# Induction of BAIAP3 by the EWS-WT1 chimeric fusion implicates regulated exocytosis in tumorigenesis

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## Summary

**Desmoplastic small round cell tumor (DSRCT) is defined genetically by the chimeric fusion of the Ewing's sarcoma and Wilms' tumor genes, generating a novel transcription factor, EWS-WT1. By using cells with inducible EWS-WT1 to screen high-density microarrays, we identified *BAIAP3* as a transcriptional target of the chimera. The *BAIAP3* promoter is specifically bound in vivo by the (−KTS) isoform of EWS-WT1, consistent with its activation in reporter assays. *BAIAP3* encodes a protein implicated in regulated exocytosis, which is colocalized with a secreted growth factor within cytoplasmic organelles. Ectopic expression of BAIAP3 in tumor cells dramatically enhances growth in low serum and colony formation in soft agar. *BAIAP3* therefore encodes a transcriptional target of an oncogenic fusion protein that implicates the regulated exocytotic pathway in cancer cell proliferation.**

## Introduction

Distinct chromosomal translocations involving the *EWS* gene at chromosome 22q12 underlie a number of primitive tumor types, including Ewing's sarcoma, malignant melanoma of soft parts, myxoid liposarcoma, myxoid chondrosarcoma, and desmoplastic small round cell tumor (DSRCT) (reviewed in de Alava and Gerald, 2000). These translocations result in the generation of novel chimeric proteins, all of which contain the N-terminal domain (NTD) of EWS, encoding a potent transactivation domain (Bailly et al., 1994; May et al., 1993; Ohno et al., 1993), which is fused to the DNA binding domains of different transcription factors. The remarkable selectivity for different cell types displayed by these oncogenic transcription factors is presumably derived to a great extent from the activation of different sets of transcriptional targets, whose identity is dictated by the DNA binding specificity of the EWS translocation partner. The susceptibility of specific cell types to the transforming effect of these genes may also contribute to their lineage restriction. Ewing's sarcoma is defined by a translocation of the EWS NTD to the DNA binding domain of one of the related ETS proteins, FLI1 and ERG (Arvand and Denny, 2001). Similarly, all cases of DSRCT carry a translocation of the EWS NTD to three of the four zinc finger domains of the Wilms' tumor suppressor WT1.

DSRCT is an aggressive neoplasm arising from the serosal surfaces of the abdominal peritoneum, primarily affecting young males (Gerald and Rosai, 1989). Tumor cells express specific epithelial, muscle, and neural markers, suggesting a pluripotent cell of origin (Gerald et al., 1991; Gerald and Rosai, 1993). The tumor itself has a remarkable histological appearance, with small nests of tumor cells surrounded by a dense reactive stroma containing fibroblasts and hyperplastic blood vessels. As such, DSRCT provides an exceptional model for paracrine interactions between the tumor and stromal cell compartments that contribute to human cancer. All cases of DSRCT carry the t(11;22)(p13;q12) translocation, fusing the NTD of EWS with the C-terminal zinc fingers 2 through 4 of WT1 (Figure 1A) (Gerald et al., 1995; Ladanyi and Gerald, 1994). *WT1* encodes a transcription factor that is essential for normal kidney development, which is inactivated in a subset of Wilms' tumors, leukemias, and mesotheliomas (reviewed in Lee and Haber, 2001). Four C-terminal $C_2$-$H_2$ zinc fingers mediate DNA binding to the consensus 5′-GCGTGGGAGTA-3′ sequence (Nakagama et al., 1995); a prevalent alternative splicing event inserts three amino acids, lysine, threonine, and serine (KTS), between zinc fingers

### SIGNIFICANCE

**Chromosomal translocations which fuse the Ewing's sarcoma gene *EWS* to different DNA binding domains result in the creation of chimeric transcription factors that define specific types of embryonal tumors. Transcriptional targets activated by these oncogenic fusion proteins drive malignant transformation, and they may identify novel pathways involved in tumorigenesis. By generating cells with inducible expression of the EWS-WT1 chimera and interrogating expression arrays, we identified a target gene, *BAIAP3*, that appears to function in regulated exocytosis, a pathway that has not previously been linked to cancer. BAIAP3 is of particular interest, since it is also expressed in breast and prostate cancers and its ectopic expression enhances malignant properties of cancer cells.**

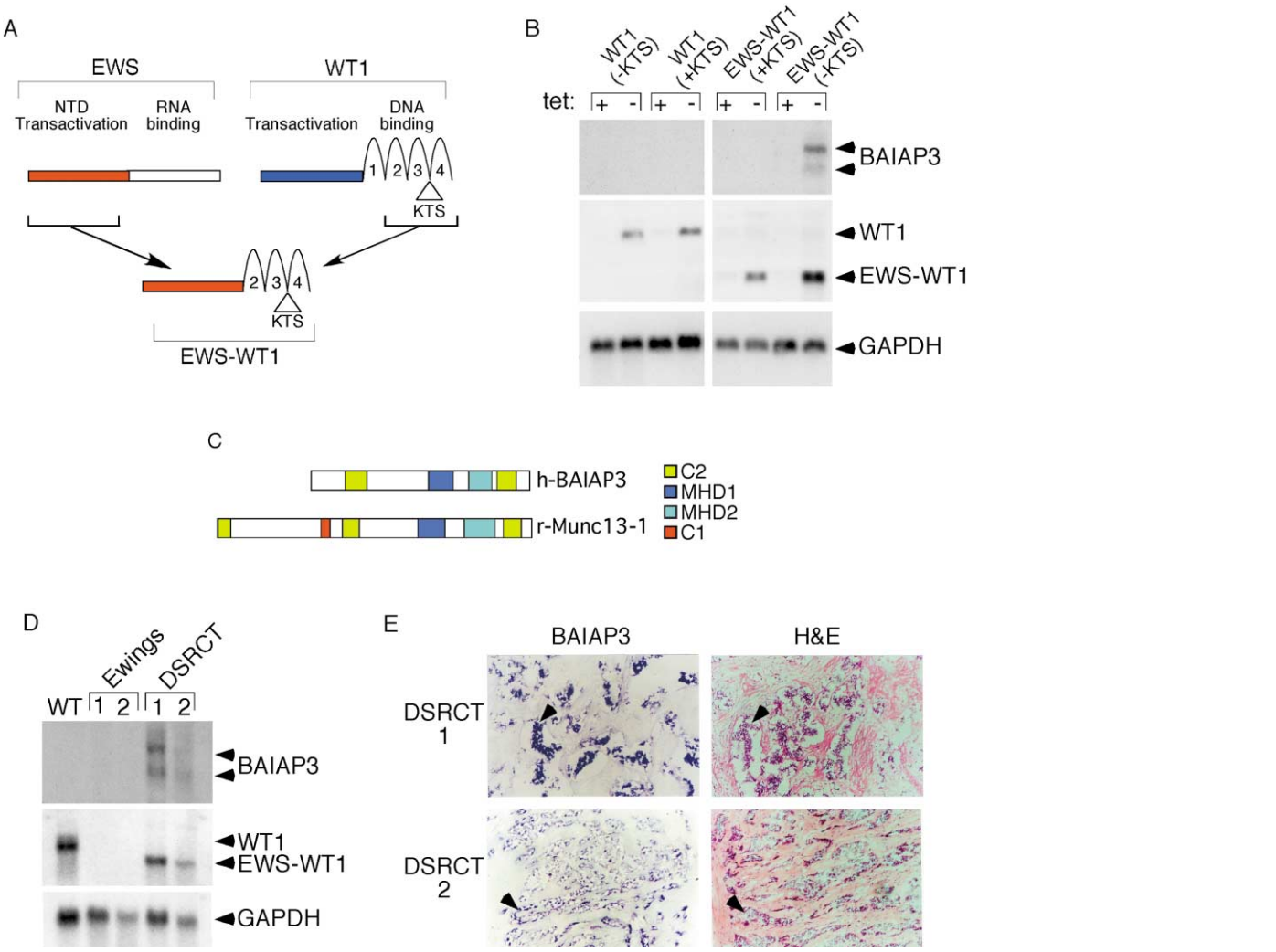


**Figure 1.** Induction of BAIAP3 by EWS-WT1 and expression in DSRCT

**A:** Schematic representation of the EWS-WT1 fusion protein. The NTD transactivation and RNA binding domains of EWS are indicated, along with the transactivation and zinc finger DNA binding domains of WT1. KTS represents the three amino acids, lysine, threonine, and serine, that are alternatively spliced within the WT1 zinc finger domain. The first zinc finger of WT1 is consistently excluded from the chimera, but the KTS splice is preserved.

**B:** Northern blot analysis of U2OS cells with tetracycline-repressible expression of WT (+/−KTS) and EWS-WT1 (+/−KTS) following growth in the presence (+) or absence (−) of tetracycline (tet) for 12 hr. Blots were probed for *BAIAP3*, *WT1* (recognizing both *WT1* and *EWS-WT1*), and *GAPDH* (loading control).

**C:** Comparison of the domain structure of human (h) BAIAP3 (NP_003924) and rat (r) Munc13-1(NP_620199) proteins, including the $C_1$, $C_2$, and Munc13 homology domains.

**D:** Northern blot analysis of representative primary specimens from Wilms' tumor, Ewing's sarcoma, and DSRCT. Variability in the intensity of *EWS-WT1* reflects differences in the relative composition of tumor and stromal cells as well as RNA integrity.

**E:** RNA in situ hybridization of *BAIAP3* in two primary DSRCT samples (40×). Adjacent sections are stained with hemotoxylin and eosin (H&E) showing the basophilic tumor cell nests (arrowheads) surrounded by dense reactive stroma. *BAIAP3* expression is restricted to tumor cells. No staining was observed with a control sense probe (not shown).

3 and 4 and disrupts binding to this element. WT1(−KTS) transactivates a number of genes implicated in cellular differentiation, whereas the function of WT1(+KTS) in transcription is less clear (reviewed in Lee and Haber, 2001). The EWS-WT1 chimera invariably loses the first zinc finger of WT1, suggesting an important difference in DNA binding specificity. However, the (+/−KTS) alternative splice is preserved, with EWS-WT1(−KTS), but not the (+KTS) variant, mediating potent transcriptional activation (Kim et al., 1998b; Rauscher et al., 1994).

Initial attempts at identifying physiological transcriptional targets of EWS-WT1(−KTS) focused on genes with WT1-responsive promoters. Indeed, Platelet-derived growth factor-A (*PDGF-A*) and Insulin-like growth factor-1 receptor (*IGF-1-R*), both of which have promoters that are modulated by WT1(−KTS) in reporter assays, were also found to be regulated by EWS-WT1(−KTS) (Finkeltov et al., 2002; Karnieli et al., 1996; Lee et al., 1997). However, despite the in vitro binding of EWS-WT1(−KTS) to promoters that are also recognized by native WT1(−KTS), few appear to be regulated by EWS-WT1 in vivo (Kim et al., 1998b; Lee et al., 1997; Rauscher et al., 1994). We therefore used cells with inducible expression of EWS-WT1(−KTS) to search for endogenous transcripts whose ex-

pression is altered following induction of the chimeric fusion. Hybridization to high-density oligonucleotide microarrays was used as an unbiased screen for novel EWS-WT1(−KTS) target genes, followed by validation using Northern blotting and promoter characterization. Here, we report a novel gene, *BAIAP3*, which is transcriptionally regulated by EWS-WT1(−KTS), but not by native WT1(−KTS). BAIAP3 appears to function in the process of regulated exocytosis, raising the possibility that EWS-WT1-dependent transformation may rely, in part, on this pathway.

## Results

### Induction of BAIAP3 by EWS-WT1(−KTS)

In order to undertake an unbiased screen for EWS-WT1(−KTS) target genes, we generated U2OS human osteosarcoma cells with tightly regulated, tetracycline-repressible expression of this isoform (Lee et al., 1997), and interrogated high-density oligonucleotide microarrays for transcripts whose expression was altered following induction of the chimera. Total cellular RNA was isolated 12 hr following drug withdrawal and compared in duplicate with RNA from cells grown under identical conditions in the presence of tetracycline. Known genes found to be induced by EWS-WT1(−KTS) have been previously reported and include interleukin-2/15 receptor β (*IL-2/15R*β), platelet-derived growth factor-A (*PDGF-A*), adrenomedulin, amphiregulin, and fibroblast growth factor receptor 4 (Wong et al., 2002). Additionally, several ESTs were also identified as putative EWS-WT1(−KTS) targets. One of these, EST (R60906), displayed the highest fold induction (79.4 and 160.8 in duplicate experiments) as compared with all other identified targets, and was therefore characterized further. By a combination of cDNA library screening and database mining, this EST was found to be derived from the 3′ untranslated region of *BAIAP3* (first described as *BAP3* [Shiratsuchi et al., 1998]), a $C_2$ domain containing protein hypothesized to function in regulated exocytosis (Figure 1C). Northern blot analysis confirmed that the 2.5 kb and 4.8 kb *BAIAP3* transcripts are below detection in U2OS cells and are strongly induced within 12 hr following expression of EWS-WT1(−KTS) (Figure 1B). In contrast, no induction of *BAIAP3* is observed following expression of either native WT1(−KTS) or EWS-WT1(+KTS). Thus, induction of *BAIAP3* is specific for the transcriptionally active EWS-WT1(−KTS) isoform of the chimera.

The use of a heterologous cell type with tetracycline-regulated expression of EWS-WT1 makes it possible to effectively screen microarrays for downstream targets, but these must be confirmed in the relevant tumor cells. We therefore screened multiple primary DSRCT samples for expression of *BAIAP3*, first by Northern blot analysis, followed by RNA in situ hybridization. Expression of the expected *BAIAP3* transcripts is readily evident in two DSRCT specimens but not in sporadic Wilms' tumor expressing wild-type WT1 or in Ewing's sarcoma containing the *EWS-FLI1* rearrangement (Figure 1D). A characteristic feature of these tumors, small nests of tumor cells surrounded by a dense reactive or "desmoplastic" stroma, is demonstrated upon histological analysis of DSRCT tumor sections. RNA in situ analysis in four DSRCT specimens indicated strong *BAIAP3* expression specifically within the nests of tumor cells and not within the surrounding reactive stroma (Figure 1E and data not shown).

### Direct regulation of the BAIAP3 promoter by EWS-WT1

The strong and rapid (<12 hr) induction of endogenous *BAIAP3* mRNA following inducible expression of EWS-WT1(−KTS) suggests the presence of a responsive element (E-WRE) within the *BAIAP3* promoter. Two isoforms of *BAIAP3* have been identified, *BAIAP3*α (AL834321) and *BAIAP3*β (AB017111). These isoforms differ in the alternative usage of exon 1α versus exon 1β, associated with different transcriptional start sites and presumptive regulatory sequences (Figure 2A). RT-PCR and 5′-RACE analyses of either U2OS cells with inducible EWS-WT1(−KTS) or normal tissues with constitutive expression of *BAIAP3* (such as fetal brain) confirmed expression of only the *BAIAP3*α isoform (data not shown). The proximal promoter for the *BAIAP3*α transcript contains multiple elements similar to the EWS-WT1(−KTS) consensus binding site, E-WRE, found in the *IL-2/15R*β promoter: 5′-(G/C)(C/G)(G/C)TGGGGG-3′ (Wong et al., 2002). Element B1 (nucleotides −443 to −458; sense 5′-GCTGTGGGCGTGGGGC-3′) contains two overlapping E-WRE sites, and element B2 (nucleotides −264 to −272; antisense 5′-GCGTGGGGT-3′) has a single site (Figure 2B). Transcriptional activation of the *BAIAP3*α promoter by EWS-WT1(−KTS) was demonstrated following transient transfection of luciferase-reporter constructs into U2OS cells. Four-fold induction was reproducibly observed using reporter plasmid pGL3-B, containing both E-WRE sites and lacking a 3′ GC-rich negative regulatory element (Figure 2C). Consistent with the EWS-WT1(−KTS)-specific induction of the endogenous *BAIAP3* transcript, the reporter constructs were not activated by either wild-type WT1(−KTS) or by the (+KTS) isoform of the chimera.

We used electrophoretic mobility shift assays (EMSA) to determine whether EWS-WT1(−KTS) binds to the B1 and B2 elements within the *BAIAP3*α promoter in vitro. Promoter regions containing either element bound efficiently to the bacterially synthesized EWS-WT1(−KTS) zinc finger domain, whereas no binding was observed with the EWS-WT1(+KTS) control (Figure 2D). To confirm this interaction in vivo, we used chromatin immunoprecipitation (ChIP) to enrich for DNA fragments bound by EWS-WT1(−KTS). Proteins bound to DNA in U2OS cells in which expression of the chimera had been induced by the removal of tetracycline were crosslinked using formaldehyde, and EWS-WT1 protein-DNA complexes were immunoprecipitated using antibodies against either the WT1 zinc finger domain or the C-terminal HA-epitope tag. With induction of EWS-WT1(−KTS), specific *BAIAP3*α promoter sequences were selectively enriched in both the anti-WT1 and anti-HA immunoprecipitates relative to control (β-*actin*) sequences (Figure 2E). No enrichment was observed in the absence of antibody or in mock immunoprecipitated samples (rabbit preimmune serum). As an additional negative control, these experiments were repeated with cells grown in the presence of tetracycline. In the absence of induction of EWS-WT1(−KTS), only minimal enrichment of the *BAIAP3*α promoter was observed (data not shown). Based on these results, EWS-WT1(−KTS) binds to the *BAIAP3* promoter both in vitro and in vivo.

### Expression of BAIAP3 in epithelial cell types and intracellular localization

BAIAP3 was originally identified as a brain specific protein, potentially involved in the regulation of neurotransmitter release (Shiratsuchi et al., 1998). Our observation that it is also expressed at high levels in primary DSRCT specimens raised the

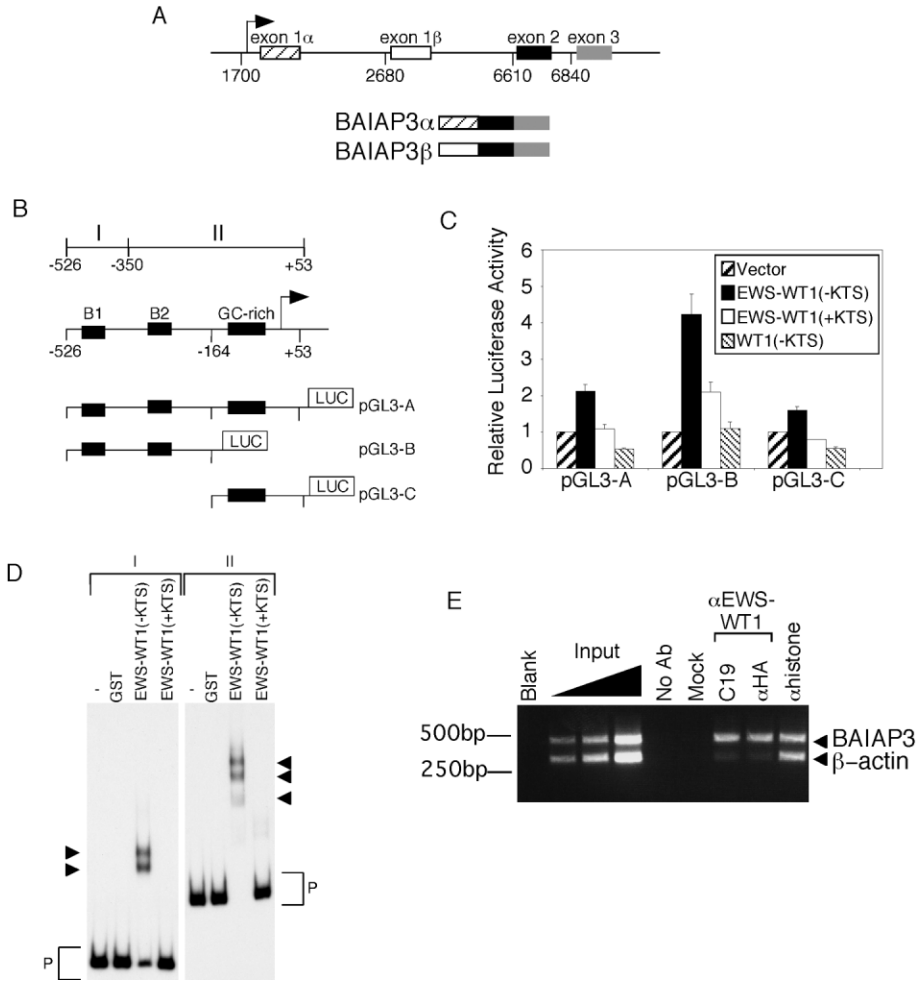


**Figure 2.** Direct transcriptional activation of BAIAP3 by EWS-WT1(−KTS)

**A:** Schematic representation of the 5′ end of the *BAIAP3* gene. *BAIAP3*α represents the transcript induced by EWS-WT1(−KTS), utilizing exon 1α. *BAIAP3*β denotes the originally published cDNA sequence for *BAIAP3*, which utilizes exon 1β, but was not detectable in cells studied here. The transcriptional start site of *BAIAP3*α (arrow) is derived from the EST database (BI546056) and 5′ RACE experiments.

**B:** Schematic representation of the *BAIAP3* promoter upstream of exon 1α denoting the positions of two potential EWS-WT1 binding sites, B1 (−443 to −458) and B2 (−264 to −272), along with a GC-rich sequence. Fragments I and II represent regions used for EMSA.

**C:** Activation of the *BAIAP3* promoter by EWS-WT1(−KTS). Luciferase activity, relative to vector-transfected cells, was measured in U2OS cells following cotransfection of CMV-driven expression constructs and the indicated reporter (see Experimental Procedures). Standard deviations were derived from three experiments.

**D:** EMSA analysis of promoter fragments I and II, following incubation with GST-conjugated zinc finger domains of EWS-WT1 (−/+ KTS) or GST alone. Migration positions of free probe (P) and DNA-protein complexes (arrowheads) are shown. Multiple shifted bands may represent the ability of >1 molecule of EWS-WT1(−KTS) to bind to each fragment.

**E:** Chromatin immunoprecipitation demonstrating in vivo association of EWS-WT1(−KTS) with the *BAIAP3* promoter. Multiplex PCR of *BAIAP3* promoter together with β-actin promoter (negative control), using templates derived from cross-linked DNA-protein complexes immunoprecipitated with either two different antibodies against EWS-WT1 (αC19 against the WT1-derived DNA binding domain or αHA against the epitope tag), nonspecific antibodies (rabbit preimmune serum, Mock), or an antibody to acetylated histone H4 (α histone; positive control). Progressive dilutions of total chromatin were also amplified to demonstrate the linearity of multiplex PCR amplification (Input).

possibility that it may be involved in additional functions in other cell types. Therefore, we examined the expression pattern of *BAIAP3* by both RNA in situ hybridization and semiquantitative RT-PCR in mouse and human tissues. As expected, specific structures of the developing brain, including the cortical plate, ventricular zone, ganglionic eminence, pyriform (olfactory) cortex, and thalamus, express high levels of *BAIAP3* (Figure 3A). However, expression of *BAIAP3* is also present in nonneuronal tissues, including placenta, lung, pancreas, spleen, and testes (Figure 3A and data not shown). Within the placenta, expression of *BAIAP3* mRNA is restricted to the syncytiotrophoblasts, cells that secrete growth factors such as chorionic gonadotropin and placental lactogen (Morrish et al., 1998). *BAIAP3* mRNA is also present in human breast and prostate tumor samples within both the normal and malignant epithelial cells. These expression studies suggest that BAIAP3 may function in a number of different secretory cell types.

A potential role for BAIAP3 in regulated exocytosis was postulated based on the sequence and spatial organization of its two $Ca^{2+}$/phospholipid binding ($C_2$) domains and two Munc13-homology domains (MHD), all of which are highly similar to those of Munc13 proteins, essential regulators of neurotransmitter release, a specialized form of regulated exocytosis (Brose et al., 2000; Koch et al., 2000; Shiratsuchi et al., 1998) (Figure 1C). Given the absence of reliable functional assays, we first tested whether BAIAP3 is physically colocalized with bona fide members of this cellular pathway. The PC12 cell line is derived from adrenal chromaffin cells of rat pheochromocytoma and contains multiple secretory vesicles that fuse with the cell membrane in response to a shift in intracellular $Ca^{2+}$ levels. Transient transfection of a plasmid encoding human growth hormone (hGH) into these cells serves as a marker for secretory vesicles (Wick et al., 1993). Indeed, cotransfection of hGH and HA-tagged BAIAP3 into PC12 cells shows colocalization of the two proteins within small cytoplasmic structures consistent with secretory vesicles (Figure 3B). This colocalization is supported by cell fractionation experiments. Differential centrifugation shows BAIAP3 to be present within the microsomal fraction (both in high-density "HDM" and low-density "LDM" microsomal fractions) with only a small amount present in the cytosolic fraction (Figure 3C). This microsomal localization is also seen for the secretory vesicle markers synaptotagmin and synaptophysin, whereas the cytosolic protein RhoGDi is almost exclusively localized within the cytosolic fraction. Further separation of HDM and LDM fractions on a discontinuous sucrose gradient demonstrates overlap of BAIAP3 with the two vesicle proteins. Taken

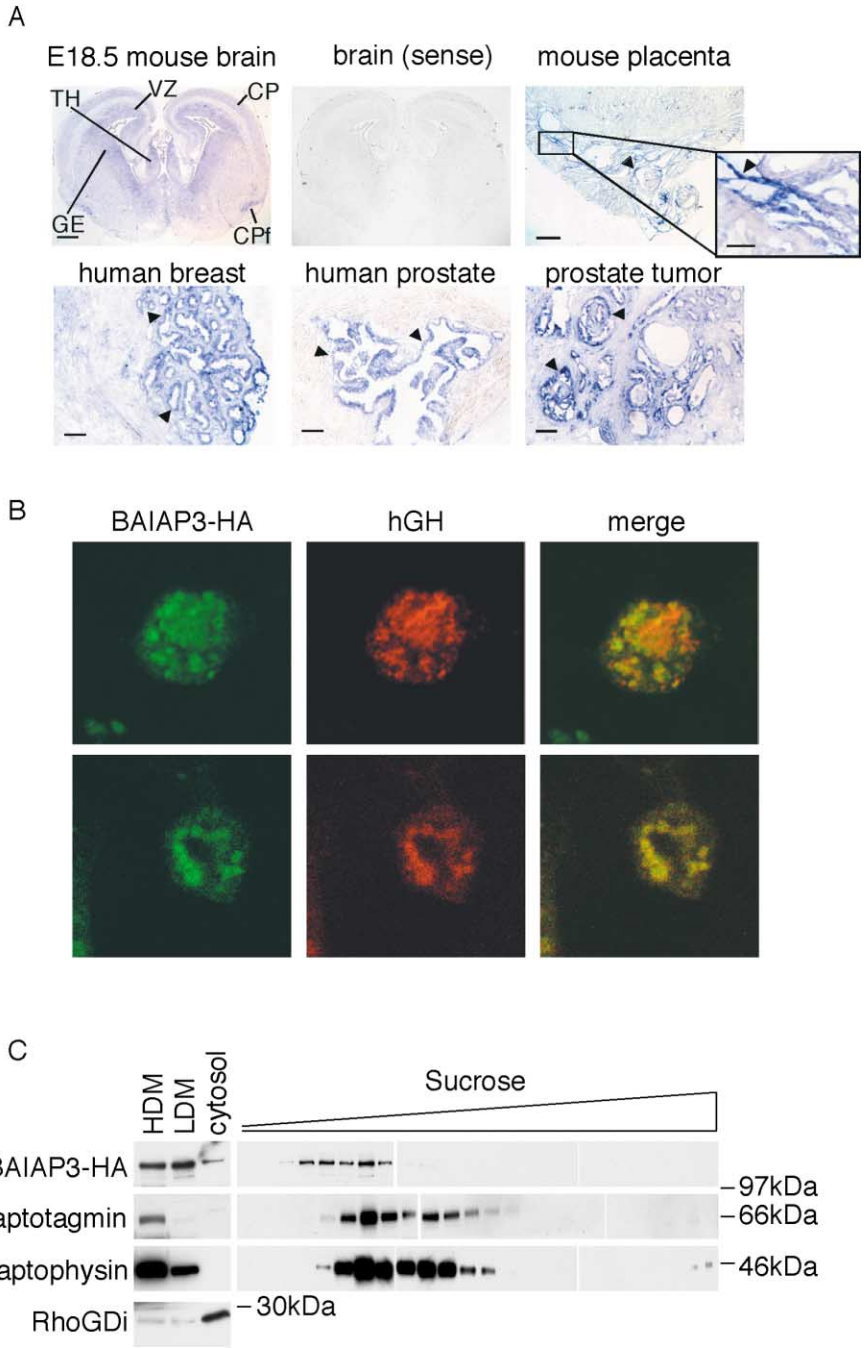


**Figure 3.** Tissue-specific expression and localization of BAIAP3

**A:** RNA in situ hybridization analysis of *BAIAP3*, demonstrating expression in E18.5 fetal mouse brain, within the ventricular zone (VZ), cortical plate (CP), thalamus (TH), ganglionic eminence (GE), and pyriform (olfactory) cortex (CPf). No staining was observed with the sense control; bar = 0.3 mm. *BAIAP3* is also expressed at high levels in the mouse placenta, specifically within syncytiotrophoblasts (arrowheads); bar = 0.4 mm. Inset shows a higher magnification of the syncytiotrophoblast layer; bar = 0.1 mm. Expression is present in normal human breast epithelium, where multiple lobules are lined with *BAIAP3* positive epithelial cells (arrowheads), and both normal and cancerous human prostate (note *BAIAP3* positive epithelial cells lining glands); bar = 0.1 mm.

**B:** Confocal imaging of PC12 cells expressing HA-tagged BAIAP3 and hGH. Cells were stained with antibody to the HA-epitope (green fluorescence) and antibody to hGH (red fluorescence), with regions of overlap (yellow) evident on the merged image.

**C:** Biochemical fractionation of BAIAP3 within subcellular fractions from PC12 cells. Cytosolic, high-density microsomal (HDM) and low-density microsomal (LDM) fractions were isolated using differential centrifugation. HDM and LDM fractions were pooled and further fractionated on a discontinuous sucrose gradient. Western blot analysis of collected fractions is shown, using antibodies to the HA-epitope (BAIAP3), synaptotagmin, synaptophysin, and RhoGDi.

together, these studies support an association between BAIAP3 and secretory vesicles, implicating BAIAP3 in regulated exocytosis pathways in neuronal cells, specialized cells of the placenta, and both normal and malignant epithelial cells.

**BAIAP3 promotes neoplastic transformation**

Ectopic expression of EWS-WT1(−KTS) has been reported to be weakly transforming in NIH3T3 cells (Kim et al., 1998a). Further studies have been limited by uncertainty as to the cell of origin of DSRCT and the inability to generate DSRCT cell lines in vitro. We therefore used ectopic expression of BAIAP3 in human tumor cells of borderline invasiveness to determine its effect on in vitro correlates of malignant transformation. For these experiments, we used the human melanoma cell line MC (Bartolazzi et al., 1994), and we generated multiple independent clones following transfection with either BAIAP3 or vector control, to ensure against clonal selection bias (Figure 4A and data not shown). Cells expressing BAIAP3 were not altered in their baseline growth properties. However, they consistently demonstrated a dramatically enhanced proliferation at limiting serum concentrations (0.5% serum), as compared with vector-transfected cells (Figure 4B). A similar effect on serum dependence was observed following transfection of BAIAP3 into NIH3T3 cells (data not shown). BAIAP3-expressing MC cells were also

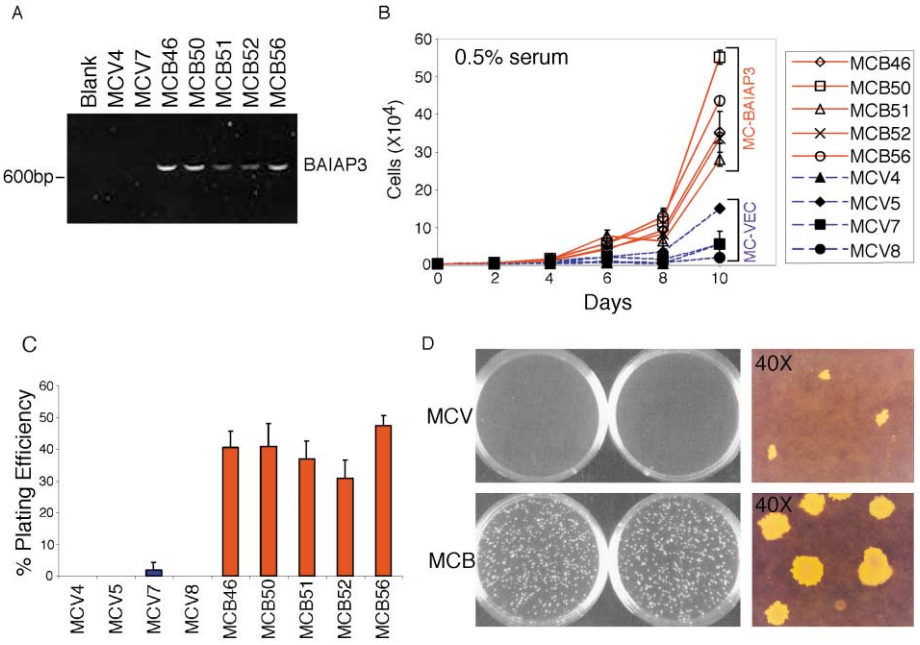


**Figure 4.** Effect of BAIAP3 on cellular proliferation and transformation

**A:** RT-PCR amplification of *BAIAP3* mRNA from five stably transfected melanoma cell lines, MCB 46, 50, 51, 52, and 56. Two control cell lines transfected with vector (MCV4 and MCV7) are negative for *BAIAP3* expression.

**B:** Growth curve of multiple independent BAIAP3- and vector-transfected cell lines maintained in medium containing 0.5% fetal bovine serum for a total of 10 days.

**C and D:** Colony formation in soft agar. **C:** Fraction of cells from multiple BAIAP3- and vector-transfected lines plated in soft agar and giving rise to colonies with >0.5 mm diameter. **D:** Representative plates are shown for vector-transfected cell lines (MCV) and BAIAP3-transfected cells (MCB), either at no magnification (left) or 40× magnification to show individual colonies (right).

tested for anchorage independent growth. Whereas vector-transfected MC cells form small (<0.15 mm) colonies following plating on soft agar, BAIAP3-expressing cells give rise to large colonies (>0.5 mm diameter) with a plating efficiency of 30%–50% (Figures 4C and 4D). Finally, inoculation of $3 \times 10^6$ vector-transfected MC cells into ten nude mice produced one tumor at 9 weeks, consistent with the reported weak tumorigenicity of this cell line (Bartolazzi et al., 1994). In contrast, by this time point, tumors were present in four out of ten mice that had been inoculated with either of two BAIAP3-transfected cell lines. Taken together, these observations support a role for BAIAP3 in reducing the need for exogenous growth factors present in serum, enhancing anchorage independent proliferation in vitro, and contributing toward tumorigenicity in vivo. The EWS-WT1(−KTS) chimeric construct itself displays modest transforming properties in heterologous cell types, a feature that is shared with other EWS-related translocation products (Arvand et al., 1998; Lessnick et al., 2002). Only few MC clones tolerated expression of EWS-WT1(−KTS) at low levels, and these formed colonies with a plating efficiency of 30% in soft agar but did not enhance tumor formation in nude mice (data not shown). The ability of MC cells to tolerate expression of the EWS-WT1(−KTS) target BAIAP3 therefore made it possible to detect its contribution to neoplastic transformation.

## Discussion

Identification of transcriptional targets for EWS-WT1, the unique chimeric transcription factor underlying DSRCT, is essential for insight into its mechanism of cellular transformation as well as its remarkable cell type specificity. As for other *EWS* translocation-dependent cancers, the absence of established in vitro models and the uncertain cell of origin for the tumor itself have complicated efforts to identify the critical downstream pathways leading to malignant transformation. Here, we have used the combination of inducible expression in a heterologous cell type with high-density microarray analysis to undertake an unbiased screen for potential targets of EWS-WT1. We demonstrate that *BAIAP3* is a transcriptional target of the chimera, that its expression pattern in primary tumor specimens is consistent with a physiologically relevant role in DSRCT, and that its ectopic expression enhances the neoplastic properties of human tumor cells. While we do not fully understand the mechanism whereby BAIAP3 modulates these in vitro correlates of malignant transformation, its apparent function within the regulated exocytosis pathway raises the possibility that growth factor secretion by tumor cells may play a role in DSRCT.

Initial in vitro studies of the EWS-WT1 chimera had suggested that the derivation of its three zinc finger domains from WT1 might result in binding to a similar set of target genes recognized by the four zinc fingers of native WT1(−KTS). However, analysis of endogenous transcripts regulated by these transcription factors has suggested that these targets are, for the most part, distinct. For instance, we have previously demonstrated that the transcripts encoding PDGF-A and the IL2/15β receptor are both induced by EWS-WT1(−KTS), but expression of these transcripts is not altered by native WT1(−KTS) (Lee et al., 1997; Wong et al., 2002). Similarly, amphiregulin mRNA is strongly induced by WT1(−KTS), but only weakly by EWS-WT1(−KTS) (Lee et al., 1999; Wong et al., 2002). Consistent with these observations, we find that *BAIAP3* is induced by EWS-WT1(−KTS), but not by native WT1(−KTS). This distinction may result in part from altered DNA binding specificity associated with the presence or absence of the first WT1 zinc finger domain, although promoter context and protein interactions involving the EWS transactivation domain may also contribute to promoter specificity. The identification of *BAIAP3* as a physiological transcriptional target of EWS-WT1(−KTS) is further supported by the demonstration of in vivo binding by the chimera to its promoter using chromatin immunoprecipitation.

BAIAP3 was originally identified from a yeast two-hybrid

assay searching for proteins interacting with the cytoplasmic domain of brain angiogenesis inhibitor-1 (BAI1), although the functional significance of this interaction is uncertain (Shiratsuchi et al., 1998). Based on its $C_2$ and Munc13 homology domains, BAIAP3 belongs to a subfamily of Munc13-like proteins, some of which are known to be essential for neurotransmitter release (Koch et al., 2000; Shiratsuchi et al., 1998). Indeed, biochemical fractionation suggests that BAIAP3 is present in microsomal fractions that also contain important components of this pathway, synaptotagmin and synaptophysin, and BAIAP3 is colocalized with a cotransfected secreted growth factor within cellular organelles. Knockout experiments have demonstrated the importance of Munc13-1 in neurotransmitter release in mouse, along with its orthologs unc-13 in *C. elegans* and dunc-13 in *Drosophila* (Brose et al., 2000). However, the only in vitro functional assay, modulation of regulated exocytosis in PC12 cells, has been inconsistent in demonstrating an effect for ectopic expression of Munc13-1 (Ashery et al., 2000; Sugita et al., 1999). Similarly, we also have not detected reproducible alterations in this assay following ectopic expression of BAIAP3 (R.E.P., K. Mukherjee, T. Sudhof, and D.A.H., unpublished data). As regulated exocytosis is a multistep process involving recruitment of secretory vesicles to the plasma membrane, followed by docking, priming, and, finally, fusion with the membrane and secretion of the enclosed molecules, the role of BAIAP3 may only be rate-limiting for selected steps in this pathway within specific cell types. Munc13-1 functions during neurotransmitter vesicle priming, a step that is conserved in endocrine, exocrine, and paracrine secretion (Ashery et al., 2000; Brose et al., 2000). Another protein family member that is closely related to BAIAP3, Munc13-4, is expressed in pulmonary goblet and alveolar type II cells and implicated in exocrine secretion of mucous and surfactant (Koch et al., 2000). The expression of BAIAP3 in secretory syncytiotrophoblast cells of the placenta and in epithelial cells of the breast and prostate suggest a broader role in growth factor secretion. This model would be consistent with the decreased serum dependence observed in melanoma cells expressing ectopic BAIAP3.

The specific induction of *BAIAP3* by the EWS-WT1 chimera suggests that the regulated exocytotic pathway may directly contribute to pathogenesis in DSRCT. In addition to potential autocrine growth pathways, the characteristic histological appearance of DSRCT, small nests of tumor cells surrounded by a dense desmoplastic reaction, also suggests paracrine interactions between tumor and surrounding stroma. A fraction (14/43) of DSRCT tumors contain abundant dense core or neurosecretory-like granules detectable by electron microscopy (Ordonez, 1998). In addition, lysosome-like vesicles, whose secretion is regulated by a similar mechanism, have been linked to the release of some leaderless secretory proteins, such as interleukin 1β (IL-1β) (Andrei et al., 1999; Andrews, 2000; Blott and Griffiths, 2002). Growth factors that lack a leader sequence, such as fibroblast growth factors 1 and 2 (FGF-1 and FGF-2), are also dependent upon regulated exocytotic pathways for their secretion (Friesel and Maciag, 1999). These growth factors have been shown to mediate cellular transformation in vitro (Blam et al., 1988; Jouanneau et al., 1991; Opalenik et al., 1995; Rogelj et al., 1988) and to be associated with enhanced tumor growth in vivo (Kandel et al., 1991; Samaniego et al., 1995). DSRCT samples express significant levels of both *FGF-1* and *2* (R.E.P. and W.L.G., unpublished data), making them potential targets for BAIAP3-dependent secretion in this tumor. In addition, growth factors that do have a leader sequence, including vascular endothelial growth factor (VEGF) and hGH, may be secreted through similar pathways in specific cell types (Valter et al., 1999; Wick et al., 1993). The establishment of DSRCT cell lines and functional assays will be required to determine which growth factors may be critical for tumor growth. Targeting the regulated exocytotic pathway may provide therapeutic options in this otherwise refractory cancer.

## Experimental procedures

### Hybridization to oligonucleotide microarrays and promoter characterization

U2OS cells with tetracycline-regulated, inducible expression of HA-tagged EWS-WT1(−KTS) were generated as described (Lee et al., 1997) and maintained in tetracycline (1 μg/ml). Expression profile analysis was performed using standard procedures for HuGeneFL chips (Affymetrix) (Lockhart et al., 1996). Briefly, poly(A)$^+$ RNA was obtained from cells grown in the presence or absence of tetracycline for 12 hr, and used to generate labeled cRNA probes that were hybridized to oligonucleotide microarrays. A total of 21 predicted targets (known genes and ESTs) were identified based on a greater than 2 fold change in duplicate experiments, and for all candidates, induction by EWS-WT1(−KTS) was analyzed by Northern blotting. RT-PCR, 5′-RACE, and database mining were used to determine the approximate transcriptional start site of the transcriptional target, *BAIAP3*. Two isoforms have been described, *BAIAP3*α (AL834321) and *BAIAP3*β (AB017111). Only products representing *BAIAP3*α were detected; therefore, this isoform was used for all subsequent analyses. For luciferase reporter assays, *BAIAP3*α promoter sequences were cloned into the promoterless luciferase reporter pGL3 (Promega), and 0.2 μg of the reporter was cotransfected into U2OS cells (24-well plates), along with 0.15 μg CMV-driven expression constructs encoding EWS-WT1(−KTS), EWS-WT1(+KTS), WT1(−KTS), or empty vector. Thymidine kinase renilla reporter (5 ng) was cotransfected as a control for transfection efficiency, and luciferase activity was assayed using the Dual-Luciferase Reporter assay system (Promega). All experiments were performed in triplicate.

### Electrophoretic mobility shift assays and chromatin immunoprecipitation

For EMSA, polynucleotide-labeled probes (50,000 cpm) were incubated for 20 min at room temperature with 500 ng of GST-conjugated EWS-WT1 zinc finger domains, in binding buffer as described (Lee et al., 1999). For chromatin immunoprecipitations, expression of HA-tagged EWS-WT1(−KTS) in U2OS cells was induced by removal of tetracycline for 24 hr, and DNA-protein crosslinking was performed as described (Meluh and Broach, 1999) with slight modifications. Formaldehyde was added to a final concentration of 1% and cells were incubated at room temperature for 20 min. Cellular lysis and DNA shearing were performed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris [pH 7.6], 1× protease inhibitor cocktail [Boeringer Mannheim]) with sonication to achieve an average DNA length of 400–500 bp. Samples were diluted to a final concentration of 0.1%SDS and incubated overnight at 4°C with either 5 μl rabbit polyclonal anti-WT1(C-19) (Santa Cruz), 2 μl rabbit polyclonal anti-HA (Sigma), 1 μl mouse monoclonal anti-acetylated histone H4, 2 μl rabbit preimmune serum (mock IP), or without an antibody. Samples were incubated with protein G sepharose and bound complexes were eluted with 1% SDS, 0.1 M NaHCO3. Samples were incubated 4 hr at 65°C to reverse crosslinking and proteins were digested with Proteinase K. Following purification of DNA fragments (Qiaquick PCR purification kit, Qiagen), promoter sequences were amplified using multiplex PCR. The following primer sets were used: *BAIAP3*, nt 1346–1761, accession AL031709; β-*actin*, nt 824–1103, accession number E00829. Coamplification of β-actin within a multiplex reaction served as an internal control for enrichment of BAIAP3 promoter sequences.

### BAIAP3 cellular localization studies

The entire open reading frame of *BAIAP3*α was cloned, along with a 3′ HA-tag, into the CMV-driven plasmid pcDNA3. This construct lacks the first N-terminal 35 amino acids of the β transcript, whose presence could not

be confirmed in the endogenous transcript of cells tested. For cellular colocalization studies, CMV-BAIAP3 was cotransfected into PC12 cells, along with a construct encoding human growth hormone (hGH) (pXGH5; Nichols Institute Diagnostics) using Lipofectamine 2000 (Invitrogen). Immunofluorescence was performed as described (Englert et al., 1995), using antibodies to the HA-epitope (12CA5) to detect HA-tagged BAIAP3, anti-growth hormone (T-20) (Santa Cruz), rhodamine conjugated anti-goat antibody (Jackson ImmunoResearch), and FITC conjugated anti-mouse antibody (Jackson ImmunoResearch). Samples were examined using a Zeiss Axiovert 100 M confocal microscope and analyzed with Laser Scanning Microscope 510 imaging system. For cell fractionation experiments, cells were lysed in water followed by the addition of 2× HES (40 mM HEPES [pH 7.1], 0.5 M sucrose, 2 mM EDTA, 2× protease inhibitor cocktail) and passed through a 28.5 gauge needle. After centrifugation at 16,000 × g for 20 min, the supernatant was collected and centrifuged at 46,000 × g for 20 min to pellet the high-density microsomes (HDM). The supernatant was centrifuged at 196,000 × g for 1 hr to separate the cytosol (supernatant) from the low-density microsomes (LDM) (pellet). Equal amounts of protein were separated by SDS-PAGE and transferred to immobilon P membranes for immunoblot analysis. For further fractionation in sucrose gradients, the HDM and LDM were collected together using a single 196,000 × g centrifugation for 1 hr. The pellet was resuspended in 1× HES, layered onto a discontinuous sucrose gradient of 0.5 M, 1 M, 1.25 M, 1.5 M, 1.7 M sucrose with 10 mM HEPES (pH 7.1) and 150 mM NaCl, and centrifuged in a swinging bucket rotor at 200,000 × g for 2.5 hr, and 0.5 ml fractions were collected. Proteins were collected and separated by SDS-PAGE for immunoblot analysis. In addition to antibody against the HA epitope, antibodies used were anti-synaptophysin SVP-38 (Sigma), anti-synaptotagmin (Transduction Laboratories), and anti-RhoGDi (Transduction Laboratories).

**RNA in situ hybridization**

In situ hybridization was performed as described (Morgan et al., 1998). SP6 and T7 flanked PCR templates were used to generate digoxigenin-labeled riboprobes (Roche Molecular Biochemicals). The following primer sequences were used: mouse *BAIAP3*, nt 45–504, accession BB609845; human *BAIAP3*, nt 3663–4195, accession AB017111. Frozen sections of mouse organs were prepared from 8-week-old FVB mice. Embryonic brain sections were prepared from E18.5 mice and provided by S. Matheson (Massachusetts General Hospital). Primary DSRCT tumor samples were obtained from the Department of Pathology, Memorial Sloan Kettering Cancer Center. Human prostate and breast primary tumor samples were obtained from C. Wu and D. Sgroi (Massachusetts General Hospital). OTC embedded samples were cut into 9 μm sections and fixed in 4% PFA, digested with Proteinase K (4 μg/ml), treated with acetic anhydride, and dehydrated in increasing concentrations of ethanol. Sections were hybridized overnight with 1 ng/μl probe. Bound probe was detected using an alkaline phosphatase conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals) followed by incubation with BM purple (Roche Molecular Biochemicals), an alkaline phosphatase substrate. Control sense riboprobes were tested for each tissue.

**Cell proliferation and transformation assays**

Serum dependence was determined by seeding equal numbers of cells ($3 \times 10^3$) from individual clones onto 60 mm plates and incubating at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS). After 24 hr, culture medium was replaced with DMEM-0.5% FBS, and viable cells were counted at sequential intervals. Multiple BAIAP3-expressing and vector-transfected clones were tested in duplicate. For colony formation in soft agarose, assays were performed in duplicate, as described (www.pmci.unimelb.edu.au/core_facilities/manual/cb2020.asp), and colony formation was assessed after 12 days. For tumor formation assays in nude mice, $3 \times 10^6$ cells in 100 μl serum-free DMEM were injected, for each cell line, into the right flank of 6- to 8-week-old nude mice (Charles River), and mice were observed for up to 90 days. Tumors were harvested when the diameter was 1 cm.

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