. It is highly possible that

gene signatures might be different in tumors growing in an animal. Thus a key in examining ZBP-89's role in specific disease states might be better characterized in an *in vivo* system modulating its expression.

ZBP-89 Function in Development

While in vitro systems have been useful in deducing possible functions for many factors, these systems are a far cry from what the actual functions may be in a living animal. Most cell culture assays utilize cells that have been transformed so they can be continually propagated in culture. As a consequence, the results are not always necessarily representative of the processes that occur in normal cells. Knock-down studies to deplete a given gene often use these same transformed cells and require the generation of stable transformants which have been selected for several generations in culture. It is easy to understand that in this situation as well the results may reflect more on the manipulations of the system rather than on the loss of the gene. For these reasons the "gold standard" on determining a gene's function has become observing it in a living organism. In this situation, all of the normal machinery is maintained not only at the cellular level but in the tissue, organ system, and whole animal. Mice in particular are a popular vehicle for such observations due to their easily manipulated genome, quick reproductive cycle, and genomic similarity to humans.

Deducing the function of ZBP-89 using in vitro methods has proven quite fruitful. However, correlating this to the role ZBP-89 plays in vivo has been

complicated. Recent studies have shown that despite the demonstration of DNA binding in vitro only a small percentage of these binding sites are occupied by their given transcription factor in vivo (16, 17). Therefore, it is necessary to generate an in vivo system to study the many roles ZBP-89 is likely to play in the body. To date several groups have generated both mouse and zebrafish models aimed at deducing these roles. While a true knock-out of ZBP-89 has not been accomplished, these models underscore the importance of ZBP-89 function in vivo.

ZBP-89 Knockdown

Takeuchi and colleagues generated embryonic stem (ES) cells heterozygous for an allele where exon 9 of mouse zfp148 was interrupted by a neomycin resistance gene with a phosphoglycerokinase promoter (PGK neo) cassette in the reverse orientation. The heterozygous ES cells showed a 50% reduction in ZBP-89 mRNA and the full-length protein was still expressed in these cells. The authors found no evidence of truncated forms of the protein as a result of the mutant allele. Haploinsufficiency for ZBP-89 in the ES cells led to impaired p53-dependent cell-cycle arrest and thus continued proliferation of the ES cells in culture. Chimeras were generated by injection of the ES cells into blastocysts; however the animals failed to transmit the targeted allele in their germ-line. Furthermore five of the nine chimeras were infertile due to apoptotic cell death of pro-spermatogonia during embryonic development. Despite problems with cell-cycle arrest, the heterozygous germ cells failed to proliferate in the gonads of the in vivo model. Thus it is likely that heterozygosity for ZBP-89 causes altered p53 function which led to abnormal growth of the fetal germ cells and triggered their apoptosis during embryonic

development. The authors conclude that two fully functional alleles of ZBP-89 are required for the survival of fetal germ cells in mice (78).

ZBP-89 Overexpression

In an effort to understand a role for ZBP-89 in suppressing cancer progression, David Law in the Merchant lab overexpressed ZBP-89 in the gastrointestinal tract of mice and examined the phenotype. A 13 kb fragment of the villin promoter was cloned upstream of ZBP-89 and thus drove its expression throughout the epithelium of the small intestine. The overexpression resulted in elevation of the apoptotic marker retinoblastoma as well as increased levels of apoptosis as measured by TUNEL assay(50). The mice showed no gross abnormalities for more than one year suggesting the overexpression and subsequent increased apoptosis do not negatively affect gastrointestinal homeostasis in vivo. The animals were bred to $Apc^{Min}/+$ mice, a line prone to tumors in the small intestine. In $Apc^{Min/+}$ animals the normal ZBP-89 expression pattern shows lower expression in the intestinal crypts but higher expression in the villus tip, where the rate of apoptosis is thought to be highest (5). On this background the overexpression of ZBP-89 caused a delay in the gastrointestinal bleeding that is normally observed with the APC^{Min} line. Furthermore there was a fifty percent reduction in the number of adenomas and a trend towards reduced size of the adenomas present. Unsurprisingly, in ZBP-89 overexpressing animals this lead to prolonged survival by more than fifty percent. Endogenous levels of ZBP-89 in the gastrointestinal tract are normally low and the authors suggest that inducible

upregulation, as demonstrated this paper, might increase the tumor suppressing ability of this gene.

ZBP-89 Function in Hematopoiesis

The role of ZBP-89 in hematopoietic cells was examined in zebrafish and mouse ES cells by Li and colleagues. Consensus binding sires for ZBP-89 found in the promoters of the genes encoding PU.1, CEBP-α, and CD11b, all of which play important roles in myeloid differentiation, first suggested a role for ZBP-89 may exist in hematopoiesis (67). Knockdown expression in developing zebrafish embryos was achieved using antisense morpholinos (MOs) to target the translation start site or the splice donor site in exon 8 of zbp-89. Data showed despite a beating heart and complete vasculature no blood cells were present 48 hours postfertilization (hpf) in the targeted embryos(55). Rescue of the phenotype was possible when the embryos were injected with wild-type human ZBP-89 mRNA in addition to the MO. This suggests the phenotype was specifically due to the loss of zbp-89 and not an aberrant result of the injection. Examination of gene expression in the depleted embryos showed reduced expression in primitive erythropoietic genes (gata1 and tif1g), primitive myeloid genes (l-plastin, mpo, pu.1), early hematopoietic genes (gata2, lmo2, scl), and definitive hematopoietic genes (c-myb, runx1). Furthermore this study localized ZBP-89 downstream of cloche, a gene essential for generation of vascular and hematopoietic progenitors, and upstream of SCL, a gene essential for direction of hematopoietic fate commitment from hemangioblasts. Staining for apoptotic cells failed to show significant changes between the knock-down and wild-type embryos, pointing to a role for ZBP-89 in fate specification of early

hematopoietic precursors as opposed to a role in their survival. Their results suggest a role for ZBP-89 in activation of genetic programs specific for the hematopoietic lineage.

In the final publication to date examining the in vivo function of ZBP-89, Woo and colleagues continue to explore the role of ZBP-89 in erythroid maturation. Using a tandem affinity purification technique the authors demonstrated that ZBP-89 is part of a complex containing GATA-1, a gene required for erythroid development, and FOG-1, a zinc finger transcriptional cofactor expressed in the hematopoietic system(85). Together this complex plays a functional role in both early megakaryocyte and erythroid development. ZBP-89 was shown to bind directly to the GATA-1 promoter and suggests that it may play a role in regulating the expression of GATA-1. Morpholino-mediated gene knock-down as well as in vitro co-immunoprecipitation experiments demonstrated a functional interaction between ZBP-89 and FOG that is not dependent on GATA-1. Mouse ES cells containing a genetrap clone that encodes a fusion protein of the first 111amino acids of ZBP-89 fused to β-galactosidase were generated. Cells heterozygous for the genetrap showed reduction of ZBP-89 mRNA levels near 50% compared to wild-type ES cells whereas homozygosity for the genetrap yielded scarcely detectable levels of ZBP-89. The genetrap containing ES cells were injected into mouse blastocysts generating mice heterozygous for ZBP-89. While homozygous genetrap animals were recovered at embryonic day 8.5 (E8.5), by E10.5 all animals were dead suggesting an embryonic lethal phenotype. At this stage the phenotype was defined by lack of a heartbeat, growth delay, open neural tube, and occasionally a lack of

red blood cells. Further studies demonstrated inadequacy in placental function led to the abnormalities seen in the embryos. This is not surprising given that ZBP-89 expression has been shown to be upregulated in the placenta. Examination of fetal liver cells from mouse chimeras showed impaired erythroid development and megakaryocyte colony formation. The authors concluded that despite a lack of viable homozygous genetrap animals there is an unmistakable function of ZBP-89 in definitive erythroid and megakaryocytic development.

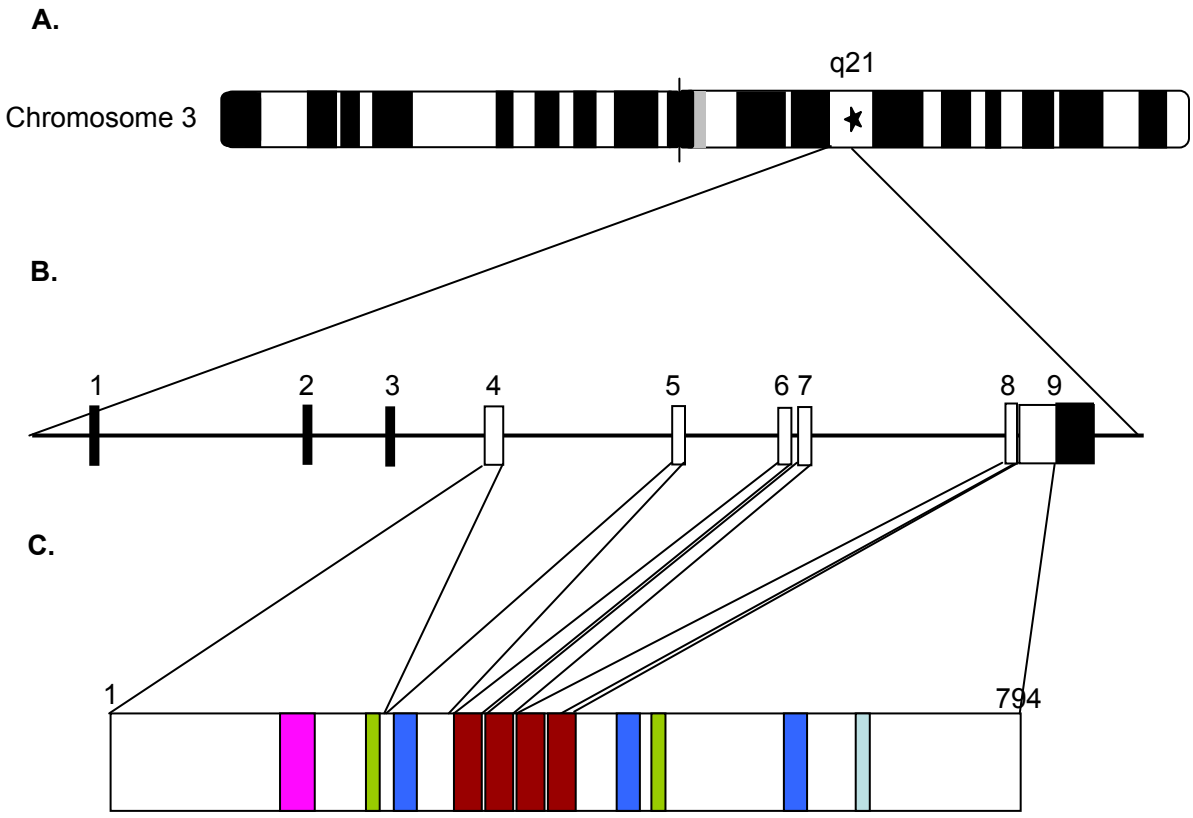
Summary

ZBP-89 is a member of a novel unit of transcription factors that contain a characteristic region of four zinc fingers and are phylogenetically conserved in mammals. Its structural complexity includes a unique set of domains which contribute to its many functional roles. This factor has been shown to regulate diverse biological functions through direct DNA promoter binding as well as protein-protein interactions. This includes its regulation of cell growth and apoptosis through mechanisms that are both dependent and independent on the tumor suppressor p53. There also appears to be a role for ZBP-89 in the development of cancer in various tissues of the body, though these exact mechanisms have yet to be identified.

Therefore this relatively novel transcriptional regulator is involved in a vast number of complex pathways as determined by in vitro methods. These functions may be confirmed or expanded upon when this factor is deleted in vivo. Achievement of this deletion has previously failed though a number of investigators

have attempted this arduous feat. The importance of studying genes in vivo cannot be stressed enough during this time when cancer prevalence and death rates are so high. The body of work depicted herein attempted to further advance the knowledge of the field regarding the function of ZBP-89 not only in the body of the animal but in the gastrointestinal tract specifically.

Figure 1-1. Chromosomal localization, genomic organization, and protein domain structure of human ZBP-89. **A**. Site q21 of chromosome 3 contains ZBP-89. **B**. The entire gene covers a region 130 kb in length. The untranslated exons are the filled rectangles where as translated exons are open rectangles. **C.** The domain structure is depicted. A = Acidic Domain, S = SC Motif, B = Basic Domain, Zn = Zinc finger region, P = PEST/Ser-rich Domain. **D.** The amino acid sequence of the full-length protein is listed.



D.

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  1 mniddklegl flkcggidem qssramvvmg gvsgqsavsg elqesvlqdr slphqeilaa
 61 devlqesemr qqdmishdel mvheetvknd eeqtdtherl pqglqyalnv pisvkqeitf
121 tdvseqlmrd kkqvrepvdl qkkkkrkqrs pakiltined gslglktpks hvcehcnaaf
181 rtnyhlqrhv fihtgekpfq csqcdmrfiq kyllqrheki htgekpfrcd ecgmrfiqky
241 hmerhkrths gekpyqceyc lqyfsrtdrv lkhkrmchen hdkklnrcai kgglltseed
301 sgfstspkdn slpkkkrqkp ekkssgmdke svldksdtkk drndylplys sstkvkdeym
361 vaeyavemph ssvggshled asgeihppkl vlkkinskrs lkqpleqsqt isplstyeds
421 kvskyafelv dkqalldseg sadidqvdnl qegpskpvhs stnyddamqf lkkkrylqaa
481 snnsreyaln vgtiasqpsv tqaavasvid enttasilds qalnveiksn hdknvipdev
541 lqtlldhysh kangqheisf svadtevtss isinssdvpe vtqsenvgss sqasssdkan
601 mlqeyskflq qaldrtsqnd aylnspslnf vtdnqtlpnp pafssidkqv yaampinsfr
661 sgmnsplrtt pdkshfgliv gdsqhpfpfs gdetnhasat stadfldqvt sqkkaeaqpv
721 hqayqmssfe qpfrapyhgs ragiatqfst angqvnlrgp gtsaefsefp lvnvndnrag
781 mtsspdattg qtfg
```

Table 1-1. ZBP-89 similarity across species

Species	Gene	Human Similarity	
H. Sapiens	ZBP-89	100%	
P. Troglydytes	ZNF148	99.5 (n)	99.6 (a)
C. Familiaris	LOC488017	94.5 (n)	98.6 (a)
M. Musculus	Zfp148	92.8 (n)	97.2 (a)
R. Norvegicus	Zfp148	91.8 (n)	96.8 (a)
G. Domesticus	ZNF148	84.6 (n)	87.7 (a)
D. Rerio	wufb06h05	74.1 (n)	61.8 (a)
D. Melanogaster	CG8474		33.3 (a)
C. Elegans	Blimp1		31.3 (a)

Table 1-2. ZBP-89 promoter binding sites

Promoter	Binding Site
βEnolase	AGTGGGGGAGGGGGCTGCG
CD11b	GAAGGGTGGGCAGGC
COX VB	GTGGGGGGGGGGTG
ENA-78	CCCCTCCCCC
gastrin	GGGGCGGGGTGGGGGGA
Growth Hormone	CACCCCGCCCCCTTCCT
HSV *oriLyt*	TCCCTCCCCCCCCCTACCCCC
IAP	CCCATTGCCTCCTCCCTCA
ODC	GCCCCTCCCCCG
$p21^{Waf1/CIP}$	CGGGTCCCGCCTCCTTG
$P21^{Waf1/CIP}$	TGGGGGAGGAGGGAAGTGCCCTCCT
Procollagen Iα2	TCCTCCCCCCTCGGC
Procollagen Iα1	TCCTCCCCCCTCTT
STAT1	GGGGGTGGG
Stromelysin	GGTTTTTTCCCCCCATCA
TCRα sil	GGGGTGGGGTGGGGGG
TCRβ	GAAGTGGGGTGGT
Vimentin	GGACCCCCCCC
CONSENSUS	gccCCT/AxCxCCC

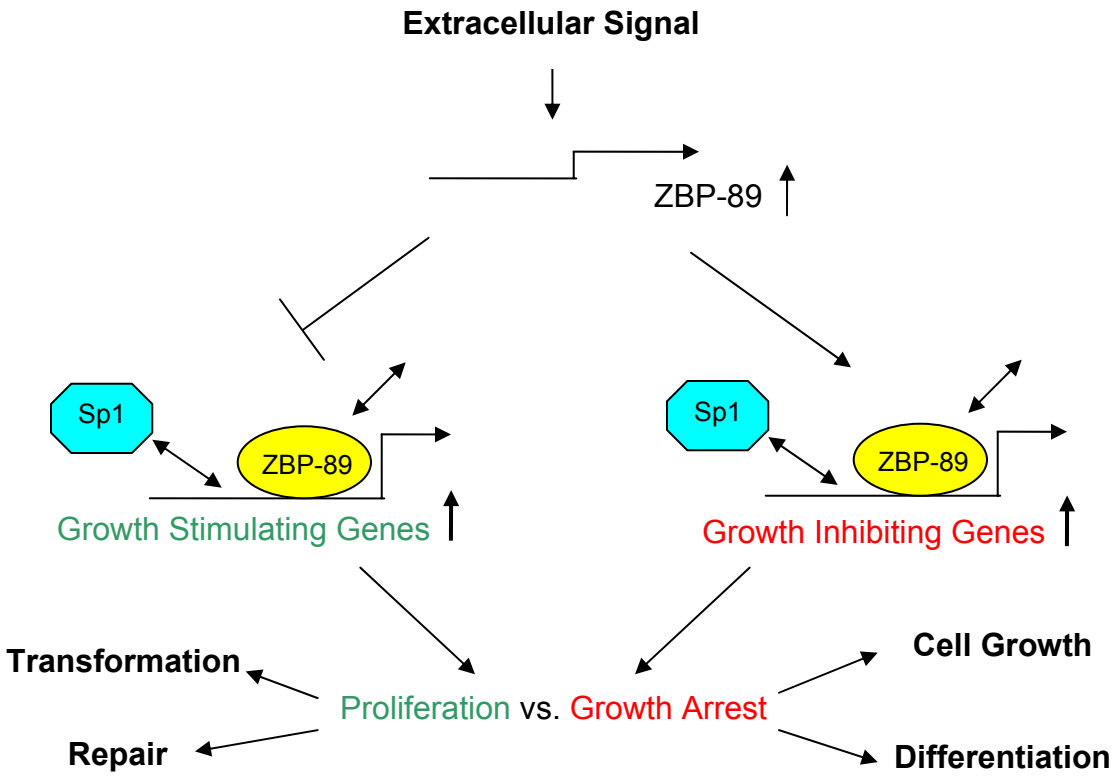

Figure 1-2. Model of ZBP-89 regulation of cell growth. Depending on the promoter context the bifunctional transcription factor ZBP-89 represses or activates genes.

References

1. **Antona, V., G. Cammarata, L. De Gregorio, T. A. Dragani, A. Giallongo, and S. Feo.** 1998. The gene encoding the transcriptional repressor BERF-1 maps to a region of conserved synteny on mouse chromosome 16 and human chromosome 3 and a related pseudogene maps to mouse chromosome 8. Cytogenet Cell Genet **83:**90-2.
2. **Archer, S. Y., S. Meng, A. Shei, and R. A. Hodin.** 1998. p21(WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. Proc Natl Acad Sci U S A **95:**6791-6.
3. **Ashkenazi, A.** 2002. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer **2:**420-30.
4. **Bai, L., J. Y. Kao, D. J. Law, and J. L. Merchant.** 2006. Recruitment of ataxia-telangiectasia mutated to the p21(waf1) promoter by ZBP-89 plays a role in mucosal protection. Gastroenterology **131:**841-52.
5. **Bai, L., C. Logsdon, and J. L. Merchant.** 2002. Regulation of epithelial cell growth by ZBP-89: potential relevance in pancreatic cancer. Int J Gastrointest Cancer **31:**79-88.
6. **Bai, L., and J. L. Merchant.** 2000. Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of p21waf1 transcription in human cells. J Biol Chem **275:**30725-33.
7. **Bai, L., and J. L. Merchant.** 2003. Transcription factor ZBP-89 is required for STAT1 constitutive expression. Nucleic Acids Res **31:**7264-70.
8. **Bai, L., and J. L. Merchant.** 2001. ZBP-89 promotes growth arrest through stabilization of p53. Mol Cell Biol **21:**4670-83.
9. **Bai, L., S. O. Yoon, P. D. King, and J. L. Merchant.** 2004. ZBP-89-induced apoptosis is p53-independent and requires JNK. Cell Death Differ **11:**663-73.
10. **Baldwin, G. S., and A. Shulkes.** 1998. Gastrin, gastrin receptors and colorectal carcinoma. Gut **42:**581-4.
11. **Bandres, E., R. Malumbres, E. Cubedo, B. Honorato, R. Zarate, A. Labarga, U. Gabisu, J. J. Sola, and J. Garcia-Foncillas.** 2007. A gene signature of 8 genes could identify the risk of recurrence and progression in Dukes' B colon cancer patients. Oncol Rep **17:**1089-94.
12. **Beales, I. L.** 2004. Gastrin and interleukin-1beta stimulate growth factor secretion from cultured rabbit gastric parietal cells. Life Sci **75:**2983-95.
13. **Billon, N., D. Carlisi, M. B. Datto, L. A. van Grunsven, A. Watt, X. F. Wang, and B. B. Rudkin.** 1999. Cooperation of Sp1 and p300 in the induction of the CDK inhibitor p21WAF1/CIP1 during NGF-mediated neuronal differentiation. Oncogene **18:**2872-82.
14. **Boopathi, E., N. Lenka, S. K. Prabu, J. K. Fang, F. Wilkinson, M. Atchison, A. Giallongo, and N. G. Avadhani.** 2004. Regulation of murine cytochrome c oxidase Vb gene expression during myogenesis: YY-1 and heterogeneous nuclear ribonucleoprotein D-like protein (JKTBP1) reciprocally

regulate transcription activity by physical interaction with the BERF-1/ZBP-89 factor. J Biol Chem **279:**35242-54.

15. **Borghaei, R. C., P. L. Rawlings, Jr., M. Javadi, and J. Woloshin.** 2004. NF-kappaB binds to a polymorphic repressor element in the MMP-3 promoter. Biochem Biophys Res Commun **316:**182-8.
16. **Bresnick, E. H., M. L. Martowicz, S. Pal, and K. D. Johnson.** 2005. Developmental control via GATA factor interplay at chromatin domains. J Cell Physiol **205:**1-9.
17. **Carroll, J. S., X. S. Liu, A. S. Brodsky, W. Li, C. A. Meyer, A. J. Szary, J. Eeckhoute, W. Shao, E. V. Hestermann, T. R. Geistlinger, E. A. Fox, P. A. Silver, and M. Brown.** 2005. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell **122:**33-43.
18. **Chen, G. G., J. L. Merchant, P. B. Lai, R. L. Ho, X. Hu, M. Okada, S. F. Huang, A. K. Chui, D. J. Law, Y. G. Li, W. Y. Lau, and A. K. Li.** 2003. Mutation of p53 in recurrent hepatocellular carcinoma and its association with the expression of ZBP-89. Am J Pathol **162:**1823-9.
19. **Chupreta, S., H. Brevig, L. Bai, J. L. Merchant, and J. A. Iniguez-Lluhi.** 2007. Sumoylation-dependent control of homotypic and heterotypic synergy by the Kruppel-type zinc finger protein ZBP-89. J Biol Chem **282:**36155-66.
20. **Crew, K. D., and A. I. Neugut.** 2006. Epidemiology of gastric cancer. World J Gastroenterol **12:**354-62.
21. **Cross, S. M., C. A. Sanchez, C. A. Morgan, M. K. Schimke, S. Ramel, R. L. Idzerda, W. H. Raskind, and B. J. Reid.** 1995. A p53-dependent mouse spindle checkpoint. Science **267:**1353-6.
22. **De Bustos, C., A. Smits, B. Stromberg, V. P. Collins, M. Nister, and G. Afink.** 2005. A PDGFRA promoter polymorphism, which disrupts the binding of ZNF148, is associated with primitive neuroectodermal tumours and ependymomas. J Med Genet **42:**31-7.
23. **Dockray, G. J., A. Varro, and R. Dimaline.** 1996. Gastric endocrine cells: gene expression, processing, and targeting of active products. Physiol Rev **76:**767-98.
24. **Dulic, V., W. K. Kaufmann, S. J. Wilson, T. D. Tlsty, E. Lees, J. W. Harper, S. J. Elledge, and S. I. Reed.** 1994. p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell **76:**1013-23.
25. **el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817-25.
26. **Elledge, S. J.** 1996. Cell cycle checkpoints: preventing an identity crisis. Science **274:**1664-72.
27. **Feo, S., V. Antona, G. Cammarata, F. Cavaleri, R. Passantino, P. Rubino, and A. Giallongo.** 2001. Conserved structure and promoter sequence similarity in the mouse and human genes encoding the zinc finger factor BERF-1/BFCOL1/ZBP-89. Biochem Biophys Res Commun **283:**209-18.

28. **Gartel, A. L., and A. L. Tyner.** 1999. Transcriptional regulation of the p21((WAF1/CIP1)) gene. Exp Cell Res **246:**280-9.
29. **Giaccia, A. J., and M. B. Kastan.** 1998. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev **12:**2973-83.
30. **Giordano, A., and M. L. Avantaggiati.** 1999. p300 and CBP: partners for life and death. J Cell Physiol **181:**218-30.
31. **Grabowska, A. M., and S. A. Watson.** 2007. Role of gastrin peptides in carcinogenesis. Cancer Lett **257:**1-15.
32. **Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge.** 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:**805-16.
33. **Harper, J. W., S. J. Elledge, K. Keyomarsi, B. Dynlacht, L. H. Tsai, P. Zhang, S. Dobrowolski, C. Bai, L. Connell-Crowley, E. Swindell, and et al.** 1995. Inhibition of cyclin-dependent kinases by p21. Mol Biol Cell **6:**387-400.
34. **Hasegawa, T., A. Takeuchi, O. Miyaishi, K. Isobe, and B. de Crombrugghe.** 1997. Cloning and characterization of a transcription factor that binds to the proximal promoters of the two mouse type I collagen genes. J Biol Chem **272:**4915-23.
35. **Hasegawa, T., H. Xiao, and K. Isobe.** 1999. Cloning of a GADD34-like gene that interacts with the zinc-finger transcription factor which binds to the p21(WAF) promoter. Biochem Biophys Res Commun **256:**249-54.
36. **Holley-Guthrie, E. A., W. T. Seaman, P. Bhende, J. L. Merchant, and S. C. Kenney.** 2005. The Epstein-Barr virus protein BMRF1 activates gastrin transcription. J Virol **79:**745-55.
37. **Hollstein, M., K. Rice, M. S. Greenblatt, T. Soussi, R. Fuchs, T. Sorlie, E. Hovig, B. Smith-Sorensen, R. Montesano, and C. C. Harris.** 1994. Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res **22:**3551-5.
38. **Hook, S. S., J. J. Lin, and A. Dutta.** 2007. Mechanisms to control rereplication and implications for cancer. Curr Opin Cell Biol **19:**663-71.
39. **Iniguez-Lluhi, J. A., and D. Pearce.** 2000. A common motif within the negative regulatory regions of multiple factors inhibits their transcriptional synergy. Mol Cell Biol **20:**6040-50.
40. **Jemal, A., R. Siegel, E. Ward, T. Murray, J. Xu, and M. J. Thun.** 2007. Cancer statistics, 2007. CA Cancer J Clin **57:**43-66.
41. **Kamangar, F., G. M. Dores, and W. F. Anderson.** 2006. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol **24:**2137-50.
42. **Keates, A. C., S. Keates, J. H. Kwon, K. O. Arseneau, D. J. Law, L. Bai, J. L. Merchant, T. C. Wang, and C. P. Kelly.** 2001. ZBP-89, Sp1, and nuclear factor-kappa B regulate epithelial neutrophil-activating peptide-78 gene expression in Caco-2 human colonic epithelial cells. J Biol Chem **276:**43713-22.

43. **Kim, Y., A. Shin, J. Gwack, J. K. Jun, S. K. Park, D. Kang, H. R. Shin, S. H. Chang, and K. Y. Yoo.** 2007. [Cigarette smoking and gastric cancer risk in a community-based cohort study in Korea]. J Prev Med Pub Health **40:**467-74.
44. **Kim, Y. S., and J. A. Milner.** 2007. Dietary modulation of colon cancer risk. J Nutr **137:**2576S-2579S.
45. **Kivinen, L., M. Tsubari, T. Haapajarvi, M. B. Datto, X. F. Wang, and M. Laiho.** 1999. Ras induces p21Cip1/Waf1 cyclin kinase inhibitor transcriptionally through Sp1-binding sites. Oncogene **18:**6252-61.
46. **Kobayashi, H., E. M. Tan, and S. E. Fleming.** 2004. Acetylation of histones associated with the p21WAF1/CIP1 gene by butyrate is not sufficient for p21WAF1/CIP1 gene transcription in human colorectal adenocarcinoma cells. Int J Cancer **109:**207-13.
47. **Ladeiras-Lopes, R., A. K. Pereira, A. Nogueira, T. Pinheiro-Torres, I. Pinto, R. Santos-Pereira, and N. Lunet.** 2008. Smoking and gastric cancer: systematic review and meta-analysis of cohort studies. Cancer Causes Control.
48. **Law, D. J., M. Du, G. L. Law, and J. L. Merchant.** 1999. ZBP-99 defines a conserved family of transcription factors and regulates ornithine decarboxylase gene expression. Biochem Biophys Res Commun **262:**113-20.
49. **Law, D. J., E. M. Labut, R. D. Adams, and J. L. Merchant.** 2006. An isoform of ZBP-89 predisposes the colon to colitis. Nucleic Acids Res **34:**1342-50.
50. **Law, D. J., E. M. Labut, and J. L. Merchant.** 2006. Intestinal overexpression of ZNF148 suppresses ApcMin/+ neoplasia. Mamm Genome **17:**999-1004.
51. **Law, D. J., S. A. Tarle, and J. L. Merchant.** 1998. The human ZBP-89 homolog, located at chromosome 3q21, represses gastrin gene expression. Mamm Genome **9:**165-7.
52. **Law, G. L., H. Itoh, D. J. Law, G. J. Mize, J. L. Merchant, and D. R. Morris.** 1998. Transcription factor ZBP-89 regulates the activity of the ornithine decarboxylase promoter. J Biol Chem **273:**19955-64.
53. **Leung, W. K., M. S. Wu, Y. Kakugawa, J. J. Kim, K. G. Yeoh, K. L. Goh, K. C. Wu, D. C. Wu, J. Sollano, U. Kachintorn, T. Gotoda, J. T. Lin, W. C. You, E. K. Ng, and J. J. Sung.** 2008. Screening for gastric cancer in Asia: current evidence and practice. Lancet Oncol **9:**279-87.
54. **Levine, A. J.** 1997. p53, the cellular gatekeeper for growth and division. Cell **88:**323-31.
55. **Li, X., J. W. Xiong, C. S. Shelley, H. Park, and M. A. Arnaout.** 2006. The transcription factor ZBP-89 controls generation of the hematopoietic lineage in zebrafish and mouse embryonic stem cells. Development **133:**3641-50.
56. **Lobrich, M., and P. A. Jeggo.** 2007. The impact of a negligent G2/M checkpoint on genomic instability and cancer induction. Nat Rev Cancer **7:**861-9.
57. **Lukas, J., C. Lukas, and J. Bartek.** 2004. Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time. DNA Repair (Amst) **3:**997-1007.

58. **Lutzker, S. G., and A. J. Levine.** 1996. A functionally inactive p53 protein in teratocarcinoma cells is activated by either DNA damage or cellular differentiation. Nat Med **2:**804-10.
59. **Malo, M. S., M. Mozumder, X. B. Zhang, S. Biswas, A. Chen, L. C. Bai, J. L. Merchant, and R. A. Hodin.** 2006. Intestinal alkaline phosphatase gene expression is activated by ZBP-89. Am J Physiol Gastrointest Liver Physiol **290:**G737-46.
60. **Merchant, J. L., L. Bai, and M. Okada.** 2003. ZBP-89 mediates butyrate regulation of gene expression. J Nutr **133:**2456S-2460S.
61. **Merchant, J. L., G. R. Iyer, B. R. Taylor, J. R. Kitchen, E. R. Mortensen, Z. Wang, R. J. Flintoft, J. B. Michel, and R. Bassel-Duby.** 1996. ZBP-89, a Kruppel-like zinc finger protein, inhibits epidermal growth factor induction of the gastrin promoter. Mol Cell Biol **16:**6644-53.
62. **Merchant, J. L., A. Shiotani, E. R. Mortensen, D. K. Shumaker, and D. R. Abraczinskas.** 1995. Epidermal growth factor stimulation of the human gastrin promoter requires Sp1. J Biol Chem **270:**6314-9.
63. **Moran, A., P. Iniesta, C. de Juan, C. Garcia-Aranda, A. Diaz-Lopez, and M. Benito.** 2005. Impairment of stromelysin-1 transcriptional activity by promoter mutations in high microsatellite instability colorectal tumors. Cancer Res **65:**3811-4.
64. **Okada, M., A. Tessier, L. Bai, and J. L. Merchant.** 2006. P53 mutants suppress ZBP-89 function. Anticancer Res **26:**2023-8.
65. **Ossina, N. K., A. Cannas, V. C. Powers, P. A. Fitzpatrick, J. D. Knight, J. R. Gilbert, E. M. Shekhtman, L. D. Tomei, S. R. Umansky, and M. C. Kiefer.** 1997. Interferon-gamma modulates a p53-independent apoptotic pathway and apoptosis-related gene expression. J Biol Chem **272:**16351-7.
66. **Owen, G. I., J. K. Richer, L. Tung, G. Takimoto, and K. B. Horwitz.** 1998. Progesterone regulates transcription of the p21(WAF1) cyclin- dependent kinase inhibitor gene through Sp1 and CBP/p300. J Biol Chem **273:**10696-701.
67. **Park, H., C. S. Shelley, and M. A. Arnaout.** 2003. The zinc finger transcription factor ZBP-89 is a repressor of the human beta 2-integrin CD11b gene. Blood **101:**894-902.
68. **Passantino, R., V. Antona, G. Barbieri, P. Rubino, R. Melchionna, G. Cossu, S. Feo, and A. Giallongo.** 1998. Negative regulation of beta enolase gene transcription in embryonic muscle is dependent upon a zinc finger factor that binds to the G-rich box within the muscle-specific enhancer. J Biol Chem **273:**484-94.
69. **Pekarsky, Y., E. Zabarovsky, V. Kashuba, H. Drabkin, A. A. Sandberg, R. Morgan, A. Rynditch, and K. Gardiner.** 1995. Cloning of breakpoints in 3q21 associated with hematologic malignancy. Cancer Genet Cytogenet **80:**1-8.
70. **Prowse, D. M., L. Bolgan, A. Molnar, and G. P. Dotto.** 1997. Involvement of the Sp3 transcription factor in induction of p21Cip1/WAF1 in keratinocyte differentiation. J Biol Chem **272:**1308-14.

71. **Reizis, B., and P. Leder.** 1999. Expression of the mouse pre-T cell receptor alpha gene is controlled by an upstream region containing a transcriptional enhancer. J Exp Med **189:**1669-78.
72. **Remington, M. C., S. A. Tarle, B. Simon, and J. L. Merchant.** 1997. ZBP-89, a Kruppel-type zinc finger protein, inhibits cell proliferation. Biochem Biophys Res Commun **237:**230-4.
73. **Rogers, S., R. Wells, and M. Rechsteiner.** 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science **234:**364-8.
74. **Ruiz-Ruiz, C., C. Munoz-Pinedo, and A. Lopez-Rivas.** 2000. Interferon-gamma treatment elevates caspase-8 expression and sensitizes human breast tumor cells to a death receptor-induced mitochondria-operated apoptotic program. Cancer Res **60:**5673-80.
75. **Sauer, F., J. D. Fondell, Y. Ohkuma, R. G. Roeder, and H. Jackle.** 1995. Control of transcription by Kruppel through interactions with TFIIB and TFIIE beta. Nature **375:**162-4.
76. **Selivanova, G., T. Kawasaki, L. Ryabchenko, and K. G. Wiman.** 1998. Reactivation of mutant p53: a new strategy for cancer therapy. Semin Cancer Biol **8:**369-78.
77. **Sowa, Y., T. Orita, S. Minamikawa-Hiranabe, T. Mizuno, H. Nomura, and T. Sakai.** 1999. Sp3, but not Sp1, mediates the transcriptional activation of the p21/WAF1/Cip1 gene promoter by histone deacetylase inhibitor. Cancer Res **59:**4266-70.
78. **Takeuchi, A., Y. Mishina, O. Miyaishi, E. Kojima, T. Hasegawa, and K. Isobe.** 2003. Heterozygosity with respect to Zfp148 causes complete loss of fetal germ cells during mouse embryogenesis. Nat Genet **33:**172-6.
79. **Tanaka, M., H. Ono, N. Hasuike, and K. Takizawa.** 2008. Endoscopic submucosal dissection of early gastric cancer. Digestion **77 Suppl 1:**23-8.
80. **Taniuchi, T., E. R. Mortensen, A. Ferguson, J. Greenson, and J. L. Merchant.** 1997. Overexpression of ZBP-89, a zinc finger DNA binding protein, in gastric cancer. Biochem Biophys Res Commun **233:**154-60.
81. **Thimmarayappa, J., J. Sun, L. E. Schultz, P. Dejkhamron, C. Lu, A. Giallongo, J. L. Merchant, and R. K. Menon.** 2006. Inhibition of growth hormone receptor gene expression by saturated fatty acids: role of Kruppel-like zinc finger factor, ZBP-89. Mol Endocrinol **20:**2747-60.
82. **Vogelstein, B., D. Lane, and A. J. Levine.** 2000. Surfing the p53 network. Nature **408:**307-10.
83. **Wang, Y., J. A. Kobori, and L. Hood.** 1993. The ht beta gene encodes a novel CACCC box-binding protein that regulates T-cell receptor gene expression. Mol Cell Biol **13:**5691-701.
84. **Wieczorek, E., Z. Lin, E. B. Perkins, D. J. Law, J. L. Merchant, and Z. E. Zehner.** 2000. The zinc finger repressor, ZBP-89, binds to the silencer element of the human vimentin gene and complexes with the transcriptional activator, Sp1. J Biol Chem **275:**12879-88.
85. **Woo, A. J., T. B. Moran, Y. Schindler, S. K. Choe, N. B. Langer, M. R. Sullivan, Y. Fujiwara, B. H. Paw, and A. B. Cantor.** 2008. Identification of

ZBP-89 as a Novel GATA-1 Associated Transcription Factor Involved in Megakaryocytic and Erythroid Development. Mol Cell Biol.

86. **Wu, Y., I. Diab, X. Zhang, E. S. Izmailova, and Z. E. Zehner.** 2004. Stat3 enhances vimentin gene expression by binding to the antisilencer element and interacting with the repressor protein, ZBP-89. Oncogene **23:**168-78.
87. **Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach.** 1993. p21 is a universal inhibitor of cyclin kinases. Nature **366:**701-4.
88. **Yamada, A., S. Takaki, F. Hayashi, K. Georgopoulos, R. M. Perlmutter, and K. Takatsu.** 2001. Identification and characterization of a transcriptional regulator for the lck proximal promoter. J Biol Chem **276:**18082-9.
89. **Yamagata, N., C. Shimazaki, T. Kikuta, H. Hirai, T. Sumikuma, Y. Sudo, E. Ashihara, H. Goto, T. Inaba, N. Fujita, and M. Nakagawa.** 1997. A translocation between 3q21 and 12q24 in a patient with minimally differentiated acute myeloid leukemia (AML-M0). Cancer Genet Cytogenet **97:**90-3.
90. **Ye, S., C. Whatling, H. Watkins, and A. Henney.** 1999. Human stromelysin gene promoter activity is modulated by transcription factor ZBP-89. FEBS Lett **450:**268-72.
91. **Zagari, R. M., and F. Bazzoli.** 2004. Gastric cancer: who is at risk? Dig Dis **22:**302-5.
92. **Zavolan, M., S. Kondo, C. Schonbach, J. Adachi, D. A. Hume, Y. Hayashizaki, and T. Gaasterland.** 2003. Impact of alternative initiation, splicing, and termination on the diversity of the mRNA transcripts encoded by the mouse transcriptome. Genome Res **13:**1290-300.
93. **Zhang, X., I. H. Diab, and Z. E. Zehner.** 2003. ZBP-89 represses vimentin gene transcription by interacting with the transcriptional activator, Sp1. Nucleic Acids Res **31:**2900-14.
94. **Zhao, Y., S. Lu, L. Wu, G. Chai, H. Wang, Y. Chen, J. Sun, Y. Yu, W. Zhou, Q. Zheng, M. Wu, G. A. Otterson, and W. G. Zhu.** 2006. Acetylation of p53 at lysine 373/382 by the histone deacetylase inhibitor depsipeptide induces expression of p21(Waf1/Cip1). Mol Cell Biol **26:**2782-90.

Chapter II

Generation of gene targeted ZBP-89 mice through insertion of loxP sites

Summary

ZBP-89 (ZNF148, Zfp148) is a transcription factor ubiquitously expressed in mice and humans. Expression levels of this factor are low in most tissues. Previous attempts to knock-out ZBP-89 in mice have failed to generate viable mice deficient in this factor. We sought to generate gene targeted animals capable of tissue-specific ZBP-89 deletion. LoxP sites were inserted flanking exons 8 and 9 of the locus. In addition, a neomycin resistance cassette flanked by FRT sites was inserted upstream of exon 8 for selection of ES cell clones. Chimeras generated from the targeting vector reproduced without complication and transmitted the targeted locus in their germline. However, the targeted allele exhibited reduced ZBP-89 expression levels. Furthermore homozygosity for this allele results in perinatal lethality presumably due to the extreme reduction in ZBP-89. Removal of the neomycin resistance cassette restores ZBP-89 expression levels and resulting homozygous ZBP-89 FLP mice are viable. These data suggest complete null ZBP-89 knockout mice will exhibit an embryonic lethal phenotype and that tissue-specific deletion is the most logical option for studying ZBP-89 function *in vivo*.

Introduction

ZBP-89 (ZNF148, Zfp148) is a ubiquitously expressed Krüppel-type transcription factor. This functionally complex protein is associated with signaling pathways involved in embryogenesis, cellular proliferation, differentiation, growth arrest, and apoptosis (1, 2, 8, 10, 16). ZBP-89 regulates these diverse biological functions through both protein-protein interactions and direct binding to GC-rich promoter elements (3, 8, 10, 18). Furthermore while ZBP-89 is known to induce cell growth arrest through direct binding to the tumor suppressor p53, it is also capable of inducing apoptosis through a mechanism that is independent of p53 (2, 3, 8, 10).

Though mouse models to study ZBP-89 function have been generated, none has been sufficient to analyze the ZBP-89 null phenotype. Heterozygosity for a null ZBP-89 allele in embryonic stem (ES) cells resulted in 50% lower transcription of this factor though translation of the full-length protein remained (16). Male chimeras produced from these ES cells were infertile due to the haploinsufficiency of ZBP-89 and subsequent failure of p53 phosphorylation in male germ cells (16). This dramatic phenotype suggests two functional alleles of ZBP-89 are necessary for germ cell development and thus fertility. Alternatively, knock-in mice lacking amino terminal residues 1-127 of the full-length protein are fertile (7). However, animals homozygous for this truncated protein experience growth delay, reduced viability, and increased susceptibility to dextran sodium sulfate (DSS) induced colitis (7). A ZBP-89 knock-down using morpholinos has been successfully generated in zebrafish, where it disrupts both primitive and definitive hematopoiesis resulting in a

bloodless phenotype (9). Taken together these findings suggest a high improbability of generating a viable whole-body knock-out of ZBP-89 in the mouse. To explore the many roles of this diverse factor, we generated a tissue specific knock-out of ZBP-89 in mice.

Materials and Methods

Targeting Vector

We constructed vector pLM10-iKO-FinalTV to replace ZBP-89 exons 8 and 9 with a loxP site by homologous recombination. High fidelity long range PCR was used to amplify the targeting arms from mouse BAC clone pBmZBP-89. The left targeting arm consisted of the distal 3.1 kb of intron 7. The right targeting arm encompassed 5.6 kb of the 3' untranslated region. A neomycin resistance cassette driven by the phosphotransferase gene (PGK) promoter is in the reverse orientation and flanked by FRT sites. This cassette allows selection of ES cells that have successfully integrated the targeting vector. A single loxP site was placed in intron 7 upstream of the neo cassette while a second loxP site was placed downstream of the termination signal in exon 9.

Generation of ZBP-89Neo/+ ES cells and chimeric mice

The targeting vector was linearized and electroporated into Bruce4 (C57BL/6 derived) ES cells. After electroporation, the cells were plated onto MEF feeder cells at a concentration of 1.0×10^6 cells/plate in culture media supplemented with G418. Media was changed every 24 hours until drug resistant colonies were apparent one week after electroporation. ES cell clones were screened by PCR for the presence of the targeting vector. Five clones were expanded in culture and underwent

analysis for cell morphology, karyotyping, and a mycoplasma screen. Four of the expanded clones were deemed suitable for injection. Chimeras were produced by microinjecting ES cells into blastocysts obtained from mating C57BL/6^{c2J} females with C57BL/6^{c2J} males. Male chimeras were then crossed to C57BL/6^{c2J} females to generate mice with germline transmission of the targeted allele. Throughout the study animals were maintained on a C57BL/6 background. All animals were housed in microisolater polycarbonate cages in non-barrier mouse rooms (conventional housing). The procedures used in this study were approved by the University of Michigan Animal Care and Use Committee, which maintains an American Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC) facility.

Genotyping

Genomic PCR was utilized to genotype targeted ES cell clones, chimeric founders, and progeny resulting after germline transmission. The Expand Long Template PCR System (Roche Indianapolis, IN) was used according to the manufacturer's instructions. Primer sequences (5' to 3') were ZBP89-5124F: AGA CCT ACG ACC CAC AGG GTG G; ZBP89-7050R: GGC TTC TCT CCA CTG TGA GTT. The wild-type allele generates a 596 bp fragment whereas the targeted allele corresponds to a 1926 bp fragment. Upon removal of the neo cassette the targeted allele corresponds to a 674 bp fragment using the same primers.

Flpe Mice

Flpe deleter strain mice use human beta actin (ACTB) to drive Flpe recombinase expression (12). Expression of this recombinase results in complete

germline excision of DNA located between FRT sites. Flpe mice were a gift from the University of Michigan Transgenic Animal Model Core who originally obtained them from The Jackson Laboratory (Bar Harbor, ME) and back-crossed them for 10 generations to C57BL/6.

Histological analysis

Adult mouse kidney and intestine were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. Sections (4 μM) were stained with hematoxylin and eosin (H&E). Sections were also immunostained with polyclonal ZBP-89 antibody (17). Antigen-antibody complexes were detected using the ABC Vectastain Kit and diaminobenzidine substrate according to the manufacturer's suggestions (Vector Labs, Burlingame, CA).

Quantitative RT-PCR

Total cellular RNA was isolated from mouse intestine, kidney, liver, lung, spleen, and stomach using TRIzol Reagent (Invitrogen, Carlsbad, CA). The samples were treated with DNase1 (Invitrogen) before cDNA synthesis using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). RT-PCR was performed using Platinum Taq DNA Polymerase (Invitrogen). Mouse ZBP-89 primer sequences (5' to 3') included Ex6-F: GGC ATG TCT TCA TTC ATA GAG G; Ex8-R: CTC ATA CCA CAT TCA TCA CAG C. HPRT primer sequences were as follows (5' to 3') HPRT forward: AGT CCC AGC GTC GTG ATT AGC; HPRT reverse: ATA GCC CCC CTT GAG CAC ACA G. Five samples from each genotype were analyzed in triplicate. RT-PCR products were purified and submitted for DNA sequence analysis (University of Michigan DNA Sequencing Core).

Western Blot Analysis

Whole cell protein extracts were prepared from mouse intestine, kidney, liver, spleen, and stomach using T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL), supplemented with Complete Mini Protease Inhibitors (Roche, Indianapolis, IN) according to the manufacturer's recommendations. Lysates were solubilized in Laemmli sample buffer (Bio-Rad Laboratories) at 95 C for 5 minutes, resolved by SDS-PAGE, and transferred to polyvinylidene diflouride (PVDF) membrane (Bio-Rad Laboratories). Blot was then blocked using Detector Block (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and probed using rabbit ZBP-89 antibody as previously described or mouse GAPDH antibody (Chemicon, Temecula, CA)(2, 10).

Results

Generation of mice

Since the ZBP-89 locus spans greater than 110 kb in the mouse, deleting the entire locus is not feasible. However, since exons 8 and 9 encode greater than 60% of the coding sequence, this region was targeted for conditional deletion. The left targeting arm contained 3.1 kb of intron 7 whereas the right targeting arm contained 5.6 kb of the 3' UTR. A PGK-neo cassette flanked by FRT sites was placed in intron 7 for selection of the ES cells. A loxP site was placed upstream of the neo cassette and a second site was added downstream of exon 9 to allow for conditional deletion (Figure 2-1A). The targeting vector was electroporated into Bruce4 ES cells

followed by G418 treatment to select for cells heterozygous for the targeting vector. Homologous recombination occurred in 18 of 480 ES clones yielding a success rate of 3.75%. After karyotyping analysis of the ES cells, 4 clones were deemed suitable for injection into blastocysts. Twenty-four chimeras were generated (16 males and 8 females) from a total of 431 injected blastocysts. Only the chimeras from ES cell clone 6073 showed germline transmission. Chimeras from the remaining 3 clones failed to transmit the targeted allele after 10 rounds of harem (one male chimera per 2 females) matings (Figure 2-1B). Germline transmission was obtained in the first lineage in 3 of 8 chimeras from clone 6073. Genotyping using PCR confirmed heterozygosity for the targeted allele, designated ZBP-89 $^{Neo/+}$ (Figure 2-1C).

Identification of animals homozygous for the targeted allele

Heterozygous animals were bred in harem matings to generate homozygous animals. A total of ten breeding cages were set up initially, however only 3 litters resulted during a 2-month period. Breeding success was achieved by switching the animals to a high fat diet, providing nestlets in the cage at all times, commencing breeding when animals were between 6 and 8 weeks of age and allowing the male to be alone in the cage for 2 days prior to the addition of females. These techniques greatly increased the success rate of mating as measured by the presence of a female vaginal plug. In addition any new litters remained undisturbed in every way for the first 4 days after birth. This helped to ensure new mothers would care for their pups and increased our numbers of weanlings. Out of 52 pups 17 wild-type and 35 heterozygous animals were obtained (Figure 2-2A). Not a single homozygous ZBP-89$^{Neo/Neo}$ animal was born in 9 litters. Timed pregnancies showed

viable mice up to embryonic day E17.5 suggesting that lethality occurred at the end stages of pregnancy.

ZBP-89$^{Neo/+}$ mice show no significant differences in growth or survival

The lack of ZBP-89$^{Neo/Neo}$ animals suggested that the targeted allele was unstable. Therefore analysis of the targeted locus was limited to wild-type and ZBP-89$^{Neo/+}$ mice. Analysis of ZBP-89$^{Neo/+}$ animals showed no significant differences in survival compared to wild-type littermates at one year of age (Figure2-2C). Also at one year of age, mice heterozygous for the targeted allele showed a trend towards a higher body weight compared to wild-type littermates (Figure 2-2B).

Reduced levels of ZBP-89 mRNA and Protein

Quantitative real-time PCR (QRT-PCR) analysis was performed to determine if ZBP-89 mRNA levels were altered in ZBP-89$^{Neo/+}$ mice. Total RNA was isolated from mouse intestine and kidney of both ZBP-89$^{Neo/+}$ and wild-type littermate controls. QRT-PCR using a forward primer located in exon 6 and reverse primer in Exon 8 showed that ZBP-89 message levels were reduced in mice heterozygous for the targeted allele (Figure 2-3A). Likewise ZBP-89 protein levels were reduced in the intestine and kidney (Figure 2-3B). Reduced protein expression was also seen in liver, lung, and stomach tissues (data not shown). Staining of the mouse tissues for ZBP-89 further demonstrated reduced levels of ZBP-89 (data not shown). Despite the fact that no part of the ZBP-89 gene has been deleted in these animals, the targeted locus was hypomorphic.

Neomycin cassette removal generates viable targeted mice

The neomycin resistance cassette was the most likely reason for the hypomorphic ZBP-89 locus. Fortunately, the targeting vector was designed to allow removal of this cassette in the event normal gene function was disturbed in the animals. The FRT sites flanking the neomycin cassette recombine in the presence of FLP recombinase, effectively removing the now superfluous neomycin cassette (Figure 2-4A). Flpe mice ubiquitously express FLP recombinase under control of the beta-actin promoter; therefore every tissue is capable of FLP mediated recombination. These animals were bred to ZBP-89$^{Neo/+}$ mice and the resulting pups were screened by PCR for the presence of the recombined allele (Figure 2-4B). This new locus was referred to as ZBP-89 FLP to distinguish from the ZBP-89 locus with the neo cassette intact. As expected, removal of the neomycin cassette allowed for generation of viable ZBP-89$^{FLP/FLP}$ mice at near Mendelian ratios. Growth analysis of ZBP-89 FLP mice revealed no significant differences when compared to wild-type littermates between weaning and 12 weeks-of-age (Figure 2-4C).

No evidence of hypomorphic allele in ZBP-89 FLP mice

Viable ZBP-89$^{FLP/FLP}$ mice were a strong indication that ZBP-89 expression levels were at least partially restored compared to the ZBP-89 Neo mice. To test this hypothesis, RT-PCR was performed on tissue samples from both ZBP-89$^{FLP/+}$ and ZBP-89$^{FLP/FLP}$ mice. mRNA levels proved unchanged compared to wild-type mice in both the intestine and kidney (Figure 2-5A). In addition levels in other organs (stomach, liver, and lung) were the same in all three genotypes (data not

shown). These results correlated with similar levels of protein expression in these organs as demonstrated by immunoblot (Figure 2-5B). This suggested that the targeted locus is no longer significantly disrupted despite the presence of loxP and FRT sites in intron 7.

Discussion

Previous studies have failed to knock-out ZBP-89 despite rational targeting strategies. Merchant and coworkers attempted targeted deletion of the ZBP-89 locus before by targeting the translational start site in exon 4, theorizing that this would block translation of ZBP-89 mRNA (7). Instead, however, alternative splicing and usage of an alternate translational start site resulted in a protein lacking the amino-terminal 127 amino acids (refer to appendix)(7). The lack of a lethal phenotype suggested that amino-terminal deletion is compatible with survival. Work by Takeuchi *et al.* targeted exon 9 for deletion (16). However, chimeras mosaic for a heterozygous deletion were infertile. This is a logical targeting site given this exon encodes roughly 60% of the overall protein, and therefore their removal was at best likely to encode a truncated protein with limited capabilities. Indeed given the extreme phenotype exhibited by the chimeras, targeting this portion of the locus is effective. However, it is important to note in these chimeras that the deletion was accomplished through insertion of a PGK neo cassette into exon 9. It is plausible that their knock-out would have a different phenotype if the deletion was accomplished without using this cassette.

Anticipating from these studies that a complete null of ZBP-89 would likely be an embryonic lethal, I generated a targeted ZBP-89 locus so as to perform tissue

specific deletion of the locus in order to spawn viable mice. Conditional gene inactivation can be achieved through the use of site specific recombinases, such as the yeast FLP recombinase or the bacteriophage P1 Cre recombinase (11, 13). Addition of a neomycin resistance cassette was chosen to select ES cells that successfully integrated the targeting vector. This selection cassette was flanked by FRT sites allowing for excision of the neomycin cassette either in the ES cells or in the chimeras in the presence of FLP recombinase. LoxP sites were added to allow for excision of both exons 8 and 9 as well as the neomycin cassette in the presence of the CRE recombinase. The neomycin cassette and upstream loxP site were placed in intron 7, as this intronic location would conserve the structure and function of the normal protein. Unfortunately this placement proved problematic and the PGK promoter of the neo cassette interfered with normal gene transcription resulting in a hypomorphic phenotype. To this end ZBP-89$^{neo/neo}$ mice were most likely embryonic lethal due to insufficient gene expression. This phenomenon has been shown in other studies where a PGK-neo cassette in the opposite orientation relative to the modified gene has been placed in an intron (4-6, 15). Unlike ZBP-89$^{neo/neo}$ mice who die near the end of gestation, mice containing a targeted β-globin LCR experience lethality during the yolk sac stage (4). These results emphasize that when homologous recombinase is utilized to target a gene locus, the inserted selectable marker should be removed to avoid influencing the resulting phenotype.

While mice heterozygous for the targeted allele also exhibited a hypomorphic phenotype, the remaining wild-type allele compensated for the reduction in gene transcription and expression thereby preventing a lethal phenotype. Furthermore

there was no alteration in growth or survival of the heterozygous mice. It can be concluded that a slight reduction in ZBP-89 gene expression in multiple organs does not have serious consequences. However, a severe reduction results in perinatal lethality. These results coincide with those shown by Takeuchi and colleagues as well as the theory that a whole body knock-out would be embryonic lethal.

Generation of a hypomorph presents a serious problem in generating true ZBP-89 knock-out animals that are both viable and whose phenotype reflects deletion of the gene not attributable to insertion of the neomycin cassette. Furthermore the lethality of animals homozygous for the neomycin cassette containing allele prevents generation of viable animals homozygous for a CRE recombinase mediated deletion of ZBP-89. Therefore it was necessary to cross targeted mice to FLPe mice, since the expression of FLP recombinase will initiate recombination between the FRT sites consequently eradicating the neomycin cassette from the targeted locus. While this step could have been performed at the ES cell stage by treating the cells with FLP recombinase in the culture media, this is not an efficient process and the prolonged culture time reduces the ES cell pluripotency (T. Saunders, personal communication)(14).

After two consecutive rounds of breeding animals homozygous for the targeted locus with the neomycin cassette removed ($89^{FLP/FLP}$) were successfully obtained. Moreover animals of the three possible genotypes (wild-type, $89^{FLP/+}$, $89^{FLP/FLP}$) were obtained at near Mendelian ratios. Analysis of both mRNA and protein levels demonstrated no evidence of a hypomorphic ZBP-89 allele. There was also no alteration in the growth rate or survival of these animals compared to

wild-type littermates. This evidence confirms the reduced ZBP-89 expression in $89^{neo/+}$ mice was the result of the PGK-neomycin cassette insertion in intron 7. The mice now contain a fully functional ZBP-89 locus ready for tissue specific deletion in the next phase.

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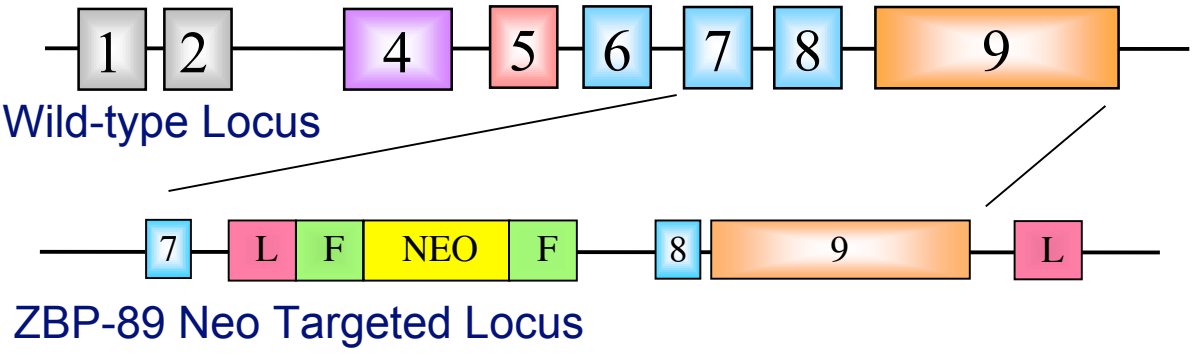
A.



B.

Clone Number	% Euploid ES Cells	Blastocysts Injected	Chimeras Produced	Germline Transmission
6071	87	108	3 Males 3 Females	No
6072	85	93	4 Males	No
6073	70	117	8 Males 4 Females	Yes
6075	80	113	1 Male 1 Female	No

C.

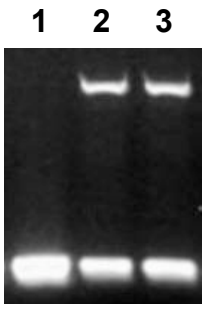
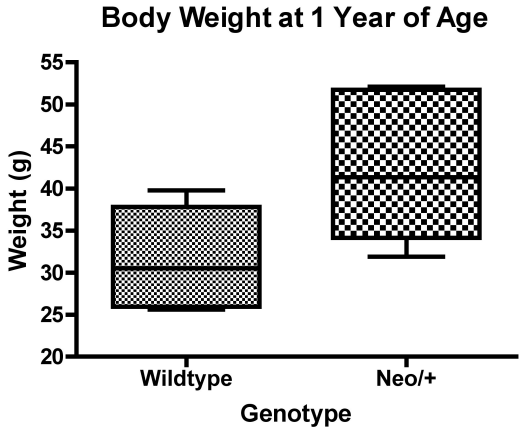


Figure 2-1. Targeting the ZBP-89 locus for conditional deletion. **A.** A loxP site (L) along with a neomycin resistance cassette (NEO) flanked by FRT sites (F) was inserted into intron 7 along with a second loxP site downstream of exon 9 of the ZBP-89 gene. **B.** Four separate ES cell clones were injected into blastocysts to produce chimeras. **C.** Genotyping demonstrated the presence of the targeted allele, wild- type allele is 596 bp where as the targeted allele is 1926 bp. Lane 1 = wild-type, 2 = Chimera, 3 = F1 progeny demonstrating germline transmission.

A.

Genotype	Estimated %	# Obtained	Actual %
WT	25	17	33
ZBP-89 $^{Neo/+}$	50	35	67
ZBP-89 $^{Neo/Neo}$	25	0	0

B.



C.

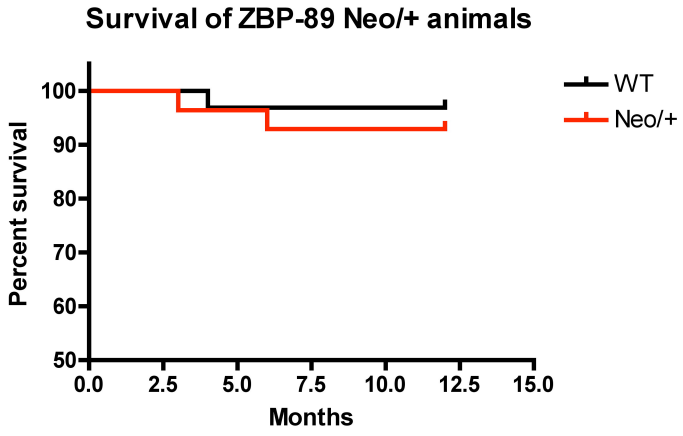
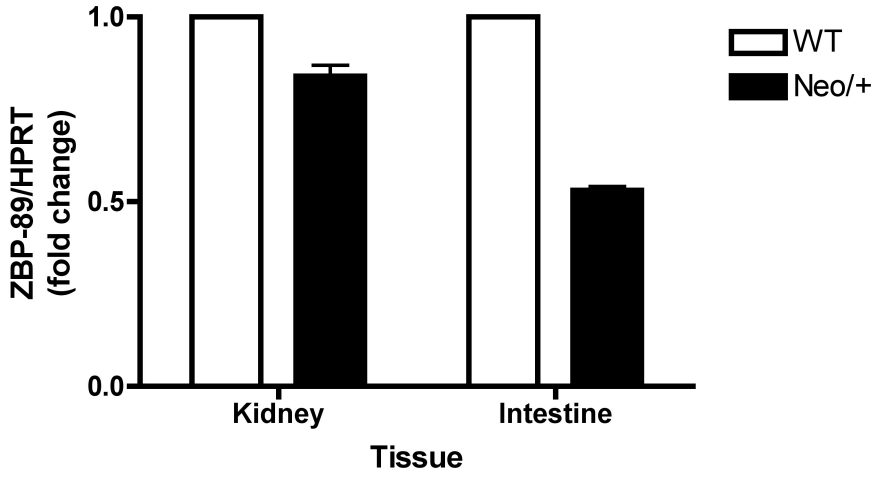


Figure 2-2. ZBP-89$^{Neo/Neo}$ mice are not viable, ZBP-89$^{Neo/+}$ mice show normal growth and survival. **A.** Three possible genotypes from ZBP-89$^{Neo/+}$ matings. Only two expected genotypes present after 9 litters. **B.** At one year of age ZBP-89$^{Neo/+}$ animals demonstrate higher body mass than their wild-type littermates. **C.** ZBP-89$^{Neo/+}$ animals exhibit no significant differences in survival compared to their wild-type littermates.

A.



B.

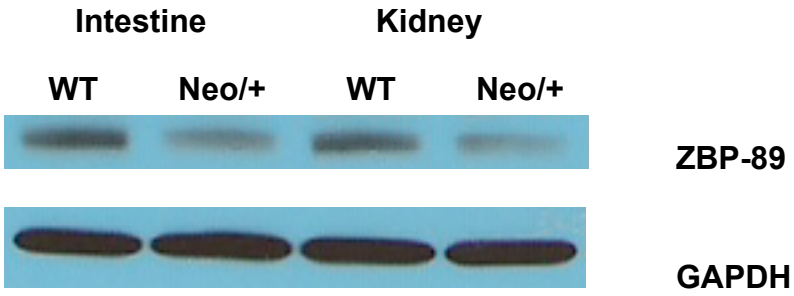
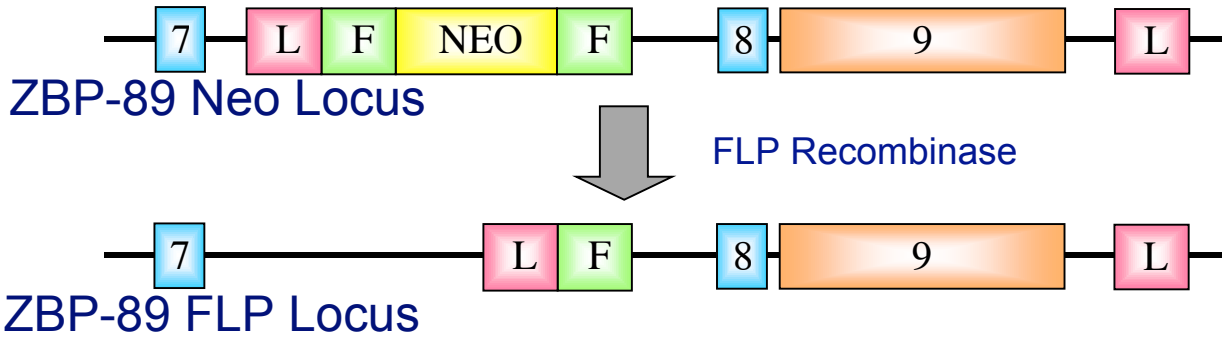


Figure 2-3. ZBP-89$^{Neo/+}$ mice show reduced mRNA and protein expression. **A.** QRT-PCR analysis of wild-type (WT) and ZBP-89$^{Neo/+}$ (Neo/+) mouse kidney and intestine tissue. Five mice per genotype were performed in triplicate for each tissue. **B.** ZBP-89 protein expression in mouse tissue.

A.



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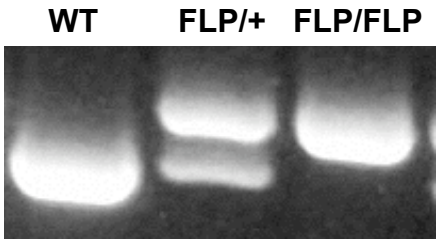


C.

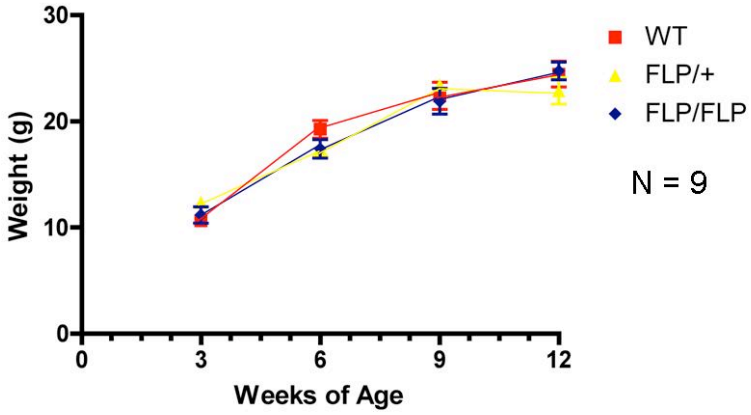
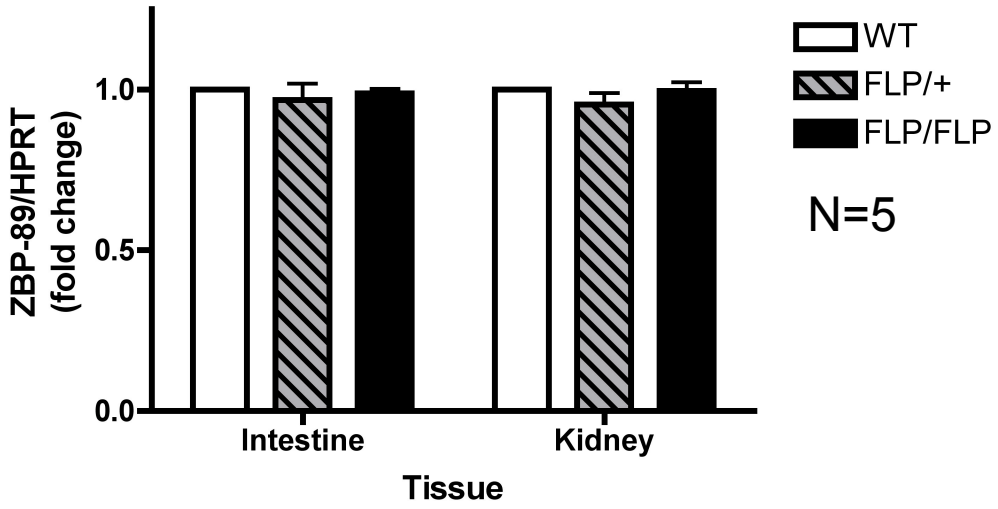


Figure 2-4. FLP recombinase mediated removal of neomycin cassette results in viable homozygous targeted mice. **A.** FRT sites (F) flank the neomycin resistance cassette (NEO), recombination occurs between the sites in the presence of FLP recombinase resulting in removal of the cassette. **B.** Genotyping demonstrating presence of FLP allele, wild-type (WT) allele is 596 bp, FLP allele is 674 bp. **C.** Growth chart displaying weight (g) of mice from weaning through 12 weeks of age. Each data point represents average weight from 12 mice.

A.



B.

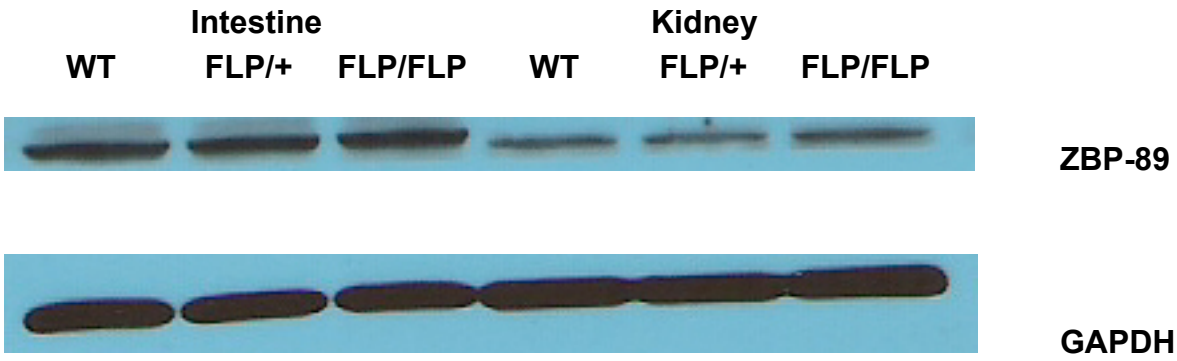


Figure 2-5. FLP recombinase restores ZBP-89 mRNA and protein expression levels. A. QRTPCR analysis of wild-type (WT), ZBP-89 FLP/+ and FLP/FLP mouse intestine and kidney tissue. B. Immunoblots for ZBP-89 and GAPDH of protein isolated from the intestines and kidneys of wild-type (WT), ZBP-89 FLP/+ and FLP/FLP mice.

References

1. **Bai, L., C. Logsdon, and J. L. Merchant.** 2002. Regulation of epithelial cell growth by ZBP-89: potential relevance in pancreatic cancer. Int J Gastrointest Cancer **31:**79-88.
2. **Bai, L., and J. L. Merchant.** 2000. Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of p21waf1 transcription in human cells. J Biol Chem **275:**30725-33.
3. **Bai, L., and J. L. Merchant.** 2001. ZBP-89 promotes growth arrest through stabilization of p53. Mol Cell Biol **21:**4670-83.
4. **Fiering, S., E. Epner, K. Robinson, Y. Zhuang, A. Telling, M. Hu, D. I. Martin, T. Enver, T. J. Ley, and M. Groudine.** 1995. Targeted deletion of 5'HS2 of the murine beta-globin LCR reveals that it is not essential for proper regulation of the beta-globin locus. Genes Dev **9:**2203-13.
5. **Fiering, S., C. G. Kim, E. M. Epner, and M. Groudine.** 1993. An "in-out" strategy using gene targeting and FLP recombinase for the functional dissection of complex DNA regulatory elements: analysis of the beta-globin locus control region. Proc Natl Acad Sci U S A **90:**8469-73.
6. **Kim, C. G., E. M. Epner, W. C. Forrester, and M. Groudine.** 1992. Inactivation of the human beta-globin gene by targeted insertion into the beta-globin locus control region. Genes Dev **6:**928-38.
7. **Law, D. J., E. M. Labut, R. D. Adams, and J. L. Merchant.** 2006. An isoform of ZBP-89 predisposes the colon to colitis. Nucleic Acids Res **34:**1342-50.
8. **Law, D. J., S. A. Tarle, and J. L. Merchant.** 1998. The human ZBP-89 homolog, located at chromosome 3q21, represses gastrin gene expression. Mamm Genome **9:**165-7.
9. **Li, X., J. W. Xiong, C. S. Shelley, H. Park, and M. A. Arnaout.** 2006. The transcription factor ZBP-89 controls generation of the hematopoietic lineage in zebrafish and mouse embryonic stem cells. Development **133:**3641-50.
10. **Merchant, J. L., G. R. Iyer, B. R. Taylor, J. R. Kitchen, E. R. Mortensen, Z. Wang, R. J. Flintoft, J. B. Michel, and R. Bassel-Duby.** 1996. ZBP-89, a Kruppel-like zinc finger protein, inhibits epidermal growth factor induction of the gastrin promoter. Mol Cell Biol **16:**6644-53.
11. **O'Gorman, S., D. T. Fox, and G. M. Wahl.** 1991. Recombinase-mediated gene activation and site-specific integration in mammalian cells. Science **251:**1351-5.
12. **Rodriguez, C. I., F. Buchholz, J. Galloway, R. Sequerra, J. Kasper, R. Ayala, A. F. Stewart, and S. M. Dymecki.** 2000. High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat Genet **25:**139-40.
13. **Sauer, B., and N. Henderson.** 1990. Targeted insertion of exogenous DNA into the eukaryotic genome by the Cre recombinase. New Biol **2:**441-9.
14. **Schaft, J., R. Ashery-Padan, F. van der Hoeven, P. Gruss, and A. F. Stewart.** 2001. Efficient FLP recombination in mouse ES cells and oocytes. Genesis **31:**6-10.
15. **Shivdasani, R. A., Y. Fujiwara, M. A. McDevitt, and S. H. Orkin.** 1997. A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. Embo J **16:**3965-73.

16. **Takeuchi, A., Y. Mishina, O. Miyaishi, E. Kojima, T. Hasegawa, and K. Isobe.** 2003. Heterozygosity with respect to Zfp148 causes complete loss of fetal germ cells during mouse embryogenesis. Nat Genet **33:**172-6.
17. **Taniuchi, T., E. R. Mortensen, A. Ferguson, J. Greenson, and J. L. Merchant.** 1997. Overexpression of ZBP-89, a zinc finger DNA binding protein, in gastric cancer. Biochem Biophys Res Commun **233:**154-60.
18. **Wieczorek, E., Z. Lin, E. B. Perkins, D. J. Law, J. L. Merchant, and Z. E. Zehner.** 2000. The zinc finger repressor, ZBP-89, binds to the silencer element of the human vimentin gene and complexes with the transcriptional activator, Sp1. J Biol Chem **275:**12879-88.