ETS1 AND ETS2 ROLE IN RAS ONCOGENIC TRANSFORMATION IN MOUSE EMBRYONIC FIBROBLASTS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

Human soft-tissue sarcoma is a malignant tumor that is usually found in children and young adults. In the United States more than 12000 new cases are diagnosed each year with soft tissue sarcomas. Oncogenic Ras has been shown to be involved in soft-tissue sarcoma development and K-Ras, H-Ras and N-Ras missense mutations at codons 12, 13 and 61 have been reported in 23 to 30% of soft tissue sarcoma tumors.

The MAPK pathway is one of the main mediators of the Ras response, and it has been shown to be deregulated in cancer in general and soft tissue sarcoma in particular. Ets1 and Ets2 are the downstream effectors of the Ras/ERK pathway and have been shown to be over expressed in cancer and activated by Ras/ERK through the phosphorylation of their conserved MAPK phosphorylation domain next to the PNT domain. This phosphorylation is essential for Ets1 and Ets2 to regulate many genes that are important for diverse biological processes.

Over expression of constitutively active Ras in mouse embryonic fibroblasts leads to cellular transformation, and injection of Ras transformed fibroblasts into nude mice leads to formation of tumors similar to fibroblastic soft tissue tumor, which makes the Ras transformation in vitro model a suitable system for studying soft-tissue sarcoma. The Ets families of transcription factors have been shown to play an essential role in Ras
transformation, but the identity of the Ets family members that are essential for Ras transformation is still unknown.

Using the Cre-lox system to delete Ets2 from Mouse Embryonic Fibroblasts (MEFs) that are Ets1-/- and have the Ets1 null allele, we showed that the deletion of both Ets1 and Ets2 is necessary to inhibit Ras oncogenic transformation and tumorigenesis in MEFs.

Here we show that Ets1 and Ets2 play a post transcriptional repression role in Ras transformation through a C-myc dependent up regulation of miR17-92 microRNA. Ets1 and Ets2 bind to the C-myc promoter and regulate C-myc RNA and protein expression. We also show that Ets2 similar to C-myc binds to the promoter of miR17-92, and Ets1 and Ets2 through transient transfection are able to upregulate the miR17-92 cluster expression in C-myc -/- MEFs. C-myc and miR17-92 Cluster over expression in Ets1/Ets2 double knockout MEFs rescued Ras tumorigenesis in vivo upon injection in nude mice.

Also we show that Ets1 and Ets2 play a transcriptional repression role through a c-myc dependent repression of Sfrp1, Fas and Lox tumor suppressor genes during Ras transformation. Fas, Lox and Sfrp1 are not repressed in Ets1/Ets2 double knockout MEFs even after Ras retroviral infection, but over expression of C-myc repressed Fas, Lox and Sfrp1 again in the Ets1/Ets2 double knockout MEFs. We found that Ets1, Ets2 and C-myc bind to the promoter of Fas, Sfrp1 and Lox genes during Ras transformation, and we show an enrichment of these promoters with H3K9 and H3K27 trymethylation markers which are usually present on silent promoters.
DEDICATION

To my family that stood by my side throughout all these years. To my father that encouraged me and motivated me to choose this path, to my Mom that prayed for me all these years, to my Sister who cheered me when I was down, to my Brother that I am waiting to see after five long years.....
ACKNOWLEDGMENTS

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I am grateful to have worked with all the great people in Dr Ostrowski’s lab. I am proud to have got to know Fu Li and Ruchika Srinivasan, I had great time working with them in the lab and sharing the good and bad moments of our PhD. I would like to thank Dr Sudu Sharma and Dr Agnieszka Bronisz for the help they provided me in my experiments. Many thanks go to Tahera Zabuawala, Haritha Mathsyaraja, Julie Wallace,
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PUBLICATIONS


FIELDS OF STUDY

Major Field: Molecular, Cellular and Developmental Biology
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<td>AKT</td>
<td>v-akt murine thymoma viral oncogene homolog</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>ATF2</td>
<td>activating transcription factor 2</td>
</tr>
<tr>
<td>bclxL</td>
<td>BCL2-like</td>
</tr>
<tr>
<td>BRCA-1</td>
<td>breast cancer 1</td>
</tr>
<tr>
<td>CaCl2</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CD</td>
<td>Common Docking</td>
</tr>
<tr>
<td>CDC42</td>
<td>cell division cycle 42 (GTP binding protein, 25kDa)</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<tr>
<td>CHOP</td>
<td>C/EBP Homologous Protein</td>
</tr>
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<td>C-myc</td>
<td>myelocytomatosis viral oncogene homolog</td>
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<tr>
<td>Cre</td>
<td>Cyclization Recombination protein</td>
</tr>
<tr>
<td>CSF1</td>
<td>colony stimulating factor 1</td>
</tr>
<tr>
<td>CXCR4</td>
<td>chemokine (C-X-C motif) receptor 4</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth factor</td>
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<tr>
<td>EIF4E</td>
<td>eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>EIF6</td>
<td>eukaryotic translation initiation factor 6</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERG</td>
<td>ets erythroblastosis virus E26 oncogene homolog</td>
</tr>
<tr>
<td>ERK1</td>
<td>Extracellular signal-regulated kinase 1</td>
</tr>
<tr>
<td>ERK2</td>
<td>Extracellular signal-regulated kinase 2</td>
</tr>
<tr>
<td>ETS</td>
<td>E-twenty six</td>
</tr>
<tr>
<td>ETV6</td>
<td>ets variant 6</td>
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<td>EWS</td>
<td>Ewing’s Sarcoma</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<tr>
<td>Fas</td>
<td>TNF receptor superfamily, member 6</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FLI1</td>
<td>Friend leukemia Virus Integration 1</td>
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</table>
Gab1  GRB2-associated binding protein 1
GABPα  GA binding protein transcription factor, alpha subunit 60kDa
GAP  GTPase activating protein
GAPDH  glyceraldehyde-3-phosphate dehydrogenase
GATA3  GATA binding protein 3
GDP  Guanosine Diphosphate
GEF  Guanine nucleotide Exchange factor
GFP  Green Fluorescent Protein
Grb2  growth factor receptor-bound protein 2
Gro1  chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
GTP  Guanosine Triphosphate
HbEGF  heparin-binding EGF-like growth factor
HBS  HEPES buffered saline
HDAC1  histone deacetylase 1
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hnRNP  Heterogeneous nuclear ribonucleoproteins
HRas  Harvey rat sarcoma viral oncogene homolog
hTERT  telomerase reverse transcriptase
IGFBP3  insulin-like growth factor binding protein 3
IL-6  interleukin 6
KCl  Potassium chloride
KRas  Kirsten rat sarcoma oncogene homolog
KSR  Kinase suppressor of Ras
LEF-1  lymphoid enhancer-binding factor 1
Lox  lysyl oxidase
loxP  locus of X-over P1
MAPK  Mitogen Activated Protein Kinase
MAPKK  Mitogen Activated Protein Kinase Kinase
MAPKKK  Mitogen Activated Protein Kinase Kinase Kinase
MEF  Mouse Embryonic fibroblast
MEK1  Mitogen activated protein kinase kinase 1
MEK2  Mitogen activated protein kinase kinase 2
miR  Micro ribonucleic acid
MMP1  matrix metallopeptidase 1
MMP9  matrix metallopeptidase 9
MSCV  Murine Stem Cell Virus
MyoD1  myogenic differentiation 1
NaCl  Sodium Chloride
NaHCO3  Sodium bicarbonate
NP40  nonyl phenoxypolyethoxylethanol-40
NRas  Neuroblastoma rat sarcoma oncogene homolog
P300  E1A binding protein p300
P53  Tumor protein 53
PAK1  p21 protein (Cdc42/Rac)-activated kinase 1
PAX5  paired box 5
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PC12</td>
<td>Pheochromocytoma cell line</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PNT</td>
<td>Pointed</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
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<td>Raf</td>
<td>murine leukemia viral oncogene homolog</td>
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<td>Ral</td>
<td>Ras like</td>
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<td>Rb</td>
<td>Retinoblastoma</td>
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<td>RBD</td>
<td>Ras binding domain</td>
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<td>RIPA</td>
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<td>ribonucleic acid</td>
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<td>RNase</td>
<td>Ribonuclease</td>
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<tr>
<td>Rpl4</td>
<td>ribosomal protein L4</td>
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<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>Serpinba1</td>
<td>serpin peptidase inhibitor, clade B (ovalbumin), member 1</td>
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<tr>
<td>Sfrp1</td>
<td>secreted frizzled-related protein 1</td>
</tr>
<tr>
<td>SHOC-2</td>
<td>soc-2 suppressor of clear homolog</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>Sno</td>
<td>Small nuclear RNA</td>
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<td>SOS</td>
<td>Son of sevenless</td>
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<td>SSX</td>
<td>Synovial Sarcoma X chromosome breakpoint</td>
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<td>STAT5</td>
<td>signal transducer and activator of transcription 5</td>
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<td>SYT</td>
<td>Synovial Sarcoma Translocation</td>
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<td>TBST</td>
<td>Tris-Buffered Saline Tween-20</td>
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<tr>
<td>Tie-2</td>
<td>Tyrosine kinase, endothelial</td>
</tr>
<tr>
<td>TIMP1</td>
<td>metallopeptidase inhibitor 1</td>
</tr>
<tr>
<td>TIMP3</td>
<td>metallopeptidase inhibitor 3</td>
</tr>
<tr>
<td>TLS</td>
<td>Also called FUS (Fused in Sarcoma)</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>uPA</td>
<td>plasminogen activator, urokinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>VEGF-R</td>
<td>vascular endothelial growth factor receptor</td>
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CHAPTER 1

INTRODUCTION

Human soft-tissue sarcoma is a malignant tumor that is usually found in children and young adults. In the United States, more than 12000 new cases are diagnosed each year (Reynoso et al., 2010). This cancer starts in the muscle, fat, fibrous tissue, blood vessels or other supporting tissue of the body and is fatal in one third of patients and usually metastasizes to the lungs. Oncogenic Ras has been shown to be involved in soft-tissue sarcoma development and K-Ras, H-Ras and N-Ras missense mutations at codons 12, 13 and 61 have been reported in 23 to 30% of soft tissue sarcoma tumors.

The MAPK pathway is one effector signaling pathway of the Ras protein, and mutations of many proteins of the Ras/MAPK cascade are responsible for cancer progression. Nuclear effectors of the Ras/MAPK pathway are ETS factory molecules, and two members of this family, Ets1 and Ets2, regulate many biological processes and are phosphorylated and activated by MAPK1/2. This phosphorylation is essential for Ets1 and Ets2 to regulate many genes that are important for diverse biological functions. Ets1 and Ets2 are overexpressed in many different types of cancer.

Over expression of constitutively active Ras in mouse embryonic fibroblasts leads to cellular transformation, and injection of Ras transformed fibroblasts into nude mice leads to formation of tumors similar to fibroblastic soft tissue tumor. Over expression of
Ets-lacZ dominant negative Ets protein inhibits Ras transformation, suggesting that the Ets transcription factors are essential mediators of Ras transformation (Langer et al., 1992). Given the high homology in the structure of Ets1 and Ets2 we believe that both Ets1 and Ets2 play a fundamental role in Ras cellular transformation. Studying the Ras/Ets1/2 signaling pathway will lead to an understanding of the mechanism of Ras tumorigenesis in soft tissue sarcoma.

Our hypothesis is that Ets1 and Ets2 are both important for Ras transformation of fibroblasts and deletion of both Ets1 and Ets2 would limit Ras transformation.

1.1. Soft tissue sarcoma

More than 12,000 Americans are diagnosed with soft-tissue sarcoma; most of them are children and young adults (Reynoso et al., 2010). Soft-tissue sarcomas arise from mesodermal tissue (connective, muscle, adipose, neural, vascular and lymphatic tissues). The common sites of soft-tissue sarcomas are the extremities like the legs and arms. Malignant fibrous histiocytoma accounts for 20 to 30% of all the tissue sarcomas in adults (Kransdorf and Murphey, 2006), followed by liposarcoma, leiomyosarcoma and synovial sarcoma. They grow in a centripetal fashion and are surrounded by pseudocapsule that includes cellular debris, inflammatory and tumor cells. Unlike carcinomas that spread to neighboring structures, sarcomas compress surrounding soft tissues and neurovascular structures as they spread. They are aggressive neoplasms that have the potential to recur after resection and spread (Eilber et al., 2005). The usual metastatic site for soft tissue sarcoma is the lung (Billingsley et al., 1999). However in 5% of the cases, metastatic spread to the lymph nodes occurs as it is the case with angiosarcoma, epithelioid sarcoma, clear-cell sarcoma, synovial sarcoma and
rhabdomyosarcoma (de Visscher et al., 2006; Dim et al., 2007; Fong et al., 1993; Spillane et al., 2000).

1.1.1. Genetic alterations in soft tissue sarcoma

1.1.1.1. Inherited mutations

Inherited genetic alterations are rare events that could lead to soft-tissue sarcoma. Synovial sarcoma is caused by t(X; 18) translocation that will lead to the formation of a SYT-SSX fusion protein causing aberrant transcriptional activation (Lazar et al., 2006). Similarly, Myxoid liposarcoma is caused by t(12;16) translocation that will lead to formation of TLS-CHOP hybrid protein that acts as a strong transcriptional activator (Lazar et al., 2006). Interestingly, The Ewing sarcoma is caused by the t(11;22) translocation that will lead to the formation of EWS-FLI1 fusion protein with the Fli-1 Ets transcription factor. The fusion EWS-FLI1 protein works as a transcriptional repressor complex that represses Insulin Growth Factor Binding Protein-3 (IGFBP-3). EWS-FLI1 knock down by siRNA leads to massive apoptosis, suggesting that IGFBP-3 protein inhibition causes Ewing Sarcoma (Prieur et al., 2004). Also, somatic mutations in the tumor suppressor genes Rb and p53 lead to soft-tissue sarcoma (Strahm and Malkin, 2006; Zahm and Fraumeni, 1997).

1.1.1.2 Ras somatic mutations in soft-tissue sarcoma

Ras somatic mutations are frequent in soft-tissue sarcoma. Soft-tissue sarcoma analysis from human tissues through the Sanger website (www.sanger.ac.uk) reveals overall 10% H-Ras and K-Ras mutations, and overall 3% N-Ras mutations, all of which mutations are at position 12, 13 and 61 leading to a constitutively active form of Ras. Indeed, in human embryonic Rhabdomyosarcoma, 20% of the tumors tested have N-Ras
missense mutations at amino acid number 61 (Martinelli et al., 2009). Also, 14% K-Ras mutations were detected in leyomyosarcoma tumors at codon number 12 (Hill et al., 1997). All the H-Ras mutations in soft-tissue sarcoma are present at codon 12 (Bohle et al., 1996; Oda et al., 2000; Sakamoto et al., 2001; Wilke et al., 1993; Yoo et al., 1999).

1.1.2. Ras role in soft-tissue sarcoma

The Ras oncogene plays a central role in soft-tissue sarcoma development. In myoblasts, Ras inhibits the MyoD1 gene expression that is necessary for myoblast differentiation and skeletal muscle development (Konieczny et al., 1989). Interestingly, oncogenic K-Ras in the absence of p53 results in the development of human Rhabdomyosarcomas in mice (Konieczny et al., 1989), suggesting that inactivation of the tumor suppressor gene p53 in addition to Ras activation is necessary for soft-tissue sarcoma development.

1.2. Ras structure and function

Ras is at the center of a large network of signal transduction pathways (Wittinghofer, 1998). It belongs to the family of GTP-binding proteins that control different biological processes including cellular proliferation, protein biosynthesis, membrane trafficking, differentiation, development, apoptosis and transformation (Saxena et al., 2008; Wittinghofer, 1998).

The Ras precursor molecule is synthesized in the cytoplasm and matures into a functional protein through a series of post translational modifications that adds a hydrophobic substrate to the CAAX domain (C: cysteine, A: aliphatic amino acid, X: any other amino acid) that is 22 to 23 amino acids long and is located at the Ras carboxy terminal end. These post translational modifications are prenylation, proteolysis,
carboxymethylation and palmitoylation that provide attachment to other proteins and to
the plasma membrane (Saxena et al., 2008). Substitution of the cysteine residues of the
CAAX motif with other residues in constitutively active mutant Ras makes it
nonfunctional and unable to transform cells since it can’t bind to the plasma membrane
(Willumsen et al., 1984). The Ras function depends on the presence or absence of the
guanine nucleotides. GEFs (Guanine nucleotide Exchange factor) catalyze the exchange
of GDP with GTP (Wittinghofer, 1998), leading to the formation of Ras-GTP active
complex. On the other hand, GAPs (GTPase Activating Protein) catalyze the hydrolysis
of Ras-GTP leading to an inactive Ras-GDP complex. The Guanine nucleotide binding
sites are present at different regions within the Ras protein sequence (two binding sites
are located within the first 65 amino acid sequences of the Ras protein, and the other two
binding sites are located between amino acids 115 and 160) (Lowy and Willumsen,
1993). Ras receives signals from tyrosine kinases receptors like growth factors and
Integrins through adaptor molecules such as Grb2, Gab1 and SOS that leads to its
activation (Mitin et al., 2005). In turn, Ras possesses an effector domain between amino
acids 26 and 45 that interacts with different Ras effector molecules which also have Ras
Binding Domains (RBD), recruiting them to the plasma membrane where they are
activated (Lowy and Willumsen, 1993).

1.2.1. Three Ras genes

There are three mammalian Ras members: H-Ras, K-Ras and N-Ras that are
located on different chromosomes. H-Ras is homologous to the viral Harvey gene
whereas K-Ras is homologous to the viral Kirsten gene. N-Ras was first discovered in a
human neuroblastoma cell line (Bar-Sagi, 2001).
K-RasB is 188 amino acids in length, while H-Ras, N-Ras and K-RasA are 189 amino acids long. K-Ras isomers A and B are derived through alternative splicing of exon number four. K-RasB transcripts are more frequent than K-RasA (Barbacid, 1987). Each member of the Ras family interact with the plasma membrane through a distinct hydrophobic anchor suggesting that the different Ras proteins are localized to different domains in the plasma membrane, One example is that H-Ras but not K-Ras localizes to the cholesterol micro domains in the plasma membrane (Apolloni et al., 2000).

In general, H, K and N-Ras transcripts expression isn’t steady and vary in different tissues. The brain, muscles and skin contain a high level of H-Ras, whereas the gut, thymus and lung produce lot of K-Ras, and the N-Ras transcript is more frequent in testis and thymus (Leon et al., 1987).

1.2.2. Ras mutations in cancer and therapeutic treatments

Besides the oncogenic role that Ras plays in soft tissue sarcomas which only represent 1% of total cancer, Ras mutations are found in 30% of all human cancers. K-Ras mutations are the most common forms of Ras mutations in cancer (around 85% of Ras mutations), whereas H-Ras and N-Ras share the remaining 15% of Ras mutations. K-Ras mutations are frequent in epithelial pancreatic, lung and colon cancers. N-Ras mutations are common in melanoma, liver and myeloid malignancies whereas H-Ras mutations are very common in bladder cancer (Karreth and Tuveson, 2009).

The mechanisms through which Ras mutations cause cancer can be explained through careful analysis of the mutant amino acid sequences. Mutations resulting in a single amino acid substitution at codons 12, 13 or 61 lead to a defect in the ability of Ras
to hydrolyse GTP, thus maintaining Ras in a continuously active state that will eventually result in persistent stimulation of the mitogenic state of the cell, aberrant proliferation and transformation (Barbacid, 1987).

Many drugs have been designed to deactivate Ras by targeting key post translational modifications that make this protein functional. One example is the use of farnesyl-transferase inhibitors like FTI-276, FTI-2148, L-739,750, BZA-2B and SCH66336 that block the transfer of a farnesyl group to Ras and consequently inhibit its function (Sebti and Der, 2003; Zhu et al., 2003).

1.2.3. The Ras effectors

Since the Ras protein plays an essential role in the transduction of variety of signals that originate from the growth factor and their receptors, it is important to know some of the important downstream effectors of Ras. Activation of Ras leads to the activation of secondary messenger molecules like Phospholipase C (Bollag and McCormick, 1991) which activates the calcium signaling pathway (Kelley et al., 2001), (Song et al., 2001) Phosphotidylinositol-3-OH kinase (PI3K) (Rodriguez-Viciana et al., 1994) which activates Akt (a member of the Protein Kinases B (PKB) family) that is important for cellular survival (Datta et al., 1999), and Protein kinase C-ζ (Diaz-Meco et al., 1994). Other Ras effectors include p120 GAP (Martin et al., 1992), neurofibromin (another Ras-GAP protein) (Martin et al., 1990) and Ral (Ras related protein) which activates phospholipase D1 and CDC42 (De Ruiter et al., 2001). But for our study, the most important Ras effector is Raf, since the Raf/MEK/ERK pathway appears to be the most critical pathway for the mitogenic and differentiation effects of Ras (Cowley et al.,
1.2.4. Ras oncogenic transformation

Tumorigenesis is a multistep process through which a cancer cell undergoes transformation by accumulating genetic alterations including genetic mutations, amplifications and deletions. Transformation of cells by oncogenes like Ras has provided an important model to study the role of these genes in human cancer. Hallmarks of cell transformation include unchecked cellular proliferation, altered cellular morphology, loss of contact inhibition, stable changes in gene expression, decreased dependence on serum for growth and anchorage independent growth. The anchorage independence growth has been shown to correlate with tumorigenicity. Cells that are able to grow in soft agar and thus grow independent of a substratum have been shown to be able to form tumors in mice. For all the above mentioned reasons, the Ras oncogenic transformation model has been adapted as an attractive tool to study Ras tumorigenesis and identifying key alterations in signal transduction that lead to cancer. In the present study, Ras tumorigenesis in mouse embryonic fibroblasts (MEFs) is a valuable in vitro tool to manipulate and study in detail the molecular mechanism of soft-tissue sarcoma development.

In primary cells, oncogenic Ras alone is not able to induce transformation. The presence of an additional oncogene or the loss of a tumor suppressor gene together with Ras are able to transform primary cells. Indeed, Ras and c-myc co-transfection were able to induce transformation in primary rat fibroblasts (Land et al., 1983). Similarly, Ras and
large T antigen polyoma virus co-transfection were also able to transform rat embryonic fibroblasts (Land et al., 1983).

In contrast, Ras alone induce transformation in immortalized cell lines. The evidence for that was discovered by Chang et al in 1982 (Chang et al., 1982). Ras alone was able to transform NIH3T3 cells that formed tumors when injected into nude mice. This was explained by the fact that immortal cells have accumulated many mutations thus making the addition of a powerful oncogene like Ras enough to fully transform cells.

1.3. The MAP Kinase pathway

MAPK (Mitogen Activating Protein Kinase) proteins constitute the intermediate molecules in the cytoplasm that transmit the signals coming from the extracellular environment to the nucleus through a series of phosphorylations carried out by the members of the MAPK cascade. The MAPK pathway targets a large number of proteins including transcription factors leading to the activation or repression of target genes expression. The cellular outcome of the MAPK activation culminates in the activation of various biological responses, ranging from proliferation, cellular movement, apoptosis, transformation, senescence, angiogenesis and differentiation. There are different MAPK cascades, and each of them includes three kinases: The MAP kinase kinase kinases (MAPKKKs) are at the top of the cascade and are activated through phosphorylation. Active MAPKKKs phosphorylate and activate MAP kinase kinases (MAPKKs) which in turn phosphorylate and activate MAPKs. Finally, MAPKs enter the nucleus and phosphorylate a significant number of proteins including transcription factors as well as activators, coactivators, repressors and corepressors molecules (Dhillon et al., 2007).
In our model, the Raf/MEK/ERK MAPK is involved in the pathogenesis of soft-tissue sarcomas and plays an essential role in Ras oncogenic transformation in MEFs.

Raf is a MAPKKK and is recruited by the active Ras-GTP complex to the cellular membrane where it is activated. In general, Ras binding to the Raf protein alleviates the inhibition which the N-terminal regulatory domain of Raf exerts over the C-terminal catalytic domain, and adaptor scaffold proteins KSR or SUR-8/SHOC-2 help in the activation of Raf at the plasma membrane as is the case with Raf-1 and A-Raf (Li et al., 2000b; Therrien et al., 1996). The activation of Raf-1 and A-Raf requires the dephosphorylation of the inhibitory site, S259, and the phosphorylation of the activating site, S338, whereas B-Raf possesses twin aspartic acids instead of S338 in the N-region that makes it constitutively active after Ras recruitment to the membrane (Galabova-Kovacs et al., 2006).

MEK1 abd MEK2 are MAPKKs activated by Raf. A-Raf, B-Raf and Raf-1 all activate MEK through phosphorylation of serine 218 and 222 contained in the MEKs activation loop (Marais et al., 1997). Furthermore, the phosphorylation of MEK by PAK1 at S298 increases the binding of Raf to MEK and its subsequent activation (Gopalbhai et al., 2003).

Finally, ERK1/2 are MAPKs that are activated by MEK. MEK phosphorylates and activates ERK1/2 at amino acids tyrosine 185 and threonine 182 (Seger et al., 1992). All the ERK proteins share a common catalytic kinase domain (that also includes the tyrosine and threonine residues phosphorylated by MEK) that is surrounded by regulatory sequences. The catalytic domain contains the Common Docking (CD) motif that is
necessary for ERK binding and activation of the target proteins through phosphorylation (Tanoue et al., 2000).

The process of ERK activation is quite fascinating and depends on many factors. The first factor is the duration and strength of the signal coming from the upstream MAPK pathway regulators. ERK phosphorylation could either be transient or sustained. In PC12 cells, EGF causes proliferation and this correlates with strong and transient activation of ERK, whereas NGF causes differentiation and this correlates with sustained activation of ERK proteins (Nguyen et al., 1993). During fibroblast oncogenic transformation, constitutively active MAPKK mutants cause sustained ERK activation leading to cellular transformation and tumor formation in nude mice (Mansour et al., 1994). Once activated, ERK activates a wide variety of transcription factors: Elk-1, c-fos, p53, Ets1, Ets2, c-jun, c-myc and many others (Yoon and Seger, 2006).

In cancer, somatic B-Raf mutations are less frequent than Ras mutations in soft-tissue sarcomas. In one study, B-Raf mutations were diagnosed in 50% of malignant fibrous histiocytoma/pleomorphic sarcoma, 3% of Rhabdomyosarcoma, and 2% of synovial sarcoma. B-Raf was more frequently mutated in 70% of malignant melanomas and in 33% of papillary thyroid carcinomas (Davies et al., 2002; Kimura et al., 2003) (Martinelli et al., 2009). The majority of those mutations have Valine 599 replaced by glutamic acid residue inside the kinase loop that makes B-Raf constitutively active (Wan et al., 2004). The Valine 599 mutation in melanocytes causes tumorigenicity in nude mice (Wellbrock et al., 2004). Successful anti-cancer drugs have been designed to target Raf activity in cancer. BAY 43-9006 is a strong and selective inhibitor of Raf-1 kinase activity (Lyons et al., 2001), and does not affect the activity of other protein kinases like
ERK1, MEK1, EGF receptors and others, but on the other hand, it inhibits some angiogenic receptor tyrosine kinases (RTKs) like VEGF-R2, VEGF-R3, Flt-3, PDGFR-beta (Wilhelm et al., 2004).

Downstream of Raf, MEK1/2 and ERK1/2 have been shown to be over expressed in many cancers. MEK1/2 and ERK1/2 play an important role in soft-tissue sarcoma development. An increase of total ERK1/2 phosphorylation is detected in histological tumor sections from pleomorphic Rhabdomyosarcoma that develops from a K-Ras, p53-/- mice model (Tsumura et al., 2006). Many drugs have been developed to inhibit MEK1/2 and ERK1/2. The ERK1/2 proteins appear to be the only targets of MEK1/2, and all signals that converge to activate MEK1/2 will eventually stimulate ERK1/2. Thus anti-cancer drugs that targets and inhibit the MEK1/2 kinase activity would also inhibit ERK1/2 activity too. PD98059 and U0126 are drugs that are frequently used to study the role of MEK1/2 and ERK1/2 in many biological processes (Dudley et al., 1995; Favata et al., 1998), and U0126 significantly reduced the growth of pleomorphic Rhabdomyosarcoma tumors in a xenograft mouse model (Marampon et al., 2009). For clinical trials, other MEK1/2 inhibitors like PD184352, PD0325901 and ARRY-142886 are currently being tested (Sebolt-Leopold et al., 1999).

1.3.1. MAPK role in Ras oncogenic transformation

The early down stream events responsible for Ras oncogenic transformation and tumorigenesis have been elucidated. As mentioned earlier, the Ras protein recruits Raf-1 to the plasma membrane where it is activated. Raf-1 over expression has been shown to mimic the Ras transformation phenotype in rodent fibroblasts (Beck et al., 1987). The Raf-1 protein activates and phosphorylates MEK1. Ras and Raf-1 dependent
transformation is blocked by a dominant negative form of MEK1 (Cowley et al., 1994). These data reveals that, downstream of Ras, Raf-1 and MEK1 are necessary for transformation. MEK1 lead to sustained ERK1/2 activation leading to cellular transformation and tumor formation in nude mice (Mansour et al., 1994).

1.4. Ets family of transcription factors

1.4.1. 30 members with common and different functions

The founder member of the Ets family was first identified as a fusion protein of three different oncogenes: gag, myb and ets that are part of the avian transforming retrovirus E26 that induces myeloblastic and erythroblastic leukemias in chicken (Leprince et al., 1983). Since then, 30 different mammalian members of Ets family have been identified and characterized. The Ets family encodes transcription factors that regulate diverse processes important for cellular movement, cellular shape, proliferation, differentiation, apoptosis, angiogenesis, development and transformation. They all share an evolutionary conserved Ets domain of around 85 amino acid residues in length which recognizes a purine rich DNA sequence with a central core GGAA/T consensus and additional flanking nucleotides (Graves and Petersen, 1998). After binding to the DNA sequence in a promoter region, an Ets protein can either repress or activate transcription depending on the Ets member as well as its cellular partners (Graves and Petersen, 1998; Li et al., 2000a).

The Ets family is divided into subfamilies depending on the Ets DNA binding domains and the similarity in their structures. Most of the Ets families of transcription factors (like Ets1 and Ets2) have their Ets DNA binding domain at the C-terminal end, whereas some of them like the Ternary Complex Factor family (TCF: Elk-1, Sap-1, Net,
Netb) have their Ets domain at the N-terminal end (Kim et al., 2001) (Fig 1.1).

Furthermore, The expression of the different Ets transcription factors doesn’t follow the same pattern and sometimes it is restricted to specific tissues (Oikawa and Yamada, 2003) (Table.1.1).

**Fig.1.1: The Ets family**

The different subfamilies of the Ets transcription factors

*Adapted from Oikawa, T 2003*
Table 1.1. Tissue distribution of Ets family proteins

<table>
<thead>
<tr>
<th>Member</th>
<th>Expressing organs and tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ets-1</td>
<td>Lymphoid organs, brain, vascular endothelial cells</td>
</tr>
<tr>
<td>Ets-2</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>Erg</td>
<td>Vascular endothelial cells, hematopoietic cells, kidney, etc.</td>
</tr>
<tr>
<td>Fli-1</td>
<td>Hematopoietic cells, vascular endothelial cells</td>
</tr>
<tr>
<td>GABPα</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>TEL</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>PEA3/E1AF</td>
<td>Epidermis, mammary gland, brain, etc.</td>
</tr>
<tr>
<td>Elf-1</td>
<td>Hematopoietic cells, liver, kidney, intestine, etc.</td>
</tr>
<tr>
<td>ESE-1/ESX</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>PU.1</td>
<td>B cells, macrophages, neutrophils</td>
</tr>
<tr>
<td>TCFs</td>
<td>Ubiquitous</td>
</tr>
</tbody>
</table>

1.4.2. Ets1 and Ets2 structures and functions

Ets1 and Ets2 share a high similarity in their structure and their amino acid sequences. In addition to the Ets DNA binding domain, they also have around 86 amino acid residues that are highly conserved in their Pointed Domain at the N-terminal end. The term “pointed” originated in the drosophila pointed Ets protein. It has been shown that Pointed is a direct target of the Ras/MAPK pathway in drosophila (Brunner et al., 1994). Interestingly, the drosophila pointed protein shares a highly evolutionary conserved MAPK phosphorylation site with both Ets1 and Ets2 (threonine 38 in Ets-1 and threonine 72 in Ets2) that is around 14 amino acids away from the PNT domain that serves as a docking site for ERK1 and ERK2 to bind, phosphorylate and activate Ets1 and Ets2 (Klämbt, 1993; Yang et al., 1996) (Fig 1.2).
Fig 1.2 Evolutionary conservation of the Ets MAPK phosphorylation site

(A) Ets1 and Ets2 proteins have three kinds of domains: The PNT domain needed for ERK1/2 docking, the DNA binding domain and the AD (activation domain) needed for protein/protein interaction and activation of transcription. The asterix points to the ERK1/2 phosphorylation sites. (B) the phosphorylation site is highly conserved in Ets1 and Ets2.

Adapted from Yang, B 1996
Ets1 knockout mouse models have revealed the importance of Ets1 in the immune system development. Ets1 is necessary for the formation of the Natural Killers cells (NK) and the development of the early as well as the mature B cells (Barton et al., 1998; Eyquem et al., 2004). Ets1 is also important for the development of the CD8+ thymocytes (Clements et al., 2006). In order to complement the interesting findings from the Ets1-/- mice models, over expression of Ets1 in different cell lines revealed the mechanism through which Ets1 is able to regulate proper development of the immune system. These over expression studies revealed that Ets1 is required to collaborate with other transcription factors in order to activate genes that are important for B cell and T cell maturation. Ets1 collaborates with LEF-1 and ATF2 transcription factors to activate the TCR alpha and beta genes that function specifically in the T lymphocytes (Giese et al., 1995; Kim et al., 1999). Other studies showed that Ets1 collaborate withSTAT5, GATA3, PAX5, c-myb and JAK3 in order to activate many genes that are necessary for B and T cell development (Aringer et al., 2003; Blumenthal et al., 1999; Maier et al., 2003; Postigo et al., 1997; Rameil et al., 2000; Sieweke et al., 1996). Ets1 is also required for the proper functioning of the heart, and a new Ets1 knockout study revealed that Ets1 directs the proper migration and differentiation of the cardiac neural crest (Gao et al., 2010).

Ets2 is essential for the embryonic development. The Ets2-/- mice are extra-embryonic lethal (Yamamoto et al., 1998). They undergo resorption and die by day 8.5. The Ets2-/- embryos show many defects. They have a small ectoplacental cone region and they lack the amnion and the chorion membranes. The Ets2-/- embryos express less matrix metalloproteinase-9 (MMP9) which is essential for the extracellular matrix
remodeling (Yamamoto et al., 1998). A previous study by Sumarsono et al suggested that the human Ets2 gene seems to be involved in the Down syndrome phenotype. In the Down syndrome patients Ets2 is located at chromosome 21 inside the minimal Down’s syndrome trizomy region, thus the Down syndrome patients have one extra Ets2 allele. The transgenic mice that overexpress Ets2 develop abnormalities similar to the mice that have trisomy 16, and Sumarsono et al suggested that the abnormalities observed in the Down syndrome patients are because of the extra Ets2 copy present in the trizomy mice. These mice have abnormalities in the immune system, the small thymus and the skeleton (Sumarsono et al., 1996). But a recent study by Hill et al suggested that an extra Ets2 copy is not sufficient to develop the abnormalities that are seen in trisomy 16 mice and consequently in Down syndrome patients. In this study, Hill et al crossed the trisomy 16 mice to Ets2+/- mice in order to generate trisomy mice that don’t have the Ets2 allele. The trisomy mice that lacked one Ets2 copy developed abnormalities similar to the trisomy mice that have all three copies of Ets2, thus dismissing Sumarsono hypothesis and suggesting that perturbation in the development of Down syndrome phenotype is due to many genes and not only Ets2 (Hill et al., 2009).

Both Ets1 and Ets2 play an important role in angiogenesis. Over expression and dominant negative studies revealed that Ets1 regulates angiogenesis genes like the VEGF receptor and the endothelial cells receptors Flk1 and Tie-2 (Dejana et al., 2007; Hashiya et al., 2004; Lelievre et al., 2001). Also Ets1 has been shown to regulate the endothelial cell migration through the regulation of the MMP1 and MMP9 extracellular proteases (Iwasaka et al., 1996). Similarly, Ets2 has been shown to regulate the endothelial cell function in vitro and knock down of Ets2 by siRNA inhibited the ability of the
endothelial cells to form *in vitro* capillary network (Petrovic et al., 2003). In our laboratory, using the Ets1 and Ets2 knockout models, Wei et al showed that both Ets1 and Ets2 are required for the endothelial cell survival during embryonic development. Deletion of both Ets1 and Ets2 caused embryonic lethality that was mostly due to defects in vascular branching. Gene analysis and chromatin immuno-precipitation showed that Ets1 and Ets2 activate and bind to the promoters of MMP9 and bclxL (antiapoptotic) genes and as a consequence, apoptosis was increased in Ets1/2 double knockout endothelial cells *in vitro*. (Wei et al., 2009).

### 1.4.3. Ets1 and Ets2 role in cancer

In human cancer, both Ets1 and Ets2 are abnormally expressed during cancer development. Ets1 and Ets2 are amplified in many leukemias, and both have been shown to be over expressed in breast cancer. Ets1 is also over expressed in the lung, colon, pancreatic and thyroid cancer. Ets2 is over expressed in the hepatic, cervical, prostate and esophageal cancer (Seth and Watson, 2005). In a recent study that could suggest that Ets1 and Ets2 are involved in ocular tumor formation, Ets1 and Ets2 are up regulated in a transgenic mouse model of pigmented ocular neoplasm (De la Houssaye et al., 2008).

Over expression studies using dominant negative Ets proteins revealed the importance of Ets1 and Ets2 in cancer. Over expression of a dominant-negative Ets-1 protein in pancreatic tumor cell lines inhibited their tumor growth *in vivo* in nude mice (Lefter et al., 2009). In thyroid cancer, over expression of a dominant-negative Ets-LacZ protein (a hybrid protein that has the DNA binding domain bound to Beta-galactosidase) into thyroid cancer cells decreased the level of Ets target genes like MMP-1 and uPA.
genes and blocked in vitro transformation of these cells when put into soft agar (de Nigris et al., 2001a).

Knock down experiments in renal carcinoma and glioma cell lines revealed that deletion of Ets1 through stable shRNA transfection inhibited tumor formation in mice (Holterman et al., 2010).

1.4.4. Ets1 and Ets2 as effectors of the Ras/MAPK pathway

Ets1 and Ets2 are activated by the ERK1/2 phosphorylation of their respective amino acids residues threonine 38 and 72 that are located around 14 amino acids far from the PNT domain where ERK1 and ERK2 bind to Ets1 and Ets2 in response to Ras signaling. Phosphorylated Ets1 and Ets2 work as transcriptional activators and they have been shown to activate many gene promoters. Both the MAPK phosphorylation domain and the PNT domains are important for the transcriptional activation of Ets1 and Ets2. Mutations of the MAPK phosphorylation sites Ets1T38 and Ets2T72 or the PNT domains in Ets1 or Ets2 abrogate the transcriptional activation of Ets1 and Ets2 to their target genes (Yang et al., 1996). ERK1/2 dependent phosphorylation of Ets1 and Ets2 recruit the CBP/p300 complex. The CBP/p300 complex is a transcriptional co-activator that has an intrinsic histone acetyltransferase activity that helps relaxing the chromatin structure and recruits the basal transcriptional machinery including RNA polymerase II to the promoter (Foulds et al., 2004; Vo and Goodman, 2001). AP-1, a transcription factor that is also activated by the Ras/MAPK pathway in response to mitogenic signals, collaborates with transcriptional activated Ets1/2 to superactivate the transcriptions of genes like uPA (urokinase) and nvl-3 (a member of the VL-30 elements). The mutation
of the AP-1 in uPA and nvl-3 abrogate the Ras/Ets1/2 superactivation of the promoter of these genes (Yang et al., 1996).

One of the biological roles of Ets2 phosphorylation by the Ras/MAPK pathway is the persistent activation of the inflammatory response in response to CSF-1 signal. One study by Wei et al, showed that Ets2T72A/Ets2T72A homozygous mutant mice showed decreased inflammation as well as increased survival after being crossed with mev/mev homozygous mice. The mev/mev homozygous mice have a mutation in the hemopoietic cell phosphatase gene, thus rendering the MAPK pathway constitutively active and leading to chronic inflammatory response through massive production of TNF alpha in response to CSF1. The Ets2T72A mutation was able to block the signal transduction cascade in macrophages started by CSF1 and CSF1 Receptor by inhibiting the production of TNF alpha. TNF alpha is an Ets target gene and a decrease in its production leads to diminished inflammatory response. Similarly, bclxL which is an anti-apoptotic molecule and an ets target gene is downregulated in Ets2T72A/T72A mutant macrophages that undergo apoptosis (Wei et al., 2004).

In the stromal tumor microenvironment of the mouse breast cancer, Ets2 activation in PTEN-/- mice (where PTEN has been deleted from the fibroblasts) lead to an increase in MMP9 production and cleavage of VEGF-A into VEGF 164 which is important for the development of the tumor angiogenesis (Trimboli et al., 2009).

1.4.5. Ets2 role as a transcriptional repressor in cancer

In addition to the well established role of Ets2 as a transcriptional activator, some studies revealed that Ets2 can work as a transcriptional repressor.
Ets2 is able to repressor BRCA1 tumor suppressor gene in MCF-7 breast cancer cell line. This repression is dependent on the physical interaction of Ets2 with Brg-1. Brg-1 is a mammalian ATPase hydrolyzing subunit that is part of the SWI/SNF chromatin remodeling complex that is required for E2F/Rb mediated repression of gene expression. Transient transfection assays show that Brg-1 and Ets2 are both required to repress the activity of BRCA1 promoter and to inhibit the BRCA1 gene expression respectively. Brg-1 GST pull down assays show that Brg-1 has more binding affinity to non phosphorylated Ets2 and co-immunoprecipitate better with non phosphorylated Ets2 (Baker et al., 2003).

In a parallel study, Ets2 was able to repress BRCA1 and uPA expression through physical interaction with BS69 co-repressor molecule. Similarly Wei et al shows that the physical interaction of Ets2 with BS69 is also phospho-dependent and there is more GST pull down of BS69 with non phosphorylated Ets2. Transient transfection assays revealed that co-expression of Ets2 and BS69 results in repression of BRCA1 and uPA genes (Wei et al., 2003).

In the tumor microenvironment, deletion of Ets2 from the tumor associated macrophages leads to a decrease in lung metastasis in spontaneous, orthotropic and tail vein injection breast cancer models, and Ets2 was able to repress the expression of extracellular modifying genes like Thrombospondin 1, Thrombospondin 2, Timp1 and Timp3. Chromatin Immunoprecipitation assays showed that Ets2 is present at the promoters of those genes together with the repressor protein HDAC1, suggesting that Ets2 repression of certain genes is also important for tumor progression and metastasis (Zabuawala et al., 2010).
1.5. Ets1 and Ets2 role in Ras oncogenic transformation

Ras activates the MAPK pathway that has been shown to be critical for the mitogenic effect of Ras and important during Ras transformation (Cowley et al., 1994; Dudley et al., 1995; Marshall, 1995). The necessary role of the Ets proteins in Ras transformation has been revealed through inhibition experiments. During Ras oncogenic transformation, over expression of any of the Ets members is able to inhibit Ras transformation even members like PU.1 that are only expressed in hematopoietic cells. This suggests that in normal cases, the binding of the Ets factor to the DNA is necessary to mediate Ras transformation, and over expression of any Ets member will make it more abundant in the nucleus compared with the important Ets members, and thus block the binding of the necessary Ets members to the DNA in order to activate or repress genes that are important for the Ras transformation. Langer et al have shown that over expression of the Ets2 DNA binding domain fused to lac Z in NIH 3T3 cells inhibited Ras oncogenic transformation (Langer et al., 1992). Over expression of the trans-dominant mutants PU.1 or Ets1 or Ets2 that only express the DNA binding domain also blocked Ras transformation phenotype (Wasylyk et al., 1994). Similarly, over expression of a full Ets2 protein or truncated portions of Ets2 that only have the N-terminal domain (that includes the MAPK phosphorylation site, PNT and the transactivation domain) was also able to suppress Ras mediated transformation by suppressing the ability of Ras to form soft agar colonies in vitro (Foos et al., 1998). This study revealed that overexpression of truncated Ets2 proteins may be recruiting ERK1 and ERK2 to their docking sites, thus driving them away from the important Ets factors that are necessary for Ras transformation.
Although these studies show the critical role of the Ets factors during transformation, but they do not address which Ets factors are required for Ras transformation. Only one group tried to address the specificity of Ets members during Ras transformation by deleting Ets2 in ES cells and surprisingly, this group found that Ets2 deletion did not inhibit Ras transformation. Furthermore, Ets2 over expression in Ets2 db Ras transformed NIH3T3 cells (Ets2 db is a null allele) did not enhance the ability of NIH3T3 cells to increase foci formation in vitro (Hever et al., 2003).

1.5.1. C-myc and Ras collaboration in Ras oncogenic transformation

C-myc is a transcription factor that plays an essential role in the control of cellular proliferation, differentiation and apoptosis. C-myc plays an oncogenic role in tumor development, and has been shown to be over expressed and amplified in soft tissue sarcomas (Barrios et al., 1994; Moritake et al., 2002; Sollazzo et al., 1999). C-myc translocation has been observed in Burkitt’s and AIDS-related lymphomas. Also, C-myc gene amplification has been shown in breast and prostate. Interestingly, C-myc transcriptional activity has been found to be enhanced in colo-rectal cancer and its translation to be high in multiple myeloma (Nesbit et al., 1999).

C-myc similar to Ras, is able to transform immortalized MEFs, but unlike Ras transformed MEFs that are morphologically transformed in culture and growth independently in soft agar, myc transformed MEFs look absolutely normal in culture and grow very poorly in soft agar, but they are tumorigenic once injected in nude mice (Keath et al., 1984).

However during Ras transformation, C-myc is necessary and critical for efficient transformation. That is supported by the fact that the Ras/Raf/MAPK pathway is able to
stabilize C-myc and increase its accumulation in the cell. The C-myc protein has a short 
have life of 30 min and it is target for degradation by ubiquitination through the 26S 
proteasome. In quiescent cells, myc is degraded rapidly, but with the presence of a 
constitutively active Ras or Raf, c-myc is stabilized and expressed, and addition of 26S 
proteasome inhibitors did not enhance its accumulation (Sears et al., 1999).

Another study by Sklar et al shows that C-myc is necessary and required for Ras 
transformation. Depletion and deletion of C-myc through continuous expression of anti-
sense nucleotides caused Ras transformed cells to lose their transformation properties. 
Ras fibroblasts that lacks C-myc are flat cells and don’t grow in soft unlike Ras wild type 
cells. Moreover, injection of these cells into nude mice failed to induce tumorigenesis 
(Sklar et al., 1991).

1.5.2. C-myc/Ets1/Ets2 interaction during transformation

Since the Ras/MAPK have been shown to be involved in the regulation of C-myc, 
the down stream effectors of Ras/MAPK have been also shown to modulate and regulate 
C-myc activity. Interestingly, Langer et al found that during Ras transformation, C-myc 
RNA expression significantly decreased in the presence of an over expressed Ets-lacZ 
dominant construct, thus suggesting that C-myc could be directly or indirectly regulated 
by Ets. Overexpression of exogenous C-myc in Ets-laZ Ras non transformed cells was 
able to rescue transformation. (Langer et al., 1992).

Similarly, during human thyroid transformation, C-myc has been shown to be 
over expressed in thyroid cancer cells that also show ever expression of Ets1 and Ets2. 
Similar to Langer et al, Nigris et al introduced the dominant negative Ets/LacZ construct 
into thyroid cancer cells and consequently found that C-myc expression is decreased. C-
myc decrease leads to apoptosis as well as reduces the anti-apoptotic Bcl-2 and BclxL proteins expression, and increases the Bax protein expression. Reintroduction and over expression of C-myc in these Ets-lacZ thyroid cancer cells rescued them from apoptosis (de Nigris et al., 2001a).

Ets1 and Ets2 have been shown to control C-myc expression through a single minimal binding site of the E2F transcription factors by the transactivation of reporter genes driven by the C-myc promoter (Roussel et al., 1994).

In endocrine resistant breast cancer cells, Ets2 has been shown to activate C-myc expression and was found to bind to the C-myc promoter by Chromatin immunoprecipitation. In these cells, knock down of Ets2 induced apoptosis and correlated with a decrease in C-myc expression (Al-azawi et al., 2008).

Finally, Ets2 and C-myc were found to physically interact and form a complex that regulates the hTERT gene expression in breast cancer cells. Ets2 and C-myc have their respective EBS and E-box binding sites in proximity on the hTERT promoter and disruption or mutation of the EBS or E-box sites disrupted the ability of either Ets2 or C-myc to bind and transactivate the hTERT promoter. Deletion of Ets lead to apoptosis in these MCF-7 breast cancer cell line (Xu et al., 2008).

1.6. MiR17-92 Cluster Role in cancer

1.6.1. MicroRNAs

MicroRNAs (miRs) are small RNA molecules of 22-24 nucleotides long that cause posttranscriptional gene silencing through binding to the 3’ untranslated regions of their target mRNAs. They regulate many biological processes: proliferation, development, cellular differentiation and apoptosis. Recently, they have been shown to be
involved in the pathogenesis of cancer and other metabolic diseases. To date, around
hundred miRNA genes have been discovered in invertebrates, and around a thousand in
vertebrates and plants. Bioinformatics and computational predictions calculate the
possible mRNA target genes for each microRNA to be many hundreds. This actually
suggests that a huge part of the transcriptome is regulated by microRNAs.

In order to perform their function in mRNA regulation, a microRNA together
with the Argonaute family proteins assemble and form a complex called miRNA-induced
silencing complex (miRISC) that is able to silence mRNA post-transcriptionally. There
are many proposed theories about microRNA posttranscriptional regulation of mRNA.
The first model proposed is called “Postinitiation mechanism”, and it suggests that
miRNAs block the translation elongation process or promotes the dissociation of
ribosomes during this process. The second model is called “Co-translational protein
degradation” and it suggests that the elongation process takes places but an unknown
protease is recruited and degrades the polypeptide chain. The third model is called “
initiation mechanism” and it suggests that miRISC interfere with the initial steps of
translation either by competing with eIF4E for binding to the cap structure of the mRNA
or miRISCs recruit eIF6 that would prevents the large ribosomal subunit from joining the
small ribosomal subunit. A fourth mechanism suggests that the miRISCs would prevent
the formation of a closed mRNA loop essential to start translation through deadenylation
of the 3’ end of the mRNA. Finally, the last mechanism is called “microRNA-mediated
mRNA decay” and during this process miRISCs would trigger deadenylation and
decapping of the mRNA and thus its degradation (Eulalio et al., 2008).
Fig1.3. Different mechanisms for miRNA-Mediated Gene Silencing

Adapted from Eulalio, A 2008
1.6.2. MiR17-92 Cluster

The human polycistronic MicroRNA-17-92 cluster is found on chromosome 13 and contains 6 microRNAs: miR-17, miR-18a, miR-19a, miR20, miR-19b and miR-92a. The cluster has a single promoter and is first transcribed as one primary transcript before further processing that will yield 6 mature microRNAs. The cluster is important in the regulation of hematopoiesis, the immune system and the cardiopulmonary system (Bonauer and Dimmeler, 2009). The cluster has been extensively studied because of its important role in cancer pathogenesis. Fig 1.4 summarizes the different functions of the miR17-92 cluster.
Fig1.4: Summary of the miR17-92 cluster function

Adapted from Bonauer, A 2009
In the immune system, miR17-92 transgenic over expression increased the proliferation and survival of the B-cells and T-cells and thus causing lymphoma and autoimmunity in mice. In these mice, two tumor suppressor genes were identified that were functional targets for the miR17-92 cluster: the phosphatase PTEN and the pro-apoptotic protein: Bim (Xiao et al., 2008). In a separate study, the cell cycle inhibitor p21 was identified as another target for the cluster in B-cell lymphoma cell lines (Inomata et al., 2009). In knockout mice that lack the miR17-92 cluster, B-cell development was inhibited and these cells showed increased apoptosis as well as increased Bim expression (Ventura et al., 2008). Using miR17-92 deficient cells for the reconstruction of mature mice immune system caused a significant decrease in mature B cells but did not have any change on pro B-cells or other types of hematopoietic cells. Finally, miR17-92 cluster control monocyte differentiation and maturation of the immune system (Fontana et al., 2007).

In development, miR17-92 deficient mice show developmental defects in the lung and the heart that eventually leads to early death of these mice after birth (Ventura et al., 2008). The miR17-92 null embryos possess serious hypoplastic lungs because of decrease in lung epithelium proliferation caused by premature differentiation of lung progenitor epithelial cells (Lu et al., 2007).

1.6.3. Regulation of the miR17-92 Cluster

As mentioned earlier, the expression of the miR-17-92 cluster is regulated from a single promoter. The first transcription factor discovered to regulate miR17-92 is C-myc. It has been shown that C-myc stimulates the transcription of the cluster in human B-cells and chronic myeloid leukemia cells (O'Donnell et al., 2005; Venturini et al., 2007). The
C-myc homologue, N-myc was also shown to regulate this cluster in neuroblastoma and medulloblastoma cells (Northcott et al., 2009).

In addition to C-myc, cell cycle regulators have been shown to regulate this cluster. E2Fs and Cyclin D1 regulates the cluster and interestingly are inhibited by miR-17 and miR20, thus forming a negative feedback loop (Sylvestre et al., 2007; Woods et al., 2007; Yu et al., 2008).

Inflammatory cytokines are also able to regulate the Cluster. In the ischemic muscles and the heart both IL-6 and VEGF increases the expression of the cluster (Brock et al., 2009; Suarez et al., 2008).

After transcription of the pre-miR17-92, Drosha and Dicer proteins bind to it leading to processing and maturation of the pre-miR into individual miRs. Interestingly, the processing of this microRNA can be selective with RNA binding proteins possibly favoring more processing of particular miRs in the cluster and thus yielding different amount of expression among individual mature miRs. One example is miR18-a whose processing is selectively facilitated by Drosha through the RNA-binding protein hnRNP A1 without affecting the processing of the other miRs (Davis et al., 2008; Guil and Caceres, 2007; Newman et al., 2008; Thomson et al., 2006; Viswanathan et al., 2008; Woods et al., 2007).

1.6.4. Role of miR17-92 Cluster in cancer

The miR17-92 cluster has oncogenic properties. The miRNAs contained in the cluster are over expressed in a variety of cancer of hematopoietic and solid tumors from many tissues: breast, lung, pancreas, colon, prostate, and stomach (Volinia et al., 2006). MiR17-92 cluster cooperate with C-myc in a mouse model of B-cell lymphoma, where
overexpression of miR17-92 increased the onset of lymphoma progression through the inhibition of the high level of apoptosis that is usually observed in C-myc induced lymphomas (He et al., 2005). And as mentioned previously, O’Donnell et al have shown that myc also transactivate the miR17-92 cluster. Inversely, Deletion of miR17-92 cluster in a C-myc lymphoma model slowed the c-myc induced oncogenesis, and reintroduction of the miR17-92 cluster rescued myc oncogenesis. Interestingly, reintroduction of the miR17-92 cluster lacking miR19 failed to rescue myc oncogenesis, indicating that the miR19a/19b is essential for myc oncogenesis. Reintroduction of only miR19a/19b in the myc lymphoma model only partially rescued tumorigenesis thus suggesting that other members of the cluster are also involved (Mu et al., 2009).

Recently, a major effort has been done to elucidate and identify miR17-92 targets in cancer, but only three targets have been identified: PTEN, E2F1 and Bim. PTEN is a miR-19 target and has been shown to be suppressed in the myc lymphoma mouse model which may help explain the oncogenic phenotype present in these mice (Olive et al., 2009).

E2F1, E2F2 and E2F3 are all targets of miR-17 and miR-20. It has been reported that overexpression of E2F1, E2F2 and E2F3 (that are know to drive G1 to S transition) induces apoptosis that protect the cell from over proliferation effects. Thus miR17 and miR-20 are able to inhibit the over production of E2Fs and thus disable the emergency break function of E2Fs and as a consequence of oncogenic cellular growth (O'Donnell et al., 2005; Sylvestre et al., 2007; Woods et al., 2007).

Bim, a pro-apoptotic molecule, was shown to suppress myc-induced mouse B cell leukemia. It is a target of many miRs in the miR17-92 cluster and thus down regulation
of this protein by the cluster has been shown to contribute to the oncogenic properties of
the miR17-92 cluster (Egle et al., 2004).

Finally the contribution of the miR17-92 cluster is still on going and the
mechanism through which miR17-92 induces cancer through different signal transduction
pathways and especially through the Ras/MAPK pathway which regulates C-myc, still
remains to be elucidated and studied carefully.
2.1. Animal Husbandry

2.1.1. Transgenic mice

The mice of the Ets-1 knockout were provided by Dr. Muthusamy (The Ohio State University, Columbus, OH) (Muthusamy et al., 1995). The conditional Ets2 loxP transgenic mice were generated by Dr Guo Wei (Wei et al., 2009).

2.1.2. Animal Care

For all our studies the mice were housed in the BRT animal facility (Biomedical Research Tower) at the Ohio State University with accordance to the National Institute of Health regulations. The mice were sacrificed in accordance with the animal resources standard operating procedures. The mice work was approved by the Ohio State University Institutional Animal Care and Use Committee.

2.1.3. Mouse Genotyping

2.1.3.1. Tail DNA Preparation

Mouse tails are cut and digested in 200ul of lysis buffer (50 mM KCl, 10mM Tris-HCl (pH8.3), 0.1 mg/ml gelatin, 0.45% NP40, 0.45% Tween20, 1mg/ml ProteinaseK), at 55°C overnight (O/N). Then the tail samples are boiled for 10 min to
inactivate the proteinaseK enzyme activity. After that, tails are stored at 4°C for future use.

2.1.3.2. Genotyping Primers and PCR Conditions

A volume of 2µl from the tail DNA is used in a total 20µl PCR reaction. The PCR program used is as follows:

Step1: 95°C for 1 minute
Step 2: 95°C (for 45 sec) followed by annealing temperature according to table 2.1 (45 sec) followed by 72°C (for 1 min). Step 2 is repeated 35 cycles.

Step 3: 1 cycle of 72°C (for 10 min). The PCR products were run on a 1.5% agarose gel.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer ID</th>
<th>Sequence</th>
<th>Annealing T°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ets1</td>
<td>Ets1-I4P2</td>
<td>CAACAACAGCAAAGAGCATCC</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Ets1-E4P2</td>
<td>ACTGTGTGCCCTGGGTAAAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PGK-P1</td>
<td>CTAAAGCGCATGCTCCAGACTGCC</td>
<td></td>
</tr>
<tr>
<td>Ets2</td>
<td>Ets2-common-F</td>
<td>TGAACCTACTGTGTGTGACGAGGA</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Ets2-flox-R</td>
<td>GGAAGAAACGGGAAATCAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PGK-P1</td>
<td>GGATTTTAGCCCCAGAAACTTAGA</td>
<td></td>
</tr>
<tr>
<td>Cre</td>
<td>WCre1-F</td>
<td>CCTGTGGTGACGATCCCG</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>WCre3-R</td>
<td>ATGCTTCTGTCCGTTGCG</td>
<td></td>
</tr>
<tr>
<td>C-myc</td>
<td>Myc2(knockout)</td>
<td>AATTAAAGCTGACCCCGCGGCA</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>3’flox</td>
<td>TACAGTCCCCAAAGCCCCAGCCAAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’flox(wild type)</td>
<td>CACCGCCTACATCCTGTCATTC</td>
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</tr>
</tbody>
</table>

Table 2.1 Genotyping primers for a list of genes used for this study
2.1.4. Animal Procedures

2.1.4.1. Mouse Embryonic Fibroblasts Dissection

13.5 days old pregnant mouse was sacrificed put in 70% ethanol. With scissors and forceps, an incision was prepared on the abdominal side of the skin. That was followed by skin removal and extensive washing of the peritoneum with 70% ethanol. Then abdominal cavity was opened and the uterus was taken and washed three times with PBS by transferring it into three consecutive 50 ml PBS filled conical. In the hood the uterus was incubated in a 100 mm dish containing 10 ml of PBS. Then, the embryos were removed and each embryo was placed in a 60 mm dish covered with 3 ml of PBS where the placenta and yolk sac were detached from the embryos, and only the yolk sac was saved for genotyping. The Red parts or tissues in the embryo like the liver, spleen and heart were totally taken out and the white part of the embryo was incubated in a 60 mm dish containing 5 ml of trypsin, and using the scissors and scalpels the embryo was well minced, and the 60 mm dish that have the embryo + trypsin was transferred into the incubator for 15 minutes. The trypsinized embryo was put into a 15 ml of 10% FBS in DMEM medium. After centrifugation, cells were resuspended in 10% FBS in DMEM medium inside a 60 mm dish. The next day the media was changed for the cells. Once confluent the cells where transferred into 100 mm dish and freeze downs were made from them.
2.2. Tissue Culture and Viral Infections

2.2.1. Mouse Embryonic Fibroblast Immortalization

Primary Mouse Embryonic Fibroblasts were genotyped first. The following genotypes were used for further passaging in order to make established cell lines: Ets1+/−, Ets2 f/f, E1+/+ E2+/+, E1−/- E2f/f, E1−/- E2−/- more cre. Three primary MEFs from three different embryos having the same genotypes were run in parallel. And using the Todaro et al protocol for establishing MEFs cell lines (Todaro and Green, 1963), these MEFs were passaged at a density of 3.5 x 10^5 cells/60 mm dish for three days in 10% FBS in DMEM for consecutive passages. Between passage 15 and 21 these cells underwent senescence, but were maintained at density of 3.5x 10^5 cells/60mm dish for 3 to 4 passages until they started growing again. All those cells were starting to grow at passage 24 and freeze downs were made of them at passage 25. Passage 26 MEFs were used for all the experiments. C-mycf/f established MEFs were a kind gift from Dr Gustavo Leone Lab. All the experiments were done with DMEM media supplemented with 10% FBS and Penicillin Streptomycin solution.

2.2.2. Retroviral Infections

2.2.2.1 Retroviral vectors used

The following retroviral vectors were used for all the experiments: pbabe-Hygro-H-Rasv12 that have a G12V mutation in the human Ras gene that makes Ras constitutively active, pbabe-Hygro-empty vector that has no Ras, pbabe-puro-cre that expresses the Cre recombinase protein, pbabe-puro-empty that does not have Cre, pMSCVpuro-miR17-92 that expresses the miR17-92 cluster and pMSCVpuro-empty that does not have the cluster, MSCV-GFP-myc-ER™ that consists of the fusion of human C-
myc with the estrogen binding domain of the estrogen receptor (ER) modified to bind to estrogen agonist tamoxifen (kind gift from Dr Leone Lab) and MSCV-GFP vectors. Induction of MSCV-GFP-myc-ER was done using 1μM of 4-hydroxyTamoxifen (4OHT). The concentration was 10 times more than what is listed in the original paper where the vector has been designed (Littlewood et al., 1995).

2.2.2.2 Pheonix cells transfection for virus packaging

1.5 X 10^6 pheonix cells were grown in 60 mm dishes. Next day, 3 ml of fresh media were added to the phoenix cells plate. Transfection was done using 500 μl of CaCl₂ (0.2 M final concentration) mixed with 6 to 10 μg of DNA of the desired retroviral vector mentioned above. That was followed by addition of 500 μl HBS (1X HBS from 2XHBS stock: 0.82 g of NaCl, 0.595 g HEPES, 0.01g Na₂HPO₄ in 50 ml water) drop by drop to the DNA/ CaCl₂ mixture. The 1ml HBS/DNA/ CaCl₂ solution was added to the phoenix cell plate. After 24 hrs media was replaced to remove CaCl₂ and replaced by fresh media. At 48 and 72 hrs the first and second virus supernatant were collected and either saved at -70°C or used directly for infection. The transfection protocol was adopted from the Stanford University website using the following link: http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html.

2.2.2.3 Retroviral infection

MEFs were seeded in 6 well plates at 1.5 x 10^5 cells/well until they reached 70% confluency. At 70% confluency 4μg/ml Polybrene was added at a final concentration to the first virus supernatant, and 2 ml of virus was added to each well. After 6 hrs fresh media was added on top of the virus supernatant. After 24 hrs the second virus supernatant was added for another 24 hrs. Next day the virus supernatant was removed.
and replaced with fresh media or passaged to a 60 mm dish if confluent. Cells were given 24 additional hrs to grow before being either selected with puromycin, or hygromycin or sorted by FACs in case of GFP retrovirus. Puromycin was added at a concentration of 4µg/ml for 3 days. Hygromycin was added at a concentration of 200 µg/ml for 5 to 6 days. GFP cells were re-suspended in PBS and brought to the FACS/Aria machine for sorting. Pure GFP population was then collected and used for the experiments. The infection protocol was adopted from the Stanford University website using the following link: http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html.

2.2.3. Transient transfection

Using the lipofectamine 2000 transient transfection protocol according to invitrogen’s manual instruction, we transfected 4 µg of FNEts1, FNEts2, FNpcDNA3, pbabe-hygro-empty, and pbabe-hygro-myc vectors into c-myc-/MEFs seeded into 60mm dish at 3.5x 10^5 cells/plate, together with 15 µl of lipofectamine. Lipofectamine and DNA were mixed together for 20 min before being added into the MEFs in plain DMEM. The transfection cocktail was added for 6 hrs than changed with 10%FBS media for 36 hrs. After 36 hrs, the cells were lysed with trizol and kept at -70 °C until further RNA processing.

2.3. RNA generation

2.3.1. RNA Extraction

MEFs were lysed in 500 µl or 1ml of Trizol (Invitrogen) and stored at -80°C. The Trizol RNA solution was thawed next day for 20 minutes at room temperature. 100 to 200 µl of Chloroform was added. The samples were centrifuged at 12000 g for 15 min, supernatant was then collected and mixed with 250 to 500 µl of isopropanol. Another
centrifugation at 12000 g for 10 min was performed to pellet the RNA. Pelleted RNA was resuspended in 75% RNase-free DEPC ethanol. A final round of centrifugation at 7500 g was performed then RNA pellet was resuspended in 30 to 50μl of RNase-free DEPC water for 5 minutes at 55°C. RNA quantification was done using the OD$_{260}$, and RNA purity was considered pure if the following ratio: OD$_{260}$/OD$_{280}$ was equal or greater than 1.8. DNA was degraded using the Ambion Turbo-DNAse free kit according to the manufacturer’s instructions.

2.3.2. Reverse Transcription

cDNA was made from 2μg of purified total RNA (RNA was purified using RNeasy kit with columns from In Vitrogen). The reverse transcription reaction to make cDNA was done as follows: 2ug of RNA was mixed with 0.2μg random hexamers and RNase-free water in a total volume of 15 µl/sample. the samples were places in the PCR machine and programmed to run at 65°C for 5 min. then the samples were kept on ice for 5 to 10 minutes. Finally, the sample volume was brought to 25 μl through the addition of 5μl 5X First strand buffer (Invitrogen), 1μl 0.1M DTT (Invitrogen), 10μM dNTPs (Roche), 1μl Superscript III and 1μl RNAse OUT (Invitrogen). The samples were put back in the thermocycler at 25°C for 10 min to allow primers to anneal to RNA, then at 50°C for 60min for reverse transcription, and at 70°C for 15min to inactivate the reaction. At the end, cDNA sample was diluted to 200μl. 4 μl in duplicates from each cDNA sample was used for each Real-Time PCR reaction.
2.3.3. Primers used for real-time PCR

Most of the RNA primers used for this study were designed using Roche Universal Probe Library System. To avoid non specificity in mature RNA detection, an intron-spanning primers were designed. This is the list of primes used for this study as shown in Table 2.2. RpL4 was used for expression normalization.
<table>
<thead>
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</tbody>
</table>
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2.3.4. micRNA gene expression

Total RNA was extracted from cells using TRizol reagent (Invitrogen). cDNA was subsequently synthesized from 5 ug RNA by oligo dT primer and superscript II (invitogen). Quantitative analysis of genes expression was done using commercial primer sets (biosystem). For normalization of expression levels 18s and GAPDH was used. Quantitative analysis of mature miRNA expression was performed by real-time PCR using Taqman microRNAs assays (Applied Biosystems). For normalization of expression levels U6 snRNA and sno RNA 202 (Applied Biosystems) were used. Real-time quantitative RT-PCR experiments were performed in the ABI Prism 7700 System (Applied Biosystems). Real time PCR was done on all of the following mature microRNAs of the miR17-92 Cluster: miR17, miR18, miR19a, miR19-b, miR20 and miR92.

2.4. Chromatin Immunoprecipitation Assay (ChIP)

2.4.1. Chromatin Preparation

ChIP assays were performed on MEFs. MEFs were seeded at a density of 1x10^6 in 100 mm dish and harvested next day for ChIP experiment. MEFs were cross-linked with 270 µl of formaldehyde at 1% final concentration at room temperature for 10 min. After 10 min the formaldehyde reaction was stopped through addition of 500 µl of 2.5M Glycine. Cells were washed twice with PBS before addition of the ChIP nuclear extraction buffer (20mM PIPES pH 8.0, 85 mM KCl, 0.5% NP40), and protease inhibitors were added too. The samples were kept on ice for 10 min to give time for cytoplasm lysis only. Then they were centrifuged at low speed (1000 rpm) for 5 min to pellet the nucleus. The supernatant was discarded and the pellet was resuspended with
ChIP lysis buffer (0.01% SDS, 1.2mM EDTA(pH8), 16.7 mMTris-HCl(pH8.0), 167mM NACl and 1.1% of Triton X100), also protease inhibitors were added to the ChIP lysis buffer. Using the Branson 250 digital sonifier (Branson Ultrasonics, Danbury, CT), the Chromatin was sonicated to generate DNA fragments of 200-800 bp range. A preclearing step was performed on these samples with tRNA-blocked Protein G-agarose. After preclearing an equivalent Chromatin of 1x10^6 cells was immunoprecipitated with 2 µg of antibodies overnight at 4 °C (Table 2.4). 10% of the pre-cleared chromatin was used as input control. Next day Protein G-agarose were used to pull down the immuno complexes formed. The samples were incubated with the Protein G-agarose for 2 hrs. After that, samples were centrifuged and the beads washed with different wash buffers. Elution was done twice through the addition of 250 µl of elution buffer (0.1M NaHCO3, 1%SDS), and reverse cross-linking reaction was done in 200mM NaCl at 65 °C overnight with 20µg of RNase A (Sigma). ProteinaseK was added to digest proteins in the immuno complex and purify DNA. DNA was precipitated with 70% ethanol and kept at -20°C till next day. Next day, samples were centrifuged, and DNA resuspended in water than purified with the Qiagen PCR purification kit according to the manufacturer's instructions.

2.4.2. Primers used for real-time PCR

SYBERGreen primers as well as Roche universal probe library (Roche Diagnostics, Indianapolis, IN) primers were used for ChIP real time PCR analysis. Here is the list of the different primers used. In this list, primers with no UPL probe are SYBERGREEN primers (table 2.3).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Left Primer</th>
<th>Right Primer</th>
<th>UPL probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-myc</td>
<td>gtecgactcgcctcactc</td>
<td>ccccctccctttttttttttt</td>
<td>16</td>
</tr>
<tr>
<td>Sfrp1</td>
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<td>getgatgcacttacttgcacg</td>
<td></td>
</tr>
<tr>
<td>Fas</td>
<td>tggteggaataatcgttaac</td>
<td>aacgcaaccttctgttgetgt</td>
<td>69</td>
</tr>
<tr>
<td>Lox</td>
<td>tagtcacacaactcecccac</td>
<td>attaggtaacccacttggga</td>
<td>10</td>
</tr>
<tr>
<td>miR17-92</td>
<td>gggcggcggaaaaagtga</td>
<td>acctacccaacctcag</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3 Real Time PCR primers for ChIP analysis of a list of gene promoter using the universal probe library system as well as SYBERGREEN.
2.5. Quantitative Real-Time PCR (qRT-PCR)

2.5.1. qRT-PCR Reaction Conditions

Real time PCR reaction was performed for RNA expression analysis and ChIP analysis. For ChIP analysis experiments 5μl of sample volume was used for each PCR reaction in duplicates. For RNA expression analysis, 4 μl of volume was used for each PCR reaction in duplicates. For each reaction we added: 10μl of FastStart Universal Probe Library Master Mix (Roche), 0.1mM of corresponding primer set and 50nM of appropriate probe. Water was added to a final volume of 20μl / reaction. And the real time PCR plate was transferred to the Icycler iQ Real-Time Detection system (BioRad) according to manufacturer’s instructions. The real time PCR protocol used for UPL primers was:

Step1: one cycle at 95°C for 5 minutes. Step2: 40 cycles at: 95°C (30sec), 54°C (30sec), 72°C (30sec). Step3: 72°C (min). Step 4: 12 °C (∞).

The real time PCR protocol used for SYBERGREEN was:

Step1: one cycle at 95°C for 5 minutes. Step2: 40 cycles at: 95°C (30sec), 54°C (30sec), 72°C (30sec). Step3: 78°C (min). Step 4: 100 cycles at 95°C (10 sec). Step5: 10 °C (∞).

2.5.2. qRT-PCR Analysis

RNA expression analysis of the different sets of genes had their threshold adjusted in accordance to RpL4 gene expression which is a ribosomal protein. Whereas for ChIP analysis, the gene threshold was adjusted to the input.
2.6. Western Blot Analysis

2.6.1. Protein Isolation

MEFs were lysed in RIPA buffer (50mM Tris-HCl (pH7.4), 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA) for 30 minutes. The following inhibitors were added to RIPA buffer (1mM PMSF, 1ug/ml Aprotinin, 1ug/ml Leupeptin, 1ug/ml Antipain, 1mM Na₃VO₄). The lysate was centrifuged at 14,000g at 4°C for 30 minutes. Aliquots were made from the supernatant and stored at -70°C.

2.6.2. Western Blot

Before proceeding to the western blot, protein concentration was measured by the Bradford assay. Around 25 to 50 μg of proteins were run on 10 to 12% SDS-Polyacrylamide gels. Nitrocellulose membrane were used to transfer the proteins from the gel. After transfer, The membrane was blocked with (5%non-fat dry milk in 0.05%TBST) for 1 hr at room temperature, then incubated with primary antibody overnight at 4°C. Next day we carried three TBST washes each for 10 minutes. Next the membrane was incubated with an HPR conjugated secondary antibody (either rabbit or mouse) for around one hour. Finally the membrane was developed using the ECL chemiluminescence system (Thermo Scientific). Here is the list of antibodies used for this study (Table 2.4).
<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>company</th>
<th>dilution</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-myc</td>
<td>Santa Cruz (sc-764)</td>
<td>1:1000 for WB 2 μg for ChIP</td>
<td>HRP α-Rabbit IgG (1:8000)</td>
</tr>
<tr>
<td>C-myc</td>
<td>Santa Cruz (sc-40)</td>
<td>1:200 for WB</td>
<td>HRP α-mouse IgG (1:5000)</td>
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<tr>
<td>Pan-Ras</td>
<td>CalBiochem(OP-40)</td>
<td>1:1000 for WB</td>
<td>HRP α-mouse IgG (1:5000)</td>
</tr>
<tr>
<td>Ets1</td>
<td>Ostrowski Lab</td>
<td>2 μg for ChIP</td>
<td>HRP α-Rabbit IgG (1:8000)</td>
</tr>
<tr>
<td>Ets2</td>
<td>Ostrowski Lab</td>
<td>10 μl for WB 2 μg for ChIP</td>
<td>HRP α-Rabbit IgG (1:8000)</td>
</tr>
<tr>
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<td>Santa Cruz (SC-350)</td>
<td>1:200</td>
<td>HRP α-Rabbit IgG (1:8000)</td>
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<tr>
<td>IgG (Rabbit)</td>
<td>Millipore</td>
<td>2 μg for ChIP</td>
<td></td>
</tr>
<tr>
<td>Trimethyl H3K27 (Rabbit polyclonal)</td>
<td>Upstate</td>
<td>2 μg for ChIP</td>
<td></td>
</tr>
<tr>
<td>Trimethyl H3K9 (Rabbit polyclonal)</td>
<td>Upstate</td>
<td>2 μg for ChIP</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4. List of WB and ChIP antibodies
2.7. Soft Agar Assays

The Soft Agar assays were done in quadruplicates for each transformation genotype. A first layer of 0.6% (w/v) of soft agar was first put to cover the surface of a 6 well plate, followed by a second layer of 0.3% (w/v) of soft agar that also contains the $1\times10^4$ cells/well. Both layers of soft agar contains 20% FBS. The colonies were let grow between 14 to 21 days before counting and scoring.

2.8. Confluency Assay

Cells were grown in 6 well plates at a $4\times10^4$ cells/well in duplicates. The media was changed every 2 days, on day 6 cells were trypsinized and counted.

2.9. Tumor formation in nude mice

Animal studies were performed with 8-10 week old male athymic nude mice. Transformed MEFs were harvested, counted and resuspended in PBS at a concentration of $1\times10^6$ cells/injection site. 100 μl of cells were injected subcutaneously into the right and left shoulders and hips (four injections per mouse), or two injections per mouse (right and left hips). Tumor formation was monitored starting at day 6 when we started to see tumor formation. Tumors were harvested after 3 to 4 weeks after tumors reached 1 cm in length.

2.10. Statistics

Experiments were repeated with a total number of n=3. Statistical analysis was done using the Standard deviation formula. For the tumors studies, the student t-test was used to determine the statistical significance between the control and experimental genotypes.
CHAPTER 3

ETS1 AND ETS2 FUNCTION IN RAS ONCOGENIC TRANSFORMATION IN MOUSE EMBRYONIC FIBROBLASTS

3.1. Introduction

The role of the Ets family in Ras oncogenic transformation is very important and essential. Expression of a dominant negative Ets-LacZ protein that have the Ets DNA binding domain fused to the Beta-galactosidase protein blocked Ras transformation in the NIH3T3 cells as well as in the thyroid cancer cell lines (de Nigris et al., 2001b; Langer et al., 1992).

Although these studies points towards the involvement of the Ets family in the Ras oncogenic transformation, and since the Ets DNA binding domain is present in all of the Ets family proteins, and given the structural complexity and variability of the Ets family, the identity of the Ets factors that are necessary and sufficient for the Ras oncogenic transformation is still unknown.

The mechanism through which the Ets proteins would be mediating Ras transformation pointed towards the involvement of the C-myc oncogene in this process. The fact that the C-myc RNA and protein are downregulated in the Ras non transformed MEFs that overexpress the dominant negative Ets protein suggested that the Ets dominant
negative protein is binding to the C-myc promoter and inhibiting the normal Ets transcription factor from binding there and transactivating it (Langer et al., 1992). Indeed, Al-Azawi el al showed that Ets2 binds to the C-myc promoter and increases both protein and RNA expression when transiently overexpressed in MCF7 cells (Al-azawi et al., 2008).

Furthermore, Ets2 could be involved in the posttranscriptional repression of some tumor suppressor genes through the regulation of certain microRNAs. One possibility is that Ets2 through the activation of C-myc would induce the expression of miR17-92, a very well known oncogenic microRNA that is activated by C-myc (O'Donnell et al., 2005).

Chromatin mediates dynamic changes in the gene function as well as in the gene expression. Covalent post-translational modifications of histones (the main Chromatin component) at the histone tails, play essential roles in the ability of the genome to store, release and inherit biological information. Histone modifications can be either highly reversible or stable. Reversible modifications are due to lysine acetylation and serine and threonine phosphorylation, whereas more stable modifications are due to lysine methylation (Fischle et al., 2003). Histone lysine methylation has a great impact on various chromatin associated functions including transcriptional regulation, heterochromatin formation, DNA repair and recombination. Histone lysine methylation recruits and regulates different biological reactions by controlling the histone protein interaction in a similar fashion as phosphorylation regulates the protein function.
H3K27 (Histone 3 Lysine 27) trimethylation presence at the promoter of many genes has been associated with gene silencing. H3K27 trimethylation recruits and stabilizes the Polycomb PRC1 complex on the chromatin which in turn mediates gene silencing. Genomic analysis suggests that the presence of H3K27 me3 at transcriptional start sites is generally correlated with the repression of gene expression. H3 K27 is methylated by the Polycomb PRC2 complex and EZH2 (Schuettengruber et al., 2007).

H3K9 methylation has also been associated with gene silencing. Methylation of H3 Lysine 9 is operated by the SET-type histone methyltransferases (HMT) like Su(Var)3-9 and G9a that lead to establishment and maintenance of heterochromatin domains (Shinkai, 2007). It has been shown that an increased trimethylation of Histone 3 at Lysine 9 is responsible for the transcription dysfunction and increased gene silencing during Huntington’s disease progression (HD). The ERG-associated protein with the SET domain (ESET) is a histone methyltransferase that leads to H3K9 trimethylation. ESET protein level and activity is high in Huntington’s disease pathogenesis and using drugs like mithramycin disrupts the transcriptional activation of ESET leading to less trimethyl H3K9 presence (Ryu et al., 2006).

Epigenetic silencing of tumor suppressor genes is an important process in cancer progression. Indeed, during Ras oncogenic transformation, many tumor suppressor genes like Sfrp1, Fas and Lox are repressed. Re-expression of Fas, Sfrp1 or Lox reversed Ras transformation. The silencing of the above tumor suppressor genes requires the activation of 28 RESEs (Ras epigenetic silencing effectors). These effectors molecules range from transcription factors like E2F1, transcriptional repressors like CTCF, RCOR2 and TRIM66, histone methyltransferases like EZH2, SMYD1 and DNA methylases like
DNMT1. Ras activation of the RESEs leads to DNA methylation and transcriptional silencing of the Fas, Lox and Sfrp1 promoters (Gazin et al., 2007).

The Ets2 transcription factor has been shown to transcriptionally repress many genes like BRCA1, Thrombospondin-1 and others through the recruitment of HDAC, Brg-1, NCOR that would form a transcriptional repressor complex. That implied that Ets2 could be involved in transcriptional repression during Ras transformation.

To study the effect of Ets1 and Ets2 on the Ras oncogenic transformation in vitro, we took a genetic approach using the Cre/loxP technology to delete Ets2 in Ets1/-/- mouse embryonic fibroblasts (MEFs) (Nagy, 2000). We made established cell lines through the consecutive passaging of MEFs according to the 3T3 protocol (Todaro and Green, 1963).

Here we show that Ets1 and Ets2 are required for Ras oncogenic transformation. Deletion of both Ets1 and Ets2 inhibited Ras oncogenic tumorigenesis and Ets1/-/- Ets2/-/- MEFs failed to form tumors when injected subcutaneously in nude mice. We also show that Ets1 and Ets2 regulate C-myc transcription. C-myc, Ets1 and Ets2 regulate the expression of miR17-92 cluster. Over expression of C-myc or miR17-92 in Ets1/2 null MEFs rescued in vivo Ras tumorigenesis by forming tumors once injected into nude mice.

In the other hand C-myc induced Ets1/2 activation lead to transcriptional repression of Fas, Lox and Sfrp1 as well as other genes. C-myc failed to activate Ets1/2 target genes like MMP9 and bclxL. Ets1, Ets2 and C-myc have enriched presence on the promoter of Fas, Lox and Sfrp1 during Ras transformation. The presence of Ets1, Ets2 and C-myc was associated with an enrichment of those promoters with H3K27 and H3K9 trimethylation in a Ras dependent manner that correlated with epigenetic silencing.
3.1.1. The Cre/loxP system:

The Cre-lox technology was discovered in the P1 bacteriophage as part of its normal viral life cycle. The bacteriophage uses the Cre-lox recombination to circularize and facilitate the replication of its genomic DNA during the reproduction cycle (Sauer and Henderson, 1988; Sternberg and Hamilton, 1981). This knowledge developed into a technology that was successfully applied in science to manipulate genetic models in mammalian cultures, yeasts, plants, mice and other organisms.

The mechanism of Cre recombination requires two components: the Cre recombinase protein and the loxP sites. The Cre recombinase protein is an enzyme that catalyzes the recombination between two loxP sites. The LoxP site is a specific 34 nucleotides sequence in length that consists of 8 nucleotides core sequence, and two flanking 13 nucleotides inverted repeats on each side of the core sequence. In the genetic system, the Lox P sites are flanking the targeted gene of interest, and the addition of the Cre recombinase enzyme completely removes the targeted gene in between the LoxP sites to create a null allele (Nagy, 2000).

3.1.2. The transgenic alleles used

For this study we used the Ets1 knockout allele from the Ets1-/- mice that lacks the Ets1 protein and thus behaves as a null allele as shown by Barton et al (Barton et al., 1998). We also used the Ets2 allele from the Ets2^{LoxP/LoxP} mice. These mice have two LoxP flanking the Ets2 exons 3 to 5. The exon 2 contains the first 24 amino acids including the ATG start codon. Deletion of the exons 3 to 5 by the Cre recombinase lead to the generation of multiple spliced RNAs that have many stop codons and thus produces no Ets2 protein (Wei et al., 2009).
3.1.3. Generation of established Mouse Embryonic Fibroblasts (MEFs)

In order to study the effect of Ets1 and Ets2 deletions during Ras oncogenic transformation we interbreed Ets1<sup>+/−</sup> Ets2<sup>loxP/loxP</sup> more Cre males with Ets1<sup>+/−</sup> Ets2<sup>loxP/loxP</sup> females. 13.5 days old embryos were genotyped and cultured in vitro to generate primary MEFs. The following genotypes were used for this study: Ets1<sup>+/−</sup> Ets2<sup>loxP/loxP</sup>, Ets1<sup>−/−</sup> Ets2<sup>loxP/loxP</sup> and Ets1<sup>−/−</sup> Ets2<sup>−/−</sup> more cre MEFs. Wild type FVB MEFs were generated later in the study and used as a control. The above mentioned genotypes were passaged in vitro every 3 days in 60 mm dishes according to the 3T3 protocol (Todaro and Green, 1963). All these cells have undergone senescence, but they were passaged continuously until they started escaping senescence and started growing again and became established cell lines around passage 23 to 25. For the following experiments, we have used immortalized MEFs from 26 passages.

3.2. Results

3.2.1 Deletion of both Ets1 and Ets2 restored contact inhibition in Ras infected Ets1/2 null MEFs

In order to generate the double knockout Ets1<sup>−/−</sup> Ets2<sup>−/−</sup> MEFs, Ets1<sup>−/−</sup> Ets2<sup>loxP/loxP</sup> MEFs were infected with the Cre-recombinase expressing retrovirus as well as the control retrovirus, than selected with puromycin for 3 days. After 3 days, the cells were digested with the proteinase K in order to generate genomic DNA. The digested samples were analyzed by PCR in order to check for the Ets2 deletion efficiency by Cre. The PCR showed a complete Ets2 deletion in Ets1<sup>−/−</sup> Ets2<sup>−/−</sup> MEFs. Since the MEFs used for this experiment were all originally Ets2<sup>loxP/loxP</sup>, the first two lanes are Ets1<sup>+/−</sup> Ets2<sup>loxP/loxP</sup> MEFs from control and Ras retroviral infections. The third and fourth lanes are Ets1<sup>−/−</sup>
Ets2\textsuperscript{floxP/floxP} MEFs from control and Ras retroviral infections that were both originally infected with the Cre-recombinase virus and selected in puromycin for the Ets2 deletion genotype. There were no traces of the Ets2 lox-P allele in these MEFs (Figure 3.1.A).

For the rest of this work we are going to refer for the control MEFs that have both Ets1 and Ets2 as E1+ E2+, and we are going to refer for the double knockout Ets1 and Ets2 MEFs as E1-E2-. In order to induce Ras transformation in MEFs, E1+ E2+ and E1-E2- MEFs were infected with either the H-Rasv12 retrovirus (that expresses the constitutively active form of the Ras protein) or the control retrovirus. After infection, cells were selected with hygromycin for 5 days then seeded in 100 mm dish at 70% confluency for three days. On day 3, photographs were taken for the Ras selected E1+ E2+ and E1- E2- MEFs as well as the control E1+E2+ and E1- E2- MEFs. The E1+ E2+ were transformed after Ras infection and grew over confluent and formed foci, whereas the E1-E2- did not transform after Ras infection and were flat, didn’t form foci, stopped growing when they reached confluency and were morphologically similar to the control non transformed E1-E2- and E1+ E2+ MEFs that were not infected with a control vector, thus suggesting that the deletion of both Ets1 and Ets2 is able to revert the morphological Ras transformation phenotype (Figure 3.1.B). It should be noted that such a phenotype is actually seen in NIH3T3 cells that overexpress Ras as well as a dominant negative Ets-lac-Z protein (a hybrid protein that have the Ets DNA binding domain linked to the B-galactosidase protein) (Langer et al., 1992). The morphology seen in the E1-E2- MEFs resembles the revertant NIH3T3 MEFs that overexpress the dominant negative Ets protein.
Figure 3.1. Deletion of Ets1 and Ets2 inhibited Ras induced foci formation
(A) PCR showing the deletion efficiency of Ets2 flox in E1-E2- generated MEFs after Cre infection. (B) E1+E2+ and E1-E2- MEFs were infected with H-Rasv12 or empty vector control retrovirus, selected with hygromycin for 5 days, than seeded at 70% confluency in 100 mm dishes and kept in culture for 3 days. Photographs were taken at day three using Olympus inverted phase contrast microscope.
3.2.2 Both Ets1 and Ets2 deletion are necessary for contact inhibition in Ras transformation

One property of the Ras transformed cells is that they are not contact inhibited and they keep on growing when they reach confluency, whereas the established (non transformed) cell lines stop proliferating when they are confluent because of contact inhibition. Non transformed cells seeded at a density of 4x10⁴ cells/well in 6 well plates reach confluency at day 3 and stop proliferating. On the other hand, the Ras transformed cells don’t stop proliferating after day 3. Thus, in order to assess which Ets factor is important for the Ras transformation, Ets1⁺⁻ Ets2loxP/loxP, Ets1⁺⁻Ets2⁻⁻, Ets1⁺⁻Ets2⁻⁻ and Ets1⁻⁻Ets2⁻⁻ MEFs were either infected with H-Rasv12 retrovirus or the control retrovirus. After selection with hygromycin, the cells were seeded in 6 wells plates at 4x10⁴ cells/well and were kept in culture for 6 days. On day 6, the cells were washed, trypsinized and counted. All the cells from the different genotypes infected with the control retrovirus scored the same number of cells on day 6. However deletion of Ets1 or Ets2 in the Ras infected MEFs was not enough to inhibit the proliferation of these cells that were over confluent at day 6 compared with the non transformed control infected cells. Upon deleting both Ets1 and Ets2, the E1-E2- MEFs infected with Ras scored similarly to the non transformed cells which indicates that both Ets1 and Ets2 deletion are necessary to restore contact inhibition during Ras transformation (Figure 3.2.).
Figure 3.2. Both Ets1 and Ets2 are necessary for contact inhibition in Ras transformation

Cells of different Ets genotypes were infected with H-Rasv12 retrovirus or the empty vector retrovirus than selected with hygromycin for 5 days. After selection, cells were seeded in 6 well plates in duplicates from two independent experiments at 40000 cells/well density and kept in culture for 6 days. On day 6 cells were trypsinized and counted.
3.2.3 Ets1 and Ets2 expression increases during Ras transformation

E1+ E2+ and E1-E2- MEFs were either infected with the H-Rasv12 retrovirus or an empty retrovirus then selected with hygromycin for 5 days. After selection, cells were seeded at 70% confluency and harvested next day for western blot analysis.

Both Ets1 and Ets2 protein levels increase during Ras transformation in E1+E2+ cells, and no protein expression of both Ets1 and Ets2 were detected in E1-E2- cells as we predicted (Figure 3.3). The Ets1 deletion allele does not produce a functional Ets1 protein. Similarly Ets2 deletion by the Cre recombinase produces stop codons in the Ets2 RNA transcript and thus no protein is produced. The first two columns in the third raw are showing the Ras expression in E1+ E2+ MEFs that were either infected with the empty vector or the H-Rasv12 expression vector. Similarly, the third and fourth columns of the third raw are showing the Ras expression in E1- E2- MEFs that were either infected with an empty retrovirus or the H-Rasv12 retrovirus. Ras is over expressed in the H-Rasv12 infected cells and its level is by far higher than the endogenous Ras that is present in the control cell lines that don’t express the H-Rasv12 protein. The increase in Ets1 and Ets2 in response to constitutive Ras expression is not surprising since Ets1 and Ets2 have been shown to be overexpressed in many cancers, and are overexpressed in thyroid carcinoma cell lines relative to normal cell lines (de Nigris et al., 2001b).

3.2.4 Ets1 and Ets2 deletion inhibited Ras transformation in vitro in soft agar

An important property of the Ras transformed fibroblasts is their ability to grow in soft agar unlike the established non transformed fibroblasts. In order to check if Ets1 or Ets2 affects the ability of the Ras transformed cells to form colonies, we used different Ets1/2 genotypes that either had Ets1 or Ets2 deletion or both. Ets2 deletion was
generated as mentioned previously by using the LoxP-Cre technique. This was followed by infecting the different MEFs genotypes with the Ras retrovirus. After selection, the Ras selected cells in addition to the control non transformed cells were put in soft agar to grow for two weeks (Figure 3.4.). The results suggest that the deletion of both Ets1 and Ets2 was able to inhibit the growth of the MEFs in the soft agar assay. The control wild type cells in the absence of Ras did not form colonies. And the few colonies that grew in soft agar from the E1-E2- MEFs were much smaller than the other MEFs genotypes. In the third column, deletion of Ets1 in Ets1\textsuperscript{-/-} Ets2\textsuperscript{ff} MEFs reduced the numbers of colonies by three fold compared with the Ets1\textsuperscript{+/+} Ets2\textsuperscript{ff} MEFs (one Ets1 copy and two Ets2 copies) in the second column. Our result is different from Hever et al who actually found that Ets2 deletion does not reduce Ras transformation in ES cells. The reason for the difference between our result and Hever el al result could be the difference in the cell lines used in our study and their study. ES cells are pluripotent cells and probably deletion of Ets2 won’t be enough to reduce Ras transformation. In the fourth raw, the Ets1\textsuperscript{+/+} Ets2\textsuperscript{-/-} MEFs were 10 times less transformed than Ets1\textsuperscript{-/-} Ets2\textsuperscript{ff} MEFs suggesting that removing one additional copy of Ets1 decreases transformation. This result suggests that Ras transformation is Ets1/2 dose dependent, and removing one copy at a time affects negatively the Ras transformation of MEFs.
Figure 3.3. Ets1 and Ets2 expression increases during Ras transformation
Western blot analysis on lysates from E1+ E2+ and E1-E2- MEFs for Ets1, Ets2 and Ras.

Figure 3.4. Ets1 and Ets2 deletion inhibited Ras transformation in vitro in soft agar.
Cells of different Ets genotypes were infected with H-Rasv12 retrovirus or the empty vector retrovirus than selected with hygromycin for 5 days. After selection, cells were seeded in 6 well plates in duplicates in soft agar from two independent expreriments.
3.2.5 Ets1 and Ets2 induce C-myc expression during Ras transformation

It has been shown that the overexpression of the Ets-LacZ dominant negative protein leads to a decrease in the level of C-myc expression (Langer et al., 1992). That suggested that probably the Ets-lacZ hybrid protein is inhibiting the binding of the actual Ets factor on the C-myc promoter and thus leading to less C-myc expression. The first evidence that Ets2 could bind and activate C-myc came from a study done by Al-Azawi et al who showed that the transient expression of Ets2 in the MCF-7 breast cancer cells lead to an increase in C-myc expression (Al-azawi et al., 2008).

Here we show that the C-myc RNA expression increases significantly during Ras transformation in the Ras infected E1+ E2+ MEFs compared with the control E1+ E2+ MEFs, but C-myc expression was low in E1-E2- MEFs even in the presence of Ras (Figure 3.5.A). That suggested that Ets1 and Ets2 regulate C-myc expression at the transcriptional level. Similarly, the C-myc protein expression increases during Ras transformation in E1+E2+ MEFs compared with the control MEFs. But C-myc expression in Ras infected E1-E2- MEFs where both Ets1 and Ets2 are deleted was significantly lower than in the fully transformed Ras infected MEFs (Figure 3.5.B). It should be noted that C-myc protein increased slightly in E1-E2- MEFs in the presence of Ras compared with the control E1-E2- MEFs, probably because of the Ras effect on the C-myc protein stability (Sears et al., 1999).
3.2.6 Ets1 and Ets2 bind to the C-myc promoter during Ras transformation

Ets1 and Ets2 have been shown to control C-myc expression through a single minimal binding site of the E2F transcription factors by the transactivation of reporter genes driven by the C-myc promoter (Roussel et al., 1994). In endocrine resistant breast cancer cells, Ets2 but not Ets1 has been shown to activate C-myc expression and was found to bind to the C-myc promoter through Chromatin immuno-precipitation (Al-azawi et al., 2008).

Here we show that we found a highly conserved Ets binding site in the C-myc proximal promoter at position -49 upstream from the start codon (Figure 3.6.A). Using primers that recognize the -49 position in the C-myc promoter, we performed ChIP with Ets1 and Ets2 antibodies and found that both Ets1 and Ets2 bind to this promoter area in the Ras transformed E1+E2+ MEFs (Figure 3.6.B). The binding of Ets1 and Ets2 to the proximal promoter of C-myc suggests that Ets1 or Ets2 are able to occupy the same binding site and activates C-myc expression. Ets1 and Ets2 could be recruiting the CBP/P300 co-activator protein through their trans-activation domain which in turn will bring the rest of the RNA polymerase transcription machinery. Our result is slightly different from Al-azawi et al finding who did not find that Ets1 binds and activates C-myc promoter. In Al-Azawi model, the MCF-7 breast cancer cells that showed Ets2 but not Ets1 binding to the C-myc promoter were resistant and insensitive to endocrine treatment. In these cells, Ets2 collaborates with the steroid receptor activators p160 to induce c-myc expression. Thus, p160 would preferentially interact with Ets2 but not Ets1 to induce C-myc expression (Al-azawi et al., 2008).
Figure 3.5. Ets1 and Ets2 lead to an increase in C-myc expression during Ras transformation.
(A) Real time analysis of C-myc RNA expression in E1+E2+ vs E1-E2- MEFs in the presence or absence of Ras. (B) Western blot analysis of C-myc expression in E1+ E2+ vs E1-E2- MEFs in the absence and presence of Ras. A-tubulin was used for equal loading.

Figure 3.6. Ets1 and Ets2 bind to the C-myc promoter during Ras transformation
(A) Map of the C-myc promoter showing conserved Ets binding sites (rectangle) and the ChIP primers binding sites (arrows). (B) ChIP was performed on E1+E2+ and E1-E2- cells that overexpress Ras with the IgG, Ets1 and Ets2 antibodies and quantified by qPCR.
3.2.7 Ets1 and Ets2 deletion inhibit Ras tumorigenesis and C-myc rescues it *in vivo*

To check if the E1- E2- MEFs that over express Ras are tumorigenic, we injected MEFs into nude mice.

Around one million cells were injected subcutaneously into the left and right shoulders and hips (four injections per mouse) of the mice. All of the 8 control injections from E1+ E2+ MEFs that over express Ras formed tumors after 3 weeks. None of the total 27 injections from E1- E2- MEFs that over express Ras formed tumors, which suggests that Ets1 and Ets2 are both required for Ras tumorigenesis. Since C-myc expression was shown to be increased during Ras transformation, and since C-myc oncogene has been reported previously to lead to tumorigenesis, putting back C-myc into the E1- E2- MEFs rescued Ras tumorigenesis. An inducible C-myc retrovirus tagged with GFP was used to infect the E1- E2- MEFs, and the cells were sorted for GFP expression. Using 1 μM of tamoxifen to induce C-myc in E1- E2- MEFs that were already infected with the Ras retrovirus (Figure.3.7.A), we injected E1- E2- MEFs subcutaneously and we injected the mice with 1 μM of tamoxifen every other day in order to make sure that C-myc is expressed. After 3 weeks, all the 8 sites injected with the E1- E2- MEFs that have the inducible C-myc expressed developed tumors (Figure.3.7.B). The average size of the E1+ E2+ MEFs transformed by Ras was around 1721 mg. No tumors were detected in the E1- E2- MEFs that have Ras. The average tumors from the E1- E2- MEFs rescued with C-myc was around 1256 mg. The difference in tumor sizes were not significant between the Ras transformed E1+ E2+ MEFs and the C-myc rescued E1- E2- MEFs (Figure 3.7.C).
Figure 3.7. Ets1 and Ets2 deletion inhibit Ras tumorigenesis and C-myc rescues it in vivo

(A) Western blot showing the C-myc expression in E1- E2- MEFs that were infected with an inducible ER-myc vector. 1 μM of 4-hydroxy-tamoxifen (4OHT) added to these MEFs in lane 2 lead to C-myc induction, Actin was used for equal loading. (B) Graph showing the percentage of tumors formed over the total number of injections for the different MEFs used. MEFs were injected in nude mice (1 million cells/injection). After 3 weeks the tumors were harvested. The ratios here represent the number of tumors over the number of total injections for the different genotypes. (C) Graph showing the tumor weight of the different tumors. The asterix indicates significant difference calculated according to the p value (p<0.001). N/A indicates that there were no tumors observed for this genotype.
3.2.8 Ets1 and Ets2 gene activation is C-myc independent:

Since C-myc transcription factor was able to activate miR17-92, we wanted to see if C-myc would be able to repress Ets1/2 target genes that are important for tumorigenesis. We looked at Ets target genes like MMP9, bclxL, HbEGF, CXCR4 and cyclin D1 that have been shown to be activated by Ets1 and Ets2 and that could be playing a role in Ras/Ets1/2 transformation. These genes were upregulated in E1+ E2+ MEFs after Ras infection. Deletion of Ets1 and Ets2 succeeded in inhibiting the activation of these genes in response to Ras transformation. The expression of these genes did not increase in E1- E2- MEFs after Ras infection. MMP9, bclxL, HbEGF, and CXCR1 expression did not increase in E1- E2- MEFs in response to Ras while cyclin D1 expression did increase, but its expression was lower compared to E1+ E2+ MEFs after Ras infection (figure 3.8.). Interestingly, over expression of C-myc did not activate Ets1/2 target genes in E1- E2- MEFs that were previously infected with Ras. This suggests that Ets1 and Ets2 activation of these genes is Ets dependent but C-myc independent. It also implies that these classical Ets1/2 target genes are not required for Ras transformation and it is necessary to identify novel targets that are essential for both Ets1/2 as well as C-myc dependent Ras transformation.

3.2.9 Ets1 and Ets2 gene repression is C-myc dependent

Ets1 and Ets2 have been shown to repress many genes like Thrombospondin-1, BRCA1 and others through recruitment of Repressor and Co-repressor molecules (Baker et al., 2003; Wei et al., 2003; Zabuawala et al., 2010). During Ras transformation, Ras drives a transcriptional repression program that lead to methylation and silencing of tumor suppress genes like Sfrp1, Lox and Fas (Gazin et al., 2007).
Here we show that Sfrp1, Fas, Lox, Gro-1 and Serpinb1a genes are repressed after Ras infection in E1+ E2+ MEFs. Deletion of Ets1 and Ets2 alleviated Ras induced repression, and there were no significant difference in the expression level of these genes in E1- E2- MEFs before and after Ras infection. The constitutive active Ras did not repress these genes when Ets1 and Ets2 were deleted. In the previous sections we showed that C-myc rescued Ras tumorigenesis of E1-E2- MEFs. Here we show that over expression of C-myc was able to repress Fas, Lox, Sfrp1, Gro-1 and Serpinb1a in the E1-E2- MEFs after Ras infection. This suggested that Ets1 and Ets2 repression of those genes is C-myc dependent during Ras transformation (figure 3.9.). Since these genes were downregulated during Ras transformation as well as C-myc rescued transformation, this suggested that Sfrp1, Fas, Lox, Gro-1 and Serpinb1a genes are tumor suppressor genes which repression is essential and necessary for the Ras/Ets1/2 transformation in mouse embryonic fibroblasts. The Sfrp1 tumor suppressor gene is one of the known targets of C-myc. It has been shown that C-myc over expression is able to repress Sfrp1 in mammary epithelial cells (Cowling et al., 2007). Amond the other targets, Gro-1 is a small cytokine belonging to the CXC chemokine family. It is secreted by human melanoma cells, has mitogenic properties and is implicated in melanoma pathogenesis and is involved in the processes of angiogenesis, inflammation, wound healing, and tumorigenesis. Similarly, Serpinb1a is a known neutrophil protease inhibitor that belongs to the largest and most broadly distributed superfamily of protease inhibitors present in humans, plants, bacteria, archaea and poxviruses.
Figure 3.8. Ets1 and Ets2 gene activation is C-myc independent
qPCR for MMP9, bclxL, HbEGF, CXCR4 and Cyclin D1 from E1+E2+ and E1-E2- in
the absence or presence of Ras retrovirus. Last lane represents E1-E2- MEFs with Ras
and myc retrovirus.

Figure 3.9. Ets1 and Ets2 gene repression is C-myc dependent
qPCR for Sfrp1, Fas, Lox, Serpinb1a and Gro1 from E1+E2+ and E1-E2- in the absence
or presence of Ras retrovirus. Last lane represents E1-E2- MEFs with Ras and myc
retrovirus.
3.2.10 C-myc deletion increases Sfrp1, Fas and Lox expression

As mentioned previously, Fas, Lox and Sfrp1 expression did not go down in E1-E2 MEFs after Ras infection, but over expression of C-myc was able to inhibit their expression (Figure 3.9). Sfrp1 has been shown to be a C-myc target gene (Cowling et al., 2007). In order to see if Fas and Lox are also C-myc target genes, we took a genetic approach by deleting C-myc from immortalized C-myc<sup>loxP/loxP</sup> MEFs using the Cre-recombinase retrovirus. The C-myc deletion was verified by western blot, and we did not find C-myc protein expression from C-myc<sup>loxP/loxP</sup> MEFs after Cre infection (figure 3.10.A). We found that Fas and Lox expression increased by three folds after Cre infection. Similarly, we show that Sfrp1 gene expression increased by three fold after Cre infection. This shows that Fas and Lox are C-myc repressed targets and deletion of C-myc is able to derepress their expression (Figure 3.10.A, B and C). Our result suggests that Ets1/2 activation during Ras transformation are able to repress Fas, Lox and Sfrp1 through C-myc. C-myc could be working as a transcriptional repressor of Fas and Lox genes similar to Sfrp1. But, that doesn’t eliminate the possibility that Ets1 and Ets2 could be also binding and repressing these genes.
Figure 3.10. C-myc deletion increases Sfrp1, Fas and Lox expression
(A) qPCR for Sfrp1 expression before and after Cre deletion of C-myc in Cmycf/f MEFs.
(B) qPCR for Fas expression before and after Cre deletion of C-myc in C-myc f/f MEFs.
(C) qPCR for Lox expression before and after Cre deletion of C-myc in C-mycf/f MEFs.
3.2.11 Ets1, Ets2 and C-myc transcriptional repression of the Fas, Lox and Sfrp1 promoters

The deletion of Ets1 and Ets2 lead to derepression of genes like Sfrp1, Fas and Lox. Ets1 and Ets2 could be binding to the promoters of these genes. Similarly, since C-myc overexpression in E1- E2- MEFs lead to the repression of these genes, this also suggests that C-myc could be binding to their promoters too. C-myc has been shown to bind to the promoter of the Sfrp1 promoter in mammary cancer cell lines (Cowling et al., 2007), which implies that C-myc could be binding to the Fas and Lox promoters too. Analysis of the promoters of Fas, Lox and Sfrp1 genes revealed Ets and C-myc conserved binding sites (Figure 3.11.A). Using specific primers that flank the Ets and C-myc binding sites on the proximal Fas, Lox and Sfrp1 promoters we performed ChIP from the following MEFs cell lines: control non transformed E1+ E2+, Ras transformed E1+ E2+ MEFs, Ras non transformed E1-E2- MEFs and Ras transformed E1-E2- MEFs that over express the C-myc protein. Using the C-myc antibody we found that C-myc binds to the Sfrp1, Fas and Lox promoters in a Ras dependent manner. There was more enrichment in C-myc on the promoters of these genes in the Ras transformed E1+ E2+ MEFs compared with the control E1+ E2+ MEFs. There was no binding of C-myc in the Ras non transformed MEFs, but there was enrichment in C-myc on the promoters of these genes in Ras transformed E1-E2- MEFs that over express C-myc (Figure 3.11.B). This suggested that C-myc increased expression during Ras transformation lead to the binding of C-myc to the promoter of tumor suppressor genes like Fas, Sfrp1 and Lox. The presence of Trimethylated H3K27 and H3K9 at the promoter of many genes has been associated with gene silencing. Using trimethylated H3K27 and H3K9 antibodies,
we found that those silencing markers are frequently present on the promoters of Fas and Sfrp1 in a Ras dependent manner. The Sfrp1 and Fas promoters were enriched with H3K27 and H3K9 trimethylated markers in the Ras transformed E1+ E2+ MEFs compared with the control non transformed E1+ E2+ MEFs. There were no enrichments on the promoters of those genes in the Ras non transformed E1- E2- MEFs, but there was enrichment with the silencing markers in the Ras transformed MEFs that over express C-myc (Figure 4.4.B). This suggests that C-myc binding to these promoters is associated with the presence of silencing markers during Ras transformation and that C-myc is working as a transcriptional repressor at the promoter of these genes. We didn’t find enrichment in trimethylated H3K27 and H3K9 with the primers associated with this particular region on the promoter of the Lox gene, and so using different primers that scan a different location would reveal the presence of those markers (Figure 4.4.B).

Finally, using Ets1 and Ets2 antibodies, we found that both Ets1 and Ets2 also bind at the Sfrp1 and Fas promoter in a Ras dependent manner, thus suggesting that Ets1 and Ets2 together with C-myc work in a big repressor complex that will lead to gene silencing of these promoters. It should be noted that we did see Ets1 and Ets2 binding at the Lox promoter which was not Ras dependent. It could be that Ets1 and Ets2 binding at this site is not important, and scanning another region of the Lox promoter may reveal an important Ets1 and Ets2 binding site (Figure 4.4.C).
Figure 3.11. Ets1, Ets2 and C-myc transcriptional repression of the Fas, Lox and Sfrp1 promoters

(A) genetic map of the Sfrp1, Fas and Lox promoters showing the position of the conserved Ets and C-myc binding sites as well as the position of the ChIP primers. (B) ChIP analysis of the Sfrp1, Fas and Lox promoters with IgG, C-myc, Trimethyl H3K27 and Trimethyl H3K9 antibodies in in four different cells lines: E1+ E2+ control MEFs, E1+E2+ Ras transformed MEFs, E1- E2- Ras non transformed MEFs and E1-E2-MEFs that overexpress both Ras and C-myc. (C) ChIP analysis of the Sfrp1, Fas and Lox promoters with IgG, Ets1 and Ets2 antibodies in three different cells lines: E1+ E2+ control MEFs, E1+E2+ Ras transformed MEFs and E1- E2- Ras non transformed MEFs.
3.2.12 Ets1 and Ets2 deletion downregulates the expression of miR17-92 cluster

MiR17-92 cluster is a direct target gene of C-myc and has been reported to be over expressed in a variety of cancers of hematopoietic and solid tumors from many tissues: breast, lung, pancreas, colon, prostate and stomach (Volinia et al., 2006). Also, it has been shown that conditional over expression of C-myc in the Ras transformed colonocytes lead to a significant increase in the miR17-92 cluster expression (Dews et al., 2006).

Since the C-myc expression increases during Ras transformation, we wanted to see if that would also lead to an increase in the oncogenic miR17-92 cluster. Here we show that the miR17-92 cluster expression increased in the Ras transformed E1+E2+ cells compared with the control E1+ E2+ non transformed cells. MiR17, miR18,miR19b and miR92 all increased significantly during Ras transformation. We also saw a decrease in the cluster expression in the E1- E2- versus the E1+ E2+ none transformed MEFs where all the individual miRs were down regulated except for miR19b. All the individual miRs were repressed in E1-E2- MEFs vs E1+E2+ MEFs in the presence of Ras (Figure 3.12). The miR17-92 expression data in this experiment correlate with the C-myc expression data (Figure 3.5), suggesting that the C-myc Ets1/2 dependent regulation is driving the increase in miR17-92 expression during Ras transformation. We don’t really know why we didn’t see an increase in miR19a and miR20 in the Ras transformed E1+E2+ MEFs versus E1+ E2+ control MEFs. One possibility is that Ras could be driving a post-transcriptional program that affects miR19a and miR20 expression.
3.2.13 Ets2 and C-myc bind to the miR17-92 promoter

It has been shown that C-myc binds to the miR17-92 promoter (O'Donnell et al., 2005). In our Ras transformation model, C-myc protein expression increases in the Ras E1+E2+ transformed cells. In the same time Ras induced an increase in the miR17-92 cluster expression. This suggested that C-myc could be binding to the miR17-92 promoter and increasing the cluster expression during Ras transformation. Also Ets1 and Ets2 expression increases during Ras transformation, and careful scanning of the C-myc promoter revealed highly conserved Ets binding sites too (Figure 3.13.A). That would suggest that Ets1 and Ets2 could be directly activating the miR17-92 cluster expression.

ChIP analysis with the Ets2 and C-myc antibodies of the miR17-92 Cluster revealed that both Ets2 and C-myc are present on the cluster promoter during Ras transformation. (Figure.3.13.B). The Ets2 and C-myc binding occurs at a distal end of the miR17-92 promoter. The ChIP primers revealed Ets2 and C-myc binding to the -700 to -900 base pairs location of the miR17-92 promoter. We didn’t see Ets2 and C-myc binding to the proximal promoter (data not shown) although there were conserved Ets and C-myc binding sites. In a previous study, O’Donnell et al saw C-myc binding in the distill region of the promoter. The C-myc binding sites were very close to the CpG island of the miR17-92 promoter that is located around -1000 base pairs from the miR17-92 cluster (O'Donnell et al., 2005). This actually suggests that the distill region of the promoter is important for miR17-92 transcriptional activation.
Figure 3.12. Ets1 and Ets2 deletion downregulates miR17-92 Cluster expression
RNA was isolated from E1+ E2+ and E1-E2- MEFs in the presence or absence of Ras
overexpression and real time PCR was performed on the different members of the
miR17-92 cluster: miR17, miR18, miR19a, miR19b, miR20 and miR92.

Figure 3.13. Ets2 similar to C-myc binds to the miR17-92 promoter
(A) Map of the miR17-92 promoter showing conserved Ets sites (rectangle) and C-myc
binding site (triangle), as well as the ChIP primers binding sites (arrows). (B) ChIP was
performed on E1+E2+ and E1-E2- cells that overexpress Ras with IgG, Ets1 and Ets2
antibodies and quantified by qPCR.
3.2.14 Ets1 and Ets2 transient expression induced miR17-92 expression

In order to study the effect of Ets1, Ets2 and C-myc on the miR17-92 expression, we knocked out C-myc using the Cre-recombinase system from C-myc f/f MEFs established cell lines. The C-myc deletion was complete as shown by western blot (Figure 3.14.A). We than did transient transfections on the C-myc -/- generated MEFs for 48 hrs by transfecting Ets1, Ets2 or C-myc alone or co-transfecting both C-myc and Ets2. Cells were than harvested for RNA extraction. By Real time PCR analysis, we made sure that Ets1, Ets2 and C-myc are over expressed during the transient transfection experiments (Figure 3.14.B, C and D).

Here we show that Ets1 or Ets2 alone were able to increase the expression of miR17-92 cluster similar to C-myc. Co-transfection of Ets2 and C-myc drastically increased the expression of the cluster compared with either Ets1 or Ets2 or C-myc alone (Figure 3.14.E). It should be noted that Ets2 lead to more increase of the cluster expression compared with C-myc or Ets-1 alone in C-myc-/- MEFs. The ability of both C-myc and Ets2 to drastically increase the expression of the cluster explains the huge increase in miR17-92 expression during Ras transformation (Figure 3.8). Constitutive Ras activation lead to an increase in Ets1 and Ets2 protein expression during Ras transformation (Figure3.3), as well as an increase in C-myc protein expression (Figure.3.5). This will lead to the binding of Ets1/2 and C-myc to the promoter of miR17-92 and drives a robust increase of the cluster transcription during Ras transformation.
Figure 3.14. Ets1 and Ets2 transient expression induced miR17-92 cluster expression

(A) Western blot showing C-myc expression in C-mycf/f MEFs before and after Cre transfection. (B) qPCR for Ets1 expression after 48 hrs of Ets1 transient transfection with Ets1 or empty vector in Cmyc-/- MEFs. (C) qPCR for Ets2 expression after 48 hrs of Ets2 transient transfection with Ets2 or empty vector in C-myc-/- MEFs. (D) qPCR of C-myc expression after 48 hrs of C-myc transient expression with C-myc or empty vector in C-myc-/- MEFs. (E) qPCR analysis of the individual miRs of the miR17-92 Cluster after transient transfection for 48 hrs with indicated vectors in C-myc-/- MEFs.
3.2.15 MiR17-92 overexpression in Ets1/2 null non transformed MEFs rescued Ras tumorigenesis

Since the expression of the miR17-92 cluster was downregulated in the Ras infected E1-E2- MEFs because of the absence of Ets1 and Ets2 (Figure 3.8), we infected the Ras E1-E2- MEFs with a 4-hydroxy-tamoxifen inducible C-myc retrovirus to conditionally express C-myc again and see its effect on miR17-92 expression. Upon addition of Tamoxifen, we saw an increase in the C-myc expression in E1-E2- Ras infected MEFs (Figure 3.7.A). Analysis of the miR17-92 cluster revealed that the over expression of C-myc lead to an increase in the expression of the individual miRs (Figure 3.15.A).

Since the deletion of Ets1 and Ets2 inhibited Ras tumorigenesis, and since C-myc and miR17-92 are downregulated as a consequence of Ets1 and Ets2 deletion, we over-expressed miR17-92 in E1-E2- Ras infected MEFs using a pMSCV-puro-miR17-92 vector. After puromycin selection, we checked if the cluster is over expressed in the E1-E2- MEFs. We saw a robust increase in the expression of the miR17-92 upon over expression (Figure 3.15.B). To check if the miR17-92 could rescue Ras transformation in E1-E2- Ras non transformed MEFs, we injected nude mice with three different MEFs: E1+ E2+ Ras transformed MEFs as a positive control, E1- E2- Ras non transformed MEFs as a negative control, and E1- E2- Ras infected MEFs that over express the miR17-92. As expected, all the positive control injected MEFs formed tumors (100%), and non of the negative control injected MEFs formed tumors (N/A). Interestingly, all of the E1- E2- Ras infected MEFs that over express the miR17-92 cluster developed tumors (100%) (Figure 3.15.C). This suggested that the miR17-92 was able to rescue Ets1/2 dependent
Ras transformation. When we compared the tumors volume of the E1+ E2+ Ras transformed MEFs with the tumors volume of the E1- E2- MEFs that over express the miR17-92 cluster, we did not see a difference a significant different. The average tumor volume was 122 for the control MEFs and 125 for the experimental MEFs (Figure3.15.D). The sizes of the positive control and experimental tumors were not different, and as we expected, we did not see tumors developing when we injected E1- E2- MEFs (Figure3.15.E).

Figure 3.15. MiR17-92 overexpression in Ets1/2 null non transformed MEFs rescued Ras tumorigenesis (A) Real-time PCR showing the miR17-92 cluster expression in E1- E2- MEFs that were infected with an inducible ER-myc vector. 1 μM of 4-hydroxy-tamoxifen (4OHT) added to these MEFs in lane 2 lead to an increase in miR17-92 cluster expression. (B) Real time PCR showing the miR17-92 cluster express in E1- E2- Ras infected MEFs in lane 1, and E1-E2- Ras infected MEFs that over express miR17-92 in lane 2. (C) Graph showing the percentage of tumors formed over the total number of injections for the different MEFs used. MEFs were injected in nude mice (1million cells/injection). After 3 weeks the tumors were harvested. The ratios here represent the number of tumors over the number of total injections for the different genotypes. (D) Graph showing the tumor weight of the different MEFs tumors. The asterix indicates significant difference calculated according to the p value (p<0.001). N/A indicates that there were no tumors observed for this genotype. (E) pictures showing the tumors formed from the different MEFs.
Figure 3.15

A

B

C

D

E

Relative Expression

Relative Expression

Number of tumors/total injections (%)

Tumor weight (mg)

Ras + + +

E1+ E2+

miR17-92

N=8

N=8

N/A

N/A

120

100

80

60

40

20

0

122

125

N=8

N=8

N/A

N/A

Ras + + +

E1+ E2+

miR17-92

+ - -

+ + +

+ - -

- - +

+ + +

+ - -

- - +

- - +
3.2.16 Ets1/2 posttranscriptional gene repression through miR17-92

Since miR17-92 was able to rescue Ets1/2 dependent Ras transformation, we did bioinformatic analysis of the different possible targets of miR17-92 cluster in our system using the following website (www.microRNA.org). We found that the tumor suppressor genes: Sfrp1, Fas and Lox which are usually repressed during Ras transformation are possible targets for the miR17-92 cluster. MiR17 seed sequence aligns with the Sfrp1 3’UTR, and miR-92 seed sequence aligns with the Fas and Lox 3’UTR, thus suggesting that these genes could be miR17-92 targets. Here we show that the over expression of miR17-92 in E1-E2- MEFs is able to repress Sfrp1, Fas and Lox genes during Ras transformation (Figure 3.16.A). Sfrp1, Fas and Lox were all repressed in the E1+ E2+ MEFs transformed with Ras compared with the control E1+ E2+ MEFs. These genes were not repressed in the E1- E2- MEFs infected with the Ras retrovirus and their expression was similar to the E1-E2- control MEFs. Interestingly, over expression of the miR17-92 cluster in the Ras infected E1- E2- MEFs drastically repressed these genes. As we are going to see in the next chapter, C-myc is able to directly repress Sfrp1, Fas and Lox genes transcriptionally. In order to see if the repression of these genes when we over express miR17-92 is not due to a feedback loop mechanism where C-myc expression would be increased and thus able to repress Sfrp1, Fas and Lox, we checked for C-myc expression in E1- E2- Ras infected MEFs that over express miR17-92. Our result shows that an increase in miR17-92 did not lead to an increase in the C-myc expression in the E1- E2- MEFs during Ras transformation, thus suggesting that miR17-92 could be directly down regulating the expression of these genes possibly through mRNA decay of Sfrp1, Fas and Lox (Figure 3.16.B).
Figure 3.16. Ets1 and Ets2 gene repression is miR17-92 dependent during Ras transformation

(A) qPCR for Sfrp1, Fas and Lox from E1+E2+ and E1-E2- in the absence or presence of Ras retrovirus. Last lane represents E1-E2- MEFs with Ras and miR17-92 retrovirus. (B)qPCR for C-myc from E1+E2+ and E1-E2- in the absence or presence of Ras retrovirus. Last lane represents E1-E2- MEFs with Ras and miR17-92 retrovirus.
3.3. Discussion

The process of carcinogenesis occurs via multiple steps that correspond to different genetic alterations. One aspect of the genetic alterations is the mutations in the Ras family of GTPases proteins. The role of Ras as a key switch in the signal transduction cascade through the integration of extracellular signals and transmission of those signals through different signal transduction cascades had made Ras the most studied protein, and revealed many important pathways that are essential for the propagation of the Ras response. Targeting of these pathways have been a consistent effort and an on going work, and the Ras/ERK pathway has been one of the essential target pathways in cancer because it mediates important biological functions like apoptosis, proliferation and differentiation. Deregulation of the Ras/ERK pathway lead to transformation and cancer.

Ets1 and Ets2 are among the effectors molecules that mediate the ERK response, and it has been shown that ERK1 and ERK2 overexpression are associated with many cancers. Similarly, Ets1 and Ets2 are over expressed in many cancers.

Functional studies on the Ets role in cancer have shown that C-myc is at least an ets2 target gene. Over expression of Ets2 in breast cancer cell lines lead to an increase in the C-myc expression. Similarly, in these cells, Ets2 was found to bind to the C-myc promoter (Al-azawi et al., 2008). In another study, over expression of an Ets dominant negative form in thyroid cancer cells lead to a decrease in the C-myc expression as well as an increase in apoptosis (de Nigris et al., 2001b). Similarly, in NIH3T3 cells, over expression of a dominant negative Ets2 in the Ras transformed cells inhibited
transformation and also lead to a decrease in the C-myc expression but not other early inducible genes like fos and jun (Langer et al., 1992).

Since previous work pointed towards the involvement of the C-myc protein in the process of Ras/ERK/Ets regulation of cellular transformation, we decided to investigate the role of Ets1 and Ets2 in regulating C-myc expression during Ras transformation. In all of the previous studies, Ets1 or Ets2 or PU.1 or the fusion Ets-lacZ proteins were over expressed, and they all share the same Ets DNA binding domain, suggesting that their artificial abundance in the cell would be blocking the binding of the actual Ets transcription factors that are necessary for the Ras transformation and that activate C-myc transcription. In our model, we took advantage of the Ets1 and Ets2 knockout mice, and generated MEFs that either have an Ets1 or Ets2 or both Ets1 and Ets2 deletions. This approach helped us understand and realize that Ets1 and Ets2 are actually sufficient and necessary for Ras transformation in mouse embryonic fibroblasts. Deletion of Ets1 and Ets2 inhibits Ras tumorigenesis. We also show that Ets1/2 deletion lead to down regulation of C-myc at the RNA and protein levels. We conclude that C-myc is an Ets1/2 target gene to which Ets1 and Ets2 bind and are able to transactivate its expression. Putting back C-myc in Ets1/2 double knockout MEFs rescues Ras tumorigenesis, thus strongly suggesting that C-myc is among the main mediators of Ets1 and Ets2 transformation and is downstream of the Ras/Ets1/2 pathway.

C-myc was not able to activate Ets1/2 classical target genes like MMP9, bclxL, CXCR4 and HbEGF. Although some reports suggested that C-myc is able to bind and activates MMP9 promoter, but this C-myc binding and activation of MMP9 was only in response to shear stress in endothelial cells that affect the development and progression
of atherosclerotic lesions in areas of vasculature that are subject to disturbed flow (Magid et al., 2003). Other reports showed that C-myc indirectly represses bclxL expression during lymphomagenesis, and even in our results we see that over expression of C-myc in Ets1/2 null Ras cells induces repression of the bclxL gene, whereas Ets1 and Ets2 are able to induce bclxL as shown here and in previous data from our lab (Wei et al., 2009). C-myc is known to activate CXCR4, it has been shown that C-myc binds to the promoter of CXCR4 and drives its expression in CD4+ T cells (Moriuchi et al., 1999), but we did not see C-myc activation of CXCR4 in our study. Also, HbEGF which is a known Ras/Ets target was not activated by C-myc (McCarthy et al., 1997). Interestingly, it has been shown that HbEGF lead to induction of C-myc in primary mouse embryonic MEFs (Nanba et al., 2008), but no reports have shown that C-myc is able to induce HbEGF expression. So it appears that C-myc does not affect the activation of Ets1/2 target genes during Ras transformation and other genes should be involved in the Ras/Ets1/2/C-myc transformation mechanism.

In addition to the role of oncogenes in cancer and tumor progression, epigenetic programs are also activated during cancer that will lead to silencing of tumor suppressor genes, and thus enhancing the role of oncogenes in the deregulation and disruption of a normal cellular program. Constitutively active Ras mutations have been shown to regulate a specific epigenetic silencing program during Ras transformation. Ras activates histone methylases, DNA methylases, Co-repressors, polycomb proteins and transcription factors that will bind to the promoter of tumor suppressor genes like Sfrp1, Fas and Lox leading to a series of epigenetic modifications, DNA methylation and transcriptional gene silencing (Gazin et al., 2007). Ets1 and Ets2 have been shown previously to work as
transcriptional repressors of genes like BRCA1, Thrombospondin-1 and others through the recruitment of Brg-1, HDAC and BS69 that have an established role in epigenetic modifications that cause transcriptional repression (Baker et al., 2003; Wei et al., 2003; Zabuawala et al., 2010).

Here we show that Ets1 and Ets2 deletion in the Ras infected MEFs didn’t repress Fas, Lox and Sfrp1 tumor suppressor genes, contrary to wild type MEFs were constitutively active Ras inhibited Fas, Lox and Sfrp1 expression. Sfrp1, Fas and Lox are powerful tumor suppressor genes that are repressed during Ras transformation, and re-expression of any of them would reverse the Ras transformed phenotypes. In mammary epithelial cells, over expression of Sfrp1 was able to reverse C-myc transformation (Cowling et al., 2007). Similarly, Fas overexpression in the Ras transformed MEFs have been shown to restore Ras sensitivity to apoptosis generated by IFN-γ (Fenton et al., 1998). Also, in Ras transformed NIH3T3 cells, Lysyl Oxidase expression lead to a decrease in colony formation (Jeay et al., 2003). The strong inhibition of Ras tumorigenesis in Ets1 Ets2 null MEFs could be explained by the high levels of Fas, Lox and Sfrp1 that unable these cells to undergo transformation. This suggests that Ets1 and Ets2 are involved in transcriptional repression of these genes. As we showed in Chapter 3, Ets1 and Ets2 regulate C-myc expression, and overexpression of C-myc rescued Ets1/2 double knockout MEFs tumorigenesis during Ras transformation. C-myc in these cells was able to repress Sfrp1, Fas and Lox genes. That again suggested the involvement of C-myc not only in transcriptional activation to microRNAs during Ras transformation, but also in the transcriptional repression of Fas, Lox and Sfrp1. Indeed, ChIP analysis shows that C-myc binds to the promoters of Fas, Lox and Sfrp1 genes during Ras
transformation. Also the RNA expression levels of Fas, Sfrp1 and Lox were higher in C-myc -/- compared with wild type MEFs which implies that these are direct c-myc target genes. Interestingly, we also show that Ets1 and Ets2 binds to the Fas and Sfrp1 promoters, thus suggesting that Ets1, Ets2 and C-myc could possibly cooperate together to repress gene transcription. The presence of Ets1, Ets2 and C-myc on the promoter of Fas and Sfrp1 correlates with the enrichment of these promoters with trimethylated H3K9 and trimethylated H3K27 that have an established role in epigenetic silencing. These results strongly suggest that Ets1, Ets2 and C-myc play a physical transcriptional repression role in the regulation of tumor suppressor genes during Ras transformation.

Our model shows that during Ras transformation, Ras is able to activate a series of repressor and Co-repressor molecules and drives them to bind to the promoters of tumor suppressor genes. Ets1, Ets2 and C-myc are activated by Ras and could be forming a strong complex that is recruiting repressor and Co-repressor molecules that strengthen the transcriptional repression of the promoter of tumor suppressor genes by additional methylation of the histones in close proximity to the promoter region, especially H3K9 and H3K27 trimethylation enrichment. EZH2 is one example of Co-repressors that are recruited by Ras to the promoter of those genes, EZH2 is methyl transferase that catalyze mono, di and trimethylation of Histone lysine 27 (H3K27), thus suggesting that C-myc/Ets1/2 could be necessary to recruit EZH2 to the promoter region.

Our data suggest that Ras induces Ets1, Ets2 that in turn induces C-myc, and that C-myc is able to bind to the promoters of many tumor suppressor genes and recruit with the help of Ets1 and Ets2 repressor and co-repressors molecules that are important for silencing tumor suppressor genes and inducing Ras tumorigenesis.
C-myc regulates a variety of targets that are important for cellular metabolism, protein biosynthesis, cell cycle, cell adhesion and the cytoskeleton. A new emerging role of c-myc has been discovered recently which is the regulation of microRNAs. C-myc regulates and activates the expression of miR17-92 cluster (O'Donnell et al., 2005). The miR17-92 Cluster has been shown, similarly to C-myc, to be amplified and overexpressed in many cancers (Volinia et al., 2006). Simultaneous overexpression of C-myc and miR17-92 cooperate to enhance the lymphoma progression (He et al., 2005).

Here we show that Ets1 and Ets2 regulation of C-myc lead to an increase in the miR17-92 cluster expression suggesting as a starting conclusion that miR17-92 is one of the indirect targets of Ets1 and Ets2. Indeed miR17-92 similarly to C-myc, rescued Ras tumorigenesis in Ets1/2 null MEFs, suggesting that miR17-92 is an important downstream oncogenic target of the Ras/Ets pathway. An earlier study has shown that the cooperation between Ras and myc lead to a more powerful induction of the miR17-92 expression compared with Ras only (Dews et al., 2006). That suggests that some Ras target pathways could be actively cooperating with C-myc on the miR17-92 promoter leading to an increase in the cluster expression. Transient transfection data reveal that in C-myc-/- MEFs (were C-myc is deleted and the miR17-92 expression is down), Ets1 or Ets2 alone are able to induce the Cluster expression, and co-expression of Ets2 with C-myc lead to a better induction of the Cluster. The Ets2-C-myc induction of miR17-92 was by far higher than C-myc or Ets1 or Ets2 alone. That could explain that Ras by activating transcription factors like Ets1 and Ets2 would be a better partner for C-myc in inducing targets similar to miR17-92. Our results go along with many reports that show that C-
myc is not the only regulator of C-myc cluster. Indeed NFKB has been shown to regulate this cluster in human biliary epithelial cells. NFKB has been found to bind to the promoter of miR17-92 in response to lipopolysaccharide (LPS) and drives the expression of the Cluster (Zhou et al., 2010). In a recent study, a certain mitotic serine/threonine kinase that is over expressed in many malignancies which is the Aurora kinase A (AURKA), has been found to increase the transcriptional activity of E2F1. E2F1 was found to bind to the miR17-92 promoter by ChIP and induces miR17-92 expression (He et al., 2010).

We also show that the Ras tumor suppressor genes (Sfrp1, Fas and Lox) that are repressed during Ras transformation in E1+E2+ MEFs and that are derepressed in E1-E2- MEFs are downregulated again in E1-E2- MEFs at the RNA level when we over express the miR17-92 cluster. The over expression of the cluster did not lead to an increase in C-myc expression, suggesting that miR17-92 is able to repress Sfrp1, Fas and Lox genes independently from C-myc probably through mRNA decay. Bioinformatics analysis shows that miR17 seed sequence aligns with the Sfrp1 3’UTR, and that miR-92 seed sequence aligns with the Fas and Lox 3’UTR, thus suggesting that these genes could be miR17-92 targets.

Our results suggest that during Ras transformation the Ras/ERK pathway activates Ets1 and Ets2, which in turn activates C-myc. Consequently, C-myc with the collaboration of Ets1 and Ets2 is able to activate the miR17-92 cluster that will lead to tumorigenesis. The activation of the miR17-92 through Ets1 and Ets2 is an example of Ets1 and Ets2 post transcriptional repression of target genes, since miR17-92 has been
shown to bind to many tumor suppressor genes and inhibit their activities. Further identification of the targets of miR17-92 would be of great importance.
CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The important role of Ras in tumor and cancer progression has stimulated intensive work to decipher and understand the different mechanisms through which abnormal Ras signaling is able to avoid normal cellular fate of differentiation, growth arrest and apoptosis into a continuous state of hyperproliferation, tumor development and progression. Genetic models have been a great biological tool that helped understand the role of Ras in cancer signal transduction and made it possible to develop pharmacological and therapeutic treatments that targeted abnormal signaling in cancer.

4.1 Complementary functions between Ets1 and Ets2 in Ras transformation

Our double knockout Ets1/Ets2 model shows that both Ets1 and Ets2 are required for Ras transformation. The reduction in the ability of MEFs to transform and form colonies in the absence of either Ets1 or Ets2 indicates that Ets1 and Ets2 could be possibly targeting different gene sets that are necessary for Ras transformation (Figure3.4.). One possibility is that Ets1 but not Ets2 regulate TGF alpha. Ets1 oncogenic function was shown to be mediated by TGF alpha and deletion of Ets1 in the renal carcinoma and glioma cell lines by stable transfection of Ets1 shRNA lead to an inhibition of tumor formation because of a decrease in the proliferation potential of these
cells that was attributed to a decrease in TGF alpha production. Stable transfection of TGF alpha rescued the proliferation phenotype of Ets1 deleted cells but failed to restore tumorigenesis (Holterman et al., 2010). A genome screening for Ets1/2 DNA binding sites through global ChIP sequencing using IgG, Ets1 and Ets2 antibodies in WT (wild type non transformed MEFs) versus RWT (the control wild type Ras transformed phenotype) MEFs and using RDKO MEFs (Ets1/2 double knockout Ras non transformed phenotype) as a negative control would allow us to identify distinct Ets1 and Ets2 targets that either bind Ets1 or Ets2 and would let us address the specificity of each one of them during the Ras transformation process.

4.2 Ets1 and Ets2 role in the regulation of miR17-92

In this work, we show for the first time a novel Ets1/2 target micro RNA that is required for Ras/Ets1/2 transformation and tumorigenesis. In addition to the ability of C-myc to induce the cluster expression, we show for the first time that Ets1 and Ets2 are also able to induce and regulate miR17-92 expression. Interestingly, over expression of both Ets2 and C-myc lead to a robust induction of miR17-92. This could be due to the fact that C-myc and Ets2 are present in the same complex with other transcription factors and co-activators that will lead to strong transcriptional activation of the miR17-92 complex. Some of these transcription factors would be NFKB and E2F that also bind to the same region as C-myc and Ets2 to activate the transcription of the cluster (Zhou et al., 2010) (Sylvestre et al., 2007). A Sequential ChIP experiment using Ets1 and Ets2 antibodies to pull down Ets1 and Ets2 at the miR17-92 promoter followed by another pull down for NFKB and the E2F would allow us to verify if NFKB and E2F are present in the same transcriptional complex with Ets1/2. The design of different luciferase reporter
vectors harboring mutations in the Ets, NFKB, E2F and myc binding sites and that are induced by Ets1, NFKB, E2F and C-myc vectors would clarify the interplay between these different transcription factors in the regulation of the miR17-92 cluster.

Each microRNA could have hundreds of targets due to the compatibility of the microRNA seeding sequence with the 3’UTR region of 100s of genes. The miR17-92 expresses 6 mature microRNAs. During Ras transformation miR17-92 was able to inhibit Fas, Lox and Sfrp1 tumor suppressor genes when over expressed in Ets1/2 double knockout MEFs, suggesting that Sfrp1, Fas and Lox repression during Ras transformation is not a pure epigenetic transcriptional repression program but also a post transcriptional repression program through the miR17-92. Cloning and mutating the 3’UTR sequence of the Sfrp1, Lox and Fas RNA region into luciferase constructs followed by the transfection of miR17 and miR92 would verify if those genes are targets of the miR17-92 cluster.

Since only 4 out of the 6 mature microRNAs are upregulated during Ras transformation (miR17, miR18, miR19b and miR92), it is necessary to design primers that recognize the premature miR17-92 and make sure that it is induced during Ras transformation. If this is the case, the failure of the active Ras oncogene to induce all of the mature miRs could be attributed to the differential processing of Ras for the different mature microRNAs which favor the induction of miR17, miR18, miR19b and miR92 versus miR19a and miR20 which expression do not change during Ras transformation.
4.3 Ets1 and Ets2 role in repression of the Ras silenced tumor suppressor genes

Previous work in our lab suggested that Ets2 repress many anti-angiogenesis genes in macrophages that lead to a decrease in angiogenesis and less tumor metastasis and growth in the lung. Ets2 with HDAC were present on the promoter of anti-angiogenesis genes like Thrombospondin-1 and Sparc (Zabuawala et al., 2010). Also the non phosphorylated Ets2 interact with the SWI/SNIF chromatin complex and Brg-1 to inhibit the activation of BRCA1 tumor suppressor gene in breast cancer cell lines (Baker et al., 2003).

Here we show that Ets1 and Ets2 induction of C-myc lead to transcriptional repression of Fas, Lox and Sfrp1 during Ras transformation. We also show that Ets1, Ets2 and C-myc bind to the Fas, Sfrp1 and Lox promoters to repress transcription, leading to epigenetic modification and enrichment of the Fas and Sfrp1 promoters with trimethyld lysine 27 and lysine 9 in the histone H3 structure. Preliminary data from our laboratory suggests that the non phosphorylated Ets2T72A mutation in immortalized mouse embryonic fibroblasts is transforming these MEFs in soft agar assays in the absence of the constitutively active Ras oncogene. Interestingly, the ets2T72A mutation in the Ets1 knockout MEFs does not cause transformation in the absence of the Ras oncogene (unpublished data). The expression of Fas, Lox and Sfrp1 tumor suppressor genes is down regulated in the Ets1+/+ Ets2T72A/72A MEFs but not in the Ets1-/- Ets2T72A MEFs. These results suggest that both the phosphorylated and the non phosphorylated forms of Ets1/2 are required for Ras transformation as well as for the repression of the Fas, Lox and Sfrp1 tumor suppressor genes.
4.4 Ras transformation in mouse versus human fibroblasts

The Ras molecule is at the center of many signal transduction pathways since it is activated by different growth factors and their subsequent tyrosine kinase receptors. Many of those growth factors and their receptors have been shown to be overexpressed in many cancers, and to mediate their tumorigenic signal through Ras. The in vitro studies using the mouse embryonic fibroblasts as a model to study Ras oncogenesis have revealed significant information about the function of Ras during carcinogenesis. Here we show that downstream of Ras, Ets1 and Ets2 activate C-myc in order to transform mouse embryonic fibroblasts. C-myc and Ets1/2 collaborate in order to either repress tumor suppressor genes like Fas and Sfrp1 or activate oncogenes like miR17-92 in order to transform MEFs in vitro. These results suggest that Ets1 and Ets2 are able to regulate a transcriptional repression and activation program that is important for Ras tumorigenesis. But we should be careful in interpreting the conclusions from these experiments. There exist substantial difference between the transformation of mouse embryonic fibroblasts and the transformation of human fibroblasts. The addition of the C-Myc or H-RAS oncogenes in the contest of p53 loss efficiently transforms murine embryonic fibroblasts but fails to transform human cells constitutively expressing hTERT, the catalytic subunit of telomerase. In contrast, transformation of human fibroblasts requires the constitutive expression of c-Myc, H-RAS, and hTERT, together with the loss of function of the p53, RB, and PTEN tumor suppressor genes (Boehm et al., 2005). These manipulations permit the development of transformed human fibroblasts with genetic alterations similar to those found associated with human cancers. It should be noted that the Ras-Raf-MEK-
ERK pathway activation induces senescence through the induction of p16 in human diploid fibroblasts. Ets1 and Ets2 activation by the Ras-ERK pathway lead to p16 accumulation in human diploid fibroblasts and senescence. P16 inhibits CDK4 and CDK6 activity and thus inhibit RB phosphorylation which leads to growth arrest and senescence. For the transformation of human diploid fibroblasts, RB inactivation is necessary to escape senescence and undergo proliferation as a first step towards transformation (Ohtani et al., 2001). Thus in the mouse system, p53/p19 inactivation is essential for fibroblasts immortalization, whereas in the human system RB/p16 pathway inactivation is necessary for fibroblasts immortalization. These differences between the human and mouse system should be taken into consideration while interpreting the role of Ets1 and Ets2 in Ras transformation. And thus associating Ets1 and Ets2 role in this model should also take into account the absence of Rb and p16 activity during the process of Ras oncogenic transformation in human fibroblasts. It seems to us that mutations in Rb or p16 are necessary for Ets1 and Ets2 to be able to induce transformation through the Ras-MAPK pathway, and thus in the presence of an intact p16 or Rb gene, Ets1 and Ets2 would be playing a protective role against cancer progression by inducing senescence. It would be interesting to use immortalized human fibroblasts that are transformed by Ras and knock down both Ets1 and Ets2 to see whether miR17-92 expression is inhibited and whether Fas and Lox expression is derepressed. MiR-17-92 is able to repress p21 tumor suppressor gene leading to more proliferation. Also miR17-92 is able to repress PTEN thus enabling cellular survival and avoiding apoptosis (Xiao et al., 2008) (Inomata et al., 2009). Thus the finding that Ets1 and Ets2 are able to
regulate miR17-92 adds new evidence towards their role in activating pro-survival pathways for the cells to survive and be able to transform.

Finally, the Ras signaling through Ets1 and Ets2 and their C-myc partner is enough to induce transformation in mouse embryonic fibroblasts but it could not be the case in human fibroblasts. The ETS family members other than Ets1 and Ets2 are frequently deregulated or mutated in human cancers through chromosomal rearrangements and they collaborate with other protein partners to regulate genes that are involved in tumorigenesis. One example is Ewing sarcoma that is caused by the fusion of the transcriptional activation domain of EWS to the Ets transcription factor Fli which contains the Ets DNA binding domain (Delattre et al., 1992). The fusion EWS/FLI functions as an aberrant transcription factor that regulates genes involved in the tumorigenic phenotype of the Ewing sarcoma. Thus ChIP sequencing using other Ets transcription factor antibodies against Fli1 as well as ERG, ETV1 and ETV4 Ets transcription factors, also shown to be translocated in prostate cancer (Tomlins et al., 2006; Tomlins et al., 2005), would allow us to identify specificity as well as redundancy among the Ets factors in the regulation of many genes that are involved in cancer tumorigenesis.

4.5 Analysis of Ets1/2 target genes in sarcomas tissues with somatic mutations

A new sarcoma microRNA database generated by the Subramanian group from the university of Minnesota revealed that most of the mature miRs of the miR17-92 cluster are over expressed in many soft tissue sarcomas. The expression pattern of these different miRs increases from fibromatosis (benign tumor from fibroblasts) to fibrosarcoma (malignant tumors from fibroblasts) (Sarver et al., 2010). It should be
verified whether over expression of miR17-92 in human fibrosarcomas is associated with somatic mutations in Ras, Raf, or over expression of tyrosine kinase receptors. That would suggest that miR17-92 could be used as a prognostic and diagnostic biomarker in fibrosarcoma patients. PTEN mutation or deletion has been shown to activate the Ras/PI3Kinase/Akt pathway leading to Ets2 over expression and activation. Current work from our laboratory suggests that PTEN deletion in tumor associated fibroblasts lead to an increase in miR17-92 expression, thus suggesting that miR17-92 could be a downstream target of the PTEN pathway. Analysis of miR17-92 expression in soft tissue sarcoma samples that have PTEN mutations would also reveal whether miR17-92 is an important marker in the PTEN mutations tumors signatures. Targeting miR17-92 cluster could be included in the treatment of cancer patients in combination with traditional and novel drugs that targets the Ras/PI3K/Akt and Ras/Raf/ERK pathway.

4.6 Ets1/2 RNA expression microarray

As shown in this work, Ets1 and Ets2 work both as transcriptional activators and transcriptional repressors during Ras tumorigenesis. The fact that Ets1 and Ets2 are able to regulate and activate C-myc and miR17-92 microRNA cluster and that miR17-92 or C-myc is able to rescue Ras tumorigenesis suggests that other genes could be involved in Ets1/2 dependent Ras tumorigenesis. A global gene analysis as well as a broader approach is needed in order to have a better understanding of the mechanism or mechanisms that regulate Ets1/2 dependent Ras oncogenic transformation.

A cDNA microarray as well as a microRNA microarray study of Ets1/2 dependent Ras transformation would be able to reveal a significant number of information about the important genetic pool that dominate this process.
The cDNA microarray should be performed on the RDKO (Ets1/2 double knockout Ras non transformed phenotype) MEFs as our experimental phenotype, versus RWT (the control wild type Ras transformed phenotype) MEFs. The analysis should also include WT (wild type non transformed MEFs). The cDNA micro array data would reveal genes that are differentially expressed in RWT versus RDKO MEFs. This list of genes should be compared to WT versus RWT MEFs. Genes that are differentially expressed in RWT versus RDKO as well as RWT versus WT could be potential Ras/Ets1/2 targets. A gene list of Ras/Ets1/2 targets would be generated. The gene list would be verified by qRT-PCR, and over expression of these genes in RDKO MEFs would test the ability of these genes to rescue Ras transformation both in vitro in soft agar as well as in vivo in nude mice. In parallel, ChIP experiments would also reveal if Ets1 and Ets2 are present at the promoter of those genes and activating or repressing their transcription.

In addition to the cDNA microarray, a microRNA microarray will give additional information about additional targets that Ets1 and Ets2 would be regulating. The same logic among the different Ets1/2 genotypes and phenotypes would be applied to identify the potential Ets1/2 microRNA targets. Over expression of microRNAs that would rescue the Ras transformation phenotype in the RDKO MEFs could be Ras/Ets1/2 activated genes. On the other hand, over expression of microRNAs in RWT MEFs that inhibit transformation would be identified as tumor suppressor microRNAs that are repressed by Ets1 and Ets2 during Ras transformation.

The previous experiments will provide us with clues to understand whether there is a predominant mechanism through which Ets1 and Ets2 regulate Ras tumorigenesis,
since the classical Ets1/2 genes like MMP9, bclxL, CXCR4, HbEGF and Cyclin D1 were not essential for Ets1/2 Ras transformation mechanism.

4.7 The oncogenic function of Ets1 and Ets2 in animal models

Using the genetic mouse model, our laboratory has shown that Ets2 plays an important role in the stroma that favors tumor progression and metastasis. The Ets2 deletion from the fibroblasts in the breast cancer stroma suggested that the Ets2 signaling from the stromal fibroblasts favors tumor progression (Trimboli et al., 2009), and Ets2 deletion from macrophages suggested that Ets2 signaling in the macrophages favors metastasis and angiogenesis (Zabuawala et al., 2010). It seems that Ets2 is the most important Ets factor in the stromal compartment. Since in our model, Ets1 and Ets2 mediate Ras oncogenesis, one question would be are Ets1 and Ets2 important for tumor progression in other cellular compartments in the stroma (the endothelial cells)? Both Ets1 and Ets2 signaling in the endothelial cellular compartment are both required during developmental angiogenesis (Trimboli et al., 2009). It could be true also that both Ets1 and Ets2 signaling are required for tumor angiogenesis. MiR17-92 increases angiogenesis through the repression of anti-angiogenic factors like thrombospondin-1 (Dews et al., 2006), and thus miR17-92 could be one of the essential Ets1/2 targets in the endothelial cell compartment in tumor angiogenesis. On the other hand, the Ets2 signaling in the epithelial cell compartment of the mammary gland is not essential for breast cancer progression (Trimboli et al., 2008; Wagner et al., 1997). Thus it would be important to study the Ets1 and Ets2 oncogenic signaling in the epithelial cell compartment of the mammary gland by combining the mouse conditional Ets1 and Ets2 models with the PyMT model in the mammary gland. Whether miR17-92 is involved in this process, this
would be addressed too in this model. MiR17-92 is able to repress PTEN protein expression, and PTEN deletion in the epithelial cellular compartment of the mammary gland lead to tumor progression (Li et al., 2002). Whether Ets1 and Ets2 activation of miR17-92 would lead to repression of PTEN in the epithelial cells and thus tumor progression would be an interesting hypothesis to follow.
BIBLIOGRAPHY


He, L., Thomson, J.M., Hemann, M.T., Hernando-Monge, E., Mu, D., Goodson, S.,

He, S., Yang, S., Deng, G., Liu, M., Zhu, H., Zhang, W., Yan, S., Quan, L., Bai, J., and
Xu, N. (2010). Aurora kinase A induces miR-17-92 cluster through regulation of E2F1


contributions of Ets2 to craniofacial and thymus phenotypes of trisomic "Down


Inomata, M., Tagawa, H., Guo, Y.M., Kameoka, Y., Takahashi, N., and Sawada, K.
(2009). MicroRNA-17-92 down-regulates expression of distinct targets in different B-cell

inducing the expression of urokinase-type plasminogen activator and matrix
metalloproteinase-1 and the migration of vascular endothelial cells. J Cell Physiol 169,
522-531.


