Association of v-ErbA with Smad4 Disrupts TGF- β Signaling

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Disruption of the transforming growth factor- β (TGF- β) pathway is observed in the majority of cancers. To further understand TGF- β pathway inactivation in cancer, we stably expressed the v-ErbA oncoprotein in TGF- β responsive cells. v-ErbA participates in erythroleukemic transformation of cells induced by the avian erythroblastosis virus (AEV). Here we demonstrate that expression of v-ErbA was sufficient to antagonize TGF- β -induced cell growth inhibition and that dysregulation of TGF- β signaling required that v-ErbA associate with the Smad4 which sequesters Smad4 in the cytoplasm. We also show that AEV-transformed erythroleukemia cells were resistant to TGF- β -induced growth inhibition and that TGF- β sensitivity could be recovered by reducing v-ErbA expression. Our results reveal a novel mechanism for oncogenic disruption of TGF- β signaling and provide a mechanistic explanation of v-ErbA activity in AEV-induced erythroleukemia.

INTRODUCTION

The transforming growth factor- β (TGF- β) signaling pathway is a major signaling network that controls cell proliferation, differentiation, and tumor suppression (Massague *et al.*, 2000; Attisano and Wrana, 2002; Wakefield and Roberts, 2002). The TGF- β cytokine signals through hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors, TGF- β type I (T β RI) and type II (T β RII) receptors (Heldin *et al.*, 1997; Massague *et al.*, 2000). On ligand binding to the T β RII receptor, T β RII phosphorylates T β RI and activates the T β RI kinase (Wrana *et al.*, 1992; Chen and Weinberg, 1995). The signal is further propagated by phosphorylation of the downstream signal transducers Smad2 and Smad3 by the type I receptor kinase (Heldin *et al.*, 1997). Phosphorylated Smad2 and Smad3 form a complex with Smad4 and translocate into the nucleus (Massague *et al.*, 2000). These Smad complexes then interact with other transcription factors and bind the promoter regions of TGF- β -responsive genes to regulate gene expression (Massague *et al.*, 2000).

Insensitivity to antigrowth signals such as TGF- β is common in cancer cells (Hanahan and Weinberg, 2000; Massague *et al.*, 2000). Multiple genetic mutations are observed in TGF- β -insensitive tumor cells, and mechanisms have been identified that explain the disruption of TGF- β 's cytosolic activity. These mechanisms include the down-regulation of TGF- β receptors, mutations in TGF- β signaling components such as Smad4, and mutations in other downstream targets (Hanahan and Weinberg, 2000; Massague *et al.*, 2000). Overexpression of oncoproteins is also implicated in the disruption of TGF- β signaling. Tumor cells insensitive to TGF- β are known to overexpress diverse oncoproteins such as Cdk4, Ski, Evi-1, Ras, Mdm2, E6/E7, E1A, and PLZF-

RAR α (Pietenpol *et al.*, 1990; Ewen *et al.*, 1993; Datto *et al.*, 1997; Kurokawa *et al.*, 1998; Sun *et al.*, 1998; Hanahan and Weinberg, 2000; Massague *et al.*, 2000; La *et al.*, 2003; Luo, 2004). Overexpression of HPV-16 E7 and E1A, for example, is shown to cause TGF- β resistance in skin keratinocytes by preventing TGF- β -induced down-regulation of c-myc transcription (Pietenpol *et al.*, 1990). Expression of E1A is also known to overcome TGF- β -regulated growth inhibition by blocking induction of the cyclin-dependent kinase inhibitors, p15^{Ink4B} and p21^{Cip1} (Datto *et al.*, 1997). Although some of the mechanisms by which tumor cells are rendered insensitive to TGF- β are known, additional mechanisms likely remain unidentified.

Although the oncogenic mechanism of erythroleukemia induced by the avian erythroblastosis virus (AEV) involves the dysregulation of a yet unidentified signaling pathway, mechanisms involving cell-growth inhibition from TGF- β pathway activation have not been previously demonstrated (Damm and Evans, 1993). AEV effectively blocks erythroid differentiation and suppresses erythrocyte-specific gene transcription (Graf and Beug, 1983; Rietveld *et al.*, 2002). AEV carries two oncogenes, *v-erbA* and *v-erbB*, that work cooperatively to induce erythroleukemia and sarcomas (Vennstrom *et al.*, 1980; Graf and Beug, 1983). *v-ErbB*, a mutated and truncated viral variant of the epidermal growth factor receptor, retains its transforming activity when expressed alone (Lin *et al.*, 1984). *v-ErbA*, on the other hand, is derived from the fusion of a fragment of the retroviral group antigen (gag) region and a mutated variant of thyroid hormone receptor α (TR α /c-ErbA; NR-1 subfamily; Damm *et al.*, 1989; Zenke *et al.*, 1990). This gag sequence is required for its biological activity (Hayman *et al.*, 1979; Zenke *et al.*, 1990). Though v-ErbA lacks the ability to transform cells when expressed alone, it is often described as a potent oncogenic enhancer of cellular transformation because of its apparent ability to block cell differentiation and proliferation pathways (Graf and Beug, 1983; Sap *et al.*, 1986; Weinberger *et al.*, 1986). Though the molecular mechanism underlying the function of v-ErbA in cellular transformation remains elusive (Thormeyer and Baniahmad, 1999; Rietveld

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et al., 2001), investigators speculate that v-ErbA antagonizes a major tumor suppressor pathway to enhance cellular transformation (Hayman *et al.*, 1979; Vennstrom and Bishop, 1982).

The purpose of this study was to determine whether v-ErbA, when stably overexpressed, dysregulates the TGF- β signaling pathway. We demonstrate that v-ErbA antagonizes TGF- β -induced cell-growth inhibition and that pathway dysregulation requires that v-ErbA complex with Smad4 and disrupt Smad4 nuclear localization. Expression of v-ErbA was found to confer TGF- β resistance in HD3 erythroleukemic cells and that increased TGF- β sensitivity was observed in these cells upon reduction of v-ErbA expression. Mutations in the zinc-finger region of v-ErbA were found to abolish v-ErbA/Smad4 association and were unable to dysregulate TGF- β signaling. We believe our work uncovers a mechanism of v-ErbA oncogenicity that underscores the importance of TGF- β signal dysregulation in oncogenic transformation.

MATERIALS AND METHODS

Tissue Culture and TGF- β -induced Growth Inhibition Assays

PE25, SW480, and 293T cell lines were incubated at 37°C at 5% CO₂ and cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. For growth inhibition assays, PE25 cells were plated at low density (approximately 15K cells/well) on six-well plates and after 8–18 h were subjected to 0, 1, 5, 10, or 50 pM concentrations of TGF- β . Medium and TGF- β were replaced every 3–4 d. After approximately 2 wk, the cells were washed with phosphate-buffered saline (PBS), fixed with 70% ethanol for 1 h, crystal violet stained for 1 h, and stored in water with 10% glycerol. HD3 cells were incubated at 37°C at 5% CO₂ and cultured in DMEM medium supplemented with 8% fetal bovine serum, 2% heat-inactivated chicken serum (1 h at 56°C), 10 mM HEPES (pH 7.4), 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Retroviral-mediated Gene Transfer and Cell Sorting

Retroviral supernatants were produced by cotransfecting the pREX-CD2-IRES-GFP, pREX-v-ErbA-IRES-GFP, pREX-delZF-IRES-GFP, pREX-C308A-IRES-GFP, pREX-GFP-Smad4, pREX-GFP-Smad4-nuc, pREX-v-ErbA-mC-CD2, pREX-delZF-mC-CD2, or pREX-C308A-mC-CD2 retroviral packaging vectors with pCL-Amp (Naviaux *et al.*, 1996) into 293T cells using FuGene 6 (Roche, Indianapolis, IN) and collecting medium 48, 72, and 96 h after transfection. PE25 or SW480 cells were plated at low density and incubated with retroviral supernatant and 10–20 μ g/ml polybrene for 8–16 h. The supernatant was replaced with fresh medium. Infected cells were enriched using a Dako Cytomation MoFlo cell sorter (Carpinteria, CA) using green fluorescent protein (GFP) or CD2 reporter expression to approximately two orders of magnitude above background fluorescence levels of uninfected cells.

Reporter Gene Construct and Luciferase Assays

The PE25 cell line used in this study was stably transfected with pGL3-PE2.1 (Hua *et al.*, 1998). This construct contains two tandem copies of a 36-base pair fragment containing the Smad-binding elements (SBEs) of the human PAI-1 promoter as previously described (Hua *et al.*, 1999). Infected and enriched PE25 cells were counted and plated at 4×10^4 cells/well in triplicates on 12-well plates. After 8–16 h, medium was replaced with low-serum (0.5%) medium for 24 h before treatment with 100 pM TGF- β for 20 h. Cells were washed with PBS (3 $\times$) and prepared using 200 μ l passive lysis buffer (1 $\times$; Promega, Madison, WI) and stored at –80°C. The manufacturer's protocol for the Luciferase Assay System (Promega) was followed using 80 μ l of substrate and 100 μ l of the prepared lysate. Activities were measured on a Dynex Technologies MLX Luminometer (Franklin, MA). Means (bars) and SEs (error bars) were reported.

Immunoblotting

Whole cell lysates were prepared from infected and enriched PE25 cells using lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% NP40, 400 mM NaCl, 15% glycerol, 50 mM NaF, 1 mM NaVO₄, 10 mM β -glycerolphosphate, and protease inhibitors). Whole cell lysates were resolved using 8% SDS-PAGE and transferred to nitrocellulose membrane. Anti-FLAG (M2; Stratagene, La Jolla, CA) primary and anti-mouse IgG HRP-conjugated secondary (Am-

ersham, Piscataway, NJ) antibodies were used with West Dura detection substrate (Pierce).

Small Interfering Knockdown of gag-Ear2 in HD3 Cells and Thymidine Incorporation Assay

Small interfering (siRNA) for the knockdown of v-erbA was prepared as described by Myers *et al.* (2003). A 509-bp fragment in the gag region of v-erbA was PCR amplified to create a DNA template using primers with that included the T7 promoter sequence: 5'- GCGTAATACGACTCACTATAGGAT-GGAAACCGTCATAAAGGTG, 3'- GCGTAATACGACTCACTATAGGG-GAGGAGCCGAGGCTGTGACG. Purified RNA was produced from the template using the MEGAscript RNAi Kit (Ambion, Austin, TX). Diced siRNA was produced using the ShortCut RNAi Kit to produce 22-base pair double-strand RNA (dsRNA) fragments that were then purified by ethanol precipitation. HD3 cells (a gift of Scott Ness, University of New Mexico) were seeded in a 12-well plate at 100,000/well 6 h before transfection in 1 ml of media. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) transfection reagent was used per the manufacturer's directions using 100 pmol of either the gag-siRNA or BLOCK-IT Fluorescent Oligo (Invitrogen) control siRNA. The media was replaced 4 h after transfection. Cells were incubated for 48 h, and the transfection was then repeated. Four hours after the second transfection, the control cells and gag-siRNA cells were each replated with new media into seven wells. Twenty-four hours after the second transfection, triplicate control and gag-siRNA wells were treated with TGF- β (100 pM). Twenty hours after TGF- β treatment, 4 μ Ci ³H-radiolabeled thymidine was added to six control wells and six gag-siRNA wells and incubated for an additional 4 h. One well each of unlabeled control and gag-siRNA cells was collected and used for quantitative RT-PCR analysis. The remaining samples were collected and washed in cold PBS. Cells were fixed in 5% trichloroacetic acid for 30 min at 4°C followed by three washes with water. Acid-insoluble materials were dissolved in 0.5 ml of 0.1 M NaOH. Samples were then counted in a Beckman LS3801 scintillation counter (Fullerton, CA) with means (bars) and SDs (error bars) reported.

In Vitro Binding Assay

The coding sequence of v-ErbA was cloned into the pRK5 vector using standard cloning techniques to provide a DNA template for in vitro translation. Mutants of v-ErbA were incorporated into pRK5-verbA using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) followed by DNA sequencing of the region of interest to confirm the desired nucleotide deletions or substitutions. Radiolabeled protein was expressed using ³⁵S-labeled methionine and the TnT Coupled Reticulocyte Lysate System (Promega) following the manufacturer's protocol. Twenty microliters of each lysate was incubated (slow rotation) at 4°C for 1 h with 0.5 μ g of purified recombinant glutathione S-transferase (GST), GST-Smad2, GST-Smad3, or GST-Smad4 proteins. Twenty microliters of glutathione Sepharose 4B (Amersham) slurry was added to each lysate and incubated at 4°C for an additional 2 h. Sepharose beads were centrifuged and washed (4 $\times$) with NETN buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM DTT, 1% NP40, and 100 mM NaCl). Bound proteins were eluted with SDS loading buffer and resolved using 8% SDS-PAGE. Signals were detected on an Amersham Biosciences Typhoon 9400 imaging system.

Fluorescence Microscopy and Nucleo-Cytoplasmic Analysis

Cells plated on glass coverslips were treated as described, fixed with 4% paraformaldehyde in PBS for 1 h, rinsed in water, and mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were acquired with a Nikon inverted microscope (Melville, NY) with a 60 $\times$ 1.4 NA oil immersion objective, GFP/YFP/RFP dichroic mirror, and single-band excitation and emission filters (Chroma Technology, Brattleboro, VT) and a CoolSNAP ES camera (Photometrics, Tucson, AZ). Excitation light was from an X-Cite 120 Fluorescence Illumination System (Mississauga, ON, Canada). Metamorph software, ver.6.3r5 (Universal Imaging, Downingtown, PA) was used for image acquisition. Images were analyzed using ImageJ (NIH, <http://rsb.info.nih.gov/ij/>) with the Multi-Measure ROI manager Plugin (<http://www.optinav.com/Multi-Measure.htm>). Background fluorescence was removed from all images before analysis. Nuclear masks were created using DAPI-stained images, and both GFP and mCherry images were used to create masks of the cell periphery. Using these masks, the mean fluorescence for each cell was measured and used to calculate the nuclear:cytoplasmic ratio. Means and SDs of these ratios from a minimum of 20 cells for each condition were reported.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare differences among groups of three or more followed by Tukey's post hoc test to assess differences within groups. Significance between two groups was demonstrated by one-tailed *t* test.

RESULTS

v-ErbA Expression Dysregulates TGF- β Signaling

It is well documented that a majority of cancer cells have lost their sensitivity to TGF- β . To gain further insight into the mechanism by which tumor cells evade the action of TGF- β , we performed a genetic screen to isolate cDNAs, that when ectopically expressed, rendered cells insensitive to TGF- β . Unexpectedly, we isolated an *ear2* sequence in a cDNA library screen that, when linked in frame to remnants of a *gag* sequence of the Moloney murine leukemia virus, exhibited resistance to TGF- β -induced cell-growth inhibition (data not shown). The nuclear orphan receptor, *ear2* (*erbA* related gene 2), is a member of the COUP-TF (NR2) subfamily of nuclear hormone receptors and shares homology with TR α (Miyajima *et al.*, 1988). Whereas *gag-ear2* fusion is artificially created during the construction of cDNA library, fusion proteins between a viral *gag* sequence and nuclear hormone receptors have been identified in nature (Grignani *et al.*, 1998; Lin and Evans, 2000; La *et al.*, 2003). An example is the oncoprotein v-ErbA, which is composed of an N-terminal *gag* sequence fused to a mutated form of thyroid hormone receptor α (TR α /c-ErbA; Vennstrom and Bishop, 1982). We were prompted to test whether v-ErbA might disrupt TGF- β signal transduction activity when we isolated an *ear2* sequence in a cDNA library screen that, when linked in frame to remnants of a *gag* sequence of the Moloney murine leukemia virus, exhibited resistance to TGF- β -induced cell-growth inhibition. The nuclear orphan receptor, *ear2* (*erbA* related gene 2), is a member of the COUP-TF (NR2) subfamily of nuclear hormone receptors and shares homology with TR α (Miyajima *et al.*, 1988). Using retroviral gene-mediated transfer, the full-length coding sequence of v-ErbA with a C-terminal-Flag-tag was stably expressed in the mink lung epithelial cell line (PE25) that was previously modified to express an ectopic receptor and the TGF- β -responsive PE2.1 luciferase reporter gene (Liu *et al.*, 1997; Hua *et al.*, 1998). Immunoblot analysis confirmed expression of the fusion protein (Figure 1A). A control cell line was also created using PE25 cells by stably expressing a fragment of CD2 and was growth inhibited above 1 pM TGF- β demonstrating that TGF- β sensitivity was not disrupted by the retroviral gene-mediated transfer technique (Figure 1B; Liu *et al.*, 1997). In contrast, PE25 cells expressing v-ErbA were resistant to TGF- β at concentrations of 10 pM (Figure 1B). Thus, stable expression of v-ErbA but not the CD2 control enables cells to escape TGF- β -induced growth arrest.

We hypothesized that resistance to TGF- β growth inhibition upon expression of v-ErbA may result from interference of TGF- β -mediated transcriptional regulation. To determine whether expression of v-ErbA affects TGF- β -regulated gene expression, we measured the luciferase activity in the PE25 cells stably expressing either CD2 (control) or v-ErbA in the absence or presence of TGF- β . The luciferase reporter gene stably transfected in the PE25 cell line is driven by a promoter containing two Smad-binding elements (SBes; Hua *et al.*, 1998). We emphasize that this reporter is stably integrated into the genome, thus ensuring that the behavior of the reporter's promoter is similar to that of an endogenous promoter within the native chromatin environment. As expected, we observed a large increase in luciferase activity in the control cells upon TGF- β treatment (Figure 1C, lane 1 vs. lane 2). In cell lines expressing v-ErbA, the luciferase activity was greatly diminished in both untreated and TGF- β -treated conditions (lanes 3 and 4). These data show that the activity of the TGF- β -responsive reporter correlates well with the escape of TGF- β -induced growth inhibition upon

Figure 1. v-ErbA expression dysregulates TGF- β signaling. (A) Immunoblot demonstrating expression of v-ErbA-Flag-tagged protein expressed in PE25 mink lung cells. (B) PE25 mink lung cells stably expressing either CD2 (control) or v-ErbA plated at low density and grown in the presence of 0, 1, 5, 10, 25, or 50 pM TGF- β for approximately 10 d. Cells were fixed and stained with crystal violet. CD2 (control) cells were unable to proliferate in the presence of 5 pM or greater TGF- β . Cells expressing v-ErbA proliferated in the presence of higher TGF- β concentrations. (C) Luciferase assay using cells (PE25) with a stably transfected reporter responsive to TGF- β . Cells lines additionally expressed CD2 (control) or v-ErbA. Means (bars) and SDs (error bars) are shown. Increased reporter response observed in CD2 (control) cells (lane 1 vs. lane 2) in the presence of TGF- β (24 h at 100 pM). Cells expressing v-ErbA demonstrated basal activity near background levels (lane 3) and greatly reduced reporter response in the presence of TGF- β (lane 4).

v-ErbA expression (Figure 1B). Specifically, the CD2 control cells were growth inhibited in the presence of TGF- β and had high levels of transcriptional activity. In contrast, cells expressing v-ErbA were both resistant to TGF- β treatment and exhibited a greatly reduced transcriptional response.

v-ErbA Expression Causes TGF- β Resistance in Erythroblasts

v-ErbA was originally isolated from chicken erythroblast cells unable to terminally differentiate in the presence of AEV (Frykberg *et al.*, 1983). Because growth inhibition is an essential step in erythroblast differentiation that leads to erythrocyte formation, we hypothesized that erythroblasts expressing AEV would be insensitive to TGF- β . HD3 cells were considered ideal to investigate this hypothesis because they are chicken erythroblasts transformed by AEV and express both native v-ErbA and v-ErbB (Beug *et al.*, 1982). To investigate the hypothesis that HD3 erythroblasts expressing AEV would be insensitive to TGF- β , we performed a thymidine incorporation assay in the presence or absence of TGF- β in HD3 cells. Cells were either left untreated or treated with 100 pM TGF- β for 24 h and then radiolabeled with [3 H]thymidine before harvesting. HD3 cells treated with TGF- β showed levels of thymidine incorporation statistically similar to untreated cells (Figure 2A). Therefore, the unchanged levels of thymidine incorporation in the presence or absence of TGF- β indicated that HD3 cells were TGF- β resistant.

Figure 2. HD3 erythroblasts with reduced *v-erbA* mRNA expression recover TGF- β sensitivity. (A) HD3 cells treated with TGF- β (100 pM, 24 h). [³H]thymidine incorporation indicates no reduction in cell growth upon TGF- β treatment ($p > 0.05$, t test). (B) HD3 cells transfected with either control-siRNA or *gag*-siRNA. Quantitative PCR shows a 31% knockdown of *v-erbA* expression in HD3 cells upon treatment with *gag*-siRNA relative to cells expressing control-siRNA ($p < 0.01$, t test). (C) Cell growth measured by [³H]thymidine incorporation. One-way ANOVA indicated statistically significant differences within the treatment group ($\pm$ TGF- β , $\pm$ *gag*-siRNA, and $\pm$ control-siRNA; $p > 0.05$). Differences within the group assessed by Tukey's post hoc test and denoted as follows: * $p < 0.05$; ** $p < 0.01$; + $p > 0.05$. Cell growth not reduced by TGF- β treatment (100 pM, 24 h; lanes 1 vs. lane 2). Cell growth was reduced 41% upon *v-erbA* knockdown only (lane 1 vs. lane 3) and reduced 58% with *v-erbA* knockdown and TGF- β treatment (lane 1 vs. lane 4). Growth reduction of 28% upon TGF- β treatment in *v-erbA* knockdown cells demonstrates acquisition of TGF- β sensitivity (lane 3 vs. lane 4).

We further hypothesized that if *v-ErbA* expression was reduced in the AEV transformed HD3 cells, the cells would both revert to a growth-inhibited state and become sensitive to TGF- β . We used siRNA-mediated gene silencing to down-regulate *v-erbA* mRNA expression. The siRNA was targeted to the *gag* sequence of *v-erbA* and produced using a published method (Myers *et al.*, 2003). We chose to target only the *gag* region to avoid potential degradation of endogenous TR α or other nuclear receptors that likely share similar sequence homologies. To demonstrate *v-erbA* down-regulation, HD3 cells were transiently transfected with either a negative-control siRNA or the siRNA duplexes targeted to *v-erbA*. Expression levels of *v-erbA* mRNA were reduced by 31% in transfected cells compared with HD3 cells transfected with an equivalent amount of control siRNA (Figure 2B). Thus, endogenous expression of *v-erbA* in an AEV-transformed cell line was down-regulated with the *gag*-targeted siRNA. We then used a thymidine incorporation assay to measure the effect of this partial depletion of *v-erbA* on the proliferation of HD3 cells in the presence or absence of TGF- β . Control siRNA or *gag*-targeted siRNA-transfected cells were either untreated or treated with 100 pM TGF- β for 24 h and then radiolabeled with [³H]thymidine before harvesting. As expected, the proliferation of HD3 cells transfected with control siRNA remained statistically unchanged upon TGF- β treatment (Figure 2C, lanes 1 and 2). In contrast, cells transfected with *gag* siRNA demonstrated a significant 41% reduction in thymidine incorporation under basal conditions (lane 1 vs. lane 3) and a significant 58% reduction in

thymidine incorporation upon TGF- β treatment (lane 2 vs. lane 4). The reduction in thymidine incorporation upon *gag* siRNA transfection without TGF- β treatment may arise from a combination of *v-ErbA*'s overall oncogenic activities. One contributing effect may be that upon *v-ErbA* reduction, the TGF- β pathway becomes reactivated by basal or autocrine signaling. Additionally, the observed reduction in thymidine incorporation may reflect a reduction of oncogenicity from other known pathways affected by *v-ErbA* such as constitutive transcriptional repression of target gene expression upon *v-ErbA* binding to *v-ErbA* response elements (Ciana *et al.*, 1998). More importantly, we observed a significant 28% decrease in thymidine incorporation upon TGF- β treatment in *gag* siRNA-transfected cells, suggesting that HD3 cells acquire TGF- β sensitivity when *v-ErbA* levels are reduced (lane 3 vs. lane 4).

In summary, we show that HD3 cells are insensitive to TGF- β and that TGF- β sensitivity is recovered in these cells by down-regulating expression of *v-erbA*. These results support our hypothesis that *v-ErbA* expression dysregulates TGF- β signaling.

v-ErbA Associates with Smad4 and Disrupts Smad4 Nuclear Localization

We hypothesized that the mechanism of TGF- β pathway disruption observed in cells expressing *v-ErbA* depends on the ability of this fusion protein to associate with one or more of the Smad transcription factors. To address this possibility, we performed direct protein-binding assays in

which GST-tagged, recombinant Smad2, Smad3, and Smad4 proteins were incubated with ^{35}S -radiolabeled v-ErbA prepared by *in vitro* translation (IVT). Little or no binding was observed between a GST-only negative control and v-ErbA and thus eliminated the possibility that v-ErbA could interact with the GST-tag of the recombinant Smad proteins (Figure 3A, lane 2). We also observed that GST-Smad2 and GST-Smad3 bound minimally to v-ErbA (Figure 3A, lanes 3 and 4). In contrast, v-ErbA efficiently bound Smad4 at a level greater than the 10% control input (lane 5 vs. lane 1). To confirm that the gag region was essential for Smad binding to v-ErbA, an ErbA-only (gag deleted) construct was created and radiolabeled. As we observed with full-length v-ErbA protein, ErbA-only demonstrated minimal association with GST-only, Smad2, and Smad3 (Figure 3A, lanes 2-4). In contrast to v-ErbA, increased binding was not observed between ErbA-only and Smad4 and thus suggests that the gag region is necessary for v-ErbA/Smad4 association (lane 5). Taken together, these *in vitro* experiments indicate that v-ErbA binds Smad4 and that v-ErbA/Smad4 association requires the presence of the gag region of v-ErbA.

Although the *in vitro* binding experiments imply that v-ErbA/Smad4 association may be involved in dysregulation of TGF- β signaling, this result does not suggest an obvious mechanism by which this interaction may convey its influence. A recent study revealed that v-ErbA is predominantly localized in the cytosol because of the presence of a CRM1-dependent nuclear export sequence within the C-terminal portion of the p10 region of gag (DeLong *et al.*, 2004). Because v-ErbA is predominately restricted to the cytoplasm, we wondered if v-ErbA/Smad4 association may disrupt Smad4 nuclear localization. Previous work demonstrates that v-ErbA, when fused to a fluorescent protein, provides an appropriate probe for studying v-ErbA localization (DeLong *et al.*, 2004).

For this study we C-terminally fused mCherry to v-ErbA (v-ErbA-mC) and used mCherry alone (mC-only) as a control. Moreover, we fused both Smad4 and a Smad4 variant that predominantly accumulates in nucleus as a result of mutations in the nuclear export signals (NES) to GFP and use these fusion proteins to characterize their subcellular localizations (Xiao *et al.*, 2000, 2003). Combinations of Smad4 and v-ErbA expression vectors were then stably expressed in colon-cancer-derived SW480 cells. We used SW480 cells because they are a Smad4-deleted cell line that regains TGF- β pathway activity upon exogenous expression of Smad4 (Schwarte-Waldhoff *et al.*, 1999). To confirm that GFP-Smad4 exhibited the subcellular localization characteristics of endogenously expressed Smad4, we first examined GFP-Smad4 in the presence of the stably expressed mC-only control with or without TGF- β treatment or with leptomycin B (LMB), a known CRM1 inhibitor (Figure 3B, a-l). As expected, we observed GFP-Smad4 in both the nucleus and cytoplasm in the absence of TGF- β treatment (Figure 3Bb), and upon TGF- β or LMB treatment GFP-Smad4 accumulated in the nucleus (Figure 3B, f and j). Thus, GFP-Smad4 nucleo-cytoplasmic distribution is similar to that of the endogenous Smad4 observed in TGF- β -responsive cell lines (Pierreux *et al.*, 2000; Xiao *et al.*, 2003). To quantitatively characterize GFP-Smad4 localization, we created nuclear and cytoplasmic masks of cell images, measured fluorescence in each region, and calculated the nuclear:cytoplasmic ratio (see *Materials and Methods* for details).

As shown in Figure 3C (lane 1 vs. lanes 3 and 5), there are statistically significant 1.24- and 1.89-fold increases in the nuclear:cytoplasmic ratio of GFP-Smad4 upon TGF- β and LMB treatments, respectively. GFP-Smad4-nuc cells exhibited a stronger nuclear localization compared with TGF- β -treated

GFP-Smad4 cells (Figure 3Bn), and we quantitatively confirmed this observation by measuring a statistically significant 2.07-fold increase in the nuclear:cytoplasmic ratio when compared with untreated cells (Figure 3C, lane 1 vs. lane 7). In agreement with the observations of others using YFP-only as a control (DeLong *et al.*, 2004), we also saw slight nuclear localization of the mCherry control for all treatment conditions. However, neither TGF- β treatment nor the expression of GFP-Smad4-nuc affects the subcellular localization of mCherry as the nuclear:cytoplasmic ratios of m-Cherry control are essentially the same under either condition (Figure 3, B, c vs. g and o, and D, lane 1 vs. lanes 3 and 7). On LMB treatment there is small, but statistically significant, 1.34-fold increase the nuclear:cytoplasmic ratio for mCherry only cells (Figure 3, B, c vs. k, and D, lane 1 vs. lane 5), similar to what has been reported for YFP-only control (DeLong *et al.*, 2004). Thus GFP-Smad4, just like the endogenous Smad4, translocates into the nucleus upon TGF- β or LMB treatments, and expression of either wild-type Smad4 or Smad4-nuc does not affect the nuclear:cytoplasmic ratio of mCherry fluorescent protein.

To investigate whether v-ErbA/Smad4 association may disrupt Smad4 nuclear localization, we analyzed Smad4 and v-ErbA subcellular localization in SW480 cells expressing both proteins under various conditions. Without TGF- β treatment, GFP-Smad4 was predominantly localized in the cytoplasm in both v-ErbA-mC and mCherry control cells (Figure 3B, b vs. r), although there was a slight decrease in the nucleo-cytoplasmic ratio of v-ErbA-mC compared with mCherry alone (Figure 3C, lane 1 vs. lane 2). v-ErbA suppressed the ability of GFP-Smad4 to translocate into the nucleus upon TGF- β treatment (a 37.1% decrease in the nucleo-cytoplasmic ratio compared with the mCherry control cells; Figure 3B, f vs. v, and C, lane 3 vs. lane 4). Unexpectedly, v-ErbA also blocks GFP-Smad4 translocation in response to LMB (a 54.5% decrease in the nucleo-cytoplasmic ratio; Figure 3, B, j vs. z, and C, lane 5 vs. lane 6). Furthermore, v-ErbA causes a significant increase in cytoplasmic accumulation of GFP-Smad4-nuc in cells expressing both v-ErbA-mC and GFP-Smad4-nuc (a 67.9% decrease in the nucleo-cytoplasmic ratio; Figure 3B, n vs. dd, and C, lane 7 vs. lane 8). We also examined the nucleo-cytoplasmic distribution of v-ErbA-mC and found no significant difference upon TGF- β or LMB treatment or when Smad4-nuc was expressed (Figure 3B, s vs. w, aa, and ee, and D, lane 2 vs. lanes 4, 6, and 8). We did observe that v-ErbA-mC is consistently more cytoplasmic than mCherry control, which shows a rather diffused localization pattern and that v-ErbA-mC subcellular localization is not affected by TGF- β or LMB treatments or the expression of Smad4-nuc (Figure 3, B and D).

Because nuclear export of both Smad4 and v-ErbA occurs through a CRM1-mediated pathway, the inability of LMB treatment to increase the nuclear localization of these proteins was unexpected (Pierreux *et al.*, 2000; DeLong *et al.*, 2004). To confirm that the v-ErbA-mCherry construct used in this investigation retained CRM1-dependent characteristics similar to wild-type v-ErbA, SW480 cells stably expressing v-ErbA-mCherry but not Smad4 were examined. As expected, v-ErbA-mCherry remains predominately cytoplasmic in the presence or absence of TGF- β (Figure 3E, b and e, and F, lanes 1 and 2). In contrast, LMB treatment induces nuclear accumulation of v-ErbA-mCherry (Figure 3, Eh and F, lane 3). Thus, in the absence of Smad4, inhibition of CRM1 by LMB treatment disrupts nuclear export of v-ErbA.

Taken together, the data support that upon expression of v-ErbA that 1) in the absence of TGF- β treatment, the nuclear:cytoplasmic distribution of Smad4 is similar to that of control cells, 2) when cells are treated with TGF- β or LMB,

Figure 3. v-ErbA/Smad4 association and nucleo-cytoplasmic distribution of Smad4 and v-ErbA. (A) Recombinant GST, GST-Smad2, GST-Smad3, or GST-Smad4 fusion proteins precipitated using GST agarose beads after incubation with in vitro-translated, 35 S-labeled v-ErbA or ErbA-only proteins. v-ErbA associated with Smad4 (lane 5) with greater affinity than with Smad2 or Smad3 (lanes 3 and 4). Only background binding was observed with the ErbA-only fragment to Smad2/3/4 proteins (lanes 3–5). (B) SW480 cells stably expressed GFP-Smad4 or GFP-Smad4-nuc and mCherry-only or v-ErbA-mCherry as indicated. Cells were either untreated, treated with TGF- β (100 pM, 1 h), or treated with LMB (50 ng/ml, 1 h) as indicated. Cells were stained with DAPI to identify nuclei. Images are representative of nucleo-cytoplasmic distribution of GFP-Smad4, GFP-Smad4-nuc, mCherry-only, or v-ErbA-mCherry for each condition. (C) SW480 cells and treatments are as indicated and as described in B. Ratios of the average nuclear and cytoplasmic fluorescence for GFP-Smad4 and GFP-Smad4-nuc were calculated for $n \geq 20$ cells for each condition to quantify changes in nucleo-cytoplasmic distribution. Means (bars) and SDs (error bars) are shown. One-way ANOVA indicated statistically significant differences ($p > 0.05$). Differences within the group assessed by Tukey's post hoc test and denoted as follows: ** $p < 0.01$; * $p > 0.05$. Smad4 nuclear localization increased in cells expressing mCherry control upon TGF- β or LMB treatment or when GFP-Smad4-nuc was expressed (lane 1 vs. lanes 3, 5, and 7). Smad4 nuclear localization was abolished upon TGF- β or LMB treatment and when GFP-Smad4-nuc was expressed in the presence of v-ErbA (lane 2 vs. lanes 4, 6, and 8). (D) Cells, treatment, and analysis methods are identical to C, but ratios of the average nuclear

indicated. Cells were either untreated, treated with TGF- β (100 pM, 1 h), or treated with LMB (50 ng/ml, 1 h) as indicated. Cells were stained with DAPI to identify nuclei. Images are representative of nucleo-cytoplasmic distribution of GFP-Smad4, GFP-Smad4-nuc, mCherry-only, or v-ErbA-mCherry for each condition. (C) SW480 cells and treatments are as indicated and as described in B. Ratios of the average nuclear and cytoplasmic fluorescence for GFP-Smad4 and GFP-Smad4-nuc were calculated for $n \geq 20$ cells for each condition to quantify changes in nucleo-cytoplasmic distribution. Means (bars) and SDs (error bars) are shown. One-way ANOVA indicated statistically significant differences ($p > 0.05$). Differences within the group assessed by Tukey's post hoc test and denoted as follows: ** $p < 0.01$; * $p > 0.05$. Smad4 nuclear localization increased in cells expressing mCherry control upon TGF- β or LMB treatment or when GFP-Smad4-nuc was expressed (lane 1 vs. lanes 3, 5, and 7). Smad4 nuclear localization was abolished upon TGF- β or LMB treatment and when GFP-Smad4-nuc was expressed in the presence of v-ErbA (lane 2 vs. lanes 4, 6, and 8). (D) Cells, treatment, and analysis methods are identical to C, but ratios of the average nuclear

Smad4 is less effectively translocated to the nucleus, 3) even when the strongly nuclear-localizing Smad4 variant is expressed, Smad4-nuc is transported into the cytoplasm, and 4) regardless of whether cells were treated with TGF- β or LMB or whether Smad4-nuc is expressed, the nuclear-cytoplasmic distribution of v-ErbA-mC remains unchanged but overall is more cytoplasmic than the mCherry control.

v-ErbA Mutants Failing to Associate with Smad4 Are Unable to Antagonize TGF- β -induced Cell-Growth and Smad4 Nuclear Localization

To confirm that the interaction between Smad4 and v-ErbA is critical for TGF- β signal disruption, we sought to identify a v-ErbA mutant unable to bind Smad4. As with other nuclear hormone receptors, v-ErbA contains a DNA-binding domain, a hinge region, and a ligand/transactivation domain (Figure 4A; Damm and Evans, 1993; Thormeyer and Banahmad, 1999). To identify the region of v-ErbA involved in Smad4 binding, we generated four v-ErbA C-terminal-deletion mutants and then tested their ability to bind Smad4 in vitro. We found binding to GST-Smad4 was retained upon deletion of residues 404-639, but observed minimal binding when residues 329-639 were deleted (Figure 4B, lane 7 vs. lane 6). These results suggest that a site located between residues 329-404 of v-ErbA, a region that includes a small fragment of the hinge region and the DNA-binding domain, is required for Smad4 association.

Having identified the general region of Smad4/v-ErbA interaction, we chose to further isolate this interaction by creating two constructs possessing mutations within the DNA-binding domain. As in TR α and other nuclear hormone receptors such as the glucocorticoid and retinoic acid receptors, v-ErbA has two tandemly repeated zinc finger motifs (Privalsky *et al.*, 1988). Each of these zinc fingers contain four invariant cysteine residues that coordinate with a single zinc ion to form a structure that enables the DNA-recognition helix within the first zinc finger to complex with its *cis*-acting element (Bonde and Privalsky, 1990). The first v-ErbA mutant deleted both zinc fingers, delZF, by eliminating residues 291-348. The second construct contained a single-point mutation, C308A, which substituted an alanine at the third Zn²⁺-coordinating cysteine within the first zinc

Figure 3 (cont). and cytoplasmic fluorescence for mCherry-only and v-ErbA-mCherry are shown to quantify changes in the nucleo-cytoplasmic ratios of these proteins. One-way ANOVA indicated statistically significant differences within the group ($p > 0.05$), but differences were not significant in the nucleo-cytoplasmic distribution of mCherry upon TGF- β treatment or the expression of GFP-Smad4-nuc (lane 1 vs. lanes 3 and 7) and were significant upon LMB treatment (lane 1 vs. lane 5). Differences in the nucleo-cytoplasmic distribution of v-ErbA-mCherry were also not significant upon TGF- β or LMB treatments or when GFP-Smad4-nuc was expressed (lane 2 vs. lanes 4, 6, and 8). (E) SW480 cells stably expressed v-ErbA-mCherry. Cells were either untreated or treated with TGF- β or LMB as indicated. Images are representative of nucleo-cytoplasmic distribution of v-ErbA-mCherry for each condition. (F) SW480 cells and treatments are as indicated and as described in E. Ratios of the average nuclear and cytoplasmic fluorescence for v-ErbA-mCherry are shown to quantify changes in the nucleo-cytoplasmic ratios of these proteins. Means (bars) and SDs (error bars) are shown. One-way ANOVA indicated statistically significant differences ($p > 0.05$). Differences within the group assessed by Tukey's post hoc test and denoted as follows: ** $p < 0.01$ and + $p > 0.05$. No increase in v-ErbA-mCherry nuclear localization was observed upon TGF- β treatment but v-ErbA-mCherry nuclear localization increased upon leptomycin B treatment.

Figure 4. The DNA-binding domain of v-ErbA is required for association with Smad4. (A) Schematic diagram of the v-ErbA oncoprotein and C-terminal deletion mutants with qualitative assessment of Smad4 binding indicated. (B) Recombinant GST-Smad4 fusion protein precipitated using GST agarose beads in the presence of in vitro-translated ³⁵S-labeled proteins v-ErbA and v-ErbA C-terminal-deletion mutants. Reduced binding to GST-Smad4 observed upon deletion of residues 329-639 (lane 1 vs. lane 6) but not deletion of residues 404-639 (lane 2 vs. lane 7).

finger. These mutants were selected based on a study using site-directed mutagenesis within the DNA-binding domain that abolishes v-ErbA's ability to induce erythroid colony formation (Bonde and Privalsky, 1990). Using these delZF and C308A v-ErbA mutants, we repeated in vitro binding assays to investigate altered Smad4 association. We found both the delZF and the C308A mutants to have reduced association with Smad4 compared with the intact v-ErbA control (Figure 5A, lane 3). The data therefore suggest that for the first zinc finger within DNA-binding domain of v-ErbA is required for Smad4 association.

If v-ErbA/Smad4 association is essential for TGF- β signal dysregulation, we would anticipate that both the delZF and C308A mutants, shown to have impaired binding to Smad4, would recover TGF- β sensitivity. On stable expression of these mutants in PE25 cells (Figure 5B), we found that though some cell growth was observed in the 5 pM-TGF- β wells, both cell line cells exhibited growth inhibition greater than cells expressing v-ErbA (Figure 5C). We also repeated the luciferase assay incorporating the PE2.1-TGF- β -responsive promoter using both the delZF and C308A mutants stably expressed in PE25 cells. The results demonstrate restored promoter activity in cells expressing these v-ErbA mutants (Figure 5D, lanes 5-8 vs. lanes 1-4). Thus, the data show that in contrast to cells expressing wild-type v-ErbA, cells expressing the delZF and C308A v-ErbA mutants are sensitive to TGF- β -induced growth inhibition and no longer repress the activity of a TGF- β -responsive promoter.

If, as proposed, TGF- β signaling is disrupted through the mechanism of v-ErbA/Smad4 interaction and perturbation of Smad4 nuclear accumulation, we would anticipate that v-ErbA mutants that are unable to associate with Smad4 would also be unable to alter Smad4 nuclear-cytoplasmic localization. To investigate this hypothesis a mCherry fusion construct was created for the C308A mutant, C308A-mC, and stably expressed in the SW480 cell lines also expressing either GFP-

Figure 5. v-ErbA mutants fail to associate with Smad4 and are unable to antagonize TGF- β -induced cell-growth. (A) Recombinant GST and GST-Smad4 fusion proteins precipitated using GST agarose beads after incubation with in vitro-translated, 35 S-labeled delZF and C308A v-ErbA mutants and v-ErbA (positive control) proteins. v-ErbA (positive control) demonstrated association with Smad4, but greatly reduced GST-Smad4 affinity observed with the delZF and C308A mutants (lane 3). (B) Immunoblot demonstrating stable expression of C-terminally Flag-tagged v-ErbA mutants in PE25 mink lung cells. (C) PE25 mink lung cells stably expressing the indicated v-ErbA mutants were plated and treated as described in Figure 1B. Cells expressing either the delZF or C308A mutants demonstrated increased sensitivity to TGF- β compared with cells expressing v-ErbA and sensitivity to TGF- β , similar to cells expressing CD2 (control). (D) PE25 cells with the luciferase reporter responsive to TGF- β and additionally expressing CD2 (control), wild-type v-ErbA, delZF mutant, or C308A mutant. Basal reporter activity recovered in cells expressing both the delZF and C308A mutants compared with cells with v-ErbA expression (lanes 5 and 7 vs. lane 3). In the presence of TGF- β (100 pM, 24 h), cells expressing the delZF and C308A mutants recovered reporter activity (lanes 6 and 8 vs. lane 4).

Smad4 or GFP-Smad4-nuc. We examined the localization of Smad4 in the presence of C308A-mC and observed that indeed, without treatment, TGF- β or LMB treatment, or upon Smad4-nuc expression, localization of control cells not expressing v-ErbA (Figure 6A, b, f, j, and n, vs. B, b, f, j, and n). In support of these qualitative observations, changes in the nuclear:cytoplasmic ratios of Smad4 for cells expressing C308A-mC in the untreated condition were compared with those with TGF- β and LMB treatments and with cells expressing Smad4-nuc. We observed statistically significant increases of the nuclear:cytoplasmic ratios of 1.62-, 2.02-, and 2.88-fold (Figure 6B, lane 1 vs. lanes 2-4, respectively). Therefore, upon expression of the C308A-mC mutant, the localization behavior of Smad4 both qualitatively and quantitatively reverted to that observed in control cell lines that did not express v-ErbA.

We postulated that the localization of the C308A mutant might be similar to that of v-ErbA because the NES of v-ErbA is located in the gag region and therefore should be unaffected by the single point mutation within the zinc finger. Because the C308A mutation was within the DNA recognition helix of the zinc finger, we also considered that the balance between nuclear retention (due to DNA binding) and the nuclear export could change. Cell lines expressing C308A-mC with GFP-Smad4 or with GFP-Smad4-nuc enabled us to observe the localization behavior of this mutant. We found that in cells without or with TGF- β and in cells expressing GFP-Smad4-nuc, the cytoplasmic localization of C308A-mC was similar to that of v-ErbA-mC (Figure 6A, c, g, and o, vs. B, s, w, and ee). Also subcellular localization of C308A-mC is indistinguishable from v-ErbA-mC (Figure 6C, lanes 1, 2, and 4). Thus, the localization behavior of the C308A mutant is similar to that of nonmutated v-ErbA for untreated, TGF- β -treated, and in Smad4-nuc-expressing cells.

For cells expressing C308A-mC and treated with LMB, the nucleo-cytoplasmic ratio was similar to that of cells expressing only v-ErbA-mC (Figure 6A, o vs. 3Eh, and Figure 6C, lane 3, vs. 3F, lane 3). This result contrasts with cells expressing both GFP-Smad4 and v-ErbA-mC, where v-ErbA nuclear localization was not increased upon LMB treatment (Figure 3Baa). These results agree with our observation that Smad4-v-ErbA interaction also disrupts nuclear localization of v-ErbA upon CRM1 inhibition by LMB. In summary, cells expressing a v-ErbA mutant that is unable to interact with Smad4 demonstrate increased nuclear localization upon LMB treatment.

Together, the results of these mutation experiments support the hypothesis that the v-ErbA oncoprotein antagonizes TGF- β signaling through its interaction with Smad4 and that v-ErbA association with Smad4 is essential for the dysregulation of TGF- β signaling. These observations additionally support a model in which v-ErbA/Smad4 interaction in conjunction with v-ErbA's predominately cytoplasmic localization disrupts Smad4 cyto-nuclear shuttling necessary for a functional TGF- β pathway.

DISCUSSION

More than two decades have passed since v-ErbA was identified as the independent oncogenic product of AEV responsible for potentiating erythroblast transformation (Hayman *et al.*, 1979; Vennstrom and Bishop, 1982). The cellular homolog of v-ErbA, TR α (c-ErbA), positively regulates target gene expression when bound to its cognate ligand, T3, and negatively regulates TR α /T3 responsive promoters in the absence of ligand. In contrast, the v-ErbA oncoprotein acts as a constitutive transcriptional repressor and is known to interfere with the function of endogenous thyroid hormone receptor as a dominant negative form of TR α (Damm *et al.*,

Figure 6. Canonical Smad4 nucleo-cytoplasmic distribution recovered in the presence of mutant v-ErbA. (A) Representative images of SW480 cells stably expressing fluorescent-fusion proteins of GFP-Smad4, GFP-Smad4-nuc, and mCherry-C308A mutant (as indicated). (B) SW480 cells with stably expressed mCherry-C308A and either GFP-Smad4 (lanes 1–3) or GFP-Smad4-nuc (lane 4) fluorescent-fusion proteins. Treatment and analysis methods are the same as described in Figure 3C. One-way ANOVA indicated statistically significant differences within the group ($p > 0.05$). Differences within the group assessed by Tukey's post hoc test and are denoted as follows: * $p < 0.05$ and ** $p < 0.01$. Smad4 nuclear localization recovered in cells expressing GFP-Smad4-C308A upon TGF- β or LMB treatment or when GFP-Smad4-nuc was expressed (lane 1 vs. lanes 2–4). (C) Cells, treatment, and analysis methods are identical to B, but show ratios of the average nuclear and cytoplasmic fluorescence for mCherry-C308A for each condition. One-way ANOVA indicated statistically significant differences within the group ($p > 0.05$). Differences within the group are assessed by Tukey's post hoc test and are denoted as follows: ** $p < 0.01$ and * $p > 0.05$. Differences in the nucleo-cytoplasmic distribution of mCherry-C308A were not significant upon TGF- β treatment or when GFP-Smad4-nuc was expressed (lane 1 vs. lanes 2 and 3) but were significant upon LMB treatment (lane 1 vs. lane 4). (D) Schematic models of canonical TGF- β signaling (top) and proposed mechanism of signal dysregulation upon v-ErbA expression (bottom).

1989). This dominant negative activity, though, does not fully explain v-ErbA oncogenicity and has lead researchers to propose that v-ErbA most likely disrupts an unidentified pathway involved in the repression of genes required to prevent cellular transformation (Damm and Evans, 1993).

TGF- β -regulated Growth Inhibition Is Disrupted by v-ErbA

In this study we hypothesized that the unidentified pathway disrupted by v-ErbA was the TGF- β signal pathway. The TGF- β pathway is a logical candidate because it regulates cell proliferation and differentiation (Massague *et al.*, 2000; Attisano

and Wrana, 2002; Wakefield and Roberts, 2002), and disruption of TGF- β signaling is observed in the majority of cancers (Hanahan and Weinberg, 2000; Massague *et al.*, 2000). Additionally, growth hormones play a critical role in erythroid progenitors in maintaining a balance between self-renewal and terminal differentiation into erythrocytes. Expression of oncogenes is often linked to the disruption of this delicate balance and results in leukemic transformation with sustained self-renewal and proliferation of erythroid progenitors (Metz, 1994). Although a previous study reports that chick embryo fibroblasts remain growth-inhibited by TGF- β upon v-ErbA expression (Piu *et al.*, 1993), it is known that TGF- β pathway activity varies among different cell lines, and we were prompted to test whether v-ErbA might disrupt TGF- β signaling in epithelial cells. Our data demonstrated that v-ErbA expression enables resistance to TGF- β -induced growth inhibition in mink lung epithelial cells and suggested that erythroblast cells expressing v-ErbA might also be insensitive to TGF- β . We found that indeed, HD3 chicken erythroblasts, which express native v-ErbA, were resistant to TGF- β -induced growth inhibition and additionally showed that TGF- β -responsiveness could be partially restored by a reduction of v-*erbA* expression using siRNA-mediated gene silencing. Interestingly, others have recently shown that in T2EC cells, which are dependent on TGF- β to induce self-renewal, v-ErbA expression causes cells to differentiate in the absence of TGF- β (Gonin-Giraud *et al.*, 2008). In contrast, our results suggest that in HD3 erythroblasts just as in mink lung epithelial cells, TGF- β functions in the more commonly observed, anti-proliferative role. We also found that reduction of v-*erbA* in HD3 cells inhibits cell growth even in absence of TGF- β . This decrease in cell proliferation may arise from a combination of several factors including a potential reactivation of the TGF- β pathway by basal or autocrine signaling and a reduction of v-ErbA's effect on previously identified regulatory pathways critical to v-ErbA oncogenic transformation. Although we demonstrate recovered TGF- β sensitivity when v-ErbA expression is reduced, further research will be required to determine the relative contributions to v-ErbA's oncogenicity for each of the known mechanisms. In summary, our data suggest that native v-ErbA disrupts the TGF- β transduction pathway in AEV-transformed erythroblast cells and triggers their escape from TGF- β -regulated growth inhibition.

v-ErbA Association with Smad4 Dysregulates TGF- β Signaling

Propagation of TGF- β signaling within the cell depends on a highly regulated combination of phosphorylation, complex formation, and nuclear translocation of the Smad proteins (Massague *et al.*, 2000). Our data demonstrate v-ErbA/Smad4 association and suggest that this interaction is involved in the dysregulation of TGF- β signaling and escape of growth inhibition. v-ErbA also remains predominately cytoplasmic due to a nuclear export signal located in the gag region (DeLong *et al.*, 2004). We therefore hypothesize that TGF- β signaling is dysregulated upon the disruption of normal cyto-nuclear shuttling of Smad4 by v-ErbA's strong cytoplasmic localization and its association with Smad4 (Figure 6D). In support of this hypothesis we demonstrated that indeed, Smad4 nuclear translocation was disrupted upon v-ErbA expression. Although *in vitro* binding assays clearly show v-ErbA/Smad4 association and fluorescence microscopy images show that Smad4 remains predominately cytoplasmic in the presence of v-ErbA, demonstration of direct v-ErbA/Smad4 interaction using *in vivo* immunoprecipitation techniques were inconsistent (results not shown). The variable outcomes of these immunoprecipitation experiments suggest

several possible scenarios: One, that unidentified conditions are required to consistently observe direct v-ErbA/Smad4 binding *in vivo*, or two, that the v-ErbA/Smad4 interaction may be transient and occur only in the nucleus or during translocation. Another possibility is that interaction of Smad4 and v-ErbA requires integrity of cytoskeleton, which is often disrupted upon cell lysis. These potential scenarios as well as other possibilities await further research efforts and may provide further understanding of v-ErbA/Smad4 interactions.

To further characterize v-ErbA/Smad4 association, we investigated two v-ErbA mutations: 1) delZF, a mutant where both zinc-fingers in the DNA-binding domain were deleted; 2) C308A, a previously discovered, single-point mutant that prevents cysteine coordination and hence structural formation of the first zinc finger in TR- α (Bonde and Privalsky, 1990). The zinc finger domain is known to mediate other protein-protein interactions. A recent publication confirms that mutations in the zinc finger domain of the MDM2 oncoprotein disrupts ribosomal protein interaction and attenuates MDM2-induced p53 degradation (Lindstrom *et al.*, 2007). Both the delZF and C308A mutations were unable to associate with Smad4 and cells stably expressing either delZF or C308A regained TGF- β -induced cell growth inhibition and TGF- β -responsive promoter activity.

Additionally, though C308A remained predominantly cytoplasmic, this mutant was unable to disrupt Smad4 nuclear localization. Thus, we observed that the ability of v-ErbA to associate with Smad4 was necessary for TGF- β signal disruption. The data from these v-ErbA mutation studies further support our hypothesis that Smad4/v-ErbA association disrupts the balance of Smad4 in the nucleus and down-regulates TGF- β -activated promoters. The fact that v-ErbA/Smad4 association renders both proteins insensitive to LMB treatment suggests that interaction between them primarily affects the nuclear import process for both proteins. However, we cannot rule out the possibility that their association enables the complex to be exported by exporting factors other than CRM1. Further experiments are needed to differentiate these mechanisms.

In conclusion, our data reinforce the importance of TGF- β signal disruption in oncogenic transformation. The evidence suggests v-ErbA, a fusion of a viral gag domain with a nuclear hormone receptor can dysregulate TGF- β signaling and disrupts TGF- β -induced growth inhibition. TGF- β pathway dysregulation correlates with the ability of cytoplasmically localized v-ErbA to bind Smad4 protein and prevent normal Smad4 nuclear translocation. We observed TGF- β pathway dysregulation in erythroblastosis cells expressing native v-ErbA and demonstrated recovery of TGF- β sensitivity and cell growth inhibition by reducing v-ErbA expression. We postulate that v-ErbA/Smad4 association, a previously unknown protein interaction, mediates TGF- β pathway dysregulation by disrupting Smad4 shuttling. These results reveal a novel mechanism for oncogenic disruption of TGF- β signaling and provide a novel explanation for v-ErbA activity in AEV-induced erythroleukemia.

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REFERENCES

- Attisano, L., and Wrana, J. L. (2002). Signal transduction by the TGF- β superfamily. *Science* 296, 1646–1647.
- Beug, H., Doederlein, G., Freudenstein, C., and Graf, T. (1982). Erythroblast cell lines transformed by a temperature-sensitive mutant of avian erythroblastosis virus: a model system to study erythroid differentiation in vitro. *J. Cell Physiol. Suppl.* 1, 195–207.
- Bonde, B. G., and Privalsky, M. L. (1990). Sequence-specific DNA binding by the v-erbA oncogene protein of avian erythroblastosis virus. *J. Virol.* 64, 1314–1320.
- Chen, F., and Weinberg, R. A. (1995). Biochemical evidence for the autophosphorylation and transphosphorylation of transforming growth factor beta receptor kinases. *Proc. Natl. Acad. Sci. USA* 92, 1565–1569.
- Ciana, P., Braliou, G. G., Demay, F. G., von Lindern, M., Baretino, D., Beug, H., and Stunnenberg, H. G. (1998). Leukemic transformation by the v-ErbA oncoprotein entails constitutive binding to and repression of an erythroid enhancer in vivo. *Embo J.* 17, 7382–7394.
- Damm, K., and Evans, R. M. (1993). Identification of a domain required for oncogenic activity and transcriptional suppression by v-erbA and thyroid-hormone receptor alpha. *Proc. Natl. Acad. Sci. USA* 90, 10668–10672.
- Damm, K., Thompson, C. C., and Evans, R. M. (1989). Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. *Nature* 339, 593–597.
- Datto, M. B., Hu, P. P., Kowalik, T. F., Yingling, J., and Wang, X. F. (1997). The viral oncoprotein E1a blocks transforming growth factor beta-mediated induction of p21/WAF1/Cip1 and p15/INK4B. *Mol. Cell. Biol.* 17, 2030–2037.
- DeLong, L. J., Bonamy, G. M., Fink, E. N., and Allison, L. A. (2004). Nuclear export of the oncoprotein v-ErbA is mediated by acquisition of a viral nuclear export sequence. *J. Biol. Chem.* 279, 15356–15367.
- Ewen, M. E., Sluss, H. K., Whitehouse, L. L., and Livingston, D. M. (1993). TGF beta inhibition of Cdk4 synthesis is linked to cell cycle arrest. *Cell* 74, 1009–1020.
- Frykberg, L., Palmieri, S., Beug, H., Graf, T., Hayman, M. J., and Vennstrom, B. (1983). Transforming capacities of avian erythroblastosis virus mutants deleted in the erbA or erbB oncogenes. *Cell* 32, 227–238.
- Gonin-Giraud, S., Bresson-Mazet, C., and Gandrillon, O. (2008). Involvement of the TGF- β and mTOR/p70S6Kinase pathways in the transformation process induced by v-ErbA. *Leuk. Res.* 32, 1878–1888.
- Graf, T., and Beug, H. (1983). Role of the v-erbA and v-erbB oncogenes of avian erythroblastosis virus in erythroid cell transformation. *Cell* 34, 7–9.
- Grignani, F., et al. (1998). Fusion proteins of the retinoic acid receptor-alpha recruit histone deacetylase in promyelocytic leukaemia. *Nature* 391, 815–818.
- Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* 100, 57–70.
- Hayman, M. J., Royer-Pokora, B., and Graf, T. (1979). Defectiveness of avian erythroblastosis virus: synthesis of a 75K gag-related protein. *Virology* 92, 31–45.
- Heldin, C. H., Miyazono, K., and ten Dijke, P. (1997). TGF- β signalling from cell membrane to nucleus through SMAD proteins. *Nature* 390, 465–471.
- Hua, X., Liu, X., Ansari, D. O., and Lodish, H. F. (1998). Synergistic cooperation of TFE3 and smad proteins in TGF- β -induced transcription of the plasminogen activator inhibitor-1 gene. *Genes Dev.* 12, 3084–3095.
- Hua, X., Miller, Z. A., Wu, G., Shi, Y., and Lodish, H. F. (1999). Specificity in transforming growth factor beta-induced transcription of the plasminogen activator inhibitor-1 gene: interactions of promoter DNA, transcription factor muE3, and Smad proteins. *Proc. Natl. Acad. Sci. USA* 96, 13130–13135.
- Kurokawa, M., Mitani, K., Irie, K., Matsuyama, T., Takahashi, T., Chiba, S., Yazaki, Y., Matsumoto, K., and Hirai, H. (1998). The oncoprotein Evi-1 represses TGF- β signalling by inhibiting Smad3. *Nature* 394, 92–96.
- La, P., Morgan, T. A., Sykes, S. M., Mao, H., Schnepf, R. W., Petersen, C. D., and Hua, X. (2003). Fusion proteins of retinoid receptors antagonize TGF- β -induced growth inhibition of lung epithelial cells. *Oncogene* 22, 198–210.
- Lin, C. R., Chen, W. S., Kruiger, W., Stolarsky, L. S., Weber, W., Evans, R. M., Verma, I. M., Gill, G. N., and Rosenfeld, M. G. (1984). Expression cloning of human EGF receptor complementary DNA: gene amplification and three related messenger RNA products in A431 cells. *Science* 224, 843–848.
- Lin, R. J., and Evans, R. M. (2000). Acquisition of oncogenic potential by RAR chimeras in acute promyelocytic leukemia through formation of homodimers. *Mol. Cell* 5, 821–830.
- Lindstrom, M. S., Jin, A., Deisenroth, C., White Wolf, G., and Zhang, Y. (2007). Cancer-associated mutations in the MDM2 zinc finger domain disrupt ribosomal protein interaction and attenuate MDM2-induced p53 degradation. *Mol. Cell. Biol.* 27, 1056–1068.
- Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A., and Lodish, H. F. (1997). Transforming growth factor beta-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. *Proc. Natl. Acad. Sci. USA* 94, 10669–10674.
- Luo, K. (2004). Ski and SnoN: negative regulators of TGF- β signaling. *Curr. Opin. Genet. Dev.* 14, 65–70.
- Massague, J., Blain, S. W., and Lo, R. S. (2000). TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 103, 295–309.
- Metz, T. (1994). Oncogenes and erythroid differentiation. *Semin. Cancer Biol.* 5, 125–135.
- Miyajima, N., Kadowaki, Y., Fukushige, S., Shimizu, S., Semba, K., Yamanashi, Y., Matsubara, K., Toyoshima, K., and Yamamoto, T. (1988). Identification of two novel members of erbA superfamily by molecular cloning: the gene products of the two are highly related to each other. *Nucleic Acids Res.* 16, 11057–11074.
- Myers, J. W., Jones, J. T., Meyer, T., and Ferrell, J. E., Jr. (2003). Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. *Nat. Biotechnol.* 21, 324–328.
- Naviaux, R. K., Costanzi, E., Haas, M., and Verma, I. M. (1996). The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J. Virol.* 70, 5701–5705.
- Pierreux, C. E., Nicolas, F. J., and Hill, C. S. (2000). Transforming growth factor beta-independent shuttling of Smad4 between the cytoplasm and nucleus. *Mol. Cell. Biol.* 20, 9041–9054.
- Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, M. R., Munger, K., Howley, P. M., and Moses, H. L. (1990). TGF- β 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* 61, 777–785.
- Piu, F., Jurdic, P., Brun, G., Samarut, J., and Castellazzi, M. (1993). Transforming growth factor beta 1-mediated growth inhibition in chick embryo fibroblasts: reversion by virally-expressed nuclear oncogenes. *CR Acad. Sci. III* 316, 772–779.
- Privalsky, M. L., Boucher, P., Koning, A., and Judelson, C. (1988). Genetic dissection of functional domains within the avian erythroblastosis virus v-erbA oncogene. *Mol. Cell. Biol.* 8, 4510–4517.
- Rietveld, L. E., Caldenhoven, E., and Stunnenberg, H. G. (2001). Avian erythro-leukemia: a model for corepressor function in cancer. *Oncogene* 20, 3100–3109.
- Rietveld, L. E., Caldenhoven, E., and Stunnenberg, H. G. (2002). In vivo repression of an erythroid-specific gene by distinct corepressor complexes. *EMBO J.* 21, 1389–1397.
- Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H., and Vennstrom, B. (1986). The c-erbA protein is a high-affinity receptor for thyroid hormone. *Nature* 324, 635–640.
- Schwarte-Waldhoff, I., Klein, S., Blass-Kampmann, S., Hintelmann, A., Eilert, C., Dreschers, S., Kalthoff, H., Hahn, S. A., and Schmiegell, W. (1999). DPC4/SMAD4 mediated tumor suppression of colon carcinoma cells is associated with reduced urokinase expression. *Oncogene* 18, 3152–3158.
- Sun, P., Dong, P., Dai, K., Hannon, G. J., and Beach, D. (1998). p53-independent role of MDM2 in TGF- β 1 resistance. *Science* 282, 2270–2272.
- Thormeyer, D., and Baniahmad, A. (1999). The v-erbA oncogene (review). *Int. J. Mol. Med.* 4, 351–358.
- Vennstrom, B., and Bishop, J. M. (1982). Isolation and characterization of chicken DNA homologous to the two putative oncogenes of avian erythroblastosis virus. *Cell* 28, 135–143.
- Vennstrom, B., Fanshier, L., Moscovici, C., and Bishop, J. M. (1980). Molecular cloning of the avian erythroblastosis virus genome and recovery of oncogenic virus by transfection of chicken cells. *J. Virol.* 36, 575–585.
- Wakefield, L. M., and Roberts, A. B. (2002). TGF- β signaling: positive and negative effects on tumorigenesis. *Curr. Opin. Genet. Dev.* 12, 22–29.
- Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruol, D. J., and Evans, R. M. (1986). The c-erbA gene encodes a thyroid hormone receptor. *Nature* 324, 641–646.
- Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F., and Massague, J. (1992). TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* 71, 1003–1014.
- Xiao, Z., Latek, R., and Lodish, H. F. (2003). An extended bipartite nuclear localization signal in Smad4 is required for its nuclear import and transcriptional activity. *Oncogene* 22, 1057–1069.
- Xiao, Z., Liu, X., Henis, Y. I., and Lodish, H. F. (2000). A distinct nuclear localization signal in the N terminus of Smad 3 determines its ligand-induced nuclear translocation. *Proc. Natl. Acad. Sci. USA* 97, 7853–7858.
- Zenke, M., Munoz, A., Sap, J., Vennstrom, B., and Beug, H. (1990). v-erbA oncogene activation entails the loss of hormone-dependent regulator activity of c-erbA. *Cell* 61, 1035–1049.