Transforming Growth Factor-Beta (TGFβ)-Mediated Post-Transcriptional Regulation of Epithelial-Mesenchymal Transdifferentiation (EMT)

Arindam Chaudhury
Cleveland State University

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TRANSFORMING GROWTH FACTOR-BETA (TGFβ)-MEDIATED
POST-TRANSCRIPTIONAL REGULATION OF EPITHELIAL-
MESENCHYMAL TRANSDIFFERENTIATION (EMT)

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Dedicated to the Inspiration, Encouragement and Perseverance of my Family
ACKNOWLEDGEMENTS

Writing this section marks the beginning of the end of my graduate life and I feel happy being on the verge of moving to the next phase of my career, but extremely remorseful that it also means an end to four most exciting years of my life as a student of science and an individual. The excitement was all due to one person, Dr. Philip H Howe (‘Phil’ as I so dearly address him)! He set the standard for me the very first day I met him (August 26, 2005) by telling that if I have to get my job done well in the lab then I should spend as much time as I can in the lab and added that if I get good publications by the end of five years then only I should think that I have utilized my time well.

Crediting Phil for my scientific and social development during my graduate school tenure will only be an understatement. His contribution to my graduate career and in fact, my future professional life is immense. He introduced me to the field of hypothesis-driven research and I am very thankful to him for giving me such an interesting and exciting project, which has been our baby for the last four and a half years. With his continued guidance and help from other colleagues in the lab, I successfully developed the project and it feels great to cross the finish line, knowing I have given my best to answer the critical questions surrounding the project. Phil is instrumental in improving my overall interpersonal and scientific skills. He gave me unbridled independence in the lab and trained me in various aspects that will help me put firm steps towards my future career aspirations.

He taught me to relentlessly work on a hypothesis ‘till the cows come home’ (as he would famously say!). I will miss everything about him and his lab. The thin line between success and failure is perhaps the presence of or lack of determination to succeed. But just being determined does not help if one does not back them up with dedicated hard work and substantial attention. This fact is going to help me for the rest of my career. Thank you Phil!
I immensely thank the Honorable Members of my Advisory Committee, Dr. Barsanjit Mazumder, Dr. Crystal Weyman, Dr. Paul Fox and Dr. Xiaoxia Li. On days when I felt tired after long hours in the lab, I would look up to Dr. Fox and draw inspiration from his relentless pursuit of science. Getting his approval after a talk was a big thing for me as he is the best scientific orator I have seen so far. His and Dr. Mazumder’s research in translational regulation always formed a benchmark for what I have been doing in my graduate school years. Dr. Mazumder perhaps revolutionized my career when I took his RNA biology course. His meticulous preparation and intriguing questioning always inspired me. He taught me how to do my experiments with the goal of attending sufficiency and not just necessity. Dr. Weyman was always there whenever I needed her for any academic or administrative needs. Her magnanimous personality has helped me to learn a lot during these years. Above all, each one of them always had time for sincere advice and I had the freedom to walk into their office with any queries that I ever had. I always walked out of my committee meetings, inspired to work even harder. Thank you Professors!

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mnemonics; it was he who inspired me to take up GRE and apply for a graduate school in USA; it was he who would constantly challenge me to work harder; it was he who will encourage me for more. Thank you Dada! My sincere gratitude to my parents-in-law, Mr. Tapan Chakraborty and Mrs. Ruby Chakraborty, for their support, well wishes and encouragement. Thanks also to my sister-in-law, Mousumi for all her help and support and my little niece, Mehaghni whose thoughts will also refresh me in tired times.

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TRANSFORMING GROWTH FACTOR-BETA (TGFβ)-MEDIATED POST-
TRANSCRIPTIONAL REGULATION OF EPITHELIAL-MESENCHYMAL
TRANSDIFFERENTIATION (EMT)

ARINDAM CHAUDHURY

ABSTRACT

TGFβ induces epithelial-mesenchymal transdifferentiation (EMT) accompanied by cellular differentiation and migration, a process fundamental during embryonic development and one that is reactivated in a variety of diseases including fibrosis and cancer. Despite extensive transcriptomic profiling, identification of TGFβ-inducible, EMT-specific genes has met with limited success. Here, we report a novel post-transcriptional pathway by which TGFβ modulates expression of EMT-specific proteins and EMT itself. We show that heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) binds a structural, 33 nucleotides (nt) TGF-beta-activated translation (BAT) element in the 3’untranslated regions (UTRs) of disabled-2 (Dab2) and interleukin-like EMT inducer (ILEI) transcripts, and repress their translation. TGFβ activation leads to phosphorylation at Ser43 of hnRNP E1 by protein kinase Bβ/Akt2, inducing its release from the BAT element and reversal of translational silencing of Dab2 and ILEI mRNAs. Further, using a genome-wide combinatorial approach involving
polysome profiling and RIP-Chip analyses we have identified a cohort of four mRNAs (Rhox5, Ube3A, Prl2c4 and IL-11Ra2) that follow the same pattern of regulation as Dab2 and ILEI. Each of the identified targets mRNA harbors a functional BAT element in the 3’-UTR and is required for TGFβ-induced EMT. Modulation of hnRNP E1 expression or its post-translational modification alters TGFβ-mediated translational activation of the target transcripts and EMT in vitro and in vivo. This cohort of mRNAs may represent a new TGFβ responsive and hnRNP E1-mediated posttranscriptional regulon that regulates TGFβ-induced EMT during development and metastatic progression of tumors in a temporal and expedited fashion. The autocrine response of cells to TGFβ-induced Akt2 activation and subsequent translational activation of EMT inducer transcripts may represent a novel mechanism through which the increased TGFβ expression in tumor cells contributes to cancer progression.
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<td>APC</td>
<td>Anaphase-promoting complex</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5′-triphosphate</td>
</tr>
<tr>
<td>BAT</td>
<td>TGF beta-activated translation element</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CAK</td>
<td>Cyclin activating kinase</td>
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<tr>
<td>Cdc25a</td>
<td>Cell division cycle 25 homolog A</td>
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<tr>
<td>CHIP</td>
<td>Carboxy terminus of Hsp70 interacting protein</td>
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<tr>
<td>CKI</td>
<td>Cyclin-dependent kinase inhibitor</td>
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<td>Common mediator Smad</td>
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<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>EpH4</td>
<td>Mouse mammary epithelial cell</td>
</tr>
<tr>
<td>EpRas</td>
<td>EpH4 cells transformed with oncogenic Ras</td>
</tr>
<tr>
<td>eRF1</td>
<td>Eukaryotic release factor 1</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAM3A-D</td>
<td>Family with sequence similarity 3A-D</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth and differentiation factor</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heterogeneous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>Hrs/Hgs</td>
<td>Hepatocytes growth factor-regulated tyrosine kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IB</td>
<td>Immunoblotting</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-11Rα2</td>
<td>IL-11 receptor alpha chain 2</td>
</tr>
<tr>
<td>ILEI</td>
<td>Interleukin like EMT inducer</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>I-Smad</td>
<td>Inhibitory Smad</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KH</td>
<td>K-homologous</td>
</tr>
<tr>
<td>Mad</td>
<td>Mothers against dpp</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCT</td>
<td>Mouse cortical tubule</td>
</tr>
<tr>
<td>MEK1</td>
<td>Mitogen activated protein kinase kinase 1</td>
</tr>
<tr>
<td>MH-1</td>
<td>N-terminal Mad homology domain-1</td>
</tr>
<tr>
<td>MH-2</td>
<td>C-terminal Mad homology domain-2</td>
</tr>
<tr>
<td>MIS</td>
<td>Mullerian Inhibiting Substance</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NMuMG</td>
<td>Normal murine mammary gland epithelial cells</td>
</tr>
<tr>
<td>PAK2</td>
<td>p21 activated kinase 2</td>
</tr>
<tr>
<td>PCBP1</td>
<td>Poly(rC)-binding protein 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3'-kinase</td>
</tr>
<tr>
<td>PID</td>
<td>Phosphotyrosine-binding/-interacting domain</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>Prl2c4</td>
<td>prolactin family 2, subfamily c, member 4</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RhoX5</td>
<td>Reproductive homeobox 5</td>
</tr>
<tr>
<td>RIP-Chip</td>
<td>RNA Immunoprecipitation-Chip analysis</td>
</tr>
<tr>
<td>R-Smad</td>
<td>Receptor-activated Smad</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SARA</td>
<td>Smad anchor for receptor activation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp1-Cul1F-box protein</td>
</tr>
<tr>
<td>Smad</td>
<td>Mammalian homologue of <em>sma</em> and <em>mad</em></td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TβRI</td>
<td>TGFβ type I receptor</td>
</tr>
<tr>
<td>TβRII</td>
<td>TGFβ type II receptor</td>
</tr>
<tr>
<td>TGFα</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TRAP-1</td>
<td>TGFβ receptor-associated protein-1</td>
</tr>
<tr>
<td>Ube3A</td>
<td>Ubiquitin protein ligase E3A</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WCL</td>
<td>Whole cell lysate</td>
</tr>
</tbody>
</table>
1.1. Transforming Growth Factor-beta (TGFβ)

TGFβ is a pleiotropic cytokine that is secreted by fibroblasts and epithelial cells in a tissue specific manner and functions in a context-dependent fashion. In the 1970s, a host of individual peptide growth factors that could confer a ‘transformed’ phenotype on nonmalignant cells were identified (Sporn, 1999). Repeated rounds of purification of extracts from virus transformed cells, which initially was used to identify sarcoma growth factor (de Larco and Todaro, 1978), identified two peptides responsible for growth of normal rat kidney epithelial (NRK) cells on soft agar (Roberts et al., 1981; Anzano et al., 1983)). These peptides were christened as transforming growth factor-alpha (TGFα) and transforming growth factor-beta (TGFβ) (Roberts et al., 1983). TGFβ was purified to homogeneity from human platelets, human placenta, and bovine kidney and
characterized as a 25-kDa homodimer (Assoian et al., 1983; Frolik et al., 1983; Roberts et al., 1983).

Structurally related peptides harboring a conserved set of cysteines characterize the TGFβ family members (Kingsley, 1994; Massague, 1998; Shi and Massague, 2003). Since the identification of TGFβ1 in 1980s, two other distinct isoforms of TGFβ have been identified in mammals, TGFβ2 and TGFβ3. Currently, this superfamily comprises 34 family members, inclusive of TGFβ, Activins, Bone Morphogenetic Proteins (BMP), Vg1, Mullerian Inhibiting Substance (MIS), Growth and Differentiation Factor (GDF) and Inhibin and is highly conserved in organisms ranging from Caenorhabditis elegans, Drosophila melanogaster, Xenopus laevis, and mammals (Massague, 1998).

Originally believed to stimulate cell proliferation and growth, TGFβ was subsequently shown to have the potential to inhibit cell growth (Tucker et al., 1984). Specifically, TGFβ1 is involved in immune suppression, angiogenesis, apoptosis, cell growth, and epithelial to mesenchymal transitions (EMT) during development and metastatic cancer progressions (Pepper, 1997; Bakin et al., 2000; Akhurst and Derynck, 2001; Derynck et al., 2001; Dennler et al., 2002; Moustakas et al., 2002; Roberts and Wakefield, 2003; Lamouille and Derynck, 2007; Massague, 2008; Xu et al., 2009).

1.2. TGFβ Signaling Cascade

Binding of TGFβ family ligands to the constitutively active TGFβ type II serine/threonine kinase receptor (TβRII) results in the recruitment of type I
receptor (TβRI) and the formation of a stable oligomeric receptor complex (Yamashita et al., 1994). Formation of the complex results in the type II receptor to phosphorylate the type I receptor at the C-terminal GS domain, a highly conserved 30 amino acid sequence with a characteristic SGSGSG sequence directly upstream of the kinase domain (Wrana et al., 1994a; Wrana et al., 1994b; Wieser et al., 1995). This phosphorylation leads to a conformational change resulting in type I receptor-kinase activation.

Recently, the structural basis for this two-step assembly process has been revealed (Groppe et al., 2008). The extracellular domains of the TβRI and TβRII fit snugly around the dimeric TGFβ as a six-piece puzzle. It was earlier shown that TβRII binds the fingertip of the extended TGFβ3 ligand structure (Hart et al., 2002), with a conserved N-terminal extension in TβRII remaining disordered. In the active complex, seven residues of this N-terminal complex become ordered resulting in active heterotetrameric complex formation. Also a five-residue finger in TβRI was shown to hydrogen bond with an aspartate in TβRII, explaining the lack of avidity of TβRI to free TGFβ ligand (Groppe et al., 2008; Massague, 2008). The activated TβRI interacts with and phosphorylates a number of proteins, thereby activating multiple downstream signaling pathways. Downstream of this the signal is broadly transduced in either a Smad-dependent (canonical) or Smad-independent (non-canonical) signaling pathway.
1.2.1. Canonical TGFβ Signaling Pathway

Smads are the central regulators: The Smads were identified as intermediates of the decapentaplegic (dpp) signaling pathway in Drosophila melanogaster (Raftery et al., 1995; Sekelsky et al., 1995). Loss of function mutations in Mothers against dpp (mad) in Drosophila melanogaster resulted in pupal lethality, gut defects, and other phenotypes similar to dpp mutant phenotypes. Genetic screens in Caenorhabditis elegans identified sma-2, sma-3, and sma-4 as genes that have mutant phenotypes similar to that observed for the TGFβ-like receptor gene, daf-4 (Savage et al., 1996). From these data, it was proposed that the mad and sma are homologous genes involved in TGFβ signaling cascade. Later, murine and human homologues to the mad and sma genes were identified and collectively called Smads (Baker and Harland, 1996; Eppert et al., 1996; Derynck et al., 1996; Macias-Silva et al., 1996; Riggins et al., 1996; Yingling et al., 1996; Zhang et al., 1996; Nakao et al., 1997).

To date eight mammalian Smad proteins have been characterized and are divided into three functional sub-groups: the receptor-activated Smads (R-Smads), common mediator Smad (Co-Smad), and the inhibitory Smads (I-Smads). Human Smad2, Smad3 and Smad7 map to chromosome 18q21-22, Smad3 and Smad6 map to chromosome 15q21-22, and Smad5, Smad1 and Smad8 map to chromosome 15q31, 15q4, and 15q13 respectively (Eppert et al., 1996; Moustakas et al., 2001). The R-Smads are directly phosphorylated by the type I receptors on their carboxy terminal Ser-Ser-X-Ser (SSXS) motif and
include Smad1, Smad2, Smad3, Smad5, and Smad8. Smad2 and Smad3 are phosphorylated in response to the TGFβs and activin, whereas Smad1, Smad5, and Smad8 are phosphorylated in response to BMP. The only mammalian Co-Smad to be identified, thus far, is Smad4 and it mediates signals from both the Thug/activin/TGFβ and BMP signaling pathways. Smad4 functions to assist in the further transduction of the signaling pathways by oligomerizing with activated R-Smad(s). The I-Smads, Smad6 and Smad7, are induced by BMP and/or TGFβ/activin, respectively and act as negative feedback to inhibit activation of the R-Smads by inducing degradation of the receptors or by competing with the R-Smads for receptor binding (Massague, 1998).

The Smads are characterized by two conserved regions known as the amino terminal (N-terminal) Mad homology domain-1 (MH1) and C-terminal Mad homology domain-2 (MH2), which are joined by a short, poorly conserved linker region. The MH1 domain is highly conserved among the R-Smads and the Co-Smad, whereas the I-Smads lack a MH1 domain. The R-Smads and Smad4 have N-terminal nuclear localization signals (NLS) and Smad4 has a nuclear export signal (NES) in the MH1 domain (Xiao et al., 2000; Kurisaki et al., 2001; Xiao et al., 2003). The MH1 domain plays a role in R- and Co-Smad nuclear import, cytoplasmic anchoring, DNA binding, and regulation of transcription. The MH2 domain is conserved among all of the Smad proteins and regulates Smad oligomerization, cytoplasmic anchoring, and transcription of target genes. The MH1 and MH2 domains bind to a number of proteins including ubiquitination adaptors and substrates, transcriptional co-activators and co-repressors, and a
A number of transcription factors (Moustakas et al., 2001). Furthermore, Smad3 has a transactivation domain in the linker region (Prokova et al., 2005). The functional roles that are assigned to the linker region of the Smads are ubiquitination and transcriptional activation.

Figure 1.1: Schematic representation of canonical and non-canonical TGFβ signaling pathways.

Binding of TGFβ to its cognate receptor initiates the signaling pathway. In the canonical pathway, activated type I receptors phosphorylates R-Smads, which subsequently form a complex with the Co-Smad, Smad4. The resulting R-Smads/C0-Smad complex translocates to the nucleus and interacts with distinct transcription factors to turn on or off transcription of many TGFβ-responsive genes that regulate cell proliferation and differentiation. Additionally, TGFβ activates different non-Smad pathways, including PI3K, Ras, Par6, Jnk/p38/MAPK pathways, which cumulatively regulate TGFβ-mediated functions.
Receptor activation of Smad2 and Smad3: The role of adaptor proteins in TGFβ Signaling: The Smad signaling cascade is initiated by C-terminal phosphorylation of Smad2 and/or Smad3 by activated TβR1 (Macias-Silva et al., 1996). However, in order for Smad2 and Smad3 to be phosphorylated by TβRI, they must be recruited to the activated receptor complex. A number of proteins have been identified to interact with Smad2 and/or Smad3 to regulate R-Smad phosphorylation. Smad anchor for receptor activation (SARA) and hepatocytes growth factor-regulated tyrosine kinase substrate (Hrs/Hgs) is FYVE domain containing proteins that present Smad2 to TβRI (Tsukazaki et al., 1998; Miura et al., 2000). SARA is associated with the plasma membrane and can interact with both non-phosphorylated R-Smads and the TGFβ receptor complex (Tsukazaki et al., 1998). When the receptors become activated, and the R-Smads are phosphorylated, the R-Smads dissociate from SARA and the receptor complex, and bind to Smad4. SARA has a higher affinity for monomeric Smads; therefore it is thought that SARA may also act to regulate Smads by inhibiting aberrant R-Smad oligomerization (Qin et al., 1996). Hrs/Hgs is localized to early endosomes and synergizes with SARA to present Smad2 to the activated receptor complex (Tsukazaki et al., 1998; Burd and Emr, 1998; Gaullier et al., 1998; Patki et al., 1998). It was earlier shown that Disabled-2 (Dab2) associates with TβRI and TβRII and functionally bridges the activated receptors to Smad2 and Smad3 through its N-terminal phosphotyrosine-binding (PTB)/-interacting (PID) domain (Hocevar et al., 2001). Additionally, TGFβ receptor-associated protein-1 (TRAP-1) (Charng et al., 1998) and the adaptor
protein embryonic liver fodrin (ELF) (Mishra et al., 2004) enable activation of R-
Smads by the activated TGFβ receptor complex. Endocytosis of the active TGFβ
receptor complex is another mechanism by which R-Smad activation is
regulated. There is sufficient evidence supporting and arguing against the
necessity for receptor endocytosis in R-Smad phosphorylation (Hayes et al.,
2002; Penheiter et al., 2002). The dependency on receptor endocytosis for R-
Smad activation may be cell-type dependent.

**The Smad Pathway:** The activated TβRI phosphorylates R-Smads at its
C-terminal SXSS motif. Phosphorylated R-Smads then form a complex with
Smad4. The resulting complex of R-Smads/Co-Smads moves to the nucleus and
functionally interacts with distinct transcription factors to turn on or off
transcription of many TGFβ-responsive genes that regulate cell proliferation and
differentiation (Massague, 1998). The L45 loop of activated type I receptor
interacts with the L3 loop of the Smad proteins (Massague, 1998). The
interaction plays an important role in determining the signaling specificity as the
structure of the L45 loop differ between receptors and dictates which Smads will
bind and be activated.

1.2.2. Non-canonical (Smad-independent) TGFβ signaling pathways

TGFβ signaling can activate the MAP kinases ERK, JNK, and p38 MAP
kinase (Hartsough et al., 1995; Atti et al., 1997; Engel et al., 1999; Hocevar et al.,
1999; Bakin et al., 2002). Evidence for this activation came from studies with
Smad4-deficient cells and cells overexpressing dominant negative Smad4 (Engel
et al., 1998). In these cells, JNK/MAPK activation was shown to be adequate to elicit TGFβ regulated responses. Conversely, it was shown that TβRIs that were incapable of activating downstream Smads could still activate p38. Recently it was shown that activated TGFβ receptors directly induce polyubiquitination via a lysine at position 63 (K63) of TRAF6, which subsequently is required for activation of JNK and p38 (Yamashita et al., 2008). The consequences of MAPK activation by TGFβ remain unclear, however evidence suggests ERK activation is involved in TGFβ-mediated Ras signaling in epithelial cells. TβRs can also directly activate RhoA to induce actin stress fiber formation in fibroblasts, albeit evidences suggest a cooperative role of Smads (Bhowmick et al., 2001; Edlund et al., 2002; Vardouli et al., 2005). TGFβ–induced EMT integrates Smad as well as non-Smad signaling, and compulsorily requires signaling through PI3K/Protein kinase B (Akt) pathway (14, 68). This happens as a follow-up of Par6 induced ubiquitination and degradation of RhoA (Ozdamar et al., 2005).

The Smad proteins can also serve as the platform for signaling crosstalk mechanisms. ERK has been shown to phosphorylate the linker region of Smad1, Smad2, and Smad3 through the Ras pathway (Kretzschmar et al., 1997; Kretzschmar et al., 1999). Phosphorylation of Smads by ERK prevents nuclear translocation of the Smad complex to the nucleus, as a result of which cells containing hyperactive Ras pathway become insensitive to TGFβ stimulation. Contrasting reports have noted nuclear translocation of Smad complex in Ras transformed cells and ERK-mediated Smad phosphorylation seems to increase the half-life of Smad, stabilize complex formation with Smad4, enhancing the
overall transcriptional activity of Smad2 (de Caestecker et al., 1998). Other kinases, like protein kinase C (PKC) can directly phosphorylate Smad3 to prevent its binding to DNA while NF-κβ and STAT signaling inhibit TGFβ signaling by increasing induction of Smad7 expression (Ullola et al., 1999; Yakymovych et al., 2001; Jenkins et al., 2005). Evidence also exists for the cooperation between the TGFβ and Wnt pathway as well as cooperation between p53 and Smads in modulating expression of TGFβ regulated genes (Cordenonsi et al., 2003; Wilkinson et al., 2005; Jiang et al., 2008). Recently, it has been shown that TGFβ acts in sync with Ras and mutant p53 to inhibit p63 and aid in metastatic progression of tumors (Adorno et al., 2009). The multi-step crosstalk of Smad and non-Smad pathways affords a complex, yet meticulous regulation of TGFβ signaling and greater understanding of these crosstalk pathways in a cell type and context specific environment will elucidate the physiological and pathological relevance of this tight regulation.

1.3. Regulation and Signal Attenuation of TGFβ Response

Attenuation of TGFβ signaling is mediated either by the I-Smads or ubiquitination and proteosomal degradation of Smad2/3. The I-Smads antagonize TGFβ signaling by competitive inhibition of Smad2/3 binding to the activated TβRI (Imamura et al., 1997; Hata et al., 1998; Nakao et al., 1997; Ebisawa et al., 2001). Additionally, Smad7 dephosphorylates activated type I receptor by initiating interactions with a complex containing GADD34 and protein phosphatase 1 (Shi et al., 2004). Smad7 also contributes in signal attenuation by
recruiting Smurf E3 ubiquitin ligase to type I receptor and initiating proteosomal degradation, thereby preventing sustained TGFβ signaling after endocytosis of the receptor complex into calveolar lipid rafts (Lin et al., 2000). Subsequent internalization of the Type II receptors occurs as a result of β-arrestin2 recruitment of TGFβ signalosome at the receptor level. Smurf2 (Lin et al., 2000) and Skp1-Cul1-F-box protein (SCF) (Fukuchi et al., 2001), the Smad2 and Smad3 E3 ubiquitin ligase, respectively, mediates the degradation of Smad2 and Smad3. Smad3 is also degraded through the carboxy terminus of Hsp70 interacting protein (CHIP) dependent degradation (McDonough and Patterson, 2003). Smad3 can interact directly with Hsp70 resulting in TGFβ independent ubiquitination and degradation of Smad3 (Xin et al., 2005). This lends credence to the homeostatic regulation of TGFβ-mediated signal amplification and subsequent attenuation.

Co-repressors, like Ski and SnoN render an additional level of regulation of TGFβ signaling. Within the nucleus, TGFβ stimulation potentiates Smad3 to bind SnoN and promote its subsequent degradation by the anaphase promoting complex (APC) or Smurf2 (Bonni et al., 2001; Stroschein et al., 1999). TGFβ signaling transcriptionally induces SnoN, allowing for a negative feedback control of TGFβ signaling (Stroschein et al., 2001). SnoN and Ski function to inhibit TGFβ signaling by disrupting the Smad complex and by recruiting histone deacetylases such as the N-CoR complex, to the chromatin (Wu et al., 2002).
Finally, attenuation of the TGFβ signaling can also occur through dephosphorylation of the Smad/Co-Smad complex. PPM1A was identified as the phosphatase responsible for dephosphorylating the Smads and their subsequent release from the nucleus (Lin et al., 2006). The dephosphorylated Smads were shown to recycle back into the cytoplasm to await the next round of signaling (Lin et al., 2006). However, whether degradation of proteins or the modulation of the protein through post-translational modifications plays the dominant role in abrogating TGFβ signaling remains to be elucidated. It is likely that ubiquitin mediated proteosomal degradation and dephosphorylation events function cooperatively and in a redundant fashion to ensure rapid kinetics and tight control of TGFβ signal attenuation.

1.4. Paradoxical Role of TGFβ Signaling - The Yin and Yang of Carcinogenesis

Extensive evidences exist for deregulated TGFβ signaling pathway as a causative agent for tumor initiation and advanced stage disease progression. TGFβ exerts antiproliferative effects and functions as a tumor suppressor during early stages of tumorigenesis, whereas at later stages it functions as a tumor promoter aiding in metastatic progression through an autocrine TGFβ loop (Bierie and Moses, 2006). Transgenic mice expressing a dominant negative TβRII in the epidermis and mammary glands show aggressive tumor formation and metastatic progression (Amendt et al., 1998). Susceptibility of TGFβ-mediated antiproliferative effects is absent in lung cancer (Yanagisawa et al., 2000), head
and neck squamous cell carcinoma (Garrigue-Antar et al., 1995), prostate cancer (Park et al., 2000), gastric cancer (Myeroff et al., 1995; Kang et al., 1999), colon cancer (Eppert et al., 1996; Markowitz et al., 2000), pancreatic cancer (Goggins et al., 1998), and breast cancers (Lucke et al., 2001). Recently it has been shown that the tumor suppressor Merlin and a trans-acting negative regulator of signaling, Erbin fine regulates the context dependent response to TGFβ signaling (Wilkes et al., 2009). It was shown that in fibroblasts, Merlin is phosphorylated and subsequently inactivated by p21 activated kinase 2 (PAK2), inducing growth and proliferation. PAK2 activity in epithelial cells promotes apoptosis. To prevent antiproliferative effects in epithelial cells Merlin recruits Erbin and disrupts activation and function of PAK2 (Wilkes et al., 2009).

1.4.1. TGFβ as a tumor suppressor: cytostatic and pro-apoptotic effects

TGFβ functions as a tumor suppressor by mediating its antiproliferative effects in a large variety of cell types. During early stages of tumorigenesis, TGFβ inhibits cell cycle promotion and evasion of TGFβ-mediated antiproliferative effects is a prerequisite for advancement of tumor progression (Akhurst and Derynck, 2001; Derynck et al., 2001; de Caestecker et al., 2000). TGFβ-mediated downregulation of c-Myc is a central event of antiproliferative regulatory effects (Mulder and Brattain, 1988; Mulder et al., 1988; Zentella et al., 1992). c-Myc functions as a transcriptional activator or inhibitor, depending on the target gene, thereby promoting cell growth through the G1 phase of the cell cycle (Alexandrow and Moses, 1995a; Facchini and Penn, 1998; Dang, 1999). Ectopic
overexpression of c-Myc results in insensitivity to the growth inhibitory effects of TGFβ (Alexandrow and Moses, 1995b). Defective repression of c-Myc and subsequent resistance to TGFβ is reported in a number of breast cancer cell lines. Repression of c-Myc by TGFβ has been shown to occur through the Smad pathway (Yagi et al., 2002).

Additionally, TGFβ induces the cyclin-dependent kinase inhibitors (CKIs) p15 and p21 (Alexandrow and Moses, 1995b; Datto et al., 1995; Reynisdottir and Massague, 1997; Iavarone and Massague, 1997; Moustakas and Kardassis, 1998; Robson et al., 1999). TGFβ transcriptionally upregulates p15 expression in a Smad-dependent fashion through inhibition of Cyclin D1/Cdk4 (Sandhu et al., 1997). TGFβ-dependent induction of p21 and/or p27 also regulates Cdk activity (Alexandrow and Moses, 1995b; Datto et al., 1995; Reynisdottir and Massague, 1997; Iavarone and Massague, 1997; Moustakas and Kardassis, 1998; Robson et al., 1999). p21 directly interacts with and inhibits Cyclin D-Cdk4/6, Cyclin E-Cdk2, and Cyclin A-Cdk2 complexes, therefore arresting progression of the cell cycle in the late G1 phase (Harper et al., 1993). TGFβ regulation of the p21 promoter involves Sp1 and the Smads (Moustakas and Kardassis, 1998). But contrasting reports have shown that lymphocytes from p27 deficient mice remain sensitive to the growth inhibitory effect of TGFβ; therefore, suggesting that p27 may not be actually necessary for TGFβ-induced cell cycle arrest (Nakayama et al., 1996). Cell division cyclin 25A (Cdc25A) mRNA and cyclin activating kinase (CAK) activity is downregulated by TGFβ (Reynisdottir and Massague, 1997;
Nagahara et al., 1999; Bhowmick et al., 2003). Cdc25A is a Cdk tyrosine phosphatase that functions to inactivate Cdns by dephosphorylating

Figure 1.2: Paradoxical effects of TGFβ signaling.

In normal epithelium TGFβ functions as a tumor suppressor through its antiproliferative and pro-apoptotic effects. But with tumor progression, autocrine loops of TGFβ are activated and in the tumor milieu resistance to the growth inhibitory effects of TGFβ is acquired. Epithelial cells transdifferentiate to mesenchymal cells through a TGFβ-dependent phenomenon, called epithelial to mesenchymal transition (EMT), with concomitant loss of adherens and tight junctions, loss of E-cadherin expression, and increase in mesenchymal cell markers such as dab2, N-cadherin, and ILEI. EMT renders mobility to the tumor cells, which is a critical pre-requisite for metastatic progression of the tumor. Both Smad-dependent and Smad-independent pathways are involved in TGFβ-mediated EMT.
threonine/tyrosine residues that are necessary for full activation of the Cdk.s. In contrast, CAK phosphorylates Cdk.s on a conserved threonine residue. Without this phosphorylation, the Cdk.s cannot be fully active. The decrease in Cdc25A expression, mediated by TGFβ, was observed in mammary gland epithelial cells (Iavarone and Massague, 1997; Bhowmick et al., 2003). As a result of TGFβ-mediated downregulation of Cdc25A and inactivation of CAK, the Cdk.s are not fully active and cell cycle progression stops during G1 phase.

Pro-apoptotic effects of TGFβ also contribute towards its cytostatic effects. TGFβ-induced apoptotic response has been seen in prostate epithelium, hepatocytes and hepatoma cell lines, B-lymphocytes and B-cell lines (Sanchez-Capelo, 2005). TRAIL and the AP-1/Smad pathway (Herzer et al., 2008), Daxx and the JNK pathway (Perlman et al., 2001), DAPK and the Smad pathway (Jang et al., 2002), GADD45b and the p38 pathway (Yoo et al., 2003), and ARTS, a mitochondrial protein that aids in caspase activation (Gottfried et al., 2004) have all been indicated to be involved in TGFβ-mediated apoptotic events. TGFβ-induced expression of the pro-apoptotic protein, Bim, induces cell death in B-lymphocytes (Wildey et al., 2003). It was also shown that stimulation of the pro-survival CD40 receptor inhibited TGFβ-mediated Bim expression and subsequent apoptosis in WEHI1231 B-lymphocytes (Patil et al., 2000). Smad3-dependent Bim induction has been shown in gastric epithelial cells undergoing TGFβ-induced apoptosis (Ohgushi et al., 2005), and in AML12 hepatocytes (Ramesh et al., 2009). Evidence suggests that Smad3 and Bim are critical mediators of TGFβ-induced apoptosis (Ramesh et al., 2003).
1.4.2. TGFβ as a promoter of metastatic progression: TGFβ-Mediated Epithelial to Mesenchymal Transition (EMT)

Alternatively, phosphorylation of the polarity protein Par6 by the activated receptor complex has also been shown to be involved in EMT. During metastatic progression, TGFβ promotes epithelial to mesenchymal transition (EMT) (Derynck et al., 2001; Moustakas et al., 2002; Zavadil and Bottinger, 2005), which is accompanied by a concomitant loss of cell-cell and cell-matrix adhesion and morphogenic changes from a polarized epithelial phenotype to an elongated fibroblastoid or mesenchymal phenotype (Zavadil and Bottinger, 2005; Thiery and Sleeman, 2006). TGFβ-induced EMT is also indispensable during embryonic development for neural crest, heart, and craniofacial structures formation (Trelstad et al., 1967; Massague, 2008). It is interesting to note that EMT during development is largely spatially and temporally regulated, whereas the EMT seen during advanced cancer progression may not reflect the order and timing of events observed during development (Larue and Bellacosa, 2005). EMT has a critical role in cancer cell motility, invasion and metastasis. In order for cancer cells to invade surrounding tissues and metastasize to distant sites it is necessary for the cells to dissociate and penetrate the basement membrane, characteristics of developmental EMT.

Evidence suggests that non-Smad signaling pathways are primarily involved in the induction of EMT by TGFβ. Signaling through integrin β1 (Bhowmick et al., 2001), p38MAPK (Bakin et al., 2002), phosphoinositide 3-
kinase (PI3K) (Bakin et al., 2000; Gotzmann et al., 2002; Kattla et al., 2008), Ras homologous (Rho) A (Bhowmick et al., 2001; Janda et al., 2002), Jagged/Notch (Zavadil et al., 2004), nuclear factor κβ (NF-κβ) (Huber et al., 2004) have all been shown to be required for TGFβ-induced EMT. TGFβ treatment of non-transformed murine NMuMG cells and mouse cortical tubule (MCT) cells resulted in an induction of EMT (Xie et al., 2004; Prunier and Howe, 2005) and treatment of the cells with the MEK inhibitor U0126 blocked TGFβ-mediated induction of EMT (Xie et al., 2004).

**Significance of Dab2 and ILEI in TGFβ-mediated EMT:** Two established in vitro models for studying TGFβ-induced EMT are normal murine mammary gland epithelial (NMuMG) cells and mouse mammary epithelial cells, EpH4, transformed with oncogenic Ras (EpRas) (Miettinen et al., 1994; Oft et al., 1996; Thuault et al., 2006). Using these models, two candidate EMT genes were defined, *Disabled-2* (Dab2) (Prunier and Howe, 2005) and *FAM3C* or interleukin like EMT inducer (*ILEI*) (Jechlinger et al., 2003; Waerner et al., 2006). Dab2 is a putative tumor suppressor gene, but modulates late stages of tumor progression by promoting EMT-dependent metastasis (Mok et al., 1994; Prunier and Howe, 2005). Dab2 protein is aberrantly low in many types of tumors, yet the molecular basis for its loss is unknown (Bagadi et al., 2007). Dab2 is involved in homeostatic balance of epithelial cell differentiation, a role confirmed by several genetic studies demonstrating a function for Dab2 in endodermal cell formation, organization, and differentiation (Sheng et al., 2000; Morris et al., 2002; Yang et al., 2002). *ILEI* was initially identified as a candidate gene for autosomal
recessive nonsyndromic hearing loss locus 17 (DFNB17) (Greinwald et al., 1998) and was subsequently identified as a member of a recently discovered gene family (FAM3A-D) (Zhu et al., 2002). In a translational-state microarray analysis (also called “polysome profiling) in which differential sedimentation is used to separate heavy, ribosome-enriched, rapidly translating mRNAs (polysomes) from light, ribosome-poor, slowly translating mRNAs (monosome), ILEI was shown to be translationally upregulated during EMT (Jechlinger et al., 2003; Waerner et al., 2006).

It has been previously demonstrated that shRNA-mediated silencing of Dab2 in NMuMG cells was sufficient to inhibit TGFβ-mediated EMT as analyzed morphologically and by loss of upregulation of N-cadherin, a mesenchymal cell marker (Prunier and Howe, 2005). More importantly, re-expression of human Dab2 in Dab2 knock-down cells restored TGFβ-mediated EMT and N-cadherin up-regulation (Prunier and Howe, 2005). Stable knockdown of ILEI has also been shown to inhibit TGFβ-mediated EMT in EpRas cells (Waerner et al., 2006), whereas ILEI expression can cause epithelial plasticity changes and tumor formation in non-tumorigenic NMuMG cells and 3T3 fibroblasts (Waerner et al., 2006). Cumulatively, it can be inferred that both Dab2 and ILEI are required, but not sufficient alone (i.e., in a TGFβ-independent fashion) to induce EMT.

Activated Ras/Raf/Mitogen activated protein kinase (MAPK) pathway has been implicated for TGFβ-mediated EMT in human, rat, or mouse epidermal, pancreas, intestine, liver, prostate, and mammary epithelial cells (Oft et al., 1996; Oft et al., 1998; Bhowmick et al., 2001; Gotzmann et al., 2002; Davies et al.,
In other models, TGFβ stimulates ERK, whose function is required for the relocalization of adherens junctions and cell motility induced by TGFβ. In addition, TGFβ activates both Snail and Slug, zinc-finger proteins that repress transcription of E-cadherin in certain cell culture models of EMT (Zavadil et al., 2001; Saika et al., 2004; Giannelli et al., 2005).

TGFβ signaling through TβRI and TβRII has also been implicated for TGFβ-mediated EMT and Smad overexpression has been shown to cause synergistic induction of EMT when combined with activated TGFβ receptors (Oft et al., 2002). Ectopic expression of Smad2 or Smad3, along with Smad4, in human and mouse non-transformed cell lines enhanced TGFβ-induced EMT, whereas the expression of a dominant negative Smad2, Smad3, or Smad4 blocked TGFβ-mediated EMT (Valcourt et al., 2005). In vivo evidence for Smad3 involvement in EMT result from experiments in Smad3 knockout mice where loss of Smad3 blocks injury-induced EMT in primary lens epithelial cells and fails to induce EMT in primary tubular epithelial cells derived from Smad3−/− mouse kidneys (Saika et al., 2004; Valcourt et al., 2005). More recently it was shown that after deletion of Smad3 in mouse hepatocytes, TGFβ induced EMT only in control hepatocytes but not in Smad3−/− hepatocytes (Ju et al., 2006), suggesting involvement of Smad-dependent signaling in TGFβ-mediated EMT.

1.5. Translational Regulation

Protein synthesis is a multi-step, multi-factorial pathway in which regulation can be exerted at many levels. Actively translating mRNAs usually
consist of a 5’ m7gpppN cap, 5’-UTR, coding region, 3’-UTR and poly (A) tail. The process of translation can be separated into three distinct stages: initiation, elongation and termination (Wickens et al., 1996; van der Kelen et al., 2009). During initiation, the 40S ribosomal subunit binds to the 5’-cap of an mRNA, scans in the 5’ to 3’ direction until the first AUG is encountered, stalls to recruit the 60S subunit, and forms the 80S ribosomal unit, which then proceeds with the translation elongation step. Elongation begins after delivery of a cognate aminoacyl-tRNA (aa-tRNA) by EF1A1 to the ribosomal A-site and translocation of the aa-tRNA to the P-site by EF-2. The α subunit of EF-1 binds aa-tRNA in a GTP-dependent manner, and this ternary complex then binds to the elongating ribosome. Once the cognate aa-tRNA is bound to the A-site, hydrolysis of GTP occurs, releasing EF1A1 from the ribosome. Termination occurs when the translating ribosome encounters stop codons in the A site. The stop codon is recognized by eukaryotic release factor 1 (eRF1), which binds to the A site and catalyzes the hydrolysis of the ester bond linking the polypeptide chain to the P site tRNA thus allowing the peptide chain to be released from the ribosome.

Translational control is an essential cellular process that governs the development and homeostasis of cells and tissues (Ruvinsky and Meyuhas, 2006) and defines a paradigm of control for different signaling pathways (Hay and Sonenberg, 2004; Lamouille and Derynck, 2007). Deregulation of the translation machinery has been implicated as causative agents responsible for alterations of cell cycle progression and cell growth during changes in nutrient
status, stress and carcinogenesis (Mazumder and Fox, 1999; Standart and Jackson, 1994; Ruggero and Pandolfi, 2003). Given the quick pace and overall energetically efficient nature, translational regulation is evolving as an important regulatory step of gene expression (Mazumder and Fox, 1999; Sampath et al., 2004).

Translational regulation can either be global through effects on eukaryotic translation initiation factor 2 α-subunit (eIF2α) (Clemens, 1996); or, transcript selective, mediated by putative cis regulatory elements in the 5’ and 3’ UTRs of mRNAs and trans factor(s) binding to the regulatory cis elements (Jackson, 1993; Standart and Jackson, 1994; Decker and Parker, 1995; Hentze, 1995; Mazumder and Fox, 1999; Sampath et al., 2004). The ‘human genome project’ reported the mean lengths of 5’-untranslated regions (UTRs) and 3’-UTRs of human mRNAs as 300nt and 770nt, respectively, compared to the mean coding length of 1340nt (International Human Genome Sequencing Consortium, 2001; Reimann et al., 2002), generating renewed interest in the 3’-UTRs of mRNAs to map translational regulatory activities. 3’-UTR’s have been shown to be involved in multiple translational regulatory mechanisms, including mRNA translation initiation (Black et al., 1997; Izquierdo et al., 1997; Ostareck et al., 1997), mRNA stability (Sachs, 1997), mRNA localization (Zoladek et al., 1995), and in control of poly(A) chain length (Sachs and Deardorff, 1992).

Transcript-selective translational control is achieved by the binding of a RNA binding protein (trans factor) to putative, regulatory cis elements in the
untranslated regions of target mRNAs. Most known examples of translational inhibition attribute a negative regulatory function to the \textit{trans} factor (Mbella et al., 2000). The mechanism by which a protein bound to the 3'-UTR inhibits ribosome assembly at the 5' end is not yet known. The current hypothesis is the existence of a closed-loop structure during mRNA translation and circular mRNA complexes have been visualized by atomic force microscopy (Wells et al., 1998). \textit{Trans} factors that interact with putative \textit{cis} elements, might affect this closed-loop model and thus affect translation efficiency (Bormann et al., 2000).

The \textit{trans} factors are RNA binding proteins harboring defined RNA binding domains. Among the various RNA binding proteins, heterogeneous ribonucleoprotein E1 (hnRNP E1) (also called poly(rC)-binding protein or \(\alpha\)-CP1) has been implicated in the translational regulation and mRNA stability of many transcripts, including gastrin (Lee et al., 2007), A2 response element (Kosturko et al., 2006), collagen I, III (Thiele et al., 2004), renin (Morris et al., 2004), folate receptors (Antony et al., 2004), and 15-lipoxygenase (Ostareck et al., 1997). hnRNP E1 is a ubiquitously expressed protein (Meng et al., 2007) and contains three copies of the RNA binding domain, KH (K-homologous) (Gibson et al., 1993; Siomi et al., 1993). hnRNP E1 preferentially binds to poly(C) region of mRNA (Aasheim et al., 1994), but has also been shown to bind poly(U) stretch (Leffers, 1995), albeit with less affinity. hnRNP E1 exists in both phosphorylated and unphosphorylated forms (Leffers, 1995; Meng et al., 2007) and it has been
predicted that the phosphorylated form has comparatively less RNA binding capacity (Leffers, 1995).
CHAPTER II

TGFβ-MEDIATED TRANSCRIPT-SELECTIVE TRANSLATIONAL ACTIVATION OF DAB2 AND ILEI IS DIRECTED BY A NOVEL STRUCTURAL ELEMENT IN THE 3'UTR OF THE MESSENGER RNAs AND HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN E1 (hnRNP E1)

2.1. Abstract

TGFβ induces epithelial-mesenchymal transdifferentiation (EMT) accompanied by cellular differentiation and migration. Despite extensive transcriptomic profiling, identification of TGFβ-inducible, EMT-specific genes has met with limited success. Here, we identify a post-transcriptional pathway by which TGFβ modulates expression of EMT-specific proteins. We show that heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) binds a structural, 33 nucleotides (nt)
TGF beta-activated translation (BAT) element in the 3’-UTR of disabled-2 (Dab2) and interleukin-like EMT inducer (ILEI) transcripts, and repress their translation.
2.2. Introduction

Epithelial-mesenchymal transition (EMT), in which cells undergo a switch from a polarized, epithelial phenotype to a highly motile fibroblastic or mesenchymal phenotype is fundamental during embryonic development and can be reactivated in a variety of diseases including fibrosis and cancer (Derynck et al., 2001; Zavadil and Bottinger, 2005; Bierie and Moses, 2006; Thiery and Sleeman, 2006; Massague, 2008). TGFβ is one of the growth factors implicated in EMT (Massague, 2008). Using normal murine mammary gland epithelial (NMuMG) cells (Miettinen et al., 1994; Thuault et al., 2006) and mouse mammary epithelial cells, EpH4, transformed with oncogenic Ras (EpRas) (Oft et al., 1996) as in vitro models for TGFβ-induced EMT two candidate EMT genes were defined, Disabled-2 (Dab2) (Prunier and Howe, 2005) and FAM3C or interleukin like EMT inducer (ILEI) (Waerner et al., 2006). Dab2 is a putative tumor suppressor gene, but modulates late stages of tumor progression by promoting EMT-dependent metastasis (Prunier and Howe, 2005). ILEI was initially identified as a candidate gene for autosomal recessive nonsyndromic hearing loss locus 17 (DFNB17) (Greinwald et al., 1998) and was subsequently shown to belong to the FAM3A-D gene family (Zhu et al., 2002). ILEI was shown to be translationally upregulated during EMT in EpRas cells (Waerner et al., 2006). Short hairpin RNA (shRNA)-mediated silencing of Dab2 in NMuMG cells inhibits TGFβ-mediated EMT and re-expression of human Dab2 in Dab2 knock-down cells restores TGFβ-mediated
EMT (Prunier and Howe, 2005). Stable knockdown of ILEI inhibits TGFβ-mediated EMT in EpRas cells, whereas ILEI expression induces epithelial plasticity changes and tumor formation in non-tumorigenic NMuMG cells and 3T3 fibroblasts (Waerner et al., 2006). These cumulatively suggest that both Dab2 and ILEI are required, but not sufficient (i.e., in a TGFβ-independent fashion) to induce EMT. However, the molecular mechanism by which expression of Dab2 and ILEI is regulated by TGFβ remains elusive.

Here, we show that post-transcriptional regulation of gene expression plays an important role in TGFβ-mediated EMT. We have identified a novel, 33nt structural element in the 3′-UTRs of Dab2 and ILEI that inhibit translation of the messages, an inhibition that is relieved following TGFβ treatment of cells. The 33nt element is sufficient to mediate translational inhibition in vitro and in vivo. Using the 33nt 3′-UTR element, we have affinity purified the mRNP complex and have identified hnRNP E1 as a critical component of this complex. Coordinate, functional regulation of EMT inducer genes by the common 3′-UTR 33nt element may represent a system similar to a prokaryotic regulon (Keene and Tenenbaum, 2002; Ray and Fox, 2007).
2.3. Materials and Methods

2.3.1. Reagents.

Rabbit reticulocyte lysate, methionine free amino acids, RNasin, Dual Luciferase Reporter Assay system and RiboMAX Large Scale RNA Production kit were purchased from Promega. MAXIscript, and mMESSAGE mMACHINE T7 Ultra kits were purchased from Ambion. Primers and oligos were purchased from Integrated DNA technologies. Translation grade $[^{35}\text{S}]$-methionine and $[^{32}\!\alpha\!]-\text{UTP}$ were purchased from Perkin Elmer Life Sciences. Mouse $\alpha$-hnRNP E1 was from Novus Biologicals, rabbit $\alpha$-hnRNP E1 was from Lifespan Biosciences. $\alpha$-FAM3C antibody was obtained from Abcam; $\alpha$-Hsp90 (H-114) and $\alpha$-hnRNP K (F45P9C7) antibodies and normal mouse and rabbit IgG were purchased from Santa Cruz Biotechnology.

2.3.2. Cell culture.

TGFβ2 was a generous gift from Genzyme Inc. and was used at a final concentration of 5 ng/ml. NMuMG cells were cultured as described previously (Prunier and Howe, 2005). EpRas were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics/antimycotics.

2.3.3. Plasmids construction and protein expression.

The conserved 575 nt. (downstream of the stop codon) in Dab2 3’-UTR was amplified using primers (mDab2-3’-UTR-F1/mDab2-3’-UTR-R2) from mouse
cDNA and cloned into pcDNA3 downstream of the T7 promoter (pcDNA3/Dab2<sub>575-UTR</sub>) via Pac1 and Not1 sites (refer to Table I for primer sequences). For synthesis of the deletion and internal fragments, 5'-and 3'-primers were designed to generate Dab2 3'-UTR fragments having upstream T7 promoter sequence. For sequences less than 30 nucleotides, oligonucleotides complementary to the desired sequence were synthesized with complementary T7 promoter sequence at the 3'-end, and were annealed to an oligonucleotide containing the T7 promoter sequence in STE buffer (0.1 M NaCl, 10 mM Tris-HCl [pH, 8.0], 1 mM EDTA). The oligonucleotides were PAGE purified before annealing.

For construction of the Luc-Dab2/BAT, Luc-Dab2/BAT-M mutant and Luc-ILEI/BAT, luciferase cDNA derived from pGL3-b vector was cloned into pcDNA3. A linker region containing 5'-EcoR1-Pac1-EcoRV-Nco-1-Xho-1-Xba-1 was inserted into the vector downstream of the luciferase gene (pCMV-LL). Synthetic Dab2/BAT, Dab2/BAT-M and ILEI/BAT were generated with 5'-EcoR1 and 3'-Xba-1 sites (pCMV<sub>Luc-Dab2/BAT</sub>, pCMV<sub>Luc-Dab2/BAT-M</sub>, pCMV<sub>Luc-ILEI/BAT</sub>).

The GST-hnRNP E1 construct was a kind gift from Dr. R. Kumar and has been previously described (Meng et al., 2007). The GST clones were maintained in E. coli BL21 (DE3)pLysS for expression. For expression, the protocol as described before was followed (Meng et al., 2007).

2.3.4. Isolation of RNA, Northern Blot and RT-PCR.

Isolation of total RNA, northern blot and RT-PCR was done as described previously (Wildey et al., 2003). Refer to Table I for primer sequence. The Dab2
probe specific for the p96 isoform was generated by PCR using primers listed in Table I.

2.3.5. Preparation of cell lysates, immunoblot analysis, immunoprecipitation and immunodepletion.

For immunoprecipitation and immunoblot analysis, cells were lysed in buffer D and immunoprecipitation carried out as previously described (Hocevar et al., 1999). For immunodepletion, indicated amounts of control cytosolic extracts from NMuMG cells were incubated with mouse α-hnRNP E1 antibody or mouse IgG coupled to Protein G agarose beads in cytosolic extraction buffer. The beads were pelleted and the supernatant was subjected to another round of immunodepletion. The supernatants were immunoblotted with rabbit α-hnRNP E1 antibody to confirm immunodepletion.

2.3.6. Preparation of cytosolic extract (S100 Fraction).

S100 fractions were prepared from control and TGF-β-treated NMuMG cells as previously described (Mazumder and Fox, 1999) with minor modifications. Briefly, the buffer used for cytosolic extraction contained 20 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail (Roche) (Hampton et al., 1998).

2.3.7. Polysome analysis.

Polysome analysis was performed as previously described (Ray and Fox, 2007) with minor modifications. For polysomes release experiments polysome
lysis buffer containing 10 mM EDTA was used as described previously (Ray and Fox, 2007).

2.3.8. Metabolic labeling.

NMuMG cells were treated with TGFβ for up to 24 hr. During the last 3 hr of treatment cells were maintained in methionine-free medium containing [\(^{35}\)S]-methionine (100 µCi/ml). To detect de novo synthesis of Dab2 protein, cell lysates were made and subjected to immunoprecipitation with mouse α-Dab2 antibody and mouse IgG as described previously (Hocevar et al., 1999). The immunoprecipitated complexes were resolved by 10% SDS-PAGE, fixed and visualized by autoradiography.

2.3.9. In vitro translation of Dab2 mRNA by a cell free translation-competent system.

In vitro translation was done as described previously (Mazumder and Fox, 1999). An aliquot of the translated products were subjected to immunoprecipitation using mouse anti Dab2 antibody and normal mouse IgG as described previously (Hocevar et al., 1999). Immunoprecipitated protein was resolved by 10% SDS-PAGE, fixed and visualized by autoradiography.

2.3.10. In vitro transcription and UV crosslinking assay.

Radiolabeled, synthetic transcripts of the 575 nt of Dab2 3'-UTR was prepared by in vitro transcription of linearized pcDNA3/Dab2\(_{575-UTR}\), using T7 RNA polymerase using MaxiScript kit in the presence of \(^{32}\)P-UTP. The transcripts were purified by passing through MicroBiospin columns (BioRad) and
were resolved on 15% Urea-acrylamide gel. The synthetic probes were gel eluted and quantitated using a scintillation counter. Synthetic probes for the various fragments were similarly made using the T7 RNA polymerase system as each of these fragments had a 5’-T7 promoter. UV crosslinking was done as described previously (Legagneux et al., 1992). Excess cold WT or mutant Dab2 33nt and 33nt ILEI RNAs were used in decoy experiments.

2.3.11. In vitro luciferase assay.

In vitro luciferase assay was performed as previously described (Mazumder and Fox, 1999). Excess (2-10 folds) Dab2 3’-UTR BAT cRNA was used in decoy experiments.

2.3.12. In vivo luciferase assay.

NMuMG cells were co-transfected with 10 µg of pCMV<sub>Luc-Dab2/BAT</sub>, pCMV<sub>Luc-Dab2/BAT-M</sub>, pCMV-LL along with 1 µg of pCMV<sub>Renilla</sub> (Promega) using Lipofectamine reagent. Cells were allowed to recover for 24 hr and then treated with TGFβ for up to 24 hr. Dual (firefly and Renilla) luciferase activities were determined by the Dual Luciferase Reporter assay system. The Renilla luciferase activity was used as an internal control for uniform transfection efficiency. The firefly luciferase activity was normalized to renilla luciferase activity and expressed as percent control.

2.3.13. PatSearch and MFold analysis.

The PatSearch syntax was used to define a query pattern based on the structure and sequence information of the Dab2 33nt element (Grillo et al., 2003;
Ray and Fox, 2007). The query pattern was used to search a nonredundant 3′-UTR sequence database. MFold algorithm was used to predict RNA secondary structures (Zuker, 2003).


Size exclusion chromatography was performed as described previously (Sampath et al., 2004).

2.3.15. RNA pull-down and isolation of mRNP complex binding to BAT element.

WT and mutant Dab2 BAT synthetic RNA (cRNA) was bound to cyanogen bromide-activated sepharose beads. Ribomax kit was used to generate milligrams quantity of synthetic 33nt RNA from the template DNA. For RNA pull down experiments, different amount (0.2-1 mg) of cytosolic extracts prepared from control and TGFβ treated NMuMG cells were pre-cleared using the Dab2/BAT-M cRNA-beads and then incubated at 4 °C for 2 hr with the Dab2/BAT cRNA beads. Following the incubation period the beads are washed with 0.3 M sodium chloride and resolved by 10% SDS-PAGE. In some experiments the mRNP complex is eluted from the beads by washing with 1 M sodium chloride and then concentrated and desalted using YM-3 desalting columns (Millipore). Size-exclusion fractions that were showing translational repression activity were subjected to RNA pull down as described above. The indicated bands were trypsinized and peptide sequences determined by capillary liquid chromatography-electrospray mass spectrometry. The data obtained were
analyzed using TurboSequest software to query the NCBI nonredundant protein database. Matching spectra was confirmed by manual interpretation using Mascot and FASTA software.

2.3.16. Determination of \textit{in vivo} interaction between hnRNP E1 and Dab2 or ILEI mRNA

\textit{In vivo} interaction was investigated as described previously (Ray and Fox, 2007).

2.3.17. Statistical analysis

Data are presented as mean ± s.d., \( n=3 \) samples per group. The renilla luciferase activity was used as an internal control for uniform transfection efficiency. The firefly luciferase activity was normalized to renilla luciferase activity and expressed as percent control.

Table I. Primers and oligonucleotide sequences for various constructs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer/oligonucleotide sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mDab2-3’-UTR-F1</td>
<td>ATTTAATTAAGTTGTATGATGACTATCCAGATGAGCAA</td>
</tr>
<tr>
<td>mDab2-3’-UTR-R2</td>
<td>AAGCGGCCGCAGTTAGCTGAGAAACGACCATCTCAAAAATGGT</td>
</tr>
<tr>
<td>hnRNP E1 shRNA 3’-UTR Top</td>
<td>GATCATGTAAGAGTGAATGTTATTCAAGAGATTGGCCCATAAGCCTTTCACTTTTTTGGAAA</td>
</tr>
<tr>
<td>hnRNP E1 shRNA 3’-UTR Bottom</td>
<td>AGCTTTTCCAAAAAAGTGAAAGGCTATATTGGGCAATCTCTTGAATAACATTTCCACTTTACAT</td>
</tr>
<tr>
<td>Primer for hnRNP E1 S43A Mutation</td>
<td>GATCCGCGAGGAGGCGGCAGGCGCGG</td>
</tr>
<tr>
<td>mActin-Exon4-F1</td>
<td>AGCTGTGCTATGTTGTGCTCTAGACTT</td>
</tr>
<tr>
<td>mActin-Exon5-R1</td>
<td>CACTTCATGATGGAATGTAAGT</td>
</tr>
<tr>
<td>mDab2-Exon9-F1</td>
<td>AAGCAGGACTTGGAAAGTTCTGT</td>
</tr>
<tr>
<td>mDab2-R2</td>
<td>CATTTGCCCTTTGAAGAGATCCAGAA</td>
</tr>
</tbody>
</table>
2.4. Results

2.4.1. Lack of correlation between Dab2 mRNA and protein expression levels in NMuMG and EpRas cells

The temporal relationship between Dab2 mRNA and protein expression levels in TGFβ-treated NMuMG and EpRas cells was investigated. Dab2 mRNA levels, as measured by Northern blot and semi-quantitative RT-PCR, were steady and only slightly induced by TGFβ in both cell lines examined (Fig. 2.1A, B). Dab2 protein expression, measured by immunoblot analysis (Fig. 2.2A, B), revealed that control cells, despite having abundant Dab2 mRNA, have low levels of Dab2 protein. Similarly, in cells treated with TGFβ for 3 hr, Dab2 protein levels are low despite abundant mRNA levels. It is only after a 3 hr TGFβ treatment that Dab2 protein levels begin to increase with maximal induction observed at ~12 hr post-TGFβ stimulation. To confirm the low rate of synthesis of Dab2 in control cells and those treated with TGFβ for short times, de novo Dab2 synthesis was measured by pulse labeling with $[^{35}\text{S}]$-methionine. Cells were treated with TGFβ for the times indicated and labeled for the last 3 hr of the incubation period, followed by immunoprecipitation of Dab2 (Fig. 2.3). As shown, little de novo synthesis occurred in control or 3 hr TGFβ-treated cells but Dab2 synthesis increased significantly between 3-6 hr of TGFβ stimulation and peaked at ~12 hr post-TGFβ treatment.
2.4.2. TGFβ translationally upregulates Dab2 expression

The absence of Dab2 protein expression in control cells was either due to the lack of translatability of Dab2 mRNA or due to attenuation in Dab2 translation. To test transcript integrity, total RNA was isolated from NMuMG cells treated with TGFβ and translated in vitro in a cell-free rabbit reticulocyte lysate system in the presence of [35S]-methionine and translated products were immunoprecipitated (IP) with α-Dab2 antibody. The in vitro translation efficiencies of RNA isolated from NMuMG cells treated with TGFβ for different times were identical (Fig. 2.4). These results indicate that the low levels of Dab2 protein expression in control cells, and early following TGFβ treatment, are not due to defective Dab2 mRNA, but perhaps due to an inhibition of Dab2 translation.

Transcript-specific translational silencing has been shown to involve translocation of regulated transcripts between non-translating non-polysomal pools containing cytoplasmic messenger ribonucleoprotein particles and translationally active polysomal complex of translating mRNA with polyribosome complexes (Aziz and Munro, 1987; Rouault et al., 1988; Mazumder and Fox, 1999). We examined whether TGFβ stimulation of NMuMG cells resulted in a translocation of Dab2 mRNA from the non-translating non-polysomal pool to the actively translating polysomal pool. Cell homogenates from control and 24 hr TGFβ-treated NMuMG cells were separated into non-polysome and polysome fractions by successive centrifugation through a 10-50% sucrose gradient and fractionation by a UV fractionater with the lighter mRNP fraction eluting first and the heavier polysomal fractions eluting last (Figure 2.5A, B; elution profiles). RNA
was isolated from the fractions and subjected to RT-PCR analyses. In control cells, where Dab2 protein is not expressed, Dab2 mRNA was absent from the polysomal fractions (Fig. 2.5A). However, after a 24 hr TGFβ treatment, when Dab2 protein expression is high, abundant Dab2 mRNA translocated to the actively translating polysomes (Fig. 2.5B). RT-PCR with β-actin specific primers showed continuous association of the mRNA with the polysomes irrespective of TGFβ treatment (Fig. 2.5A, B). This indicates that the translational repression of Dab2 mRNA observed in control cells is a transcript specific effect and not due to a global inhibition of translation. Control experiments demonstrated that treatment of cell homogenates with EDTA (10 mM) prior to fractionation completely disrupted polysomal complex formation and resulted in the translocation or release of the Dab2 mRNA, isolated from TGFβ-treated cells, into the mRNP fractions (Fig. 2.6A, B, C). The polysome release experiment validates the authenticity of the polysome analyses and rules out the observed polysome association of Dab2 mRNA to non-specific interaction or heavy molecular weight aggregates. Taken together, these data suggest that Dab2 is translationally regulated in a TGFβ-dependent fashion.

2.4.3. Identification of a novel structural element in the 3’-UTR of Dab2 that mediates translational regulation

We postulated that the conserved first 575 nt of Dab2 3’-UTR harbors a cis regulatory element which regulates its expression. UV-crosslinking analysis using this region as a probe revealed two proteins, which showed TGFβ-dependent loss of binding (Fig. 2.7). Fine mapping subsequently defined a 33-nt
region as the *cis* element (Fig. 2.8A, B). We named this region ‘BAT’ for TGFβ-activated translational element and this region carries a stem loop structure with an asymmetric bulge. A U10A mutant was predicted to destroy this secondary structure using ‘Mfold’ analysis (Fig. 2.8B) (Zuker, 2003). A ‘PatSearch’ algorithm driven search of a non-redundant 3'-UTR database for similar structures reconfirmed the Dab2 3'-UTR to harbor the BAT element (UTRdb ID: 3MMU027375), and additionally identified the 3'-UTR of ILEI (UTRdb ID: 3MMU039724) (Fig. 2.8B) (Grillo et al., 2003). Examination of the temporal relationship between ILEI mRNA and protein expression levels showed a pattern similar to Dab2 (Fig. 2.1B, 2.2B, 2.9A, B) and polysome profiling reaffirmed that TGFβ translationally upregulates ILEI (Fig. 2.10). UV-crosslinking analysis and decoy experiments using *Dab2*/BAT, its U10A mutant and *ILEI*/BAT showed that the binding of the 50 and 40 kDa proteins were TGFβ-dependent (Fig. 2.11A) and confirmed the specificity of the element (Fig. 2.11B).

**2.4.4. The 33nt element confers translational silencing activity to a heterologous transcript in vitro and in vivo**

Control cytosolic extracts inhibited the translation of a chimeric luciferase construct carrying wild-type BAT (Luc-Dab2/BAT) (Fig. 2.12A) but not that of the construct carrying the U10A mutant (Luc-Dab2/BAT-M) in a dose-dependent fashion, suggesting that proteins in these extracts bind the identified BAT element and functionally repress translation (Fig. 2.12B). *In vitro* translation repression of Luc-Dab2/BAT and Luc-ILEI/BAT was relieved after 3 hr of TGFβ stimulation, (Fig. 2.13A). Decoy experiments further confirmed the BAT-specific
translational repression of the chimeric luciferase cRNA by control cytosolic extracts (Fig. 2.13B). Similarly, in vivo translation was found to be repressed in control cells using the WT BAT chimera (Luc-BAT), but not the mutant chimera (Luc-BAT-M), or the luciferase construct with no 3’-UTR (Luc-alone) (Fig. 2.14). TGFβ relieved translation repression as early as 3 hr and by greater than 80% at 24 hr. These results established BAT as a novel, structural element sufficient to mediate translational inhibition in vitro and in vivo.

2.4.5. Identification of hnRNP E1 as a protein binding to the Dab2 33nt structural element

Size-exclusion chromatography of control extracts was used to isolate the BAT binding mRNP complex responsible for translational inhibition. Fractions #36-38 showed maximum translation silencing activity (Fig. 2.15A, B). The fractions with maximum translation silencing activity were affinity purified by precipitation with Dab2/BAT cRNA and visualized by silver staining (Fig. 2.16). The lower band (Fig. 2.16, arrowhead), present in both the active chromatographic fractions and control cytosolic extracts, was identified as heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) through mass spectrometric analysis. Immunoblot analysis confirmed the presence of hnRNP E1 in the fractions with maximal translation silencing activity (Fig. 2.17A). TGFβ induced the loss of binding of hnRNP E1 to both Dab2 and ILEI BAT element after 3 hr of treatment (Fig. 2.17B), and the kinetics of hnRNP E1 release from the BAT element correlated with the kinetics of de-repression of translational
silencing *in vitro* (Fig. 2.13A) and Dab2 and ILEI protein induction by TGFβ (Fig. 2.2A, 2.9B).

hnRNP E1, together with heterogeneous nuclear ribonucleoprotein K (hnRNP K), bind to poly r(C) regions, called differentiation control elements (DICE) in 3'-UTR of 15-lipoxygenase and L2 mRNAs and mediate their translational regulation (Ostareck et al., 1997). However, in pull down experiments, despite both hnRNP E1 and hnRNP K being present in inputs, only hnRNP E1 from control extracts bound the WT BAT cRNA, whereas hnRNP E1 in extracts from TGFβ-treated cells did not bind. The BAT-M did not pull down either hnRNP E1 or hnRNP K, whereas a DICE cRNA pulled down both proteins in a TGFβ-independent fashion (Fig. 2.17C). Immunodepletion of hnRNP E1 from control cytosolic extracts caused loss of translational silencing activity as assayed by *in vitro* translation of Luc-Dab2/BAT (Fig. 2.18A, B).

2.4.6. **hnRNP E1 interacts with the 33nt element *in vitro and in vivo***

*In vitro* binding assays showed that GST-hnRNP E1 could be precipitated in a dose-dependent manner by both Dab2 and ILEI BAT elements, but not by the mutant (Fig. 2.19). *In vivo* interaction studies revealed that although Dab2 and ILEI mRNAs were steadily expressed, hnRNP E1 interacted with them only in control cells (Fig. 2.20A, B, C). Hence, hnRNP E1 is a functional component of the mRNP complex, binding to the BAT element in a TGFβ-dependent manner, which correlates with the kinetics of translational activation of ILEI and Dab2.
**Figure 2.1:** *Dab2* and *ILEI* transcription is not significantly induced in NMuMG and EpRas cells post TGFβ treatment.

(A) Northern blot analysis examining *Dab2* expression levels in NMuMG cells treated with TGFβ for the durations indicated. The lower panel shows a quantification of band intensities analyzed by NIH Image J software. The *Dab2* band intensity was normalized to that of *cyclophilin* (1B15), then normalized to the t=0 unstimulated value.

(B) Semi-quantitative RT-PCR examining *Dab2*, *ILEI*, and *β-actin* expression levels in EpRas cells treated with TGFβ for the durations indicated.
Figure 2.2: Dab2 and ILEI protein expression levels are significantly induced post TGFβ treatment in NMuMG and EpRas cells.

(A) Immunoblot analyses examining Dab2 protein levels in NMuMG cells treated with TGFβ for the indicated durations. The lower panel shows a quantification of band intensities analyzed by NIH Image J software. Dab2 band intensity was normalized to Hsp90, then normalized to the t=0 unstimulated value.

(B) Immunoblot analyses examining Dab2 and ILEI protein levels in EpRas cells treated with TGFβ for the indicated durations.

(A) Immunoblot analyses examining Dab2 protein levels in NMuMG cells treated with TGFβ for the indicated durations. The lower panel shows a quantification of band intensities analyzed by NIH Image J software. Dab2 band intensity was normalized to Hsp90, then normalized to the t=0 unstimulated value.

(B) Immunoblot analyses examining Dab2 and ILEI protein levels in EpRas cells treated with TGFβ for the indicated durations.
Figure 2.3: TGFβ induces the *de novo* synthesis of Dab2.

The *de novo* rate of Dab2 synthesis post-TGFβ stimulation was measured by metabolic labeling. NMuMG cells were treated with TGFβ (5 ng/ml) for the times indicated and metabolically labeled with [35S]-methionine in methionine-free medium for the last 3 hr of incubation. WCLs were immunoprecipitated (IP) with α-Dab2 antibody, resolved by SDS-PAGE and detected by autoradiography.

Figure 2.4: Dab2 mRNA is stable even under control conditions.

Dab2 mRNA stability analysis by *in vitro* translation (IVT) of total RNA isolated from NMuMG cells treated with TGFβ for the times indicated followed by immunoprecipitation (IP) with α-Dab2 antibody and mouse IgG.
Figure 2.5: TGFβ translationally upregulates Dab2 expression.

(A) & (B) Polysome profiling in unstimulated and TGFβ treated NMuMG cells. Translocation of Dab2 mRNA from the non-polysomal to polysomal pool was analyzed by semi-quantitative RT-PCR of RNA isolated from each fraction following polysome profiling.
Figure 2.6: Polysome release experiment confirmed authentic polysome isolation. (A) NMuMG cells were stimulated with TGFβ for 0 and 24 hr and treated with cycloheximide (100 µg/ml) for the last 15 min of the incubation time. Cells were homogenized in either buffer containing cycloheximide (100 µg/ml) or EDTA (10 nM) and centrifuged at low speed. The post-mitochondrial supernatants were layered on a 10-15% (w/v) sucrose gradient and centrifuged and the non-polysomal and polysomal fractions were isolated through a fractionater. The panels show representatives UV absorbance peaks during polysome fractionation with and without EDTA. (B) & (C) RNA associated with each fraction was isolated and equal amounts of RNA were subjected to RT-PCR with primers specific for Dab2 (B), and β-actin (C).
Figure 2.7: UV crosslinking analysis reveals TGFβ-dependent loss of binding of proteins to 3'-UTR of Dab2 mRNA.

UV crosslinking (X-link) analysis to characterize regulatory element(s) in the 3'-UTR of Dab2 mRNA using [α-32P]-labeled Dab2 3'-UTR 575-nt probe (10 fmol) and S100 cytosolic extract from NMuMG cells treated with TGFβ for the times indicated.
Figure 2.8: Translational silencing is mediated by a novel structural element.

(A) Schematic representation of deletion mapping used to identify the minimal binding BAT element. The binding activity of the different transcripts and the sequence of the predicted Dab2/BAT element have been summarized.

(B) Secondary structure of the mouse $Dab2$/BAT ($\Delta G = -5.0$ Kcal/mol) and $ILEI$/BAT ($\Delta G = -2.5$ Kcal/mol) elements as predicted by the Mfold algorithm. Substituted nucleotide (U10A) represents a mutant form. $ILEI$/BAT element was folded under $(F\ 5\ 0\ 2)/ (F\ 9\ 0\ 2)/ (P\ 11\ 0\ 2)$ constraints.
Figure 2.9: Uncoupled transcription and translation of ILEI in NMuMG.
(A) Semi-quantitative RT-PCR and (B) immunoblot analyses examining mRNA and protein expression levels of ILEI in NMuMG cells treated with TGFβ.

Figure 2.10: TGFβ translationally upregulates ILEI expression.
Translocation of ILEI mRNA from the non-polysomal to polysomal pool in control and TGFβ-stimulated NMuMG cells was analyzed by semi-quantitative RT-PCR of RNA isolated from each fraction following polysome profiling.
Figure 2.11: The 33 nt BAT element binds 2 proteins in a TGFβ-dependent fashion and the binding is highly specific to the BAT element.

(A) X-link analysis was performed with [α-32P]-labeled Dab2/BAT probe (10 fmol) and S100 cytosolic extract from NMuMG cells treated with TGFβ. The arrows indicate the positions of two proteins that fail to bind the probe following TGFβ treatment.

(B) Specificity of the BAT element was examined by decoy X-link using [α-32P]-labeled Dab2/BAT probe and a 2- or 10-fold molar excess (2X or 10X) of unlabeled Dab2/BAT, ILEI/BAT, and mutant (U10A) Dab2/BAT-M cRNA.
Figure 2.12: The translational silencing capacity of the 3’-UTR element can be conferred to a heterologous transcript in vitro.

(A) Schematic representation of the chimeric luciferase construct used for the in vitro translation assays and the overall procedure adapted for the experiments.

(B) Proteins in the cytosolic extract can functionally repress translation. In vitro translation was performed using wild-type (top panel) and mutant chimeric luciferase constructs (bottom panel) (Luc-Dab2/BAT and Luc-Dab2/BAT-M) in the presence of increasing amounts of unstimulated cytosolic extracts (50 ng to 10 µg) to assess in vitro functional translation silencing activity. Capped, Xenopus elongation factor-1 (X. EF-1) cRNA was added to each reaction as specificity and loading control.
Figure 2.13: Translational silencing conferred by the Dab2 and ILEI 3′-UTR elements is relieved by TGFβ in vitro.

(A) IVT analyses with chimeric Luc-Dab2/BAT, Luc-ILEI/BAT and Luc-Dab2/BAT-M shows that TGFβ treatment relieves translational silencing conferred by the WT and not the mutant BAT element following 3 hr of TGFβ treatment.

(B) Functional specificity of the BAT element was examined by loss of translational inhibition activity conferred by TGFβ-untreated cytosolic extract in a decoy IVT assay using 1-10 fold (1X-10X) molar excess of BAT cRNA as cold competitors.
Figure 2.14: Translational silencing conferred by the Dab2 and ILEI 3'-UTR elements is relieved by TGFβ in vivo.

Dual-luciferase assay examining the in vivo translational silencing activity conferred by the BAT element by co-transfecting with wild-type, mutant (Luc-BAT, Luc-BAT-M) or luciferase alone (Luc-alone) and CMV-driven renilla luciferase constructs. The firefly luciferase values were normalized to renilla luciferase values (which were checked for uniformity to monitor equal transfection efficiency). Results are shown as means ± s.d. for three independent sets of experiments (n=3), each experiment done in triplicates.
Figure 2.15: Isolation of the mRNP complex that binds the BAT element.

(A) Purification of mRNP complex (BAT complex) binding to the BAT element by size exclusion chromatography of unstimulated S100 cytosolic extract (5 mg). Protein content of chromatographic fractions was quantitated at 280 nm (■) and compared to protein standards (top line); translation inhibition was quantitated by NIH ImageJ software and compared to inhibitory capacity of unfractionated, unstimulated S100 extract (▲). The open and filled bars on the right hand side are quantitative representation of translational inhibitory activity of unstimulated and TGFβ-treated extracts seen in Fig. 3a (last 2 lanes on the right side).

(B) IVT assay for translation inhibitory activity of chimeric Dab2/BAT-Luc cRNA using size exclusion chromatographic fractions.
Figure 2.16: hnRNP E1 is an integral functional component of the mRNP complex.

Chromatographic fractions (# 36-38) harboring translational silencing activity were subjected to pull-down with Dab2/BAT cRNA bound to cyanogens bromide (CNBr)-activated sepharose beads after pre-clearing with U10A Dab2/BAT-M cRNA. Precipitated mRNP complex was visualized by silver staining (left panel) and the band (arrowhead) which migrated similarly to the band that does not bind the BAT element after TGFβ treatment (shown by arrowhead in right panel) was analyzed by LC-MS.
Figure 2.17: RNA affinity chromatography identified hnRNP E1 as one of the proteins binding the BAT element.

(A) IB analysis of chromatographic fractions with α-hnRNP E1 antibody exclusively detected hnRNP E1 in fractions harboring translational silencing activity.

(B) RNA affinity pull-down and IB analyses using S100 cytosolic extracts for the times indicated to define the temporal association of hnRNP E1 with the Dab2 and ILEI BAT element.
Figure 2.18: hnRNP E1 is required for translational silencing.

(A) *In vitro* translation with specific immuno-depleted cytosolic extracts confirmed that hnRNP E1 is a functionally important trigger responsible for the observed translational silencing. Control cytosolic extracts (S100 fractions) (100 to 400 µg) were immuno-depleted with 5 µg of α-hnRNP E1 antibody or 5 µg of mouse pre-immune serum. Immuno-depleted extracts were assayed for translational silencing activity *in vitro* using the chimeric Luc reporter cRNA.

(B) Confirmation of the immuno-depleted status of the cytosolic extracts. Pellets from the immunodepletion were resolved by SDS-PAGE and immunoblotted with α-hnRNP E1 antibody. Immunodepleted extracts were also probed with α-hnRNP E1 antibody to confirm their relative immunodepletion of hnRNP E1.
Figure 2.19: hnRNP E1 interacts with the BAT element \textit{in vitro}.

IB analyses of GST-hnRNP E1 or GST proteins precipitated \textit{in vitro} by Dab2/BAT cRNA (\textit{top panel}) and ILEI/BAT cRNA (\textit{bottom panel}).
Figure 2.20: hnRNP E1 interacts with the BAT element in vivo.

hnRNP E1 interacts with the BAT element in vivo. Immunoprecipitation with α-hnRNP E1 (A) or mouse IgG (B) of cytosolic extracts from NMuMG cells treated with TGFβ for the times indicated followed by semi-quantitative RT-PCR (using Dab2, ILEI, and β-actin specific primers) analyses of RNA isolated from the immunoprecipitates to examine in vivo association of hnRNP E1 with the BAT element. RNA isolated from input extracts were also analyzed by semi-quantitative RT-PCR (C).
2.5. Discussion

TGFβ-mediated EMT is an important prerequisite for metastatic progression (Derynck et al., 2001; Zavadil and Bottinger, 2005; Thiery and Sleeman, 2006). But the precise mechanism(s) regulating TGFβ-mediated EMT remain(s) elusive. We now report that TGFβ stimulates translational upregulation of Dab2 and ILEI expression. A novel 33nt cis-element (TGFβ activated translation element or BAT element) was identified to be regulating translational silencing of Dab2 and ILEI. We have purified and identified hnRNP E1 as a component of the mRNP complex binding to the BAT elements in the 3′-UTRs of Dab2 and ILEI mRNAs. Prolonged stimulation of TGFβ causes release of hnRNP E1 from the BAT element allowing their translation. Although TGFβ is known to cause global translational upregulation by activation of the mTOR pathway (Lamouille and Derynck, 2007), to our knowledge, this is the first demonstration of transcript-specific translational activation of transcripts required for EMT by TGFβ.

Translational Regulation by UTRs Represent a Novel Paradigm for Regulation of Gene Expression. Regulation of gene expression at the posttranscriptional level seems to render a more well-defined and rigorous regulatory checkpoint for cellular processes that governs the development and homeostasis of cells and tissues (Ruvinsky and Meyuhas, 2006). Concurrently, deregulation of this regulatory machinery has been implicated in alterations of cell cycle progression and cell growth associated with stress, inflammation and carcinogenesis (Standart and Jackson, 1994; Mazumder and Fox, 1999;
Ruggero and Pandolfi, 2003). Regulatory elements located within the 5’ or 3’ UTRs have been implicated for the translational regulation of different mRNAs. For example, 5’-UTR sequences control amino acid metabolism in *Saccharomyces cerevisae* (Dever et al., 1992; Hinnebusch, 1994). Similarly, binding of iron regulatory protein 1 to the ferritin 5’-UTR prevents translational initiation by inhibiting recruitment of the 40S ribosomal subunit (Muckenthaler et al., 1998). Interestingly, the 3’-UTRs has assumed more importance as mediators of transcript-specific translational regulation. In fact, it is now believed that 3’-UTRs more often contains regulatory sequences than 5’-UTRs (Ostareck et al., 1997). 3’-UTR mediated translational regulation seems to be important in developmental regulation in *C. elegans, Drosophila, Xenopus*, and mammals or in disease conditions like inflammation (Huarte et al., 1992; Jackson, 1993; Wickens et al., 1993, 1996; Evans et al., 1994; Curtis et al., 1995; Mazumder and Fox, 1999). In addition to this, translational regulation is mediated by cooperative regulation of regulatory elements in the 5’ and 3’-UTR as in the case of *Drosophila* male sex lethal-2 (MSL-2) (Beckmann et al., 2005). We have identified a novel 33nt structural element in the 3’-UTR of two bonafide EMT inducer transcripts. Structurally, the element forms a 12-nt terminal loop separated from an asymmetric bulge by a short 3-nt stem and a proximal 7-nt double-helical stem supports the entire structure. The 33 nt element is sufficient to mediate translational inhibition of a heterologous chimeric reporter transcript both *in vitro* and *in vivo*.  

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We have identified hnRNP E1 as a critical component of the mRNP complex that binds the 33nt element in the 3’-UTR of Dab2 and ILEI mRNA and uncouples it from the polysomes in control cells thereby repressing their translation. hnRNP E1 (also called poly(rC)-binding protein or α-CP1) has been implicated in the translational regulation and mRNA stability of many transcripts, including gastrin (Lee et al., 2007), A2 response element (Kosturko et al., 2006), collagen I, III (Thiele et al., 2004), renin (Morris et al., 2004), folate receptors (Antony et al., 2004), and 15-lipoxygenase (Ostareck et al., 1997). Recent studies have revealed that a distinct subset of hnRNP E1 shuttle between the nucleus and the cytoplasm, suggesting unexpected functions of these protein in nucleo-cytoplasmic transport and/or in the cytoplasm (Pinol-Roma and Dreyfuss, 1993; Meng et al., 2007). hnRNP E1 preferentially binds to poly(C) regions of mRNA (Aasheim et al., 1994). As is evident from the sequence of the 33nt element, it is not poly(C) rich. hnRNP E1 has also been shown to bind poly(U) stretch (Leffers, 1995), albeit with less affinity. Decoy experiments with a putative DICE element (containing 2 subunits of the 19 nt DICE element) showed that it could not compete the binding affinity shown by the 33nt Dab2 element in a UV crosslinking assay (data not shown). Also, RNA pull down with the 33nt and DICE synthetic RNAs showed that while the former showed a TGFβ-dependent temporal association with hnRNP E1, the later associated with hnRNPs E1 and K in a TGFβ-independent fashion. Hence, it can be rationalized that while the hnRNP E1 show relatively broad specificity for CU-rich sequences in biochemical
binding assays (Matunis et al., 1992; Kiledjian et al., 1995), they are exquisitely discriminatory in their functional specificity (Ostareck et al., 1997).
CHAPTER III

TGFβ-MEDIATED PHOSPHORYLATION OF hnRNP E1 IS CRITICAL TO TRANSLATIONAL ACTIVATION OF DAB2 AND ILEI AND INDUCTION OF EMT

3.1. Abstract

We have earlier shown that TGFβ induces epithelial-mesenchymal transdifferentiation (EMT) accompanied by cellular differentiation and migration through transcript-selective translational activation of Dab2 and ILEI. Heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) binds a structural, 33 nucleotides (nt) TGF \_beta-activated translation (BAT) element in the 3'-UTR of disabled-2 (Dab2) and interleukin-like EMT inducer (ILEI) transcripts, and repress
their translation. We now show that TGFβ activation leads to phosphorylation at Ser43 of hnRNP E1 by protein kinase Bβ/Akt2, inducing its release from the BAT element and translational activation of Dab2 and ILE1 mRNAs. Modulation of hnRNP E1 expression or its post-translational modification alters TGFβ-mediated translational activation of the target transcripts and EMT in vitro and in vivo. These results suggest the existence of a TGFβ-inducible post-transcriptional regulon that regulates EMT during development and metastatic progression of tumors.
3.2. Introduction

TGFβ-mediated epithelial-mesenchymal transition (EMT), in which cells undergo a switch from a polarized, epithelial phenotype to a highly motile fibroblastic or mesenchymal phenotype is fundamental during embryonic development and can be reactivated in a variety of diseases including fibrosis and cancer (Derynck et al., 2001; Zavadil and Bottinger, 2005; Bierie and Moses, 2006; Thiery and Sleeman, 2006; Massague, 2008). TGFβ is one of the growth factors implicated in EMT (Massague, 2008). Disabled-2 (Dab2) (Prunier and Howe, 2005) and FAM3C or interleukin like EMT inducer (ILEI) (Waerner et al., 2006) were identified as two candidate genes required for TGFβ-induced EMT. We have earlier shown in Chapter II that heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) binds a structural, 33 nucleotides (nt) TGF β-activated translation (BAT) element in the 3′-UTR of disabled-2 (Dab2) and interleukin-like EMT inducer (ILEI) transcripts, and repress their translation. Prolonged TGFβ stimulation caused a release of hnRNP E1 from the BAT elements and concurrent translational activation of Dab2 and ILEI. We next wanted to investigate the effect of TGFβ stimulation on hnRNP E1 and mechanism by which TGFβ was promoting translational activation of Dab2 and ILEI.

Here, we report identification of the single site of hnRNP E1 phosphorylation responsible for its release from the mRNP complex and concurrent activation of Dab2 and ILEI translation in TGFβ-treated NMuMG and EpRas cells. Additionally, we show that TGFβ activates a cascade in which
protein kinase Bβ/Akt2 is activated and subsequently phosphorylates hnRNP E1 at Ser43 residue, culminating in its release from the transcripts and concurrent translational activation of the two EMT inducers. Remarkably, ectopic overexpression of hnRNP E1 in NMuMG cells results in total inhibition of TGFβ-mediated EMT, whereas shRNA-mediated silencing of hnRNP E1 renders mesenchymal properties to NMuMG cells irrespective of TGFβ treatment. Re-introduction of wild type, but not a Ser43 to Ala mutant of human hnRNP E1 can rescue the epithelial phenotype of the shRNA-silenced hnRNP E1 cells. We also report that modulation of hnRNP E1 expression levels or its phosphorylation status modulates migration and invasive potential in in vitro and in vivo xenograft assays. The autocrine response of cells to TGFβ-induced Akt2 activation and subsequent translational activation of EMT inducer transcripts may represent a novel mechanism through which the increased TGFβ expression in tumor cells contributes to cancer progression.
3.3. Materials and Methods

3.3.1. Reagents.

Rabbit reticulocyte lysate, methionine free amino acids, RNasin and Ribonuclease Large Scale RNA Production kit were purchased from Promega. MAXIscript, mMESSAGE mMACHINE T7 Ultra kits, siPORT and pSilencer™ neo vector were purchased from Ambion. Primers and oligos were purchased from Integrated DNA Technologies. Translation grade [35S]-methionine and [γ-32P]-ATP were purchased from Perkin Elmer Life Sciences. LY294002 was obtained from Alexis-Biochemicals, SB431542 from Sigma and Akt IV inhibitor from EMD Biosciences. Mouse α-hnRNP E1 was from Novus Biologicals, rabbit α-hnRNP E1 was from Lifespan Biosciences. Antibodies against phospho-Akt (Ser-473), total Akt, Akt1, Akt2, Akt3, phospho-Akt substrate (RXRSS/T) (110B7E), PAK1, vimentin and recombinant GSK-3 fusion protein and recombinant Akt1 and Akt2 kinase were purchased from Cell Signaling Technology. α-FAM3C and N-cadherin antibodies were obtained from Abcam, α-phospho-serine (clone PSR-45) antibody from Sigma-Aldrich, and α-ZO-1 from Zymed Laboratories. α-Hsp90 (H-114), α-p-Threonine (H-2) and normal mouse and rabbit IgG were purchased from Santa Cruz Biotechnology. Secondary antibodies Alexa Flour 568 phalloidin, Alexa Flour 468 and Oregon Green 468 were purchased from Molecular Probes, Invitrogen. Vectashield mounting medium with DAPI was purchased from Vector Laboratories.
3.3.2. Cell culture and treatments.

TGFβ2 was a generous gift from Genzyme Inc. and was used at a final concentration of 5 ng/ml. NMuMG cells were cultured as described previously (Prunier and Howe, 2005). EpRas were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics/antimycotics. For stimulation with insulin, the cells were maintained in a serum-free media for 6 hours and were then stimulated with 10 nM insulin. Where indicated, cells were treated with DMSO (vehicle, control) or 10 µM of SB431542, LY294002 or Akt IV 30 min before TGFβ treatment.

3.3.3. Plasmids construction and protein expression.

For construction of the Luc-Dab2/BAT, Luc-Dab2/BAT-M mutant and Luc-ILEI/BAT, luciferase cDNA derived from pGL3-b vector was cloned into pcDNA3. A linker region containing 5’-EcoR1-Pac1-EcoRV-Nco-1-Xho-1-Xba-1 was inserted into the vector downstream of the luciferase gene (pCMV-LL). Synthetic Dab2/BAT, Dab2/BAT-M and ILEI/BAT were generated with 5’-EcoR1 and 3’-Xba-1 sites (pCMV_{Luc-Dab2/BAT}, pCMV_{Luc-Dab2/BAT-M}, pCMV_{Luc-ILEI/BAT}).

The GST-hnRNP E1 construct was a kind gift from Dr. R. Kumar and has been previously described (Meng et al., 2007). The GST clones were maintained in E. coli BL21 (DE3)pLysS for expression. For expression, the protocol as described before was followed (Meng et al., 2007). The mouse pCMV14-hnRNP E1-FLAG and psiRNA-hH1neo-mouse hnRNP E1 (shRNA against hnRNP E1 ORF) were kind gifts from Dr. T. Kobayashi and has been previously described
(Nishinakamura et al., 2007). pSilencer neo-shRNA-mouse hnRNP E1-3’-UTR (shRNA against 3’UTR of hnRNP E1) was constructed by annealing shRNA template oligonucleotides (target selected through engine at Ambion and cloned into pSilencer neo vector (refer to Table I for oligonucleotide sequences). The S43A mutation was introduced into GST-hnRNP E1 and pCMV14-hnRNP E1-FLAG using Quick Change Site Directed Mutagenesis Kit, Stratagene (refer to Table I for primer sequence). pSUPER-Dab2si construct has been generated in the lab and described previously (Prunier and Howe, 2005). The ILEI siRNA (m), a pool of 3 target-specific 19-25 nt. siRNAs, and control siRNA-A, a non-targeting 20-25 nt. siRNA, were brought from Santa Cruz Biotechnology Inc.

3.3.4. Preparation of cell lysates, immunoblot analysis, immunoprecipitation and immunodepletion.

For immunoprecipitation and immunoblot analysis, cells were lysed in buffer D and immunoprecipitation carried out as previously described (Hocevar et al., 1999).

3.3.5. Preparation of cytosolic extract (S100 Fraction).

S100 fractions were prepared from control and TGFβ-treated NMuMG cells as previously described (Mazumder and Fox, 1999) with minor modifications. Briefly, the buffer used for cytosolic extraction contained 20 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail (Roche) (Hampton et al., 1998).
3.3.6. **In vitro** luciferase assay.

*In vitro* luciferase assay was performed as previously described (Mazumder and Fox, 1999).

3.3.7. **In vitro** kinase assays.

*In vitro* kinase assays were performed using both recombinant Akt kinase and kinases immunoprecipitated from whole cell lysates as described previously (Qi et al., 2003).

3.3.8. **Immunofluorescence.**

Protocol followed was previously described (Lamouille and Derynck, 2007) with minor modifications. The primary antibodies used were: anti-E-cadherin (diluted 1:400), anti-ZO-1 (diluted 1:200). The secondary antibodies used were Alexa Flour 468 (diluted 1:1500) or Oregon Green 468 (diluted 1:1500). The slides were then incubated in Alexa Fluor 568 Phalloidin (diluted 1:1000) for 20 min at room temperature to visualize actin filaments. The slides were mounted in Vectashield with DAPI and visualized using a Leica DMIRB inverted microscope (Leica Microsystems) equipped with Retiga Exi Cooled CCD Camera (Q Imaging, Burnby). Excitation at 490 nm and standard fluorescent filter were used to take images.

3.3.9. **Cell proliferation assay.**

$10^5$ cells/well of a particular cell type were plated in triplicates in a 6-well tissue culture plate. Cells were trypsinized and resuspended in 1 ml of media, before being counted through a hemocytometer chamber upto 12 days following
initial seeding. The experiment was repeated thrice and the results are represented as means ± s.d.

3.3.10. Anchorage-independent growth assay (soft-agar colony formation assay).

Anchorage-independent growth assay was performed as described earlier (Pietenpol et al., 1989). Approximately $10^4$ cells were suspended in 2 ml of 0.4% soft-agar in DMEM containing 10% fetal bovine serum and were overlaid on 2 ml of 0.8% soft agar ± TGFβ in the same medium in 35-mm-diameter dishes. Each cell line was tested in triplicate wells. Colonies were visualized under an inverted light microscope after 3 weeks. Data are represented as means ± s.d. of single experiment done in triplicates.

3.3.11. Wound healing (migration) assay.

Wound healing assay was done as described previously (Lamouille and Derynck, 2007) with brief modifications. Cell monolayers were wounded with a plastic tip after 24 hr of seeding and images obtained using a phase-contrast microscope at x5 magnification. The cells were incubated in a humidified chamber with 5% carbon dioxide ± TGFβ for 24 hr at $37^\circ$C before being photographed again at x5 magnification.

3.3.12. Invasion assay.

Cell invasion assay was done using CytoSelect™ 96-well Cell Invasion Assay Kit (Cell Biolabs, San Diego, CA) as per the manufacturer’s protocol. The experiment was done with the addition of the $2 \times 10^5$ cells to the membrane chamber and in the absence and presence of serum and TGFβ in the feeder tray.
Cell invasion was assayed by using the provided cell lysis buffer and CyQuant® dye fluorimetrically at 480 nm/520 nm. The data is represented as means ± s.d. of three independent experiments.

3.3.13. Experimental tumorigenesis and metastasis assay.

Tumor formation and metastatic ability of parental NMuMG cells and the different NMuMG clones that were generated were determined by subcutaneous injection of $10^5$ cells on the hind flank (each side) into six weeks old BalbC athymic nude mice ($nu/nu$), according to approved protocols of Institutional Animal Care and Use Committee (IACUC), Cleveland Clinic. Tumor volume ($\text{mm}^3$) was determined by using the standard formula $a^2 \times b/2$, where ‘$a$’ is the width and ‘$b$’ is the length of the horizontal tumor perimeter, determined thrice a week with a vernier caliper. Twelve animals were used for each cell type but the data represented here is representative of three animals per group as the other animals have still not been harvested. The data is represented as mean ± s.e.m.

After determination of tumor weight and/or photography, excised tumors and lung, liver and colon tissues were fixed with paraformaldehyde (4%, 18 hr, 4°C) and post fixed (70% ethanol, 16 hr) before dehydration and paraffin embedding. Paraffin sections were stained with hematoxylin/eosin according to standard protocols.
3.4. Results

3.4.1. TGFβ driven phosphorylation of hnRNP E1 releases the translational silencing

We observed that pre-treatment with calf intestinal alkaline phosphatase (CIP) renders translational silencing activity to TGFβ-treated extracts, suggesting necessity of TGFβ-dependent phosphorylation for the release of translational inhibition (Fig. 3.1). We next evaluated TGFβ-mediated phosphorylation of hnRNP E1 as a possible mechanism for loss of translational silencing following TGFβ treatment. TGFβ induced phosphorylation of hnRNP E1 at serine residue(s), with phospho-hnRNP E1 detected as early as 30 min after TGFβ treatment and maximally at 3 and 6 hr (Fig. 3.2).

3.4.2. Akt2 phosphorylates hnRNP E1 in TGFβ-stimulated NMuMG cells

Sequence analysis revealed that mouse hnRNP E1 contains an Akt consensus phosphorylation site at Ser43 (Fig. 3.3). We therefore postulated that hnRNP E1 might be a substrate of Akt. As shown by others (Bakin et al., 2000; Kattla et al., 2008; Kato et al., 2009), TGFβ was found to activate Akt (Fig. 3.4); furthermore, using a substrate-directed phospho-specific antibody, Akt-mediated phosphorylation of hnRNP E1 was demonstrated to be TGFβ-dependent (Fig. 3.5A). Use of PI3K inhibitor, LY294002 showed robust inhibition of TGFβ-induced phospho-hnRNP E1 (Fig. 3.5B) and attenuated release of hnRNP E1 from the Dab2/BAT element following TGFβ treatment (Fig. 3.5 C). Selective inhibition of
either TGFβ signaling with the type I receptor inhibitor SB-431542 and of Akt kinase with Akt IV inhibited TGFβ-dependent hnRNP E1 phosphorylation and Akt activation, without affecting total Akt levels (Fig. 3.6), confirming the direct correlation between TGFβ signaling and hnRNP E1 phosphorylation. Importantly, inhibiting either TGFβ signaling (with SB-431542) or Akt (with LY294002 and Akt IV) also inhibited the reversal of translational silencing (Fig. 3.6, **bottom panel**), hence suggesting a direct relationship between hnRNP E1 phosphorylation and translational activation post-TGFβ stimulation.

Additionally, recombinant Akt phosphorylated GST-hnRNP E1, but not GST in an *in vitro* kinase assay (Fig. 3.7A) and *in vitro* Akt-phosphorylated GST-hnRNP E1 no longer bound the Dab2/BAT element (Fig. 3.7B). *In vivo* phosphorylation of hnRNP E1 by Akt was investigated by using immunoprecipitated Akt (pan Akt antibody) as the kinase source to phosphorylate hnRNP E1. TGFβ activated Akt was capable of phosphorylating a WT hnRNP E1 fusion protein but not a S43A mutant, confirming Ser43 as the Akt phosphorylation site (Fig. 3.8). Since p21-activated kinase 1 (PAK1) can phosphorylate hnRNP E1 on Thr60 and Thr127 (Meng et al., 2007), we examined the phosphorylating effects of PAK1 following TGFβ stimulation. PAK1 immunoprecipitates phosphorylated both WT and the S43A mutant of hnRNP E1 indicating that phosphorylation at Ser43 is specific to TGFβ signaling (Fig. 3.8).

We investigated if phosphorylation of hnRNP E1 by activated Akt was specific to TGFβ stimulation. Both insulin (previously shown to activate Akt (Datta
et al., 1999; Brazil et al., 2001) and TGFβ induced Akt activation in NMuMG cells, albeit with different kinetics (Fig. 3.9A, top and middle panels), but insulin-mediated Akt activation did not result in hnRNP E1 phosphorylation (Fig. 3.9A, bottom panel). Insulin stimulation also failed to induce either Dab2 or ILEI protein expression (Fig. 3.9B) or reverse in vitro translation silencing activity (Fig. 3.10). To determine whether different Akt isoforms (Kato et al., 2007) were activated by insulin and TGFβ, we immunoprecipitated lysates with the three Akt isoforms, Akt1, Akt2 and Akt3 and probed them with α-p-Akt (pSer473) antibody (Fig. 3.11). Insulin selectively activated Akt1 (Fig. 3.11A, top panel), whereas TGFβ activated Akt2 (Fig. 3.11A, third panel). Neither insulin nor TGFβ activated Akt3 (data not shown). Similar isoform specific Akt activation was observed in EpRas cells (Fig. 3.11B). Substrate specificity of Akt2 for hnRNP E1 was further demonstrated by in vitro kinase assay of GST-hnRNP E1 by using immunoprecipitated Akt2 or Akt1 from TGFβ-treated cells as the kinase source to phosphorylate hnRNP E1. TGFβ activated Akt2, and not Akt1, was only capable of phosphorylating the GST-hnRNPE1 protein in vitro (Fig. 3.12). The fact that immunoprecipitated Akt1 fails to phosphorylate hnRNP E1 suggests that it is not being activated by TGFβ since both Akt1 and Akt2 share the same phosphorylation target sequence and purified Akt1 and Akt2 can phosphorylate GST-hnRNP E1 in vitro (data not shown). Hence, phosphorylation of hnRNP E1 on Ser43 by TGFβ-activated Akt2 kinase disrupts its binding to the BAT element and causes loss of translation silencing.
3.4.3. Modulating hnRNP E1 levels in NMuMG Cells directly affects the translational regulatory mechanism and EMT

We investigated whether modulating hnRNP E1 levels altered TGFβ-mediated EMT. We stably overexpressed (E23) or silenced (E2KD) hnRNP E1 in NMuMG cells (Fig. 3.13), and compared effects on EMT. NMuMG cells underwent EMT after TGFβ treatment (24 hr), while the process was blocked or constitutively active in E23 and E2KD cells, respectively (Fig. 3.14). Expression of Dab2, ILEI and mesenchymal cell markers N-cadherin and vimentin were constitutively active in E2KD cells and completely blocked in E23 cells (Fig. 3.15). Parental NMuMG cells showed classical mesenchymal cells features following a 24 hr TGFβ treatment, including loss of E-cadherin expression, actin reorganization at cell junctions and re-localization of ZO-1 from tight junctions (Massague, 2008), whereas such changes were absent in E23 cells and visible in E2KD control cells, demonstrating that hnRNP E1 is an important component of the TGFβ-mediated translational regulation of Dab2 and ILEI, and EMT (Fig. 3.16).

To confirm that hnRNP E1, and specifically the Ser43 phosphorylation of hnRNP E1, was regulating TGFβ-mediated EMT, we knocked in either WT (KIWT6 cells) or a phospho-mutant (KIM2 cells) version of hnRNP E1 into stable hnRNP E1 knockdown cells, SH14 (shRNA directed against the 3'-UTR of hnRNP E1) (Fig. 3.17A). Stable knockdown of hnRNP E1 rendered mesenchymal phenotype to cells even in the absence of TGFβ, whereas knock-in of either the WT or S43A mutant hnRNP E1 rescued the epithelial phenotype.
TGFβ stimulation caused EMT in KIWT6, but blocked it in KIM2 cells (Fig. 3.17B). Correspondingly, TGFβ-induced hnRNP E1 phosphorylation in KIWT6 cells, but not in KIM2 cells (Fig. 3.18, top panel) further confirming Ser43 as the phosphorylation site. In both KIWT6 and KIM2 cells, TGFβ activated Akt like in parental cells (Fig. 3.18, third panel). Vimentin, N-cadherin, Dab2 and ILEI expression corroborated the morphological analysis (Fig. 3.19). Cytosolic extracts from these cells confirmed that Ser43 phosphorylation of hnRNP E1 also regulates translational silencing activity (Fig. 3.20A). In addition, RNA pull-down with Dab2/BAT cRNA showed that hnRNP E1 is not released from the BAT element following TGFβ treatment in KIM2 cells as in NMuMG and KIWT6 cells (Fig. 3.20B). These results confirm that TGFβ-activated Akt2 phosphorylates hnRNP E1 at Ser43, a prerequisite for its release from the BAT element and concurrent translational activation of Dab2 and ILEI mRNAs.

Since Dab2 and ILEI are required but not sufficient to induce EMT it is difficult to precisely define their function downstream of hnRNP E1. Overexpression of Dab2 or ILEI alone does not induce any morphological changes associated with EMT (Fig. 3.21 & data not shown) or up-regulate N-cadherin expression (Fig. 3.22) independent of TGFβ stimulation. We postulated that if Dab2 and ILEI are required for EMT, then silencing the expression level of either one will rescue epithelial cell properties in the SH14 cells. si-RNA mediated silencing of either Dab2 or ILEI attenuated induction of EMT as evident by loss of expression of mesenchymal cell markers, N-cadherin and vimentin (Fig. 3.23) and loss of morphological features associated with mesenchymal cells.
Cumulatively, these results clearly support our hypothesis that the role of hnRNP E1 on EMT is mediated through induction of Dab2 and ILEI and that they are critical mediators of EMT.

3.4.4. Modulating hnRNP E1 levels in NMuMG Cells or its derivative clones affect in vitro migration and invasive capacity

Based on our model, and as we observed with knock-down and reconstituted NMuMG cells, we predict that the S43A hnRNP E1 mutant will not be released from the BAT element of Dab2, ILEI, and other EMT inducer mRNAs, resulting in lower rates of proliferation, invasion, migration, and soft agar growth. We functionally analyzed the parental NMuMG and the derivative clones by measuring changes in the proliferative, tumorigenic, migration and invasive potential using a combination of in vitro assays including proliferation rates in monolayer, colony formation on soft agar, wound healing (migration) and invasion assays, respectively. Knock-down of hnRNP E1 (SH14 cells) showed an increase in the proliferation (Fig. 3.24), soft agar growth (Fig. 3.25), migration (Fig. 3.26) and invasion of the cells (Fig. 3.27). Reconstitution with WT hnRNP E1 (KIWT6 cells), did not result in any significant difference in these parameters compared to the parental NMuMG cells. Reconstitution with S43A mutant hnRNP E1 (KIM2 cells) resulted in attenuation in these indexes.

3.4.5. Modulating hnRNP E1 levels in NMuMG Cells or its derivative clones affect in vivo tumor formation and metastasis

Based on the in vitro findings, we performed preliminary xenograft tumor growth studies in which we sub cutaneously injected either parental NMuMG
cells, the E23 overexpressing, or the SH14 knock-down clones into the hind flank of Balbc athymic nude mice (12 mice/cell types). A representative animal from each group is depicted in Fig. 3.28, demonstrating that parental NMuMG (right) form small orthotopic tumors (36 days post injection), whereas E23 cells (middle) do not develop any detectable tumors. In contrast, animals injected with the hnRNP E1 knock-down SH14 cells (left) develop tumors with a mean volume of $153 \pm 46 \text{ mm}^3$. Fig. 3.29 presents graphically the data of tumor growth over time for the 3 different cell types. Interestingly, we observed that animals injected with the SH14 knock-down cells metastasized to lung (Fig. 3.30A) and liver (Fig. 3.30B). We did not observe any metastatic lesions in the colon (data not shown).
Figure 3.1: TGFβ-mediated phosphorylation is involved in loss of translational silencing.

IVT assay of Luc-Dab2/BAT cRNA with unstimulated and 24 hr TGFβ-treated cytosolic extracts in the presence and absence of phosphatase. Where indicated, the cytosolic extracts were pre-treated with calf intestinal phosphatase (CIP), CIP and sodium orthovanadate, or heat-denatured CIP before addition to the IVT assay.

Figure 3.2: TGFβ phosphorylates hnRNP E1.

IB analysis of immunoprecipitates derived from NMuMG WCLs with α-phospho-serine (p-ser) antibody (top panel) and α-hnRNP E1 antibody (bottom panel) to examine TGFβ-dependent hnRNP E1 phosphorylation.
Figure 3.3: Schematic representation of the Akt consensus phosphorylation site at Ser43 in the KH1 domain of hnRNP E1.

Figure 3.4: TGFβ activates Akt kinase.

NMuMG cells were treated with TGFβ for the times indicated and WCLs were prepared and analyzed by immunoblotting with α-phospho-Akt antibody (top panel). The blot was stripped and re-probed with α-Akt antibody (bottom panel).
Figure 3.5: Akt is the kinase that phosphorylates hnRNP E1 after TGFβ stimulation.

(A) IB analysis of α-hnRNP E1 immunoprecipitates derived from NMuMG WCLs were probed with the phospho-Akt substrate antibody that recognizes the RXRXXpS/pT motif.

(B) IB analysis of α-hnRNP E1 immunoprecipitates from LY294002 treated and untreated WCLs with α-phospho-serine (p-ser) antibody (top panel) and α-hnRNP E1 antibody (bottom panel) to confirm Akt as the kinase.

(C) RNA affinity pull-down and IB analysis of cytosolic extracts from unstimulated and LY294002-treated cells to examine temporal association of hnRNP E1 and the BAT element.

Figure 3.6: Akt is the kinase for hnRNP E1 and Akt-mediated phosphorylation of hnRNP E1 is required for reversal of translational silencing.

IB analysis of immunoprecipitates (with α-hnRNP E1) (top panel) and WCLs (b, middle panels) derived from NMuMG cells treated with SB-431542, LY294002, and Akt IV to further confirm Akt as the kinase and the TGFβ-dependency of the hnRNP E1 phosphorylation event. IVT assay of Luc-Dab2/BAT with cytosolic extracts to examine the relationship between hnRNP E1 phosphorylation and de-repression of translational inhibition (lower panel).
Figure 3.7: Phosphorylation of hnRNP E1 by TGFβ-mediated activation of Akt disrupts its binding to the BAT element.

(A) Akt phosphorylates hnRNP E1 \textit{in vitro}. Increasing amounts of recombinant Akt kinase was incubated with 5 µg of GST-hnRNP E1 or GST in the presence of [γ-^{32}P]-ATP. The kinase reaction products were resolved by SDS-PAGE, and phosphorylation was detected by autoradiography.

(B) Phosphorylated hnRNP E1 does not bind the BAT element. Increasing amounts of phosphorylated-GST-hnRNP E1 protein was subjected to pull-down with \textit{Dab2/BAT} cRNA. The precipitates and the supernatants post pull-down were analyzed by IB.
Figure 3.8: Phosphorylation of hnRNP E1 at serine-43 by TGFβ-mediated activation of Akt.

Akt phosphorylates hnRNP E1 at Ser43. Activated kinases were recovered by anti-p-Akt (pSer473) or PAK1 immunoprecipitation and incubated with 5 µg of GST-hnRNP E1 or serine-43-alanine (S43A) mutant GST-hnRNP E1 in the presence of [γ-32P]-ATP. The kinase reaction products were detected by autoradiography. Coomassie stain (bottom panel) reveals equal amount of GST-hnRNP E1 was present in each reaction.
Figure 3.9: TGFβ stimulation, and not insulin stimulation induces Dab2 and ILEI protein expression.

(A) IB analysis of WCLs derived from NMuMG cells post insulin and TGFβ stimulation to examine insulin and TGFβ-mediated Akt activation (top panel). IB analysis of immunoprecipitates derived from NMuMG WCLs with α-phospho-serine (p-ser) antibody (bottom panel) to examine insulin and TGFβ-dependent hnRNP E1 phosphorylation.

(B) IB analysis of WCLs derived from NMuMG cells post insulin and TGFβ stimulation to examine insulin and TGFβ-mediated induction of Dab2 and ILEI expression.
Figure 3.10: TGFβ stimulation, and not insulin stimulation induces loss of translational silencing.

IVT analysis of chimeric luciferase transcripts in the presence of cytosolic extracts made from insulin or TGFβ treated NMuMG cells for the indicated times.
Figure 3.11: TGFβ causes isoform specific activation of Akt2 in NMuMG and EpRas cells.

(A) IB analysis of Akt1 and Akt2 immunoprecipitates derived from NMuMG WCLs with α-phospho-Akt (pS473) antibody to examine insulin and TGFβ-dependent isoform specific Akt activation.

(B) Isoform specific Akt activation is not cell type specific. WCLs post-TGFβ and insulin stimulation was prepared from EpRas cells and immunoprecipitated with α-Akt1 or α-Akt2 and the blot was probed with α-p-Akt (pS473) antibody.
Figure 3.12: TGFβ activated Akt2 specifically phosphorylates hnRNP E1.

Activated Akt1 or Akt2 was recovered by anti-Akt1 or anti-Akt2 immunoprecipitation following TGFβ stimulation and incubated with 5 µg of GST-hnRNP E1 in the presence of [γ-32P]-ATP.
Figure 3.13: Confirmation of ectopic overexpression and sh-RNA-mediated silencing of hnRNP E1.

IB analysis of WCLs derived from wild-type (WT, NMuMG), stable FLAG-hnRNP E1 overexpressing (E23) and stable shRNA-mediated hnRNP E1 knockdown (E2KD; harboring shRNA directed against the ORF) NMuMG cells to confirm overexpression and knockdown of hnRNP E1, respectively. (Endo represents the endogenous hnRNP E1 band; FL represents the flagged-tagged hnRNP E1 band).
Figure 3.14: Modulation of hnRNP E1 expression alters sensitivity of NMuMG cells to TGFβ-induced EMT.

Phase contrast images of unstimulated and TGFβ-treated (24 hr) WT, E23 and E2KD cells examining morphological changes post TGFβ-stimulation. Images were taken at 10X magnification.
Figure 3.15: Modulation of hnRNP E1 expression alters expression of Dab2 and ILEI and induction of mesenchymal cell markers.

IB analysis monitoring Dab2, ILEI, N-cadherin, vimentin and β-actin protein levels in WT, E23 and E2KD cells treated with TGFβ for the times indicated.
Figure 3.16: hnRNP E1 expression levels regulate sensitivity of NMuMG cells to TGFβ-induced EMT.

Immunofluorescence of E-cadherin, ZO-1, F-actin in unstimulated and TGFβ-treated (24 hr) WT, E23 and E2KD cells. DAPI was used to stain the nuclei. Images were obtained at original magnification of 63X. Scale bars, 50 µm.
Figure 3.17: Modulation of hnRNP E1 phosphorylation changes sensitivity of NMuMG cells to TGFβ-induced EMT.

(A) IB analysis of WCLs derived from wild-type (WT), stable shRNA-mediated hnRNP E1 knockdown (SH14; harboring shRNA directed against the 3'-UTR), stable knock-in of wild-type FLAG-hnRNP E1 expressing (KIWT6) and stable knock-in of S43A mutant FLAG-hnRNP E1 expressing (KIM2) NMuMG cells to confirm knockdown and re-expression of hnRNP E1, respectively.

(B) Phase contrast images of unstimulated and TGFβ-treated (24 hr) SH14, KIM2 and KIWT6 cells examining morphological changes post TGFβ-stimulation. Images were taken at 10X magnification.
**Figure 3.18: In vivo validation of Ser43 as the hnRNP E1 phosphorylation site.**

WCLs derived from NMuMG, KIM2 and KIWT6 cells were immunoprecipitated with α-hnRNP E1 antibody and analyzed by IB with α-phospho serine antibody *(top panel)* and α-hnRNP E1 antibody *(second panel)*. TGFβ-dependent Akt activation analyzed by IB analysis of WCLs derived from NMuMG, KIWT6 and KIM2 cells treated with TGFβ for the times indicated *(third and bottom panel)*.
Figure 3.19: Modulation of phosphorylation of hnRNP E1 expression alters expression of Dab2 and ILEI and induction of mesenchymal cell markers.

IB analysis examining Dab2, ILEI, N-cadherin, vimentin and β-actin protein levels in cells treated with TGFβ for the times indicated.
Figure 3.20: Modulation of phosphorylation of hnRNP E1 expression alters reversal of translational silencing with TGFβ and temporal association of hnRNP E1 with the BAT element.

(A) IVT and (B) RNA pull-down assays with cytosolic extracts from SH14, KIWT6 and KIM2 cells treated with TGFβ for the times indicated to examine translational silencing of chimeric Luc-Dab2/BAT cRNA and temporal association of the modified hnRNP E1 with the Dab2/BAT cRNA, respectively.
Figure 3.21: Dab2 is required, but not sufficient for TGFβ-induced EMT.

Phase contrast images of unstimulated and TGFβ-treated (24 hr) NMuMG/Dab2 cells (NMuMG cells overexpressing Dab2) examining morphological changes post TGFβ-stimulation. Images were taken at 10X magnification.

Figure 3.22: Ectopic Dab2 overexpression in NMuMG cells does not induce expression of mesenchymal cell marker.

WCLs made from NMuMG/Dab2 cells were probed with α-mouse Dab2 and α-rabbit N-cadherin antibodies. One of the blots was stripped and re-blotted with α-rabbit Hsp90 antibody as a loading control.
Figure 3.23: Role of hnRNP E1 on EMT is mediated by Dab2 and ILEI.

IB analysis of WCLs derived from SH14 cells, un-transfected or transiently transfected with ILEI, Dab2 or control-A siRNA to confirm knockdown of Dab2 and ILEI, respectively (first and second panel, respectively). IB analysis examining N-cadherin, vimentin and Hsp90 protein levels in these cells (third, fourth and bottom panel, respectively).
Figure 3.24: shRNA-mediated silencing of hnRNP E1 results in increased proliferation of NMuMG cells.

$10^5$ cells/well were seeded in triplicates in 6-well tissue culture plates. Cells were trypsinized and resuspended in 1 ml of media, before being counted through a hemocytometer chamber upto 12 days following initial seeding. The experiment was repeated thrice and the results are represented as means ± s.d.
Figure 3.25: shRNA-mediated silencing of hnRNP E1 in NMuMG cells renders anchorage independent growth.

$10^4$ cells were suspended in 2 ml of 0.4% soft-agar in DMEM containing 10% fetal bovine serum and were overlaid on 2 ml of 0.8% soft agar ± TGFβ in the same medium in 35-mm-diameter dishes. Each cell line was tested in triplicate wells. Data are represented as means ± s.d. of single experiment done in triplicates.
Figure 3.26: Modulating hnRNP E1 levels in NMuMG Cells or its derivative clones affect *in vitro* migration capacity.

Cell monolayers were wounded with a plastic tip after 24 hr of seeding and images obtained using a phase-contrast microscope at x5 magnification (*Control, 0h*). The cells were incubated in a humidified chamber with 5% carbon dioxide ± TGFβ for 24 hr at 37 °C before being photographed again at x5 magnification (*Control, 24h, TGFβ, 24h*).
Figure 3.27: Modulating hnRNP E1 levels in NMuMG Cells or its derivative clones affect in vitro invasion capacity.

The experiment was done with the addition of the $2 \times 10^5$ cells to the membrane chamber and in the absence and presence of serum and TGFβ in the feeder tray. Cell invasion was assayed by using the provided cell lysis buffer and CyQuant® dye fluorimetrically at 480 nm/520 nm. The data is represented as means ± s.d. of three independent experiments.
Figure 3.28: Modulating hnRNP E1 levels in NMuMG Cells or its derivative clones affect in vivo tumor formation.

$10^5$ cells were sub cutaneously injected on the hind flank (each side) into six weeks old BalbC athymic nude mice ($nu/nu$), according to approved protocols of Institutional Animal Care and Use Committee (IACUC), Cleveland Clinic.
Figure 3.29: Tumors formed from SH14 cells showed steady tumor growth.

Tumor volume (mm$^3$) was determined by using the standard formula $a^2 \times b/2$, where ‘$a$’ is the width and ‘$b$’ is the length of the horizontal tumor perimeter, determined thrice a week with a vernier caliper. The data represented here is representative of three animals per group. The data is represented as mean ± s.e.m.
Figure 3.30: Tumors formed from SH14 cells showed metastatic progression to lung and liver tissues.

Excised tumors and lung, liver and colon tissues were fixed with paraformaldehyde (4%, 18 hr, 4°C) and post fixed (70% ethanol, 16 hr) before dehydration and paraffin embedding. Paraffin sections were stained with hematoxylin/eosin according to standard protocols. Images were obtained at 40X magnification.
3.5. Discussion

We have identified a transcript-selective translational regulation pathway by which TGFβ modulates expression of mRNAs required for EMT. TGFβ activates a kinase cascade terminating in phosphorylation of Ser43 of hnRNP E1, by isoform-specific stimulation of protein kinase Bβ/Akt2, inducing its release from the BAT element and loss of translational silencing of Dab2 and ILEI mRNAs. Modulation of hnRNP E1 expression, or of its Ser43 site, alters TGFβ-mediated loss of translational silencing and EMT. Additionally, modulation of hnRNP E1 levels significantly alters the *in vitro* and *in vivo* proliferation, tumorigenic and metastatic potential of the NMuMG cells.

Translational regulatory pathways are laced with examples of phosphorylation-dependent regulation. Global translational regulation is regulated by phosphorylation of eIF2α, eIF4B, eIF4E and eIF4G (Dever, 2002; Raught et al., 2004). On the other hand, *trans* factor-mediated, e.g., hnRNP K, EPRS, maskin, L13a, phosphorylation has been shown to be important in transcript-selective translational regulation (Ostareck-Lederer et al., 2002; Sampath et al., 2004; Barnard et al., 2005; Mukhopadhyay et al., 2008). hnRNP E1 has been shown to exist in both phosphorylated and unphosphorylated forms (Leffers, 1995; Meng et al., 2007) and it has been predicted that the phosphorylated form has comparatively less RNA binding capacity (Leffers, 1995). p21 activated kinase 1 (PAK1) has been previously shown to be constitutively associated with hnRNP E1 and regulate its RNA binding activity.
(Meng et al., 2007). Here, we have identified a cascade whereby TGFβ activates Akt2, which in turn is responsible for the single site phosphorylation of hnRNP E1 at serine 43. This is the first evidence of isoform specific Akt activation by TGFβ and Akt2-mediated phosphorylation of hnRNP E1, and confirms recent findings that Akt2 is involved in promoting EMT, invasiveness and metastasis (Irie et al., 2005). We also show that hnRNP E1 phosphorylation is responsible for its release from the mRNP complex and concurrent activation of Dab2 and ILEI translation in TGFβ-treated NMuMG and EpRas cells.

The autocrine response of cells to TGFβ-induced Akt activation and subsequent translational activation of EMT inducer transcripts may represent a novel mechanism through which the increased TGFβ expression in tumor cells contributes to cancer progression and provides avenues for novel anti-cancer therapeutic strategies.
CHAPTER IV

GENOME-WIDE EXPRESSION PROFILING REVEALS hnRNP E1 MEDIATES TGFβ-INDUCED EMT VIA A POSTTRANSCRIPTIONAL REGULON

4.1. Abstract

TGFβ induces epithelial-mesenchymal transdifferentiation (EMT) accompanied by cellular differentiation and migration. EMT has emerged as a fundamental process governing embryonic development, adult tissue homeostasis and metastatic progression. Transcriptional array analyses have failed to identity and validate ‘EMT signature genes’ because the transcriptome does not mirror the proteome. We have previously shown that TGFβ post-transcriptionally regulates EMT by causing increased expression of two transcripts required for EMT, \textit{Dab2} and \textit{ILEI}, by modulating hnRNP E1 phosphorylation. Ectopic expression of ILEI and Dab2 do not render
mesenchymal properties to cells in a TGFβ independent fashion suggesting involvement of other mRNAs in the pathway. Using a genome-wide combinatorial approach involving polysome profiling and RIP-Chip analyses using hnRNP E1, we have identified a cohort of translationally regulated mRNAs required for TGFβ-induced EMT. Coordinated translational regulation by hnRNP E1 constitutes a post-transcriptional regulon inhibiting the expression of related EMT genes, thus enabling the cell to rapidly and coordinately suppress multiple EMT genes and downregulate metastatic progression.
4.2. Introduction

The epithelial-mesenchymal transition (EMT), in which cells undergo a developmental switch from a polarized, epithelial phenotype to a highly motile fibroblastic or mesenchymal phenotype, has emerged not only as a fundamental process during normal embryonic development and in adult tissue homeostasis, but is also essential for metastatic progression (Derynck et al., 2001; Zavadil et al., 2005; Thiery et al., 2006). EMT is associated with changes in cell-cell adhesion, remodeling of extracellular matrix, and enhanced migratory activity, all properties that enable tumor cells to metastasize (Derynck et al., 2001; Zavadil et al., 2005; Thiery et al., 2006). Numerous cytokines and autocrine growth factors, including TGFβ, have been implicated in EMT (Bierie and Moses, 2006; Massague, 2008). TGFβ exerts antiproliferative effects and functions as a tumor suppressor during early stages of tumorigenesis, whereas at later stages it functions as a tumor promoter aiding in metastatic progression (Bierie and Moses, 2006; Massague, 2008).

We have earlier shown that regulation of gene expression at the post-transcriptional level plays an important role in TGFβ-mediated EMT. A transcript-selective translational regulatory pathway exists in which a ribonucleoprotein (mRNP) complex, consisting of heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1), binds to a 3′-UTR regulatory BAT (TGFβ activated translation) element and silences translation of Dab2 and ILEI mRNAs involved in mediating EMT. Ribonucleoprotein complexes (mRNPs) are diverse macromolecular assemblies consisting of both protein and RNA components and possessing
indispensable roles in the maturation of most RNAs and in the translation of messenger RNAs (Varani et al., 1998; Cusack, 1999). We have shown that TGFβ activates a kinase cascade terminating in the phosphorylation of hnRNP E1 by isoform-specific stimulation of protein kinase Bβ/Akt2, inducing the release of the mRNP complex from the 3'-UTR element, resulting in the reversal of translational silencing and increased expression of Dab2 and ILEI transcripts and subsequently mediates EMT.

Despite intensive transcriptional array analysis of human tumors, the identity and validation of ‘EMT signature genes’ remains elusive (Pradet-Balade et al., 2001; van’t Veer et al., 2002; Ramaswamy et al., 2003; Kang and Massague, 2004; Vyas et al., 2009), partially because the transcriptome does not mirror the proteome (van der Kelen et al., 2009). An alternative is expression profiling on a genome wide scale, whereby non-translating and actively translating pools of mRNAs are isolated by sucrose density gradient fractionation and subsequently subjected to microarray analysis (Zong et al., 1999; Arava et al., 2003; Bjorklid et al., 2003; Hofacker et al., 2003; Jechlinger et al., 2003; Vyas et al., 2009). RNA-Binding Protein Immunoprecipitation-Microarray (Chip) Profiling is an advanced high-throughput analysis of mRNAs that coimmunoprecipitate with particular mRNA-binding proteins (Penalva et al., 2004). An mRNA-binding protein of interest is immunoprecipitated, and the associated mRNA is isolated and subsequently subjected to microarray analysis (Johannes et al., 1999; Tenenbaum et al., 2000; Brown et al., 2001; Miyashiro et al., 2003; Gerber et al., 2004; Inada and Guthrie, 2004; Penalva et al., 2004). A
combinatorial approach involving expression profiling and RIP-chip analysis on a genome-wide basis will yield definitive information on a particular regulatory pathway.

shRNA-mediated silencing of Dab2 and ILEI in NMuMG cells is sufficient to inhibit TGFβ-mediated EMT as analyzed morphologically and by loss of upregulation of N-cadherin and vimentin, mesenchymal cell markers, whereas their overexpression does not induce constitutive EMT, independent of TGFβ signaling (Prunier and Howe, 2005). Thus Dab2 and ILEI are required, but not sufficient, for TGFβ-induced EMT. Hence, we hypothesized that there are other mRNAs which are being silenced by hnRNP E1 in a similar fashion, and which cumulatively contributes to TGFβ-induced EMT. Hence, we adopted a combinatorial approach involving polysome profiling and RIP-Chip analyses using hnRNP E1 and filtered the array data based on the regulatory mechanism of Dab2 and ILEI, i.e., enrichment in the polyribosome and loss of temporal association with hnRNP E1 with 24 hs of TGFβ treatment. This led to the identification of a cohort of four mRNAs that follow the same pattern of regulation as Dab2 and ILEI and were further validated using qRT-PCR and immunoblot analysis. Each of the identified target mRNA harbors a functional BAT element in the 3'-UTR as revealed by functional testing using chimeric luciferase reporter constructs and are required for TGFβ-induced EMT as evidenced by failure to EMT in cells where these mRNAs are silenced. This cohort of mRNAs may represent a new TGFβ responsive and hnRNP E1 mediated posttranscriptional
regulon regulated directly at the posttranscriptional level in order to mediate TGFβ-induced EMT in a temporal and expedited fashion.
4.3. Materials & Methods

4.3.1. Cell culture and treatments

TGFβ2 was a generous gift from Genzyme Inc. and was used at a final concentration of 5 ng/ml. NMuMG cells were cultured as described previously (Prunier and Howe, 2005).

4.3.2. Plasmids construction

For construction of the chimeric luciferase constructs, luciferase cDNA derived from pGL3-b vector was cloned into pcDNA3. A linker region containing 5’-EcoR1-Pac1-EcoRV-Nco-1-Xho-1-Xba-1 was inserted into the vector downstream of the luciferase gene (pCMV-LL). Synthetic Dab2/BAT, ILEI/BAT, Rhox5/BAT, Prl2c4/BAT, Ube3A/BAT and IL-11Ra2/BAT were generated with 5’-EcoR1 and 3’-Xba-1 sites. pSilencer neo-shRNA-mouse Rhox5 and Ube3A were constructed by annealing shRNA template oligonucleotides (target selected through engine at Ambion and cloned into pSilencer neo vector (refer to Table S3 for oligonucleotide sequences). pSUPER-Dab2si construct has been generated in the lab and described previously (Prunier and Howe, 2005). The ILEI siRNA, a pool of 3 target-specific 19-25 nt. siRNAs were brought from Santa Cruz Biotechnology Inc.

4.3.3. Preparation of cytosolic extract (S100 Fraction)

S100 fractions were prepared from unstimulated and TGFβ-treated NMuMG cells as previously described (Mazumder and Fox, 1999) with minor modifications. Briefly, the buffer used for cytosolic extraction contained 20 mM
Hepes (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail (Roche) (Hampton et al., 1998).

4.3.4. RNA immunoprecipitation

RNA immunoprecipitation was performed as described previously (Penalva et al., 2004), the cytosolic extract was incubated with 10 µg of rabbit α-hnRNP E1 antibody or rabbit α-IgG at 4°C overnight. 100 µL of protein A-Sepharose (Sigma) suspension (50% packed Sepharose in Buffer C) was added and the incubation was continued overnight as described. The beads were pelleted by 2 min centrifugation at 240 g at 4°C; the pellet was briefly washed three times with 1 ml of IP Wash Solution (150 mM NaCl, 50 mM Tris pH 7.5, 0.5% NP40) and the Sepharose was transferred for elution into a fresh plastic tube and pelleted again. The immunoprecipitated RNAs were isolated by Trizol extraction as per manufacturer’s instructions. The isolated RNA was treated with RNase-free DNase I to get rid of any contaminating DNA and used for the Illumina MouseWG-6 v2.0 expression BeadChips.

4.3.5. Polysome profiling

Polysomes were analyzed from cytosolic fractions made in polysome lysis buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 50 U of recombinant RNasin containing 100 µg/ml cycloheximide) as described previously (Ray and Fox, 2007). Cytoplasmic extract was carefully layered over 10 to 50% (w/v) linear sucrose gradients and centrifuged at 1,50,000g for 4 hr. Gradients were fractionated using a TELEDYNE ISCO gradient fractionation
system equipped with a UA-6 detector following an upward displacement method. Light RNP fractions, 40S, 60S, monosomes and heavy polysome fractions were monitored by continuous UV absorption profiles at 264 nm, and 12 tubes of 1 ml fractions were collected. The fractions collected in the first four tubes, representing light RNP and free ribosomes, were used to isolate a translationally inactive pool of mRNAs, and fractions numbered five to ten, representing polysomes, were used as a source of translationally active mRNAs. Fractions 11 and 12 were discarded as heavy molecular weight aggregates. Total RNA was isolated from these fractions by extraction with Trizol and purified with RNeasy minikit, following the manufacturers’ protocols. The RNA was quantitated and checked for purity by agarose formaldehyde gel and used for the Illumina MouseWG-6 v2.0 expression BeadChips.

4.3.6. cDNA amplification and labeling

TotalPrep RNA amplification kit (Ambion, Foster City, CA, USA) was used to generate biotin-16-UTP-labeled cRNA from 400 ng of total RNA. The hybridization mix was prepared based on the guidelines provided in the Illumina BeadStation 500X System Manual (Illumina, San Diego, CA, USA) using the supplied reagents. Hybridization to the Illumina Mouse-6 expression BeadChip was done for 16 h at 55°C on a BeadChip Hyb Wheel. The slide was washed for 15 min on an orbital shaker at RT in a staining dish with 250 ml of E1BC wash solution, followed by incubation in 100% ethanol for 10 min at RT, and washed again in E1BC for 2 min. For the blocking reaction the slide was placed in a BeadChipWash Tray containing 4ml of Block E1 buffer on a rocker mixer for 10
min. Staining was performed for 10 min in a tray with 2 ml of buffer E1 and 1 µg/ml Cy3-streptavidin (Amersham Biosciences, Piscataway, NJ, USA), followed by incubation in E1BC for 5 min. The slide was dried by centrifugation (275 rpm, 5 min) and kept in the dark until ready to scan. Scanning used the Illumina BeadArray Reader software together with the Illumina BeadStation 500 platform and was read by the Illumina BeadXpress™ Reader.

4.3.7. Data Analysis

Illumina data were analyzed by using the Illumina BeadStation 1.5.1.3. Software. After clustering, results were screened. The data were subtracted for background and normalized using the rank invariant option. A RefSeq gene was accepted as expressed if the detection value was ≥0.99. The ratios of the raw signal intensity values of the mRNAs in the different test groups of the selected genes were subsequently determined.

4.3.8. Isolation of total RNA and RT-PCR

Isolation of total RNA and RT-PCR was done as described previously (Wildey et al., 2003). Refer to Table II for primer sequences.

4.3.9. Quantitative Real Time PCR Analysis

qRT-PCR was done as described previously (Vyas et al., 2009). Please refer to Table II for primers used. The results were expressed after normalization to β-Actin expression levels from three different experiments. Data has been represented as means ± S.D.
4.3.10. Preparation of cell lysates and immunoblot analysis

For immunoprecipitation and immunoblot analysis, cells were lysed in buffer D and immunoprecipitation carried out as previously described (Hocevar et al., 1999).

4.3.11. Prediction of putative BAT elements in the potential target mRNAs

3′-UTR sequence of the target mRNAs were examined for structural similarities with Dab2/BAT and ILEI/BAT elements using Foldalign program (http://foldalign.ku.dk/), which allowed clustering of RNA sequences independent of sequence homology (Vyas et al., 2009). Various lengths of identified sequences were expanded to 33 nucleotides randomly and folded using the Mfold algorithm (Zuker, 2003). Structures that resembled the Dab2/BAT were chosen by visual selection and were screened for structure-based homology with the Dab2 and ILEI BAT elements using the Foldalign program, version 2.0.3 (Vyas et al., 2009). Statistical relevance of predicted structure existence was predicted by Sfold algorithm (Ding and Lawrence, 1999). Finally, structures were evaluated with UNAFold algorithm to check for hybridization characteristics and melting pathways to confirm uniform predictive function (Markham and Zuker, 2005).

4.3.12. In vitro luciferase assay

In vitro luciferase assay was performed as previously described (Mazumder and Fox, 1999).
4.3.13. RNA pull-down

WT and mutant BAT synthetic RNA (cRNA) was bound to cyanogen bromide-activated sepharose beads. Ribomax kit was used to generate milligrams quantity of synthetic 33nt RNA from the template DNA. For RNA pull down experiments, different amount (0.5 mg) of cytosolic extracts prepared from unstimulated and TGFβ treated NMuMG cells were incubated at 4 °C for 2 hr with the cRNA beads. Following the incubation period the beads are washed with 0.3 M sodium chloride and resolved by 10% SDS-PAGE.

4.3.14. Statistical analysis

Data obtained from qRT-PCR are presented as mean ± s.d., n=3.
Table II. Primers and oligonucleotide sequences for constructs used.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer/oligonucleotide sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-11Rα2 shRNA 5’-UTR Top</td>
<td>GATCCATCCGGGTAGGTATTAGTAGTTCCCCAGACTCTAATACCTACCCGGATTATTGGAAA</td>
</tr>
<tr>
<td>IL-11Rα2 shRNA 5’-UTR Bottom</td>
<td>AGGTTTCCCAAAAAATCCCGGTAGGTATTAGTAGTTCCCCAGACTCTAATACCTACCCGGATTATTGGAAA</td>
</tr>
<tr>
<td>Prl2c4 shRNA 5’-UTR Top</td>
<td>GATCCGGTACGCCTAGAAATAATTCAAGAGATTATTTCTGAGAGCTACTTTTTGGAAA</td>
</tr>
<tr>
<td>Prl2c4 shRNA 5’-UTR Bottom</td>
<td>AGGTTTCCCAAAAAAGTAGCTCTAGAAATAATTCAAGAGATTATTTCTGAGAGCTACTTTTTGGAAA</td>
</tr>
<tr>
<td>Rhox5 shRNA 5’-UTR Top</td>
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<tr>
<td>Rhox5 shRNA 5’-UTR Bottom</td>
<td>AGGTTTCCCAAAAAAGTAGCTCTAGAAATAATTCAAGAGATTATTTCTGAGAGCTACTTTTTGGAAA</td>
</tr>
<tr>
<td>Ube3A shRNA 5’-UTR Top</td>
<td>GATCCGGTACGCCTAGAAATAATTCAAGAGATTATTTCTGAGAGCTACTTTTTGGAAA</td>
</tr>
<tr>
<td>Ube3A shRNA 5’-UTR Bottom</td>
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</tr>
<tr>
<td>mDab2-Exon9-F1</td>
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</tr>
<tr>
<td>mDab2-R2</td>
<td>CATTTGCCTTTGAAGAGATCCAGA</td>
</tr>
<tr>
<td>mILEI-F1</td>
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</tr>
<tr>
<td>mILEI-R1</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>mRhox5-R1</td>
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</tr>
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<tr>
<td>mβ−Actin-Exon5-R1</td>
<td>CACTCCATGGAATTGGGATGAG</td>
</tr>
</tbody>
</table>
4.4. Results

4.4.1. Experimental design to identify potential target mRNAs that are translationally upregulated by TGFβ and differentially regulated by hnRNP E1 in a TGFβ-dependent manner

We have previously shown in Chapter II that NMuMG cells undergo EMT following TGFβ stimulation for 24 hs. Hence, for the expression profiling we isolated non-translating, non-polysomal (40S, 60S, and monosomes) and actively translating polysomal fractions from cells treated ± TGFβ for 24 hs by sucrose gradient centrifugation (Fig. 4.1A). We have earlier shown in Chapter II that hnRNP E1 binds the BAT elements of Dab2 and ILEI and inhibits their translation in control cells and TGFβ treatment leads to loss of temporal association with hnRNP E1 and subsequent translational activation of the two mRNAs. For the RIP-Chip profiling, we immunoprecipitated cytosolic extracts made from NMuMG cells treated ± TGFβ for 24 hs with anti-hnRNP E1 antibody and IgG (Fig. 4.1A). The protocol was repeated for cytosolic extracts made from E23 cells, NMuMG cells stably overexpressing hnRNP E1, which were previously shown to be refractory to TGFβ-induced EMT in Chapter III. The rationale behind using the E23 cells for the RIP-Chip analysis was to use it as an external control for normalization of the microarray data obtained from NMuMG cells (mRNAs in which TGFβ treatment leads to loss of temporal association with hnRNP E1 in NMuMG cells will still be associated with hnRNP E1 in the E23 cells). mRNA was isolated from the different fractions and the immunoprecipitates. Total, unfractionated RNA was also obtained from NMuMG cells treated ± TGFβ for 24 hs. The different
pools of mRNAs were subjected to cDNA synthesis and amplification before being subjected to microarray analysis using the Illumina MouseWG-6 v2.0 Chip.

4.4.2. Identification of the ‘EMT signature genes’ panel

The data obtained from the microarray was subjected to statistical analysis and normalization by the Illumina BeadStudio software. Cut-off intensity for significant detection for polysome profiling was fixed at 5.0 folds \([(P/NP)_{24h}/(P/NP)_{0h}]\). For RIP-Chip, the cutoff was fixed at < 1.0 fold \((IP_{0h}/IP_{24h})\). As depicted in Fig. 4.1B, the data revealed 83 genes that are translationally upregulated following TGFβ stimulation and 23 genes that selectively interact with hnRNP E1 under unstimulated conditions and subsequently lose their temporal association following TGFβ addition. A complete list of the different target mRNAs identified by the expression profiling and RIP-Chip is presented in Tables III and IV, respectively. Self-organizing maps (SOM) clustering analysis and dendogram (Fig. 4.2A) confirms that the obtained data conforms to external controls and normalization platforms adopted for data analysis. As an example, total mRNA isolated from unstimulated and TGFβ-treated cells (S5 and S6, respectively in Fig. 4.2A), grouped closely as is normally expected, whereas mRNAs from non-polysomal and polysomal pools (S3 and S4, respectively in Fig. 4.2A) from TGFβ treated cells mapped to distant loci in the dendogram.

The EMT signature genes were obtained by intersecting the data obtained from polysome profiling and RIP-Chip analyses and identified 5 target mRNAs (Fig. 4.2B). Both approaches identified \(ILEI\) mRNA, thus confirming that the experimental approach adopted was valid. The other target genes that were identified were interleukin-11 receptor alpha, chain 2 (\(IL-11Ra2\)), reproductive homeobox 5 (\(Rhox5\)), prolactin family
2, subfamily c, member 4 (Prl2c4) and ubiquitin protein ligase E3A (Ube3A). But we did not identify Dab2 mRNA. This can be either due to the high (>5.0) cut-off limit of detection that we used for the analysis or because of inefficient hybridization between the samples and the Dab2 probes on the array. The ratios of the raw signal intensity for all the samples that were subjected to microarray analysis for each of the five potential target mRNAs are represented as heat maps and bar diagrams (Fig. 4.3A, B). As depicted, the color progression scale distinctively shows that even though the pattern of regulation for the five potential targets is same, the fold induction levels are different.

4.4.3. Validation of potential target mRNAs

The presence of ILEI, Rhox5, Ube3A, IL-11Ra2 and Prl2c4 mRNAs in each sample subjected to microarray analysis was determined by semi-quantitative RT-PCR analysis (Fig. 4.4). Presence of Dab2 was also determined in the samples. The expression of all the potential targets corroborated with the microarray data analysis. Similar results were obtained for Dab2, even though it was not detected in the microarray analysis. For each target, message was found to translocate to the actively translating polysome fractions with TGFβ treatment, while they were found sequestered to the non-translating pools in the unstimulated cells. The targets also showed differential temporal association with hnRNP E1 in unstimulated and TGFβ treated NMuMG cells, but not in E23 cells. We further validated the targets by monitoring their steady state mRNA and protein expression levels. Total mRNAs were isolated from NMuMG cells treated with TGFβ for the indicated times and subjected to quantitative Real Time PCR (qRT-PCR) to assess steady state mRNA expression levels of the target genes (Fig. 4.5). As shown, none of the mRNAs showed >2.5 fold induction in the
transcript expression levels. But TGFβ treatment of NMuMG cells led to increased expression levels of protein for all the potential targets (Fig. 4.6). Taken together, the results suggest that each of the identified target mRNAs is translationally upregulated by TGFβ and further validates the identified targets.

4.4.4. In silico analysis of the 3’-UTRs of potential target mRNAs for translational silencing

A 33nt BAT element mediating TGFβ-induced translational regulation of Dab2 and ILEI has been previously defined in Chapter II. As shown in Fig. 4.5A, the BAT element consists of a stem-loop structure with an asymmetric bulge and similarity in Dab2/BAT and ILEI/BAT is based on secondary structure and not sequence information contained within the structure. It was imperative to determine whether the Ube3A, Prl2c4, Rhox5 and IL-11Ra2 also contain the BAT element in their 3’-UTRs. Putative BAT elements in the target mRNAs were predicted using structure information contained within the BAT element as detailed in Materials & Methods. Putative BAT elements were identified in Rhox5 (nt 27-60) (UTRdb ID: 3MMU062400), Prl2c4 (nt 22-54) (UTRdb ID: 3MMU060233), Ube3A (nt 1098-1130) (UTRdb ID: 3HSA011848) and IL-11Ra2 (nt 153-183) (UTRdb ID: 3MMU026940). The resultant structures appeared to contain significant folding similarity as identified by the stem-loop and asymmetric bulge (Fig. 4.7). It should be noted that Ube3A was not detected in the mouse 3’-UTR database and hence the corresponding human sequence was used to predict putative BAT element.
4.4.5. The predicted BAT elements in the target mRNAs interacts with hnRNP E1 \textit{in vitro} and can functionally repress translation

Temporal association of hnRNP E1 with the predicted BAT elements in the target mRNAs was investigated using a RNA pull-down from cytosolic extracts from unstimulated and TGF\(\beta\)-treated NMuMG cells (Fig. 4.8). The data demonstrate that the predicted 33nt elements could precipitate hnRNP E1 from unstimulated extracts but that TGF\(\beta\) induced the loss of binding of hnRNP E1 to the RNA. hnRNP E1 could not bind the U10A or the bulge mutants suggesting the indispensability of the stem-loop and the asymmetric bulge for functional specificity. We next investigated if the predicted elements can functionally repress translation of chimeric luciferase transcripts, where the predicted BAT elements were cloned downstream of a luciferase (Luc) reporter gene. Unstimulated cytosolic extracts inhibited the translation of all the chimeric luciferase constructs tested (Fig. 4.9). \textit{In vitro} translation repression of Luc-Dab2/BAT and Luc-ILEI/BAT was relieved after 3 hr of TGF\(\beta\) stimulation and similar results obtained for the chimeric constructs for \textit{Rhox5}, \textit{Prl2c4}, \textit{Ube3A} and \textit{IL-11Ra2} (Fig. 4.9). Together, the results show that the predicted structures bearing secondary structure similar to Dab2/BAT and ILEI/BAT elements are effective for translational silencing and reversion of translational silencing is seen following 3 hs of TGF\(\beta\) treatment.
4.4.6. Modulation of hnRNP E1 expression or its posttranslational modification in NMuMG cells alters expression of the identified targets

We have previously generated NMuMG cells either stably overexpressing hnRNP E1 (E23 cells) or silenced hnRNP E1 expression (SH14 cells). We had also knocked in either WT (KIWT6 cells) or a phospho-mutant (Ser43Ala) (KIM2 cells) version of hnRNP E1 into SH14 cells (Chapter III). SH14 cells constitutively EMT even in the absence of TGFβ, E23 cells was refractory to TGFβ-induced EMT. Knock-in of either the WT or Ser43Ala mutant hnRNP E1 rescued the epithelial phenotype and TGFβ stimulation caused EMT in KIWT6, but blocked it in KIM2 cells (Chapter III).

We hypothesized that if the identified targets are true modulators of TGFβ-induced EMT like Dab2 and ILEI, their expression levels should mimic the later in the NMuMG, E23, SH14, KIWT6 and KIM2 cells. Whole cell lysates were prepared from these cells following TGFβ stimulation for the indicated times and then probed with the respective antibodies. As shown in Fig. 4.10, in NMuMG and KIWT6 cells, TGFβ induced the protein expression levels in a time dependent fashion, whereas in E23 and KIM2 cells, none of the target mRNAs were being expressed even after TGFβ treatment. Correspondingly, constitutive expression of the target mRNAs were detected in the SH14 cells. Overall, the target mRNAs were being expressed in a fashion similar to Dab2 and ILEI and was suggestive of an equivalent role in TGFβ-induced EMT.
We postulated that if the target mRNAs are required for EMT, then silencing the expression of even one of the mRNAs will rescue epithelial cell properties in the SH14 cells. sh-RNA mediated silencing of either *Rhox5* or *Ube3A* attenuated induction of EMT as evident by loss of expression of mesenchymal cell markers, N-cadherin and vimentin (Fig. 4.11) and loss of morphological features associated with mesenchymal cells (data not shown). Cumulatively, these results clearly support our results that the role of hnRNP E1 on EMT is mediated through induction of a cohort of six mRNAs and that they are each critical mediator of EMT.
Figure 4.1: Schematic representation of experimental design for combinatorial polysome profiling and RIP-Chip analyses.

(A) For expression profiling, cell lysates from untreated and TGFβ-treated (24 hs) NMuMG cells were fractionated by sucrose gradient (10-50%) centrifugation and RNA was isolated from the non-translating and actively translating pool, designated as NP and P, respectively. For the RIP-Chip analysis, cytosolic extracts made from untreated and TGFβ-treated (24 hs) NMuMG and E23 cells were immunoprecipitated with α-hnRNP E1 antibody or an isotype control and RNA was isolated from the immunoprecipitates. Following cDNA synthesis and amplification step, the cDNA was hybridized to Illumina Mouse WG-6 v2.0 chip (with capacity for 6 samples) and analyzed by Illumina BeadStudio™ Reader. Data obtained was analyzed by Illumina BeadStudio Software. (B) Flow chart representing the scheme followed for data filtering and normalization to arrive at the ‘EMT signature genes’ (annotates as translationally upregulated by TGFβ and differentially regulated by hnRNP E1 in a TGFβ-dependent manner). X and Y represent translationally upregulated and hnRNP E1 differentially regulated genes, respectively.
Figure 4.2: Combinatorial polysome profiling and RIP-Chip identifies an EMT signature profile.

(A) Raw expression data were exported from Illumina BeadXpress™ Reader and subjected to self-organizing maps (SOM) clustering analysis to confirm rigorosity of data obtained with respect to external controls and normalization platforms adopted for data analysis. S1 through S12 represent the sample identifications used for the array analysis and definition of each has been included.

(B) Venn diagram summarizing the results of the genome wide analysis performed. Identity of the 5 genes translationally upregulated by TGFβ and differentially regulated by hnRNP E1 in a TGFβ-dependent manner has been included.
Figure 4.3: Quantitative representation of data analysis as obtained from Illumina BeadStudio Software.

(A) Heat map generated from the Illumina Mouse WG-6 v2.0 chip analysis of the genes translationally upregulated by TGFβ and differentially regulated by hnRNP E1 in a TGFβ-dependent manner. The color progression scales represent the relationship between different colors and relative quantities of the particular mRNA.

(B) Bar diagram representing the raw intensity profiles obtained for each sample in the Illumina analysis.
Figure 4.4: Validation of the potential target mRNAs.
Semi-quantitative RT-PCR analysis using gene specific primers for the potential targets and β-Actin (control) on the samples that were subjected to illumina analysis.
Figure 4.5: The identified targets are not significantly induced transcriptionally by TGFβ.

Total mRNAs were isolated from NMuMG cells treated with TGFβ for the indicated times and subjected to quantitative Real Time PCR (qRT-PCR) to assess steady state mRNA expression levels of the potential target genes. qRT-PCR was carried out using SYBR Green PCR Master Mix and ABI Thermo Cycler. The value depicted is representative of relative amount of test mRNA normalized to β-Actin from three different experiments.
Figure 4.6: Expression of all the identified target transcripts are induced by TGFβ.

Immunoblot (IB) analysis examining protein expression levels of the potential targets in NMuMG cells treated with TGFβ for the indicated times. One of the blots was stripped and re-probed for β-Actin as a loading control.
Figure 4.7: The 3'-UTR of the identified mRNAs contain the BAT element.

Secondary structures of the target mRNAs with similarities to Dab2/BAT element. Specific regions of the 3'-UTR were selected and the structures were generated as described in Materials & Methods (dG\textsubscript{Dab2/BAT} = -5.0 Kcal/mol; dG\textsubscript{ILEI/BAT} = -2.5 Kcal/mol; dG\textsubscript{Rhox5/BAT} = -0.10 Kcal/mol; dG\textsubscript{Prl2c4/BAT} = -4.7 Kcal/mol; dG\textsubscript{Ube3A/BAT} = -1.9 Kcal/mol; dG\textsubscript{IL-11R\alpha2/BAT} = -10.4 Kcal/mol). ILEI/BAT element was folded under constraints (F 5 0 2)/ (F 9 0 2)/ (P 11 0 2) and Prl2c4/BAT element was folded under (F 2 0 1)/ (F 29 0 1) constraints.
Figure 4.8: The BAT elements in the target mRNAs temporally associates with hnRNP E1.

RNA affinity pull-down and IB analyses using S100 cytosolic extracts for the times indicated to define the temporal association of hnRNP E1 with the different BAT elements. Neck (U10A) and bulge (lacking asymmetric bulge) mutants represent BAT element forms that loose the secondary structure.
Figure 4.9: The BAT elements in the target mRNAs can functionally repress translation.

IVT analyses with chimeric luciferase constructs of the BAT element present in Dab2 and the identified targets show that TGFβ treatment relieves translational silencing conferred by the WT following 3 hr of TGFβ treatment.
Figure 4.10: Modulation of hnRNP E1 expression or its posttranslational modification alters expression of the identified targets.

IB analysis monitoring Dab2, ILEI, Rhox5, Prl2c4, Ube3A, IL-11Rα2 and Hsp90 protein levels in WT, E23, SH14, KIWT6 and KIM2 cells treated with TGFβ for the times indicated.

<table>
<thead>
<tr>
<th>NMuMG</th>
<th>E23</th>
<th>SH14</th>
<th>KIWT6</th>
<th>KIM2</th>
<th>TGFβ (hr)</th>
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<tbody>
<tr>
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<td>3</td>
<td>24</td>
<td>0</td>
<td>3</td>
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</table>

blot: α-Dab2

blot: α-ILEI

blot: α-Rhox5/Pem

blot: α-Prl2c4

blot: α-Ube3A

blot: α-IL-11Rα2

blot: α-Hsp90
Figure 4.11: Role of hnRNP E1 on EMT is mediated by the identified transcripts.

IB analysis of WCLs derived from SH14 cells, un-transfected or transiently transfected with ILEI and Dab2 siRNA and Rhox5 and Ube3A shRNA to confirm knockdown of targets. IB analysis examining N-cadherin protein levels in these transfected cells to confirm rescue of epithelial phenotype in the SH14 cells (fifth panel).
Table III  The list of the potential target mRNAs showing significant increase in translational competence in NMuMG cells following TGFβ stimulation for 24 hours. The ratios of polysomal vs. non-polysomal abundance of mRNAs before and after TGFβ treatment were measured by analyzing the raw signal intensity data using the Illumina BeadStation software. mRNAs presented in this Table show a significant induction (>5 folds) in translational potency. The data was normalized to fold induction of total mRNA.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<th>Fold Induction of Total mRNA = (mRNA\textsubscript{24hs} / mRNA\textsubscript{0hs})</th>
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Table IV. The list of the potential target mRNAs showing significant decrease in temporal association with hnRNP E1 in NMuMG cells following TGFβ stimulation for 24 hours. The ratios of abundance of mRNAs before and after TGFβ treatment were measured by analyzing the raw signal intensity data using the Illumina BeadStation software. mRNAs presented in this Table show a significant induction (>5 folds) in hnRNP E1 association in control cells and ≤1 fold in TGFβ treated cells. The data was normalized to fold induction in E23 cells and for IP: IgG in NMuMG cells.

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</table>
4.5. Discussion

We have identified a cohort of translationally regulated mRNAs required for TGFβ-induced EMT by using a combinatorial approach involving polysome profiling and RIP-Chip analysis using hnRNP E1 as the bait. Rigorous data analyses have identified the targets, which have been further validated using a combination of molecular and functional approaches. mRNA translation state change was considered significant only if the following two criteria were met: (a) the signal intensity of the non-translated RNA filter of resting cells and the translated RNA filter of activated cells exceeded the medial signal intensity of all the array elements, and (b) the value of the change in translation state was >5.0-fold of median value of the change of mRNA distribution of all the genes on the arrays. Similarly, for the RIP-Chip analysis, the ratios of raw signal intensities were normalized to the mean signal level, internal standards (housekeeping genes), and to the levels of external standards (E23 cells and IgG isotype control). The validity of the predicted targets are further ascertained by the facts that (a) our approach identified ILEI proving the validity of the approach; (b) presence of functional BAT elements, resembling a pattern similar to Dab2 and ILEI BAT elements, in the 3’-UTRs of the identified target mRNAs; and (c) indispensability of the target in TGFβ-induced EMT as evidenced by failure to EMT post shRNA-mediated silencing of Rhox5 and Ube3A in the SH14 cells.

Protein expression levels depend on the rate of transcription, as well as other defined control mechanisms, such as mRNA stability (Garcia-Martinez et al., 2004), nuclear export and mRNA localization (Hieronymus and Silver, 2004),
translational regulation (Beilharz and Preiss, 2004), and finally protein degradation (Beyer et al., 2004). Post-transcriptional regulation is mainly controlled by the binding of RBPs to regulatory regions in the UTRs of mRNAs. An advantage of genome-wide approaches, like the one adapted for the current study, is their unbiased nature and rigorous testing, which enables the discovery of unexpected connections.

Prediction of putative BAT elements in the identified target mRNAs was based on structure-based homology. Validation of the BAT elements was performed by a functional loss-of-silencing assay using chimeric luciferase reporter transcripts. All the putative BAT elements in Fig. 4.5A have a stem-loop structure with an asymmetric bulge; however, the structures show considerable diversity, which can be attributed to the somewhat arbitrary nature of RNA structure prediction itself (Vyas et al., 2009).

The BAT element in the 3’-UTR of Dab2, ILEI and the identified transcripts provides further insights into the importance of regulatory elements in maintenance of homeostasis. Coordinated translational regulation by hnRNP E1 constitutes a post-transcriptional regulon inhibiting the expression of related EMT genes (Keene and Tenenbaum, 2002). Eukaryotic regulons are defined as higher-order genetic units (quasi genome) consisting of monocistronic mRNA subsets under the control of a regulatory RNA binding protein (Keene and Lager, 2005). RNA binding proteins have been shown to specifically bind transcripts encoding functional and colocalized protein classes (Brown et al., 2001; Miyashiro et al., 2003; Waggoner and Liebhaber, 2003; Gerber et al., 2004).
Post-transcriptional operons or regulons may have evolved as mechanisms to rapidly and coordinately suppress multiple EMT genes and downregulate metastatic progression.
CHAPTER V

SUMMARY AND PERSPECTIVE

We have identified a transcript-selective translational regulation pathway by which TGFβ modulates expression of mRNAs required for EMT, and EMT itself in vitro and tumorigenesis and metastasis in vivo. hnRNP E1 binds to a structural, 33-nucleotide TGFβ-activated translation (BAT) element in the 3’-UTR of a cohort of mRNAs (Dab2, ILEI, Rho5, Ube3A, IL-11Ra2 and Prl2c4), thereby repressing their translation. TGFβ activates a kinase cascade terminating in phosphorylation of Ser43 of hnRNP E1, by isoform-specific stimulation of protein kinase Bβ/Akt2, inducing its release from the BAT element and loss of
translational silencing of the target mRNAs. Modulation of hnRNP E1 expression, or of its Ser43 site, alters TGFβ-mediated loss of translational silencing and EMT.

The BAT element in the 3′-UTR of these transcripts provides further insights into the importance of regulatory elements in maintenance of homeostasis. Coordinated translational regulation constitutes a post-transcriptional regulon inhibiting the expression of related EMT genes. Post-transcriptional regulons may have evolved as mechanisms to rapidly and coordinately suppress multiple EMT genes and downregulate metastatic progression. The autocrine response of cells to TGFβ-induced Akt2 activation, and subsequent translational activation of transcripts involved in EMT, may represent a mechanism by which increased TGFβ expression in tumor cells contributes to cancer progression and provides new avenues for novel anti-cancer therapeutic designs.

Rigorous investigation leading to the identification of the other components of the mRNP complex binding the BAT element and the underlying mechanism of translational activation of the identified transcripts following TGFβ stimulation will surely be one of the avenues of future research. Future work will also delve into the iterative cycle of system modeling, hypothesis generation and systematic experimentation that will address if this hnRNP E1 posttranscriptional regulon is involved in TGFβ-induced EMT and metastatic progression of tumors and delineate how each transcript contributes to the EMT process.
Even though research spanning over the last two decades has candidly outlined details of TGFβ signaling pathway in physiological and pathological conditions, much remains to be elucidated about the precise mechanisms of its deregulation in different forms and stages of cancer. Furthermore, detailed mechanistic explanation is still unavailable for the apparent paradoxical observation that TGFβ can induce cell cycle arrest and cytoskeletal alterations in the same cell. Answers to these questions will undoubtedly lead not only to many interesting and surprising observations that reveal additional regulatory complexities, but hopefully lead to the development of TGFβ-dependent anti-cancer therapies. Right now we are at a very exciting phase having all the tools for this next phase of transition into translational research.
Figure 5.1: BAT element: battling TGFβ-induced EMT along with hnRNP E1.

As shown in Chapters II, III, and IV a novel post-transcriptional pathway exists by which TGFβ through hnRNP E1 phosphorylation modulates expression of EMT-specific proteins and EMT itself through regulatory BAT elements in transcripts required for EMT.


Johannes G, Carter MS, Eisen MB, Brown PO, Sarnow P (1999) Identification of eukaryotic mRNAs that are translated at reduced cap binding complex


Keene JD, Tenenbaum SA (2002) Eukaryotic mRNPs may represent posttranscriptional operons. Mol Cell 9, 1161-7.


Mazumder B, Fox PL (1999) Delayed translational silencing of ceruloplasmin


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