CHAPTER V

FUTURE DIRECTIONS

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SUMMARY OF RESULTS

The broad aims of this research project were to define the expression patterns of *Met* in the developing mammalian forebrain and to understand the transcriptional regulatory networks involved in establishing them. This work was motivated by a series of recent findings suggesting that the Met receptor tyrosine kinase is involved in multiple facets of neurodevelopment and by a set of human genetics studies demonstrating that a functional variant in the gene's 5'-regulatory region is associated with susceptibility for autism spectrum disorders (ASD) (Campbell et al., 2007; Campbell et al., 2006; Judson et al., 2009; Judson et al., 2010b).

Although reports addressing the functional roles for Met indicate that it is a pleiotropic molecule with putative roles in multiple neurodevelopmental processes (Birchmeier et al., 2003; Ma et al., 2003), the actual roles for the protein *in vivo* are poorly understood. We anticipated that insight into these roles could be achieved by characterizing *Met* expression throughout forebrain development, and that this understanding would provide the bases for future experiments designed to elucidate the *in vivo* roles for the receptor and to clarify the biological underpinnings of the Met-ASD association. Comprehensive mapping of Met expression throughout peri- and post-natal development (as detailed in Chapter II), led to the hypothesis that Met is involved in neurite outgrowth, synaptogenesis, and, broadly, the early establishment of forebrain connectivity, with a more prominent

role in circuits related to social and emotional dimensions of behavior (Judson et al., 2009).

Our efforts to characterize the regulatory network involved in coordinating *Met* expression were fueled by two key findings. First, the mapping analysis revealed remarkably restricted patterns of expression in both time and space, thus highlighting the requirement for precise regulation of the gene's transcription. Second, the discovery of a *MET* promoter variant conferring ASD susceptibility suggested that tight regulation of *MET* expression is required for normal brain development and underscores the importance of defining the transcriptional network controlling this expression. Our approach for evaluating transcriptional control of *Met* expression combined in-depth validation and analysis of one *Met*-regulating transcription factor (TF), Sp4, with a screen intended to identify additional regulators for later study. The work on Sp4 points toward this TF as a bona fide *Met*-regulating protein, and the screen identified 157 putative regulators from postnatal day (P)7 cortical tissue. We propose a series of experiments designed to validate and further characterize both Sp4 and the newly identified putative regulators both in affecting *Met* expression and, more generally, contributing to forebrain development.

VALIDATION OF PUTATIVE *MET*-REGULATING FACTORS

Using a DNA pulldown-mass spectrometric approach, we identified 157 putative *Met*-regulating proteins from P7 cortical tissue., of which we considered 49

to be of particular interest. Because mass spectrometry (MS) carries significant risk of false positive results (Mallick and Kuster, 2010), we will initially validate candidate regulators by separating the eluted fractions by gel electrophoresis and probing for the candidates using specific antibodies. This verification process serves a secondary purpose in establishing antibody specificity, a prerequisite for the chromatin immunoprecipitation (ChIP) experiments described below.

The western blot experiments can validate the subset of proteins identified by MS that bind, either directly or in a complex, to the 5'-*Met* promoter *in vitro*. Further experiments will be necessary to determine 1), whether this binding occurs *in vivo* and 2), whether this binding is functionally relevant. To address the former question, we propose ChIP experiments in tissue harvested from P7 cortex. In these experiments, protein-DNA complexes are cross-linked with formaldehyde, and the DNA is sheared into fragments approximately 200-600 base pairs (bp) in length. The protein of interest is immunoprecipitated, and after reversing the cross-links, the captured DNA is subjected to polymerase chain reaction using primers that amplify segments of the *Met* promoter. This approach offers several advantages over the pulldown approach. First, DNA is found in its native state instead of existing as a short oligonucleotide. The three dimensional structure allows distant regions of the sequence to be brought into close proximity, facilitating interactions among proteins that bind to disparate segments of the DNA, and permitting single proteins to bind regions that are physically close despite large stretches of intervening sequence. Second, *in vitro* experiments can facilitate interactions that are not biologically relevant due to prohibitive stoichiometric or physical

constraints imposed by the *in vivo* environment. ChIP experiments circumvent this problem.

While the ChIP experiments can establish that binding occurs *in vivo*, they do not give insight into whether these interactions are functional. To address this issue, we propose reporter gene assays in which activity at the *Met* promoter is measured following manipulation of the levels of candidate regulators by transfection of either overexpression constructs or siRNA in immortalized mouse cell lines. We have designed a reporter construct that contains 1388bp of *Met* sequence (1051bp upstream and 336bp downstream of the transcription start site) upstream of the luciferase gene. Measurement of luminescence in cells 24 hours after transfection of this construct serves as a proxy for activity at the *Met* promoter since luciferase expression is being driven by the promoter, and is directly proportional to luminescence. Preliminary experiments using this reporter construct in the EL4 mouse lymphoblastic cell line demonstrate that the included region of the *Met* promoter acts as a repressor in this context. Over five trials, the *Met*-containing vector produced luminescence levels 17% +/-4% of those produced by the empty vector (Bergman and Levitt, unpublished observations). In other culture systems, the same sequence may act as a repressor or an activator and must be determined empirically. After establishing the effect of the *Met* promoter in a cell line of choice, we will determine the effect of manipulating candidate regulator levels prior to reporter transfection as described above. While the finding that a particular protein functions to regulate *Met* expression in one cell type does not imply the same function, or any function, in another cell type, it does provide

evidence that the protein is capable of exerting a functional influence on the *Met* promoter.

CHARACTERIZATION OF *MET*-REGULATING FACTORS

The validation experiments proposed above seek to identify the candidate regulators that 1) bind the *Met* promoter *in vivo* in the tissue of interest and 2) are capable of exerting a functional influence on its transcription *in vitro*. After establishing this set of regulators, we aim to better characterize the way in which each of these proteins regulates Met expression in the developing forebrain.

To inform subsequent studies, we plan first to generate maps of these regulators' expression patterns in the developing forebrain by immunohistochemistry. These data will enable us to predict the specific regions and developmental epochs during which each factor is most relevant to *Met* expression. We do not expect that TF-*Met* expression will be entirely identical or entirely nonoverlapping, but the TF must be expressed to have an effect – activating or repressing – in a particular cell. By assessing expression *in situ* over a wide developmental period, we will obtain information critical to planning subsequent experiments, and we will gain insight into the regions and processes that might be affected by dysregulation of *Met* by a particular regulatory protein. It will be interesting to repeat the DNA pulldown experiments and subsequent western blots using starting material harvested from different regions of the brain or at different developmental time points in accordance with the expression data. These

experiments will provide information regarding the spatial and temporal dynamics of each transcription factor's contribution to *Met* expression.

The DNA pulldown experiments as performed limit the resolution of the DNA binding site to about 200bp, based on the length of the oligonucleotide probes. Honing in on a more narrow region where binding occurs would be beneficial in establishing regions of the promoter where sequence integrity is more critical and other regions where variation can be tolerated. Additionally, knowledge of specific factors' binding sites would inform predictions regarding the consequences of single nucleotide polymorphisms (SNPs) on protein-DNA binding. To achieve this level of resolution, we propose complementary DNA pulldown experiments and luciferase assays using mutated versions of the oligonucleotide bait and reporter construct, respectively. For transcription factors whose binding sites are well-characterized, and for those belonging to a family whose site is well-characterized, mutating two or three critical bases should be sufficient to significantly disrupt binding. For other transcription factors, generating truncated versions of the existing probes and constructs is more practical.

NETWORK OF GENES REGULATED BY SP4

Just as *Met* expression is controlled by a combination of factors, each *Met*-regulating factor similarly acts on an array of downstream genes. Any perturbation to the transcription factor itself would be expected to affect the expression of many, if not all, of these targets. It would be interesting to know which genes are co-

regulated with *Met*, as the effects of *Met* disruption may occur in the context of these other disruptions. To identify Sp4 targets that may be co-regulated with *Met*, we suggest ChIP using P7 mouse cortical tissue. The DNA that is precipitated with Sp4 will be hybridized against a genome-wide array to identify bound target genes. Given recent studies implicating Sp4 as an etiological contributor to several neurodevelopmental disorders including major depressive disorder (Shi et al., 2010; Shyn et al., 2009), bipolar disorder (Zhou et al., 2009), schizophrenia (Tam et al., 2010; Zhou et al., 2010; Zhou et al., 2009), and Alzheimer's disease (Boutillier et al., 2007), these findings will be particularly valuable. It is possible that several of the same Sp4 targets will be involved in multiple disorders, and that the differential outcomes will result from the timing of the insult or from the presence or absence of co-occurring disruptions.

IN VIVO **MANIPULATION OF SP4 DURING DEVELOPMENT**

To better understand the role of Sp4 in regulating *Met* and contributing to forebrain development *in vivo*, we propose *in utero* electroporation to introduce expression plasmids or siRNA into the developing embryonic cortex. By enhancing or reducing, respectively, expression of Sp4 at an early stage, we will be able to evaluate the *in vivo* contribution of Sp4 to *Met* expression and the overall impact of Sp4 perturbations on cortical development. These manipulations typically yield changes in expression that persist for several weeks postnatally (Tabata and Nakajima, 2008), thus allowing the study of an extended alteration in transcription

factor expression rather than an acute change. Analysis of *Met* expression will indicate whether Sp4 functions as an activator or a repressor in this context. Additionally, these studies will provide a more detailed understanding of the relationship between Sp4 and *Met* in the cortex than could be achieved from the DNA pulldown experiments. In the pulldown experiments, cortex was considered *en masse*, but by evaluating changes in *Met* expression by *in situ* hybridization, we will be able to appreciate layer-specific and region-specific differences in the regulatory relationship. To assess the broad influences of Sp4 disruption on cortical development we will examine cell differentiation, laminar differentiation, and anterior-posterior (A-P) patterning by evaluating expression of well-described markers that distinguish neurons from progenitors and glia or deep layers from superficial layers, or that show A-P gradients. Given the network of downstream genes that Sp4 is predicted to regulate, any changes along these dimensions cannot necessarily be attributed to changes in *Met* expression. However, such changes may track with alterations in *Met* expression when Sp4 is the causative agent due to the variety of downstream targets affected by changes in its expression.

POSSIBLE ROLES FOR *MET*-REGULATING FACTORS IN CONTRIBUTING TO ASD RISK

It has been established in five independent cohorts that the *MET* (human ortholog of *Met*) single nucleotide polymorphism rs1858830 (G/C) C allele is associated with increased risk of developing an ASD (Campbell et al., 2008; Campbell et al., 2006; Jackson et al., 2009; Sousa et al., 2009; Thanseem et al., 2010).

This variant is found in the gene's 5'-regulatory region, and several pieces of evidence suggest that it is a functional variant that affects gene transcription. Campbell et al. (2006) demonstrated by electromobility shift assay (EMSA) that the complex of proteins binding to the promoter varies depending on the nucleotide at that site and used luciferase assays to show that transcription driven by the *MET* promoter decreases when the promoter contains a C rather than a G at the SNP. Further, expression of MET protein in the superior temporal gyrus is reduced in individuals with a C/C genotype at this locus relative to those with a G/G genotype.

Given that aberrant *MET* expression is associated with increased risk of ASD, it is logical to propose that alterations in the factors that control *MET* expression may also confer risk for the disorder. Such alterations include changes in the level, timing, or place of expression, aberrant post-translational modifications, or mutations affecting interaction of the protein with other proteins or with promoter DNA. We propose using preexisting data sets to evaluate whether the *Met* regulators show relevance to ASD susceptibility. Loci associated with ASD due to the presence of linkage peaks or overrepresented gains or losses of genetic material in individuals with the disorder are found on every chromosome (reviewed in Abrahams and Geschwind, 2008). Determining whether the putative *Met* regulators are located at any of these loci provides a simple test of potential relatedness to ASD risk and may aid in prioritizing candidates for future study. A more explicit approach would use microarray data to compare gene expression between individuals with ASD and matched neurotypical controls. Although this type of analysis carries caveats, most notably the inability to choose the brain region and

developmental age of interest for study and the difficulty in matching samples precisely, it also provides a powerful method for identifying changes in the ASD brain, and consistent expression differences are likely meaningful.

The proposed analyses assume that the factors regulating *Met* expression in the mouse are homologous to those regulating *MET* expression in the human. While it is unlikely that the set of regulatory factors is identical between the species, three pieces of evidence suggest that there may be substantial overlap. First, the 5'-regulatory regions of mouse *Met* and human *MET* share 68% homology over the ~1kb segment (-771 - +225 relative to the mouse TSS) covered in the pulldown experiments used to generate the candidate regulators. Second, a recent study comparing Met/MET expression in the mouse and macaque forebrains revealed many similarities in the two species, suggesting evolutionary conservation of the mechanisms regulating its patterning. Specifically, in both species, expression was maximal during the period of synaptogenesis (P7 in the mouse and gestational day 150 in the macaque), and enriched in limbic nodes and pathways, suggesting a role in emotional information processing (Judson et al., 2010a). It should be noted that this study analyzed protein expression, and it is unknown whether the underlying transcript expression is correspondingly similar, but the results remain suggestive of conserved regulatory mechanisms. Finally, a preliminary informatics analysis of SP binding sites reveals significant overlap in predicted sites between the mouse and human *Met/MET* promoters (Fig. 1). Although this example considers a single TF subfamily, it demonstrates that the notion of shared transcriptional regulatory mechanisms between the promoters is realistic.

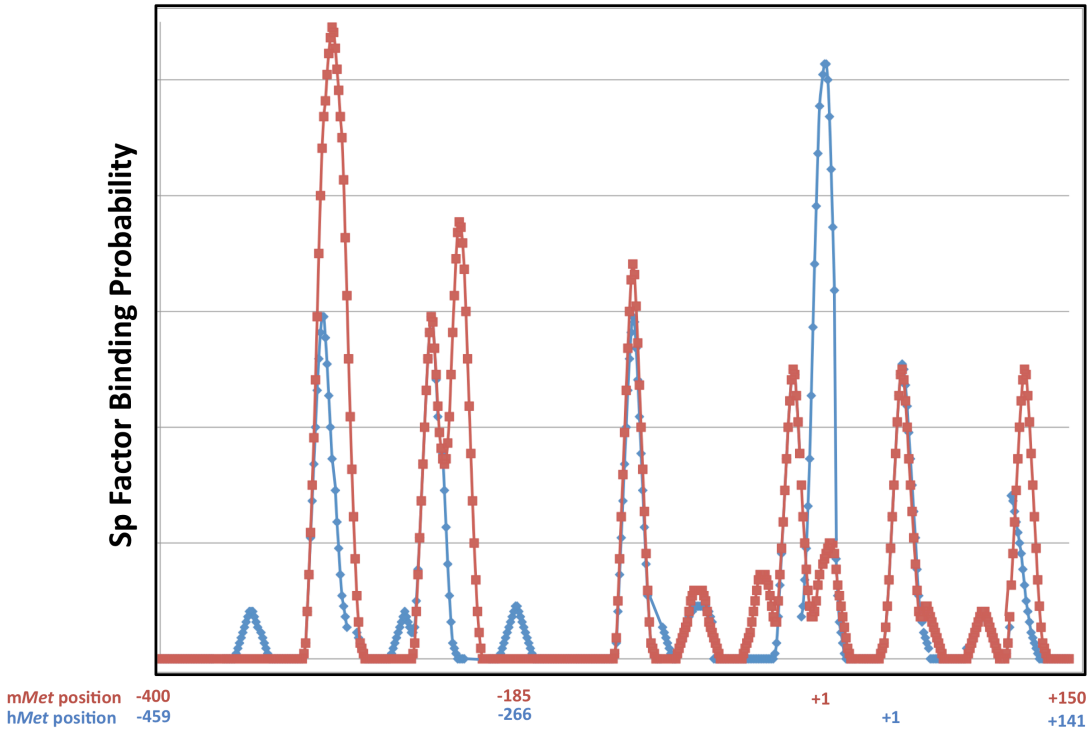

Figure 1. TFScan predictions of Sp factor binding sites along the mouse (pink) and human (blue) *Met/MET* promoters. The promoter sequences were aligned based on homology, and positions are relative to the transcription start site (+1). With a few exceptions, the locations and relative strength of predicted Sp factor binding sites are very similar between the two species' promoters.

ENVIRONMENTAL INFLUENCES ON *MET* REGULATION

Disruption to the transcriptional regulation of a gene may be rooted in genetic alterations to the gene itself or to the regulator, as discussed above. Additionally, environmental factors may shape the ways in which regulatory proteins interact with their target genes to affect transcription. This phenomenon has been demonstrated in the literature numerous times. For example, in cultured

cerebellar granule neurons, toxic concentrations of glutamate alter the binding profile of transcription factor YY1 to its consensus sequence in EMSA. Specifically, the binding of one regulatory complex is reduced, whereas the binding of a second regulatory complex is enhanced (Korhonen et al., 2005). Similar effects can be seen *in vivo*. Hood et al. demonstrated that *in utero* exposure to benzo(a)pyrene (BaP), a toxin associated with automobile exhaust fumes and coal tar, among others, results in altered Sp-factor binding in early postnatal rat pups. In the cerebellum of BaP-exposed pups, Sp-binding peaks earlier and remains at maximum intensity for a longer period relative to unexposed control animals (Hood et al., 2000). Especially in light of the recent expansion in our awareness and understanding of gene-environment interactions in the pathogenesis of autism (reviewed in Herbert, 2010), investigation of environmental effects on individual TF-*Met* binding will be of great interest in the future.

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