Identification of Novel Crk-associated Substrate (p130Cas) Variants with Functionally Distinct Focal Adhesion Kinase Binding Activities

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Background: The tumor promoter p130Cas utilizes its SH3 domain to control cell adhesion and migration.

Results: We identified N-terminal extended p130Cas variants with enhanced SH3 domain binding activity which modulates cell migration and invasion.

Conclusion: Naturally occurring p130Cas variants exhibit differential biological activities.

Significance: Knowledge on these variants may explain how p130Cas controls cellular programs and drives carcinogenesis.

Elevated levels of p130Cas (Crk-associated substrate)/BCAR1 (breast cancer antiestrogen resistance 1 gene) are associated with aggressiveness of breast tumors. Following phosphorylation of its substrate domain, p130Cas promotes the integration of protein complexes involved in multiple signaling pathways and mediates cell proliferation, adhesion, and migration. In addition to the known BCAR1-1A (wild-type) and 1C variants, we identified four novel BCAR1 mRNA variants, generated by alternative first exon usage (1B, 1B1, 1D, and 1E). Exons 1A and 1C encode for four amino acids (aa), whereas 1D and 1E encode for 22 aa and 1B1 encodes for 50 aa. Exon 1B is non-coding, resulting in a truncated p130Cas protein (Cas1B). BCAR1-1A, 1B1, and variant 1C mRNAs were ubiquitously expressed in cell lines and a survey of human tissues, whereas 1B, 1D, and 1E expression was more restricted. Reconstitutions of all isoforms except for 1B in p130Cas-deficient murine fibroblasts induced lamellipodia formation and membrane ruffling, which was unrelated to the substrate domain phosphorylation status. The longer isoforms exhibited increased binding to focal adhesion kinase (FAK), a molecule important for migration and adhesion. The shorter 1B isoform exhibited diminished FAK binding activity and significantly reduced migration and invasion. In contrast, the longest variant 1B1 established the most efficient FAK binding and greatly enhanced migration. Our results indicate that the p130Cas exon 1 variants display altered functional properties. The truncated variant 1B and the longer isoform 1B1 may contribute to the diverse effects of p130Cas on cell biology and therefore will be the target of future studies.
to FAK. Overexpression in a p130Cas-null background leads to altered cell morphology and differential migratory and invasive potential. Importantly, the naturally occurring truncated variant and the longest isoform exhibited greatly diminished and significantly increased functionality, respectively.

**EXPERIMENTAL PROCEDURES**

**Generation of Expression Constructs**

I.M.A.G.E. clones for human full-length p130Cas cDNAs including exon 1A (Cas1A), Cas1C, and Cas1E ID 6428300, ID 6428300, and ID 4940045, respectively, were obtained from Open Biosystems. All expression constructs for N-terminal p130Cas variants were generated by PCR using Pfu polymerase (Stratagene) and subcloned into pcDNA4/V5-HisA vector (Invitrogen).

Expression constructs for full-length p130Cas were generated by PCR and subcloned into the retroviral expression vector pCXbsr (20). Primer sequences are provided in Table 1.

**Database Analysis**

**BCARI** and the exon 1 mRNA and protein variants were analyzed using database resources and software from AceView (21), the European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute (EBI), Genomatix (22), and the National Center for Biotechnology Information (NCBI) (23). Expressed sequence tags (ESTs) and cap analysis of gene expression (CAGE) tags for each variant were identified at AceView, Genomatix (ElDorado), and NCBI (BLAST, EST, and UniGene) databases. To further support the existence of the variants, the genes, potential promoters, complements of transcripts, and proteins in different species were analyzed with software and databases at Genomatix (Gene2Promoter, MatInspector, and ElDorado) and NCBI (Nucleotide and Protein). Putative functional protein motifs in the N-terminal part of each variant up to the end of the SH3 domain were identified using the ELM server (24) and Scansite 2.0 (25).

**Tissue Samples, Cell Lines, and Culture Conditions**

**Tissue Samples**—Historical human tissue samples from normal breast, breast carcinoma, normal colon, colon carcinoma, stomach, liver, kidney, and rectum were used.

**Cell Lines**—Cell lines were purchased from the American Type Culture Collection (ATCC) or obtained as indicated. The following cell lines were used: human foreskin fibroblast, BJ1; untransformed mammary epithelium, MCF-10A; normal breast, breast carcinoma, normal colon, colon carcinoma, stomach, liver, kidney, and rectum were used.

**Cell Lines**—Cell lines were purchased from the American Type Culture Collection (ATCC) or obtained as indicated. The following cell lines were used: human foreskin fibroblast, BJ1; untransformed mammary epithelium, MCF-10A; normal breast, breast carcinoma, normal colon, colon carcinoma, stomach, liver, kidney, and rectum were used.

**Culture Conditions**—For Cas−/− cells, the culture conditions were: DMEM, 5% calf serum. For MCF-7 cells, the culture conditions were: RPMI 1640, 5% FBS, and 4 mM glutamine.

**Real Time RT-PCR**

RNA was extracted using RNeasy kit including the DNase treatment step (Qiagen), and RT-PCR was performed as described (10, 27, 28). cDNA was treated with RNase H (Life Technologies). Real time PCR was performed in 20-μl reactions in triplicate using ABI PRISM 7900HT sequence detection sys-
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Migration and Invasion Assays

Cas variant cells (migration, $7.5 \times 10^4$; invasion, $5 \times 10^4$) were layered in the upper compartments of a Transwell (migration) chamber or invasion chambers (both chambers: 6.5-mm-diameter polycarbonate filter; 8-µm pore size; Costar) and incubated at 37 °C for 6 h (migration) or 22 h (invasion). Culture medium (20% calf serum) was added to the lower compartment. Migration and invasion of the cells to the underside of the filter were evaluated by staining with crystal violet and quantified by counting the cells in three random fields per experiment. Three independent assays were performed in triplicate.

Phosphatase Treatment

Cells were washed twice with 1× PBS, lysed in PBS/0.5% SDS, and incubated at 95 °C for 10 min. Cell lysates (60 µg) were treated with 20 units of calf intestinal phosphatase (New England Biolabs) or water control in New England Biolabs buffer 3 for 1 h at 37 °C and subjected to immunoblotting as described.

RESULTS

BCAR1 Has Multiple First Exons—We recently described two BCAR1 exon 1 variants (BCAR1-1 and BCAR1-1'), which we refer to here as Cas1A and Cas1C, respectively (10). Based on these findings, we investigated whether additional p130Cas variants with different functions exist. Subsequent comprehensive database analyses revealed that BCAR1 contains five additional exons upstream of exon 1 (1A, first identified WT, RefSeq NM_014567.3) (Fig. 1). ESTs and cap analysis of gene expression tag databases suggested that these exons represent alternative first exons, hence designated by their genomic position related to 1A as exons 1B (XM_005256260.2), 1B1 (NM_001170714.1), 1C (NM_001170718), 1D (NM_001170716.1), and 1E (NM_001170715.1). Exons 1A and 1C encode for four aa, whereas 1D and 1E translate into 22 aa (Fig. 1). Exon 1B1 encodes for 50 aa. Exon 1B is non-coding, leading to a truncated form of the p130Cas protein (Cas1B) starting at aa 30 in reference to Cas1A, thereby lacking 24 aa of the SH3 domain (aa 6–64). The variant proteins 1A and 1C are evolutionarily conserved in mammals, whereas the longer variants 1B, 1D, and

Retroviral Transduction and Transient Transfection

Retroviral transduction and transient transfection were carried out as described previously (10, 11, 30).

Protein Analysis and Immunoprecipitation

Whole cell extracts were prepared and analyzed by immunoblotting as described (31). Some proteins were separated on low-bis (ratio 149:1) SDS-polyacrylamide gels and subsequently blotted as described (31). The following antibodies with different functions exist. Subsequent comprehensive database analyses revealed that BCAR1 contains five additional exons upstream of exon 1 (1A, first identified WT, RefSeq NM_014567.3) (Fig. 1). ESTs and cap analysis of gene expression tag databases suggested that these exons represent alternative first exons, hence designated by their genomic position related to 1A as exons 1B (XM_005256260.2), 1B1 (NM_001170714.1), 1C (NM_001170718), 1D (NM_001170716.1), and 1E (NM_001170715.1). Exons 1A and 1C encode for four aa, whereas 1D and 1E translate into 22 aa (Fig. 1). Exon 1B1 encodes for 50 aa. Exon 1B is non-coding, leading to a truncated form of the p130Cas protein (Cas1B) starting at aa 30 in reference to Cas1A, thereby lacking 24 aa of the SH3 domain (aa 6–64). The variant proteins 1A and 1C are evolutionarily conserved in mammals, whereas the longer variants 1B, 1D, and
1E were only identified in primates (Tables 2 and 3). The truncated 1B isoform was detected in Primates (Hominidae only) and Cetacea. Once complete genetic information of lower mammalian species or other classes is available, the variants may also be identified in additional species. Motif analysis of the NT region of p130Cas revealed that Cas1A and 1C and the novel variant proteins Cas1B1, 1D, and 1E contain a YDNV motif representing a putative phosphorylation site (Tyr-12/58/30) at aa 12–15, 58–61, and 30–33, respectively, and a SPDEL/SFR motif at aa 18–25/64–71/36–43 predicted for phosphorylation at Ser-23/69/41. Therefore, the longest variant Cas1B1 contains additional potential interaction motifs at aa 4–8, 6–13, 15–19, and 40–46. Therefore, BCAR1 has six first exons resulting in protein isoforms that possibly possess altered functions.

**BCAR1 Exon 1 Variants Are Expressed in Human Cell Lines and Tissues**—To reveal the expression pattern of the BCAR1 exon 1 variants, real time RT-PCR was performed on mRNA isolated from immortalized mammary epithelial (MCF-10A), estrogen receptor-positive (ER+) MCF-7 and T47D, ER-negative (ER-) BT-20 breast carcinoma, and human fibroblastic (BJ1) cells (Fig. 2A). The highest relative expression was detected for BCAR1-1A, 1C, and 1E in all cell lines, whereas 1B, 1B1, and 1D levels were lower. When compared with MCF-10A in T47D and BT-20 cells, significantly higher levels of 1B (4.8- and 4.3-fold, respectively), 1B1 (3.8- and 8.7-fold), 1C (2.1- and 3.2-fold), and 1E (4.4- and 2-fold) were found (Fig. 2A, right panel).

To further analyze the abundance of the isoforms, real time RT-PCR was conducted on major human organs (breast, colon, stomach, liver, kidney, and rectum) and breast carcinoma and colon carcinoma (Fig. 2B). Three samples from each tissue were investigated. As observed for the cell lines, relative expression of BCAR1-1A, 1C, and 1E was stronger when compared with 1B, 1B1, and 1D. The variants 1A, 1B1, and 1C were ubiquitously expressed. A more restricted pattern was observed for 1B (detected in only 15 out of 24 samples), 1D (17 out of 24), and 1E (18 out of 24), especially in normal colon and kidney. No trends in expression differences of the variants between normal breast and breast carcinoma were found. Interestingly, isoform 1E was not expressed in normal colon, whereas all colon carcinoma samples had high levels of 1E. These data suggest that the BCAR1 variants are differentially expressed in human tissues and cell lines.

**Reconstitution of p130Cas-deficient Fibroblasts with the p130Cas Variants Alters Cell Morphology**—The aa sequences encoded by the p130Cas exon 1 variants border the N-terminal end of the SH3 domain, which binds to several proteins including FAK (1). To compare the influence of the exon 1 variants on p130Cas phosphorylation, cell morphology, and growth pattern, p130Cas-deficient (Cas−/−) murine fibroblasts (26) were stably transduced with p130Cas variants Cas1A, 1B, 1B1, 1C, 1D, 1E, or empty vector (ev) (Fig. 3A). In addition, we transduced a Cas1Δ deletion construct starting at exon 2 while maintaining the initiating methionine. Expression and size of each variant were confirmed by Western blotting by utilizing low bis-acrylamide gels (32) (Fig. 3B). The multiple p130Cas bands are potentially related to posttranslational modifications and/or cleavage products (1).

Equal cell numbers of each transductant were seeded, and the morphology was investigated after 2 (Fig. 3C) and 4 days (Fig. 3D). Reconstitution of Cas−/− cells with each variant except for Cas1B restored a phenotype characterized by lamellipodia formation accompanied by membrane ruffling (Fig. 3C, shown for Cas1A, Cas1B1, and Cas1E). At 2 days in culture, Cas1A and Cas1E transductants exhibited a spindle-like polarized (single leading edge) morphology, whereas cells expressing the longest variant Cas1B1 displayed a rounded cell body and multiple lamellipodia. A different morphology was observed in the Cas1B transductants as most of these cells lacked lamellipodia (Fig. 3C). At 4 days, the morphology was investigated after 2 (Fig. 3C) and 4 days (Fig. 3D). Reconstitution of Cas−/− cells with each variant except for Cas1B restored a phenotype characterized by lamellipodia formation accompanied by membrane ruffling (Fig. 3C, shown for Cas1A, Cas1B1, and Cas1E). At 2 days in culture, Cas1A and Cas1E transductants exhibited a spindle-like polarized (single leading edge) morphology, whereas cells expressing the longest variant Cas1B1 displayed a rounded cell body and multiple lamellipodia. A different morphology was observed in the Cas1B transductants as most of these cells lacked lamellipodia but instead displayed spike-like protrusions (Fig. 3C). Cas1B reconstituted cells primary grew in clusters. In contrast, cells expressing Cas1A or the other variants showed cell scattering (Fig. 3D). Phosphorylation of the SD is of major importance for p130Cas-mediated cell migration (17, 33). Thus, cells expressing Cas1A, Cas1B, Cas1B1, Cas1C, and Cas1E were harvested after sodium vanadate treatment to accumulate phosphorylated proteins and analyzed by immunoblotting using a mixture of three phospho-Tyr-specific p130Cas abs (Fig. 3E). Reconstitution of Cas−/− cells with the variants did not significantly alter SD tyrosine phosphorylation when compared with WT Cas1A. This suggests that alterations in tyrosine phosphorylation on these sites do not correlate with the observed morphological changes.
p130Cas Exon 1 Variants with Extended N Termini Show More Efficient Binding to FAK—To test whether the N-terminal variations of p130Cas mediate different interactions with FAK, we generated NT expression constructs of p130Cas (Fig. 4A) and performed IP (Fig. 4B). The sequences encoding for the different exon 1 variants and the SH3 domain were subcloned in-frame with a 3’ V5 tag. In addition, we generated a NT-1 deletion construct. We could not detect NT-1B expression in these studies (not shown). Cell extracts from 293T cells transfected with NT-1A, NT-1C, or the novel variants NT-1B1, NT-1D, NT-1E, or NT-1Δ or ev control were mixed with equal amounts of cell extracts from FAK-transfected 293T cells, immunoprecipitated with V5 abs, and subjected to immunoblotting with FAK (95% of total IP) or V5 (5% of total IP) abs. Equal amounts of NT-1A and the other variants were precipitated (Fig. 4B, 5% IP). The longer variants NT-1B1, NT-1D, and NT-1E exhibited considerably more efficient binding to FAK in comparison with all the other isoforms or NT-1Δ (95% IP). Binding of NT-1D and 1E was significantly increased when compared with NT-1B1. NT-1D separated into a faster and slower migrating band (15 and 20 kDa) on an 18% acrylamide gel. Phosphatase treatment greatly reduced the 20-kDa band, indicating that this band is a phosphorylated form of NT-1D (Fig. 4C).

FIGURE 2. BCAR1 exon 1 variant mRNA expression in human cells and tissues. mRNA levels of 1A and the variants 1B, 1B1, 1C, 1D, and 1E were determined by real time RT-PCR. Shown are the results after normalization to GAPDH and SD from triplicate. #, not detected. A (left panels) and B, relative expression levels are shown. Different scales are used. // indicates that parts of the scale were omitted. A, right panel, average levels relative to the expression in MCF-10A cells (black bar, set to 1) are shown. p values were calculated using Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001 of MCF-10A versus the indicated cell line. Error bars indicate means ± S.D.
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FIGURE 3. Expression of p130Cas variants alters cell morphology. Cas−/− fibroblasts were stably transduced with Cas1A, variants 1B, 1B1, 1C, 1D, 1E, or 1Δ, or ev. Results are presented from one experiment out of three performed with similar results. A, schematic representation of the full-length p130Cas variants. B, whole cell extracts (WCE, 10 μg) were separated on low bis-acrylamide gels and subjected to immunoblotting (IB) with p130Cas and actin abs (control). C and D, transduced Cas−/− cells (10,000) were seeded in 6-well plates and analyzed by phase contrast microscopy after 2 days (20× magnification; scale bars, 50 μm) (C) and 4 days (10× magnification; scale bars, 250 μm) (D). Black arrows, lamellipodia and membrane ruffling; white arrows, spike-like protrusions. E, cells were pretreated with sodium vanadate, and cell extracts were separated on a low bis-acrylamide gel and subjected to IB with total p130Cas abs or a mixture of p130Cas-specific phospho-Tyr (phospho) abs.

FIGURE 4. p130Cas variants exhibit differential binding activity to FAK. A and D, schematic representations of the constructs: the N-terminal p130Cas variants fused to a V5 tag (A) and the full-length p130Cas variants (D). B, WCE from 293T cells transfected with the N-terminal variants or ev control were mixed with WCE from FAK-transfected 293T cells, immunoprecipitated with V5 abs and probed with FAK abs. C, WCE (60 μg) from 293T cells transfected with p130Cas−/− NT-1D or ev control were treated with calf intestinal phosphatase or control-treated and subjected to IB analysis with V5 abs. Results are presented from one representative experiment out of two performed. WB, Western blot. E, WCE from Cas−/− transductants expressing the full-length variants were mixed with WCE from FAK-transfected 293T cells, immunoprecipitated with p130Cas−/− abs, and probed with FAK abs. h.c., heavy chain. Equal expression of the Cas variants was assessed by IB of 0.5% of WCE. B and E, 5% of each IP reaction was used to assess equal precipitation of the variants by IB with p130Cas−/− abs. Results are presented from one experiment out of three performed with similar results.

To elucidate whether altered binding to FAK can also be seen with the full-length p130Cas−/− variants, the interaction of Cas1A, the novel Cas1B, Cas1B1, and Cas1E with FAK was investigated by IP (Fig. 4, D and E). Whole cell extracts from Cas−/− transductants were mixed with cell extracts from FAK-transfected 293T cells, immunoprecipitated with p130Cas−/− abs, and exam-
ined for FAK or p130Cas expression (Fig. 4E). Equal amounts of each full-length isoform were used as input and precipitated (Fig. 4E, right panel). Importantly, almost no association with FAK was detected for truncated Cas1B (Fig. 4E, left panel). Cas1E showed an increased binding to FAK when compared with Cas1A. Contrary to the N-terminal construct results (Fig. 4B), binding of full-length Cas1B1 was significantly enhanced in comparison with Cas1E (Fig. 4E). Together, these results indicate that the longer N-terminal extensions present in Cas1B1, Cas1D, and Cas1E increase the binding of the p130Cas SH3 domain to FAK.

*p130Cas* Exon 1 Variants Influence the Migratory and Invasive Potential of Cas−/− Fibroblasts—To investigate whether increased binding of the p130Cas variants to FAK alters the migratory and invasive behavior, serum-stimulated migration and invasion assays were performed with Cas−/− cells expressing Cas1A, 1B, 1B1, or 1E (Fig. 5). Cas1A reconstituted cells were used as reference cells. Expression of the truncated variant Cas1B significantly reduced migration by 73% (p < 0.01) and reduced invasion by 75% (p < 0.05) when compared with WT Cas1A. Reconstitution with the longest variant Cas1B1 increased migration by 39% (p < 0.05), whereas the invasion rate was not altered. Cas1E expression did not influence migration, but invasion was reduced by 37% (p < 0.01).

Taken together these results indicate that the p130Cas exon 1 variants display altered functional properties. Importantly, the truncated variant Cas1B, lacking parts of the SH3 domain, and Cas1B1, comprising the longest N-terminal extension, exerted greatly reduced and increased functionality, respectively.

**DISCUSSION**

Here we identified four novel p130Cas/BCAR1 mRNA variants encoded by alternative first exons, resulting in functionally different protein isoforms. The presence of two alternative first exon variants of p130Cas was first identified in mice (15) and recently also by us in humans (10). Database searches and detailed analysis of BCAR1 revealed four additional mRNAs that are transcribed from alternative first exons (1B, 1B1, 1D, and 1E). Exons 1A and 1C correspond to the previously described mouse variants (15). Our studies show that the BCAR1 isoform mRNAs are commonly co-expressed in cell lines and in major human organs. The more widely distributed BCAR1 mRNAs are BCAR1-1A, 1B1, and 1C, whereas expression of the other mRNAs is more restricted. Highest relative expression was observed for BCAR1-1A, 1C, and 1E, whereas lower levels were found for the other variants.

These results and the database analysis for BCAR1 suggest the utilization of multiple promoters to drive the expression of the exon 1 variants. Alternative promoter usage can result in the expression of different non-coding first exons (34, 35) and also in the expression of functionally distinct N-terminal protein variants (36, 37). Most of the BCAR1 exon 1 variants belong to the latter. They encode for aa sequences flanking the N-terminal end of the SH3 domain. The p130Cas SH3 domain is important for binding to FAK and several other proteins (1, 3) and the translocation of p130Cas to FAs and leading edges of the cell (17, 19, 38). Exon 1B is non-coding, producing a truncated p130Cas variant (Cas1B) that lacks a portion of the SH3 domain. The longer protein variants Cas1B1, 1D, and 1E showed significantly more efficient binding to FAK when compared with the shorter Cas1A (WT) and 1C. How this occurs is not clear, although data suggest that these additional sequences positively regulate the interaction of p130Cas with FAK. All of the

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Footnote:

4 J. Kumbrink and K. H. Kirsch, unpublished data.
longer N-terminal extensions are likely unstructured\(^5\) but may provide additional binding surfaces for FAK. This could result in increased FAK binding to p130\(^{Cas}\) or a more stable complex formation as described for myosin-2 (39) and phospholipase Cg-1 (40). Moreover, our results suggest that additional domains in the C-terminal part support efficient binding of the longest variant Cas1B1 to FAK. The C-terminal domain contains a second focal adhesion targeting domain (41), which may contribute to this observation. The stronger binding could also be mediated by posttranslational modifications because the Cas1D N terminus is in part modified by phosphorylation.

Reduced association with FAK was observed for Cas1B, likely due to its truncated SH3 domain. Most of the Cas1B-expressing cells lacked lamellipodia and did not form a distinct leading edge. These cells grew in tight clusters, whereas cells expressing the most studied Cas1A or the other variants showed a high degree of cell scattering. The partial deletion of the SH3 domain in Cas1B corroborates the importance of the SH3 domain in establishing a polarized phenotype associated with cell motility and FA formation (1, 42, 43). Importantly, reconstitution of Cas1B in Cas\(^{−/−}\) cells significantly reduced cell migration and invasion when compared with CasA1 cells. These characteristics suggest that the novel Cas1B variant is a naturally occurring p130\(^{Cas}\) isoform with greatly decreased functionality. Deletion of the SH3 domain has been shown to significantly impair the localization of p130\(^{Cas}\) to FAs, accompanied by a substantial reduction of tyrosine phosphorylation within the SD (17). Phosphorylation of the SD is of major importance for p130\(^{Cas}\)-mediated cell migration (33). Unexpectedly, cells expressing the truncated Cas1B show no alteration in tyrosine phosphorylation of the SD. Thus, the phosphorylated SD of Cas1B may act as a decoy for common p130\(^{Cas}\) SD-binding proteins without mediating SH3 domain function.

In contrast, the longest variant Cas1B1 exhibited greatly enhanced functionality. This is indicated by its most efficient FAK binding among all variants and a significantly enhanced potential to drive cell migration. The SD tyrosine phosphorylation of Cas1B1 was not influenced. However, the N-terminal extension of 50 aa contains several potential additional interaction motifs, which may recruit other factors and/or stabilize FAK binding to enhance and potentially alter the functionality of Cas1B1. Our studies have identified novel p130\(^{Cas}\) variants that exhibit functional differences including naturally occurring isoforms with greatly diminished and increased functionality.

How the isoforms that are often co-expressed in various organs affect p130\(^{Cas}\) function (for example, the truncated Cas1B1 by acting dominant negative or single variants by activating distinct cellular programs) is not clear. Interestingly, Cas1E was not detected in normal colon samples, whereas all colon carcinomas had high levels. However, the low sample number does not allow for any statistical correlation but allows for a trend. The higher expression levels of Cas1A, 1C, and 1E may imply a greater importance of these isoforms than of the other variants. Nevertheless, we have recently shown that

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\(^{5}\) P. C. Simister and S. M. Feller, personal communication.

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cancer cells by random insertional mutagenesis using defective retroviruses: identification of bcar-1, a common integration site. Mol. Endocri-

15. Polte, T. R., and Hanks, S. K. (1995) Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. Proc. Natl. Acad. Sci. U.S.A. 92, 10678–10682

16. Nakamoto, T., Sakai, R., Honda, H., Ogawa, S., Ueno, H., Suzuki, T., Aizawa, S., Yazaki, Y., and Hirai, H. (1997) Requirements for localization of p130Cas to focal adhesions. Mol. Cell. Biol. 17, 3884–3897

17. Donato, D. M., Ryzhova, L. M., Meenderink, L. M., Kaverina, I., and Hanks, S. K. (2010) Dynamics and mechanism of p130Cas localization to focal adhesions. J. Biol. Chem. 285, 20679–20779

18. Meenderink, L. M., Ryzhova, L. M., Donato, D. M., Hochberg, D. F., Kaverina, I., and Hanks, S. K. (2010) p130Cas Src-binding and substrate domains have distinct roles in sustaining focal adhesion disassembly and promoting cell migration. PLoS One 5, e13412

19. Deramaudt, T. B., Dujardin, D., Noulet, F., Martin, S., Vauchelles, R., Kaverina, I., and Hanks, S. K. (2010) p130Cas Src-binding and substrate domains have distinct roles in sustaining focal adhesion disassembly and promoting cell migration. PLoS One 5, e13412

20. Akagi, T., Murata, K., Shishido, T., and Hanafusa, H. (2002) v-Crk activates the phosphoinositide 3-kinase/akt pathway by utilizing focal adhesion kinase and H-Ras. Mol. Cell. Biol. 22, 7015–7023

21. Thierry-Mieg, D., and Thierry-Mieg, J. (2006) AceView: a comprehensive database resource. Nucleic Acids Res. 34, D5–D16

22. Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M., and Werner, T. (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 21, 2933–2942

23. Sayers, E. W., Barrett, T., Benson, D. A., Bolton, E., Bryant, S. H., Canese, K., et al. (2010) Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 38, D15–D16

24. Gould, C. M., Diella, F., Via, A., Puntevoll, P., Gemünd, C., Chabanis-Davidson, S., Michael, S., Sayadi, A., Bryne, J. C., Chica, C., Seiler, M., Davey, N. E., Haslam, N., Weatheritt, R. J., Будд, А., Hughes, T., Pas, J., Rychlewski, L., Trév, G., Aasland, R., Helmer-Citterich, M., Linding, R., and Gibson, T. J. (2010) ELM: the status of the 2010 eukaryotic linear motif resource. Nucleic Acids Res. 38, D167–D180

25. Obenauer, J. C., Cantley, L. C., and Yaffe, M. B. (2003) Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res. 31, 3635–3641

26. Honda, H., Oda, H., Nakamoto, T., Honda, Z., Sakai, R., Suzuki, T., Saito, T., Nakamura, K., Nakao, K., Ishikawa, T., Katsuki, M., Yazaki, Y., and Hirai, H. (1998) Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. Nat. Genet. 19, 361–365

27. Zhao, Y., Kumbrik, J., Lin, B. T., Bouton, A. H., Yang, S., Toselli, P. A., and Kirsch, K. H. (2013) Expression of a phosphorylated substrate domain of p130Cas promotes PyMT-induced e-Src-dependent murine breast cancer progression. Carcinogenesis 34, 2880–2890

28. Kumbrik, J., Kirsch, K. H., and Johnson, J. P. (2010) EGR1, EGR2, and EGR3 activate the expression of their coregulator NAB2 establishing a negative feedback loop in cells of neuroectodermal and epithelial origin. J. Cell. Biochem. 111, 207–217

29. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>−ΔΔCT</sup> method. Methods 25, 402–408

30. Soni, S., Lin, B. T., August, A., Nicholson, R. I., and Kirsch, K. H. (2009) Expression of a phosphorylated p130Cas substrate domain attenuates the phosphatidylinositol 3-kinase/Akt survival pathway in tamoxifen resistant breast cancer cells. J. Cell. Biochem. 107, 364–375

31. Kirsch, K. H., Georgescu, M. M., and Hanafusa, H. (1998) Direct binding of p130Cas to the guanine nucleotide exchange factor C3G. J. Biol. Chem. 273, 25673–25679

32. Makkinkie, A., Nair, R. I., Infusini, G., Vanden Borre, P., Bloom, A., Cai, D., Costello, C. E., and Lerner, A. (2009) AND-34/BCAR3 regulates adhesion-dependent p130Cas serine phosphorylation and breast cancer cell growth pattern. Cell. Signal. 21, 1423–1435

33. Huang, J., Hamasaki, H., Nakamoto, T., Honda, H., Hirai, H., Saito, M., Takato, T., and Sakai, R. (2002) Differential regulation of cell migration, actin stress fiber organization, and cell transformation by functional domains of Crk-associated substrate. J. Biol. Chem. 277, 27265–27272

34. Wang, H., Li, R., and Hu, Y. (2009) The alternative noncoding exons 1 of aromatase (Cyp19) gene modulate gene expression in a posttranscriptional manner. Endocrinology 150, 3301–3307

35. Kos, M., Reid, G., Denger, S., and Gannon, F. (2001) Minireview: genomic organization of the human ERα gene promoter region. Mol. Endocrinol. 15, 2057–2063

36. Streb, J. W., Kitchen, C. M., Gelman, I. H., and Miano, J. M. (2004) Multiple promoters direct expression of three AKAP12 isoforms with distinct subcellular and tissue distribution profiles. J. Biol. Chem. 279, S6014–S6023

37. Kogerman, P., Krause, D., Rahnama, F., Kogerman, L., Undén, A. B., Zaphiropoulos, P. G., and Toftgård, R. (2002) Alternative first exons of PTCH1 are differentially regulated in vivo and may confer different functions to the PTCH1 protein. Oncogene 21, 6007–6016

38. Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M., and Weed, S. A. (2000) Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. Oncogene 19, 5606–5613

39. Fujita-Becker, S., Tsaiavalis, G., Ohkura, R., Shimada, T., Manstein, D. J., Laborda, J., and Bonvini, E. (1998) Identification of two focal adhesion targeting sequences in the adapter protein Cas: signal convergence and the determination of cellular responses. J. Biol. Chem. 273, 6448–6458

40. Jánosiák, R., Brabek, J., Auernheimer, V., Tatárová, Z., Lautscham, L. A., Dey, T., Gemperle, J., Merkel, R., Goldmann, W. H., Fabry, B., and Rösel, D. (2014) CAS directly interacts with vinculin to control mechanosensing and focal adhesion dynamics. Cell. Mol. Life Sci. 71, 727–744