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PROSPECTS

Scaffold Attachment Factors SAFB1 and SAFB2: Innocent Bystanders or Critical Players in Breast Tumorigenesis?

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Abstract Scaffold attachment factor B1 (SAFB1) and SAFB2 are large, multifunctional proteins that have been implicated in numerous cellular processes including chromatin organization, transcriptional regulation, RNA splicing, and stress response. While the two homologous proteins show high similarity, and functional domains are highly conserved, evidence suggests that they also have unique properties. For example, SAFB2 can be found in both the nucleus and cytoplasm, whereas SAFB1 seems to be mainly localized in the nucleus. In breast cancer cells, SAFBs function as estrogen receptor corepressors and growth inhibitors. SAFB protein expression is lost in approximately 20% of breast cancers. Interestingly, the two genes reside in close proximity, oriented head-to-head, on chromosome 19p13, a locus which is frequently lost in clinical breast cancer specimens. Furthermore, SAFB1 mutations have been identified in breast tumors that were not present in adjacent normal tissue. The possibility that *SAFB1* and *SAFB2* are novel breast tumor suppressor genes, and how they might function in this role, are discussed. J. Cell. Biochem. 90: 653–661, 2003.

Key words: scaffold attachment factor; breast cancer; tumor suppressor gene; ERα corepressor

SCAFFOLD ATTACHMENT FACTOR B1 (SAFB1) AND SAFB2—INTRODUCTION AND HISTORICAL PERSPECTIVE

We originally cloned HET (SAFB1) as a protein binding to an estrogen response element (ERE) flanking a TATA box in the promoter of the heat shock protein hsp27 (HET = Hsp27 ERE TATA) [Oesterreich et al., 1997]. Renz and Fackelmayer [1996] cloned the same protein based on its ability to bind to matrix/scaffold attachment regions (S/MAR) and termed it SAFB. Subsequently, Weighardt et al. [1999] identified SAFB/HET in a yeast two hybrid screen using hnRNP A1 as bait. They introduced the terminology "HAP" (hnRNP A1 associated protein). HET, SAFB, and HAP are identical.

More recently we have discovered that SAFB exists in a protein family—two proteins which share 75% similarity at the amino acid level are encoded by two separate genes [Townson et al., 2003]. We have proposed that the two proteins should be named SAFB1 (the original SAFB) and SAFB2. This nomenclature has recently been approved by the Human Genome Organization (HUGO) Gene Nomenclature Committee.

SAFBS AS MULTIFUNCTIONAL PROTEINS

Functional Domains

SAFB1 and SAFB2 are large proteins (130 kDa) with a number of putative functional domains which are highly conserved between the two proteins (schematically illustrated in Fig. 1). The N-terminus contains a SAF-Box [Kipp et al., 2000] (also called SAP domain), which is a homeodomain-like DNA-binding motif that interacts with S/MAR, and is often found in proteins which are involved in chromatin organization and apoptosis [Aravind and Koonin, 2000]. The central region harbors an

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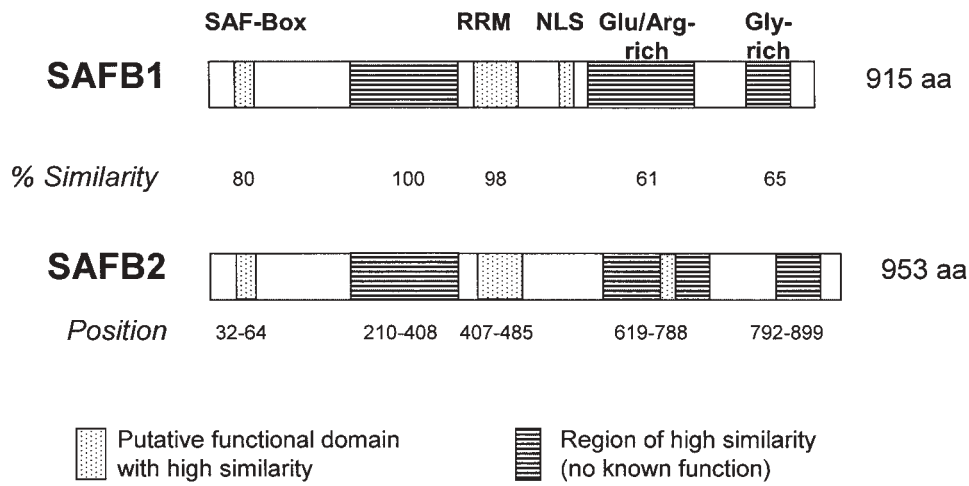

Fig. 1. *SAFB1* and *SAFB2* are homologous genes with a number of functional domains. Putative functional domains including a scaffold attachment factor-box (SAF-box), an RNA recognition motif (RRM), and a nuclear localization signal (NLS) are indicated. The C-termini contain Glu/Arg- and Gly-repeat regions of yet to be determined functions. The numbers between the SAFB1 and SAFB2 diagrams indicate percentage similarity at the amino acid level.

RNA recognition motif (RRM), and a nuclear localization signals (NLS). The C-termini of SAFB1 and SAFB2 contain Glu/Arg- and Gly-rich regions, which are likely to be involved in protein–protein interactions.

As suggested by the existence of multiple independent functional domains, the SAFB proteins have been implicated in a wide variety of cellular processes (Fig. 2). Due to its identification at an earlier time point, more is known about SAFB1, however functions associated with the conserved domains are likely to be shared by SAFB2 as well. Each of these functions will now be discussed.

Chromatin Organization and S/MAR Binding

Using biochemical fractionation experiments, we and others have shown that SAFB1 is a nuclear protein which copurifies with chromatin [Renz and Fackelmayer, 1996] and nuclear matrix protein fractions [Oesterreich et al., 1997]. Indeed, it binds to S/MARs which are AT-rich regions involved in providing the basis for a higher-order nuclear architecture. Although the concept of a "nuclear matrix" has been much debated and is still controversial, it is generally accepted that S/MARs can function as architectural elements which help to partition chromatin into distinct and topologically independent loops [Bode et al., 1996]. Thus, the SAFB proteins may regulate chromatin organization though S/MAR binding. Interestingly, S/MARs have also been implicated in the regulation of gene expression. We have found that SAFB can regulate gene expression, and this will be discussed in the next section.

Transcriptional Regulation

We initially identified SAFB1 by screening an expression library with an hsp27 promoter probe, and subsequently showed that it functions as a repressor of the hsp27 promoter. Consistent with SAFB1's ability to regulate transcriptional activity of the hsp27 promoter, we have also shown that SAFB1 can regulate ERα transcriptional activity [Oesterreich et al., 2000]. Importantly, we have found that SAFBs can repress ERα-mediated transcriptional activation, and that this is mediated via an intrinsic C-terminal transcriptional repression domain [Townson et al., submitted] (discussed in detail in SAFBs repress ERα activity).

Fig. 2. SAFB1 and SAFB2 are multifunctional proteins. Nuclear (SAFB1 and SAFB2) and cytoplasmic (SAFB2) processes in which SAFB proteins are proposed to play a role are indicated in the dark gray squares. The light gray ovals indicate the specific characteristics of SAFB1 and SAFB2 involved in the respective cellular functions. While a few of these SAFB functions have been analyzed in detail, many are still speculative, as indicated by the question marks.

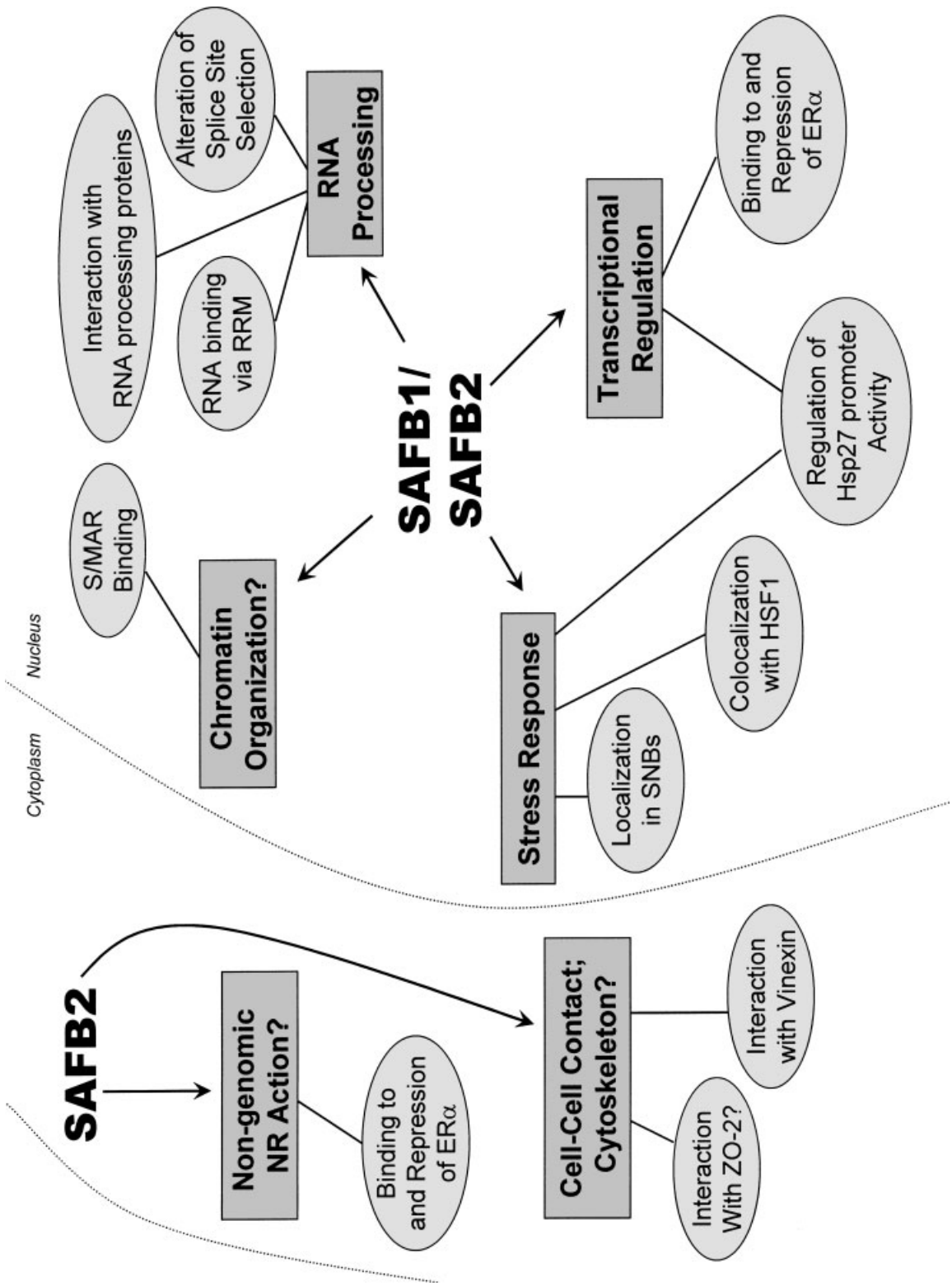
Cytoplasm
Nucleus
SAFB2
Non-genomic NR Action?
Binding to and Repression of ERα
Cell-Cell Contact; Cytoskeleton?
Interaction With ZO-2?
Interaction with Vinexin
SAFB1/ SAFB2
Chromatin Organization?
S/MAR Binding
RNA Processing
Interaction with RNA processing proteins
Alteration of Splice Site Selection
RNA binding via RRM
Stress Response
Localization in SNBs
Colocalization with HSF1
Transcriptional Regulation
Binding to and Repression of ERα
Regulation of Hsp27 promoter Activity

Fig. 2.

RNA Metabolism

SAFB1 has been shown to interact both with RNA processing proteins [Weighardt et al., 1999; Arao et al., 2000] and with the C-terminal domain of RNA polymerase II [Nayler et al., 1998], and thus has been suggested to be part of a "transcriptosome" complex, bringing transcription and RNA processing together. We hypothesized that SAFB1-mediated repression of ERα would involve its RNA-binding properties. This idea was based on the identification of a number of ER cofactors that were involved in various aspects of RNA metabolism, e.g. RTA (repressor of tamoxifen transcriptional activity) [Norris et al., 2002], and the RNA helicase DP97 [Rajendran et al., 2003]. While we were able to show that the putative RRM in SAFB1 can bind RNA, it was not required for SAFB1's function as an ERα corepressor in transient reporter assays [Townson et al., submitted]. Although more experiments are needed, our data suggest that there is a separation between SAFB1's function in some aspects of RNA metabolism and its role in transcriptional regulation.

Stress Response

We originally identified SAFB1 as a protein binding to and regulating activity of a heat shock gene promoter. Not surprisingly, there is now more evidence that SAFB1 indeed plays a role in the cellular stress response. Upon stress treatment, SAFB1 relocates into nuclear speckles termed "stress-induced SNBs" (previously also called hnRNP A1-associated protein (HAP) bodies). SNBs are sam68 nuclear bodies which are distinct from other nuclear structures such as coiled bodies, gems, and promyelocytic nuclear bodies [Chen et al., 1999; Chiodi et al., 2000]. A colocalization of SAFB1 and sam68 (and YT521-B) was originally described by Stamm's group [Hartmann et al., 1999]. Subsequently, Biamonti's group showed that SAFB1 and sam68 localization in both SNBs and stress-induced SNBs temporally coincides [Denegri et al., 2001], suggesting a close relationship between these two subnuclear structures. SNBs contain both proteins and nucleic acids, but are most likely not sites of transcription. It has been suggested that these nuclear bodies present central depots for controlling posttranscriptional modification of RNA and for regulating protein activity [Denegri et al., 2001]. A candidate protein for this regulation is the heat shock factor HSF1 which has been shown to colocalize with SAFB1 in stress-induced SNBs [Weighardt et al., 1999]. Interestingly, in cell lines, the formation of SNBs has been correlated with the rate of neoplastic transformation [Chen et al., 1999]. It is not understood why SNBs do not form in normal cells, and whether SNBs are causatively involved in, or are a consequence of transformation. These are interesting questions which will have to be answered before we can fully comprehend the role of SAFB1 and SNBs in transformation.

Cytoplasmic Functions of SAFB2

The cytoplasmic localization of SAFB2 suggests a non-nuclear function, however, this is yet to be determined. Our recent finding of a SAFB2-vinexin interaction [Townson et al., 2003] suggests that SAFB2 is involved in converging pathways for cell signaling and cytoskeletal organization. Vinexin is a member of the SH3 domain-containing adaptor proteins that regulate growth factor signaling, cell adhesion, and cytoskeletal organization [Kioka et al., 1999]. Alternatively, it is possible that SAFB2 plays a role in connecting tight intercellular junctions with the cytoskeleton. This speculation arises from the recent finding by Traweger et al. [2003], who isolated a mouse clone with 95% similarity to rat SAFB in a yeast two hybrid screen using the tight junction protein ZO-2 as a bait. ZO-2 belongs to the family of *Zonula occludens* proteins which connect junctional transmembrane proteins to the cytoskeleton, and thereby play a role in the organization of intercellular tight junctions [Gonzalez-Mariscal et al., 2000]. Traweger et al. [2003] proposed that ZO-2 not only plays a role in the cytoplasm but may also have a novel role in the nucleus, potentially through its interaction with SAFB1. It is possible however, that ZO-2 also interacts with SAFB2 in the cytoplasm, a hypothesis yet to be tested.

Interestingly, ZO-2 shows homology to the lethal(1)discs-large (*dlg*) gene of *Drosophila*. While *dlg* was originally discovered as a tumor suppressor in *Drosophila*, there is recent evidence that it is also important in cancer progression in humans [Humbert et al., 2003]. A dlg-related gene, *lp-dlg* (large type of p-dlg), was recently shown to bind to vinexin at sites of cell–cell contact [Wakabayashi et al., 2003], leading to the provocative hypothesis that SAFB2 might

function as a scaffold for proteins involved in cell adhesions.

It is also speculated that SAFB2 is involved in regulating the activity of cytoplasmic ERα, similar to what has been described for the ERα cofactor MTA1s [Kumar et al., 2002].

Future Analysis of SAFB Functions

SAFB proteins have multiple functional domains and can clearly affect a diverse array of cellular functions. However, the study of SAFB functions is complicated by the presence of two homologous *SAFB* genes which may have additive, synergistic, or opposing actions. Future studies need to dissect out the individual roles of SAFB1 and SAFB2, but these experiments are inherently difficult to conduct and interpret. First, we have yet to identify a cell line model which only expresses one SAFB protein and not the other. Second, even if we could delete one gene in vivo, the other one might be upregulated by compensation. Thus, further investigations of both members of the *SAFB* gene family need to be carefully designed in order to understand the interplay between SAFB1 and SAFB2.

SAFB PROTEINS IN HUMAN BREAST CANCER

SAFBs Repress ERα Activity

ERα is a steroid receptor which dimerizes upon ligand binding, binds to so-called ERE in estrogen-responsive promoters, and activates transcription. Transcriptional regulation of target genes by ERα is a complex, multistep, and tightly regulated process. One of the major breakthroughs in understanding the regulation of ERα's activity was the discovery of the interacting coregulator proteins that can either positively (coactivators) or negatively (corepressors) modulate the activity of nuclear receptors (NR) (reviewed in [McDonnell and Norris, 2002]). We have shown that SAFB1 directly interacts with the DNA-binding/hinge region of ERα, and that SAFB1 and SAFB2 can inhibit ERα transcriptional activity. Thus, the SAFB proteins function as ERα corepressors [Oesterreich et al., 2000]. The SAFB1 and SAFB2-mediated repression is mediated via a C-terminal repression domain [Townson et al., submitted], and preliminary data suggest that the repression involves both histone deacetylase-dependent and -independent mechanisms.

Interestingly, the tumor suppressor gene *BRCA1* which causes approximately 50% of heritable breast cancers which in turn account for 5–10% of all breast cancer, functions as an ERα corepressors [Fan et al., 1999]. Data using breast cancer cell line studies and fibroblasts from BRCA1-knockout mice elegantly showed that BRCA1 is involved in the regulation of ligand-dependent and -independent activity of ERα. Importantly, in contrast to wildtype BRCA1, BRCA1 derivatives carrying familial breast cancer-derived missense mutations fail to repress ERα [Zheng et al., 2001]. Some of these mutations map to the exon 11-encoded region that binds Rad50, a domain previously shown to be involved in DNA damage repair. These data reflect the challenge to distinguish between ERα-dependent and -independent tumor suppressive functions of tumor suppressor/ERα corepressor genes.

SAFB Expression in Human Breast Cancer

There is overwhelming evidence for ERα being a central player in breast cancer development and progression [Osborne et al., 2001]. Considering that SAFB proteins function as ERα corepressors, and that they are involved in a number of other important cellular processes, we asked whether they would also play a role in breast cancer. We therefore measured SAFB1 and SAFB2 expression in human breast tumor samples.

We generated a monoclonal antibody (6F7; Upstate Biotechnology Incorporated, Lake Placid, NY) that was raised against a peptide (345–357) mapping to a region of 100% homology between SAFB1 and SAFB2. This antibody detects both proteins which run as a very close doublet on SDS–PAGE. We measured SAFB protein expression by immunoblotting in 61 primary breast tumors [Townson et al., 2000] and found that expression varied widely, with some tumors expressing high amounts, some moderate, and in 10 tumors, none at all that we could detect. While there was a trend for low SAFB-tumors to have high S-phase, it did not reach significance. However, we detected a significant association between SAFB protein levels and aneuploidy. More recently, we have measured SAFB protein levels in invasive breast tumors, and found that low levels were significantly associated with worse overall survival of the patients [Oesterreich et al., 2002]. These clinical studies are somewhat difficult to

interpret since we used the antibody which detects both SAFB proteins, and which is not suitable for immunohistochemistry. Studies are ongoing in our laboratory which aim at the independent analysis of SAFB1 and SAFB2 in clinical breast cancer specimens with clinical follow up.

Overexpression of SAFB Inhibits Breast Cancer Cell Growth

As expected of an ERα corepressor, overexpression of SAFB1 in ERα-positive breast cancer cell lines ([Townson et al., 2000] and unpublished data) resulted in inhibition of cell proliferation concomitant with a decrease of cells in S-phase. Notably, we were also able to detect growth inhibition in ERα-negative cell lines [Townson et al., 2000], suggesting that SAFB1 can inhibit cell growth independent of its repression of ERα activity. Whether this ERα-independent activity is connected to SAFB's involvement in chromatin organization, RNA processing, stress response, or other proposed functions (illustrated in Fig. 2), is an active area of investigation in our laboratory.

Inactivation of SAFB in Human Breast Tumor Specimens

Low or absent SAFB1/2 expression is likely to be a result of alterations at its chromosomal locus on 19p13.3. We have performed loss of heterozygosity (LOH) studies using DNA from microdissected invasive breast cancers, the polymorphic marker D19S216, which resides just distal to the SAFB locus, and three other nearby markers [Oesterreich et al., 2001]. Briefly, the LOH peak (78%) was detected at the marker D19S216 colocalizing with SAFB1 and SAFB2, with the other nearby markers displaying LOH frequencies ranging from 22 to 47%. There are reports which describe similar high LOH rates at the same locus in cervical cancer [Lee et al., 1998] and in certain brain metastases [Sobottka et al., 2000]. Interestingly, the LOH frequency for brain metastases differed depending on the primary tumor origin—it was high for breast and lung cancer, and low for colorectal cancer, renal cell carcinoma, and melanoma.

This rate of LOH at the 19p13 locus in breast cancer is one of the highest so far described. Since LOH is a hallmark of tumor suppressor genes, the results suggest that this region harbors a tumor suppressor gene that plays an important role in breast cancer. While the region is gene-rich, and therefore contains a number of potential candidates, we hypothesized that *SAFB1* and *SAFB2* are tumor suppressor genes in human breast cancer, and have initiated studies to prove this hypothesis.

First of all, it is important to determine whether, in LOH-positive tumors, the second allele is inactivated. While a few mutations have been identified in microdissected tumor DNA which were not found in adjacent normal tissue [Oesterreich et al., 2001], our data indicate that mutational inactivation of SAFB1/2 is infrequent. However, mutational studies are labor-intensive and expensive, and our analysis has been limited due to the large size of the genes, *SAFB1* and *SAFB2* contain 21 exons each, and span a region of approximately 45 and 36 kb, respectively. Additional studies are necessary to form solid conclusions on mutational inactivation of the two genes in clinical specimens.

Inactivation of tumor suppressor genes occurs not only through mutations but also through other mechanisms, including mislocalization, altered protein stability, and gene silencing, mostly through promoter hypermethylation. Interestingly, the *SAFB1* and *SAFB2* genes are oriented in head-to-head orientation (Fig. 3), and their expression is

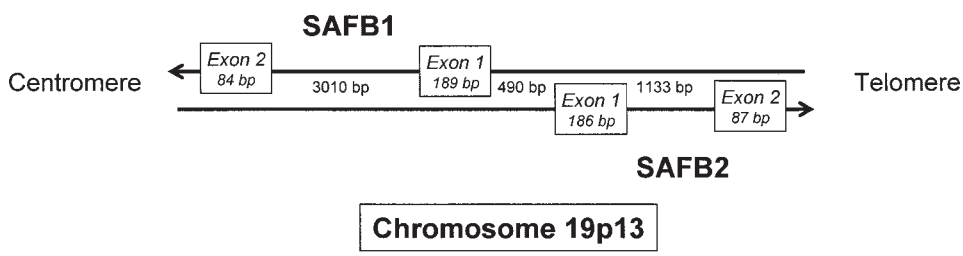

Fig. 3. Chromosomal arrangement of SAFB1 and SAFB2 on chromosome 19p13. SAFB1 and SAFB2 are arranged in head-to-head orientation, separated by a 490 bp region which can function as a bi-directional promoter.

driven by a bidirectional promoter. A recent study of genes found in such bidirectional loci showed that a common feature is the presence of CpG-rich areas [Adachi and Lieber, 2002]. Indeed, the SAFB1/2 promoter harbors a number of CpG islands which extend into the first exons of both genes. We are currently analyzing whether hypermethylation occurs at the shared SAFB1/SAFB2 promoter, which could result in concurrent inactivation of both genes.

Alternative Views

Clearly, more studies such as the analysis of SAFB1/2 in human clinical specimens and generation of knockout mouse models are necessary to prove (or disprove) the hypothesis that *SAFB1/2* are breast tumor suppressor genes. Meanwhile, alternative hypotheses should be considered, and I will briefly discuss these below.

Like all LOH events, the S216 LOH event involves more than one gene, and there may be important players other than SAFB1 and SAFB2 which drive the high rates of LOH. For instance, the putative tumor suppressor gene *LKB1*/*STK11*, a serine threonine kinase, also maps to D19S216. Germline mutations of this gene are associated with the Peutz–Jeghers syndrome (PJS), an inherited disease characterized by a predisposition to mucocutaneous pigmentations, as well as to several cancer types, including breast [Boardman et al., 2000]. However, germline mutations of this gene have not been identified in hereditary [Chen and Lindblom, 2000] or sporadic breast cancers [Bignell et al., 1998; Forster et al., 2000]. APC2, a homologue of the adenomatous polyposis coli (APC) tumor suppressor gene known to be inactivated primarily in colon cancer, also maps to chromosome 19p13.3. APC2 was recently fine-mapped to a small region containing D19S883, a locus frequently lost in ovarian cancer [Jarrett et al., 2001]. Although this region is distal to the peak of LOH in our breast cancer study, at this point we can not completely rule it out as a candidate gene.

We actually believe that regions with very high LOH rates contain a number of critical genes. Thus, just like "amplicons" which mostly harbor more than one gene giving the tumor a growth advantage, LOH regions might contain a number tumor suppressor genes which function at different steps and which affect different processes during cancer evolution.

SUMMARY AND CONCLUSIONS

SAFB1 and SAFB2 are multifunctional proteins involved in numerous cellular functions. Besides their proven (or in some instances proposed) roles in chromatin organization, stress response, RNA metabolism, and cell–cell contact, SAFB1 and SAFB2 also regulate the activity of ERα, one of the most critical players in breast cancer. They function as growth inhibitors in breast cancer cells, and their locus on chromosome 19p13 displays one of the highest LOH rates reported in clinical breast cancer. Together with clinical studies correlating SAFB1/2 with important biomarkers and survival, these data leave little doubt that the two proteins are involved in breast tumorigenesis, though on the basis of our current knowledge it is impossible to predict to what extent, and whether they indeed are breast tumor suppressor genes. The further elucidation of the role of these proteins in tumorigenesis is likely to involve manipulation via gene knockout, both in cell lines and mouse models. Efforts should also be directed at the further analysis of clinical breast cancer specimens. Foremost, we need to know how frequently and in what ways SAFB1 and/or SAFB2 are inactivated. Therefore, large scale mutation and/or SNP (single nucleotide polymorphism) studies are needed, which should be complemented with the analysis of alternative mechanisms of inactivation, such as promoter inactivation and protein mislocalization.

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