CHAPTER III

THE TRANSCRIPTION FACTOR SP4 BINDS TO THE 5'-REGULATORY REGION OF THE MET RECEPTOR TYROSINE KINASE GENE IN THE DEVELOPING MOUSE CORTEX

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INTRODUCTION

The Met receptor tyrosine kinase is expressed in the developing and adult nervous systems and based *on in vitro* and *in vivo s*tudies, has the potential to mediate a variety of neurodevelopmental and neurophysiological processes, including peripheral and central neuron survival (Hamanoue et al., 1996; Zhang et al., 2000), neuron migration (Garzotto et al., 2008; Giacobini et al., 2007; Powell et al., 2001), axon guidance (Caton et al., 2000; Ebens et al., 1996; Powell et al., 2003), dendritic arborization (Gutierrez et al., 2004; Judson et al., 2010b; Lim and Walikonis, 2008), and spine and synapse maturation and activity (Akimoto et al., 2004; Judson et al., 2010b; Tyndall et al., 2007; Tyndall and Walikonis, 2006). Detailed mapping analysis of the *Met* transcript in the developing mouse forebrain reveals remarkably restricted patterns of expression in both the spatial and temporal domains (Judson et al., 2009), implying both tight regulation of transcription and specific function for the protein product. Temporally, *Met* levels peak dramatically during the first two postnatal weeks, suggesting a role for the protein in neurite outgrowth and synapse formation. Localization of the transcript to projection neurons within the neocortex and specific limbic structures suggests that it is involved in establishing connectivity between these structures. In fact, a recent study in which Met is deleted selectively from neocortical neurons reports selective effects on dendritic arborization and spine head volume of intrinsic pyramidal neurons and postsynaptic medium spiny striatal neurons (Judson et al., 2010b). Consequently, deviations in the regulated expression of *Met* may

predispose to alterations in the formation and functioning of these circuits. Although several transcription factors have been identified that regulate Met *in vitro* (see Chapter I, Table 1), mostly using cancer cell lines, the complement of transcription factors controlling its expression in the nervous system is poorly understood. Yet knowledge of these regulatory factors is critical to understanding how *Met* expression patterns are established *in vivo*, and gives insight into sources of vulnerability to aberrant circuit patterning. This is particularly relevant in the context of recent studies of neurodevelopmental disorders. Three different alleles in the 5' regulatory region of the human ortholog, *MET*, increase risk for Autism Spectrum Disorder (ASD) (Campbell et al., 2008; Campbell et al., 2006; Jackson et al., 2009; Sousa et al., 2009; Thanseem et al., 2010), and there is a reduction in MET protein expression in postmortem brain tissue from subjects with ASD (Campbell et al., 2007). Clues to one potential regulatory factor came from the initial genetic discovery of *MET* as an ASD risk gene, in which the risk allele disrupted binding of SP1 to the *MET* promoter (Campbell et al., 2006).

The Sp-family of transcription factors is comprised of nine zinc-finger domain proteins. While they share a characteristic, GC-/GT-box consensus binding motif, these proteins differ in their precise binding motifs, expression patterns, and cofactors (reviewed in Bouwman and Philipsen, 2002; Suske, 1999; Wierstra, 2008). The best-studied member of the group, Sp1, has been implicated repeatedly in regulating the transcription of *Met*/*MET in silico* and *in vitro* in cancer cell lines (Seol et al., 2000; Zhang et al., 2003). Importantly, Sp1 shares a binding motif with Sp3 and Sp4 (Hagen et al., 1992). Consequently, the results of the Sp1 studies may be

similarly applicable to Sp3 and Sp4, and the relevant transcription factor or factors may be context-dependent. In this study, we show that Sp4, but not Sp1, is expressed in the mouse cortex at the time of peak *Met* expression and further demonstrate that Sp4 binds the 5' regulatory region of the *Met* promoter, thus suggesting that it may participate in regulating *Met* expression in the developing forebrain. Given the emerging role of Sp4 in neuropsychiatric disorders including bipolar disorder, schizophrenia, major depressive disorder, and Alzheimer's disease (Boutillier et al., 2007; Shi et al., 2010; Shyn et al., 2009; Tam et al., 2010; Zhou et al., 2010; Zhou et al., 2009), these results suggest a convergence of potential genetic risk through transcriptional regulation of key genetic elements.

MATERIALS AND METHODS

Mice and Tissue Collection

C57Bl/6J mice were purchased from Charles River Laboratories (Wilmington, MA). All research procedures using mice conformed to NIH guidelines and were approved by the Institutional Animal Care and Use Committee at University of Southern California. All animals were housed on a 12 hr light:dark cycle with full access to food and water. Efforts were made to minimize animal suffering and to reduce the number of animals used for the studies reported here.

To harvest brains, mice were anesthetized with isofluorane prior to decapitation and brain removal. Harvested brains were immediately immersed in ice-cold Hanks' balanced salt solution (Sigma, St. Louis, MO) with the aid of an MZ-6

stereozoom microscope (Leica). The cerebral cortex from each hemisphere was rapidly dissected and weighed. Nuclear extracts were prepared immediately. Tissue to be used for total protein preparation or chromatin immunoprecipitation was snap-frozen in liquid nitrogen and stored at -80°C.

Nuclear extract preparation

The following protocol was modified from a number of previously published methods (Dignam et al., 1983; Dobi et al., 1997; Lewis and Konradi, 1996). Following dissection, tissue was immediately homogenized in 800ul ice-cold phosphate-buffered saline (PBS) with fresh protease inhibitor cocktail (P8340; Sigma, St. Louis, MO) using a glass tissue homogenizer (Wheaton, Millvale, NJ). Homogenate was spun at 1500g for 5 minutes at 4°C, and supernatant was removed. Pellet was resuspended in Buffer A (10mM HEPES pH 7.9; 1.5 mM MgCl2; 10mM KCl with fresh 0.5 mM DTT and protease inhibitor cocktail). Volume of buffer A was approximately 10-fold of packed cell volume. After buffer addition, samples were vortexed for 15 seconds and incubated on ice for 10 minutes. NP-40 was added to each sample for final concentration 0.55%, and samples were vortexed for 5 seconds, incubated on ice for 1 minute, vortexed an additional 5 seconds, and centrifuged at 16,000g for 5 minutes at 4°C. The supernatant (cytoplasmic fraction) was discarded, and the nuclear pellet was resuspended in buffer C (20 mM HEPES pH 7.9; 0.42 M NaCl; 1.5 mM MgCl2; 0.2 mM EDTA; 25% glycerol with fresh protease inhibitor cocktail), using a volume equal to approximately ¼ of the volume of buffer A. Samples were vortexed for 15 seconds and then incubated on ice for

approximately 40 minutes, with vortexing every 10 minutes. Samples were then centrifuged at 16,000g for 10 minutes at 4°C. Supernatants were collected, DTT added to a final concentration of 0.5mM, and stored at -80°C until used for analyses.

Total protein preparation

Frozen tissue was thawed on ice and homogenized in 10-fold (volume per mass) ice-cold homogenization buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 0.5 mM DTT, 2 mM EDTA, pH 8.0, 2mM EGTA with fresh protease inhibitor cocktail) using a glass tissue homogenizer. Homogenates were spun at 16,000g for 20 minutes at 4°C, and supernatants were collected and stored at -80°C.

Western blotting

The following primary antibodies (diluted as indicated in 3-5% Blotto) were used for Western blotting experiments to detect specific protein expression: rabbit anti-Sp1 (Santa Cruz, sc-59, 1:500, 0.05% Tween-20), rabbit anti-Sp3 (Santa Cruz sc-644, 1: 1000, 0.05% Tween-20), and rabbit anti-Sp4 (Santa Cruz, sc-645, 1:500). Protein concentrations were determined using the Dc protein assay (Bio-Rad, Hercules, CA). Samples were fractionated by SDS-PAGE and transferred to supported nitrocellulose membranes. The membranes were blocked in Blotto (3-5% nonfat Carnation dried milk in PBS) for 1 hr at room temperature followed by incubation in primary antibody overnight at 4°C or 1.5 hr at room temperature. Membranes were then washed repeatedly with vigorous shaking in PBS followed by

incubation in horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) at 1:5000 for 1-1.5h at room temperature. Membranes were again washed repeatedly in PBS and subsequently reacted with SuperSignal West Dura Substrate (Pierce, Rockford, IL). Specific signal was detected using a Bioimaging system (UVP, Upland, CA).

Bioinformatics

We created a program, TFScan, which predicts transcription factor binding to DNA sequence by combining the predictions of three independent search engines (Promo 3.0, Consite, and Genomatix MatInspector). The sequence of interest is screened by each of the search engines for binding sites recognized by a particular transcription factor, and each nucleotide is given a binding probability score accordingly. TFScan sums the score at each nucleotide across the search engines and generates a moving average along the sequence that is the composite binding probability score. By comparing the TFScan predictions with published reports of transcription factor-DNA binding, we have demonstrated that TFScan predictions provide a more integrated set of data than those from the individual search engines alone (data not shown).

In this study, the sequence of interest in the mouse *Met* promoter extended from 1000 bp upstream of the transcription start site (TSS) to 500 bp downstream of the TSS. The transcription factor Sp1 was analyzed. These data serve additionally as an *in silico* proxy for Sp3 and Sp4, due to their identical consensus binding motifs (Hagen et al., 1992). The individual programs use different scoring matrices and algorithms, which

contribute to the differences in their binding predictions. The appropriate threshold scores, therefore, differ between programs, and we relied on the programs' default cutoff values rather than attempting to normalize between the programs.

Individual search engines for transcription factor binding sites can be found at the following URLs:

Consite: http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite/ (Sandelin et al., 2004)

Genomatix: http://www.genomatix.de (Cartharius et al., 2005)

Promo 3.0: http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3 (Farre et al., 2003; Messeguer et al., 2002)

Streptavidin-pulldown assays

Dynabeads MyOne Streptavidin T1 superparamagnetic beads with a surface streptavidin monolayer (Invitrogen, Carlsbad, CA) were used to detect DNA-nuclear protein interactions *in vitro*. Beads (375 ug) were combined in 1.5 ml tubes with 100ul 1X binding and washing (B&W) buffer (5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1M NaCl, 0.1% Tween-20). Tubes were placed on a magnetic strip (New England Biolabs, Ipswich, MA), and supernatant was removed. Beads were then washed three times with 1X B&W buffer and resuspended in 200 ul 2X B&W buffer. Individual 5'-biotinylated probes (10-50pmol in 200ul water), representing specific 5' upstream regions of the *Met* gene sequence, were added to the beads individually and incubated at room temperature for 30 minutes with end-over-end rotation. After rotation, tubes were placed on a magnetic strip to facilitate removal of unbound probe. Beads were then washed twice with 1X B&W buffer and once with

Buffer C-150 (20 mM HEPES pH 7.9; 0.15 M NaCl; 1.5 mM MgCl2; 0.2 mM EDTA; 25% glycerol) with 0.1% Tween-20, followed by resuspension in 400ug nuclear extract in Buffer C-150 with 0.1% Tween-20. Beads and protein were then incubated at room temperature for 30 minutes with end-over-end rotation. After rotation, tubes were placed on a magnetic strip to facilitate removal of unbound protein. Beads were then watched 4 times with Buffer C-150 with 0.1% Tween-20. Contents were transferred to clean tubes at the fourth wash to reduce protein leeching. Bound proteins were eluted in 10 mM EDTA with 95%formamide at 90oC for 10 minutes and collected off of the beads using the magnetic strip.

In order to evaluate specific *Met* promoter - SP protein interactions, 5'-biotinylated probes representing 5' regulatory sequence of the mouse *Met* gene used were as follows, where the numbers indicate the position relative to the *Met* TSS, which is located on mouse chromosome 6, position 17413957.

IDT1 : -695/-496

IDT2: -515/-316

PCR2: -395/-43

IDT4: -145/+45

IDT5: +25/+225

Chromatin Immunoprecipitation

The cerebral cortex was dissected from P7 mice as described above. Sp4 occupancy of specific genomic regions was tested by GenPathway, Inc. (San Diego, CA) using a rabbit polyclonal anti-Sp4 antibody (Santa Cruz sc-645) and

quantitative PCR according to their protocols (Alexiadis et al., 2007). Binding was tested in triplicate for five regions of the *Met* promoter and one negative control, untranscribed genomic region, Untr6. For analysis, binding at each *Met* site was ratioed to binding at the Untr6 site.

RESULTS

Sp4, but not Sp1, is expressed in the mouse forebrain during the period of peak *Met* expression

Although several studies have identified Sp1 as a regulator of *Met*/*MET* transcription (Campbell et al., 2006; Seol et al., 2000; Zhang et al., 2003), there are no data that address this regulation in the developing brain *in vivo*. Sp1 is largely regarded as a ubiquitously expressed protein, but in fact expression varies by cell type and developmental time period (Bergman and Levitt, unpublished observations, Mao et al., 2007; Mao et al., 2009). As a result, the relevance of *in vitro* findings and *in silico* predictions of Sp1 binding and regulation depends on the particular system under consideration, and whether or not Sp1 is expressed. Moreover, because Sp1, Sp3, and Sp4 share a common, highly conserved binding motif for which each protein has equal affinity (Hagen et al., 1992), *in vitro* and *in silico* data for Sp1 may instead or additionally apply to Sp3 and/or Sp4 in cells that express these factors.

In the developing mouse forebrain, *Met* expression is first detected at E15.5, and peaks during the first two postnatal weeks, before declining significantly at P21 to reach low levels in adulthood (Judson et al., 2009). To determine which of the Sp-

family of transcription factors might be most relevant to forebrain *Met* expression, we performed Western blots on nuclear extracts from mouse cortex harvested at various time points during postnatal development. Sp1 was not expressed at detectable levels at any postnatal or adult time point that was assayed (Fig. 1a). As a positive control for the antibody, nuclear extract from the mouse n2A neuroblastoma cell line, which expresses Sp1, was included in the experiment, and a specific band at the predicted molecular mass (~105kD) was observed (Fig. 1a). In contrast, Sp4 was detected at high levels at P7. This protein showed dramatic reduction by P14 and was not detectable by P21 and through adulthood (Fig. 1c). Sp3 was also expressed in the neocortex, but showed less temporal variation than Sp4; substantial expression was observed at all ages probed, though moderate enrichment was observed at P7 and P14 relative to P21 and in the adult (Fig. 1b).

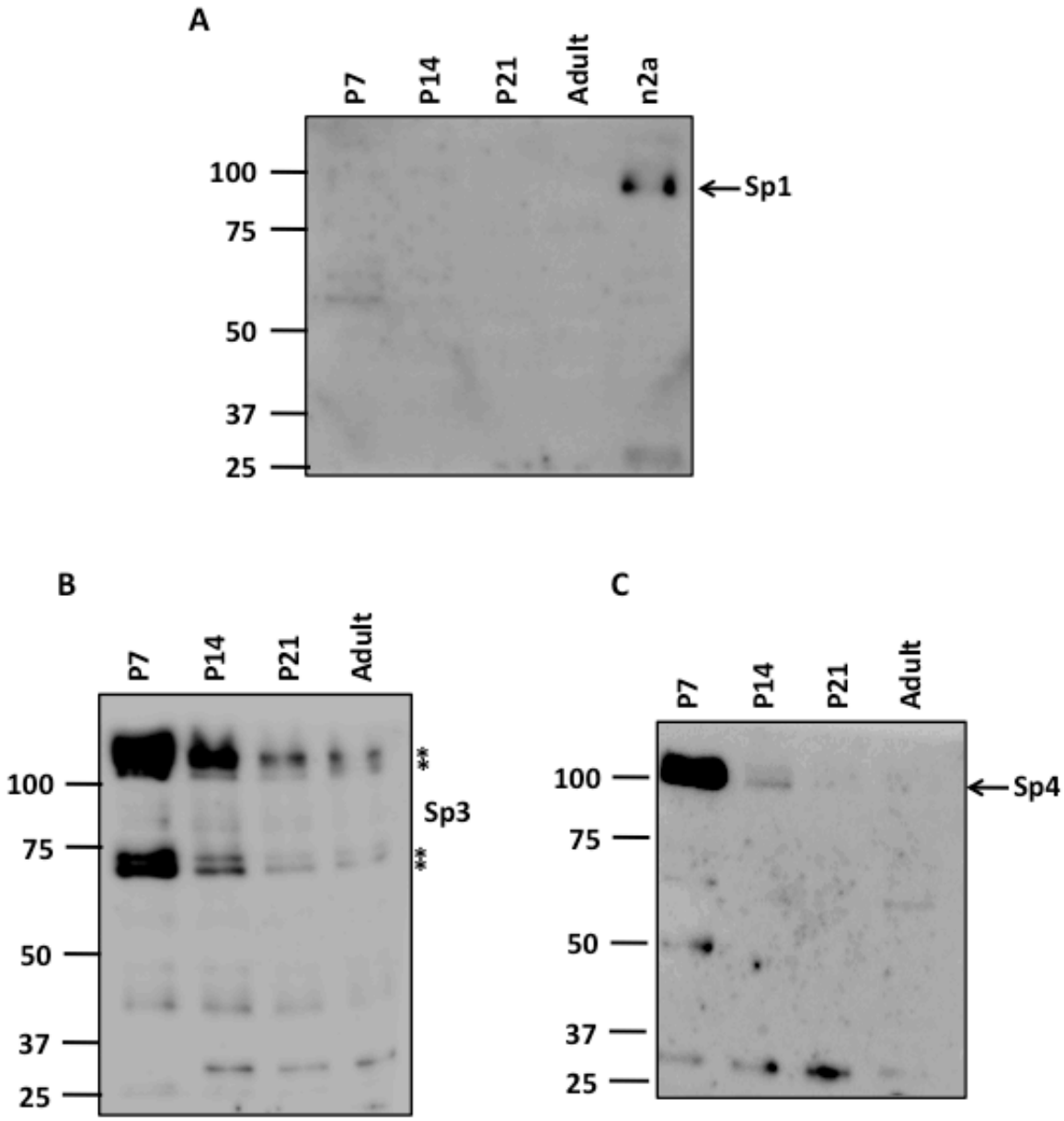

Figure 1. Expression of Sp1, Sp3, and Sp4 in the developing mouse neocortex. 35ug nuclear protein prepared from mouse neocortex at the indicated age was loaded onto gels. **A.** Sp1 is not expressed at detectable levels at any age tested. Nuclear extract from the n2a neuroblastoma cell line was run as a positive control. **B.** Sp3 is expressed from P7 through adulthood, but progressively decreases in level through this period. * = Sp3 isoform. **C.** Sp4 is expressed robustly at P7 and at very low levels at P14. It was not detected in the P21 and adult samples.

In silico predictions of Sp-factor binding to the *Met* promoter

We used a novel informatics program, TFScan, to predict Sp factor binding along the mouse *Met* promoter, from position -1000 to position +500 relative to the transcription start site (TSS). Figure 2a shows the predicted Sp binding sites across the entire region. It is clear that most of the predicted Sp activity falls in the 450 bp stretch between about -300 and +150 relative to the TSS. Figures 2b and 2c provide higher resolution of this region of interest, showing progressively more narrow regions of the *Met* promoter. The data from the analyses suggest that the most probable site of Sp-mediated regulation along the *Met* promoter lies about 300bp upstream of the TSS. In addition, there are two additional sites with high binding probability that reside approximately 225 and 115 bp upstream of the TSS. The three significant peaks further downstream (-18, +48, +122) also represent sites with high likelihood of playing a role in SP regulation at this promoter.

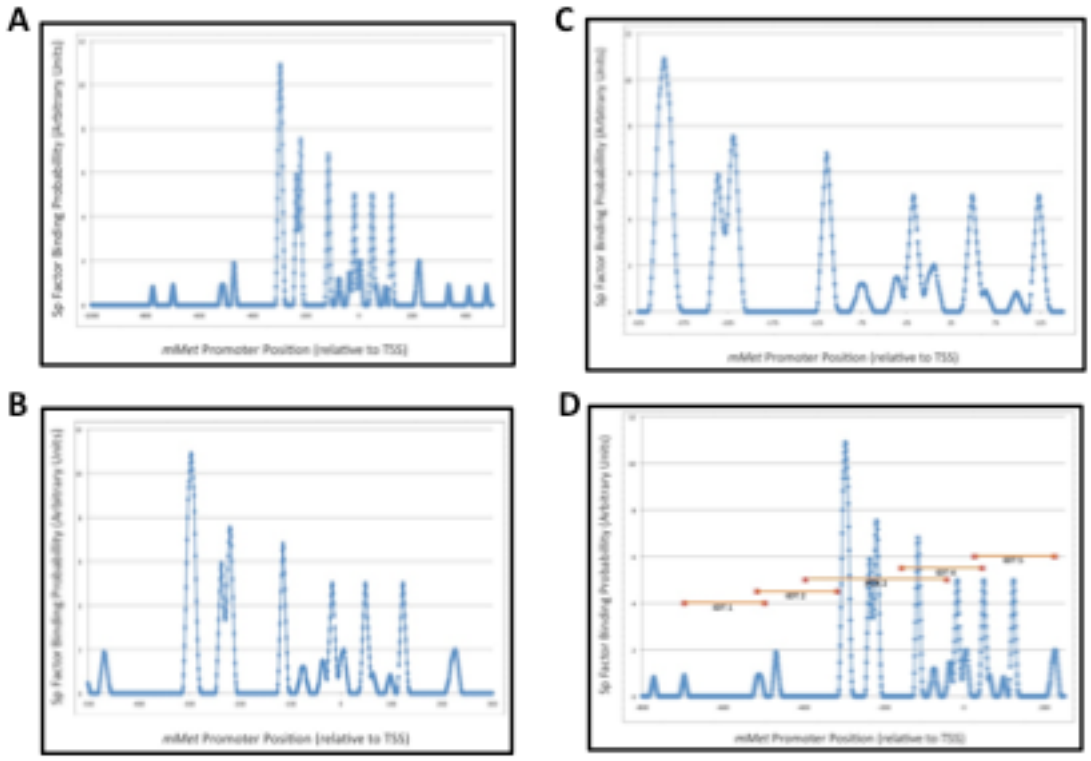

Figure 2. Graphs demonstrating TFScan predictions of Sp factor (Sp1, Sp3, or Sp4) binding sites along the mouse *Met* promoter. A covers the region extending from 1000bp upstream to 500bp downstream of the transcription start site (TSS), and **B and C** cover progressively more narrow regions. In **D**, the oligonucleotide probes used in the pulldown assays are aligned onto the display of predicted Sp binding sites.

Sp4 binding to the mouse *Met* promoter

In silico analyses predict Sp1 binding to the mouse and human *Met* promoters (Liu, 1998; Seol and Zarnegar, 1998)), and *in vitro* experiments confirm this result (Campbell et al., 2006; Seol et al., 2000; Zhang et al., 2003). In the mouse cortex, however, we showed that Sp1 is not expressed, and therefore cannot be directly involved in regulating *Met* expression. Sp4, on the other hand, is an attractive candidate regulator; it shares a consensus binding motif with Sp1 (Hagen

et al., 1992), rendering the *in silico* and *in vitro* results similarly applicable, and it is robustly expressed in the cortex at P7 (Fig. 1b). The ability of a protein to bind DNA and regulate gene expression, however, depends on the presence and absence of other proteins. It is therefore not possible to generalize results observed in one context to a novel context wherein a different set of proteins is expressed. That is, the ability of Sp1 to bind *Met* in a particular cell type or tissue does not guarantee that Sp4, or even Sp1, will bind the same promoter in a different cell type or tissue.

To assess the capacity of Sp4 to bind the *Met* promoter in P7 neocortex, we performed a streptavidin-pulldown assay, using nuclear extract prepared from this tissue. The protein mixture was incubated under conditions conducive to protein-DNA binding, using synthetic ultramers and a PCR-generated amplicon representing the *Met* promoter region. To achieve greater resolution in this experiment, five overlapping DNA probes were used, which extended 695 bp upstream, and 225 bp downstream of the TSS, and covered the entire CpG island associated with this region (Fig. 3). The minimal promoter has not been defined in the system of interest, but the region under consideration encompasses the minimal promoters defined in other systems (Tsuda et al., 2007; Verras et al., 2007).

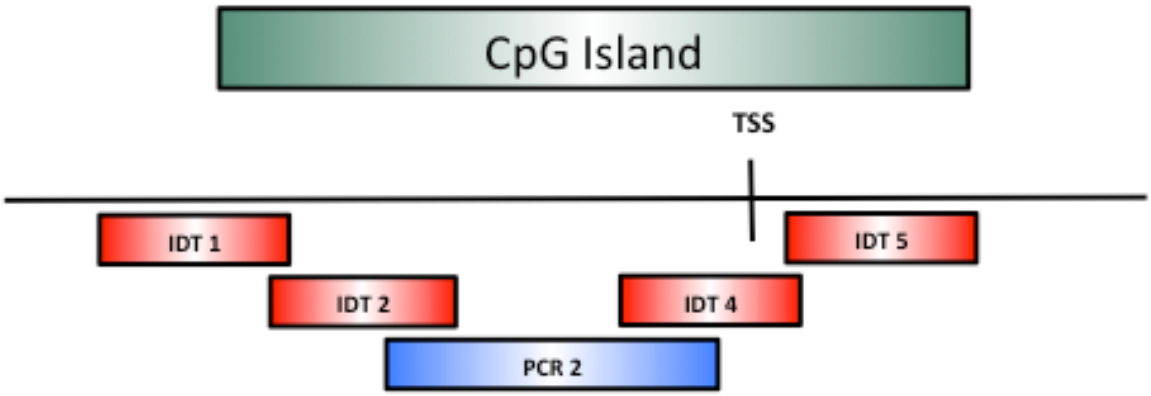

Figure 3. Location of oligonucleotide probes used in the streptavidin-pulldown assays. The black line represents mouse *Met* DNA sequence, and the transcription start site is indicated by the TSS designation. The probe designated "PCR" represents the amplicon generated by PCR, whereas the probes labeled "IDT" represent synthetic ultramers.

The pulldown experiments revealed that Sp4 in the P7 cortex binds the *Met* promoter (Fig. 4). Further, binding is non-uniform along this regulatory region; Sp4 binds strongly to probes 3, 4, and 5, which correspond to bases -395 - +225 relative to the TSS, but shows relatively weak, if any, specific binding to probes 1 and 2, which correspond to bases -695 - -316 relative to the TSS. These data provide evidence that Sp4 in the mouse brain can bind the Met promoter and demonstrate the particular regions of DNA that may be most relevant for regulation through this transcription factor.

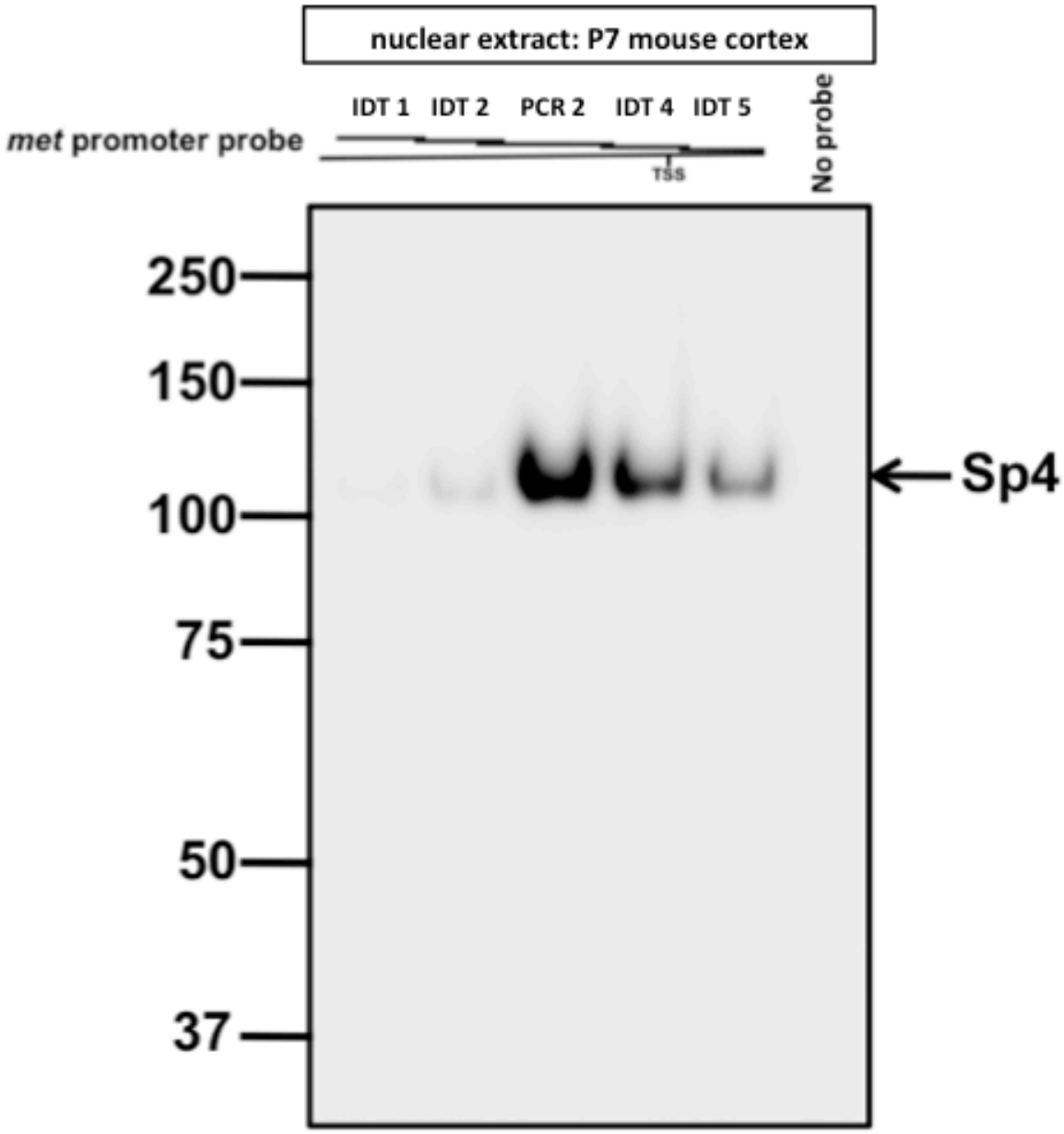

Figure 4: In streptavidin-pulldown assays, Sp4 binds *Met* promoter oligonucleotide probes in a region-specific manner. Sp4 binds with greatest affinity to *Met* promoter probes PCR 2, IDT4, and IDT 5, and among these, shows most robust binding to the PCR 2 probe

We aligned the probes used in the pulldown experiments onto the display of predicted SP binding sites generated by TFScan (Fig 2d). The *in vitro* pull down data exhibited striking similarity to the predicted peaks of binding based on the *in silico*

analysis. Thus, the most intense Sp4 band in the Western blot analysis was seen with the PCR 2 probe, which aligns with the highest peak on the TFscan plot. Relatively intense Sp4 bands were also observed following pull-down with the IDT 4 and IDT 5 probes, both of which lie over predicted Sp binding sites. Finally, probes IDT 1 and IDT 2 did not pull down significant quantities of Sp4 protein. Correspondingly, these probes fall only on weakly-predicted Sp binding loci.

Sp4 binds the *Met* promoter *in vivo* in the P7 mouse cortex

The pulldown assays show that Sp4 is able to bind the *Met* promoter *in vitro*, but do not indicate whether this binding is relevant *in vivo*. We used chromatin immunoprecipitation (ChIP) to trap protein-DNA complexes in tissue samples and determine whether Sp4 binds the Met promoter. The technique does not distinguish between direct DNA binders and proteins that bind to DNA indirectly as a member of a complex, but the assay is used to define putative regulatory proteins that can exert an effect on transcriptional activity directly or indirectly within a complex.

Five pairs of experimental primers were used in this study, each of which amplified a region of the *Met* promoter, and together covered from 974bp upstream of the TSS to 338bp downstream of the TSS. We additionally performed qPCR using primers designed to amplify a gene desert within chromosome 6. Gene deserts are regions of the genome to which no transcription factors are expected to bind, so this amplification served as a negative control. Binding to this site was considered to be

background, and binding obtained at the *Met* promoter was normalized to that obtained using the desert site.

Using P7 neocortical tissue as starting material, ChIP experiments revealed binding of Sp4 to the *Met* promoter at all sites under consideration. Compared to binding events at the gene desert site, binding events at the *Met* promoter were increased 1.79 – 2.56 fold (Fig. 5). While these levels of enrichment are considered low, they nonetheless indicate a real, and replicable effect, and provide initial evidence in support of Sp4 binding the *Met* promoter *in vivo* in the P7 mouse cortex.

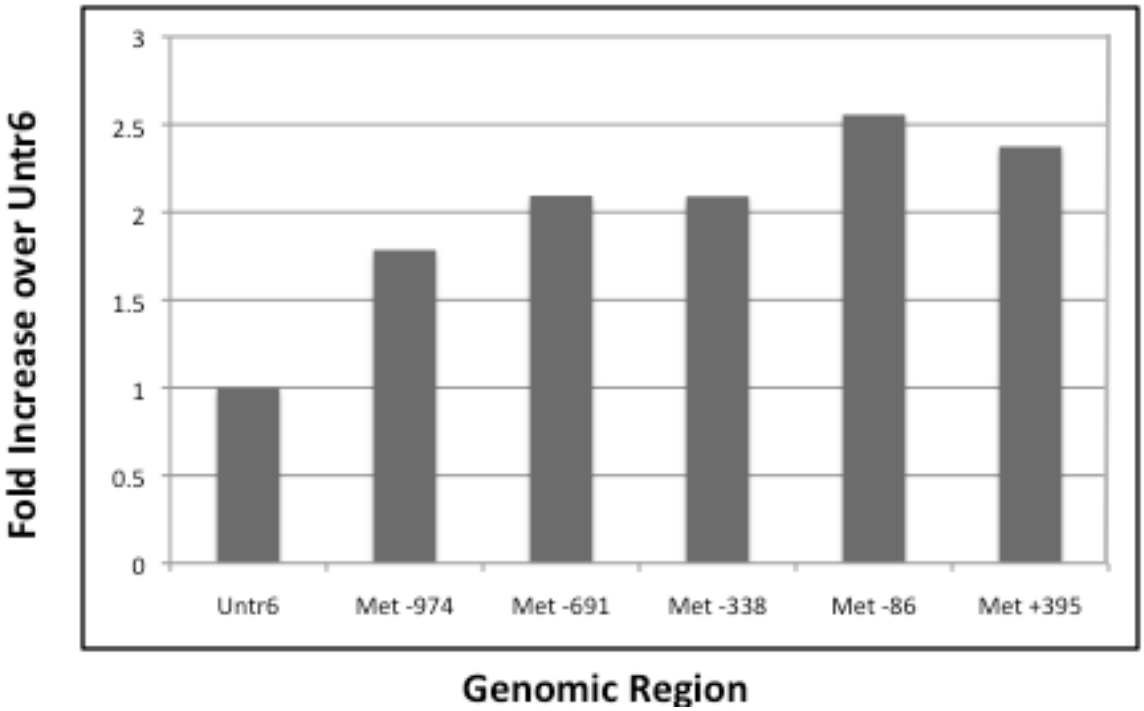

Figure 5: Chromatin immunprecipitation (ChIP) analysis of Sp4 binding to sites along the *Met* promoter in P7 mouse cortex. The graph demonstrates Sp4 binding events at the indicated genomic regions relative to Sp4 binding events at Untr6, an untranscribed genomic region. Data represent the average of two technical replicates.

DISCUSSION

This study identifies the transcription factor Sp4 as a regulator of *Met* expression in the developing mouse cortex. We demonstrate that Sp4 binds the *Met* promoter *in vitro*, and provide preliminary evidence for *in vivo* binding in the mouse forebrain. Given the emerging role of Met as a replicated ASD risk gene, and its newly defined roles in circuit formation *in vivo*, delineating the set of proteins that act in concert to control its expression is particularly important.

Sp4 as a member of a complex of proteins involved in regulating *Met* expression

The current study establishes Sp4 as a regulator of *Met* expression in the developing mouse neocortex, but it is clear that a one-to-one relationship between transcription factor and transcript does not exist; gene promoters tend to be controlled combinatorially by multiple transcription factors acting in concert or in competition (e.g. Milagre et al., 2008; Schweizer et al., 2002). Here, we show that while the expression of Sp4 and *Met* are overlapping, they are not identical, indicating that other regulatory proteins must be contributing to the observed patterns of expression. For example, although Sp4 is expressed in both the neocortex and striatum (Bergman and Levitt, unpublished observations), *Met* transcript is only observed in the neocortex (Judson et al., 2009). Additionally, Met is expressed in a layer-specific cortical pattern, with the highest levels in supra- and infragranular laminae. This pattern is not mimicked by Sp4 expression. These discrepancies likely result from regional differences in the expression of other

regulatory proteins including transcription factors and/or chromatin modifiers. This type of mechanism for generating cell-specific regulation by Sp4 has been reported in other systems. In the mouse retina, for example, Sp4 is highly expressed in multiple cell types, but activates rod *opsin* exclusively in the photoreceptors. Luciferase assays and chromatin immunoprecipitation experiments were used to demonstrate that the photoreceptor-specific transcription factor Crx can synergize with Sp4 to increase activation of the *opsin* promoter (Furukawa et al., 1997; Lerner et al., 2005). Sp4 thus plays a direct role in cell-specific transactivation of the promoter within the context of a more generalized pattern of expression. Another mechanism for refining Sp4-mediated regulation of downstream targets is via the stoichiometric contribution of factors binding to the same consensus sequence (Kwon et al., 1999; Lerner et al., 2005; Ross et al., 2002). Using drosophila SL2 cells, which lack endogenous Sp-factors, Kwon et al. (1999) demonstrated that expression of Sp1 drives transcription of the human alcohol dehydrogenase 5/formaldehyde dehydrogenase (*ALD5/FDH*) gene, but that coexpression of Sp3 or Sp4 prevents this activation, presumably by occupying promoter binding sites. In a parallel fashion, Lerner et al. (2005) demonstrated in HEK cells that Sp4 drives expression of the β-subunit of rod-specific cGMP-phosphodiesterase gene (*β-PDE*), but that this activation is inhibited by co-transfection of Sp1 or Sp3 (but not additional Sp4). Although Sp1 is not expressed appreciably in the P7 cortex, given that many members of the large Sp/XKLF family of transcription factors share the same or a similar binding sequence (Hagen et al., 1992; Suske, 1999; Wierstra, 2008), it is

conceivable that a similar mechanism is in place utilizing one or more of these additional proteins.

Investigators using human cancer cell lines have identified several transcription factors that regulate *Met* expression, including the androgen receptor, Daxx, TFE3, and others (see Chapter I, Table 1). Given that homology between the human and mouse *Met* promoters is nearly 70%, it is likely that a similar subset of transcription factors can bind the mouse promoter. Additional studies will be necessary to determine whether these factors are expressed in the developing mouse forebrain and bind to *Met* in that context.

Functional implications of Sp4 as a *Met*-regulating transcription factor

The restricted expression of *Met* in the developing forebrain underscores the need for exquisite control of its transcription, and implies specific roles for the receptor during development. As a *Met*-regulating transcription factor, Sp4 is a part of the network of proteins responsible for establishing *Met* patterning. As such, it is positioned to effect changes in the level, timing, and location of *Met* expression, and in so doing, may give rise to a wide array of downstream consequences. Given the peak in *Met* expression during the second postnatal week in the mouse, we have suggested that the protein plays a prominent role in terminal field ramification of axons, dendritic and spine maturation and ultimately synaptogenesis. Moreover, we predict that the mediation of these stereotyped neurodevelopmental events will occur particularly in the formation of circuits involved in socio-emotional processing and memory, based on the localization of the transcript and protein to

classically defined limbic structures, and trafficking of the protein product to their interconnecting fiber pathways (Judson et al., 2009). Mapping of MET in the non-human primate indicates conserved expression in subcortical limbic structures, but an even more dramatic restriction to neocortical regions involved in assigning emotional valence to visual stimuli, including faces (Judson et al., 2010a). Given the importance of vision for social interactions in primates, this differentially regulated pattern is consistent with a role for *MET* in impacting ASD risk.

Perturbations of levels of *Met* expression – temporally or spatially – are, therefore, expected to result in altered developmental trajectories and forebrain connectivity. While studies addressing these changes are ongoing, initial reports indicate that the predictions are borne out. For example, electrophysiological studies in our laboratory indicate that local connectivity between superficial and deep layers is altered following constitutive deletion of *Met* (S. Qiu, C. Anderson, P. Levitt, G. Shepherd, unpublished observations).

In a conditional knockout mouse in which *Met* is effectively removed from neurons derived from the dorsal pallium, dendritic architecture is affected, suggesting consequent alterations in forebrain circuitry. Based on reconstructed dendritic arbors following Lucifer Yellow injections, Judson et al. (2010b) report that the dendrites of Met-deficient neurons in layer 5 neurons of the anterior cingulate cortex sample a smaller volume of cortical space relative to wild-type neurons, and that spine head diameter is increased approximately 20% in layer 2/3 neurons from the same cortical region (Judson et al., 2010b). Further, the authors report increased dendritic arbor length and spine head volume in striatal medium

spiny neurons, which do not express Met. These data imply that Met disruption additionally causes cell-nonautonomous defects in wiring.

The observed influence of Met on forebrain circuit formation may, in the human, predispose to disorders of altered neural connectivity, including ASD or schizophrenia. Postmortem analyses reveal that individuals with autism express less Met protein in the superior temporal gyrus compared to unaffected controls. While this association is correlative, together with the replicated genetic association studies (Campbell et al., 2008; Campbell et al., 2006; Jackson et al., 2009; Sousa et al., 2009; Thanseem et al., 2010), the data collectively imply a relationship between *MET* disturbances and ASD, and suggest that altered levels of MET expression might contribute to neuropathology.

The Met signaling system relies on precise expression of the receptor during neurodevelopment. Changes in the timing, location, or intensity of expression can affect forebrain connectivity and may result in the emergence of disorder. Because Sp4 acting in *trans* on the *Met* promoter may drive altered expression in each of these dimensions, the transcription factor itself may represent a point of vulnerability in predisposing to aberrant formation of neural circuitry.

Role for Sp4 in neurodevelopmental disorders

Over the past two years, genetic studies have linked Sp4 to multiple neuropsychiatric disorders including bipolar disorder, schizophrenia, major depressive disorder, and Alzheimer's disease(Boutillier et al., 2007; Shi et al., 2010; Shyn et al., 2009; Tam et al., 2010; Zhou et al., 2010; Zhou et al., 2009). This

convergence is not surprising; there is considerable overlap in the processes that are disrupted in neurodevelopmental disorders, including those that have adult onset such as bipolar and schizophrenia (Carroll and Owen, 2009; Harrison and Weinberger, 2005). The nature of the disruption is likely to vary widely. Thus, factors affecting circuit wiring are vulnerable targets in predisposing to aberrant neurodevelopment generally, with the particular disorder or phenotype dependent on the parameters of the insult. For example, minicolumns, considered to be the fundamental unit of neocortical information processing (Mountcastle, 1997), show pathology in multiple disorders, though the quality of this pathology is disorder-dependent. Minicolumns in the brains of dyslexic individuals are wider than those in brains from typically-developed individuals, whereas brains from subjects diagnosed with either schizophrenia or ASD have narrower minicolumns (Buxhoeveden and Casanova, 2002; Casanova, 2006; Casanova et al., 2002). Similarly, an identical insult can result in different pathology depending on the location or timing of that insult. The impact of timing on neurological phenotype is illustrated well in animal models by *in utero* exposure studies with the viral mimic, poly(dI-dC). While aberrant development is evident in all instances, the timing of the exposure has profound implications for the nature of the phenotype. Exposure at E9, compared to E17, results in vastly different, non-overlapping, deficits in the mature mice: impaired sensorimotor gating in those exposed during early-mid gestation but impaired performance on working memory tasks in those exposed during late-gestation(Fatemi, 2009; Meyer et al., 2008).

Context-dependent roles for Sp4 have also been demonstrated. For example, germline deletion of Sp4 results in reduced dendritic branching in the hippocampal dentate gyrus (Zhou et al., 2007), the region of focus of their study. siRNA-mediated knockdown of Sp4 in cerebellar granule neurons results in exuberant dendritic branching (Ramos et al., 2007). These findings suggest that Sp4 contributes to promoting dendritic branching in one system, but that it limits branching in another, a discrepancy which could be explained by several mechanisms including 1) differential Sp4 target activation/repression owing to the other transcription factors and chromatin modifiers expressed in the cells under consideration, and 2) expression of other proteins that interact with Sp4 targets to elicit cellular consequences.

We suggest that Sp4 may be viewed as an additional, potential contributor to ASD risk. Sp4 has been implicated in the development of several other neuropsychiatric disorders, many of which share features with, or involve disruption of, phenotypic dimensions that are also disrupted in ASD (e.g. prepulse inhibition in both schizophrenia and ASD). Moreover, as a regulator of *Met*, Sp4 is primed to evoke the changes in expression that may underlie the contribution of *MET* to ASD risk.

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