CHAPTER I

INTRODUCTION

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THE MET RECEPTOR TYROSINE KINASE IN NEUROBIOLOGY

Met and its ligand, hepatocyte growth factor/scatter factor (HGF/SF): discovery, structure, and signaling

The Met proto-oncogene is a receptor tyrosine kinase that was identified as a transforming gene after treatment of a human osteosarcoma cell line with a chemical carcinogen resulted in the formation of a DNA species capable of transforming a second cell line (Cooper et al., 1984). The mouse ortholog, Met, was subsequently cloned, and due to the high level of amino acid homology (88.1% overall), has facilitated the study of this molecule (Iyer et al., 1990). Met has been implicated in the formation, progression, and metastasis of many types of cancers and has well-defined roles in the development of the kidneys, liver, muscles, and placenta (reviewed in Birchmeier et al., 2003).

The Met receptor tyrosine kinase is an α, β-heterodimer comprised of an extracellular domain, a transmembrane domain, and an intracellular tyrosine kinase domain. At the C-terminal end of the kinase domain, the receptor contains a multiprotein docking site that provides an attachment platform for a wide array of downstream signaling molecules (Park et al., 1987; Ponzetto et al., 1994). The receptor has one known endogenous ligand, the plasminogen-related growth factor hepatocyte growth factor/scatter factor (referred to as HGF hereafter) (Bottaro et al., 1991; Nakamura et al., 1984; Stoker et al., 1987). HGF is secreted as a signaling-

deficient, single-chain precursor that is cleaved by a serine protease to assume its active, α, β-heterodimeric form (Lokker et al., 1992; Mars et al., 1993).

Signaling through Met is initiated by HGF binding to the receptor's sema domain (Gherardi et al., 2003), which results in receptor dimerization and kinase domain autophosphorylation (Ferracini et al., 1991; Longati et al., 1994; Naldini et al., 1991). This phosphorylation event promotes subsequent phosphorylation of downstream tyrosines, which must be activated to allow functioning of the Met multisubstrate docking site (Ponzetto et al., 1994). These molecules either bind directly to one of the phosphoryated tyrosines or associate with an adapter protein, which is bound to one of these sites. Most of these pathways have been discovered and described in the cancer literature (for review see Birchmeier et al., 2003; Bolanos-Garcia, 2005; Furge et al., 2000; Ma et al., 2003; Maulik et al., 2002; Peschard and Park, 2007; Rosario and Birchmeier, 2003).

Termination of Met signaling is critical to modulating the duration and extent of the receptor's activity. The primary mechanism for activity-induced downregulation is ubiquitylation by the Cbl E3 ubiquitin ligase, which promotes Met internalization and degradation following stimulation (Jeffers et al., 1997) and can bind directly to the receptor or indirectly via Met-bound Grb2 (Peschard et al., 2001). Subsequent ubiquitylation marks Met for recognition by the endocytic pathway and consequent clathrin-coated endoyctosis and lysosome-mediated degradation (Hammond et al., 2003; Jeffers et al., 1997). Dephosphorylation of activated tyrosine residues is another mechanism by which Met signaling can be attenuated, and phosphatases implicated in this process include density-enhanced

phosphatase 1 and leukocyte common antigen-related protein-tyrosine phosphatase (Machide et al., 2006; Palka et al., 2003).

Roles for Met in nervous system biology *in vitro*

Over the past two decades, numerous studies have probed the ways in which HGF and Met contribute to nervous system development and function. The signaling system has been implicated in a wide array of processes including neuron survival, differentiation, and migration, axon guidance, dendrite outgrowth and elaboration, and synaptic activity. Although the set of neurodevelopmental events in which the signaling system has been implicated is large, the number of studies that focus on any particular aspect of development are relatively small in number. Moreover, the majority of these studies was performed *in vitro*, and most were restricted to a single cell type and time point. Thus, while they demonstrate the capacity of HGF/Met signaling to mediate these processes, there is a paucity of studies that clarify specific roles played by this ligand and receptor *in vivo* in the developing nervous system. Until the present study reported here, there has been little mapping of gene expression for either the ligand or receptor, and the constitutive knockout mouse is embryonic lethal at midgestation, prior to the onset of production of most central nervous system neurons (Bladt et al., 1995).

Neuron Survival

HGF is a neurotrophic factor that promotes the survival of several classes of neurons. Ebens et al. (1996) and Yamamoto et al. (1997) demonstrated a pro-

survival effect of exogenously applied HGF on cultured E15 mouse and E14 rat motor neurons, respectively, and Ieraci et al. (2002) reported similar results in P8 mouse cerebellar granule cells. In sensory neurons, HGF does not independently promote survival, but it is capable of enhancing the effects of particular growth factors, including nerve growth factor and ciliary neurotrophic factor (Davey et al., 2000; Maina et al., 1998).

In the neocortex, conclusions regarding HGF-mediated survival are mixed. Using propidium iodide/fluroscein diacetate double staining in cultured E18 rat cortical neurons, Sun et al. (2002) demonstrated a dose-dependent increase in neuronal survival with HGF application. In contrast, DAPI staining of E15.5 rat cortical neurons revealed no HGF-related change in staining, suggesting that the factor is not functioning as a survival factor in these neurons (Powell et al., 2003). It is possible that this difference results from a difference in the age or region of the two cultures, or in technical differences between the assays.

Neuron Differentiation

HGF/Met signaling has been shown to trigger differentiation in a variety of neurons. In mouse superior cervical ganglion cultures, application of anti-HGF antibodies reduces differentiation of sympathetic neuroblasts into mature neurons (Maina et al., 1998), suggesting that endogenous HGF/Met signaling facilitates differentiation in these cells. HGF/Met signaling also contributes to the differentiation of calbindin D+ pyramidal cells in the rat hippocampus and to the establishment of PEA3+ spinal motor neuron pools in mice (Helmbacher et al., 2003;

Korhonen et al., 2000). Despite these observations, no clear mechanism has been shown to be responsible for the impact of HGF/Met signaling on specification of neuron types.

Cell migration

Met has been shown to transduce a motogenic signal in a variety of neurons. Garzotto et al. (2008) showed that HGF stimulates olfactory interneuron precursor migration *in vitro* as a chemoattractant, and similarly, Giacobini et al. (2007) demonstrated a role for HGF/Met signaling in the migration of hypothalamic gonadotropin hormone-releasing hormone-1 neurons. Cortically, Segarra et al. (Segarra et al., 2006) and Sun et al. (Sun et al., 2002) independently demonstrated an attractive motogenic effect of HGF on embryonic neocortical neurons.

Neurite outgrowth

Met involvement in this area of neurodevelopment is perhaps the best studied. The HGF/Met signaling system is involved in neurite outgrowth in a wide variety of neurons. Using E17 rat neocortical explants and exogenous, recombinant human HGF, Hamanoue et al. (1996) first demonstrated a role for the factor in promoting neurite outgrowth. Subsequent work has supported the finding that Met promotes dendrite growth and branching in neocortical neurons, and extended this finding to both GABAergic interneurons and pyramidal cells in dissociated and tissue slice hippocampal cultures (Gutierrez et al., 2004; Korhonen et al., 2000; Lim and Walikonis, 2008).

HGF has dramatic effects on neurite outgrowth and branching in peripheral sensory neurons, but these effects typically depend on concurrent stimulation by another growth factor. In fact, the synergistic effects are similar in nature to the cooperative activity reported between HGF and other growth factors in the survival experiments (Davey et al., 2000; Maina et al., 1997). In sympathetic neurons, too, HGF promotes neurite growth and branching, and in this system, the requirement for an additional growth factor depends on the age of the cultured cells (Thompson et al., 2004). Finally, HGF/Met signaling has been shown to enhance neurite outgrowth in spinal and cranial motor neurons (Caton et al., 2000; Ebens et al., 1996).

In the cultured thalamus, HGF promotes neurite outgrowth and acts as a chemoattractant, and the Met signaling system is a critical mediator of axonal guidance, but only in specific populations of neurons. When E15.5 rat thalamic explants were grown in the presence of a point source of HGF, neurite length increased relative to controls, but E15.5 somatosensory cortical explants did not exhibit a similar response. Division of the thalamic explant into four quadrants revealed regional differences in the extent of thalamic neurites' outgrowth responses, but consistent among neurites showing outgrowth was the directionality of the response toward the point source of HGF. Thus, *in vitro*, HGF is a potent inducer of neurite growth in a subset of thalamic neurons, and serves as a chemoattractant for responsive thalamic neurons (Powell et al., 2003).

Synaptic Activity and Plasticity

Roles for HGF and Met in hippocampal synaptic activity and plasticity have been established. In the rodent, expression of Met mRNA and protein in the hippocampus is evident throughout embryonic and postnatal development, and through adulthood (Jung et al., 1994; Korhonen et al., 2000). In the mature hippocampus, Akimoto et al. (2004) reported overlapping expression of Met and MAP-2, suggesting that Met is expressed in the dendrites of hippocampal pyramidal cells.

There is a reciprocal relationship between synaptic activity and HGF/Met signaling: pharmacologic activation of neural activity by glutamate or bicuculline application induces activation of HGF and Met (Tyndall and Walikonis, 2007), and Met activation plays a role in modulating activity at the synapse. Application of HGF to hippocampal neuron cultures imparts multiple changes at the molecular level that may enhance synaptic activity. HGF stimulation results in increased expression and clustering of primary synaptic proteins, including the NR2B subunit of the NMDA receptor, the GluR1 subunit of the AMPA receptor, and the α subunit of CaMKII. Additionally, it has been demonstrated that HGF stimulation increases calcium uptake along hippocampal dendrites, and that both CamKIIα and NR2B show increased phosphorylation following this stimulation. It is likely that these events occur in series, as increased calcium promotes CamKIIα activity, and CamKIIα acts at the examined NR2B phosphorylation (Tyndall et al., 2007; Tyndall and Walikonis, 2006; Tyndall and Walikonis, 2007).

These *in vitro* data suggest a bidirectional relationship between HGF/Met signaling and activity at the synapse that is consistent with the neurotrophin hypothesis of neural plasticity, whereby synaptic activity induces the activity of growth factors, which subsequently affect synaptic structure and function (McAllister et al., 1999; Poo, 2001).

Emerging roles for Met in nervous system biology *in vivo*

To date, few studies have investigated the roles of HGF/Met signaling in the nervous system *in vivo*. These studies, however, suggest important roles for this signaling system in mammalian brain development. In the early postnatal rat striatum, Met activation promotes the proliferation of oligodendrocyte progenitor cells, but inhibits their differentiation into oligodendrocytes. The reduction of mature, myelin basic protein (MBP)-expressing oligodendrocytes translates into reduced myelination of striatal axons (Ohya et al., 2007), which could have severe consequences for striatally-derived information processing. In the hippocampus, alterations in the numbers and ratios of interneuron subtypes have been observed following genetic deletion of Met from proliferating or postmitotic cells in the ganglionic eminence. Specifically, early Met disruption resulted in decreased calretinin-expressing cells in the dentate gyrus, CA1, and CA3, and region-specific changes in parvalbumin-expressing cells: increased numbers in the dentate gyrus, unchanged numbers in CA1, and a reduction of this population in CA3 (Martins et al., 2007).

Recent work in our laboratory is focused on understanding the *in vivo* contributions of Met to establishing neuronal connectivity in the forebrain using conditional Met mutant mice (Emx1^{cre}/Met$^{fx/fx}$) in which Met function is specifically ablated in cells arising from the dorsal pallium (Judson et al., 2009; Judson et al., 2010). To clarify the roles of Met in forming and maintaining forebrain circuits, Judson et al. (2010) injected neurons with Lucifer Yellow (LY) to facilitate morphometric analyses of the cells' dendritic properties. These experiments focused on the pyramidal neurons of the anterior cingulate cortex (ACC), given their involvement in limbic circuit function, and on the medium spiny neurons (MSN) of the dorsolateral striatum, because these neurons do not express Met transcript or protein, but receive corticostriatal afferents that express high levels of the receptor protein presynaptically (see Chapter II). In the ACC, two major changes were observed in the Met-deficient neurons. First, in layer V, the cells' dendrites sampled a smaller volume of cortical space relative to their wild-type counterparts. This finding is consistent with the notion that these neurons receive more limited cortical input, and suggests alterations in cortical circuitry in these animals. Second, in layer II/III, Met-deficient neurons displayed increased spine head volume of approximately 18-22% relative to wild-type neurons, with a corresponding increase in stable spines relative to smaller, more labile ones. This phenotype might result in decreased plasticity in this population of inputs, with consequent deficits in information processing. Similar single neuron, LY analyses in striatal MSN revealed increased dendritic arbor length and spine head volume. These neurons do not express Met transcript (see Chapter II), but as noted above, receive Met-expressing

afferents from the neocortex. Thus, the statistically significant changes in dendrite and spine morphology in the conditional *Met* mutant mice reveals a cell-nonautonomous effect at the synapse, and speaks to the importance of receptor availability for the fine details of circuit formation (Judson et al., 2010). Electrophysiological experiments in the Emx1^{cre}/Met$^{fx/fx}$ mice in our laboratory reveal additional contributions of Met to forebrain circuits. Using laser scanning photostimulation to map local circuitry in the prefrontal cortex, we have observed two-fold increases in input from layer II/III to layer V in the conditional Met knockout mice relative to wild-type animals, with evidence for reduced synaptic input of long-distance connections on the pyramidal cells (Qiu et al., unpublished observations). Overall, these data are consistent with a role for Met activation in establishing the quantitative details of synaptic connectivity in neurons that normally express Met. This is consistent with the restricted spatial and temporal expression patterns in the mouse forebrain (see Chapter II).

MET IN AUTISM SPECTRUM DISORDERS (ASD)

Autism as a disorder of altered connectivity

The laboratory's focus on the Met receptor tyrosine kinase in neurodevelopment originates from our discovery of human *MET* polymorphisms as increasing risk for autism spectrum disorder (ASD) (Campbell et al., 2006). This will be discussed in more detail below.

ASD is a highly heterogeneous neurodevelopmental disorder with onset before the age of three and persistence of key core clinical features throughout life. The triad of atypical social behavior, impaired verbal and non-verbal communication, and patterns of restricted interests and repetitive behavior are highly reliable phenotypes that are used to by clinicians to diagnose ASD. Decades of studies, however, have clarified that the quality and extent of symptoms varies dramatically between patients. In the United States, the Center for Disease Control (CDC) reports prevalence of the disorder to be 1 in 110 (CDC, 2009). That ASD shows a heritability of 90% (Bailey et al., 1995; Le Couteur et al., 1996; Steffenburg et al., 1989) speaks to the importance of genetic factors underlying the disorder. The heterogeneity of patient presentations, however, argues against the existence of a single causal factor, and genetic studies suggest that many genes may contribute to ASD risk (Abrahams and Geschwind, 2008; Levitt and Campbell, 2009).

Our laboratory and others have suggested that the unifying core deficit in ASD is atypical development of cortical connectivity resulting in an impaired ability to integrate complex information (Courchesne and Pierce, 2005; Frith, 2004; Geschwind and Levitt, 2007; Rubenstein and Merzenich, 2003). We note, however, that the etiology, nature, and outcome of this cortical disconnection may vary widely among patients. Mechanistically, such disconnection could include a weakening of already formed connections or a failure of certain connections to establish correct organization. Prenatally, this could result from alterations in migration of projection neurons or interneurons, or from dysregulated axon pathfinding, which is required to establish proper positioning and patterning of basic connectivity.

Postnatally, such disconnection could result from alterations in dendritic development, synaptogenesis, or synaptic pruning.

Consistent with the cortical disconnection hypothesis is the replicated finding that postmortem brains from patients with ASD show minicolumnar pathology (Casanova et al., 2002a; Casanova et al., 2002b; Casanova et al., 2006). Minicolumns are fundamental units of neocortical information processing that are composed of excitatory pyramidal neurons, inhibitory interneurons, and the connections both between these neurons and with neurons outside of the assembly (Courchesne and Pierce, 2005; Courchesne et al., 2005; Mountcastle, 1997). Disruptions to their normal structure may result in an impaired ability to process information from distant systems (emotional, autonomic, sensory, language, etc.) within the brain (Casanova et al., 2006; Courchesne and Pierce, 2005). Although we have important clues regarding the underlying neuropathologies in ASD, the etiologies of these alterations are not well understood.

Highly replicated genetic association of MET with ASD

Based on twin studies and recurrence rates in siblings, the heritability of ASD is estimated to be about 90% (Bailey et al., 1995; Le Couteur et al., 1996; Steffenburg et al., 1989), rendering it one of the most heritable human disorders. The genetic mechanisms contributing to ASD etiology include rare mutations, common polymorphisms, and copy number variations (CNVs), and the specific mechanism or mechanisms involved in each ASD case are likely to vary (Abrahams and Geschwind, 2008; Geschwind, 2009). Rare events, including CNVs and

syndromic disorders in which ASD is highly correlated (Rett Syndrome, for example) may account for 5-15% of cases in which the mutations are causal. However, idiopathic ASD may involve a large number of common variants that each contribute modestly to risk and may combine with environmental influences to cause the disorder. It also should be noted that both environmental and genetic factors may potentiate or reduce particular phenotypic components, contributing to disorder heterogeneity.

Although many genes have been identified to contribute to autism risk (for review see Abrahams and Geschwind, 2010), few have been successfully replicated (Buxbaum and Baron-Cohen, 2010). The association of *MET* with ASD risk is a notable exception; three single nucleotide polymorphisms (SNPs) in the 5' region of the *MET* gene have been associated with ASD, and in sum, nine independent cohorts have shown statistically significant *MET*-ASD correlations. The original report of *MET* as an ASD susceptibility gene identified a SNP, rs1858830 (G/C) in the gene's 5'-regulatory region and used family based association tests (FBAT) to determine whether one allele was preferentially transmitted from parents to ASD-affected individuals. Indeed, the C allele was over-transmitted to individuals with ASD in both the original 204 family sample ($p=0.0005$), and in the 539-family replication sample ($p = 0.001$, combined $p = 0.00005$) (Campbell et al., 2006). Using FBAT and case-control tests, the *MET* rs1858830-C association with ASD was replicated in two additional cohorts (Campbell et al., 2008; Jackson et al., 2009). Two additional *MET* SNPs, rs38845 (G/A) and rs38841 (A/G), both of which are located in intron 1, have been associated with ASD. FBAT and case control analyses revealed that the

rs38845 A allele is associated with ASD in two separate populations (Sousa et al., 2009), and a transmission disequilibrium test showed that the rs38841 A allele is overtransmitted to individuals with ASD (Thanseem et al., 2010).

Interestingly, rs1858830 is a functional variant that affects gene expression both *in vitro* and *in vivo.* In luciferase reporter assays, a G-containing *MET* promoter construct drove transcription at least two times more efficiently than a C-containing *MET* construct in three independent cell lines (Campbell et al., 2006). Electromobility shift assays demonstrated that different protein complexes bind the promoter depending on the rs1858830 allele, offering a potential mechanism for this difference in transcription rate (Campbell et al., 2006). *In vivo*, analyses of postmortem temporal neocortex revealed reduced MET protein expression in subjects with ASD. Moreover, the distribution of genotypes at this locus in control subjects showed that individuals with a C/C genotype expressed less MET relative to individuals with a G/G genotype, revealing an endophenotype that is disorder-independent. The allele distribution in ASD cases was overwhelmingly C/C, precluding a similar analysis. Although functional data has not been ascertained for the other two *MET* SNPs, the fact that they are located in intron 1, which often contains gene regulatory elements (Kleinjan et al., 2001; Lettice et al., 2003), suggests that these variants should be examined for additional involvement in controlling *Met* expression levels.

Motivation for Developmental Met Mapping Studies

Our current understanding of the roles of *Met* in neurodevelopmental processes, along with the accumulating evidence that dysregulated expression of the receptor predisposes to ASD, informed our decision to pursue a large-scale effort to map the expression of *Met* transcript and protein in the mouse forebrain throughout perinatal and postnatal development (Chapter II). We reasoned that from the perspective of basic biology, this type of neuroanatomical analysis should give insight into the *in vivo* roles for the receptor. A major mechanism for influencing neurodevelopmental processes is through modification of regulatory mechanisms through which gene expression is controlled spatially and temporally. For example, regional specification and laminar patterning in the mammalian cortex are controlled in part by the transcription factor Tbr1. Perturbations to Tbr1 result in significant alterations to the spatial and temporal expression of downstream targets, and this dysregulation yields subsequent aberrations in axon growth and pathfinding (Bedogni et al., 2010). This type of mechanism is consistent with our hypothesis of a role of MET in ASD risk: disruption to a *Met*-regulating transcription factor could result in misexpression of the receptor and subsequent effects on neurodevelopmental processes influenced by Met. The normal *in vivo* expression patterns, therefore, are important for determining the specific neurodevelopmental events that Met is likely to mediate. As noted above, while much is known about the capacity of *Met* to influence neurodevelopmental and neurophysiological processes, there remains a significant gap regarding the actual functions of the receptor *in vivo*.

This understanding is essential for connecting the clinical findings to a biological framework of disorder etiology.

TRANSCRIPTIONAL REGULATION OF MET

Known transcriptional regulators of Met

Because it is a proto-oncogene, understanding how levels of *Met* are controlled and which factors result in uncontrolled increases in expression is of paramount importance. Thus, from the context of understanding its contribution to a variety of cancers, transcriptional regulation of Met is an active area of research. At least 13 *Met*-regulating transcription factors have been identified, and are listed in Table 1. It is important to note that the data displayed here are cell context-dependent. Whether a transcription factor is relevant to *Met* transcription in another context must be determined empirically. That said, knowledge that a particular transcription factor is capable of regulating *Met* in one context provides a platform for analysis in additional cellular contexts. And, information regarding the families of transcription factors already confirmed to have a role in *Met* regulation provides useful insight for considering additional factors that might be relevant.

Transcription Factor	Effect	Species	Assays	Reference
Androgen Receptor	Repress	Human	Manipulation-Correlation, EMSA, Reporter Assay	Verras et al., 2007
Ap1	Unknown	Mouse	EMSA	Seol et al., 2000
Daxx	Repress	Mouse	Manipulation-Correlation, Reporter Assay, ChIP	Morozov et al., 2008
Ets1	Activate	Human	Manipulation-Correlation	Gambarotta et al., 1994
Macc1	Activate	Human	Manipulation-Correlation, EMSA, Reporter Assay, ChIP	Stein et al., 2009
Mitf	Activate	Human	Reporter Assay	Mascarenhas et al., 2010
p53	Activate	Mouse	EMSA, Reporter Assay	Seol et al., 1999
Pax3	Activate	Human	EMSA, Reporter assay	Epstein et al., 1996
Pax3	Activate	Human	Reporter Assay, ChIP	Mascarenhas et al., 2010
PC4	Unknown	Human	EMSA	Campbell et al., 2006
Sox10	Activate**	Human	Reporter Assay, ChIP	Mascarenhas et al., 2010
Sp1	Block Ap1	Mouse	EMSA	Seol et al., 2000
Sp1	Activate	Mouse	EMSA, Reporter Assay	Zhang et al., 2003
Sp1	Unknown	Human	EMSA	Campbell et al., 2006
Sp3	Block Ap1	Mouse	EMSA	Seol et al., 2000
Sp3	Activate	Mouse	EMSA, reporter assay	Zhang et al., 2003
TFE3	Activate	Human	Manipulation-Correlation, Reporter Assay, ChIP	Tsuda et al., 2007

Table 1: *Met*-regulating Transcription Factors

* Manipulation-Correlation indicates that the transcription factor was under- or over-expressed, and *Met* expression was subsequently measured.

* ChIP – chromatin immunoprecipitation assay

* EMSA – electromobility shift assay

** Sox10 was shown to activate *Met* expression if Pax3 and/or Mitf were also expressed

SPECIFICITY FACTOR 4 (SP4)

Sp Transcription Factor Family: Discovery and Structure

The findings in the initial *MET*-ASD genetic study revealed the possibility that the rs1858830 risk allele disrupts binding of an Sp1 family transcription factor (Campbell et al., 2006). The Sp/KLF transcription factor family comprises a set of transcription factors whose DNA-binding domains consist of three Cys2-His2 zinc fingers that share at least 65% identity and have significantly overlapping binding specificities. The Sp-family has nine known members (Sp1-Sp9), which show particular affinity for the GC-box, 5'-GGGGCGGGG-3' and related DNA sequences, and can be further subdivided into Sp1-4, which contain an N-terminal glutamine-rich transactivation domain, and Sp5-9, which do not (reviewed in Bouwman and Philipsen, 2002; Suske, 1999; Wierstra, 2008). The archetypal family member, Sp1, was identified as a component in HeLa cell extract necessary for *in vitro* transcription of the SV40 polyomavirus promoter (Dynan and Tjian, 1983). Sp2 and Sp3 were later identified in a screen of a human cDNA library for factors binding to an Sp1 zinc finger domain probe (Kingsley and Winoto, 1992). Sp3 was independently identified, along with Sp4, in a screen designed to identify factors binding to a known Sp1 target within the rabbit uteroglobin promoter (Hagen et al., 1992). Electromobility shift assays demonstrated that Sp1, Sp3, and Sp4 bind to the same GC-box target sequence with equal affinity (Hagen et al., 1992), whereas Sp2 does not bind that sequence, and instead shows preference for a GT-box (Kingsley and Winoto, 1992). This difference in affinity may be mediated by a histidine-to-

leucine substitution in the first zinc finger domain within a trio of amino acids predicted to be those that bind DNA directly (Fairall et al., 1993; Pavletich and Pabo, 1991; Suske, 1999).

Sp4 in the brain

Sp1 and Sp3 are widely considered to be ubiquitously expressed, whereas Sp4 is enriched in the brain, heart, reproductive system, and some epithelial tissue (Hagen et al., 1992; Supp et al., 1996). In the brain, Sp1 and Sp3 are indeed expressed, though expression is not as general as is often presumed. *In situ* hybridization studies in our laboratory indicate that Sp1 is restricted in expression from mid-embryonic development through adolescence (E12.5 – P35), showing robust expression in the ventricular zone, with relatively limited expression elsewhere in the forebrain (Bergman and Levitt, unpublished observations). Moreover, studies in the adult rat hippocampus and frontal cerebral cortex demonstrate that Sp1 expression is primarily detected in astrocytes, whereas Sp4 expression is localized to neurons, and Sp3 is found in both populations (Mao et al., 2007; Mao et al., 2009).

Constitutive knockout of Sp4 yields viable mice, though two thirds of the offspring die by P28, of which half die by P10. Additionally, the knockout animals show growth retardation for the first four weeks of life, both overall and in specific organs including the thymus, spleen, testis, and uterus. Both sexes exhibit deficits in reproduction; male knockout animals do not breed, and female animals show

delayed onset of puberty (Göllner et al., 2001). There are no reports of brain or neuronal phenotype assessment in these animals.

To study the role of Sp4 in the brain *in vivo*, Zhou et al. (2005) generated an Sp4 hypomorphic mouse in which Met protein level is reduced to about 3-5% wild-type levels in all cells. These mice show severe hippocampal phenotypes, both morphologically and behaviorally. Structurally, vacuoles are observed in the CA3 region of the hippocampus by two months of age, and similar vacuoles are observed in the hilus of the dentate gyrus (DG) and the stratum oriens by four months of age (Zhou et al., 2005). The DG also shows decreased cell proliferation at early postnatal stages (e.g. P7), resulting in decreased DG cell density and reduced DG width in the adult hippocampus (Zhou et al., 2007). As expected, the Sp4 hypomorphic mice show deficits in hippocampus-driven learning and memory behavior as well. At a cellular level, these mice show impairment in long term potentiation (LTP), and behaviorally, a deficit in spatial memory is suggested based on their performance on the Barnes maze, in which they demonstrate increased error rate and escape latency times relative to wild-type littermates (Zhou et al., 2010).

In vitro, two groups have assessed the role of Sp4 in dendritic arborization. In cerebellar granule cells in culture, Sp4 contributes to dendrite maturation by promoting pruning. Following transfection of RNAi targeting Sp4, dendrites in these cultures show excessive and persistent branching, whereas overexpression of Sp4 promotes pruning (Ramos et al., 2007). Interestingly, in cultured hippocampal DG cells, the opposite effect was observed; dendrites on neurons cultured from Sp4

hypomorphic mice were reduced in length, number, and branching relative to those cultured from wild-type controls, suggesting that Sp4 *promotes* dendritic growth and branching. Interestingly, both groups identified neurotrophin-3 (NT-3) as a transcriptional target of Sp4 relevant to the observed dendritic phenotype. In the cerebellar granule cell cultures, RNAi-mediated knockdown of Sp4 resulted in increased NT-3 expression (Ramos et al., 2009), whereas in the hippocampal cultures, Sp4 hypomorph-derived DG cells showed decreased NT-3 expression. Thus, Sp4 appeared to repress NT-3 expression in the cerebellar system and to activate NT-3 expression in the hippocampal system, but between the two systems, the correlation between NT-3 expression and dendritic phenotype was constant. The observation that the effect of Sp4 on NT-3 varies by cellular context underscores the importance of the transcription factor milieu in affecting the downstream effect of Sp4 on a particular target.

Sp4 in human disease

To date, Sp4 has been linked to multiple neuropsychiatric disorders, including bipolar disorder, schizophrenia, major depressive disorder (MDD), and Alzheimer's disease. Using transmission disequilibrium tests, Zhou et al. (2009) demonstrated that the A allele in SNP rs12673091 within Sp4 intron 5 is overtransmitted to individuals with bipolar disorder in a Caucasian population, and replicated the result using case-control analysis in a Chinese population. Interestingly, an association of this allele was additionally seen in a set of 325 Chinese trio families with schizophrenia (Zhou et al., 2009). Two other studies have

implicated Sp4 in schizophrenia risk. Using data from the International Schizophrenia Consortium (ISC), Zhou et al. (2010) analyzed 3391 cases and 3181 controls for the presence of copy number variations (CNVs) in the *SP4* gene. While no deletions or duplications were found in the controls, two deletions were discovered in the cases, both of which excised exons 1-3 and a segment of the gene's 5' regulatory region. Tam et al. (2010) also reported evidence of CNVs in the *SP4* gene in individuals with schizophrenia. Beginning with a Scottish population of 91 schizophrenia cases and 92 controls, they identified a deletion in *SP4*, which was validated by qPCR. To cross validate, the authors scanned the ISC and found two additional deletions. One might suspect, however, that these were the same deletions identified by Zhou et al. Evidence of a genetic link between Sp4 and MDD comes from a genome-wide association study comparing SNP frequencies in a population of individuals with recurrent early-onset MDD to controls, and from a meta-analysis of three similar studies (Shi et al., 2010; Shyn et al., 2009). The association of Sp4 with Alzheimer's disease was not based on genetics, but rather on postmortem analyses. The level of neuronal Sp4 expression was increased in CA1 and entorhinal cortex in individuals with Alzheimer's disease, particularly in those cells expressing the pathognomonic neurofibrilliary tangles (Boutillier et al., 2007).

That one transcription factor, which is likely to regulate many genes, may play a role in multiple neuropsychiatric disorders is unsurprising. There is significant overlap in the neurodevelopmental and neurophysiological processes that are disrupted in schizophrenia, bipolar and autism (Carroll and Owen, 2009; Levitt et al., 2004), and the differences in disorder manifestation are likely to result

from the timing and location of the disruption, along with the presence or absence of co-occurring insults and genetic and environmental modifiers.

The following chapters report data that attempt to provide initial insight regarding the specificity of *Met* gene and protein expression in the mouse, and a first attempt at deciphering what may be complex mechanisms for *Met* transcriptional regulation. We used a variety of methods to generate candidates that will be followed up in future studies. In some instances, the requisite validations were initiated in the context of this thesis, but remain to be proven unequivocally. This will be discussed in the final chapter, 'Future Directions.'

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