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Nuclear and Cytoplasmic Roles of the Transcription factor REST

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Author
Vu, Anthony

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Nuclear and Cytoplasmic Roles of the Transcription Factor REST

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Anthony Vu

Committee in charge:

Professor Fred H. Gage, Chair
Professor Lorraine Pillus, Co-chair
Professor Gene W. Yeo

2009

This Thesis of Anthony Vu is approved, and it is acceptable in quality and form for

publication on microfilm and electronically:

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Co-chair

_____

Chair

University of California, San Diego

2009

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ACKNOWLEDGEMENTS

ABSTRACT OF THE THESIS

Nuclear and Cytoplasmic Roles of the Transcription Factor REST

by

Anthony Vu

Master of Science in Biology

University of California, San Diego, 2009

Professor Fred H. Gage, Chair

Professor Lorraine Pillus, Co-chair

The neuron-restrictive silencer factor (NRSF), also known as repressor element -1 (RE1) silencing transcription factor (REST), is known to act as a transcriptional repressor of neuron-specific genes in nuclei of non-neuronal cells. REST binds a DNA sequence known as neuron-restrictive silencer elements (NRSEs/RE1s) and recruits co-repressors to carry out silencing chromatin modifications. Recent REST ChIP-seq publications have shown that REST binding is not limited to the canonical RE1 sequence, but binds multiple variations of it across the genome.

This study focuses on determining REST's capacity to act as a repressor based on the binding site's degree of correspondence to the canonical RE1 motif. I show that the RE1 right half site acts as a REST-recruiting repressive element. I have also begun studying differential recruitment of REST protein and its cofactors between human embryonic stem cells (hESCs) and hESC-derived neural progenitor cells (NPCs) on a

genome-wide scale using chromatin immunoprecipitation followed by massive parallel sequencing of DNA tags (ChIP-seq).

I also present preliminary data suggesting that REST may have a second function as a post-transcriptional activator. Increased luciferase expression is observed when reporter constructs were cotransfected with high levels of REST in mouse Neuro2A (N2A) cells. I show that this concentration dependent function of REST is due to the cell's limited capacity to transport and/or retain REST in the nucleus, where it acts as a repressor. With increasing concentrations of REST, a large fraction remains in the cytoplasm, where REST may bind and stabilize the luciferase transcript and/or enhance its translation.

Introduction

In 1990, both Gail Mandel's and David Anderson's labs independently encountered an upstream *cis*-acting silencer element responsible for down-regulating the two neuronal genes SCG10 and type II sodium channel (NaII) in non-neuronal cells (Maue et al., 1990; Wuenschell et al., 1990). Two years later, the sequence of this element was identified by both groups and called repressor element-1 (RE1) by Mandel or neuron-restrictive silencer element (NRSE) by Anderson. In 1995, both labs independently isolated a *trans*-acting silencing factor from a murine cDNA clone that bound RE1 to repress neuronal genes in non-neuronal cells. They named this protein repressor element 1 silencing transcription factor/neuron restrictive silencer factor (REST/NRSF) (Chong et al., 1995; Schoenherr and Anderson, 1995). Since then, a plethora of RE1 sites have been discovered in promoter regions of various neuron-specific genes (Lonnerberg et al., 1996; Kallunki et al., 1997; Li et al., 1993). As these genes are lowly expressed in non-neuronal cells where REST levels are high, and vice versa in neuronal cells, it is believed that REST may be a master regulator of the neuronal phenotype. Thus, the protein continues to be of great interest in the field of developmental biology and neuroscience.

REST Gene Structure

The REST gene, comprising three alternative first exons in the 5'UTR, three coding exons, and a 28 bp alternatively spliced exon, is present and highly conserved in all vertebrate genomes (Mortazavi et al., 2006). Inclusion of the alternatively spliced

exon yields the most common splice variant, REST4. This prematurely terminated transcript retains only the first five zinc fingers (ZFs) and was found to be neuron specific. Alternatively, full length REST transcript yields a ~120-kDa protein with two repressor domains, separated by a DNA binding domain (DBD) consisting of eight ZF motifs. The first repressor domain, located near the amino terminus of the protein, recruits the corepressor mSin3A, which in turn, binds multiprotein complexes containing histone deacetylases and other chromatin modifying enzymes (Grimes et al., 2000). Containing a C_2H_2 class ZF, the second repressor domain resides near the carboxyl terminus of REST and recruits the corepressor CoREST (Andres et al., 1999). Additionally, REST includes a lysine-rich region and a proline-rich region between the DBD and the C-terminal repressor domain; however, the function of these elements is unknown (Figure 1).

After protein synthesis, REST is recognized by its nuclear localization signal (NLS) and shuttled into the nucleus, where it represses transcriptional activity of neuronal genes in non-neuronal cells. It was initially thought the NLS sequence was located at residues 512-522, between the eighth and ninth ZFs, as a mutation in this region prevented nuclear localization of REST in COS-1 cells when analyzed by immunoflourescence (Grimes et al., 2000). However, more recent studies in which various sets and individual ZFs were mutated, concluded that the ZF domain 5 is necessary and sufficient for nuclear targeting (Shimojo et al., 2000; Shimojo, 2005).

During neuronal differentiation from ESCs, REST levels decline to allow expression of neuron-specific genes (Ballas et al., 2005). To maintain the low levels of REST cellular protein during this transition, a degradation signal sequence (degron)

located near the C-terminus of REST is recognized via the ubiquitin ligase SCF$^{\beta\text{-TrCP}}$ and

directs the protein for proteasome-mediated degradation (Guardavaccaro et al., 2008).
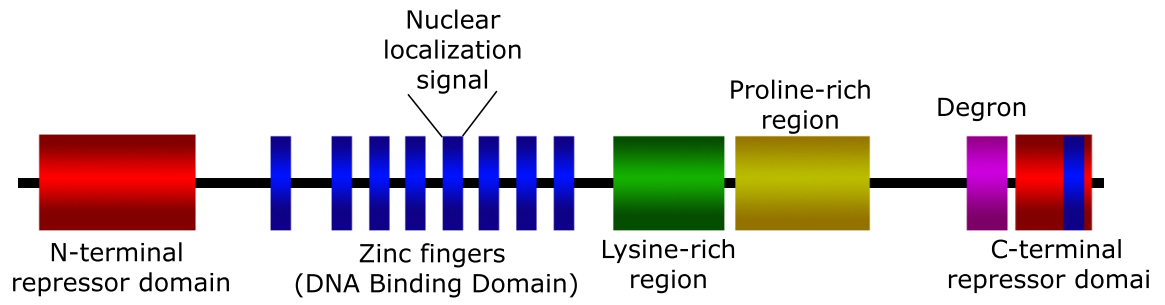
Figure 1. REST Protein Diagram.

The REST protein contains multiple functional domains.

RE1 Binding Site

Approximately half of the reported transcription factors in the human genome include a C_2H_2 ZF motif, with each ZF usually capable of binding to a specific triplet of DNA base-pairs (Pavletich and Pabo, 1991; Emerson and Thomas, 2009). Since REST has eight ZFs in its DNA binding domain, not all may be involved in binding the 21-bp canonical RE1 simultaneously. Furthermore, recent evidence from large-scale chromatin immunoprecipitation experiments has shown that the canonical RE1 comprises two half sites (Figure 2), which appear to be able to recruit REST independently. These observations suggest that REST may utilize alternative sets of ZFs in a manner dependent on the type of binding site.

Due to its well-defined and relatively large binding sequence, REST and the RE1 have been of interest to many research groups attempting to predict binding sites and target genes on a genome wide scale. For example, Mortazavi et al. (2006) were able to generate a database of 842 evolutionarily conserved binding sites associated with 733 genes in mammals, merely by use of computational analysis of aligned genomes. With advanced sequencing technology such as chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq), researchers have identified as many as 5,813 binding sites for REST in the human genome. Moreover, 17% of these sites contain only one of the half sites, with a majority being right half sites (Jothi et al., 2008). Interestingly, Bruce et al. (2009) were able to show that a REST binding site's degree of agreement with the canonical RE1 sequence determines its affinity for, and thus occupancy level of, REST protein. Thus, at a given cell-type specific level of REST, the

degree of repression that each RE1-containing gene experiences is determined by its precise RE1 sequence.

Figure 2. Canonical RE1 Motif.

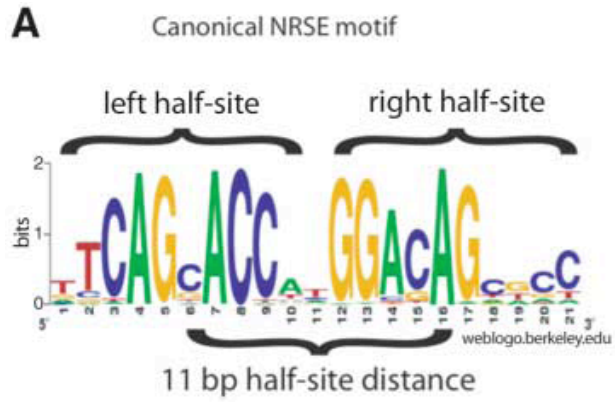The 21bp conserved RE1 binding site of REST, comprised of two separate left and right half sites separated by two degenerate nucleotides (from Johnson et al., 2007).

Function in Development

Neuronal differentiation during development depends on global changes in transcriptional activity across the genome, to modulate a network of genes that give rise to a particular cell type. One factor contributing to the regulation of this lineage determination during embryonic neurogenesis is REST. REST levels decrease during neuronal differentiation to alleviate repression, and therefore promote expression, of genes required in neurons. For that reason, scientists initially believed REST determines neuronal cell fate based on decreased protein concentration levels. In support of this, homozygous REST knockout mouse embryos show forebrain malformation during the start of neurogenesis, i.e. on embryonic day 9 (E9) and embryonic lethality with 100% penetrance by E11.5, stressing the importance of the protein in early neuronal development. Surprisingly, however, de-repression was only seen for two REST target genes out of several examined. Additionally, inhibition of REST function failed to produce neurons from muscle cells (Chen et al., 1998). Therefore, the results of these *in vivo* experiments suggest that REST down-regulation is not the sole determining factor for neuronal commitment, but instead, is required at a later stage to repress a subset of genes and to prevent neuronal differentiation.

The function of REST is not restricted to embryonic development, but has been found to regulate mature neurons as well. Studies on rodent hippocampus during kainate-induced epilepsy have shown that these insults lead to increased REST transcript levels (Palm et al., 1998). Therefore, it has been suggested that the presence of REST serves to down-regulate the expression of the genes during such a nervous system disorder, perhaps in order to protect these cells from further injury. Similarly, REST levels have

also been discovered to increase during global ischemia that leads to CA1 pyramidal neuron cell death (Formisano et al., 2007). As a result, it has been suggested that by its mechanism of regulating the expression of REST target genes, such as GluR2 and MOR-1, REST is actively involved in controlling gene expression in the adult brain as well.

Function in ES cells

More recently, experiments in mouse ESCs (mESCs) have suggested a possible alternative function. Specifically, REST has been demonstrated to maintain ESC pluripotency and self-renewal by preventing microRNA-mediated down-regulation of ESC factors Oct4, Sox2, Nanog, and c-Myc. Singh and colleagues showed that in mESCs, REST actively represses miR-21, a miRNA which in turn targets these pluripotency factors for down-regulation (Singh et al., 2008). In support of a role for REST in maintenance of pluripotency, REST loss-of-function results in differentiation towards all lineages. However, two groups have since challenged these findings, and the role for REST in maintenance of pluripotency of ESCs remains controversial (Buckley et al., 2009; Jorgensen et al., 2009).

REST Function as a Repressor

REST has been known to have two different functions: to block expression of neuronal genes through the recruitment of silencers and repressors in terminally differentiated non-neuronal cells and to be expressed at low but detectable levels in certain neuronal cells, presumably to allow dynamic regulation of neuronal genes. The defined mechanism for repression of neuronal genes is: REST's two repressor domains

function to recruit corepressors CoREST at its C-terminus and mSin3A at its N-terminus, which then bind histone deacetylases (HDACs) and other chromatin modifying enzymes. HDACs remove acetyl groups on the N-terminal tails of histones H3 and H4, and the targeted chromosomal region assumes a condensed state that inhibits transcription activity. Additionally, silencing proteins found to bind CoREST following REST recruitment are MeCP2, SUV39H1, and HP1 (Lunyak et al., 2003). Similar to HDACs, the complex recruited by CoREST methylates neighboring nucleosomes on histone tails resulting in chromosome condensation (Figure 3). Generally, both repressor domains are necessary for full repression of their target; however, an individual domain is sufficient to suppress type II promoter in non-neuronal cells (Tapia-Ramirez et al., 1997). On the other hand, low levels of REST/NRSF prevent this protein-DNA interaction and thus allow transcription of genes, designating the path for neuronal cells.
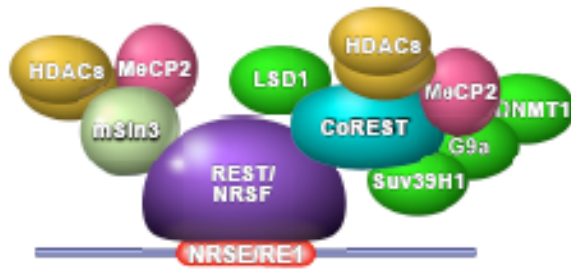
Figure 3. REST Complex.

When bound to RE1 site, REST recruits two distinct repressor complexes. The N-terminus recruits mSin3a and the C-terminus recruits CoREST. The corepressors act as anchors for binding of chromatin modifying proteins.

REST as an Activator

Since its discovery, REST has been widely studied for its role as a transcriptional repressor. However, reports have emerged hinting at an additional function, namely one as a transcription activator. In 1997, Bessis and colleagues observed an increase in transcription of a synthetic promoter driving the luciferase gene when the RE1 binding site was located in the 5'UTR or less than 50bp upstream of the TATA box in neuronal cells. When positioned >50bp away from the TATA box, REST acted as a repressor (Bessis et al., 1997). In non-neuronal cells, however, RE1 was consistently a silencer regardless of the binding element's position. A similar effect was later observed in 2001, when Yoo and colleagues sought to determine the molecular mechanism regulating dynamin I in neuronal cells. After characterizing the promoter region of the gene, they discovered a RE1-like sequence, and showed that co-expressing a reporter gene directed by dynamin I's promoter along with REST plasmid in neuronal cells increased transcription (Yoo et al., 2001). Neither study, however, addressed whether the increase in expression was due to increased transcription, or due to post-transcriptional or post-translational effects.

Indeed, REST's functions may not be limited to the nucleus. Several studies have hinted at a possible novel REST function, that is, one for post-transcriptional regulation. REST has shown to be localized to the inside and outside of the nucleus in multiple cell lines including human embryonic stem cells, human neuroblastoma cells, and hippocampal primary neurons (Sun et al., 2005). Regulation of the subcellular localization of REST has been shown to control the availability of REST to RE1 sites in the nucleus, thereby modulating target gene expression. For example, wild-type

huntingtin protein binds and targets REST to the cytoplasm to relieve repression of RE1-regulated genes, including brain derived neurotrophic factor (BDNF), a survival factor for neurons that die in Huntington's disease (Zuccato et al., 2003). However, the observation of REST in the cytoplasm of multiple cell lines raises questions about its function in this cellular compartment beyond sequestration from the nucleus. To begin addressing the issue, Kim and colleagues showed that neuroblastoma cells transfected with REST expression plasmid, along with a reporter plasmid containing luciferase cDNA under the control of the RE1 from the mu opioid receptor (MOR), had twice as much luciferase output as cells with no overexpressed REST, suggesting REST-mediated activation (Kim et al., 2008). Together, these observations exemplify the complexity to understanding the range of functions a single protein may serve.

Overview of Research

In recent ChIP-seq studies, REST has been shown to bind numerous noncanonical sites. In the first part of my thesis work (Chapter 1), I assess the function of the various binding sites in a dual luciferase reporter assay system by cotransfecting cells with REST and luciferase reporter gene constructs containing the newly discovered REST binding sites. The results were then compared to cells without transfected REST plasmid. Furthermore, I performed a genome-wide analysis of REST binding and function in hESC differentiation to NPCs by ChIP-seq. I prepared ChIP libraries for REST and cofactors mSin3a and CoREST in both cell lines. Although the bioinformatic analysis and experimental validation of this experiment are still ongoing at this time, I expect these data to yield insights into the function of the noncanonical binding sites, i.e.,

whether they are capable of recruiting corepressors for active repression. In addition, the analysis may also sharpen our understanding of REST's role during the early steps of hESC differentiation towards the neuronal lineage.

In the second part of my thesis work (Chapter 2), I present preliminary data of an ongoing study of REST's potential function as an activator when expressed at high concentrations. I showed in luciferase reporter assays that high levels of REST led to activation of the reporter gene in a RE1-independent manner. Moreover, when REST is expressed at high levels, a large fraction of the protein localizes to the cytoplasm. Given these observations and recent results from lab into consideration, I hypothesize that cytoplasmic REST may act as an activator of translation.

Materials and Methods

Cell Culture

HEK 293T cells were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, from Gibco) supplemented with 10% fetal bovine serum. Mouse Neuro2A (N2A) cells were also cultured at 37°C in 5% CO_2, in alpha-MEM (Cellgro) media supplemented with 10% fetal bovine serum.

HUES6 and NPCs were cultured as previously described (Yeo et al., 2007). NPCs were derived from HUES6 cells as previously described (Yeo et al., 2007).

Plasmid Construction

Wild type REST was kindly provided by D. Anderson. REST constructs containing an individual mutation in one of the nine ZFs were kindly provided by Marcial Vilar (Riek lab).

Reporter plasmids for luciferase assays were constructed as follows. The pGL3-TK reporter plasmid was constructed by sub-cloning the thymidine kinase (TK) promoter from the pRL-TK *Renilla* expression vector into pGL3-basic (both from Promega) with *Bgl*II/*Hind*III. RE1 sites consisting of the canonical RE1 sequence, a canonical RE1 with two point mutations in two highly conserved nucleotides, left half site, right half site, a scrambled RE1 sequence, and split RE1 site with seven nucleotide spacer between both halves, were generated by TOPO-mediated ligation of annealed oligos into in pCR-Blunt II-TOPO (Invitrogen). The RE1 sequences were excised by restriction digest with *Kpn*I

and *XhoI* restriction enzymes (Figure 5) and inserted into the same sites upstream of the TK promoter in the engineered pGL3-TK vector.

Coimmunoprecipitation (Co-IP) analysis

Using Invitrogen's Lipofectamine 2000 reagent, 293T cells grown in 10cm plates were transfected with 25µg of DNA encoding myc-tagged and 25µg FLAG-tagged wild type REST or one of the REST constructs that contain an individual point mutation in one of the nine ZFs. Two days post-transfection, nuclear extracts were prepared from cells using a kit (Active Motif) and their protein concentration quantified by Bradford assay. Forty µg (protein) of each nuclear extract was incubated overnight in 400µl binding buffer (200mM NaCl, 50mM tris-HCl pH 8.0, 1% NP-40, and 1mM DTT) at 4°C. Five percent of this sample was saved as input control. The following day, half of each sample was immunoprecipitated with either 40µl anti-FLAG M2 agarose beads (Sigma) or 40µl Protein G agarose (Upstate) and 1µg anti-c-myc 9E10 antibody (Santa Cruz) for 1 hour with rotation at 4°C. The supernatant was removed and the beads were washed three times in 1ml binding buffer prior to eluting by heating to 95°C in SDS-PAGE loading buffer. Samples were run on 4-12% bis-tris gels (Invitrogen). After transfer to PVDF membrane, the blots were probed with either mouse anti-FLAG antibody (Sigma, M2) at a dilution of 1:10,000 or anti-myc antibody (Santa Cruz, 9E10) at a dilution of 1:1,000, followed by anti-mouse HRP (Jackson) at 1:1,000. The input membrane was probed with Upstate anti-REST 07-579 at 1:1,000, followed by anti-rabbit HRP (Jackson) at 1,1000. Bands were visualized by enhanced chemiluminescence.

Luciferase Reporter Assays

Luciferase assays were conducted with Promega's Dual Luciferase Reporter Assay System using Neuro2A (N2A) cells, a REST-deficient mouse neuronal cell line. Cells were transfected in triplicate with reporter constructs and FLAG-tagged REST or control vectors: green fluorescent protein (GFP) under the CMV promoter (CMV GFP, gift from Lynne Moore) or empty vector control (pcDNA3.1, Invitrogen). A plasmid driving *Renilla* luciferase under the TK promoter (phRL TK, Promega) served as a transfection control. Transfections were done in 24-well plates, using 1.0μg of total DNA per well and Lipofectamine 2000 (Invitrogen). Cells were harvested two days later, lysed in 200μl of 1x Passive Lysis Buffer (Promega) and assayed for firefly and *Renilla* luminescence activities as per Promega's protocol. Relative luciferase activity was expressed as the ratio of firefly output to by *Renilla* output.

Luciferase assays using psiCheck-2 vector (Promega) were executed similarly to the protocol mentioned above. However, transfections were carried out in 96-well plates, using 0.2 μg of total DNA per well. Transfection reagent and lysis buffer were scaled accordingly and *Renilla* and firefly luminescence activities were determined as above.

REST Chromatin IP (ChIP) and Solexa sequencing

For REST-ChIP in ES Cells library:

Following Upstate's EZ ChIP protocol and scaling accordingly, HUES6 cells grown on a 10cm plate to 90% confluency were harvested for chromatin immunoprecipitation. We used one 10 cm plate of cells per immunoprecipitation. Briefly, cells were sonicated in a water bath sonicator (Diagenode Bioruptor) at 'high'

setting for 0.5 min on/0.5 min off, for 30 min total. This sonication time emerged as optimal to achieve uniform chromatin fragments of ~200bp in length. Ten μg REST antibody (H290, Santa Cruz) and 600μl of blocked protein A agarose beads (Upstate) were used for immunoprecipitation. After washing, de-cross-linking and purification of the immunoprecipitates, samples were subjected to real-time PCR using primers (listed below) of known REST targets to determine library quality. The final product was purified using a nucleotide removal kit (Qiagen). Fifty ng of DNA was used for library preparation according to the Illumina Genomic DNA Sample Preparation protocol. After linker ligation and PCR amplification (20 cycles), products of 150-300bp in length were isolated, quantified by PicoGreen assay (Invitrogen) and sequenced on a Solexa 1G Genome Analyzer.

For the remaining libraries:

Cells were cross-linked and chromatin and a nuclear extract was prepared as described (Johnson et al., 2007). The lysate was sonicated under the same conditions as above, and the remainder of the procedure performed according to Upstate's EZ ChIP protocol and Illumina Genomic DNA Sample Preparation protocol. Two plates of cells were used for each immunoprecipitation, with 2 μg of Santa Cruz H290 REST antibody, Santa Cruz AK-11 mSin3a antibody, or Upstate 07-579 CoREST antibody.

Solexa sequencing (35 cycles), adaptor trimming, quality filtering and genomic mapping to the genome (assembly hg18) were performed by Yujing Liang (Yeo lab, UCSD).

qPCR primer sequences used are as follows:

ATP2B2 forward 5'-CAAGGGCACAGTGCTGATT-3'

ATP2B2 reverse 5'-GCACTTAATAACATCCTGCTCTG-3'

BAI3_positive forward 5'-TTTGAAACAATCCGATGAGC-3'

BAI3_positive reverse 5'-CCTATGCGAGGAACAGCAG-3'

BAI3_negative forward 5'-AAATCCACCAATTGCAGCTT-3'

BAI3_negative reverse 3'-CAACATGGTGATTGGCATTT-3'

RT-PCR analysis for *Renilla* and firefly luciferase mRNA expression

RNA was extracted using RNA-Bee (AMS) as per the supplier's protocol from 50μl of transfected cell extract produced for luciferase assays. Approximately 675ng of RNA was DNase-treated (Turbo DNA free kit, Ambion), followed by Invitrogen reverse transcription (Superscript III First-Strand Synthesis kit, Invitrogen). Real-time PCR was carried out using cDNAs with Applied Biosystems SYBR Green PCR master mix in triplicate. PCR products were normalized to levels of GAPDH, which was quantified using Taqman probes (ABI).

qPCR primers sequences used are as follows:

Firefly luciferase forward 5'-GCAGCCTGCAAGACTACAAA-3'

Firefly luciferase reverse 5'-TCTCGTGCAGGTTAGACAG-3'

Renilla luciferase forward 5'-CTCCTACGAGCACCAAGACA-3'

Renilla luciferase reverse 5'-CTTGATCAGGGCGATATCCT-3'

Immunoflourescence

N2A cells were grown on 2-well glass slides. 1.8 μg or 20 ng of FLAG-mREST plasmid were transfected with Lipofectamine 2000 at 30% confluency, to simulate activation and repressor conditions, respectively. Cells were then fixed with paraformaldehyde the next day and incubated in primary antibody [Upstate anti-REST 07-579 (1:200) or anti-REST 12C11 (gift from David Anderson, 1:10)] overnight at 4°C. Secondary antibodies were anti-rabbit Alexa Fluoro 488 and Cy5 labeled donkey anti-mouse IgG (both from Jackson Immuno Research, both at 1:500).

Images were captured using a Blue-Diode confocal microscope (Nikon) under two settings and then further processed using MetaMorph (Molecular Devices). Setting A, for high REST levels, used reduced diode, iris, and gain settings compared to Setting B.

Chapter 1 – Characterization of REST Interaction With the Genome

Introduction

Studies employing ChIP-seq have shown that REST is associated not only with the canonical RE1, but also with sequences comprising subsets of the RE1 (Johnson et al., 2008; Valouev et al., 2008; Jothi et al., 2008; Figure 4). However, little is known about the mechanism of REST binding to these sites and their functional significance. To explore these details, I investigated the possibility of REST binding as a dimer, and tested whether this interaction is ZF mediated. I also performed promoter luciferase reporter assays to assess whether the noncanonical binding sites function as repressive sequence elements similar to the RE1. I also performed a genome-wide study of REST's role during hESC differentiation to NPCs, using ChIP-seq.

Figure 4. REST Noncanonical Binding Sites.
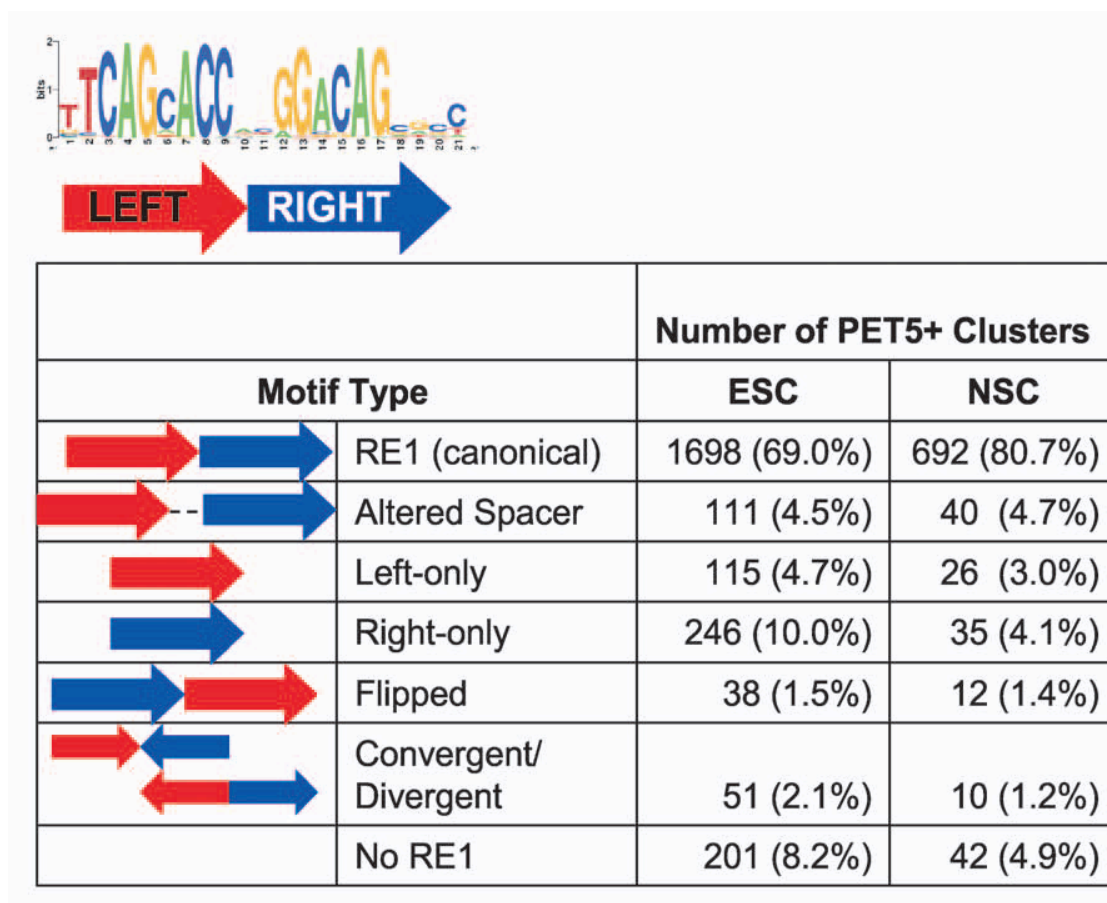
Following ChIP, immunoprecipitated DNA fragments receive paired end tags (PET) that allow sequencing and mapping from both ends. Genomic regions containing an overlap of five or more nonredundant tags are defined as a cluster. Shown here are the noncanonical REST binding sites identified in mESCs and mESC-derived NPCs from this technique (ChIP-PET) (from Johnson et al., 2008).

REST Oligerimzation Is ZF Independent

The RE1 binding motif is divided into two half sites. A position weight matrix of the canonical motif shows each half site to include six highly conserved nucleotides required for REST binding (Johnson et al., 2008; Figure 2). Since ZF motifs are known to bind triplets of nucleotides, only four ZFs would be required for a single REST protein to bind RE1. Therefore, the function of the remaining four is a mystery. Since each half site may recruit REST independently and since ZFs are not only DNA-binding modules but can also mediate protein-protein interactions, we hypothesized that only a subset of ZFs might be required for DNA binding, leaving the remaining ZFs available to interact with another REST protein recruited nearby (Figure 5). The possibility of REST dimerization may support a previous study by Bruce and colleagues, which showed that the half sites have lower occupancy for REST when compared to a full RE1 site (Bruce et al., 2009), and REST dimerization on the RE1 may provide additional stabilization energy.

To test whether REST dimerizes and whether this interaction is ZF mediated, I performed reciprocal co-immunoprecipitation studies with differentially tagged ZF mutants of REST (Figure 6). First, constructs were made in which each of the nine ZFs was individually inactivated by mutating the first cysteine residue of the ZF to an arginine residue. These constructs, tagged N-terminally with five tandem repeats of the myc epitope, were a gift from Marcial Vilar (Riek laboratory). I then subcloned these REST ZF mutants into a vector providing an N-terminal FLAG tag instead of myc. Next, I cotransfected the myc- and FLAG-tagged versions of each ZF construct into 293T cells, prepared nuclear extracts, and analyzed association by immunoprecipitation. Western

blots, probing for the opposite tag of that used in the Co-IP, showed that REST does indeed associate with other REST molecules (lane 10) and that the ZFs have little or no role in this interaction (Figure 7). More specifically, deletions of ZFs 1, 6, and 9 slightly impair REST self-association, since mutation in these domains result in a noticeably diminished signal when probed for the opposite tag. On the other hand, ZF 3 failed to express properly as shown by the weak or absent signal when probed with the same antibody used for immunoprecipitation. Therefore, the effect of this domain on REST self-association remains inconclusive and will need to be tested again. All other ZF are not essential for self-association of REST, because mutations did not greatly impact the REST co-immunoprecipitation levels.
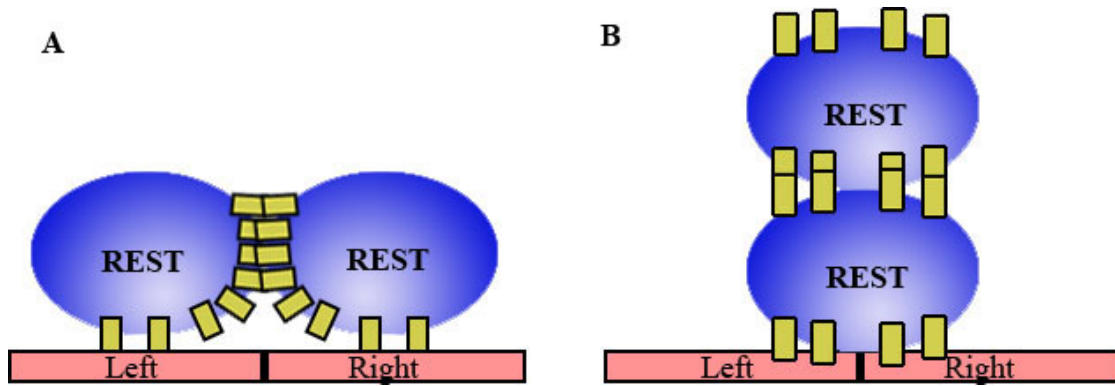
Figure 5. Possible REST Dimerization Scenarios.

(A) RE1 half sites require only two REST ZFs, thus allowing the other ZFs to bind and dimerize adjacent REST proteins that are recruited on the other half site. (B) REST uses four ZFs to bind canonical RE1, allowing other ZFs to recruit free REST.
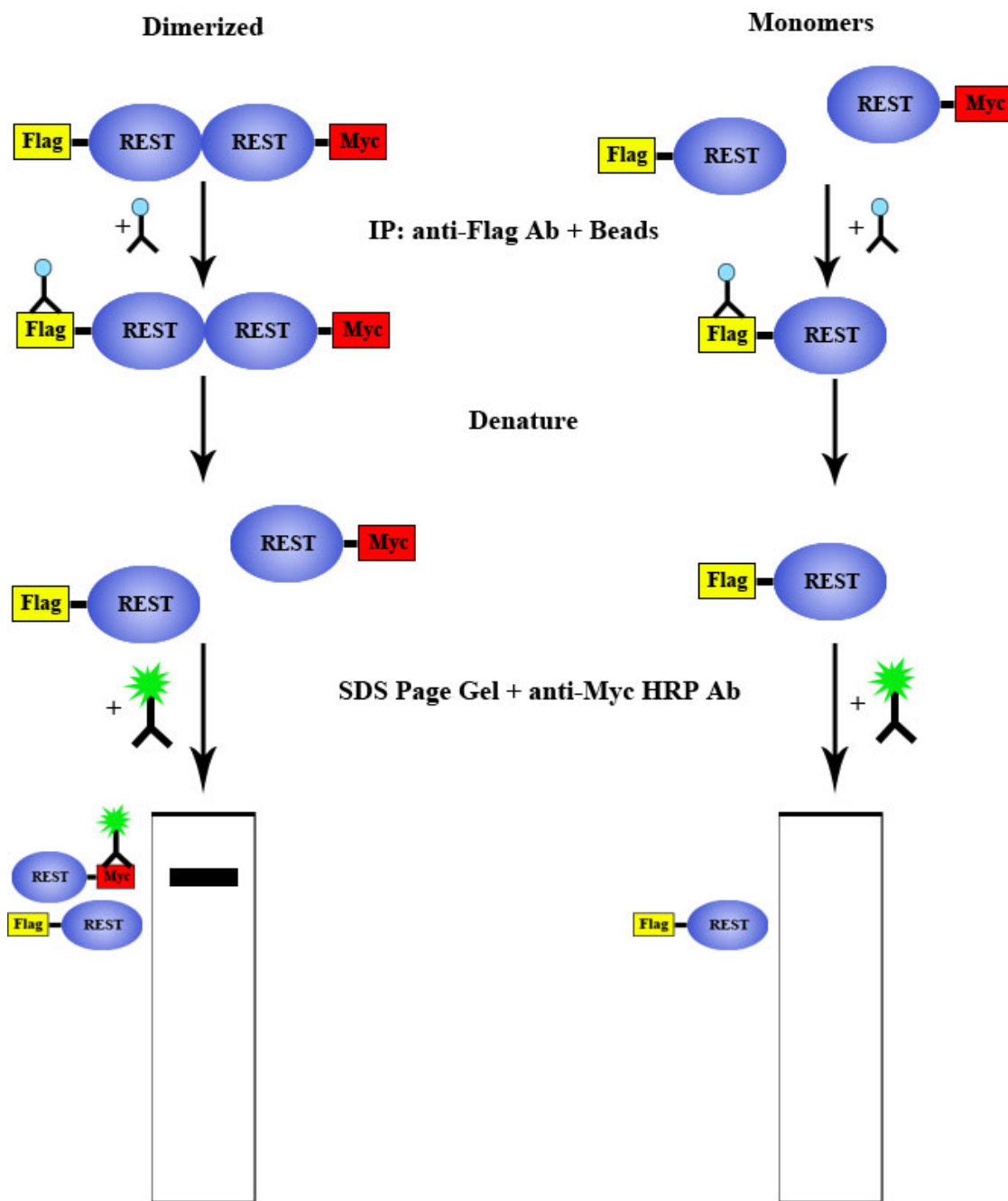
Figure 6. Co-IP Sequence Diagram.

Diagram of the co-IP protocol. Left lane depicts dimerized REST and results. Right lane depicts REST monomers that do not interact.
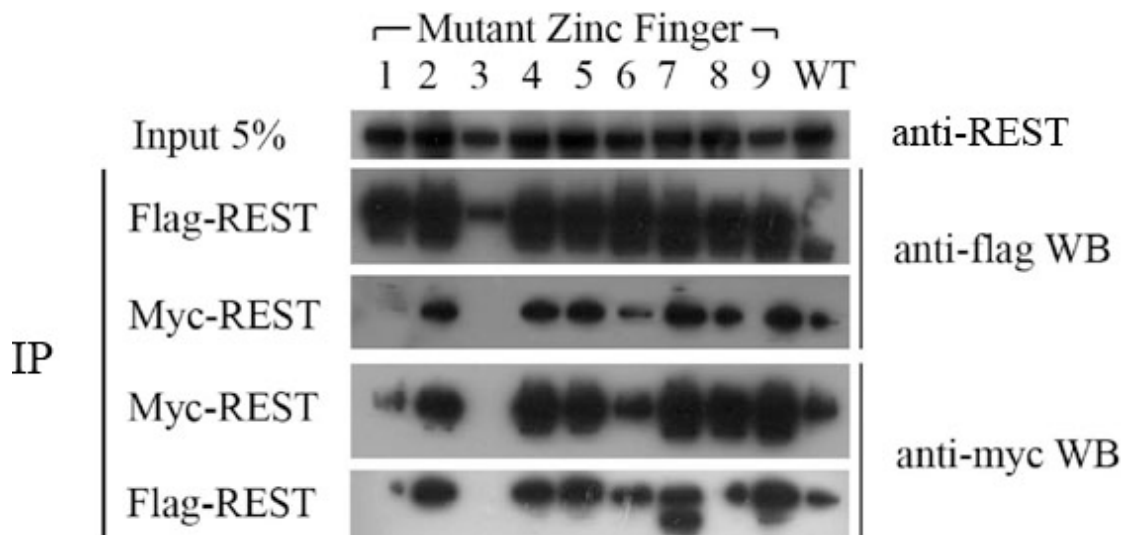
Figure 7. REST Self-Association Through REST ZFs

Reciprocal co-IP/Western blot experiments showing that inactivation of ZFs has little or no effect on REST self-association. When differentially myc- and FLAG-tagged REST was probed for the opposite tag used in the immunoprecipitation, it was evident that mutations in ZFs 1, 6, and 9 decreased REST co-immunoprecipitation efficiency. ZF 3 mutant expressed poorly and will need to be retested. When probed for the opposite tag, REST was detectable at roughly equal levels in all lanes other lanes: 2, 4, 5, 7, and 8, suggesting no effect on oligomerization of REST.

Function of Alternative REST Binding Site by Dual Luciferase Assay

To test the functionality of variants of the RE1, I conducted luciferase reporter assays in N2A cells, a mouse neuroblastoma cell line with undetectable endogenous levels of REST (Kallunki et al., 1997). The simultaneous expression and measurement of two luciferase genes allowed us to examine firefly luciferase activity under the control of the repression system, while using *Renilla* luciferase as a transfection control. To assess repression activity of REST, I engineered a reporter construct in which the thymidine kinase promoter from the herpes simplex virus (HSV-TK) was placed upstream of the firefly luciferase gene along with the different RE1 sites to be tested (Figure 8).

A plasmid with the CMV promoter driving either REST, or GFP as a negative control, was transfected along with the following RE1 reporter constructs (Figure 8): wt canonical site (canonical), the left half site (left), the right half site (right), and 'split' (split) site in which the two half sites are separated by seven nucleotides; this version of a split RE1 was one of the most commonly seen in ChIP-seq experiments (Johnson et al., 2007; Figure 9). As negative controls, I generated a canonical site with two nucleotide changes in conserved residues of right half site (2mt), and a sequence-scrambled canonical site (scr). As expected, samples containing the canonical and split site showed luminescence activity that was reduced by 67% and 62%, in samples cotransfected with REST versus those cotransfected with pcDNA control plasmid, respectively. In contrast, those transfected with the 2mt construct or a scrambled site failed to show repression activity, demonstrating the specificity of the assay. The RE1 right half site reduces luciferase expression by 25%, indicating that it is able to act as a REST-recruiting repressive element by itself, albeit more weakly so than the canonical sequence (Figure

10). Surprisingly, the left site did not show reproducible repression despite the fact that this sequence was identified as REST-associated by ChIP-seq. It will be interesting to find out whether the absence of repression in our assays is due to a failure of REST-recruitment, or, alternatively, whether REST is recruited to this site but is incapable of repression. Our genome-wide analyses of REST association described below may shed a light on this question.
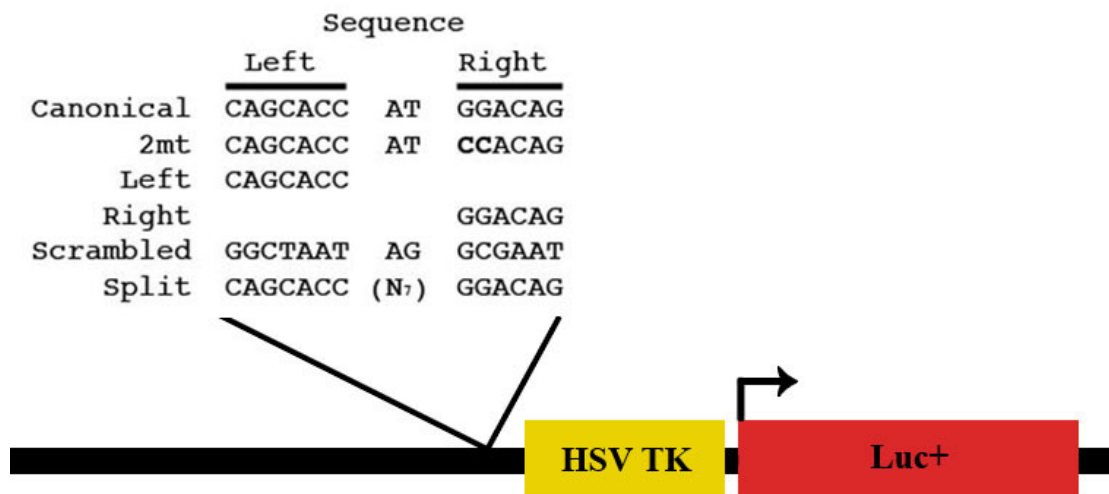
Figure 8. Luciferase Reporter Plasmid

Common sites across the genome found to recruit REST by chromatin immunoprecipitation as well as scrambled and two pairs of point mutations (2mt) sequences were cloned upstream of a firefly luciferase reporter gene driven by the HSV-TK promoter.

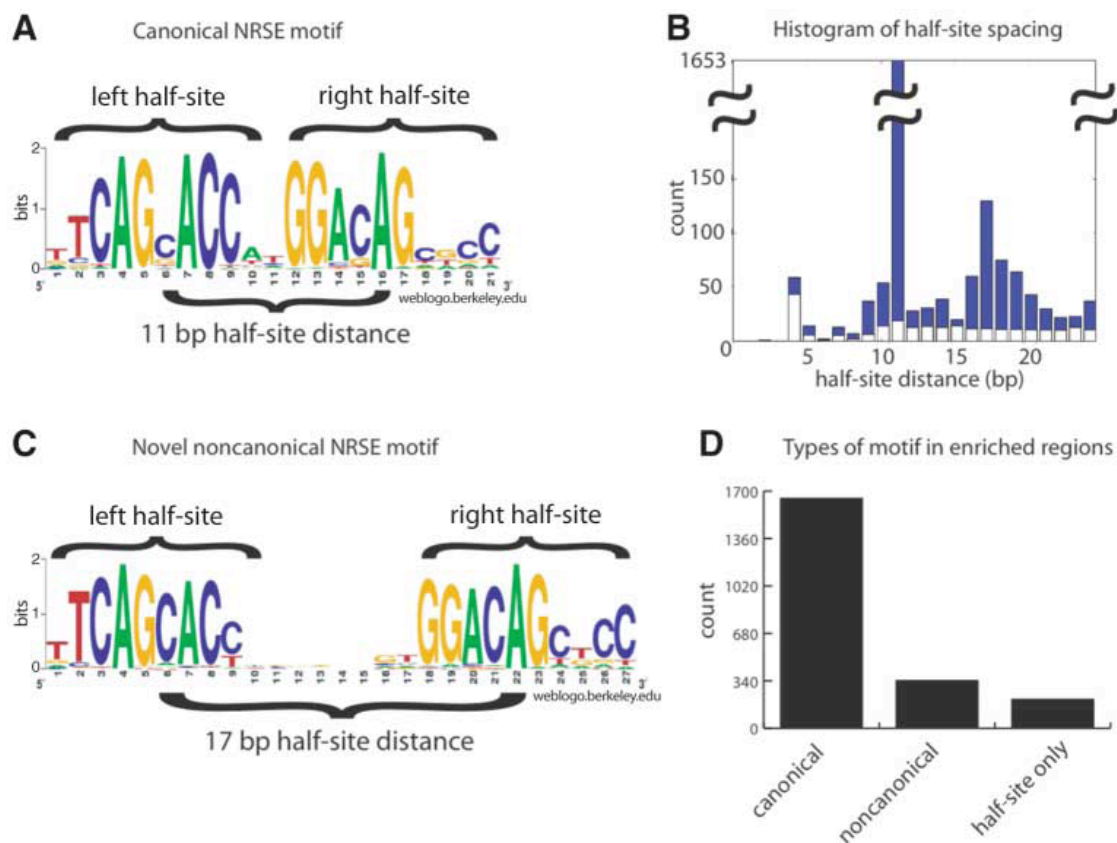**Figure 9. Frequency of Noncanonical RE1 Motifs Based on Half Site Spacing.**

Histogram of half-site distances in ChIP-seq-enriched regions, showing the observed (blue) and expected (white) counts (based on frequency in the genome). In addition to the expected canonical peak at distance 11 bp, there is also significant enrichment of half sites with noncanonical distances of 16 to 20 bp (from Johnson et al., 2007).

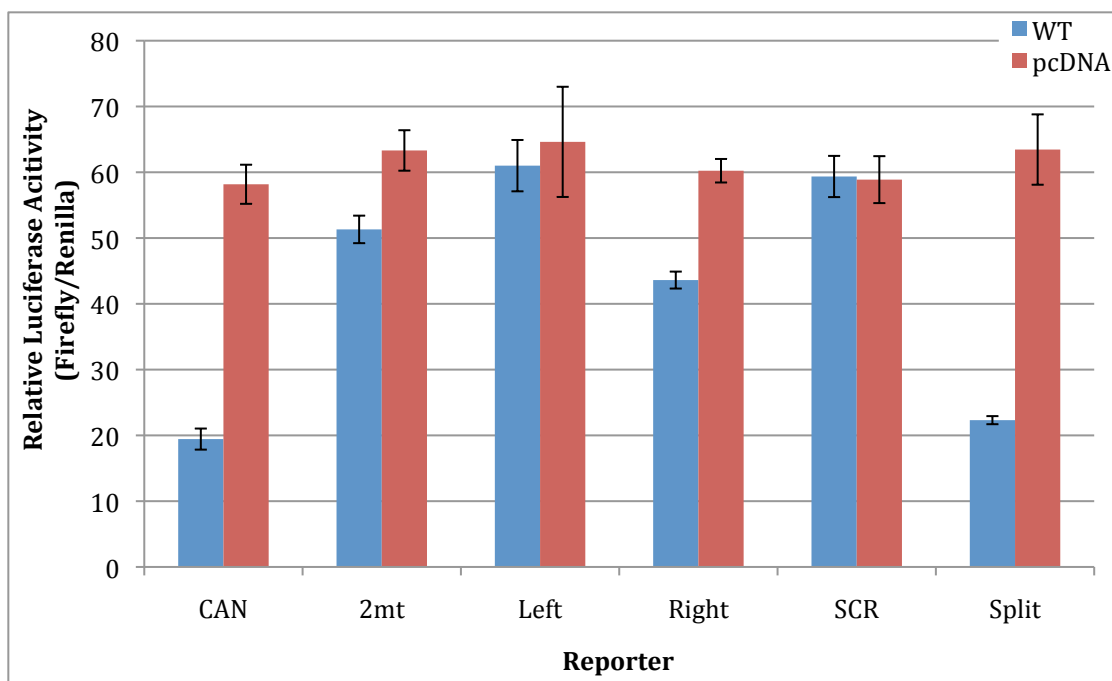**Figure 10. REST and RE1 Dual Luciferase Optimization Assay**

In vivo, WT REST successfully suppresses canonical and split RE1 as expected. In the presence of a mutated or half RE1, suppression levels are limited. However, wt REST inhibits luciferase expression levels by 25% when recruited to the right half site. The data represents the average of biological triplicates, and error bars indicate the standard deviation.

REST ChIP-Seq in hESCs and hESC-derived NPCs

Chromatin immunoprecipitation followed by Solexa high throughput sequencing (ChIP-seq) has recently emerged as a powerful tool to identify sites of protein-DNA interactions in vivo on a genome-wide scale. Typically, these interactions are captured by formaldehyde cross-linking in living cells and the chromatin is then subjected to sonication to reduce the fragments to 200bp in length. The protein of interest is then immunoprecipitated using a targeting antibody and antibody complexes captured with protein A/G beads. Bound DNA is eluted and de-cross-linked by high salt concentrations and heat. Before submitting for sequencing on the Solexa 1G Genome Analyzer for sequencing, adaptors are ligated onto the DNA fragments and the libraries are amplified by PCR to increase the population of reads (Figure 11). When sequenced reads are mapped onto the genome, a global picture of protein occupancy emerges. Not only can novel genomic sites of interaction be determined, but the number of sequence reads clustering onto a particular site is indicative of the degree of occupancy (see examples below). This approach has been used on a wide range of proteins, including REST in mESCs and mESC-derived NPCs (Johnson et al., 2007; Johnson et al., 2008; Valouev et al., 2008). We are interested in assessing REST and REST cofactor occupancy in human ESC and NPCs, and correlating this data with gene expression data collected in the Yeo lab.

In preliminary tests, where I performed REST ChIP with three antibodies followed by qPCR with known REST targets, I have determined Santa Cruz anti-REST H290 antibody to bind its target with the highest specificity along with the greatest degree of enrichment over samples incubated with IgG control antibody (Figure 12).

Using this antibody, as well as corepressor antibodies that have been used successfully for standard ChIP in published data (Wiper-Bergeron et al., 2003; Chingy and Liem, 2009), I have prepared ChIP libraries from hESCs and from hESCs-derived NPCs (Yeo et al., 2007) for REST and its corepressors mSin3a and CoREST (i.e., a total of six libraries). In collaboration with the Yeo lab, we have begun sequencing and genome-mapping of these libraries, as well as computational analysis of the data.

So far, I have successfully prepared all six libraries. Three libraries, namely REST ChIP in ES, REST ChIP in NPC, and mSin3a ChIP in ES, have been sequenced and mapped, and are undergoing computational analysis; the remainder await Solexa sequencing (Table 1).

When sequence reads are aligned with the genome using UCSC's online genome browser, there are distinct clusters of reads on sites that have been previously shown to be highly associated with REST. For example the mu opioid receptor gene (MOR or OPRM1; Kim et al., 2008), shown in Figure 14A, has a cluster of reads precisely on REST's predicted binding site of interaction. In addition, other known and published REST-associated genes, such as BDNF and ATP2B2, also have multiple reads mapped onto their RE1 site, providing positive controls to assess the quality of the libraries (Figure 14B, 14C, 14D) (Johnson et al., 2007).

Interestingly, we found a cluster of REST ChIP reads on the Lin28 gene, indicating REST occupancy. LIN28 is one of the four factors discovered to efficiently reprogram human somatic cells to pluripotent stem-like cells (Yu et al., 2007). It was previously shown that the Lin28 promoter has two conserved REST sites that are REST-occupied in mESCs (Johnson et al., 2008). Given its vital role in induction and

maintainance of pluripotency, it is surprising to find not only an enrichment of reads from REST-ChIP in ES cells on the two RE1 sites of Lin28, but to also see mSin3a occupancy on the same region as well (Figure 15). The presence of the two factors suggests active repression of this gene by REST-recruited mSin3a corepressor complexes. However, Lin28 is highly expressed in hESCs. Perhaps REST serves to dampen and maintain expression levels of Lin28. Alternatively, mSin3a may recruit activators of gene transcription. Our observations suggest a role for both REST and mSin3a in regulation of Lin28 expression, and we will employ gain and loss of function experiments for REST and mSin3a to address this question.

It appears that the technique used to isolate DNA sequences associated with transcriptions factors and their cofactors, and the computational analysis has provided a great tool in studying REST-DNA interaction on a global scale. With the information that will be gathered from these libraries, we may provide a novel analysis on REST's occupancy and function across the genome. For example, we will be able to determine the quality of the RE1 site in relation to its conservation and degree of occupancy. We will do this by comparing how similar a REST binding site is to the canonical RE1 motif, the amount of tags mapped to it, and whether this occupancy for the transcription factor is retained as REST levels begin to decrease during ESC differentiation to NPCs (Figure 13). In addition, we will also determine the fraction of sites that are REST-occupied in one of the two cell types, in both, or in none. This may provide insight as to which binding sites lose occupancy in NPCs due to lower REST levels, or if there are any sites that increase occupancy despite the decrease in REST levels.

At the moment, there are no published datasets for REST cofactor occupancy across the genome. Therefore, our CoREST and mSin3a ChIP-seq may provide a strong contribution to assessing REST's activity globally. From these experiments, we will overlap binding sites from the CoREST and mSin3a ChIP datasets to those recovered in REST-ChIP in order to distinguish which REST sites are capable of recruiting only one, both, or none of the cofactors. This will allow us to determine if the level of corepressor occupancy controls the degree of repression on the target gene. Also, this test for cofactor co-occupancy may ascertain whether those binding sites that are retained during differentiation to NPCs continue to actively repress or not. Instead, REST may continue to bind those sites in NPC and recruit other factors for an alternative function.

The ChIP-seq datasets from the corepressors may also shed light onto the reasons for weak or absent repression activity from the half sites that I tested in my luciferase activity assays. Perhaps the right site has less corepressor occupancy, and therefore weaker repression. Under the same logic, the left half site may have little or no repression activity because it simply does not recruit any cofactors. In this case, it may be interesting to see if the failure to recruit corepressors on these sites is a direct consequence of a differential binding mode (ZF recruitment) of REST to these half sites. By overlaying binding sites identified from the corepressor ChIP-seq data to known half sites extracted from the REST-ChIP data, we may provide evidence for this theory. If true, I would expect to see a majority of canonical RE1 sites to recruit both corepressors, the right half site to recruit one of the two corepressors, and the left half site to recruit none.

As an additional step to verify corepressor occupancy and the strength of repression, we will overlap the occupancy data with gene expression data to determine whether corepressor occupancy is inversely related to expression level. More specifically, genes that have both corepressors recruited on them would be expressed at lower levels than those with one or none. The plethora of data that will emerge from these experiments will undoubtedly provide important novel insights, both for our other studies of REST in particular, as well as other transcription factors in general.

Overall, the techniques employed and data gathered serve as a powerful tool in studying REST's DNA interactions globally, and the strong peaks of reads on known genes provide evidence of the high specificity and reliability of the assay. This provides great confidence in our future assays on the remaining REST cofactors and the data that may emerge from these assays. We are confident that our analyses will lead to a better understanding of REST's function in maintenance of hESC pluripotency and differentiation.
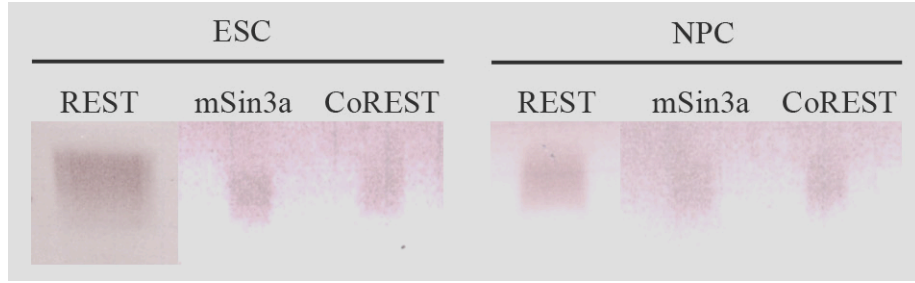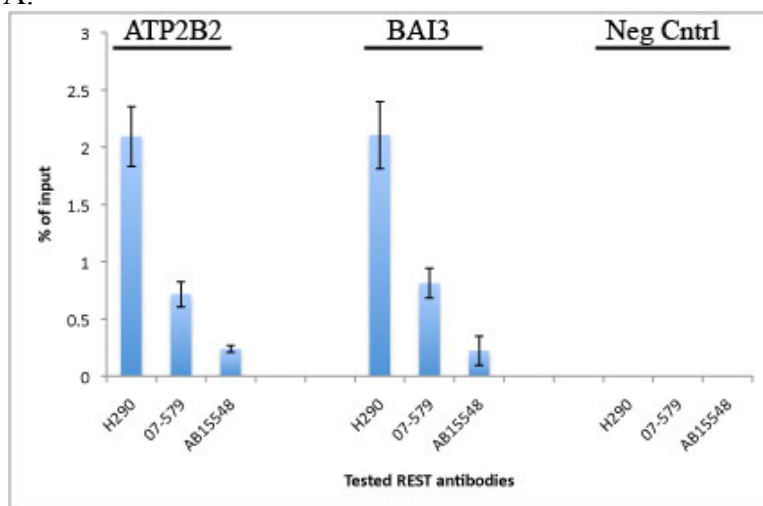
Figure 11. ChIP Library Amplified PCR Products.

After adaptor ligation, ChIP DNA libraries were amplified by PCR and run on an agarose gel for visualization (shown here). Bands typically range from 150-300bp.
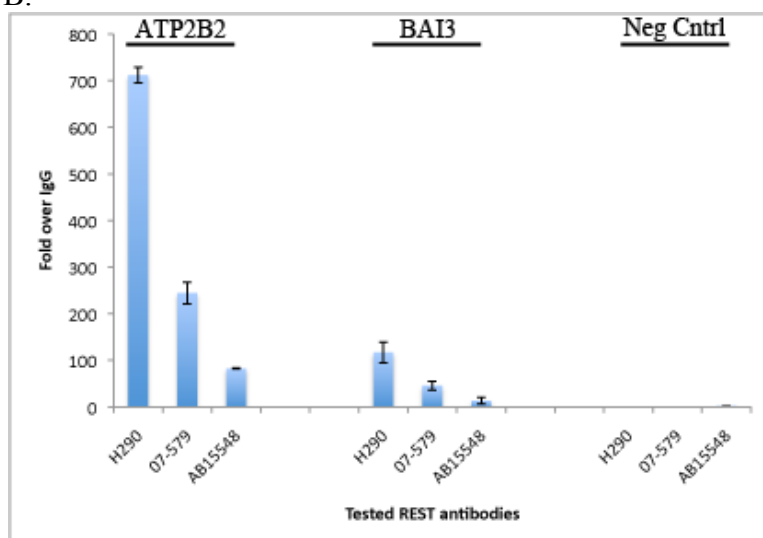
A.

B.

Figure 12. REST-ChIP Antibody Test in 293T Cells.

Three antibodies were tested for REST-ChIP efficiency in 293Ts cells. They include: Upstate H290, Millipore 07-579, and Millipore Ab15548. qPCR primers amplifying a known RE1 site on ATP2B2 and BAI3 genes were used as positive controls. In conclusion, Upstate H290 antibody pulled down 2.09% ± 0.26% of specifically immunoprecipitated DNA compared to input; in addition, the antibody also yielded a 712 ± 17 fold enrichment of RE1 associated binding sites on ATP2B2 gene over IgG-ChIP control. When tested with primers amplifying the RE1 site on BAI3 gene, the same REST antibody returned a 116 ± 22 fold enrichment of positive reads over IgG-ChIP control. Having the greatest values, Upstate H290 was determined the best antibody to use among its competitors. The data shown represent the average of technical triplicates and the error bars indicate the standard deviation.
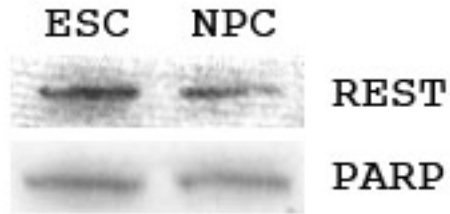
Figure 13. REST Levels in HUES6 hESCs and NPCs.

REST levels decline marginally as ESCs differentiate into NPCs. REST was probed with Upstate anti-REST 07-579 at 1:5,000, followed by anti-rabbit HRP (Jackson) at 1:10,000. The membrane was stripped and re-probed Santa Cruz anti-PARP (F-2) at 1:5,000, followed by anti-mouse HRP (Jackson) at 1:10,000 for loading control.

Table 1. ChIP-Seq Library Status

Three libraries have been successfully sequenced and mapped to the genome browser. The remaining three libraries have been prepared and will be sequenced in the near future.

| Target Protein	Cell Line	Antibody	Status
REST	HUES6	Santa Cruz NRSF (H290)	Sequenced. Mapped to genome browser. Undergoing computational analysis.
REST	NPC	Santa Cruz NRSF (H290)	Sequenced. Mapped to genome browser. Undergoing computational analysis.
mSin3a	HUES6	Santa Cruz mSin3a (AK-11)	Sequenced. Mapped to genome browser. Undergoing computational analysis.
mSin3a	NPC	Santa Cruz mSin3a (AK-11)	Awaiting sequencing
CoREST	HUES6	Upstate CoREST 07-455	Awaiting sequencing
CoREST	NPC	Upstate CoREST 07-455	Awaiting sequencing
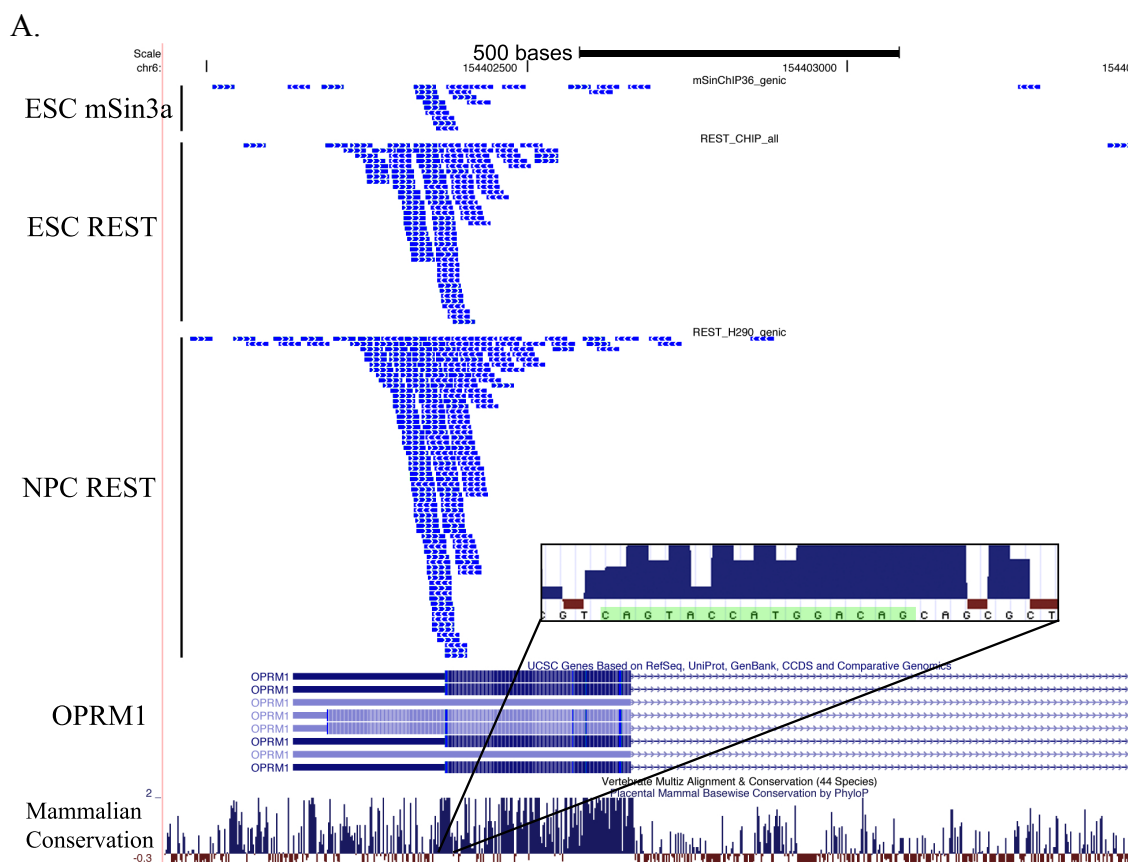
Figure 14. ChIP-Seq Reads Alignment to Known REST Targets.

ChIP-seq reads were aligned to UCSC's genome browser (assembly hg18) by Yujing Liang, Yeo lab. Known targets of REST such as OPRM1 (A), BDNF (B), and ATP2B2 (C, D) are shown. Reads shown near the top of the screenshots represent mSin3a-ChIP in ES cells, middle reads represent REST-ChIP in ES cells, and reads near the bottom represent REST-ChIP in NPCs derived from ES cells. Peak heights (shown in blue) are an indication of the number of ChIP mapping to a given site. The insets show close-ups of the RE1 sequences (highlighted in green).
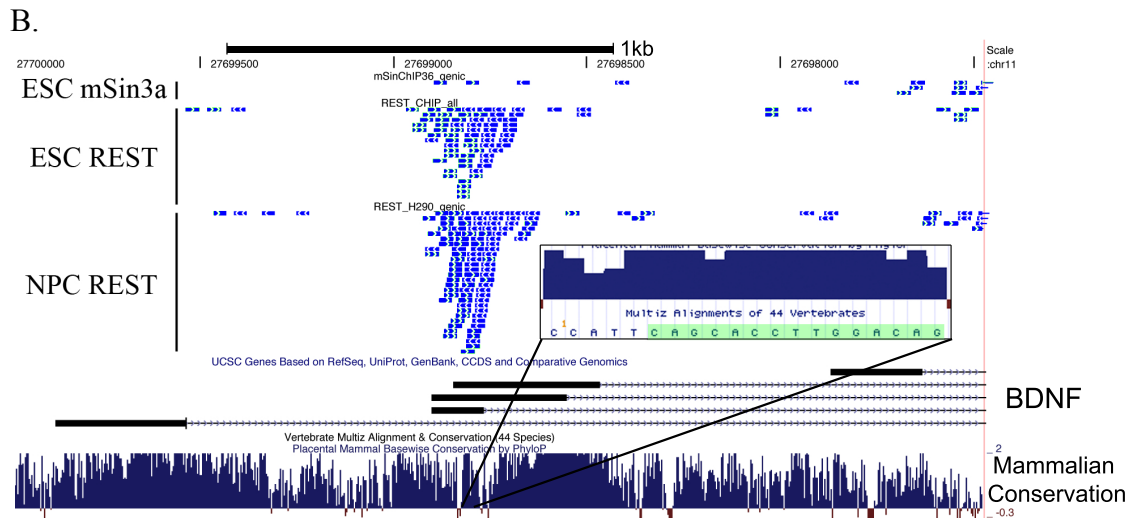
Figure 14. ChIP-Seq Reads Alignment to Known REST Targets.

Figure 14 continued.

C.

Figure 14. ChIP-Seq Reads Alignment to Known REST Targets.

Figure 14 continued.

D.
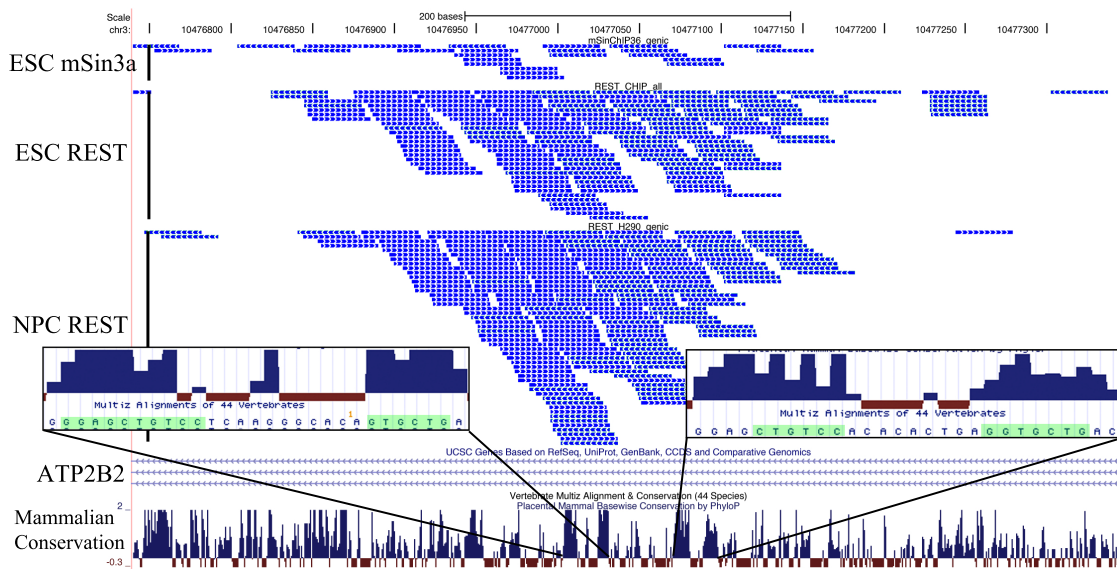
Figure 14. ChIP-Seq Reads Alignment to Known REST Targets.

Figure 14 continued.

A.
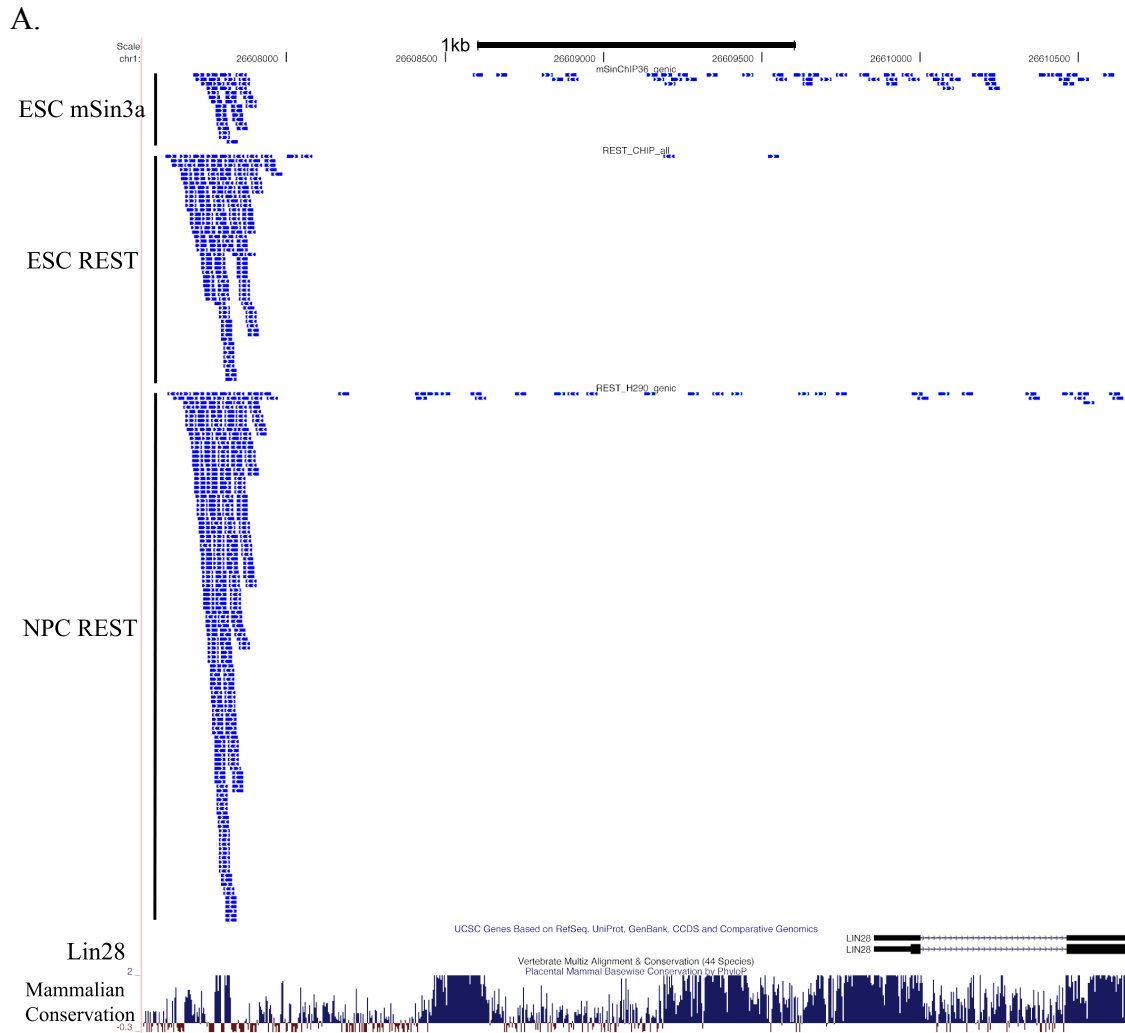
Figure 15. ChIP-Seq Read Alignments to the Lin28 Gene.

ChIP-seq sequences were aligned to UCSC's genome browser (hg18). Peaks found in all three libraries: REST-ChIP in hESC, mSin3a-ChIP in hESC, and REST-ChIP in NPCs near the Lin28 gene (A). The inset shows a close-up of the two RE1 sequences (highlighted in green).

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Figure 15. ChIP-Seq Read Alignments to Lin28 Gene.
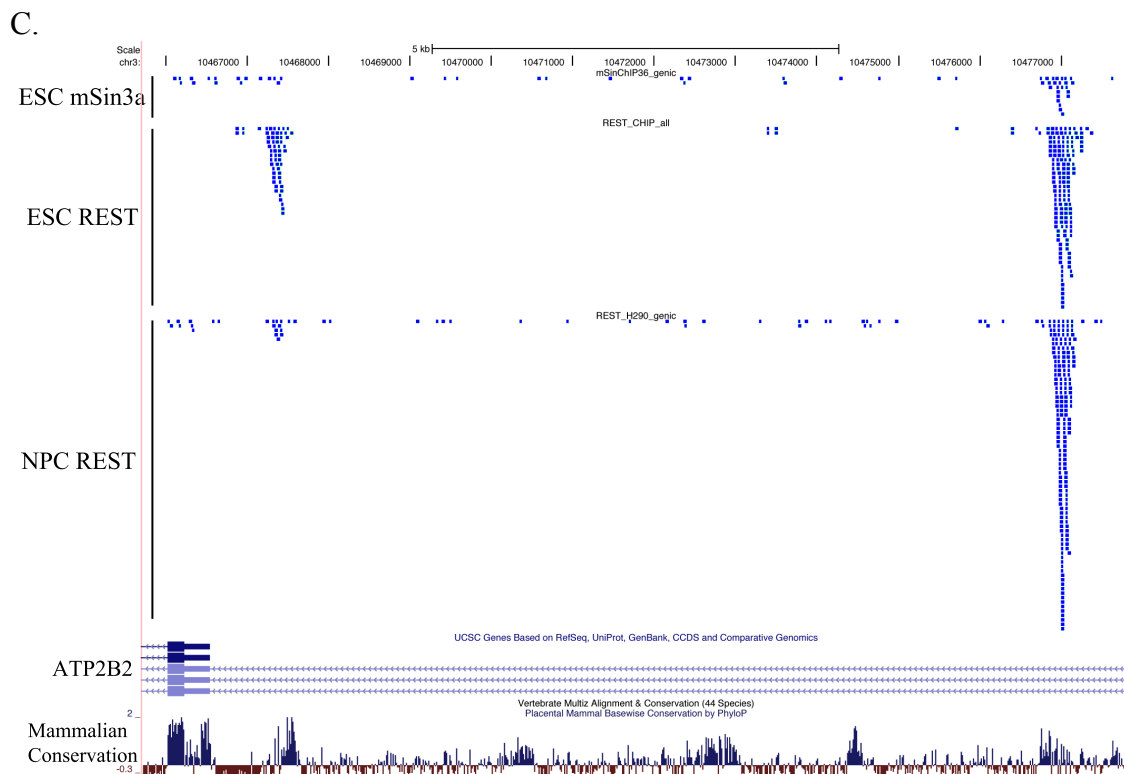
Figure 15 continued.

Chapter 2 – A Novel Function for REST Based on Cellular Localization

Concentration Dependence of REST Function

In the process of titrating REST levels to optimize repression activity in the dual luciferase reporter assay described previously, we unexpectedly discovered that an increase in REST concentrations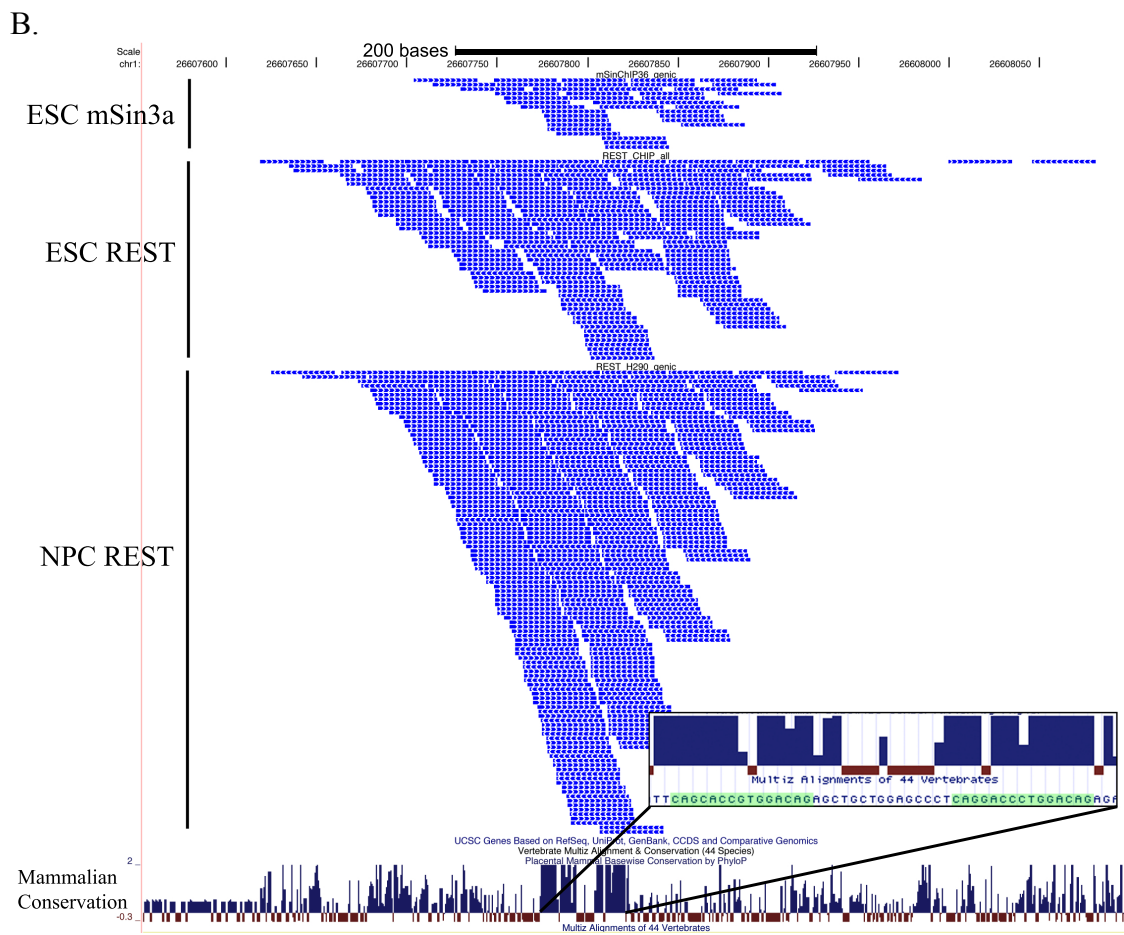 resulted in a progressive loss of repression. Conducted in N2A cells, we demonstrated that a 10:1 ratio of REST: reporter plasmid was sufficient to achieve optimal 'activation' activity. Under these conditions, an 88.5% ± 2.3% increase in luciferase activity was seen compared to the control in which a reporter containing a scrambled RE1 sequence was transfected along with GFP plasmid. Likewise, a 1:100 ratio of REST: reporter plasmid was used as the optimal condition for REST repression; here, a 44.2% ± 3.1% decrease in luciferase output was observed compared to vector controls (Figure 16). Importantly, REST's function as an activator is RE1-independent because a scrambled RE1 reporter plasmid showed a significant increase in luciferase output compared to GFP control, similar to the RE1 reporter.

The same effect was observed using Promega's psiCheck-2 vector as a reporter plasmid. When cotransfected with the same ratios of REST expression plasmid and reporter plasmid as described in the previous assay, an 8.64 ± 0.87 fold increase in *Renilla* and a 3.52 ± 0.24 fold increase in firefly luciferase output was observed when compared to GFP control (Figure 17A and C). To determine whether this enhancement in expression was at the transcriptional or translational level, RNA from the cellular lysate used to measure luciferase output was extracted, DNase treated, and reverse transcribed prior to being quantified by qPCR. These data revealed only a 4.84 ± 1.93

fold increase in *Renilla* mRNA in the cells transfected with REST plasmid vs those transfected with GFP plasmid. The fact that I saw a ~9-fold increase in *Renilla* luciferase levels but only a ~5-fold increase in its mRNA levels implies a post-transcriptional component to the activation observed (Figure 17B). Likewise, firefly luciferase mRNA was found to be only 1.91 ± 0.46 times greater in cells cotransfected with REST plasmid than in cells cotransfected with GFP plasmid (Figure 17D). Again, this unaccounted 1.8-fold increase (~3.5 fold vs. 1.9-fold) in expression enhancement may be a result of REST interaction on the RNA or protein level.

The observed increase in gene transcription levels upon transfection of high levels of REST may be due to the fact that such high amounts of REST may compete with other DNA-bound transcription factors for repressor cofactors, such as mSin3a or CoREST, and thereby sequester such corepressors away from the DNA. The SV40 early promoter and the HSV-TK promoter used to drive the expression of the reporter genes used in these assays are quite likely contain binding sites for numerous endogenous transcription factors. Therefore, overexpression of REST may prevent recruitment of cofactors for repression and thus the de-repression effect seen here. Nonetheless, the increase of reporter gene expression, even when normalized for reporter transcript levels, is intriguing and may very well be due to REST acting at the post-transcriptional level, such as REST-aided recruitment of mRNAs to the ribosome or nuclear export.

Taken together, our results from reporter assays with overexpressed REST suggest that this protein may have a function aside from being a transcriptional repressor when present at high levels.
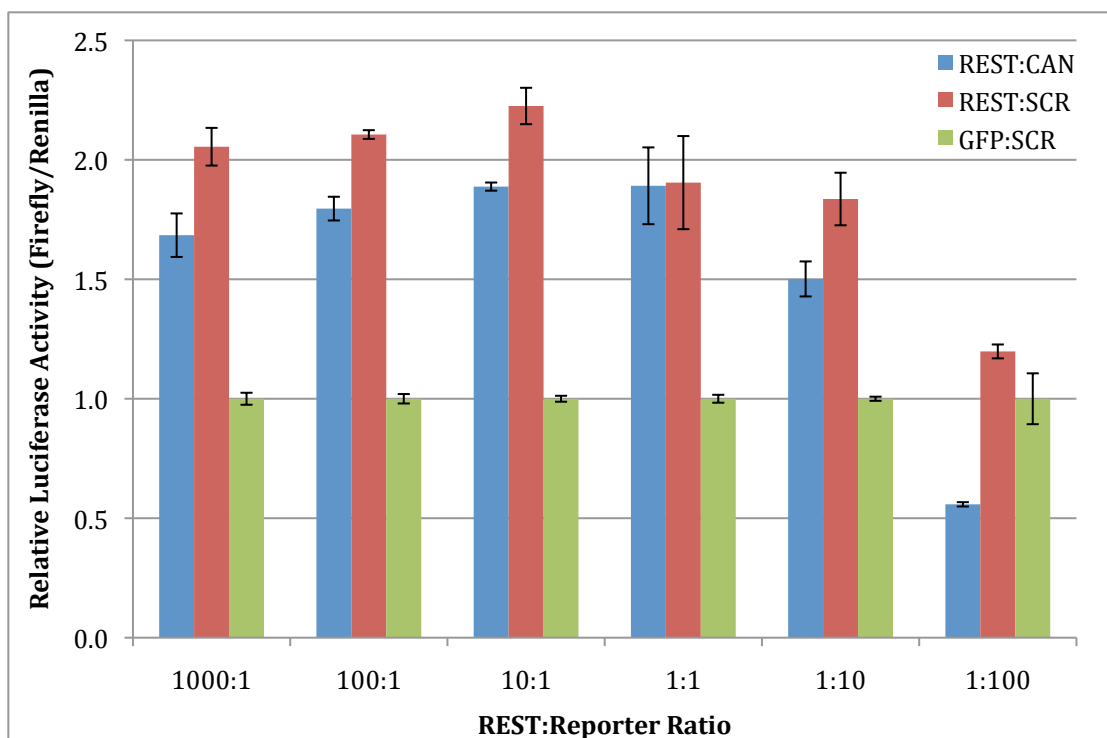
Figure 16. REST Activation and Repression Optimization.

All samples were normalized to output from cells transfected with GFP plasmid and scrambled RE1 reporter. A ratio of 10:1 emerged as optimal for REST activation. In contrast, a 1:100 ratio was further used for REST repression activity. The data represents a representative transfection experiment done in triplicate. Bars show the average and error bars indicate standard deviation.
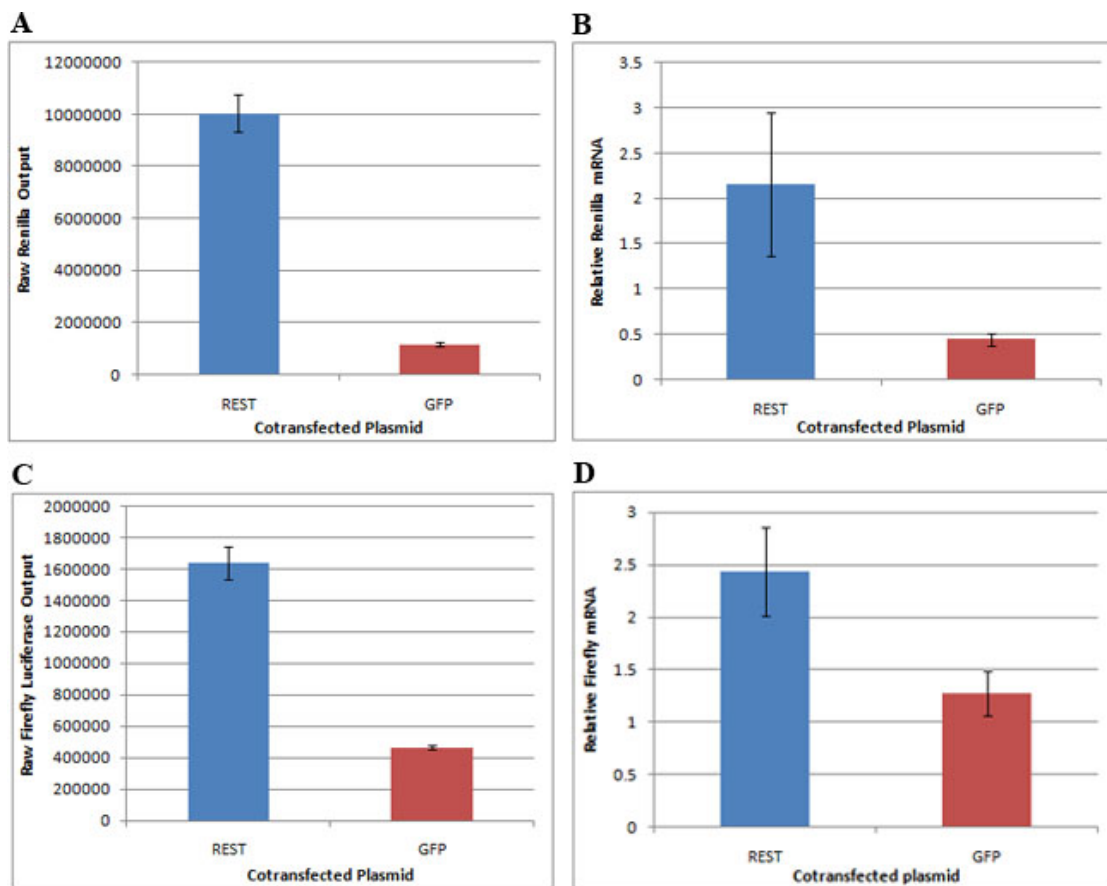
Figure 17. REST Increases Reporter Gene Expression in psiCheck-2 Vector

N2A cotransfected with firefly and *Renilla* luciferase expression vector psiCheck-2 and REST or GFP control under activation conditions (10:1). (A and C) Raw luminescence outputs were recorded. *Renilla* showed a 8.64 ± 0.87 fold increase in expression in the presence of REST; firefly luciferase showed 3.52 ± 0.24 fold increase under the same conditions. *Renilla* (B) and firefly luciferase. (D) mRNAs were quantified by qPCR and normalized to GAPDH. High REST concentrations increased *Renilla* gene transcription by 4.84 ± 1.93 fold, while increasing firefly gene transcription by a 1.91 ± 0.46 fold. The data shown represents the average of technical triplicates and the error bars indicate the standard deviation.

Visualization of Cellular Localization of Overexpressed REST in N2A cells via Confocal Microscopy

Inspired by my findings that high concentrations of REST may have an activating role in translation, and knowing that translation is a cytoplasmic activity, I were interested in finding out whether REST localizes to the cytoplasm when overexpressed at high levels. I used confocal microscopy to visualize REST protein in N2A cells, which are thought to have extremely low levels of REST. Briefly, the N2A cells were transfected with the same REST plasmid concentration as the optimal activator (REST expression plasmid: reporter plasmid, 10:1 by mass) and repressor (1:100) conditions established in the dual luciferase assay. In order to control for nonspecific binding, I used two different REST antibodies, a polyclonal (Upstate cat. #07-579) and a monoclonal (12C11, gift from David Anderson, UCSD), raised against REST's C- and N-terminus, respectively. I determined which antibody has less background by comparing signal intensity between transfected and untransfected cells. In addition, because both antibodies detect different regions of REST, the use of the two may reveal if REST isoforms, such as C-terminally truncated REST4, exist, and if so, where they localize within the cell. Because staining intensities for REST were dramatically different between the two transfection conditions, I used two different settings to capture fluorescence while avoiding overexposure: a low sensitivity setting to visualize highly expressed REST levels (Setting A) and a high sensitivity setting for detection of low REST levels (Setting B).

When transfecting with concentrations similar to those used in the repressor condition of the luciferase assays, both antibodies detect REST primarily in the nucleus

(Figure 18A, B). There appears to be cytoplasmic REST protein when probed with Upstate 07-579 antibody, but this may be nonspecific binding because nearby untransfected cells show signal of similar intensity as well. Additionally, these images of the cells with low REST levels indicate that 12C11 REST antibody is more specific, and I will therefore use it in future immunoflourescent staining experiments. On the other hand, when transfecting with concentrations suggesting gene activation in luciferase assays, both antibodies strongly detect REST in the cytoplasm (Figure 18C, D). Although there appears to be little or no REST in the nucleus, a higher setting (Setting B) confirmed the protein to be present, albeit at lower levels, in the nucleus as well (Figure 19).

Taken together, the images confirm that at low expression levels, REST is mainly confined in the nucleus where it acts in its canonical role as a repressor. In contrast, activation conditions show REST to be localized in both compartments of the cell, with the majority in the cytoplasm, where it may bind and interact with translational proteins.
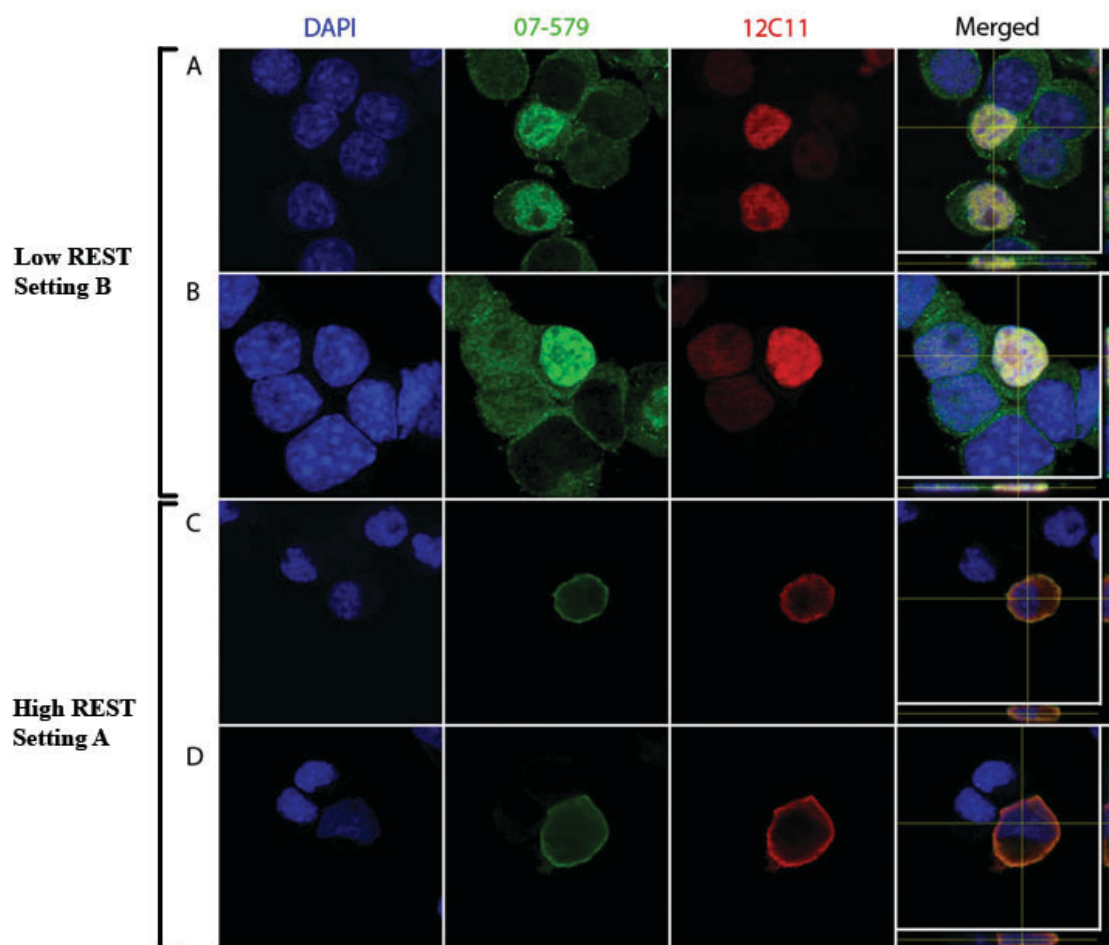
Figure 18. REST Localization in Transfected N2A Cells.

(A and B) Confocal microscopy images showing nuclei stained with DAPI (blue), immunofluorescent detection of the REST C-terminus using Upstate 07-579 antibody (green), and REST N-terminus using 12C11 antibody (red) in N2A cells transfected with low concentrations of REST plasmid. Visualized under Setting B, REST localizes only in the nucleus. (C and D) In contrast, high REST concentrations, used for activation in luciferase assays, showed REST primarily in the cytoplasm. Images were acquired with Setting A. The last column of images represent image stacks from 22 optical sections over a z-distance of 6 μm, which were then reconstructed in the yz-direction (right panels) and xz direction (bottom panels). This allows confirmation of overlap within labeled regions.
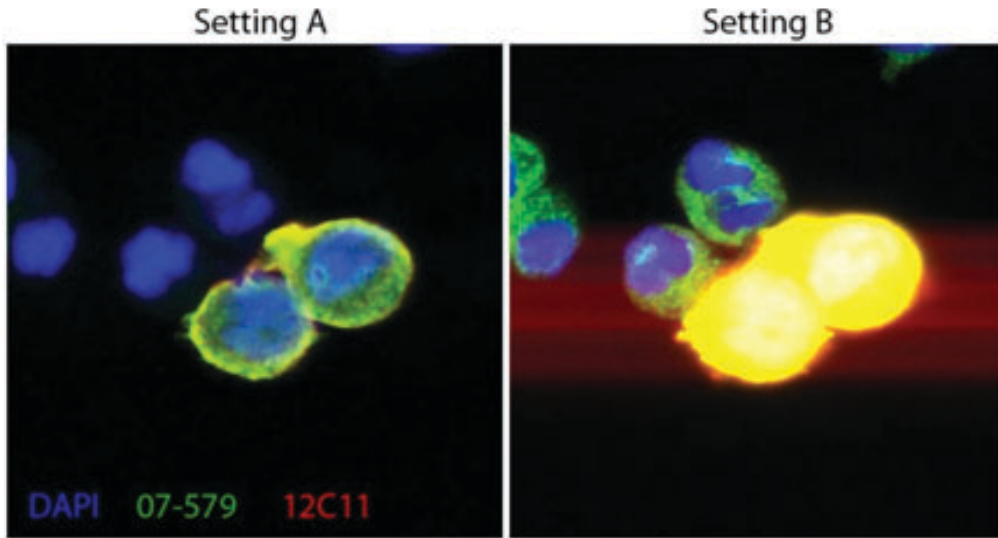
Figure 19. High Concentrations of REST in the Nucleus and Cytoplasm.

Confocal microscopy images showing N2A cells transfected with high levels of REST under Setting A (low sensitivity setting) and Setting B (high sensitivity setting), confirming REST is expressed in both compartments; however, it is most abundant in the cytoplasm.

Concluding Remarks

Although much of the work on REST in the field revolves around its function as a repressor on the transcriptional level, there are still newly introduced functions to explore. Based on data gathered from my experiments, there is evidence suggesting that REST may be able to stabilize transcripts at the post-transcriptional level. This activity seems to be dependent on its expression levels, which in turn, govern its localization in the cell. Experimental data suggests that nuclear REST levels may be saturable at relatively low levels of REST, which in turn leads excess REST to localize to the cytoplasm, where it may bind mRNA to stabilize or enhance translation. In addition to the results from my luciferase assay reports and confocal microscopy, recent data gathered from our collaborators, the Yeo lab and Mohamedi Kagalwala (Gage lab), are in agreement with a translation-stimulatory function for REST in the cytoplasm.

REST-CLIP, an assay which involves REST immunoprecipitation from cultured cells followed by Solexa high throughput sequencing of associated RNAs, performed by the Yeo lab, showed REST to associate with the 3' untranslated regions (3'UTRs) of several mRNAs in 293T cells (data not shown). 3'UTRs are commonly targeted by protein complexes that regulate mRNA stability, such as ARE-binding proteins and the microRNA-induced silencing complex (miRISC). Therefore, REST may bind these regions to stabilize the mRNA and prevent degradation. In addition, the protein may bind 3'UTRs simply to recruit translation initiation factors to the mature transcript for translation.

In support of REST playing a role to enhance gene expression, Mohamedi Kagalwala's data from REST immunoprecipitation in mESCs followed by mass-spectrometric identification of candidate REST-interacting proteins revealed specific association with proteins known to be involved in translation (data not shown). Notable are eukaryotic translation initiation factors: eIF3A, eIF3C, eIF3D, and eIF1. Because these proteins are known to promote pre-initiation complex (PIC) assembly onto mRNA (Sonenberg and Hinnebusch, 2009), REST may act by bridging mRNA and initiation factors to facilitate translation efficiency.

Collectively, our data are consistent with a possible role for REST as a mediator between mRNA and eukaryotic translation initiation factors in the cytoplasm to stabilize or enhance translation efficiency. Such a function seems contradictory to its role in the nucleus; however, the discovery would definitely provide a better understanding of the molecular basis of the multifunctional transcription factor and neuronal cell regulation as a whole.

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