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Regulation of anoikis by oncogenic pathways

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Regulation of anoikis by oncogenic pathways

By

Sun Hee Park

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in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Cancer Cell Biology

Michael Schaller, Ph.D, Committee Chairperson

John Michael Ruppert, M.D., Ph.D

Alexey Ivanov, Ph.D

Lisa Salati, Ph.D

Laura Gibson, Ph.D

Steven M Frisch, Ph.D, Mentor

Cancer Cell Biology Program
Morgantown, West Virginia
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ABSTRACT

Regulation of anoikis by oncogenic pathways

Sun Hee Park

Detachment of epithelial cells from matrix or attachment to an inappropriate matrix engages an apoptotic response known as anoikis, which prevents metastasis.
Anoikis-resistance of tumor cells is critical for anchorage-independent growth and metastasis. Here we show two novel oncogenic pathways that confer resistance to anoikis in tumor cells. One pathway (chapter 2) signifies a new link between EMT and anoikis-resistance. In chapter 3, we present a new connection between inflammatory signaling pathways and anoikis-resistance. Cellular sensitivity to anoikis is compromised during the oncogenic epithelial-to-mesenchymal transition (EMT), through various mechanisms.

Chapter 2 introduces a novel mechanism (NRAGE/TBX2/p14ARF) that is induced upon loss of E-cadherin and EMT, mediating anoikis resistance.

The inflammatory-response transcription factor, NF-κB, contributes to anoikis-resistance and metastatic progression, through mechanisms that are understood incompletely. Deleted in Breast Cancer (DBC1) is over-expressed in several tumor types and correlates with a poorer prognosis in some cases. DBC1 suppressed anoikis in normal epithelial and breast cancer cell lines. Chapter 3 introduces a novel mechanism: DBC1 suppresses anoikis through enhancing canonical NF-κB signaling. This study will provide mechanisms for DBC1 to act as a tumor promoter, by providing a link between inflammation and anoikis-resistance and providing a potential target for anti-NF-κB/anti-cancer drugs.

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CHAPTER 1

Introduction and Literature Review

PART I

Inflammation and cancer

Inflammation is a protective reaction activated when body tissue becomes infected, irritated, or injured [1]. When inflammation becomes chronic, it can lead to the development of many types of cancer including bladder, cervical, gastric, ovarian, prostate, thyroid, intestinal and esophageal cancer [1, 2]. Chronic inflammation has been linked to numerous steps of tumorigenesis, including tumor cell survival, proliferation, invasion, angiogenesis, and metastasis [1]. The cytokines released from the activated immune cells stimulate tumor growth and progression [3]. In addition, the immune cells are recruited to the tumor microenvironment through interaction with stromal and malignant cells and the pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-17, produced from both immune and malignant cells further promote proliferation, invasion, and metastasis [1, 4].

Diseases that have been associated with chronic inflammation includes autoimmune diseases, such as Rheumatoid Arthritis (RA), as well as diabetes, obesity, and the aging process [5-8]. All involve the elevation of pro-inflammatory cytokines, interleukin-6 (IL-6) and/or TNF-α that lead to activation of NF-κB signaling which is known to promote inflammatory processes and enhance tumor development and progression [9]. For instance, obesity and insulin resistance have been established as risk factors for cancer development due to dysfunctional adipocytes, which release IL-6 and TNF-α. Increased levels of the cytokines then recruit macrophages to the adipose tissues and activate NF-κB signaling to further promote inflammatory responses [10, 11]. This persistent cycle of inflammatory response and NF-κB activation can lead to cancer development.

NF-κB and Cancer

NF-κB: structure and family members

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) was first identified as a B-cell nuclear transcriptional factor that is ubiquitously expressed in all animal cell types. It functions as a major player in immune and inflammation response as well as in cell proliferation, survival and apoptosis [12]. There are five members of the NF-κB family, including the class I family: p50/p105, and p52/p100 and the class II family: p65/RelA, c-RelA, and RelB, which homo- and hetero-dimerize through their conserved N-terminal Rel homology (RH) domain that is required for interaction with the inhibitor of NF-κB (IκB) protein and DNA binding and dimerization [13]. The most commonly expressed form is a heterodimer of the p65/RelA and p50 subunits. In addition to the RH domain, the class II family has a C-terminal transcription activation domain that is not conserved, but activates transcription in variety of species. NF-κB heterodimers recognize distinct DNA sites (9-10 base pairs) known as κB sites, resulting in regulation of distinct, but overlapping, sets of target gene expression upon a variety of signals in different cell types [14, 15].

Regulation of canonical and non-canonical pathways of NF-κB

NF-κB activation is tightly controlled by canonical and non-canonical pathways that regulate proteolysis of IκB and IκB-related proteins. The canonical NF-κB pathway is stimulated by various immune receptors, such as the Toll-like receptors (TLRs), interleukin-1 receptor (IL-1R), tumor necrosis factor receptor (TNFR) and antigen receptors [16]. In addition, a canonical pathway involves the activation of NF-κB through proteasomal degradation of IκBα. In unstimulated normal cells, the NF-κB heterodimer complexes are localized in cytoplasm in an inactive form through binding to an IκB protein. Upon stimulation, IκBα at two N-terminal

Serine 32/36 residues become phosphorylated mainly by IKKβ, a subunit of IκB kinase (IKK) complex, which also includes IKKα and IKKγ (NEMO). Phosphorylated IκB is then degraded in 26S proteasomes which uncovers the nuclear localization signal of NF-κB, resulting in free NF-κB complex to translocate into the nucleus. In the nucleus, the NF-κB complex binds to the κB DNA binding sites in the promoter region of target genes and activates transcription [12, 15]. Therefore, when this pathway is not controlled properly, prolonged NF-κB activation leads to chronic inflammation and cancer [17].

The non-canonical pathways are induced by a subset of TNF family members and cell-differentiating or developmental stimuli, such as lymphotoxin β (LTβ), B cells activating factor (BAFF), CD40 ligand and receptor activator of NF-κB ligand (RANKL) [17]. Upon stimulation, IKKα activated by NF-κB induces Kinase (NIK) to phosphorylate p100 whose function is analogous to IκBα. This results in ubiquitination and subsequent partial proteolysis by the proteasome to produce p52, which translocates into the nucleus as a complex with RelB [16]. The non-canonical NF-κB pathway regulates important biological functions, such as lymphoid organogenesis, B-cell survival and maturation, dendritic cell activation, and bone metabolism and deregulated non-canonical NF-κB signaling is associated with lymphoid malignancies [18, 19].

Role of NF-κB signaling pathways in inflammatory responses
The body's initial immune response to the presence of cellular stress conditions, including bacterial lipopolysaccharides (LPSs), microbial and viral pathogens, and growth factors, is initially mediated by proinflammatory cytokines, such as TNFα and IL-1 and various pathogen-

associated molecular patterns (PAMPs), leading to an inflammatory response with the activation of NF-κB playing a major role [20]. Through the canonical pathway, activated NF-κB induces expression of genes encoding chemokines (Monocyte chemotactic protein-1 (MCP-1), Macrophage inflammatory protein-1 (MIP-1), IL-8, and eotaxin), cytokines (TNF-α), adhesion molecules (Intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial–leukocyte adhesion molecule 1 (ELAM)), and enzymes such as cyclooxygenase II (COX2), which are involved in innate immune response and migration of the inflammatory and phagocytic cells to the site of tissue that has been infected [21, 22]. In addition, the canonical NF-κB pathway is also critical for the survival of macrophages, lymphocytes and B cells during bacterial infections or against LPS and TNF-α induced apoptosis through activating expression of anti-apoptotic genes including Bcl-2 [21, 23]. While the innate immune response is under control of the classical IKKβ-dependent NF-κB signaling pathway, the IKKα-dependent alternative NF-κB pathway plays a role in adaptive immune response by generating chemokines such as the secondary lymphoid tissue chemokine (SLC), Epstein–Barr virus-induced molecule 1 ligand CC chemokine (ELC) and stromal cell-derived factor-1α (SDF-1α), which are required for migration of antigen presenting cells (APCs) to the spleen and lymph nodes where T cells and B cells induce further immune response upon encountering with APCs [21, 24].

Functional significance of IKK and IKK-related kinase, IKKi/IKKε, in inflammation and cancer
Binding of all the inflammatory cytokines and PAMPs to their receptors such as TNFR1 [25], IL-1R [26] and Toll-like receptors (TLRs) [27] results in activation of the IκB kinase (IKK) complex, the endogenous activator of NF-κB [28]. IKK complex includes two catalytic subunits,

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IKKβ and IKKα that are structurally similar, and a regulatory subunit, IKKγ (NEMO) that lacks kinase activity [29]. In mammalian cells, IKKα and IKKβ form a stable heterodimer that is tightly associated with the IKKγ (NEMO) [29, 30].

Studies have shown that depletion in IKKγ (NEMO) expression causes a major defect in IκB degradation and NF-κB activation in response to pro-inflammatory stimuli [31]. Therefore, this regulatory subunit is critical for mainly the activation of IKK complex and NF-κB [32].

Although IKKβ and IKKα has highly similar structures and in vitro kinase activities, their functions in inflammation and cancer is very different [33]. For instance, IKKα-deficient cells and tissues showed no defect in IKK activation and IκBα degradation in response to TNF-α, IL-1, or LPS, which suggests that IKKβ and IKKα have different physiological functions and that IKKα cannot substitute for IKKβ [33, 34]. IKKα mainly regulates an alternative NF-κB pathway that is important for lymphoid organogenesis [35]. In addition, IKKα also controls epidermal keratinocyte differentiation and skeletal morphogenesis independent of NF-κB pathway [36, 37]. However, IKKβ has been known as the major kinase critical for activating the canonical NF-κB pathway, which is critical for the anti-apoptotic pathway and innate immune response. In fact, mice deficient in IKKβ showed massive liver apoptosis mediated by TNF-α signaling and were very susceptible to infections, which led to their death a short time after birth [38]. Moreover, both the T- and B-cell receptors, as well as TLR4, use this IKKβ mediated NF-κB pathway to mediate survival and various inflammatory and immune functions [39-41]. Therefore, IKKβ/NF-κB is important for the activation of immune cells and the duration of the period through which they remain functional and viable [38].

While initial phosphorylation of IκBα is critical for the translocation of NF-κB complex, DNA-binding ability and transactivation activity of NF-κB is additionally regulated through the post-

translational modification such as acetylation and phosphorylation by IKK. For instance, phosphorylation at serine 536 site in the active loop has been shown to be critical for transactivation activity of NF-κB [42]. Interestingly, a number of studies showed that phosphorylation at serine 536 of RelA is mainly regulated by IKKβ upon TNF-α or LPS stimulation [42-44]. Importantly, the RelA subunit phosphorylated at serine-536 is an independent prognostic factor in colorectal cancer patients [45]. In addition, increased expression of phosphorylation at Ser536 of RelA was positively correlated with more malignant features of colorectal tumors [46] .

In addition to IKKβ, which is overexpressed in some cancer cells [47], IKKi/IKKε has shown to be expressed in several cancer cells and maintain constitutive activation of NF-κB through phosphorylation of RelA at Ser-536 [48]. However, unlike IKKβ, IKKi/IKKε is not responsible for phosphorylation of IκBα, suggesting that IKKi/IKKε regulates a later stage of NF-κB response [49]. IKKi/IKKε is an IKK-related kinase that has been discovered through sequence analysis study. Although detailed functions of these two kinases are not completely understood, IKKi/IKKε has been suggested as a potential therapeutic target in inflammation and cancer [49, 50]. In addition, IKKi/IKKε has been shown to be critical for invasiveness and growth of breast cancer cells [51].

Link between inflammation and Cancer through NF-κB

The link between inflammation and cancer is understood incompletely. There are many cases where infections (HBV and HCV, and IBDs) and inflammatory cytokines (TNF, MIF and interleukins) cause development of cancers such as colorectal, breast, lung, and liver cancers. Many studies show that the processes that contribute to the malignant progression involve

uncontrolled proliferation, invasion, angiogenesis, metastasis, and evasion of apoptosis, which are all regulated by IKK/NF-κB signaling pathways. Therefore, these studies suggest that IKK/NF-κB is the critical molecule that links inflammation and cancer [52, 53].

Overexpression/activation/mutation of NF-κB in lymphoid and solid cancers

Continuous activation and overexpression of NF-κB factors has been emerging as a hallmark of various types of solid tumors including breast [54], ovarian [55], colon [55], pancreatic [56], thyroid [57], and melanomas [58]. In addition, gene amplification and rearrangement of *c-Rel* and *RelA* are found in leukemia and carcinomas [59]. Constitutive activation of NF-κB results in increased phosphorylation of IκBα following accumulation of NF-κB in nucleus. Therefore, elevated NF-κB activity was attributed to defective IκB function. In fact, there are some studies showing inactivating mutations in *IκBα* and *IκBε* in lymphomas [60-62] indicating the critical function of IκB molecules in the regulation of Rel/NF-κB function.

Hyperactive NF-κB because of upstream pathways

In addition to the amplification or mutation of NF-κB pathway molecules, there are many different signaling pathways/molecules that lead to constitutive activation of NF-κB in cancers [15]. First, NF-κB activity is deregulated by upstream oncogenes and kinases such as Ras [63] and Raf [64]. For instance, oncogenic Ras and Raf, which induce transformation through NF-κB, are also frequently mutated and/or mutated in many human tumors, resulting in constitutive activation of NF-κB through PI3K/Akt and MAPK/MEKK-1 signaling pathways, causing IκBα phosphorylation and nuclear NF-κB accumulation. Secondly, overexpression of upstream receptors such as EGFR and Her2/ ErB2 that are frequently overexpressed in breast cancers,

leads to constitutive induction of NF-κB through a PI3-kinase/Akt kinase pathway in a IKK-IκBα independent manner [65]. In addition, IKKβ/NF-κB signaling is required for EGFR induced proliferation and survival in the EGFR+/ER- subtype of breast cancers [66-68]. Lastly, chronic infections with hepatitis B virus (HBV), hepatitis C virus and chronic inflammatory bowel diseases (IBDs) lead to developing hepatocellular carcinoma (HCC), gastric cancers and colorectal cancers through constitutive activation of NF-κB pathway.

Inflammatory cytokines secreted from the tumor microenvironment promote survival and proliferation while inhibiting apoptosis through the NF-κB pathway

The tumor microenvironment includes immune cells that release inflammatory cytokines such as TNF-α and IL-1 and oxidative stress molecules like reactive oxygen species (ROS) during chronic inflammation. These molecules react with tumor cells to activate NF-κB signaling pathways. Increased activation of NF-κB in these tumor cells leads to enhanced expression of genes that are involved in many different process, such as proliferation, cell survival, invasion, and angiogenesis, resulting in tumor progression and malignancies. For instance, activated NF-κB induces the expression of the genes involved in anti-apoptotic pathways such as c-FLIP [69] and Bcl-xL [70] to promote tumor cell survival. Expression of matrix metalloproteases (such as MMP9) and angiogenic factors like vascular endothelial growth factor (VEGF) that are involved in invasion and angiogenesis also promote a malignant phenotype [71, 72]. Moreover, TNF-α and ROS have been shown to induce epithelial-to-mesenchymal transition (EMT), an oncogenic process that leads to invasive and metastatic phenotype, through increasing expression of Snail while downregulating E-cadherin through NF-κB pathway in a breast tumor cell line [73, 74] .

Deleted in Breast Cancer 1 (DBC1)

Basic information about its history, structure, and post-translational regulation

DBC1/KIAA1967 was mistakenly identified as a tumor suppressor because it was originally found in a region of chromosome 8p21 that was frequently deleted in some breast cancer cell lines [75]. However, many recent studies show that DBC1 protein is not deleted in tumor cells, but overexpressed in various cancers [76, 77] . DBC1 is composed of N-terminal coiled-coil (zipper) region and multiple conserved globular domains, including a C-terminal inactive EF-hand module (Fig. 1).

DBC1 oligomerizes and exhibits a dynamic expression pattern during the cell cycle [78, 79]. In addition, DBC1 has the Nudix domain (Fig. 1) that is catalytically inactive, but might be necessary for binding of ligands such as ADP-sugars, generated by SIRT1 activity [80, 81]. DBC1 mainly resides in the nucleus except in Hela cells where DBC1 can localize to mitochondria following

Figure 1. Domain structure of DBC1.
NLS: Nuclear localization signal, EF: EF-hand:
LZ: Leucine zipper, NUDIX: nudix domain, and
CC: Coiled-coiled region
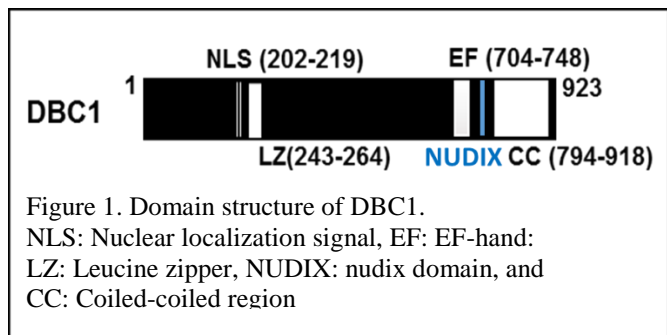
caspase-dependent processing, sensitizing the cells to TNFα mediated apoptosis [82]. There is evidence showing that DBC1 undergoes posttranslational modification in response to DNA damage: DBC1 becomes phosphorylated at threonine 454 by ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases, which is critical for the interaction with SIRT1 deacetylase [83].

Biological functions of DBC1

The function of DBC1 was initially discovered as a SIRT1 inhibitor [79]. SIRT1 is a histone/protein deacetylase involved in several cellular functions such as longevity, metabolism, cellular stress response and tumorigenesis, which implies that DBC1 might also play a role in these functions through regulating SIRT1 activity [77]. DBC1 directly interacts with and inhibits SIRT1, stabilizing the activity of p53 transcription factor, resulting in an increase in the expression of genes that are involved in apoptotic pathways [79, 84]. DBC1 regulates heat shock response (HSR), the cellular response to protein damaging stress, which is critical in maintaining proteostasis. Through inhibition of SIRT1 activity, DBC1 increased acetylation of HSF1, a known transcriptional factor regulating expression of genes such as Hsp70, which repairs cellular damage due to stress. Acetylated HSF1 then loses its DNA binding ability, which results in decreased transcription of Hsp70, suggesting DBC1 as a new target available for therapeutic regulation of the heat shock response [85]. Secondly, DBC1 interacts with ZNF326 to form part of DBIRD complex and regulates alternative splicing through affecting the transcript elongation rate and density of RNA polymerase II (RNAPII) at the transcription site. DBIRD complex links RNAPII and a messenger ribonucleoprotein (mRNP) particle, integrating transcript elongation with mRNA splicing [86]. Thirdly, DBC1 appears to associate with the COUP-TFI transcriptional factor that is mainly known to function in embryonic development on the promoter of COUP-TFI target genes, and functions to stabilize interaction between Nuclear Receptor co-repressor (NCoR) and COUP-TFI, thus contributing to COUP-TFI-mediated transcriptional repression [87]. DBC1 also stimulated the transcriptional activities of the nuclear receptors such as GR (glucocorticoid receptor) and TR (thyroid hormone receptor) in a hormone-dependent manner [88]. In addition to these functions, DBC1 is also known to induce high-fat

diet-induced liver steatosis through inhibiting SIRT1 activity in a mouse model [89]. Lastly, DBC1 is important for DNA damage signaling pathways by stabilizing Chk1, which is a critical effector molecule in the DNA damage signaling pathway. Thus, DBC1 may be an important regulator of a wide variety of cellular processes including gene expression, chromatin remodeling, splicing, apoptosis and metabolism.

Role of DBC1 in cancer

DBC1 overexpression in cancer

Recent studies have shown through DNA microarray and protein analysis that DBC1 is upregulated in esophageal, prostate, colorectal, lung, and breast cancers [90-94]. Moreover, overexpression of DBC1 correlates with a poorer prognosis of gastric, colorectal, and esophageal cancers [90, 91, 95]. Likewise, breast carcinomas that over-express DBC1 showed a more frequent DMR (distant metastatic relapse rate) and significantly lower OS (Overall Survival) and RFS (Relapse free survival) among patients receiving endocrine therapy [96]. Therefore, DBC1 would be a critical poor prognostic marker for breast cancer patients. Expression of DBC1 was also higher in metastatic breast carcinoma than at the primary site and lung cancer cells show higher DBC1 expression than in normal lung fibroblasts. Interestingly, the level of DBC1 protein in normal cells differs from that found in lung and breast cancer cells [78]. This aberrant expression of DBC1 in cancer suggests that DBC1 may serve as a potential diagnostic marker.

Interactions of DBC1 with cancer relevant proteins

DBC1 binds to several nuclear receptors and enzymes, including the estrogen receptors α and β, androgen receptors, the transcription factor BRCA1, and the deacetylase HDAC3. Through

interacting with these molecules, DBC1 regulates proliferation, apoptosis and histone modification that are important processes for tumorigenesis in SIRT1 dependent or SIRT1 independent manners. Interestingly, depending on the type of cancer, interacting partner, and cancer-inducing stress type, DBC1 can function either as a tumor promoter or suppressor.

DBC1 functions as a tumor suppressor

Several studies have shown that DBC1 can function as a tumor suppressor in certain types of cancer cells mainly through inhibiting SIRT1 activity. For instance, through inhibiting SIRT1 activity, DBC1 induces acetylation of p53, FOXO, and BRCA1 transcriptional factors, promoting apoptosis [79]. In addition, DBC1 directly interacts with HDAC3, which is known to be overexpressed in many different cancers and to inhibit its deacetylase activity, leading to an increase in cell-cycle arrest and apoptosis of the cancer cells [97-99]. Therefore, these studies suggest a tumor suppressive activity of DBC1. However, SIRT1 itself can function either as a tumor promoter or a tumor suppressor. For instance, knock down of SIRT1 in colon cancer induces tumor progression in xenograft models [100] while SIRT1 expression is elevated in some prostate tumor cells [101]. In addition, in liver cancers, DBC1 does not act as a SIRT1 regulator, further illustrating that the tumor suppressive effect of DBC1 is cancer type-dependent [102].

DBC1 functions as a tumor promoter

DBC1 function as a tumor promoter through interacting with different nuclear receptors in SIRT1 independent manner. For instance, DBC1 interacts with estrogen receptor alpha (ERα) to stabilize its expression and promotes proliferation and cell cycle progression dependent upon

estrogen [88] . In addition, in a ligand-dependent manner, DBC1 interacts with ER-β to repress its transcriptional activity and enhances the expression of cell survival genes such as Bcl2 [103]. DBC1 interacts with and induces Androgen receptor transcriptional activity and enhances prostate specific antigen (PSA) expression in a hormone-dependent manner [104]. DBC1 interacts with SUV39H1 to inhibit this tumor suppressor effect in lung cancer cells [105]. SUV39H1 methyltransferase functions as a tumor suppressor by enhancing Ras induced spontaneous senescence. It also interacts with pRb to inhibit E2F1 target promoters through methylation, inducing cell cycle arrest. DBC1 can also function as a tumor promoter in a SIRT1-dependent manner. For instance, through inhibiting SIRT1, DBC1 enhances survival against UV irradiation, protecting against genotoxic-stress induced cell death in breast cancer cell line [106]. Lastly, through inhibiting SIRT1 activity, DBC1 interacts with cell cycle and apoptotic response 1 (CCAR1) and enhances ERα DNA binding to increase target gene expression such as PgR, CTSD, and GREB1 that are known to promote tumor cell survival and metastasis of breast cancer cells [88]. Taken together, these findings clearly demonstrate that DBC1 functions as a tumor promoter in both SIRT1 dependent and independent manners.

Regulation by AMPK and PKA- potential link between metabolism and cancer

Impaired glycolysis or aerobic respiration has been suggested as a metabolic hallmark of cancer development [107, 108]. Protein kinase A (PKA) and AMP-activated protein kinase (AMPK) are kinases that are involved in metabolic regulation [109], whose overexpression is frequently detected in some cancers. In addition, these proteins also confer anoikis-resistance [110, 111]. Determining the molecular mechanism of how these metabolic proteins regulate oncogenic molecules will be critical, but it is largely unknown. Nevertheless, recently, Veronica et al.

show how PKA and AMPK regulate the activity of SIRT1 deacetylase, which is known to function in tumorigenesis. Under a fasting condition, the level of cellular cAMP increases, resulting in activation of PKA and AMPK [109, 112] . The study shows that activated AMPK increases the activity of SIRT1 through dissociation of DBC1 from the SIRT1-DBC1 complex [113]. This result provides the possible link between metabolic signals and oncogenic pathways that are regulated by SIRT and/or DBC1, linking metabolism and cancer.

Other DBC1 interacting proteins relevant to cancer

Overexpressed c-Myc sequesters DBC1 to induce SIRT1 activity, which stabilizes c-Myc through a positive feedback loop to regulate proliferation and survival in cancer cells [114]. Interestingly, SIRT1 and c-Myc also has been shown to suppress transformation, cell growth, and metabolism through negative feedback [114, 115]. These studies suggest that DBC1 might be critical in regulating the functions of c-Myc in cancer.

PART II

Anoikis

Definition of anoikis and significance of anoikis resistance in metastasis

Extracellular matrix (ECM) is critical to the development and stabilization of tissue structure. Anoikis is form of apoptosis that occurs when normal epithelial cells lose their contact with the ECM, or when cells are misplaced onto an inappropriate matrix [116]. Carcinoma cells are known to be resistant to anoikis, which allows the tumor cells to survive upon detachment and travel through lymphatic and circulatory systems resulting in metastasis. Anoikis resistance therefore represents a critical stage of metastasis [117] and understanding the mechanisms regulating anoikis sensitivity will prove an important first step towards targeting metastatic malignancies.

Regulation of Anoikis

Survival signals are induced by attachment of epithelial cells to ECM through the integrin and other growth factor receptors. When these signals are disrupted, anoikis will be induced. Several key factors and pathways that regulate anoikis are summarized below, but more detailed information is reviewed in Frisch et al. [118].

Integrin and growth factor Receptors

Integrins are transmembrane receptors activated upon attachment of ligands such as fibronectin, collagen, laminin, and thrombospodin, which compose the ECM. Integrins exist as heterodimers including different alpha and beta subunits, resulting in about 24 unique integrins made through various subunit combinations [119]. In addition to functioning as a bridge between ECM and

cytoskeletal proteins, integrins also regulate cell migration, survival, differentiation, cell cycle progression and apoptosis [119]. Moreover, integrins have been shown to regulate anoikis through different survival molecules and pathways [120]. For instance, integrins suppress anoikis through activating either Shc/MAPK and/or ILK/PI3K/Akt survival signaling pathways [121]. In addition, integrins can partly regulate anoikis through control of metabolic pathways including the pentose phosphate pathway and molecules that are important for survival such as NADPH and reactive oxygen species (ROS) whose level is induced by depletion of ATP upon detachment [122, 123].

Integrins also are known to crosstalk with growth factors to regulate anoikis through activating survival signaling pathways. Resistance to anoikis in tumor cells may be caused by a loss of integrin- growth factor coupling, allowing inappropriate survival signals to be maintained upon detachment. In colon cancer, for example, increased expression of EGF induces Src-Fak association through EGFR and leads to anoikis resistance[124]. Through crosstalk with growth factor receptors such as EGFR, integrins also regulate Bim (pro-apoptotic, BH3-only protein) expression and anoikis via the MEK/Erk pathway [125]. In addition, Insulin and Insulin-like growth factor receptors (IGFRs) are also known to induce anoikis resistance through PI3K/Akt signaling pathway [126] .

Pro-/anti-apoptotic molecules

Upon loss of association of integrin with ECM through detachment, expression of pro-apoptotic BH3-only Bcl-2 family members such as Bax, Bim, and Bid is increased which inactivates anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-xL, resulting in stimulation of intrinsic apoptotic pathways to induce anoikis [125]. Upon detachment, Bax and Bid localize to the

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cytoplasm to induce permeablization of mitochondria and the release of cytochrome c and apoptotic molecules while Bim translocates to the mitochondria, where it interacts with Bcl-xL, neutralizing the pro-survival function of Bcl-xL [125]. Downregulation of an anti-apoptotic, endogenous caspase-8 inhibitor c-FLIP, leads to activation of extrinsic apoptotic pathway, which involves Fas ligand (FasL)-Fas-Associated protein with Death Domain (FADD) complex formation and activation of caspase-8 [119]. In fact, some cancer cells that overexpress Bcl-2, c-FLIP, and/or Bcl-xL protect the cells from anoikis through blocking the transmission of death signaling to the mitochondria upon detachment [127, 128].

Rho family GTPase signaling

Small GTPase proteins like Rac and cdc42 are also important for adhesion-dependent signaling pathways and migration of cells through regulating actin cytoskeletons. In MDCK cells, it has been shown that Rac and cdc42 suppress anoikis in the PI3K/Akt signaling dependent manner. In addition, cdc42 can regulate anoikis through a positive feedback loop involving Rac and PI3K [129-131].

In addition to these pathways and molecules, epithelial-to-mesenchymal transition (EMT) has been recognized as a critical factor in regulation of anoikis sensitivity.

Epithelial-to-mesenchymal transition (EMT)

Intracellular adhesion proteins like E-cadherin (adherent), occludins (tight), claudins (tight), desmogleins (desmosome) and desmocollins (desmosome) promote and maintain the integrity of epithelial layers by regulating cell polarity [115]. In addition, epithelial-specific cytosolic

proteins such as Ankyrin-G and Rab25 maintain the appropriate localization of these junction proteins [116, 117]. Several molecules that regulate epithelial gene expression program have been identified, such as GATA3 [132], GRHL2 [133, 134], mir200 family of micro-RNAs [135], ESRP1 and ESRP2 [136]. The detailed mechanism of these molecules is illustrated in Frisch et al. 2013 [118].

During EMT, epithelial cells lose their polarized structure and cell–cell junctions, undergo cytoskeletal re-organization and gain mesenchymal characteristics, such as fibroblast-like cell morphology and increased cell migration and invasion [137]. The EMT process is important for many different cellular processes such as embryonic development, wound healing, inflammation and cell motility in normal epithelial cells [137]. However, when epithelial tumor cells undergo EMT, they can convert into migratory mesenchymal cancer cells that endure anchorage-independent survival, which leads to cancer metastasis. Therefore, anoikis resistance has been recognized as a hallmark of oncogenic EMT [138].

Mechanisms that link EMT and anoikis

Several key mechanisms that link EMT and anoikis-resistance are described here. More detailed information is also reviewed in Frisch et al.[118].

Polarity proteins through TGF-β, Wnt, and hippo pathways

Loss of cell polarity proteins such as Scribble, Crumbs, and Par, or translocation of these proteins to wrong places during EMT, can lead to activation of signaling pathways causing

anoikis resistance. These polarity molecules regulate anoikis through association with different signaling molecules involved in EMT, mainly the Wnt, TGF-β, and Hippo pathways.

Two co-activators in Hippo-pathway, Yes-associated protein (YAP) and Transcriptional coactivator with PDZ-binding motif (TAZ) are key oncogenic transcriptional cofactors that bind to Scalloped (Sd) transcriptional factor in the active/un-phosphorylated form to induce expression of genes promoting cell growth, proliferation and inhibition of apoptosis [139].   In normal epithelial cells, YAP/TAZ is in a phosphorylated/inactive state interacting with Smad in the nucleus. Upon stimulation, phosphorylated YAP/TAZ localizes to the cytoplasm and binds to the polarity proteins Crumbs and Scribble in the membrane, sequestering Smad protein thus inactivating both TGF-β and Hippo signaling pathways [140]. Inactivation of these signals would result in an increase in anoikis sensitivity and this might be a mechanism by which normal epithelial cells maintain anoikis sensitivity.   During EMT, mislocalized Scribble protects against anoikis through activating TAZ [141, 142] or by inhibiting c-Myc induced Rac-JNK-c-Jun-Bim apoptotic pathway in breast cancers [143]. Lastly, Dvl (Dishevelled homolog), a segment polarity protein, regulates anterior and posterior polarities by affecting the transmission of Wnt and Hedgehog pathways. During EMT, YAP and TAZ proteins activate the Wnt signaling pathway through inhibiting Dvl protein, which results in an increase in anoikis resistance [144].

EMT inducing transcriptional factors

Snail, Slug, Twist, and ZEB

Several different bHLH and Zn-Finger transcription factors such as Snail, Slug, Twist and ZEB [137] mediate oncogenic EMT and anoikis resistance through transcription repression of genes involved in apoptotic pathways and genes maintaining epithelial phenotype [118].

 A number of studies show that Snail, Slug, Twist and ZEB transcription factors induce EMT and anoikis resistance through downregulation of E-cadherin in response to various stimuli, such as TGF-β [145]. In addition, these molecules can regulate EMT and anoikis through other mechanisms. For instance, ZEB1 regulate EMT and anoikis through repressing expression of p73, a member of p53 family protein involved in maintaining mammary epithelial cell polarity by up-regulation of E-cadherin [146, 147].   In addition, a study utilizing human adenoid cystic carcinoma (ACC) shows that Slug induces EMT and anoikis resistance through affecting EGFR/PI3K/Akt pathway [148]. Twist cooperates with c-Myc to induce hyper-proliferation and transformation while inhibiting the c-Myc-ARF mediated apoptotic pathway [149]. c-Myc induces apoptosis upon cell-cell contact inhibition, which suggests that Twist induces EMT and anoikis resistance through inactivating the c-Myc mediated apoptotic pathway while activating c-Myc mediated proliferation [143].   Snail has been known to induce EMT and enhance the metastatic potential of noninvasive breast epithelial cells such as MCF7 in response to overexpression of the transcription/translation regulatory protein Y-box binding protein-1 (YB-1) through downregulation of epithelial and growth-related genes and activation of mesenchymal genes [150].

CtBP

C-terminal binding protein (CtBP) was found as a co-repressor whose activity is inhibited by E1a, which is known to induce anoikis sensitivity, through direct interaction. CtBP confers anoikis resistance through repressing expression of p53-target genes such as p21, PERP, Bax and Noxa [151]. In addition, CtBP interacts with EMT inducting transcriptional factors such as ZEB1 and ZEB2 to co-repress the promoter of epithelial phenotype related genes such as E-cadherin, desmoglein-2, and gap junction-β3 in some tumor cells [152]. These studies suggest that CtBP co-represses epithelial and pro-apoptotic gene expression programs, potentially contributing to EMT, anoikis resistance and tumor metastasis [153]. Moreover, CtBP1/2 are known to be the targets in p14ARF-mediated p53-independent apoptosis in colon cancer. Interaction with p14ARF mediates proteasomal degradation of CtBP, which allows the induction of apoptosis [154].

NF-κB

There are several studies showing the significance of NF-κB in EMT and anoikis resistance. Especially in intestinal epithelial cells, NF-κB activity is induced upon detachment and it happens through inducing expression of downstream target genes regulating survival signaling pathways such as the IAP family proteins, XIAP, survivin, c-FLIP, osteoprotegerin, and Bcl-xL [155, 156]. In addition to these targets, NF-κB also regulates genes such as TNFα and IL-6 which are involved in inflammatory pathways that further activate NF-κB signaling to induce EMT and metastatic progression [157, 158]. In addition, survivin and XIAP complex is known to activate NF-κB, which leads to increased expression of fibronectin, a mesenchymal marker. This

in turn establishes integrin clustering under detached conditions and results in rescue from anoikis [159].

FAK, ILK, and trkB

The anoikis suppressing effect of focal adhesion kinase (FAK) and integrin linked kinase (ILK) is well established [118]. FAK and ILK are intracellular adaptor kinases that link the cell-adhesion receptors, integrins and growth factors to the actin cytoskeleton and with a range of signaling pathways [121]. FAK and ILK colocalize with integrins at the site of cell attachment to ECM [160, 161] . Therefore, upon ligand (collagen)-integrin interaction or growth factor (TGF-β)-receptor binding, FAK and ILK are activated to regulate EMT. Several studies suggest that FAK induces EMT through PI3K-MAPK pathway by either up-regulation of KLF8, which is known to repress E-cadherin promoter [162-164], or EMT-inducing transcription factors like Snail1, ZEB1, and Twist2 [165] . In addition, FAK has been shown to induce EMT through either downregulation or internalization of E-cadherin in fibroblasts both in vitro and in vivo [166, 167]. ILK is also known to regulate EMT through up-regulation of EMT inducing factors like Snail and Slug upon TGF-β stimulation and down-regulation of E-cadherin [168, 169].

TrkB is a neurotrophic tyrosine kinase receptor known to be overexpressed in many different cancers such as pancreatic and ovarian cancers [170, 171]. TrkB has been known to contribute to disease progression by inducing EMT and inhibiting anoikis. Overexpression of TrkB induced EMT [172, 173], while inhibiting anoikis through PI3K/Akt pathway [171, 174].

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CHAPTER 2

Regulation of anoikis through a novel pathway induced by loss of E-cadherin and EMT

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*Co-First authors

INTRODUCTION

Anoikis is apoptosis that is induced upon cell-matrix detachment or attachment to inappropriate matrix. While normal epithelial cells are sensitive to anoikis, tumor cells are known to be resistant due to genetic mutations or defects in the signaling pathways leading to constitutive activation of survival signaling and inactivation of the apoptotic pathway [1-5].

The oncogenic epithelial-to-mesenchymal transition (EMT) is critical for tumor development and metastasis [6, 7]. Decrease in cell adhesion proteins like E-cadherin is the hallmark of EMT. E-cadherin loss induces multiple signaling pathways during EMT which makes cells resistant to anoikis [8]. In fact, downregulation of E-cadherin expression through specific up-regulation of EMT mediating transcriptional factors like ZEB results in induction of EMT, cancer progression, and metastasis [9, 10]. In addition to ZEB, other oncogenic transcription factors such as Twist and Snail1/2 can also suppress anoikis both by regulating apoptosis control genes and by suppressing E-cadherin expression [11, 12].

Onder et al. showed that stable knock down of E-cadherin regulates anoikis through decreased phosphorylation of β-catenin, which results in activation of Wnt signaling [13]. In this study, a novel molecular mechanism in which E-cadherin regulates anoikis sensitivity through Ankyrin-G-NRAGE-TBX2 pathway is investigated.

NRAGE (Neutrophin Receptor-interacting MAGE protein), also known as MAGE D-1, is a type II MAGE (melanoma antigen) protein which is broadly expressed in embryonic and adult somatic tissues [14]. NRAGE is known to function in various cellular process such as Dlx/Msx mediated

transcription, cell cycle progression and myogenic differentiation [14, 15]. MAGE family members share a highly conserved MAGE homology domain that interacts with various membrane proteins such as p75 neurotrophin receptor and Unc5 netrin receptor to facilitate apoptosis [16, 17]. The fact that NRAGE mediates p38 activation and neural progenitor apoptosis, and NRAGE knockout mice have a developmental defect in brain neuronal apoptosis, supports NRAGE as a pro-apoptotic factor [18, 19]. Nevertheless, NRAGE is shown to be overexpressed in several cancers such as head and neck, lung, and kidney cancers [20-22]. In addition, quantitative RT-PCR and quantitative immunofluorescence data showed that NRAGE is overexpressed in breast, lung, colorectal, and metastatic skin cancers [23]. Moreover, NRAGE expression was induced by EMT mediated by Twist and depletion of E-cadherin in Human mammary epithelial cells (HMLE) [23]. However, the molecular mechanism of NRAGE as a pro-tumorigenic factor has not been well studied. It has been shown that MAGE domain of NRAGE directly interacts with UPA (Unc5-PIDD-ankyrin) component of supermodule [23, 24]. NRAGE localization is regulated by Ankyrin-G: NRAGE is sequestered in cytoplasm in normal epithelial cells that express Ankyrin-G whereas NRAGE mainly localizes in nucleus in cells that have undergone EMT, thus down-regulating ankyrin-G, or cells that have depleted level of Ankyrin-G by siRNAs [23].

TBX2 is a member of the T-Box (TBX) family of transcriptional repressor proteins that are known to regulate embryonic development, cell cycle progression, senescence and apoptosis. In addition, TBX2 has been implicated in tumor development through downregulating the p14ARF (Alternative reading frame) tumor suppressor, which is frequently mutated in human carcinomas and promotes apoptosis through p53 dependent and independent, c-Myc and CtBP signaling pathways [25-28]. In fact, TBX2 is known to be overexpressed in melanomas, breast, bladder,

and pancreatic cancers [25, 29-31]. TBX2 promoted anchorage independent survival which was independent of p53 in SW13 cells [32] . However, the role of TBX2 or p14ARF in anoikis regulation has not been previously studied.

In this chapter, we will demonstrate that during oncogenic EMT, cytoplasmic NRAGE localized to the nucleus and interacted with TBX2 to co-repress p14ARF conferring anoikis resistance.

MATERIALS AND METHODS

All materials and methods for this chapter have been published elsewhere [33].

RESULTS

The loss of the cell-cell adhesion protein E-cadherin through transduction with shRNA directed against this molecule has been associated with induction of the oncogenic epithelial to mesenchymal transition (EMT) in normal human mammary epithelial cells (HMLE) which are immortalized with SV40 T antigen and telomerase [34]. Likewise in these HMLE cells, ectopic expression of Twist, a known cellular repressor of E-cadherin, has been shown to promote this EMT process [34]. A significant resistance to the cell death phenomenon known as anoikis has been associated with this oncogenic EMT. We have confirmed these findings, both in the context

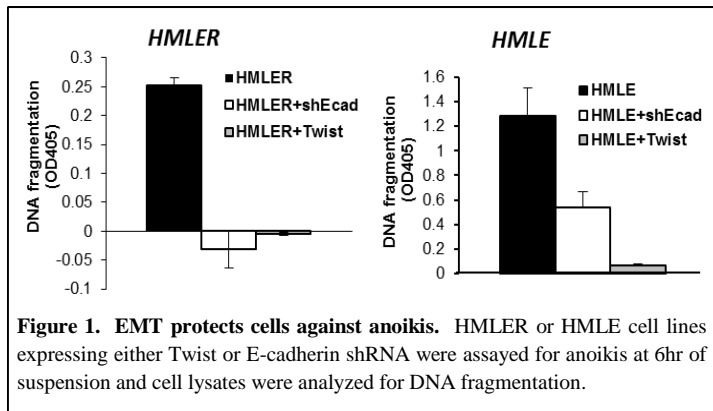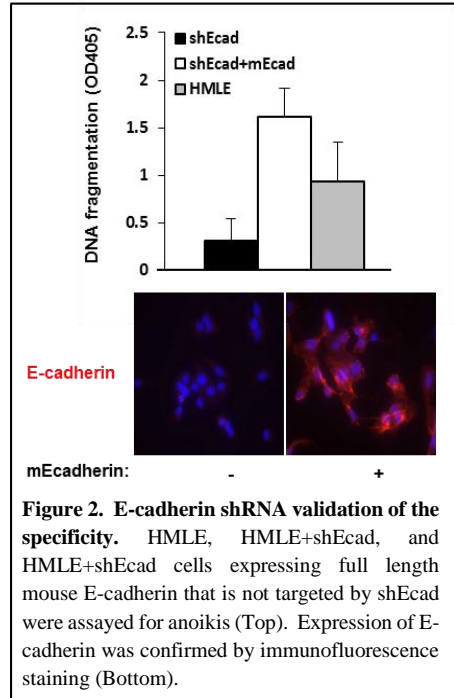**Figure 1. EMT protects cells against anoikis.** HMLER or HMLE cell lines expressing either Twist or E-cadherin shRNA were assayed for anoikis at 6hr of suspension and cell lysates were analyzed for DNA fragmentation.

mentioned before as well as in both "HMLER cells," [13] a cell line expressing activated RAS, as well as in cell lines expressing other EMT associated genes (Fig. 1). Expression of a mouse

"shRNA-resistant" form of E-cadherin (Fig. 2), was shown to ameliorate the effect that E-cadherin loss has on anoikis resistance, and in effect, confirming the specificity of the E-cadherin knockdown.

Ankyrin-G is known to co-localizes with E-cadherin [34], suggesting that ankyrin-G participates in E-cadherin signaling. Ankyrin-G expression is downregulated in claudin-low subclass of breast cancer tumors which suggest that Ankyrin-G is regulated with EMT [23, 35]. In fact, experimental induction of EMT suppressed Ankyrin-G expression [23]. Functionally, overexpression of AnkΔMBD-CAAX (smaller version of ankyrin-G made by deleting N-terminal membrane binding domain containing a C-terminal CAAX box that leads to membrane localization) resulted in sensitization of HMLE+shEcad cells to anoikis (Fig. 3 and 4). These results indicate that loss of Ankyrin-G in context of human cancer and EMT may be understood in terms of a cell survival effect.

Figure 2. E-cadherin shRNA validation of the specificity. HMLE, HMLE+shEcad, and HMLE+shEcad cells expressing full length mouse E-cadherin that is not targeted by shEcad were assayed for anoikis (Top). Expression of E-cadherin was confirmed by immunofluorescence staining (Bottom).

Figure 3. Ankyrin-G is an anoikis promoter. HMLE+shEcad cells expressing either control vector or ankyrin ΔMBD-CAAX were assayed for anoikis and lysates were measure for DNA fragmentation.

Figure 4. Validation of the specificity of the effect of re-expressed ankyrin-G on anoikis. HMLE+shEcad cells expressing either control vector or ankyrin ΔMBD-CAAX were assayed for anoikis using the trypan blue/ cell permeability assay.

Ankyrin-G regulated the localization of NRAGE through direct interaction, suggesting that the interaction between Ankyrin-G and NRAGE is functionally significant, because NRAGE interacts with both receptors and transcription factors. The potential role of NRAGE in anoikis was tested by depleting it in cells that had undergone EMT such as HMLE+shEcad and HMLE+Twist cells.

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The cell lines that have transiently transfected with NRAGE siRNAs became significantly more sensitive to anoikis (Fig. 5 and 6). Consistent effect of NRAGE on suppressing anoikis was also

Figure 5. NRAGE protects cells against anoikis. HMLE+shEcad cells transfected with a pool of four different NRAGE siRNAs were assayed for anoikis (Left). HMLE+shEcad cells transfected with an additional siRNA targeting different sequences of NRAGE were assayed for anoikis (Right).

Figure 6. Depletion of NRAGE sensitizes HMLE+twist cells to anoikis. HMLE+twist cells transfected with a pool of four different NRAGE siRNAs were assayed for anoikis (Left). HMLE+twist cells infected with a doxycycline-inducible NRAGE shRNA were incubated for 72hrs with or without doxycycline and assayed for anoikis (Right).

shown both in the lung and breast carcinoma cells, NCI-H1299 and MDA-MB-435 respectively (Fig. 7). In addition, depletion of NRAGE in MDA-MB-435 cells decreased formation of anchorage-independent colony formation (Fig. 7).

Figure 7. Depletion of NRAGE sensitizes tumor cell lines to anoikis. MDA-MB-435 cells treated with either control or NRAGE siRNA were assayed for anoikis at 48hr suspension (Left), and were also assayed for soft colony formation (p<0.0002) (Middle). The efficiency of NRAGE siRNA in MDA-MB-435 cells is shown in figure 13. NCI-H1299 cells transfected with either control or NRAGE siRNA (Smartpool) were assayed for anoikis (Right). The efficiency of NRAGE knockdown in NCI-1299 cells was confirmed by immunoblotting as shown here.

To investigate how NRAGE suppressed anoikis, a yeast two-hybrid interaction was performed [23]. Among the several candidate proteins that were identified, TBX2, was investigated. The interaction between TBX2 and NRAGE was confirmed by immunoprecipitating endogenous NRAGE from HMLE+shEcad cell lysates (Fig. 8).

Figure 8. TBX2 and NRAGE endogenous interaction. HMLE+shEcad cells were immunoprecipitated with either NRAGE antibody or normal IgG antibody. The immunoprecipitates and total lysates were probed for both NRAGE and TBX2. NR: NRAGE, Con: Control.

Figure 9. The repression domain of TBX2 is required for the interaction with NRAGE. 293T cells were co-transfected with either FLAG-TBX2, FLAG-TBXΔRD, or control vector. The cell lysates were immunoprecipitated with FLAG beads and the precipitates were analyzed by western blotting.

The reverse co-IP was unreliable for technical reasons (data not shown). The repression domain of TBX2 was critical for the interaction with NRAGE (Fig. 9). These results suggested that NRAGE might regulate anoikis through interacting with the oncogenic transcriptional repressor TBX2, and that NRAGE might participate in gene

Figure 10. The interaction of NRAGE and TBX2 protects cells against anoikis. NRAGE knock out mouse embryo fibroblasts (MEFs) rescued with the wild-type or the mutant NRAGE (ΔC) construct were assayed for anoikis (Left). The expression of the constructs was confirmed by immunoblotting (Right).
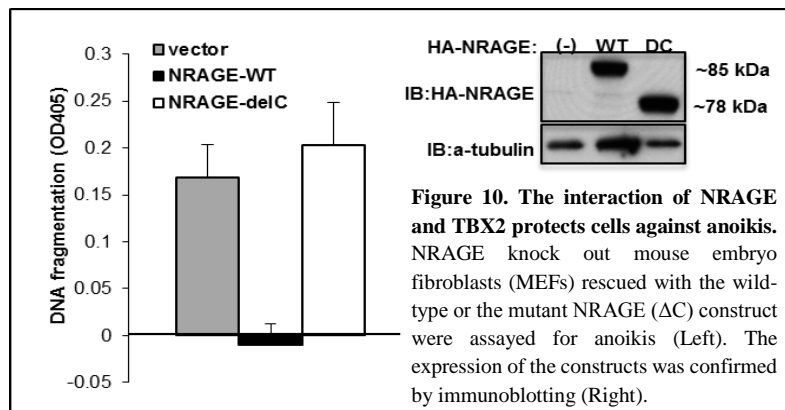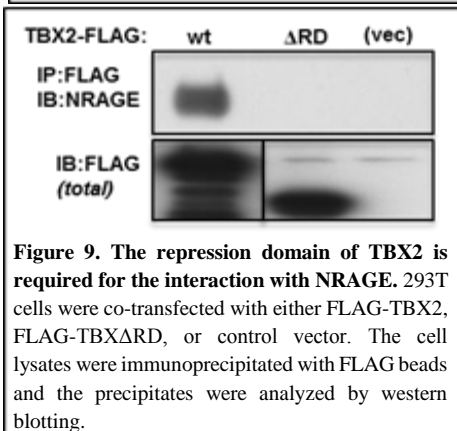
repression by TBX2. Consistently, the rescued expression of wild-type NRAGE protected NRAGE-knockout mouse embryo fibroblasts (MEFs) against anoikis while a mutant NRAGE lacking the TBX2-interaction domain had no e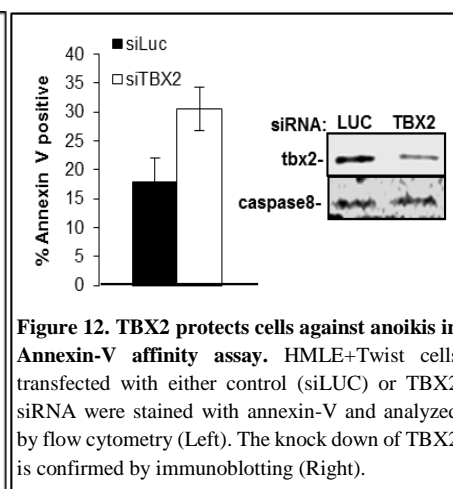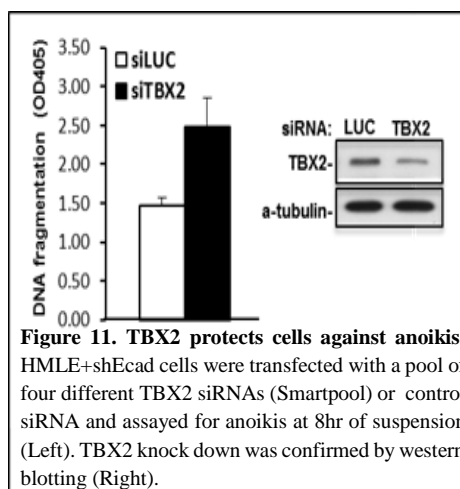ffect on anoikis (Fig. 10). Moreover, depletion of TBX2 using multiple siRNAs sensitized both HMLE+shEcad and HMLE+Twist cells to anoikis, suggesting that TBX2 was an anoikis suppressor (Fig. 11 and 12).

Figure 11. TBX2 protects cells against anoikis. HMLE+shEcad cells were transfected with a pool of four different TBX2 siRNAs (Smartpool) or control siRNA and assayed for anoikis at 8hr of suspension (Left). TBX2 knock down was confirmed by western blotting (Right).

Figure 12. TBX2 protects cells against anoikis in Annexin-V affinity assay. HMLE+Twist cells transfected with either control (siLUC) or TBX2 siRNA were stained with annexin-V and analyzed by flow cytometry (Left). The knock down of TBX2 is confirmed by immunoblotting (Right).

The effect of NRAGE in TBX2-mediated transcriptional repression was tested. TBX2 is known to repress p14ARF. The level of P14ARF protein was increased by the depletion of NRAGE protein both in HMLE+shEcad and HMLE+Twist (Fig 13). Similar effect was observed
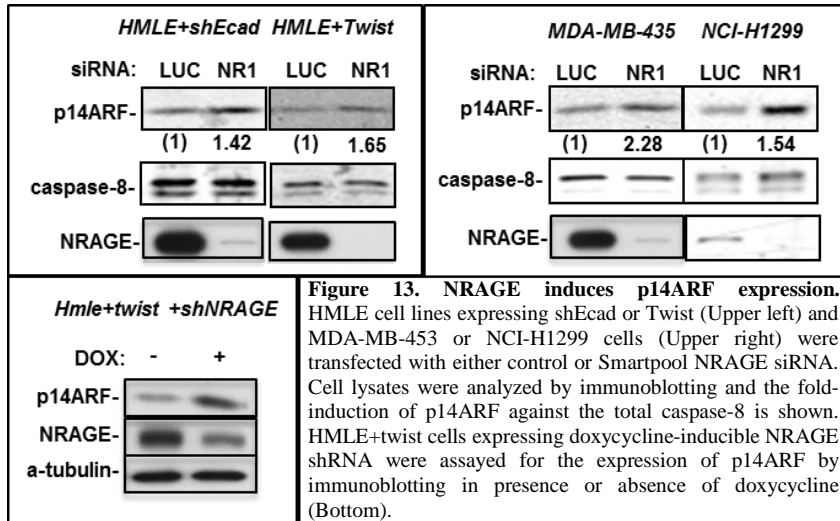
Figure 13. NRAGE induces p14ARF expression. HMLE cell lines expressing shEcad or Twist (Upper left) and MDA-MB-453 or NCI-H1299 cells (Upper right) were transfected with either control or Smartpool NRAGE siRNA. Cell lysates were analyzed by immunoblotting and the fold-induction of p14ARF against the total caspase-8 is shown. HMLE+twist cells expressing doxycycline-inducible NRAGE shRNA were assayed for the expression of p14ARF by immunoblotting in presence or absence of doxycycline (Bottom).

in tumor cells such as MDA-MB-435 (breast carcinoma) and NCI-H1299 (lung carcinoma) (Fig. 13). In addition, to eliminate off-target effects, and to show that the mechanism of p14ARF induction is transcriptionally mediated, RT-PCR and western blotting were performed using different siRNAs and cell lines. The results showed that p14ARF protein (Fig. 14) and also mRNA [23] were found to be inducible by NRAGE siRNA, suggesting

Figure 14. Confirmation of the specificity of the NRAGE knockdown effect on p14ARF. HMLER+shEcad cells transfected with either control or NRAGE siRNA (Smartpool) were analyzed for p14ARF induction (Left). HMLER cells transfected with control siRNA or two different NRAGE siRNAs (NR-D3 and NR-D4) were analyzed for p14ARF by immunoblotting (Right). The p14ARF fold-inductions normalized against the tubulin are shown in the lower portions of the top panel.
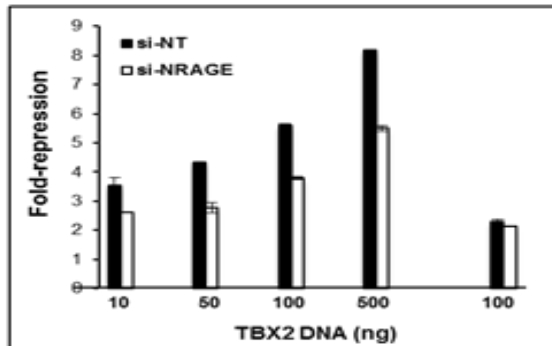

Figure 15. TBX2 requires NRAGE to repress the promoter activity of p14ARF. 293T cells pre-transfected with either a non-targetting (NT) or NRAGE siRNA (Smartpool) were co-transfected with different amounts of a TBX2 vector and a p14ARF promoter-luciferase vector containing either wild-type (first four pairs) or mutant TBX2 binding sites (last pair).
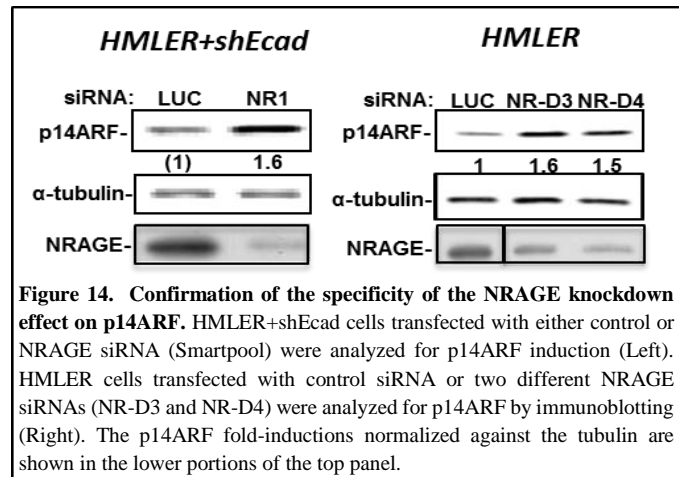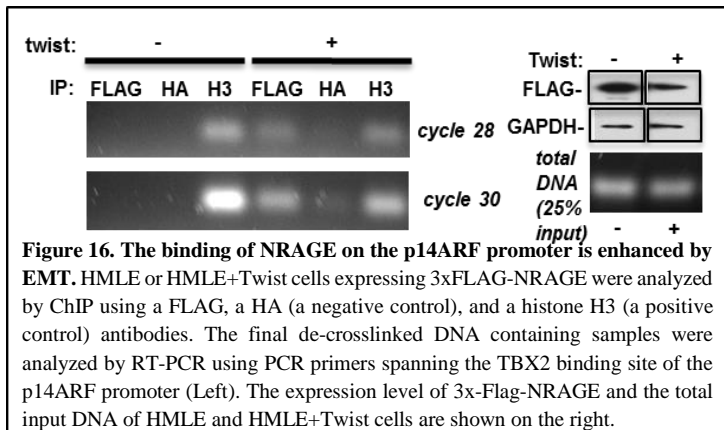
that NRAGE function as a co-repressor for TBX2. To further show that NRAGE is the co-repressor of TBX2 on regulating p14ARF expression, reporter assays were performed to measure the effect of NRAGE on TBX2 repressor activity. Depletion of
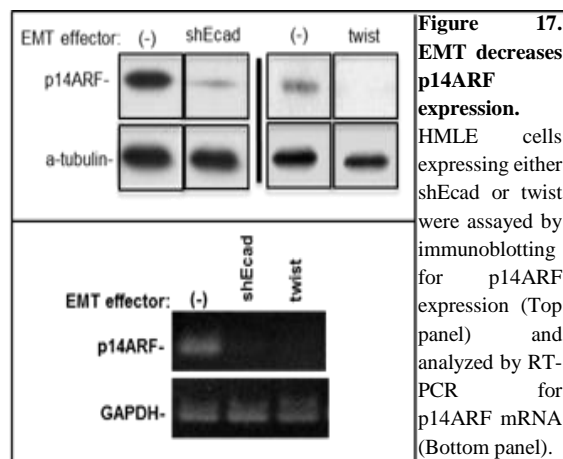
endogenous NRAGE in the TBX2-deficient cell line 293T impaired the ability of co-transfected TBX2 to repress the p14ARF promoter (Fig. 15).
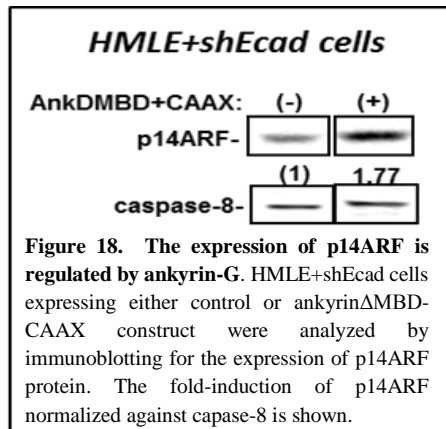
The effect of EMT on interaction of NRAGE with the p14ARF promoter was tested. ChIP with FLAG antibody (on chromatin from cells that stably expressed 3XFlag-NRAGE) showed that significantly more NRAGE was bound to the ARF promoter in cells that had undergone EMT due to Twist expression than in the control HMLE cells (Fig. 16). Consistent with this, twist expression

Figure 16. The binding of NRAGE on the p14ARF promoter is enhanced by EMT. HMLE or HMLE+Twist cells expressing 3xFLAG-NRAGE were analyzed by ChIP using a FLAG, a HA (a negative control), and a histone H3 (a positive control) antibodies. The final de-crosslinked DNA containing samples were analyzed by RT-PCR using PCR primers spanning the TBX2 binding site of the p14ARF promoter (Left). The expression level of 3x-Flag-NRAGE and the total input DNA of HMLE and HMLE+Twist cells are shown on the right.

also promoted the nuclear localization of NRAGE and the repression of p14ARF [23]. These results suggested that ankyrin-G could regulate p14ARF, by binding NRAGE protein, thus regulating anoikis-sensitivity.

Endogenous p14ARF expression was downregulated both in both HMLE+shEcad and HMLE+Twist cells compared to the parental HMLE cells (Fig. 17). However, when NRAGE was sequestered by the ectopic expression of ankyrinΔMBD-CAAX protein [23], the expression of p14ARF was increased (Fig. 18), indicating that Ankyrin-G alleviated the repressive effect of NRAGE. Therefore, these results demonstrate that expression of p14ARF is regulated by Ankyrin-G during EMT.

Figure 17. EMT decreases p14ARF expression. HMLE cells expressing either shEcad or twist were assayed by immunoblotting for p14ARF expression (Top panel) and analyzed by RT-PCR for p14ARF mRNA (Bottom panel).

Figure 18. The expression of p14ARF is regulated by ankyrin-G. HMLE+shEcad cells expressing either control or ankyrinΔMBD-CAAX construct were analyzed by immunoblotting for the expression of p14ARF protein. The fold-induction of p14ARF normalized against capase-8 is shown.

P14ARF promoted anoikis-sensitivity. Depletion of p14ARF at both mRNA and protein levels via siRNA transfection moderately protected against anoikis, suggesting that the p14ARF tumor suppressor gene is criti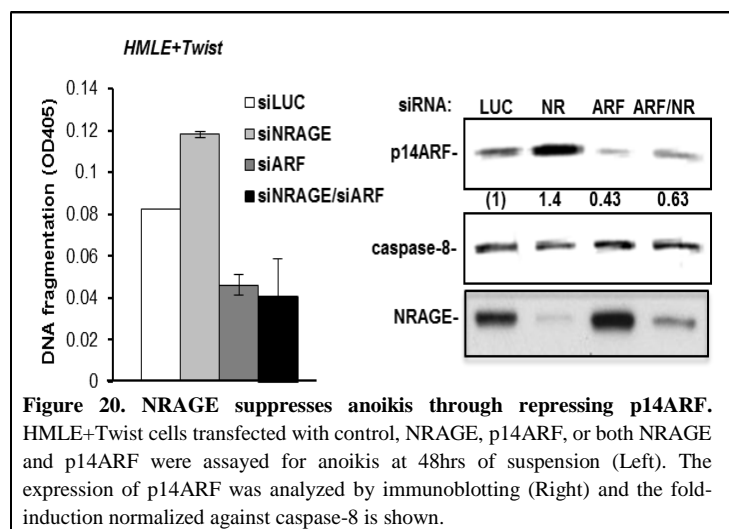cal in the induction of anoikis (Fig. 19). These results led to a hypothesis that the NRAGE-TBX2 complex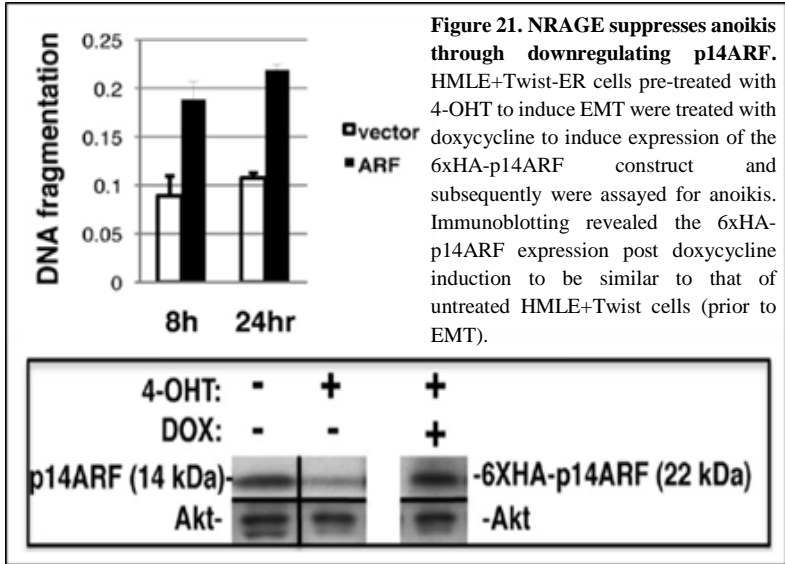 suppressed anoikis by suppressing expression of p14ARF. To further explore this possibility, HMLER+Twist cells were transfected

Figure 19. p14ARF promotes anoikis. HMLER cells transfected with either control or p14ARF siRNA (exon1 beta-specific) were suspended for 24hrs and assayed for anoikis (Left). The knock down of p14ARF is confirmed by western blotting (Right).
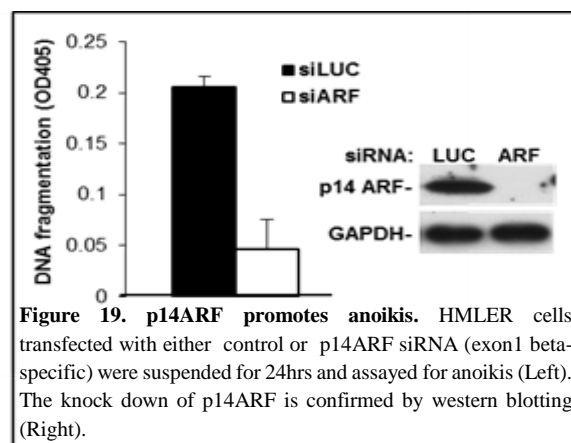
with either NRAGE siRNA, p14ARF siRNA or both (Fig. 20). The depletion of NRAGE led to an increase in anoikis sensitivity and up-regulation of p14ARF. However, the effect of NRAGE depletion was abolished when cells were pre-treated with p14ARF siRNA, confirming that the mechanism of NRAGE regulation of anoikis-

sensitivity is through p14ARF. Furthermore, when p14ARF was overexpressed in HMLE+Twist cells, sensitivity to anoikis was increased. These results demonstrate that regulation of p14ARF by NRAGE is critical for mediation of anoikis sensitivity (Fig. 21).

Figure 20. NRAGE suppresses anoikis through repressing p14ARF. HMLE+Twist cells transfected with control, NRAGE, p14ARF, or both NRAGE and p14ARF were assayed for anoikis at 48hrs of suspension (Left). The expression of p14ARF was analyzed by immunoblotting (Right) and the fold-induction normalized against caspase-8 is shown.

Figure 21. NRAGE suppresses anoikis through downregulating p14ARF. HMLE+Twist-ER cells pre-treated with 4-OHT to induce EMT were treated with doxycycline to induce expression of the 6xHA-p14ARF construct and subsequently were assayed for anoikis. Immunoblotting revealed the 6xHA-p14ARF expression post doxycycline induction to be similar to that of untreated HMLE+Twist cells (prior to EMT).

DISCUSSION

Loss of E-cadherin is critical for induction of EMT, leading to anoikis resistance, and metastasis [13, 36]. In fact, several oncogenic transcription factors such as Twist, Snail, and ZEB1/2, are known to induce EMT, and they confer anoikis resistance through downregulation of E-cadherin [12, 37, 38]. E-cadherin loss resulted in transcriptional change of various molecules, mediated by EMT [13]. Therefore, E-cadherin can act as a critical modulator of the cell biological phenotypes and the molecular factors that lead to anoikis resistance and metastatic dissemination of carcinomas. In light of its well-established function in maintaining adherent junctions, E-cadherin loss ostensibly promotes metastasis by enabling the first step of the metastatic cascade: the disaggregation of cancer cells from one another and the induction of anoikis resistance.

In this study, we demonstrated that depletion of Ankyrin-G, which has shown to interact with NRAGE, allowed the fraction of NRAGE to localize to nucleus where it interacted with TBX2 to repress the p14ARF promoter in cells that have undergone EMT. Repression of p14ARF promotes anoikis resistance allowing the cells to survive upon detachment from the extracellular matrix.

We observed that NRAGE is an anoikis suppressor. This data has been supported by the fact that NRAGE is overexpressed in lung, kidney, and head and neck cancers as well as colon, melanoma, and prostate tumors [20, 22, 23]. On the other hand, NRAGE has been shown to inhibit metastasis in melanoma and pancreatic cancer cell lines. In addition, overexpression of NRAGE inhibited malignant phenotypes, such as proliferation, migration, and invasion in breast cancer cell lines [39, 40]. This contradicting function of NRAGE could be due to its differential function based on its localization. MAGE-D1/NRAGE induces apoptosis through interacting with receptors such as

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Unc5 and p75NTR [24, 41]. In fact, nuclear localization of NRAGE and interaction with TBX2 were increased in cells that have undergone EMT [23]. To further evaluate the function of NRAGE as an anoikis suppressor and a pro-tumorigenic factor, the use of a genetically engineered mouse model may provide valuable insight for future research.

TBX2 is frequently amplified in pancreatic and overexpressed in melanomas and breast cancers and plays an important role in tumorigenesis [25, 29, 30]. Consistently, we have demonstrated here that TBX2 suppresses anoikis and permits anchorage independent growth [23], which further supports TBX2 as a tumorigenic factor. TBX2 is known to interact with several co-repressors to promote metastasis such as KAP-1, Ajuba, and CtBP [42-45] . However, the mechanism of repression by TBX2 is not well understood. Our data showed that NRAGE is a novel co-repressor of TBX2. As with other co-repressors, NRAGE likely targets multiple genes for repression, which remain to be identified.

Our results demonstrated that p14ARF, which is frequently deleted in human cancers [27], is an anoikis promoter. This indicates a novel function of the tumor suppressor gene. P14ARF haploinsufficiency shows a pro-tumorigenic phenotype, consistent with the idea that even a two-fold decrease of ARF expression (as observed here) has important biologic consequences.

These data demonstrated a novel pathway, ankyrin-G/NRAGE/TBX2/p14ARF, that confers anoikis-resistance in response to EMT (Figure 22).
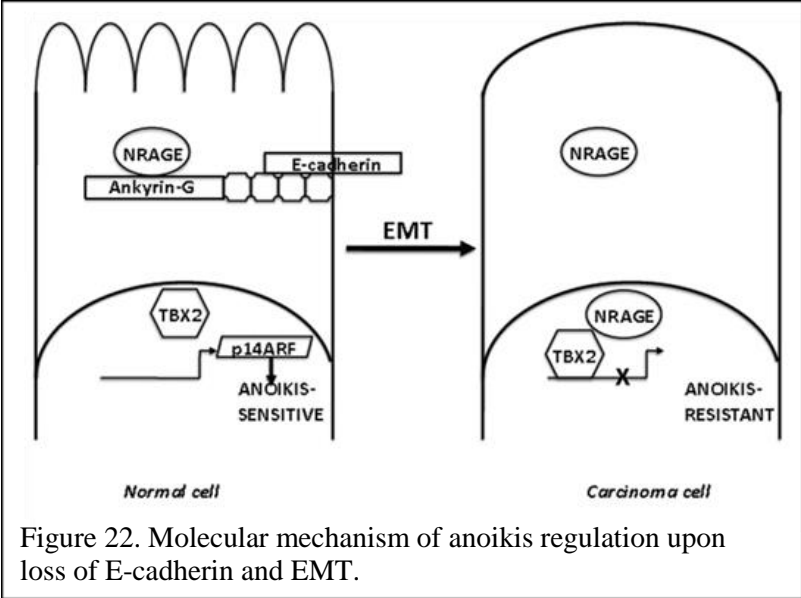
Figure 22. Molecular mechanism of anoikis regulation upon loss of E-cadherin and EMT.

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CHAPTER 3

Regulation of anoikis by Deleted in Breast Cancer-1 (DBC1) through NF-κB

Sun Hee Park, Philip Riley IV, Steven M Frisch

ABSTRACT

Anoikis-resistance of tumor cells is critical for anchorage-independent growth and metastasis. The inflammatory-response transcription factor NF-κB contributes to anoikis-resistance and tumor progression through mechanisms that are understood incompletely. Deleted in Breast Cancer-1 protein (KIAA1967) is over-expressed in several tumor types, and correlates with a poorer prognosis in some cases. We report here that DBC1 suppressed anoikis in normal epithelial and breast cancer cell lines. DBC1 interacted with IKKβ, stimulating its kinase activity, promoting NF-κB transcriptional activity through the phosphorylation of RelA serine-536 and enhancing the expression of the NF-κB target genes, c-FLIP and Bcl-xL. Our results indicate that DBC1 is an important co-factor for the control of the IKKβ-NF-κB signaling pathway that regulates anoikis.

INTRODUCTION

Anoikis is defined as apoptosis that is rescued by cellular interaction with an appropriate extracellular matrix [1]. Physiologically, it is critical for cellular homeostasis and development. Anoikis-resistance is a hallmark of metastasis, because it is required for anchorage-independent growth during tumor dissemination. Identification of the factors and mechanisms that control anoikis is a high priority in cancer cell biology and developmental therapeutics. Such factors may control either the set-point, i.e., the gene expression program controlling sensitivity vs. resistance of cells (which may exist prior to detachment from matrix) or the apoptosis-triggering mechanisms that occur in detached cells, or both. Indeed, oncogenes or tumor suppressor proteins affecting both functions have been identified [2-4].

Among the signaling and transcription factor pathways involved in regulating anoikis, NF-κB is notable because it links anoikis with inflammatory signaling between and within cells [5-7]. Consistent with this, several NF-κB target genes including, c-FLIP, survivin, Bcl-2, Bcl-xL, cIAP-2, xIAP, PLK1, and trkB protect tumor cells against anoikis [8-14]. Accordingly, NF-κB signaling is widely up-regulated in diverse tumor types [5, 7]. This up-regulation is attributed mainly to hyperactivation of upstream signaling pathways, except in a subclass of leukemias in which activating RelA mutations occur. These pathways include Akt, inflammatory cytokines, TNF and, interestingly, cell-matrix detachment of tumor cells, but not normal cells [6, 14].

Phosphorylation and acetylation regulate the NF-κB activation process in several respects, including nuclear translocation, DNA binding, and potency of transcriptional activation [15, 16]. The kinase IKKβ plays a particularly significant role in that it both promotes the translocation of NF-κB to the nucleus through the phosphorylation of IκB-α, as well as enhancing the ability of RelA to activate transcription

by phosphorylation of sites within the RelA activation domain [17-20]. The mechanisms linking the regulation of NF-κB to the control of anoikis are understood incompletely, however.

Deleted in Breast Cancer (DBC1) is a nuclear protein encoded by a gene on 8p21 that was originally believed to reside within a deleted region in breast cancer, a deletion assignment that was later found to be inaccurate [21]. In fact, DBC1 over-expression has been observed in colorectal, esophageal and breast cancers, where its over-expression correlates, in some cases, with poor prognosis [22-25]. These observations suggest a potential role of DBC1 in tumor progression, although paradoxical roles as a tumor suppressor have been proposed as well [26].

In this study, we demonstrate that DBC1 suppresses anoikis by activating IKKβ through a direct interaction, increasing NF-κB activity and enhancing the expression of key anoikis-relevant cell survival genes.

MATERIALS AND METHODS

Antibodies

Antibodies used in this study were from the following sources: DBC1 (pAb, Bethyl laboratory and mAb Cell signaling); RelA (Santa cruz biotechnology); RelA acetyl-lys310 (Abcam); β-Actin (Sigma); IKK-β (rabbit mAb, Cell signaling); p-RelA S536 (Cell signaling); β-Tubulin (Santa Cruz biotechnology); α-Tubulin (Millipore Mab DM1A); human c-FLIP (NF-6, Enzo life sciences, Inc); mouse c-FLIP clone DAVE-2 (Axxora), HA (Covance HA. 11 mAb (Ascites)); FLAG (Sigma mAb M2); GADPH (Sigma-Aldrich pAb G9545); Bcl-xL (rabbit pAb, Cell Signaling); cleaved caspase 3 (Cell Signaling); p-IκB-α (mouse mAb, Cell Signaling); NRAGE (Santa Cruz pAb sc-28243); Normal rabbit IgG (Covance).

Reagents

Reagents were from the following sources: TNFα (R&D System); Bay 11-7082 (Sigma-Aldrich); recombinant GST-IκBα and active IKK-β (Signalchem); S-protein HRP Conjugate (Novagen).

ShRNAs and siRNAs

DBC1 siRNA duplexes from Sigma Aldrich Sense: 5'-AAACGGAGCCUACUGAACAUU-3' Anti-Sense: 5'-AAUGUUCAGUAGGCUCCGUUU-3'. Non-targeting control siRNA (Dharmacon RNAi Technology). The siDBC1 sequences are adapted from [27]. DBC1 shRNAs in the vector pTRIPZ were from Open Biosystems: A6: 193-0178-A-6 GGTTCCACTTAACAACTA (in 5'UTR); B2: 193-0195-B-2 CGGCTCTACCTAGAGAAC (in coding sequence).

Protein Expression and Purification

Human DBC1 was expressed in E.coli BL21 using a clone in the GST vector pGEX-6P3 (generated as

described below). BL21 cells were grown in Luria broth supplemented with 50 µg/mL ampicilin at 37 °C overnight. Protein expression was induced by adding 1 mM isopropyl thio-β-D-galactopyranoside (IPTG) for 2hrs. Cells were pelleted by centrifugation at 5,000 rpm and washed with 1x PBS once and resuspended in bufferA (50 mM sodium phosphate buffer (pH 7.4), 500 mM KCl, 1 mM DTT and a protease inhibitor mixture (Pierce)). The cells were lysed by French Press (twice), and the lysate was centrifuged at 100,000 rpm for 1 hr to obtain the soluble fraction of the cell extract. The supernatant was incubated with 0.3 ml of 50% slurry of gluthione-sephorose (Pharmacia-pre-equilibrated with PBS) for 2 hours at 4 degrees on rotating wheel. Beads were washed once with wash buffer (50 mM Tris-HCl, pH8, 300 mM NaCl, 1 mM DTT/PBS+protease inhibitors) and washed 3 times with cleavage buffer (50 mM Tris-HCl, pH8, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol + 0.01% Triton X-100). To elute protein, 160 uL of cleavage buffer containing Protease (Pierce, 27-0843-01) mixture (12.8ul [80unit] of PreScission protease and 147.2ul of cleavage buffer) was added to 160ul of bead solution. The mixture was incubated for 5 hours at 4^0C on rotating wheel, spun down for 15 seconds at full speed, and the supernatant was transferred to a new tube. The protein sample was dialyzed with kinase assay buffer (see below) overnight using Slide-A-Lyzer MINI Dialysis Units (Thermo Scientific).

qRT-PCR

The primers used for RT-PCR were as follows:

CIAP-1 (BIRC2) 5-TTGAGGTGTTGGGAATCTGG-3, 5-GGCCTTTCATTCGTATCAAGAAC-3;

XIAP(BIRC4) 5-GCACGGATCTTTACTTTTGGG-3, 5-GGGTCTTCACTGGGCTTC-3; Survivin 5-CAAGGAGCTGGAAGGCTG-3, 5-TTCTTGGCTCTTTCTCTGTCC-3; BCL2 5-GTGGATGACTGAGTACCTGAAC-3, 5-GCCAGGAGAAATCAAACAGAGG-3; BCLXL 5-GACATCCCAGCTCCACATC-3, 5-GTTCCCATAGAGTTCCACAAAAG-3; FLIP 5-

CTTGGCCAATTTGCCTGTAT-3, 5-TCTTTGGCTTCCCTGCTAGA-3. Beta2M: 5-

GGCATTCCTGAAGCTGACAG-, 5-TGGATGACGTGAGTAAACCTG-3.

For real–time quantitative RT-PCR, RNA was purified from cell lines using RNeasy Plus mini kit (Qiagen, Germantown, MD) and cDNA was synthesized with invitrogen 18080-051 Superscript III First-strand Synthesis System for RT-PCR using 1-3 ug of RNA template with oligo-dT primers. Reactions were run in total 20 uL utilizing 10ul of 2x Applied Biosystems (Foster City, CA) SYBR green PCR Master Mix, 8 uL of 10 uM primer mix and 2 uL of 50X diluted cDNA.

Expression constructs

3xHA-DBC1-pCDNA3.1: DBC1 coding sequence from pCMV-SPORT6 vector (obtained from Open Biosystems) was ligated into the BglII-EcoRI site of 3xHA-pRc/CMV2 vector (D. Anderson, University of Saskatchewan). The 3xHA-DBC1 fragment is then subcloned by PCR into the HindIII-EcoRI site of pCDNA3.1.

pCDNA-IKK-β-Flag WT expression construct was obtained from Addgene (plasmid 23298).

DBC1/pGEX: Full length DBC1 coding sequence (aa1-923) amplified from DBC1-pCMV-SPORT6 vector by PCR was subcloned into pGEX-6P3 vector (GE Healthcare) that was cut with EcoRI and XhoI.

RelA-cFlag-pcDNA3 (mouse) vector was obtained from Addgene (Plasmid 20012).

RelAS536A-cFlag-pCDNA3 was generated by performing mutagenesis the Quick-change IIXL kit (Agilent).

HA-DBC1/pBabe-puro: DBC1 fragment was amplified by PCR from pCMV-Sport6 vector (Open Biosystems) using a forward primer that had an HA sequence. The fragment was ligated into the SalI site of pBabe-puro retroviral vector (addgene).

HA-FLIP-pMIG: c-FLIP coding sequence was amplified by PCR from the pcDNA3-Myc-FLIP vector

(kindly provided by J. Tschopp, University of Lausanne) with inclusion of a single HA tag on the forward primers and ligated into the XhoI-EcoRI site of pMIG (Addgene).

IKK-β-S-mscv-puro: Synthetic double stranded oligonucleotides (S-tag-f: CTAGC CCACC ATG AAG GAG ACCGCC GCT GCC AAG TTC GAG AGA CAG CAC ATG GAC TCC GGC GGC A; S-tag-r: agcttGCCGCCGGAGTCCATGTGCTGTCTCTCGAACTTGGCAGCGGCGGTCTCCTTCATGGTGG g) were annealed and ligated into the NheI-HindIII site of pcDNA3.1+ to generate the vector S-tag/pcDNA3.1. The NheI-EcoRI fragment from this vector was ligated together with an EcoRI-NotI fragment generated by PCR from pcDNA-IKK-β-Flag-WT vector (Addgene plasmid 23298). The ligated product was then re-amplified with primers spanning the S-tag and the IKK-β sequence, carrying XhoI and NotI sites respectively. This PCR product was subcloned into the XhoI-NotI site of pMSCV-IRES-puro.

3xFlag-IκBmutant–pBabe-hygro (super-repressor): pBabe-puro-IκBmutant was obtained from Addgene (plasmid 15291). Full length IKBmt fragment was amplified by PCR and ligated into 3xFlag-CMV-10 (Invitrogen) that was cut with NotI-BglII. The 3xFlag-IKBmt from 3xFlag-IKBmt-CMV10 vector was amplified by PCR and ligated into pBabe-hygro (Addgene) that was cut with BamHI and EcoRI.

IKK-β-pMXS-ires-puro: The IKK-β-Flag fragment from IKKb-Flag-pcDNA3 vector by PCR using NotI primers and subcloned into the Not I site of pMXS-ires-puro-FF (R. Carstens, University of Pennsylvania), creating three C-terminal Flag sequences.

NF-κB-luc reporter vector: pNF-κB-Luc contains five copies of a consensus NF-kB site in a pGL2 luciferase reporter vector (Promega).

TK-LacZ vector: constructed by A. Ivanov used as an internal control for luciferase assays.

Cell lines

In this study, we used MCF10aPG2, a subclone of the human mammary epithelial cell line MCF10a developed in our lab that undergoes anoikis more rapidly than the parental line (S.H.-P. and S.M.F., unpublished data), except for acinar morphogenesis assays, where MCF10aneoT cells (F. Miller, Karmanos Cancer Center) were used. These cells were maintained in MCF10a medium (DME/F12+5% horse serum +1x penicillin-streptomycin-glutamine (PSG) + 10 ug/ml insulin, 10 ng/ml EGF, 0.5ug/ml hydrocortisone, and 0.1ug/ml cholera toxin). MDA-MB231 and MDA-MB-468 (provided by A. Ivanov) were maintained in DMEM +10% fetal bovine serum + 1X PSG. All derivative cell lines were mixed populations generated by drug selection or flow sorting after viral infection (except the HA-DBC1-expressing MCF10a cells, where high-expressing clones were selected). BT-549 cells were provided from E. Pugacheva (WVU) and maintained in RPMI + 10% fetal bovine serum + 1X PSG. Inducible DBC1 shRNA knockdown cell lines were generated by infection with the A6/pTRIPZ or B2/pTRIPZ lentiviruses (described above), followed by selection with 2 μg/ml puromycin, and after 24 hours induction with 1 μg/ml doxycycline, cells were then flow-sorted for RFP. Retroviruses were packaged by co-transfecting 60mm dishes of 293T+GP2 cells (Clontech) using Mirus LT1 reagent, with 4.5 μg of retroviral plasmid and 2.5 μg of pCMV-VSV-G. Lentiviral constructs were packaged by co-transfecting 60mm dishes of 293T cells, using Mirus LT1, with 3.3 μg of lentiviral vector, 2.2 μg of sPAX2 and 1.2 μg of CMV-VSV-G. Viral supernatants were cleared about 48 hours post-transfection by filtration through 0.45 micron cellulose acetate filters (Whatman), aliquoted and stored at -80 degrees. To improve the transfection efficiency, cells were infected by spinning down the cells with virus stock at 1,400 rpm for 1.5 hr at room temperature followed by overnight incubation at 37^0. After infection, and selection in puromycin (2 μg/ml), and/or flow-sorting for GFP or RFP expression, expression of the transgenes or knockdown was verified by western blotting. To induce shRNA expression, 1 μg/ml of

doxycycline was added to growth media in cells with pTRIPZ-based shRNA and incubated for 48 hours before performing any experiment.

Derivative cell lines used in this study were generated as follows:

MCF10a with inducible DBC1 shRNA knockdown: MCF10a cells were infected with the A6/pTRIPZ or B2/pTRIPZ lentiviruses (described above), selected with puromycin (2 µg/ml), and after 24 hours induction with 1 µg/ml doxycycline, cells were flow-sorted for RFP.

Breast cancer cells with inducible DBC1 shRNA knockdown: MDA-MB-231 and BT-549 cells were infected with the A6/pTRIPZ lentiviruses, and selected for puromycin (2 µg/ml). After 24 hours induction with 1 µg/ml doxycycline, cells were flow-sorted for RFP.

MCF10a+DBC1 over-expression: MCF10a cells were infected with HA-DBC1/pBabe-puro and selected for puromycin (2 µg/ml). Empty vector control cells lines were generated by infecting with pBabe-puro and selecting for puromycin.

DBC1-KO MEFs with rescued DBC1 expression: DBC1-KO mouse embryo fibroblasts [27], kindly provided by Z. Lou (Mayo Clinic College of Medicine), were immortalized with SV40 T-antigen by infection with the ZipTer retrovirus (provided by P. Soriano, Hutchinson Cancer Center). Cells were then infected with HA-DBC1/pBabe-puro vector and selected for puromycin (2 µg/ml). Empty vector control cells lines were also generated by infecting immortalized DBC1-knock out MEFs with pBabe-puro and selecting for puromycin (2µg/ml).

MCF10a over-expressing IKK-β: MCF10a cells were infected with 3xFlag-IKKβ-pMXS-puro and selected for puromycin (2 µg/ml). Empty vector control cells lines were also generated by infecting with pMXS-puro and selecting for puromycin.

MCF10a with inducible DBC1 shRNA and c-FLIP over-expression: MCF10a cells expressing DBC1 shRNA (A6/TripZ) were infected with HA-FLIP/pMIG and selected for GFP-positive cells by flow cytometry. Empty vector control cells were also generated by infecting with empty pMIG vector and selecting for GFP-positive cells by flow cytometry.

Mouse embryo fibroblasts expressing IκBmt: Immortalized DBC1-KO and WT MEFs were infected with 3xFlag-IκBmt/pBabe-hygro vector and selected for hygromycin (200 μg/ml). Empty vector control cells lines were also generated by infecting immortalized DBC1-KO and WT MEFs with pBabe-hygro and selecting for hygromycin.

MDA_MB-231 cells overexpressing S-tag-IKK-β: MDA-MB-231 cells were infected with S-tag-IKK-β/MSCV-IRES-Puro vector and selected for puromycin (2 μg/ml). Empty vector control cells lines were also generated by infecting MDA-MB231 cells with mscv-puro and selecting for puromycin.

Western blots

Novex/Invitrogen 4-12% gradient gels were transferred under standard conditions in Tris-glycine/5% methanol buffer and transfer was run overnight at 45V or at 80V for 3 hours with stirring. Secondary antibodies anti-mouse-hrp and anti-rabbit-hrp were from BioRad; Clean-Blot secondary antibody (Pierce/Thermo) was used to avoid detection of precipitated IgG on blots used for co-immunoprecipitation experiments.   Blots were developed using either enzyme-linked chemiluminescence (ECL, West Pico, Pierce), or, for the more quantitative applications, using fluorescently tagged secondary antibodies, buffers and scanning instrument from LiCor; quantitation generated by the software was based on raw pixel integrations.

siRNA transfection and anoikis assays

1.5×10^5 cells were plated in wells of 6-well collagen-coated dishes. Two duplicate wells with target siRNA and two duplicate wells with control siRNA, using 500 µL of Opti-MEM containing 5 µl of 20 µM siRNA (or 1 µl of 100 µM DBC1 siRNA) and 5 µl of lipofectamine RNAi-max, added to a well containing 2.0 ml of Opti-MEM. After 4-6 hours, cells were re-fed with regular growth medium, and, 24 hours later, each well was split into one 60mm dish. These were further incubated forty-eight hours, trypsinized, and resuspended in 3ml of growth medium. Cells were counted and 100,000 cells were plated in a 35mm Ultralow Attachment well and incubated for specified periods of time in presence of 0.5% of methylcellulose. At each time points, including time zero, cells were spun down at 4,000 rpm for 1.5 minutes in a microfuge tube, washed in ice-cold D-PBS once and lysed in 100 uL of phosphate buffered saline (PBS) containing 0.5% Triton-X100 and 10 mM EDTA. Lysates were incubated on ice for 15 minutes and cleared at 13,200 rpm for 12 minutes and, 5-15ul were assayed in a total of 100ul of Roche Cell Death ELISA Lysis/Incubation buffer in the Roche system and read in a Perkin-Elmer Envision/Excite. Values measured at a suspension time of zero (time-zero), which were generally unaffected by the siRNAs we transfected in this study, were subtracted from the final values shown in the figures. MCF10a cells that were pre-treated with either curcumin (25 µM) for 4 hours were suspended for indicated time and collected to measure DNA fragmentation using Roche Cell Death ELISA lysis kit as described above.

Cell permeability-based cell death assays

DBC1-KO MEF (~100,000 cells) expressing either vector or HA-DBC1 vector treated with BAY 11-7082 (10 µM) for 1 hour were plated on low attachment 6-well plates containing 2 ml of growth medium. Cells were collected at indicated time points, spun down at 3,200 rpm for 15 seconds,

resuspended in 100 µL of Accumax (Innovative Cell Technologies) and incubated (~5-10 minutes) at room temperature to generate single cell suspensions. Equal volumes of trypan blue staining solution (Invitrogen) were added to the cell suspension and the percentage of dead cells was counted on a hemacytometer in duplicate; time-zero cell death values were subtracted.

RelA acetylation assay

MCF10a cells expressing control shRNA or DBC1 shRNA, or mouse embryo fibroblasts (DBC1 knocko ut or DBC1-rescued) were plated on 6 well plates. When cells were about 80% confluent, cells were trea ted with TNF (20ng/ml for MCF10a, 40ng/ml for MEF) for 30 or 120 minutes. Cells were lysed with 5 % βME/1xSDS sample buffer, boiled and analyzed on western blots using acetyl-lys310 relA antibody.

Reporter Assays

50,000 MCF10ap+DBC1 shRNA cells were plated on 12 well plates coated with collagen in duplicates and shRNA was induced with 1 µg/ml of doxycyclin. Next day, cells were co-transfected with 600ng NF-kB- luciferase construct, 150 ng TK-lacZ and either 250 ng of HA-DBC1-pCDNA3.1 or pCDNA3.1 vector by using Lipofectamine 2000 reagent (Invitrogen) at a ratio of 1 µg of DNA to 2 µL of Lipofectamine reagent. MEF DBC1 WT and KO expressing 3xFlag-IκBα mutant construct were plated on 6 well plates coated with collagen. Next day, cells were transfected with either DBC1 or control siRNA. After 24hrs, cells were re-plated on 12 well plates coated with collagen in duplicates. Following day, cells are co-transfected with ug of NF-κB-luciferase construct and TK-LacZ (1 µg of total DNA) by using Lipofectamine 2000 reagent. After 28hrs, cells were treated with TNF (20ng/ml) for 90mins and lysed with 200ul of 1x cell culture lysis buffer (Promega). The cell lysates were centrifuged at 4C for 12mins at 13,200rpm and assayed for luciferase and beta-galactosidase activity in 100ul of luciferase

assay reagent or 150ul of 1x galactosidase assay buffer (both Promega), which were analyzed in a Perkin-Elmer Envision/Excite apparatus. Light units were normalized to b-galactosidase activity to control for transfection efficiency. For MDA-MB 231 cells, 60,000 cells plated on 6-well dishes were transfected with 750 ng NF-κB-luciferase construct and 250 ng TK-LacZ. After 24 hours, cells were re-plated on low- attachment 6-well plates. Cells were harvested at time points indicated and assayed for luciferase and β-galactosidase activity. MCF10a cells plated on 6 well plates coated with collagen were transfected with either control or DBC1 siRNA. After 24 hours, cells were split and plated onto 12 well plates coated with collagen. Following day, cells were transfected with 600 ng NF-κB-luciferase construct, 150 ng TK-LacZ, and either 250 ng of pCDNA3.1 or 3xFlag-IKKβ-pCDNA3.1 vector by using Lipofectamine 2000 reagent. After 28 hours, cells were treated with TNF (20 ng/ml) for 1.5hr and assay assayed for luciferase and beta-galactosidase activity.

Effect of Bay 11-7082 on RelA localization

Cells grown on poly-L-lysine coated coverslips were treated with 10 ng/ml of Bay 11-7082 for 1 hour at 37^0 followed by 20 ng/ml of TNF for various times. At each time point, cells were fixed in PBS containing 4% paraformaldehyde and permeabilized with PBS containing 0.2% Triton X-100. Cells were blocked in PBS containing 10% normal goat serum, 0.1% Tween-20, and 0.1% BSA (blocking solution) for 60 minutes at room temperature. A 1:500 dilution of RelA antibody to blocking solution was then applied overnight at 4°C. Slides were washed with 0.1% PBS/Tween-20 three times and incubated for 1.5 hour with Alexa 488-conjugated goat anti-rabbit reagent (Molecular Probes) diluted 1:1000 in blocking solution. Coverslips were mounted with ProLong Gold (Molecular Probes) containing 4', 6-Diamidino-2-phenylindole. Target proteins were detected fluorescently using microscope (Zeiss Axiovert).

Acinar Morphogenesis assay

MCF10aneoT cells expressing either control or DBC1 shRNA were assayed for morphogenesis following the protocol indicated in [28].

RelA S536 phosphorylation assay

MCF10a cells expressing either control or DBC1 shRNA were plated on 6 well plates. Cells were treated with TNF (20 ng/ml) for 1.5 hours or 3 hours and lysed with 1x SDS sample buffer +5% βME. Western blots were performed using relA phospho-S536 specific antibody or total relA antibody; buffer containing 1X TBS+0.1% Tween+ 5% BSA was used for phospho-specific antibody incubation and blocking. Three independent experiments were performed. Image J was used to measure the relative phosphorylation level against total RelA expression. The results from three different experiments were averaged and indicated in the bottom of the blots with standard deviation.

S-tag pulldown assay

MDA-MB-231 cells expressing either S-tag-IKK-β or empty vector (one 100ml dish for each sample) were pre-treated with TNF (20 ng/ml) for 1.5hr or suspended for 24 hours on low attachment plates. Cells were lysed in IP buffer (600 µL), followed by preclearing the lysates at 13,200 rpm for 10 minutes. Lysates were precipitated with 40 µL of a 50% slurry of S-protein beads (Novagen/EMD Biosciences) that had been pre-equilibrated with IP buffer containing 10 mg/ml of bovine serum albumin and washed. After overnight incubation at 4°C with rotation, beads were washed three times with IP buffer. Samples were boiled in 2x SDS sample buffer for 5 minutes and analyzed by Western blotting.

In vitro kinase assay

To each 20 µL reaction, recombinant proteins GST-IKK-β, GST-IκBα and DBC1 protein was added at the indicated amounts. The final concentrations of components in the kinase assay buffer were: 25 mM Tris-HCl, pH8, 50mM KCl, 10mM Mgcl2, 1mM Na3VO4, 1mM DTT and 500 µM ATP. After 30 minute incubation at 30^0, the reactions were stopped by the addition of 20 µL of 2X SDS sample buffer+βME to the tube and boiling. Samples were analyzed on western blots for IκBα phospho-S32/36.

In vivo kinase assay

MDA-MB-231 cells that express either control or DBC1 shRNA were plated on 60mm dishes. Cells (Either attached or suspended for 24 hours in complete growth medium) were lysed in 600ul of standard IP buffer (25mM Tris pH8, 150mM NaCl, 0.5% Triton-X100, 10% glycerol, 1 mM EDTA, 1 mM beta-glycerophosphate, and Pierce complete protease inhibitor). After centrifugation at 13,200 rpm for10 minutes at 4°C, supernatants were incubated with either 1 µg of IKK-β or normal IgG antibodyovernight at 4°C with rotation, followed by incubation with 40 µl of pre-equilibrated protein Asepharose for another hour. Beads were washed three times with IP buffer and washed three times with kinase assay buffer (25mM Tris-HCl pH8, 50mM KCl, 10mM MgCl2, 1mM Na3VO4, and 1mM DTT). Kinase assay buffer (40 µL) containing GST-IκBα (50 ng) fusion protein and ATP (1mM) was added to the beads, which were incubated at 30°C for 30 minutes. Following removal of the beads by centrifugation, the supernatant was diluted with 2S SDS sample buffer, heated and analyzed by western blotting and probed for phospho-IκBα, IKK-β, and DBC1.

Endogenous co-IP

The co-immunoprecipitation of DBC1 and NRAGE was performed in HMLE+Twist cells following the

protocol described in [29].

Co-transfection and pull down assay

293T cells co-transfected with 3xHA-DBC1 and 3xFlag-NRAGE ([29]) were assayed for their

interaction following the protocol described in [29].

RESULTS

DBC1 suppresses anoikis

To investigate the potential role of DBC1 in anoikis, full-length DBC1 was over-expressed in the human
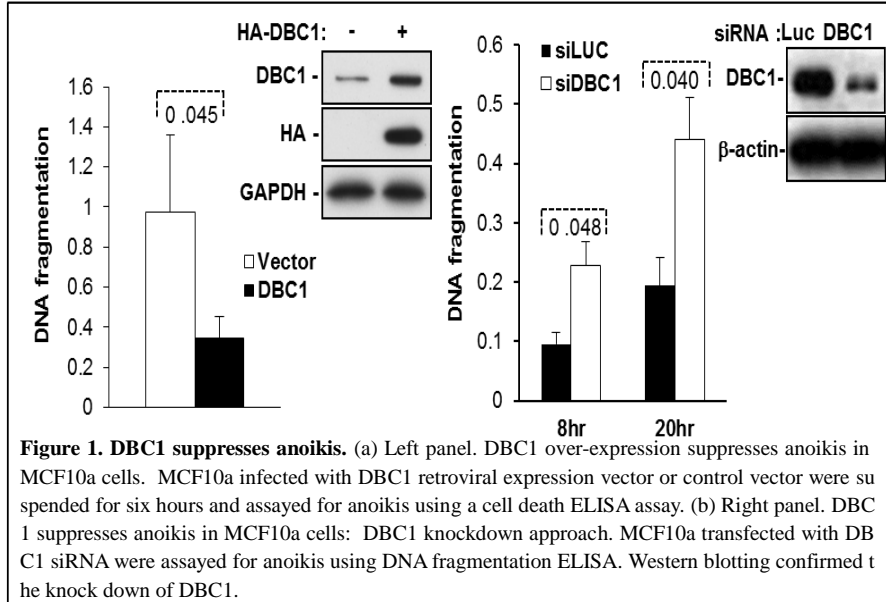

Figure 1. DBC1 suppresses anoikis. (a) Left panel. DBC1 over-expression suppresses anoikis in MCF10a cells. MCF10a infected with DBC1 retroviral expression vector or control vector were suspended for six hours and assayed for anoikis using a cell death ELISA assay. (b) Right panel. DBC1 suppresses anoikis in MCF10a cells: DBC1 knockdown approach. MCF10a transfected with DBC1 siRNA were assayed for anoikis using DNA fragmentation ELISA. Western blotting confirmed the knock down of DBC1.

mammary epithelial cell line MCF10a. Over-expression (~3-fold) of DBC1 conferred significant (~3-fold) resistance to anoikis after six hours of suspension, although anoikis was not permanently suppressed (Fig. 1a). Conversely, the partial

depletion of DBC1 via siRNA transfection sensitized MCF10a cells to anoikis (Fig. 1b). Similar results

were obtained using MCF10a cells stably transfected with DBC1 shRNA targeting a sequence distinct

from the siRNA targets, ruling out off-target effects (Fig. S1). The effect of DBC1 siRNA on anoikis

was further validated using a cell permeability assay for percent cell death as well as a three-dimensional

acinar morphogenesis assay (Fig. S2, S3).

The generality of the protective effect of DBC1 on anoikis was then addressed in several diverse cell lines. First, mouse embryo fibroblasts (MEF) derived from DBC1$^{-/-}$ mice [27] were found to be more sensitive to anoikis than DBC1$^{+/+}$ MEF (Fig. 1c). To exclude potential artifacts due to spurious differences between
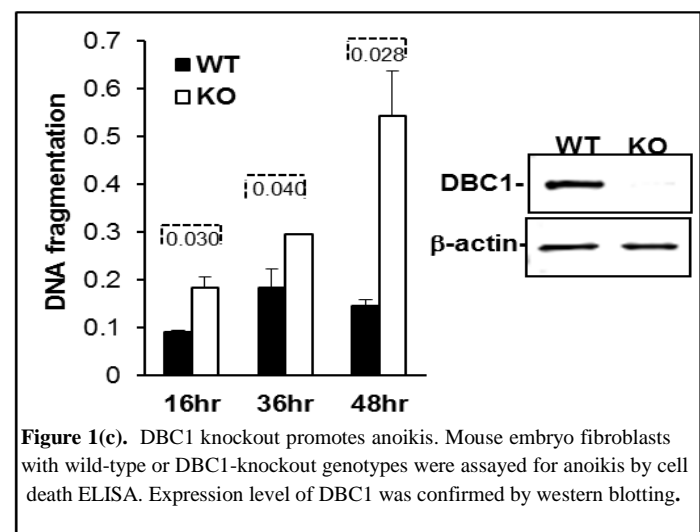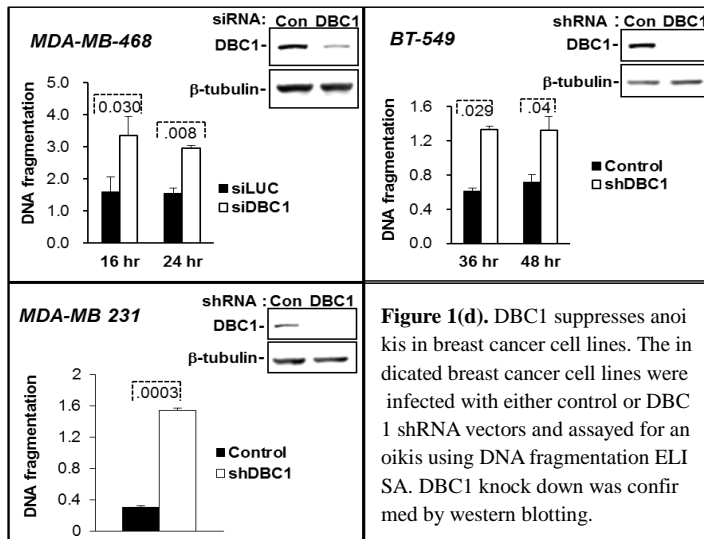

Figure 1(c). DBC1 knockout promotes anoikis. Mouse embryo fibroblasts with wild-type or DBC1-knockout genotypes were assayed for anoikis by cell death ELISA. Expression level of DBC1 was confirmed by western blotting.

MEF cell lines, DBC1$^{-/-}$ MEFs were rescued with DBC1 retroviral expression vector, which reverted

them to the wild-type level of anoikis-sensitivity (Fig. S4). The anoikis-sensitization effect of DBC1 depletion was also observed in three breast cancer cell lines: MDA-MB-231, BT-549 and MDA-MB-468 (Fig. 1d). These data indicate that DBC1 functions as a generalized anoikis suppressor.

Figure 1(d). DBC1 suppresses anoikis in breast cancer cell lines. The indicated breast cancer cell lines were infected with either control or DBC1 shRNA vectors and assayed for anoikis using DNA fragmentation ELISA. DBC1 knock down was confirmed by western blotting.

DBC1 affects the expression of NF-κB target genes

We hypothesized that DBC1 might affect NF-κB, in light of the documented importance of this factor

for anoikis regulation [8, 12, 14]. To test this, we initially probed for several NF-κB target genes in cells with or without DBC1 expression. MCF10a cells with DBC1 depletion were probed by qRT-PCR, showing
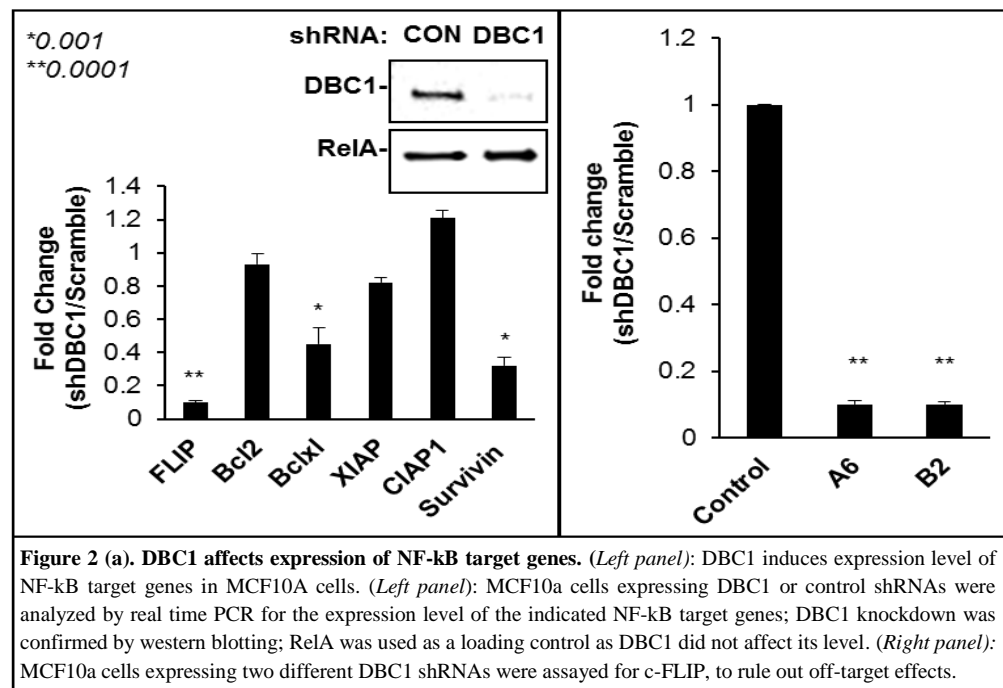
Figure 2 (a). DBC1 affects expression of NF-kB target genes. (*Left panel*): DBC1 induces expression level of NF-kB target genes in MCF10A cells. (*Left panel*): MCF10a cells expressing DBC1 or control shRNAs were analyzed by real time PCR for the expression level of the indicated NF-kB target genes; DBC1 knockdown was confirmed by western blotting; RelA was used as a loading control as DBC1 did not affect its level. (*Right panel*): MCF10a cells expressing two different DBC1 shRNAs were assayed for c-FLIP, to rule out off-target effects.

significant decreases in the levels of the NF-κB target genes c-FLIP, Bcl-x-l, and survivin; other target genes, Bcl-2, xIAP, and cIAP, were unaffected (Fig. 2a). Positive regulation of both c-FLIP and bcl-xl

proteins by DBC1 was observed in both attached and detached MCF10a cells (Fig. 2b), as well as in the

comparison of wild-type vs. DBC1 knockout MEFs (Fig. S5).

These data indicated that DBC1 protein enhanced the expression of some NF-κB target genes, under

attached or detached conditions.

Figure 2(b). DBC1 knockdown reduces c-FLIP and Bcl-xl expression in MCF10A. Expression levels of c-FLIP and Bcl-xl in attached or suspended MCF10A cells were assayed by western blotting.
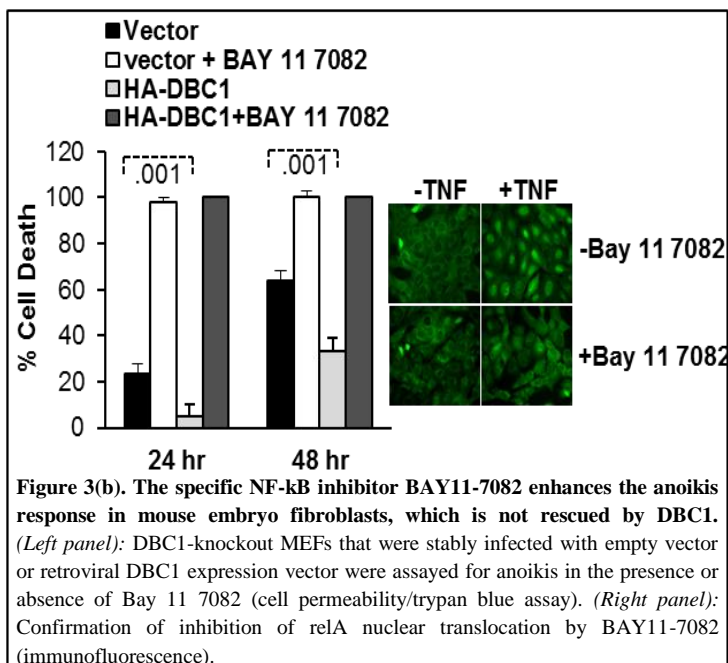
DBC1 regulates anoikis through NF-kB

Initially, the role of NF-κB in anoikis of MCF10a cells was

tested by the use of the dietary, non-specific inhibitor,

curcumin and a highly specific pharmacologic inhibitor of IKK-β activity, Bay 11-7082 [30, 31]. Both

compounds, used at similar concentrations to those previously reported for cell lines, sensitized MCF10a

cells and mouse embryo fibroblasts to anoikis (Fig. 3a, b). The anoikis-hypersensitive state in BAY 11-

7082-treated mouse embryo fibroblasts was not reversed by DBC1 expression, indicating that DBC1

protects primarily through the NF-κB

pathway (Fig. 3b).

Figure 3(a). DBC1 regulates anoikis through NF-kB. Curcumin enhances the anoikis response in MCF10a cells (DNA fragmentation ELISA).

Figure 3(b). The specific NF-kB inhibitor BAY11-7082 enhances the anoikis response in mouse embryo fibroblasts, which is not rescued by DBC1. *(Left panel):* DBC1-knockout MEFs that were stably infected with empty vector or retroviral DBC1 expression vector were assayed for anoikis in the presence or absence of Bay 11 7082 (cell permeability/trypan blue assay). *(Right panel):* Confirmation of inhibition of relA nuclear translocation by BAY11-7082 (immunofluorescence).

Further supporting the role of NF-κB in anoikis, over-expression of IKK-β, which has been shown previously to activate the NF-κB pathway [32], protected MCF10a against anoikis efficiently and induced higher levels of c-FLIP expression, both in attached and, especially, detached cells (Fig. 3c).

Figure 3(c). IKK-β over-expression suppresses anoikis and induces c-FLIP expression in MCF10a cells. *(Left panel):* MCF10a cells over-expressing Flag-IKK-β were assayed for anoikis (DNA fragmentation ELISA). *(Right panel):* Expression of c-FLIP in MCF10a cells upon detachment was analyzed by western blotting.

Among NF-κB target genes, c-FLIP is particularly noted for its ability to regulate anoikis [11]. To test the role of c-FLIP in the suppression of anoikis by DBC1, MCF10a cells that express inducible DBC1 shRNA were infected with a constitutive c-FLIP retroviral construct. This was found to reverse the effect of DBC1 knockdown (i.e., confer resistance to anoikis; Fig. 3d). These data indicate that DBC1 promoted the expression of multiple NF-κB target genes, including c-FLIP, suppressing anoikis.

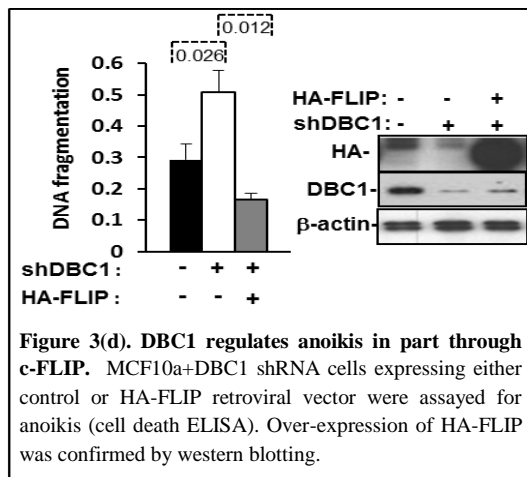**Figure 3(d). DBC1 regulates anoikis in part through c-FLIP.** MCF10a+DBC1 shRNA cells expressing either control or HA-FLIP retroviral vector were assayed for anoikis (cell death ELISA). Over-expression of HA-FLIP was confirmed by western blotting.

DBC1 activates NF-kB-mediated transcription

The results described above suggested that DBC1 activated the transcriptional activity of NF-κB. To test this, an NF-κB-responsive reporter construct was co-transfected into MCF10a cells stably bearing a doxycycline-inducible DBC1 shRNA construct. The depletion of DBC1 inhibited NF-κB-driven luciferase expression significantly (Fig. 4a). As a control, cells were co-transfected with DBC1 cDNA expression vector to rescue DBC1 expression (because the shRNA was targeted to the 3' UTR), which rescued the NF-κB activity, ruling out off-target effects. The effect of DBC1 on NF-κB activity was
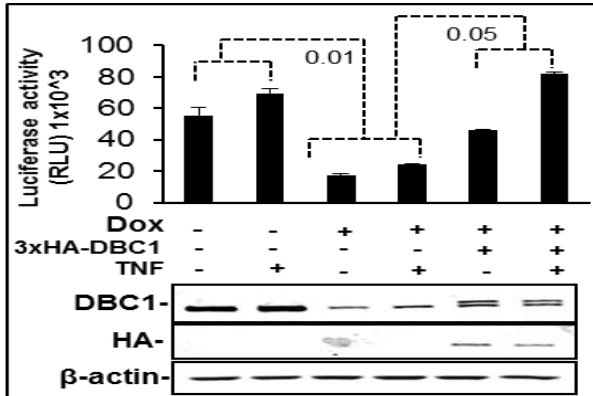
Figure 4(a). DBC1 promotes NF-κB activation in MCF10a cells. MCF10a+shDBC1 cells were transfected with 3X-NF-κB-luc, and either empty pCDNA3.1 or 3xHA-DBC1-pCDNA3.1. The cells were then assayed for luciferase activity following stimulation with TNF in the indicated samples. Knockdown and re-expression of (shRNA-resistant) DBC1 was confirmed by western blotting.

observed in cells with or without stimulation by TNF-α. The effect of DBC1 was confirmed by additional experiments in DBC1 knockout vs. DBC1 wild-type MEFs, which were co-transfected with the NF-κB-luc reporter vector, in the presence or absence of a dominant-negative mutant of IκB-α ("IκBmt") that constitutively inhibits relA translocation regardless of stimulatory signals [33]. DBC1 enhanced NF-κB reporter activity in this system, which was inhibited to below baseline level by the IκBmt construct, indicating the dependence of DBC1-stimulated NF-κB activity on the canonical kinase pathway (Fig. 4b).

Breast cancer cell lines, but not normal mammary epithelial cells, respond to detachment conditions
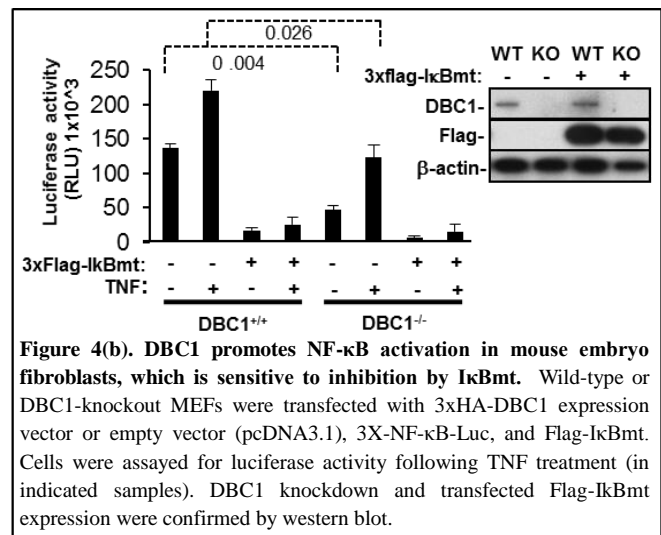
Figure 4(b). DBC1 promotes NF-κB activation in mouse embryo fibroblasts, which is sensitive to inhibition by IκBmt. Wild-type or DBC1-knockout MEFs were transfected with 3xHA-DBC1 expression vector or empty vector (pcDNA3.1), 3X-NF-κB-Luc, and Flag-IκBmt. Cells were assayed for luciferase activity following TNF treatment (in indicated samples). DBC1 knockdown and transfected Flag-IkBmt expression were confirmed by western blot.

by activating NF-κB activity, contributing to anoikis-resistance [14]. To determine whether this stimulation was DBC1-dependent, MDA-MB-231 cells with or without DBC1 shRNA expression were transfected with the NF-κB reporter construct and assayed for NF-κB activity following detachment. Control cells activated NF-κB almost linearly over a 24-hour period, as reported previously [14]. By contrast, cells with DBC1 knockdown
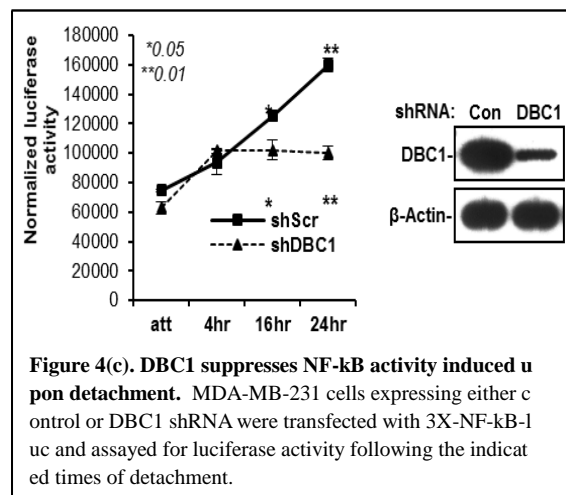

Figure 4(c). DBC1 suppresses NF-kB activity induced upon detachment. MDA-MB-231 cells expressing either control or DBC1 shRNA were transfected with 3X-NF-kB-luc and assayed for luciferase activity following the indicated times of detachment.

showed significantly reduced NF-κB activity, especially at time points after four hours (Fig. 4c). This indicated that the detachment-induced increase in NF-κB activity in a breast cancer cell line was partially dependent upon DBC1 protein.

DBC1 interacts with IKK-β and stimulates its activity

By immunofluorescence, we confirmed previous reports that DBC1 was a nuclear protein [27], and, consistent with this, the translocation of relA to the nucleus upon stimulation with TNF was not affected by DBC1 (Fig. S6). This excluded mechanisms involving the canonical activity of the IKK complex in promoting relA translocation from cytoplasm to nucleus. We also addressed the possibility that DBC1 – an endogenous inhibitor of SIRT1 deacetylase activity [34, 35]—activated the transcriptional activation domain of relA through acetylation, but relA K310 acetylation was not detectable in MCF10a cells (data

Figure 5(a). DBC1 promotes IKK-b induced NF-κB activation. MCF10a cells that were pretreated with either control or DBC1 siRNA were transfected with Flag-IKK-β and 3X-NF-κB-luc and assayed for luciferase activity. Knockdown of DBC1 and expression of Flag-IKK-β were confirmed by western blotting. Samples were assayed in duplicate and the data shown are representative of two experiments.

not shown), and there was no significant effect of DBC1 acetylation in mouse embryo fibroblasts (Fig. S7).

Interestingly, DBC1 stimulated the activation of NF-κB activity by co-transfected IKK-β in reporter assays (Fig. 5a). Moreover, the major phosphorylation site for IKK-β on relA, S536, potentiated transactivation by relA using a co-transfection assay (Fig. S8), as reported previously in other systems [17-20]. We reasoned that perhaps DBC1 stimulated the kinase activity of IKK-β on relA S536. Consistent with this, the phosphorylation of relA S536 was suppressed by shRNA-mediated depletion of DBC1 (Fig. 5b).

By high-throughput proteomics analysis of the NF-κB pathway, DBC1 was identified previously as a candidate interactor of IKK-β [36]. To demonstrate an interaction of DBC1 with IKK-β, S-tagged IKK-β expressed in MDA-MB-231 cells was precipitated with S-protein agarose and probed for DBC1. Interactions clearly above a background control (i.e., a sample of MDA-MB-231 cells without the S-tagged construct) were observed, with highest efficiency in cells that were detached for twenty-four hours prior to lysis (Fig. 5c).

Figure 5(b). DBC1 promotes the phosphorylation of RelA S536. *(Left panel):* MCF10A stably expressing control or DBC1 shRNA were analyzed by western blotting for phospho-RelA S536 and total relA upon TNF stimulation. The numbers on the bottom represents the average of three independent experiments.
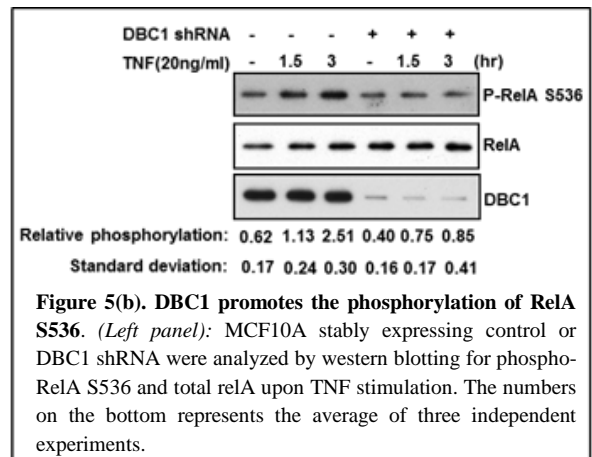
A fully endogenous co-immunoprecipitation of IKK-β with DBC1 was observed as well, but these results were not as consistently reproducible as those using the S-tagged construct, perhaps reflecting the low abundance of nuclear IKK-β expression [37](data not shown).
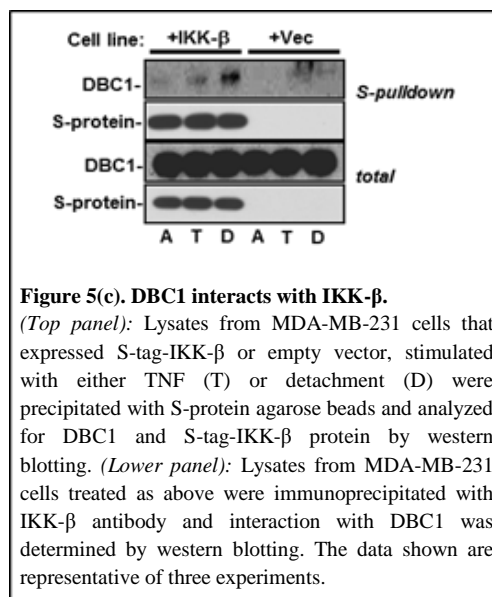
Figure 5(c). DBC1 interacts with IKK-β.
(Top panel): Lysates from MDA-MB-231 cells that expressed S-tag-IKK-β or empty vector, stimulated with either TNF (T) or detachment (D) were precipitated with S-protein agarose beads and analyzed for DBC1 and S-tag-IKK-β protein by western blotting. *(Lower panel):* Lysates from MDA-MB-231 cells treated as above were immunoprecipitated with IKK-β antibody and interaction with DBC1 was determined by western blotting. The data shown are representative of three experiments.

DBC1 stimulated NF-κB transcriptional activity and the phosphorylation of relA S536 and interacted with IKK-β as well. To test the effect of DBC1 on IKK-β kinase activity, recombinant proteins were assayed in vitro. The results showed a dose-dependent stimulation of IKK-β activity by DBC1 protein (Fig. 5d), with maximal stimulation at a DBC1: IKK-β molar ratio of approximately 1.0-1.5. Correspondingly, the knockdown of DBC1 decreased the enzymatic activity of IKK-β that was immunoprecipitated from detached MDA-MB-231 cells and assayed (Fig. 5e).

These observations confirmed that DBC1 interacted with and stimulated the activity of IKK-β, resulting in enhanced NF-κB activity and protection from anoikis.

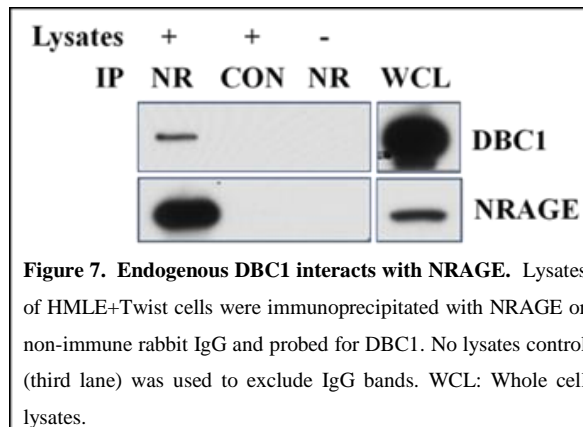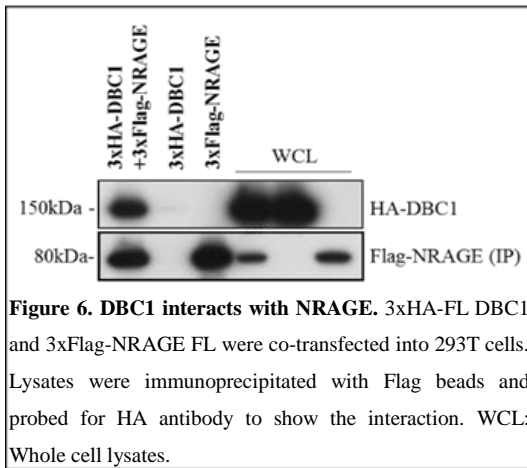**Figure 5(d). DBC1 stimulates IKK-β kinase activity (in vitro).** Kinase activity of recombinant IKK-β was assayed on recombinant GST-IκB-α substrate in the presence of the indicated amounts of recombinant DBC1 protein. Kinase activity was detected using a phospho-IκB-α antibody. The data shown are representative of three experiments.

Figure 5(e). DBC1 stimulates IKK-β kinase activity (in vivo). MDA-MB-231 cells with control or DBC1 shRNAs (see figure 4c) were assayed for IKK- β under attached or suspended conditions; phosphorylation of substrate was normalized to total IKK-β to generate the values shown, which were averaged together over two experiments.

DBC1 interacts with NRAGE

Our yeast-two-hybrid study showed that DBC1 is a candidate interactor with NRAGE. To confirm their interaction, 293T co-transfection assay was performed using 3X-HA tagged DBC1 and 3X-Flag tagged NRAGE (Fig. 6). In addition, to further confirm their interaction, an endogenous co-IP using HMLE+Twist cells was performed (Fig.7). The results showed that DBC1 interacts with NRAGE.

Figure 6. DBC1 interacts with NRAGE. 3xHA-FL DBC1 and 3xFlag-NRAGE FL were co-transfected into 293T cells. Lysates were immunoprecipitated with Flag beads and probed for HA antibody to show the interaction. WCL: Whole cell lysates.

Figure 7. Endogenous DBC1 interacts with NRAGE. Lysates of HMLE+Twist cells were immunoprecipitated with NRAGE or non-immune rabbit IgG and probed for DBC1. No lysates control (third lane) was used to exclude IgG bands. WCL: Whole cell lysates.

DISCUSSION

The key finding of this study is that DBC1 protein contributes to the protection of normal and transformed cells against anoikis. Notably, DBC1 is not invariably anti-apoptotic. For example, the interaction of DBC1 with SIRT1 is enhanced by genotoxic and/or oxidative stress, through ATM-mediated DBC1 phosphorylation, enhancing p53 acetylation – a pro-apoptotic effect of DBC1 with respect to genotoxic/oxidative stress-induced apoptotic responses, in contrast with its role in anoikis [21].

The novel pathway by which DBC1 protein promotes NF-κB activity and anoikis-resistance is depicted in figure 8. DBC1 may be conceptualized as a co-factor for IKK-β that stimulates its kinase activity on relA (S536), promoting the transcriptional activation of NF-κB target genes that enhance anoikis-resistance. Among the most robustly DBC1-sensitive genes were c-FLIP and Bcl-xl, both of which have significant roles in anoikis-resistance of tumor cells, documented both here and elsewhere [11, 38]. In this connection, novel drugs that act by destabilizing c-FLIP expression are

Figure 8.
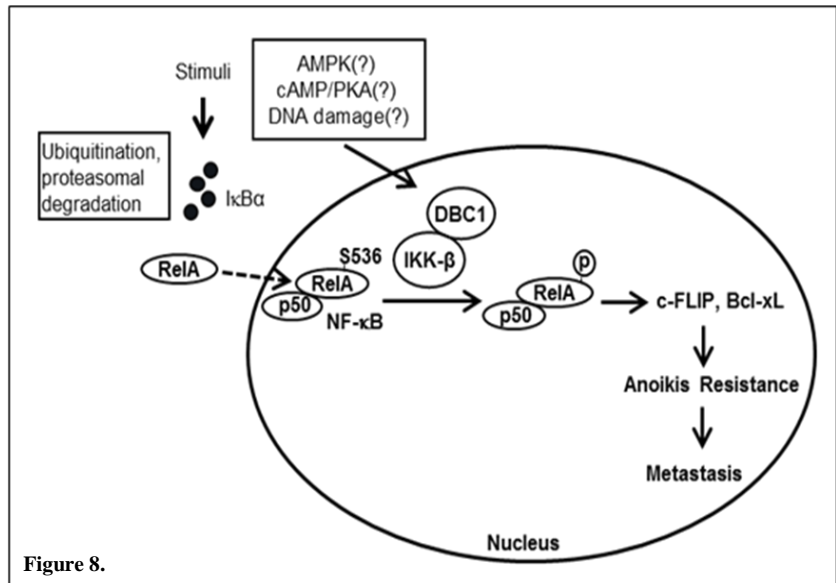
being developed with the goal of sensitizing tumor cells to anoikis [39].

Notably, IKK complexes can promote cell survival by additional, NF-κB-independent mechanisms as well, for example, through the phosphorylation of Foxo3A, p53, and mTORC (the latter resulting in survivin up-regulation) [5]. Although our data indicate a dominant role of NF-κB in the DBC1 cell

survival effect, we cannot exclude that other IKK-dependent, NF-κB-independent effects might play additional roles. In this connection, IKK-I/IKK-ε appear to be responsible for increased relA S536 phosphorylation in some tumor cells [40]; the potential of DBC1 to affect additional IKK complexes will be interesting to explore. Note also that while IKK-β clearly is important also for the phosphorylation of IκB-α, DBC1 did not affect the phosphorylation of this substrate in vivo, consistent with the primarily nuclear localization of DBC1, the stimulus-responsive nuclear and cytoplasmic localization of IKK-β and the constitutive cytoplasmic localization of IκB-α [27, 41].

Recent studies indicate that increased relA S536 phosphorylation and DBC1 over-expression each correlate with worse prognosis in colorectal cancer, a cancer type that is influenced dramatically by inflammatory stimuli that activate NF-κB [25, 42]. Correlation of more aggressive disease with DBC1 over-expression has been reported in breast cancer also [22, 23], and our data here show that DBC1 is important for the detachment-induced NF-κB activation that is observed in breast cancer cell lines, reported by the Richer laboratory [14]. These observations suggest that DBC1 over-expression, which has been demonstrated in esophageal cancer as well [24], promotes tumor progression through the pathway that we have elucidated here.

Biochemically, DBC1 was previously shown to regulate several transcription factors or regulators of transcription factors. As an endogenous inhibitor of SIRT1 deacetylase activity [34, 35], DBC1 enhanced the acetylation and stability of the SIRT1 substrate p53, potentially enhancing apoptosis in certain contexts. However, a recent study in liver cancer cells showed an effect of DBC1 neither on SIRT1 activity nor p53 acetylation, complicating the interpretation of this phenomenon [43]. We did not observe an effect of DBC1 on the acetylation of relA in mouse embryo fibroblasts, and failed to detect

significant relA acetylation in MCF10a cells, diminishing the relevance of this as a potential mechanism for DBC1 to regulate NF-κB, at least in our cell lines. DBC1 regulates several other factors as well, including the SUV39H1 histone methyltransferase, histone deacetylase-3, BRCA1, estrogen receptor and androgen receptor, thus having multiple pleiotropic effects on transcription [44-49]. In fact, DBC1 may also modulate alternative splicing, as it is a component of the DBIRD complex that integrates mRNA splicing with RNA polymerase II-mediated transcription [50]. Thus, it is not only possible but likely that DBC1 affords anoikis-resistance by mechanisms additional to the NF-κB mediated one described here, which remain to be identified. In this connection, though, microarray analysis identified c-FLIP plus a surprisingly short list of other DBC1-regulated genes, suggesting that NF-κB and/or additional c-FLIP regulatory proteins may be the most significant DBC1 targets (data not shown).

The interaction of DBC1 with IKK-β signifies that NF-κB is positioned for activation by novel mechanisms that have not yet been suggested. For example, the interaction between SIRT1 and DBC1 is dissociated by cAMP/PKA, acting through AMP-dependent protein kinase (AMPK), suggesting a novel mechanism for the metabolic control of SIRT1 [51]. Although it is not yet clear how this dissociation is achieved, it is intriguing to note that NF-κB is inhibited by AMPK. This raises the possibility that the interaction of DBC1 with IKK-β may also be regulated by AMPK or other signal transducers, providing novel metabolic control mechanisms for NF-κB and anoikis.

The interaction between DBC1 and NRAGE suggests that crosstalk exists such that DBC1 and NRAGE affect each other's functions and signaling pathways. This potential crosstalk is discussed in chapter 4.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

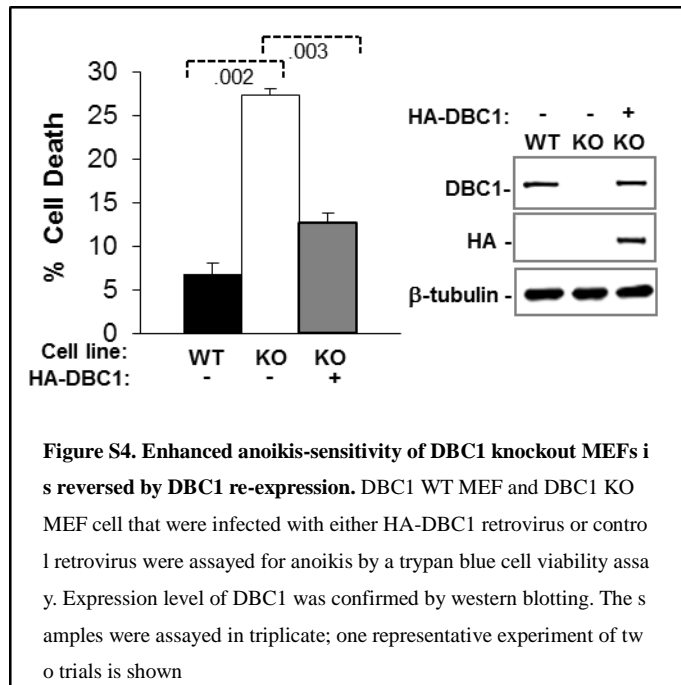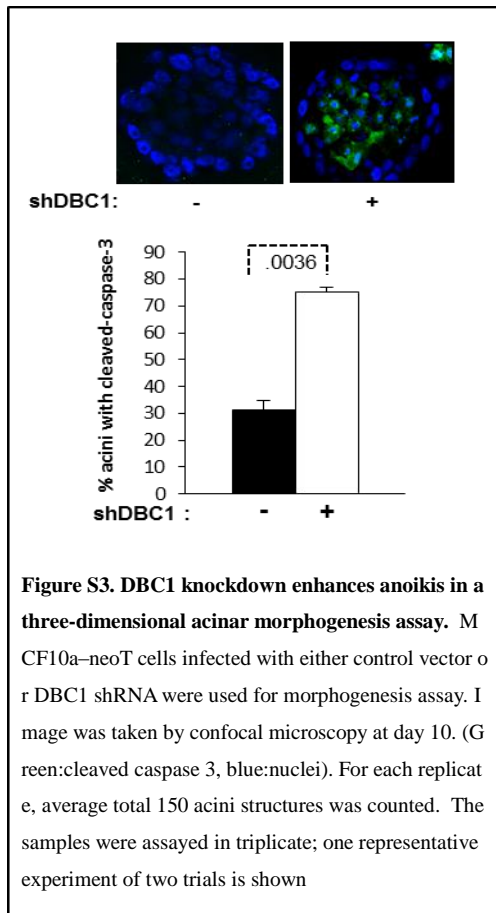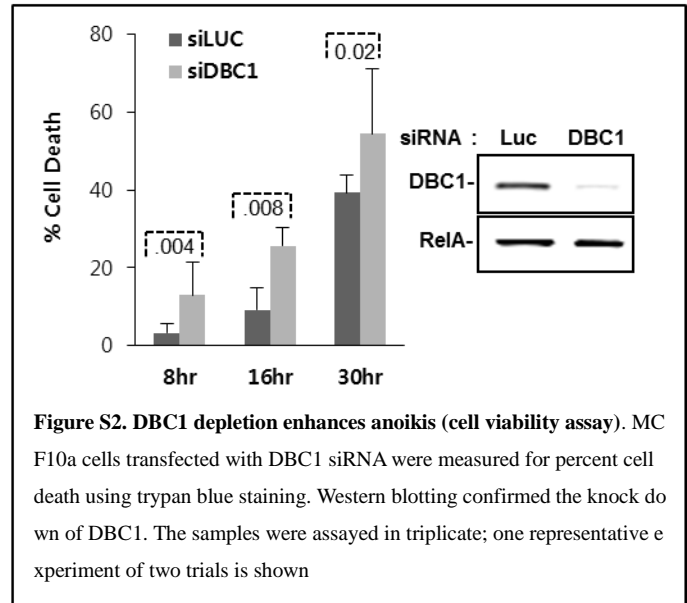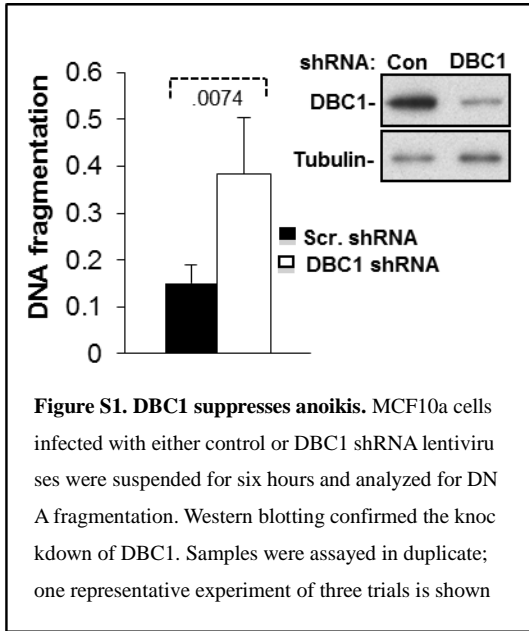The authors declare no conflict of interest.

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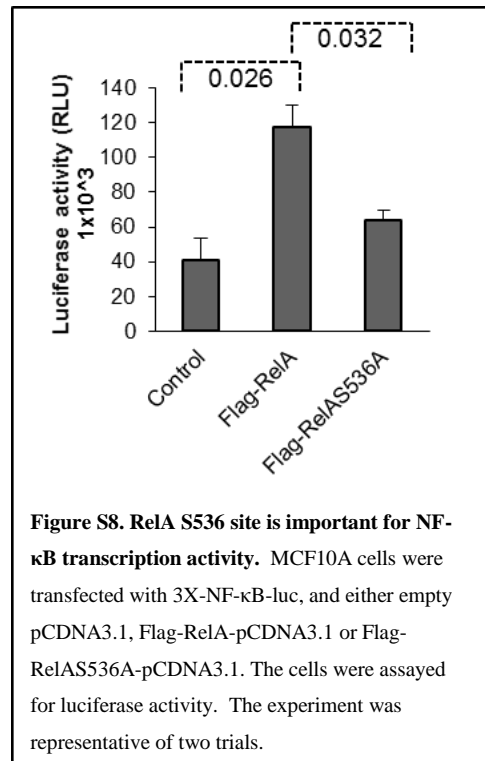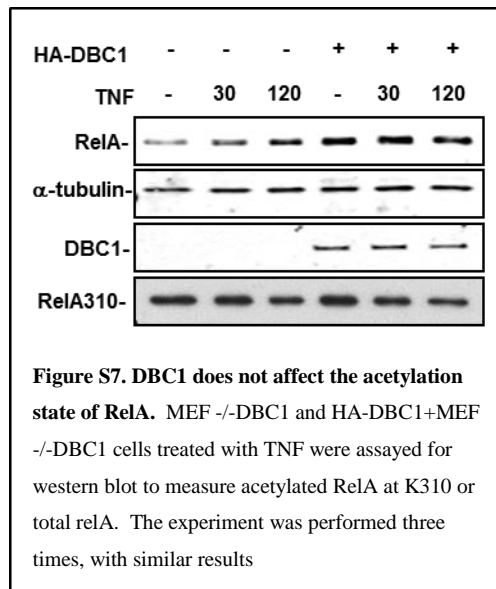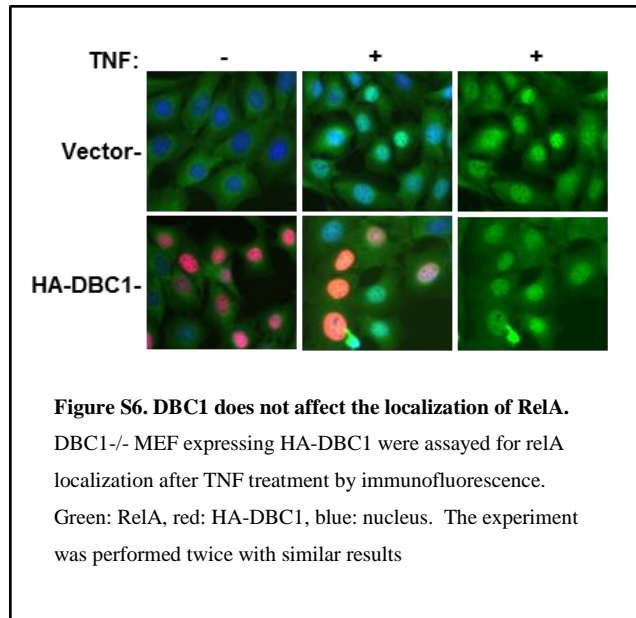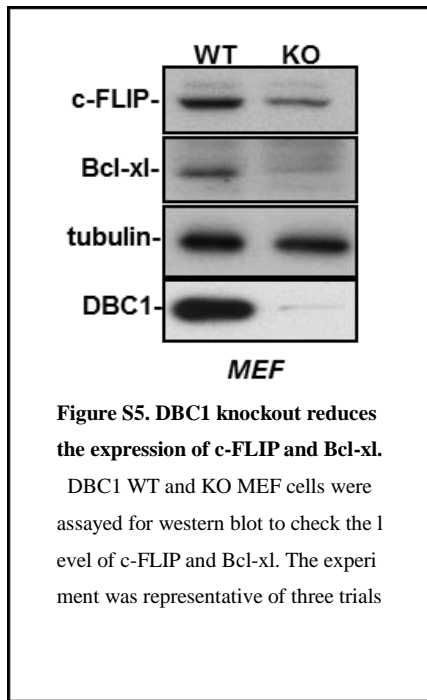10.1158/1078-0432.CCR-10-3236

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SUPPLEMENTARY DATA

Figure S1. DBC1 suppresses anoikis. MCF10a cells infected with either control or DBC1 shRNA lentiviruses were suspended for six hours and analyzed for DNA fragmentation. Western blotting confirmed the knockdown of DBC1. Samples were assayed in duplicate; one representative experiment of three trials is shown

Figure S2. DBC1 depletion enhances anoikis (cell viability assay). MCF10a cells transfected with DBC1 siRNA were measured for percent cell death using trypan blue staining. Western blotting confirmed the knock down of DBC1. The samples were assayed in triplicate; one representative experiment of two trials is shown

Figure S3. DBC1 knockdown enhances anoikis in a three-dimensional acinar morphogenesis assay. MCF10a–neoT cells infected with either control vector or DBC1 shRNA were used for morphogenesis assay. Image was taken by confocal microscopy at day 10. (Green:cleaved caspase 3, blue:nuclei). For each replicate, average total 150 acini structures was counted. The samples were assayed in triplicate; one representative experiment of two trials is shown

Figure S4. Enhanced anoikis-sensitivity of DBC1 knockout MEFs is reversed by DBC1 re-expression. DBC1 WT MEF and DBC1 KO MEF cell that were infected with either HA-DBC1 retrovirus or control retrovirus were assayed for anoikis by a trypan blue cell viability assay. Expression level of DBC1 was confirmed by western blotting. The samples were assayed in triplicate; one representative experiment of two trials is shown

Figure S5. DBC1 knockout reduces the expression of c-FLIP and Bcl-xl. DBC1 WT and KO MEF cells were assayed for western blot to check the level of c-FLIP and Bcl-xl. The experiment was representative of three trials

Figure S6. DBC1 does not affect the localization of RelA. DBC1-/- MEF expressing HA-DBC1 were assayed for relA localization after TNF treatment by immunofluorescence. Green: RelA, red: HA-DBC1, blue: nucleus. The experiment was performed twice with similar results

Figure S7. DBC1 does not affect the acetylation state of RelA. MEF -/-DBC1 and HA-DBC1+MEF -/-DBC1 cells treated with TNF were assayed for western blot to measure acetylated RelA at K310 or total relA. The experiment was performed three times, with similar results

Figure S8. RelA S536 site is important for NF-κB transcription activity. MCF10A cells were transfected with 3X-NF-κB-luc, and either empty pCDNA3.1, Flag-RelA-pCDNA3.1 or Flag-RelAS536A-pCDNA3.1. The cells were assayed for luciferase activity. The experiment was representative of two trials.

CHAPTER 4

Overall discussion

This thesis presents the mechanisms of anoikis regulation by two distinct oncogenic signaling pathways: Chapter 2 introduces the novel oncogenic pathway, NRAGE/TBX2/p14ARF, which is critical for linking EMT and anoikis resistance. Chapter 3 introduces the regulation of anoikis by DBC1 through IKKβ/NF-κB, which is a critical signaling pathway that links inflammation and cancer.

**PART I: Regulation of anoikis through the cytoskeletal protein ankyrin-G during EMT**

The regulation of anoikis through a novel pathway induced upon EMT was established [1]. Cells undergoing EMT, with loss of Ankyrin-G expression in the lateral membrane, allows NRAGE proteins to localize to the nucleus and directly interact with TBX2. NRAGE-TBX2 complex co-represses p14 ARF, a tumor promoter which results in anoikis resistance.

Cell-cell interaction mediated by E-cadherin is important for anoikis sensitivity, as illustrated in our study and others [1-4]. There is evidence that loss of E-cadherin induces activation of β-catenin and Wnt signaling which causes EMT and leads to anoikis resistance [3]. Although β-catenin was necessary for EMT induced by E-cadherin loss, increased β-catenin alone was not sufficient to cause anoikis-resistance. In our paper, we demonstrated a novel pathway where loss of E-cadherin was also associated with a drop in Ankyrin-G levels. However, knock down of ankyrin-G alone was not found to alter β-catenin localization (Unpublished data). This implies these pathways are potentially induced in a parallel fashion upon E-cadherin loss, although crosstalk at some other level is possible.

There are some studies demonstrating the potential link between E-cadherin and the polarity protein, scribble: scribble is sequestered to the cell-to-cell junctions in E-cadherin dependent manner and depletion of scribble is known to reduce E-cadherin expression leading to change in

morphology and migration by inhibiting cell-cell adhesion [5, 6]. These studies indicate that there

is a potential crosstalk between loss of E-cadherin- and Scribble- mediated signaling pathways

inducing EMT. Loss of scribble inhibits apoptosis by blocking c-Myc mediated Rac/JNK/Bim

pathway and promotes breast cancer development [7]. Therefore, the NRAGE/TBX2/p14ARF

pathway might be regulated by scribble to regulate anoikis sensitivity during EMT.

Although presented as two independent studies, a recent report indicates a possible molecular

linkage between the NRAGE and DBC1 pathways elucidated here. NRAGE is required for the

activation of NF-κB through phosphorylation of IKKα/β in

a bone morphogenic protein-4 (BMP-4) signaling pathway,

which was previously shown to be critical for cellular

process of EMT during embryonic development as well as

in pancreatic cancers [8-10]. Therefore, these studies and

our interaction data suggest that during EMT, NRAGE

Figure 1. Potential mechanism of anoikis regulation by NRAGE and DBC1
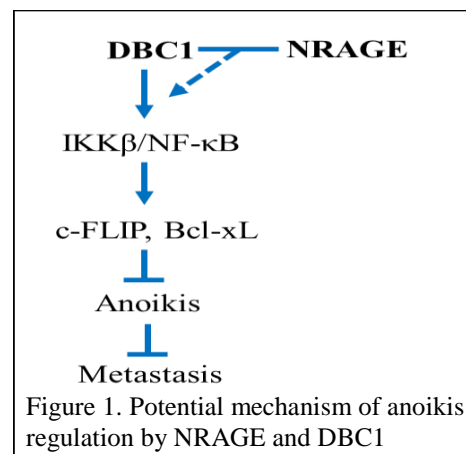
could interact with DBC1 to induce the DBC1/IKKβ/NF-κB pathway (chapter 3), leading to

anoikis resistance (Fig. 1). Moreover, the potential NRAGE/DBC1/NF-κB pathway could provide

us with an important connection between embryonic or/and cancer developmental pathways and

anoikis regulation through EMT.

A recent study showed that TBX2 induces cell motility and invasiveness, and promotes metastasis

of breast carcinoma cells such as MDA-MB435 cells. This fits conceptually with our earlier

observation (chapter 2 and [1]) showing that NRAGE, a novel co-repressor of TBX2, was also

critical for anchorage-independent growth of tumor cells that arose from transformation with

TBX2 ([11] and chapter 2). Therefore, these studies suggest that NRAGE might be important in

tumor metastasis through interacting with TBX2, which may be addressed in the future by the use

of the appropriate xenograft, transgenic or knockout models. These data further support that co-expression of NRAGE and TBX2 could be developed as an important prognostic marker for some breast cancers and targeting the interaction between NRAGE and TBX2 could be developed as an important anti-cancer therapy.

The transcription repression domain of TBX3 overlaps with that of TBX2 which was important for the interaction with NRAGE. Therefore, there is potential interaction between NRAGE and TBX3. Similar to TBX2, TBX3 is also known to be overexpressed in breast cancers and promote tumorigenesis [12, 13]. In addition, TBX3 has known to be important in stem cell generation through targeting p14ARF [14, 15]. These findings implicate that NRAGE might also interact with TBX3 and induce generation of cancer stem cells phenotype, which is the critical cause of recurrence and resistance to therapeutic drugs, the subject of another interesting future study.

The INK4a/ARF gene is frequently inactivated in human cancers by deletion, mutation, or transcriptional silencing [16], abrogating critical cell senescence and apoptotic pathways [17]. Therefore, the novel discovery of p14ARF as an anoikis promoter [1] further supports previous findings and also provides an explanation as to why cancer cells that have depleted p14ARF develop into malignancies. Although the exact mechanism of p14ARF regulation of anoikis is unknown, several studies demonstrating its role in apoptosis provides the potential

Figure 2. Potential mechanisms of anoikis regulation by p14ARF
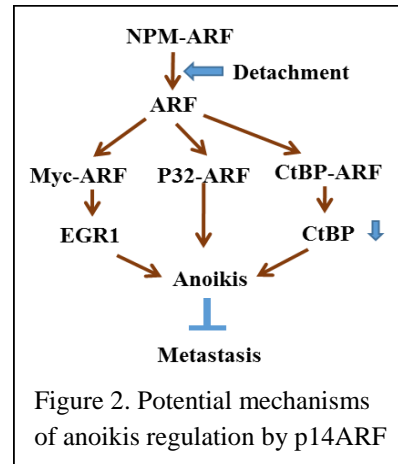
molecular mechanisms by which ARF might regulate anoikis (Fig. 2). First, upon detachment, p14ARF might interact with co-repressor CtBP, which is introduced as an important oncogenic molecule that links EMT and anoikis resistance [18, 19], Through direct interaction, p14ARF destabilizes CtBP expression to induce p53 independent apoptosis [20]. Secondly, p14ARF is

known to regulate apoptosis through interacting with p32 (mitochondria-anchored protein) by reducing mitochondrial membrane potential [21]. Lastly, p14ARF has been known to interact with c-Myc to inhibit c-Myc ubiquitination and promotes c-Myc mediated apoptosis through p53-independent pathway by up-regulation of EGR1 (Early growth response 1) transcription factor [22, 23]. All of these studies provide a potential explanation of the frequent mutations of the p14ARF gene in various cancers and their molecular mechanisms leading to anoikis resistance.

In conclusion, this novel pathway of anoikis regulation mediated by EMT and loss of E-cadherin will provide targets for anti-metastatic drugs and should help to advance the development of cancer therapeutics.

PART II: Regulation of anoikis by DBC1 through NF-κB pathway

In chapter 3, DBC1 enhanced IKKβ mediated phosphorylation and transcriptional activation of NF-κB, leading to increased expression of known anoikis suppressors, including c-FLIP and Bcl-xL. Interestingly, this effect was achieved through interaction of DBC1 with IKKβ that was enhanced upon cell-matrix detachment [24]. This pathway will provide the first links between inflammation and anoikis, currently not well established.

As an interesting future study, DBC1 might regulate anoikis through interactions with additional IKK proteins. IKKα, another regulatory subunit of the IKK complex, is shown to be critical for NF-κB activation mediated by the PI3K/Akt pathway in human salivary adenoid cystic carcinoma (ACC) cells [25]. In addition, phosphorylation of IKKα was induced upon detachment, which activated NF-κB in suspended intestinal epithelial cells [26]. In addition, IKKε, which is composed of similar structure to IKKβ and IKKα, is an IκB kinase involved in non-canonical NF-κB pathway

[27]. IKKε is overexpressed in multiple cancers (Breast, ovarian, prostate, and glioma) and IKKε induced NF-κB activity is critical for proliferation, survival and malignant transformation of breast cancer cells [27, 28]. IKKε may contribute to enhanced NF-κB activity and tumorigenesis by directly or indirectly phosphorylating RelA/p65 at serine 536 site [29, 30]. Moreover, up-regulated IKKε induced the expression of Bcl-2, a known anoikis suppressor [31], through activation of NF-κB signaling [32]. Therefore, these studies suggest the possibility that DBC1 might also interact with IKKε to regulate anoikis through the NF-κB pathway in certain contexts.

The fact that expression of oncogenic NRAGE is induced upon EMT and the constitutively activated NF-κB promotes tumor formation [8] and metastasis through EMT in breast cancer [9] suggest that DBC1 could be a potential tumorigenic factor through interaction with NRAGE and activate NF-κB signaling pathway. Therefore, testing the effect of DBC1 on anchorage independent survival and tumor metastasis in a xenograft mouse model, would be an interesting future study.

Survivin, a member of IAP family, is one of the downstream target proteins of NF-κB that is known to be overexpressed in several solid tumors as well as lymphomas and inhibit apoptosis and cell cycle progression. Consistently, survivin whose expression was regulated by DBC1 (chapter 3) is also known to protect cells against anoikis in cell type and context dependent manner [33, 34]. Interestingly, survivin interacts with other IAP members such as XIAP to activate IKK/NF-κB signaling pathway and suppress anoikis and induce tumor cell invasion and metastasis [35, 36]. Therefore, it is possible that increased expression of survivin, due to the constitutively active NF-κB signaling, might regulate interaction between DBC1 and IKK through interaction with other IAPs to further activate the NF-κB pathway to suppress anoikis by up-regulating Bcl-xL and c-FLIP.

It is crucial to determine the unknown pathways or molecules affecting the interactions between DBC1 and its binding partner, as these interactions govern the function of DBC1 in anoikis regulation. A recent study showed that the interaction between DBC1 and SIRT1 is regulated by activated AMPK, an important metabolic regulator, upon stimulation [37] . Although the direct mechanism of AMPK action on dissociation of DBC1 and SIRT1 binding is not well understood, this finding provides the possibility that the interaction between DBC1 and IKKβ might also be regulated by AMPK. Interestingly, recent publications showed that AMPK suppresses anoikis in breast cancer cells and a family of AMPK protein regulates NF-κB activity through IKK in endothelial cells [38, 39]. Therefore, these studies indicates that determining whether the interaction between DBC1 and IKKβ can be affected by AMPK in suspension would be useful for discovering a novel pathway of anoikis regulation by metabolic signaling molecules.

Colorectal, esophageal, and breast cancers are known to have overexpressed DBC1 [40-42]. In addition, several studies showed that NF-κB is constitutively activated and localized in the nucleus [43-45]. This overexpression was correlated with poor prognosis and reduced overall survival. Moreover, phosphorylated RelA at serine 536 was detected in colorectal cancers and was correlated with poor prognosis [46, 47]. A direct study looking at the correlation between DBC1 expression and NF-κB activity in tumors is clearly warranted.

Although our project focused on cancer, DBC1 has also been shown to be important in other human diseases related to chronic inflammation, such as liver steatosis. Additionally, greater knowledge of the role of DBC1 in this pathway may prove useful to better understanding the progression of inflammatory diseases in general.

This thesis demonstrated two novel oncogenic pathways that regulate anoikis in two different contexts. Moreover, the interaction between NRAGE and DBC1 suggests potential crosstalk

between these and additional pathways that might prove critical for anchorage independent survival of tumor cells upon EMT (Fig. 3).

At certain stages of development and tissue repair, cells are required to migrate in an anchorage independent manner, suggesting that anoikis should be regulated stringently. In addition, defects in anoikis regulation during the cellular transformation can lead to metastatic phenotypes. Therefore, appropriate anoikis

Figure 3. Summary of the potential pathways
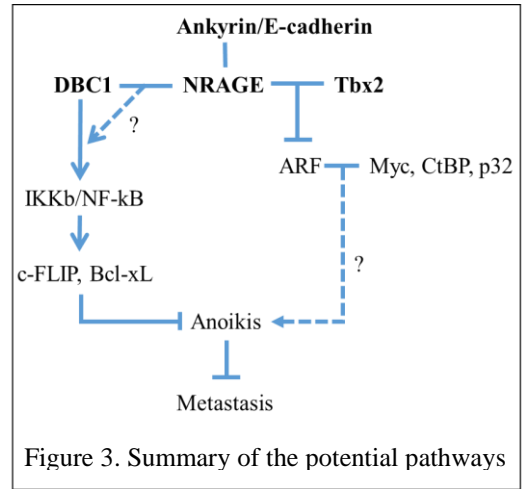
regulation is important. Our novel pathways, both enhance our understanding of this process and provide additional targets for anti-metastatic therapy.

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SUN PARK

5 Silver Creek Dr, Morgantown, WV, 26505 | 443-235-4904 |spark7@mix.wvu.edu

EDUCATION

West Virginia University, Morgantown, WV   2007-2013
Ph.D Cancer Cell Biology
Salisbury University, Salisbury, MD
B.S Chemistry (biochemistry track)   2002-2007

AWARDS AND RECOGNITIONS

WVU Doctoral Student Travel Award ($400)   2012

Tri Beta National Biological Honor Society   2006-2007

Salisbury University Dean's List   2005-2007

Junior Chemistry Awards ($500)   2005

PUBLICATIONS

Journal Article:

Kumar S*, **Park SH***, Cieply B*, Schupp J, Killiam E, Zhang F, Rimm DL, Frisch SM. 2011 A Pathway for the Control of Anoikis Sensitivity by E-Cadherin and Epithelial-to-Mesenchymal Transition. Molecular and Cellular Biology.19:4036-51.

*Co-first authors

Park SH, Riley P IV, Frisch SM. 2013 Regulation of Anoikis by DBC1 (Deleted-in-Breast Cancer 1) through NF-κB. Apoptosis.

PRESENTATIONS

Sun Park, Philip Riley IV, Steven M Frisch. *Oral presentation*. Regulation of anoikis by DBC1 through NF-κB. Health Sciences Van Liere Memorial Convocation and Research Day, Morgantown, WV, March 2013.

Sun Park, Philip Riley IV, Benjamin Cieply, Steven M Frisch. *Poster presentation*. Regulation of anoikis by DBC1 through NF-κB. American Association for Cancer Research (AACR), Chicago, IL, April 2012.

Sun Park, Philip Riley IV, Benjamin Cieply, Steven M Frisch. *Poster presentation.* Regulation of anoikis by DBC1 through NF-kB. Health Sciences Van Liere Memorial Convocation and Research Day, Morgantown, WV, March 2012.

Sun Park, Steven M Frisch. *Poster presentation.* Regulation of anoikis by DBC1. School of Pharmacy Research Day. Morgantown, WVU, October, 2011

Sun Park, Steven M Frisch. *Poster presentation.* Regulation of anoikis by DBC1. 14th Annual Meeting of the Translational Research Cancer Centers Consortium, Seven Springs Mountain Resort, Seven Springs, PA, February, 2011

Sun Park, Steven M Frisch. *Poster presentation.* Regulation of anoikis by DBC1. School of Pharmacy Research Day. Morgantown, WVU, October, 2010

Sun Park, Steven M Frisch. *Poster presentation.* Regulation of anoikis by DBC1 (Deleted in Breast Cancer 1). Health Sciences Van Liere Memorial Convocation and Research day, Morgantown, WV, March 2010.

Sun Park, Katherine R. Miller. *Poster presentation.* Seasonal microbial community diversity in a forested wetland using FAMEs, University of Maryland at Baltimore Annual Convocation, UMBC, October, 2006

COMMUNITY SERVICE AND TEACHING EXPERIENCE

Worship team, Morgantown, WV   2010-2013
Playing as a drummer at Morgantown Korean Church

Muscular Dystrophy Association (MDA) Summer Camp Volunteer   2006
Camp Maria Retreat Center, Leonardtown, MD

Private tutor, Salisbury, MD   2005-2007
Teaching math and general science for middle school students

Volunteer in Peninsula Regional Medical Center, Salisbury, MD   2003-2005
 At pharmacy department, pathology laboratory and Emergency Room

References

1. Steven M. Frisch, Ph.D
   Professor at West Virginia University School of Medicine
   Department of Biochemistry and Mary Babb Randolph Cancer Center
   Phone: (304) 293-2980
   E-Mail: sfrisch@hsc.wvu.edu
   Address: Health Sciences Center 2836HSS
         West Virginia University
         Morgantown, WV 26506

2. William Tse, MD, FACP
   Associate Professor at West Virginia University School of Medicine
   Hematology/Oncology
   Phone: (304) 293-4980
   E-Mail: wtse@hsc.wvu.edu
   Address: 2nd floor Mary Babb Randolph Cancer Center
         P.O. Box 9162
         Morgantown, WV 26506

3. Micheal D. Shaller, Ph.D
   Professor and Chair of Biochemistry at West Virginia University School of Medicine
   MBR Cancer Center, Center for Cardiovascular and Respiratory Sciences
   Phone: (304) 293-9514
   E-Mail: mschaller@hsc.wvu.edu
   Address: Health Sciences Center 3124HSN
         West Virginia University,
         Morgantown, WV 26506