TRANSLATIONAL INVESTIGATIONS OF GASTROINTESTINAL COMORBIDITIES IN CHILDREN WITH AUTISM

By

Phillip Gorrindo

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

December, 2011

Nashville, Tennessee

Approved:

Nancy Brown, M.D.

Pat Levitt, Ph.D.

Karoly Mirnics, M.D.

James Sutcliffe, Ph.D.

ACKNOWLEDGEMENTS

This work was supported by NICHD grant R21HD065289, and in part by NIMH grant R01MH067842, NIGMS grant T32GM07347 for the Vanderbilt Medical-Scientist Training Program, NCRR grant TL1RR024978, the Vanderbilt Clinical and Translational Science Award UL1RR024975 from NCRR, and the Marino Autism Research Institute.

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CHAPTER I

INTRODUCTION

Biological and Phenotypic Complexity in Autism Spectrum Disorders

Autism is behaviorally defined by severe deficits in some of our most human abilities: social understanding and engagement, an elaborate repertoire of language and communication skills, and flexibility of thought, behavior and interests (Volkmar, Lord, Bailey, Schultz, & Klin, 2004). In an original and seminal case series in 1943, Leo Kanner first reported on a collection of impairments in 11 children, describing what he termed “autistic disturbances of affective contact” (Kanner, 1943). Kanner’s rich descriptions of these children included both the common triad of impairments that now define autism spectrum disorders (ASDs) — such as a failure to develop appropriate social relationships with caregivers or peers, profoundly affected language abilities including abject mutism, echolalia, literalness, and first-/third-person pronoun reversal, and an insistence on sameness manifest as strong aversion to changes in daily routines — as well as idiosyncratic complexity in each child, with each case presenting, in his words, “individual differences in the degree of their disturbance, the manifestation of specific features, the family constellation, and the step-by-step development in the course of years.”

In the seven decades since Kanner's original report, our understanding of ASDs as neurodevelopmental disorders has grown a great deal. As defined by the 4th Edition of the *Diagnostic and Statistical Manual of Mental Disorders*, Pervasive Developmental Disorders include Autistic Disorder, Asperger's Syndrome (AS), Pervasive Developmental Disorder — Not Otherwise Specified (PDD-NOS), Rett's Syndrome, and Childhood Disintegrative Disorder (American Psychiatric Association, 1994). ASDs in particular, however, are the focus of this thesis and include only autism, AS and PDD-NOS. Current estimates of prevalence of ASDs are 0.9 to 1.1% in the US (Autism and Developmental Disabilities Monitoring Network, 2009; Kogan et al., 2009), and a recent report found a prevalence of 2.6% in a South Korean community (Kim et al., 2011) — placing ASDs among the most common neuropsychiatric disorders worldwide. The annual societal cost for caring with individuals with ASDs in the US was estimated to be $34.8 billion (including medical and non-medical needs, and lost productivity; using 2003 dollars and prevalence data) (Ganz, 2006); extrapolating to contemporary prevalence estimates (i.e., 1.1% (Kogan, et al., 2009)) suggests the cost to in fact be near $139 billion (in 2003 dollars) each year. As a lifelong condition, these costs are relatively consistent from childhood through adulthood (Ganz, 2007). Heritability of ASDs has been estimated at approximately 92% (Bailey et al., 1995), strongly implicating genetic risk as a primary etiology. The advent of ASDs-specific diagnostic instruments with high sensitivity, specificity, and both inter-rater and test-retest reliability (the Autism Diagnostic Observation

Schedule, ADOS (Lord et al., 1989) and the Autism Diagnostic Interview — Revised, ADI-R (Lord, Rutter, & Le Couteur, 1994)), highlights the homogeneity of the core triad of ASD impairments. Although individuals with ASDs, as a spectrum, vary in degrees of impairment (Volkmar & Pauls, 2003), the core features remain true to Kanner's original description.

Yet Kanner's appreciation of individual differences in his original case series has proven prophetic, for our modern, nuanced understanding of ASDs reveals a far more complex and heterogeneous disorder. In search of biomarkers and endophenotypes, a number of studies have reported various neuro-centric findings. Macroscopic brain over-growth in the first years of life, measured grossly by head circumference or in more neuroanatomical detail with structural magnetic resonance imaging (MRI), showed group effects of ASD enlargement relative to controls, but importantly, a variable distribution within individuals with ASDs such that some affected individuals were within the normative range (Courchesne, Carper, & Akshoomoff, 2003; Hazlett et al., 2005). Macrocephaly, defined by head circumference greater than the age- and sex-normed 97th percentile, has been reported in approximately 15 to 17% of individuals with ASDs (Fombonne, Rogé, Claverie, Courty, & Frémolle, 1999; Lainhart et al., 2006). Functional MRI studies showed focal hypoactivation of the "fusiform face area" (i.e. the fusiform gyrus of the ventral temporal lobe) in passive viewing tasks of static faces by individuals with ASDs compared to controls (Schultz et al., 2000), although interestingly more recent findings suggest only for unknown

(stranger) faces but not familiar (caretaker) faces (Pierce & Redcay, 2008). Looking at the long-range functional connectivity of different regions, numerous studies have shown hypoconnectivity across brains of individuals with ASDs (Geschwind & Levitt, 2007; Just, Cherkassky, Keller, & Minshew, 2004). For example, resting-state, interhemispheric-correlated activity is decreased in individuals with ASDs compared to controls, in ASDs-relevant regions, including the fusiform gyrus and anterior insula (Anderson et al., 2011). These functional data are supported by structural data, such as reduced volume and fractional anisotropy (measured by diffusion tensor imaging) of the corpus callosum in some individuals with ASDs (Alexander et al., 2007). Abnormalities at the microscopic level have also been reported in ASDs. Although inherently limited by small sample sizes, multiple reports have shown abnormalities in the post-mortem cytoarchitecture of neocortical minicolumns, including increased number, decreased size and altered spacing, suggesting local hyperconnectivity in the ASD brain (Casanova, Buxhoeveden, Switala, & Roy, 2002; Casanova, El-Baz, Vanbogaert, Narahari, & Switala, 2010; Casanova et al., 2006). This seeming contradiction of simultaneous hyper- and hypoconnectivity in the ASD brain has been synthesized in unifying hypotheses regarding brain dysfunction in the ASDs, hypothesizing that local and overactive networks become disconnected from distant cortical areas which would, in individuals without ASDs, allow for higher order integration of information (Casanova & Trippe, 2009; Geschwind & Levitt, 2007). Biomarkers for ASDs have also been examined outside of the

brain. Hyperserotonemia has been reported in approximately 35 to 60% of individuals with ASDs (Cook & Leventhal, 1996; Mulder et al., 2004). Various genetic findings (discussed in detail below) have been increasingly reported in recent years. Finally, the preponderance of males (a male-to-female ratio of 4.3:1) affected with ASDs is perhaps the oldest — and yet, still unexplained — biomarker in ASDs(Fombonne, 2003).

The biological complexity of ASDs is met with equally vexing clinical and behavioral heterogeneity (Geschwind, 2009; Gillberg & Billstedt, 2000; Levy, Mandell, & Schultz, 2009). ASDs vary in degrees and domains of impairment. For example, individuals with AS may have no intellectual disability, intact functional language capacities, and good outcomes; whereas greater than 50% of individuals with prototypical autism have intellectual disability, are often nonverbal, and have poor outcomes (Volkmar & Pauls, 2003). In fact, there is evidence that the triad of impairments fractionate along heritability, with each core deficit being, by itself, highly heritable, but correlating only modestly with each of the other two deficits (i.e., pairwise correlation coefficients ranging from 0.23 to 0.38) (Ronald et al., 2006). Individuals with ASDs are often diagnosed with co-occurring mental health and behavioral disorders (Levy et al., 2010), including anxiety (42% (Simonoff et al., 2008)), aggression (22% (Hartley, Sikora, & McCoy, 2008), and obsessive-compulsive disorder (37% (Leyfer et al., 2006)). High prevalences of various medical comorbidities have also been reported in ASDs (Boulet, Boyle, & Schieve, 2009; Gurney, McPheeters, & Davis, 2006).

Seizure disorders have been reported in 21% of individuals with ASDs, a significant increase compared to a non-ASD population (Volkmar & Nelson, 1990), with some estimates ranging as high as 38% prevalence (reviewed in (Tuchman & Rapin, 2002)). Sleep problems have been reported in 53% of children with ASDs (Krakowiak, Goodlin-Jones, Hertz-Picciotto, Croen, & Hansen, 2008; Malow et al., 2006). Gastrointestinal problems have also been reported in some individuals with ASDs, discussed in depth below.

The preceding survey of biological and phenotypic heterogeneity serves to illustrate a key, foundational point: individuals with ASDs are highly complex, and this complexity has important implications. Aside from the triad of core impairments (which, by definition, must be present for a diagnosis of an ASD), no other feature is invariant and completely penetrant in all individuals with ASDs. This heterogeneity has ramifications for the diagnosis and treatment of individuals with ASDs (Bauman, 2010b). It has been reported that children with co-occurring medical conditions and ASDs are diagnosed later than children with only an ASD diagnosis, suggesting that co-occurring conditions can mask core impairments and delay accurate ASD diagnosis (Levy, et al., 2010). Moreover, this heterogeneity has hindered efforts to understand the genetic basis of risk for ASDs, discussed below.

Pleiotropy — defined as a single genetic element affecting multiple phenotypes — is one possible explanation for this heterogeneity (Bill & Geschwind, 2009). Genes can be expressed in diverse cells and tissues across

an organism, at different times in those tissues during development, and in response to different stimuli in a cell-specific manner. A pathogenic or susceptibility genetic variant, therefore, can affect multiple organ systems in a single individual (i.e., the *DSCR1* gene is implicated in both congenital heart defects and Down Syndrome (Fuentes et al., 1995)). An appreciation of this simple fact affords an opportunity to gain insight into the ASDs: by better understanding the complexity of ASDs, heterogeneity can be leveraged for benefit. It has been proposed that genetic variants conferring risk for ASDs are enriched in populations stratified by pleiotropic phenotypes (Campbell et al., 2009). Investigating biological differences in stratified populations can therefore enable insight into ASD risk and pathogenesis. With a richer understanding of such differences, a natural extension is an appreciation for — and hopefully insight into — individualized strategies for clinical care. As a case study for this paradigm, the work reported in this thesis focuses on co-occurring gastrointestinal dysfunction (GID) in ASDs. The remainder of this chapter will proceed with a survey of the genetics of ASDs, highlighting progress and challenges. Then the focus will turn to one particular ASD susceptibility candidate gene, *MET*, examining supporting evidence for its role in risk for ASDs, as well as GID. Finally, this chapter will conclude with an in-depth description of GID in ASDs, returning to the clinical aspects of this disorder, and providing the keystone to the compelling rationale for undertaking this thesis.

The Genetics of Autism Spectrum Disorders: Progress and Challenges

As a profound, prevalent and highly heritable condition, the interrogation of the genetics of ASDs has a rich history. Classic studies of concordance for ASDs in monozygotic (MZ) compared to dizygotic (DZ) twins established the strong heritability of the disorders. Using a less-stringent definition of ASDs (including broader cognitive and social deficits), DZ twin pairs were 10% concordant for diagnoses, compared to 92% of MZ pairs; using a strict definition of autism, 60% of MZ pairs compared to 0% of DZ pairs were concordant (Bailey, et al., 1995; Folstein & Rutter, 1977). Sibling recurrence risk for autism is high, reported at 4.5% (Jorde et al., 1991). Building on this foundation, numerous studies in recent years have implicated a variety of genetic insults contributing to risk for ASDs (Abrahams & Geschwind, 2008).

However, before moving to an examination of more recent genetic findings in the ASDs, a discussion of the two major, prevailing hypotheses regarding the genetic architecture of ASDs must be elaborated to provide necessary context. The two hypotheses that serve as framework for ASD risk and pathogenesis, which are neither necessarily mutually exclusive nor specific to ASDs, are the Common Variant-Common Disease (CVCD) and the Rare Variant-Common Disease (RVCD) hypotheses (El-Fishawy & Matthew W State MD, 2010). The CVCD hypothesis proposes that multiple common genetic variants (greater than 1% prevalence in a population), each with small effect sizes, interact in an oligogenic manner to contribute to disease risk (Chakravarti, 1999; Reich &

Lander, 2001). The out-of-Africa theory of evolution is key to the CVCD hypothesis, for the theory proposes that a relatively small population of ancestral humans (on the order of 10,000 individuals) rapidly grew into the current world population over a relatively brief evolutionary period (on the order of 100,000 years). In this ancestral population, the small size presumably allowed for only a small diversity of variants at any genetic locus, and therefore disease alleles for common diseases were common, whereas disease alleles for rare diseases were rare. With a rapid population expansion, although new mutations were introduced into the larger population's collective genome, the original relative frequencies (common versus rare) were maintained during expansion, leaving common alleles as the prime source of variation for common diseases in the current human population. A secondary hypothesis consistent with the CVCD hypothesis is that a sub-threshold number of disease-associated common variants will be present in family members of probands, with subclinical manifestations of disease in these family members. Studies on the broad autism phenotype (BAP (Piven, 2001)) support such a hypothesis, for example reporting increased social and communication impairment in the undiagnosed parents and siblings of probands (Bishop et al., 2004; Constantino et al., 2006).

In contrast, the RVCD hypothesis posits that rare polymorphisms (less than 1% population prevalence), each with much larger effects than is seen in the CVCD hypothesis, are the predominant contributors to disease risk. A primary rationale for the RVCD hypothesis is that early-onset disorders (such as the

ASDs) which negatively affect an individual's reproductive fitness (i.e., social, communicative, and often cognitive, impairments in the ASDs) would be subject to negative selection over evolutionary timescales, therefore only allowing such deleterious variants to exist, maximally, at low frequency in a population (El-Fishawy & Matthew W State MD, 2010). Rare variants can be inherited or occur *de novo*, and recent reports (discussed below) provide support for both categories. Additional support for the RVCD hypothesis comes from, unexpectedly, reports that used the CVCD hypothesis as an intellectual foundation. Recent studies in search of common variants have found relatively few significant findings, with modest effect sizes and explaining a small fraction of disease variability, suggesting that common variants may not play a large role in risk, and thus implicating, indirectly, rare variants. However, although the two hypotheses represent contrasting models of allelic architecture, one hypothesis does not necessarily preclude the other's existence. While a recent report stated that "the hypothesis that autism results from an unfortunate combination of common low-risk variants can be safely rejected," this conclusion seems myopic (Levy et al., 2011). Instead, a more balanced view is one which appreciates biology's complexity, and seeks to gain insight into disease by integrating findings grounded in both CVCD and RVCD hypotheses, transcending the historical dichotomy (and sometimes antagonism (Levy, et al., 2011)) (Iyengar & Elston, 2007). Additionally, an implicit concept in this discussion of genetic risk for complex disorders is that genes do not operate in a biological vacuum;

epistatic, epigenetic, experiential, environmental, and stochastic modifiers can all exert significant effects.

In recent years, multiple genome-wide association (GWA) studies of ASDs, explicitly designed to test the CVCD hypothesis, have been reported. One allele of a single nucleotide polymorphism (SNP) in a 300 kilobase (kb) intronic region of the MACRO domain containing 2 (*MACROD2*) gene on chromosome 20p12.1 was reported as significantly associated with an ASD diagnosis (Anney et al., 2010). Other variants on chromosome 5p15.2, approximately 80 kb upstream from the semaphorin 5A (*SEMA5A*) gene*,* were also reported (Weiss, Arking, Daly, & Chakravarti, 2009). Another report implicated chromosome 5p14.1, with loci in a 2.2 megabase (Mb) intergenic region between the cadherin 10 (*CDH10*) and cadherin 9 (*CDH9*) genes (Wang et al., 2009). The 5p14.1 association was reported again by the same group (Ma et al., 2009). Interestingly, a subsequent report based on the 5p14.1 findings showed significant association of the rs4307059 risk allele with lower social communication functionality in a general population sample, consistent with the CVCD hypothesis (St Pourcain et al., 2010).

In a critical appraisal of these GWA studies, several points are worth noting. Although the 5p14.1 findings were initially replicated, subsequent studies have failed to replicate the associations with 5p14.1, 20p12.1 or 5p15.2. The loci implicated are often presented without rigorous empirical data that clarifies the biological roles or implications of the disease-associated variant, leaving a void

where a reader would hope to find a biological context that could ground further studies of the locus. Instead, the nearest annotated gene is reported, guilty-by-proximity. In these reports, it remains to be demonstrated that a locus 80, 300, or 2,200 kb away from an exon of a nearby gene can have an effect on that particular gene's transcription or function, with concomitant biological implications for the resulting disease. With thousands of subjects in these GWA studies, only a small number of loci, with modest effect sizes (ranging from 1.2 to 1.9 (Anney, et al., 2010; Ma, et al., 2009; Wang, et al., 2009; Weiss, et al., 2009)), have been identified. These criticisms, however, are opportunities for improvement in future studies and highlights of the difficulties inherent in GWA studies. Although modest, the findings are presumably valid, and consistent with some contribution of common variants to risk for ASDs. As unbiased, non-hypothesis-driven investigations, they serve as starting points for further studies that may reveal aspects of the pathobiology of ASDs.

Additional insight into risk for and pathogenesis of the ASDs has also come from study of rare variants. One approach to studying low frequency genetic insults is to focus on monogenic disorders with large phenotypic overlap with ASDs. In these rare disorders, such as fragile X syndrome (FXS) and tuberous sclerosis (TSC), as high as 25% of individuals with the syndrome also have a diagnosable ASD (Muhle, Trentacoste, & Rapin, 2004). These disorders are rare (approximately 1 in 6,000 for TSC (O'Callaghan, Shiell, Osborne, & Martyn, 1998) and 1 in 2,500 for FXS (Crawford et al., 2002)), and each disorder

accounts for only 1-2% of ASD cases (Abrahams & Geschwind, 2008). Although some have expressed concern regarding the relevance of cases of so-called syndromic ASDs to the understanding of core pathogenesis in non-syndromic, idiopathic ASDs (particularly in the context of severe intellectual disability) (Moss & Howlin, 2009), as highlighted below, elucidating the genes involved (*FMR1* for FXS; *TSC1* and *TSC2* for TSC) in these monogenic disorders may provide insight into molecular mechanisms of pathogenesis in the ASDs.

Other rare variant findings are also important contributions to understanding the genetics of ASDs. Maternal duplications of the 15q11-15q13 chromosomal region, with the maternally-expressed *UBE3A* gene thought to be critically involved, are common cytogenetic and chromosomal microarray findings in the ASDs (Abrahams & Geschwind, 2008; Cook et al., 1997; Matsuura et al., 1997). Deletions involving 22q13 have also been reported, with the *SHANK3* gene now implicated in pathogenesis (Durand et al., 2007; Manning et al., 2004; Moessner et al., 2007). Other studies have reported major effects of rare mutations in the *NLGN3*, *NLGN4*, and *NRXN1* genes (Feng et al., 2006; Jamain et al., 2003). Although each of these rare sequence and structural abnormalities are present in no more than 1 to 2 % of individuals with ASDs, collectively they account for an estimated total of 15% of cases (Abrahams & Geschwind, 2010).

The field has recently focused on larger structural variants (greater than 1,000 bp, including both gains and losses) across the genome, collectively referred to as copy-number variants (CNVs) (Merikangas, Corvin, & Gallagher, 2009).

Noteworthy findings include CNVs at 16p11.2, initially reported to account for approximately 1% of cases of ASD (Kumar et al., 2008; Weiss et al., 2008). In a recent meta-analysis of seven studies of 16p11.2 in the ASDs, prevalence was found to be a more modest 0.76% (with deletions approximately two-fold more common than duplications at the locus) (Walsh & Bracken, 2011). Other studies have replicated some genes and regions that were already implicated in risk for ASD, including *UBE3A*, *NRXN1*, and 16p11.2 (Glessner et al., 2009), and 15q11-13 (Christian et al., 2008), while also suggesting novel loci to be further studied. Some research groups have developed a particular focus on *de novo*, and thus presumed pathogenic, CNVs. Multiple studies have reported increased frequency of *de novo* CNVs in simplex (7-10%; only one child affected with an ASD) compared to multiplex (2-3%; more than one child affected with an ASD) families, or compared to controls (1%) (Marshall et al., 2008; Pinto et al., 2010; Sebat et al., 2007). This enrichment of signal in simplex families served as a strong rationale for focusing exclusively on simplex families in larger cohorts and with state-of-the-art assay arrays, as two recent reports did (Levy, et al., 2011; Sanders et al., 2011). As the most current CNV data published, these two studies suggest a range of 100 to 400 genes and loci are implicated in risk and pathogenesis for ASDs, and account for approximately 5% of simplex cases. However, as a still-developing method of inquiry, the CNV literature has unresolved issues of specificity and presumed pathogenicity. Otherwise normal and unaffected individuals have, on average, 11 CNVs each (Sebat et al., 2004),

strongly suggesting that presence of a CNV is, by itself, insufficient for causing disease. Moreover, association between a recurrent CNV and ASDs as a specific phenotype, is variable. For example, 16p11.2 deletions have been reported to also be associated with obesity and intellectual disability, irrespective of social disability (Walters et al., 2010).

Although the field has learned a great deal about genetic susceptibility for ASDs, two conspicuous points persist, with particular relevance to case-control studies of association. Namely, the vast majority of idiopathic cases of disease remain unexplained (by neither putatively pathogenic *de novo* mutations, rare mutations of large effect, nor monogenic insults) (Schaaf & Zoghbi, 2011), and large consortia-based studies have often not replicated previous findings for genome-wide analyses (Anney, et al., 2010; Weiss, et al., 2009). Pointing to underlying genetic heterogeneity as the root cause, calls for larger samples sizes in response — to detect even lower frequency rare variants, and common variants with even smaller effect sizes — have been made (Anney, et al., 2010; Marshall, et al., 2008; Wang, et al., 2009). However, although the phenotypic heterogeneity of ASDs is often mentioned in reports of genetic studies, curiously, it is mentioned seemingly only as a formality, without giving the concept much attention in the study (Wang, et al., 2009). As a field, it is an improvement to have consensus that gold standard diagnostic instruments (i.e., ADOS and/or ADI-R) must be used to confirm a categorical diagnosis of an ASD, facilitating inter- and intra-study comparisons of ASD case status. However, by including all subjects

meeting ASD categorical criteria, and given the deep heterogeneity of ASDs, genetic studies of association are, possibly, diluting disease-associated signal, as that signal may only be present in a subset of the study's entire population. For example, in a recent GWA study, a significant association was found for rs4141463 on chromosome 20p12.1 (Anney, et al., 2010). However, this association was only significant when considering cases that met strict criteria for ASD diagnosis and were of European ancestry ($P = 2.10 \times 10^{-8}$; i.e., meeting the conventional Bonferroni-corrected GWA threshold for significance of $P < 5 \times 10^{-8}$). For all cases, including those meeting a less strict diagnosis for the broader autism spectrum, and those of all ancestries, this locus was no longer significant ($P = 4.30 \times 10^{-5}$). Unfortunately, the current practice is to report minimal phenotypic information on study subjects (and, when more detailed information is offered, it is relegated to a publication's online supplement, seemingly as an afterthought (Marshall, et al., 2008; Wang, et al., 2009)). Phenotype is not supplemental; phenotype is substantive. However, hopefully representative of the field's evolving priorities, a recent report does look at relationships between *de novo* CNVs and more nuanced phenotypes beyond categorical ASD diagnoses, such as IQ and externalizing behaviors (Sanders, et al., 2011). Broad support for, and NIH-mandated compliance with, data-sharing in the National Database for Autism Research (NDAR, (National Institute of Mental Health)) may help move the field in a positive direction, as granular phenotype data will be available to investigators through this centralized database and enable follow-up phenotype

analyses even if such analyses were not included in original reports. As the field quickly progresses towards exome (O'Roak et al., 2011) and whole genome sequencing, there is an opportunity to move beyond the challenges of categorical diagnoses in ASDs, by leveraging phenotypic differences in stratified subpopulations to enrich for, potentially, a more genetically homogenous subset of individuals. The calls for larger sample sizes, therefore, may in fact prove useful, as they will allow for stratifying of populations based on phenotype.

In a broader view, the variety of loci now implicated in the ASDs — common and rare, single sequence variants and larger CNVs, *de novo* and inherited — suggest an emerging, central hypothesis for risk and pathogenesis. Proteins of several of the loci implicated in the ASDs, including *NLGN3*, *NLGN4*, *NRXN1*, and *SHANK3*, share a common neuroanatomical geography: the excitatory synapse (Gilman et al., 2011; Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011; Zoghbi, 2003). Extending the model from synapse structure genes to downstream intracellular signaling molecules, the PI3 Kinase-AKT pathway is further implicated, which integrates the monogenic, syndromic ASD genetic defects (such as *FMR1*, *TSC1* and *TSC2*, *NF1*, and *PTEN*) (Bill & Geschwind, 2009; Levitt & Campbell, 2009). As discussed in the following section, a key player involved in upstream signaling of this pathway, MET, is a receptor tyrosine kinase with multiple, convergent lines of evidence in support of its role in ASD susceptibility (Judson, Eagleson, & Levitt, 2011).

The Genetics of Autism Spectrum Disorders: Focus on MET Signaling

An association between a common polymorphism in the *MET* gene, and risk for developing an ASD, was first reported in 2006 (Campbell et al., 2006). The reasons for first investigating *MET* as an ASD candidate risk gene were numerous. A broad linkage peak on chromosome 7q — encompassing many genes, including *MET* — had been reported multiple times (International Molecular Genetic Study of Autism Consortium, 1998, 2001; Lamb et al., 2005; Philippe et al., 1999; Schellenberg et al., 2006). At the time, although MET signaling was primarily known to be involved in the biology of cancer due to its original discovery as a proto-oncogene (Cooper et al., 1984; Trusolino, Bertotti, & Comoglio, 2010), there was a growing literature which implicated MET signaling in neurodevelopment. Using mouse models and cell culture assays (in which the mouse form is indicated by Met instead of MET), multiple studies had demonstrated *in vitro* evidence of Met signaling's involvement in both axonal and dendritic outgrowth of cultured neurons (Gutierrez, Dolcet, Tolcos, & Davies, 2004; Powell, Mühlfriedel, Bolz, & Levitt, 2003), as well as regulation and functioning of synapses (Akimoto et al., 2004; Tyndall & Walikonis, 2006). Moreover, it was also reported that Met signaling increased motility of cells migrating out of the ganglionic eminence en route to the neocortex (Powell, Mars, & Levitt, 2001). Implicating Met signaling in interneuron migration, and thus potentially the overall inhibitory tone of the developed cortex, was consistent with hypotheses of imbalanced excitation-inhibition as a possible etiology of the ASDs

(Casanova, Buxhoeveden, & Gomez, 2003; Levitt, Eagleson, & Powell, 2004; Rubenstein & Merzenich, 2003). Although at the time this was an additional rationale for exploring *MET* as an ASD candidate gene, more recent findings, it should be noted, have suggested that in fact Met signaling does not have a direct effect on interneuron motility *in vivo*, and that initial findings reported in 2001 were possibly confounded by ectopic expression of Met induced by cell culture conditions (Eagleson, Campbell, Thompson, Bergman, & Levitt, 2010). Finally, beyond the central nervous system, Met signaling is also implicated in immune and gastrointestinal processes, abnormalities of which are both reported in the ASDs (Beilmann et al., 1997; Beilmann, Vande Woude, Dienes, & Schirmacher, 2000; Numata et al., 2005; Tahara et al., 2003). The role of Met in gastrointestinal function, as well as gastrointestinal issues in ASDs, will be discussed below in greater detail.

Since the original report in 2006 of association between a functional common polymorphism in *MET* and risk for ASDs, additional findings on the neurobiological roles played by Met have corroborated the initial rationale and hypothesis of MET signaling playing an important role in the ASDs (reviewed in (Judson, et al., 2011)). Comprehensive and detailed neuroanatomical mapping studies of Met expression during neurodevelopment suggest Met signaling is involved in the development of forebrain and limbic circuits involved in various aspects of social and emotional behavior and which are thought to play important roles in the etiology of the ASDs. In the mouse, *Met* mRNA transcript is detected

in neocortical projection neurons of cingulate, prefrontal, temporal and sensory cortices and traditional limbic structures such as the amygdala, hippocampus, and septum, whereas protein is detected in axon fiber tracts connecting those regions (such as the cingulum, fornix, and internal capsule) (Judson, Bergman, Campbell, Eagleson, & Levitt, 2009). Moreover, peak expression of Met is during the second postnatal week in the mouse, which corresponds to extensive neurite outgrowth and synaptogenesis, suggesting Met signaling plays important roles in developing appropriate wiring connections in these circuits (Judson, et al., 2009). A subsequent study comparing Met expression in the developing mouse to MET expression in the developing rhesus macaque brain provides additional support for the receptor's roles in development of particular circuits. The timing of MET protein expression was found to be highly conserved between mouse and macaque, with the primate expression also coinciding with synaptogenesis (Judson, Amaral, & Levitt, 2010a). Although expression in limbic structures was also conserved, there was a difference in neocortical expression between mouse and macaque, with restricted expression of MET in cortical areas of species-specific relevance (such as the posterior cingulate, inferior temporal, posterior parietal and visual cortices) (Judson, et al., 2010a). Although divergent, these findings are consistent with the hypothesis that MET signaling is involved in the ontogeny of limbic and neocortical circuits implicated in species-specific communication and social behavior. Along with developmental expression studies that increased understanding of the normative roles played by Met/MET

signaling, there have also been reports of the implications of a specific loss of Met signaling in a mutant model system. In Met conditional null mice (in which the kinase domain, critical for proper receptor signaling, is ablated specifically in forebrain excitatory neurons arising from the dorsal pallium), dendrite and spine morphology changes have been reported and are presumed to be cell-nonautonomous (Judson, Eagleson, Wang, & Levitt, 2010b). Using the same conditional mutant model, the physiological impact of loss of Met signaling in neurons derived from the dorsal pallium was studied. In *Met* null mice, local excitatory input from layer 2/3 to layer 5 pyramidal neurons was two-fold stronger compared to controls (Qiu, Anderson, Levitt, & Shepherd, 2011). This specific alteration of local circuit connectivity is consistent with hypotheses of differentially-altered local versus long-range connectivity in the ASDs (Casanova & Trippe, 2009; Geschwind & Levitt, 2007). Together, these reports on dendritic, synaptic and connectivity findings all suggest that MET signaling may be an important component to include in synaptic models of ASD pathobiology (Gilman, et al., 2011; Penzes, et al., 2011; Zoghbi, 2003). In summary, the experimental data elucidating the developmental and neurobiological roles of the MET signaling system provide multiple lines of convergent evidence to support *MET* as an ASD risk gene.

Multiple reports of human genetic findings complement the basic neurobiological data implicating MET signaling in ASD pathobiology. The original study of an association between a common variant, the *C* allele of rs1858830, in

the promoter of the *MET* gene and risk for ASDs reported a relative risk of 2.27 (Campbell, et al., 2006). The rs1858830 *G/C* SNP resides in the 5' untranslated region (UTR), 20 bp upstream of the transcriptional start site of *MET*. The association between rs1858830 and risk for ASDs was replicated in two independent cohorts, one by another research group (Campbell, Li, Sutcliffe, Persico, & Levitt, 2008; Jackson et al., 2009). Using gel-shift and cell-based transcriptional assays, it was also reported that the *C* allele at rs1858830 resulted in altered transcription factor binding to the *MET* promoter and decreased transcription of a reporter signal (Campbell, et al., 2006). These *in vitro* data were consistent with previous reports of the functional significance of promoter variants (Masotti et al., 2005). A subsequent study reported a two-fold reduction in MET protein in post-mortem temporal cortex of ASD compared to matched control brains (Campbell et al., 2007), data which was suggestive of the *in vivo* functional consequence of rs1858830. These data, combined with the neurobiology of Met/MET signaling discussed above, support the hypothesis that the rs1858830 *C* allele is a common variant which predisposes specific neural circuitry to an altered developmental trajectory and functional capacity, which ultimately places the individual at risk for developing an ASD.

In addition to the rs1858830 findings, additional reports have implicated altered MET signaling in risk for ASDs. Two other SNPs in *MET*, rs38841 and rs38845, have also been reported to be significantly associated with risk for ASDs, in independent cohorts and by separate research groups (Sousa et al.,

2009; Thanseem et al., 2010). Both SNPs reside in intron 1 of *MET*, and although direct evidence of functional significance of either SNP has not been reported, a bioinformatics analysis of rs38845 alleles suggests the sequence variation may impact transcription factor binding (Sousa, et al., 2009). Additionally, two rare missense variants in exon 14 of *MET*, rs56391007 and rs34589476, were reported with increased prevalence in cases (1.8%) versus controls (0.6%), although not to a statistically significant degree (Campbell, et al., 2006). These variants reside in the juxtamembrane domain of the protein and have been reported to increase protein function (Ma et al., 2003). The linkage disequilibrium (LD) block structure of the 5' region of *MET* is shown in Figure 1, with relative positions of rs1858830, rs38841, and rs38845. Each 5' SNP, according to this block structure, resides in a distinct block in Caucasians. Figure 2 shows a schematic representation of the 5' and exon 14 rare variants. Multiple research groups using independent sample data, with different races and ethnicities, in both case-control and family-based (thereby addressing concerns of population stratification) study designs have reported significant association between polymorphisms in the 5' region of *MET* and risk for ASDs. Moreover, rare *de novo* CNVs of chromosome 7 that encompass *MET* have been observed in ASD (Marshall, et al., 2008; Pinto, et al., 2010). Variants of two other genes with protein products that are involved in the MET signaling cascade, *PLAUR* and *SERPINE1*, have also been associated with risk for ASDs (Campbell, et al., 2008). Finally, a recent gene expression study using a large sample of

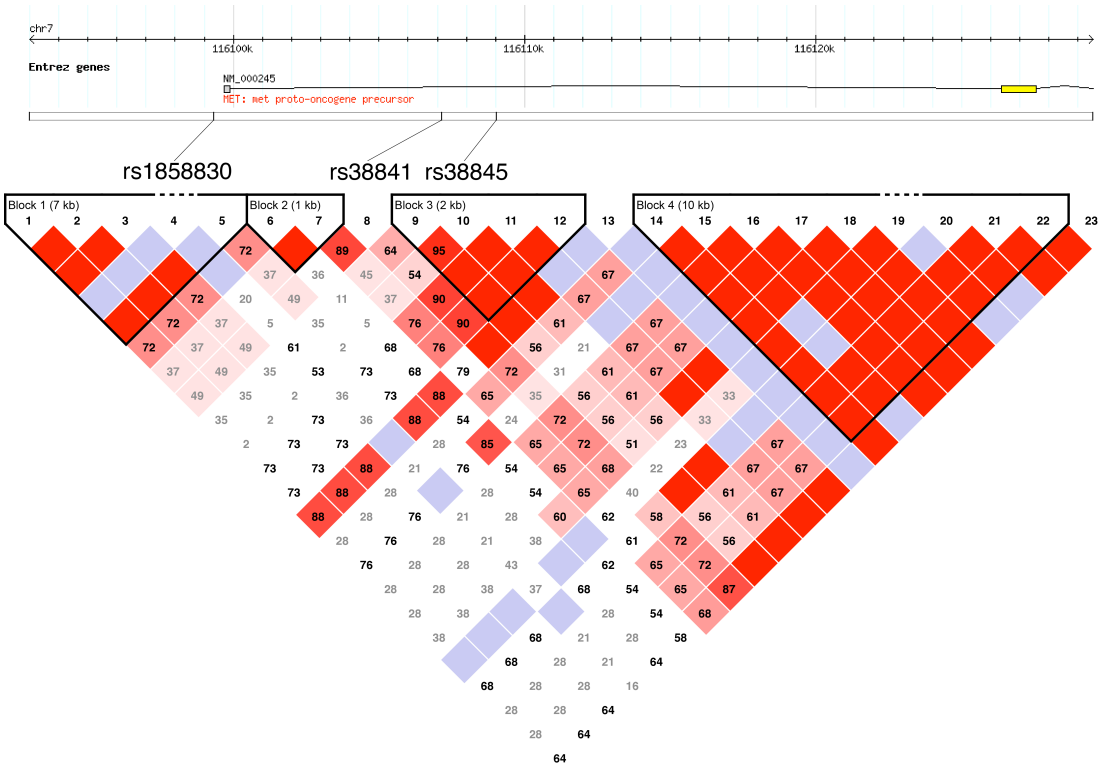

Figure 1. Structure of ASD-associated loci in the *MET* gene. LD block structure of 5' region of MET in Caucasians, showing 3 ASD-associated SNPs (rs1858830, rs38841, and rs38845) and their locations in three distinct blocks. Figure modified from output generated in Haploview 4.2 <Barrett Bioinfor 2005>

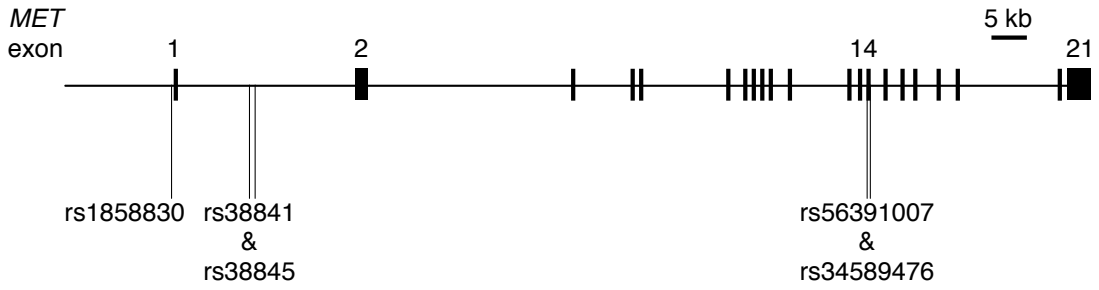

Figure 2. Schematic structure of ASD-associated loci in the *MET* gene. Schematic representation of MET, showing relative locations of common SNPs in the 5' UTR (rs1858830) and intron 1 (rs38841 and rs38845), and rare variants distantly in exon 14 (rs56391007 and rs34589476).

postmortem brains reported decreased expression of MET in temporal neocortex of individuals with ASD (Voineagu et al., 2011). In sum, the data and reports linking altered MET signaling to ASDs are comprehensive, rigorous, and replicated — and taken together, make a strong and compelling case for *MET* as a risk gene for ASDs.

Met as a Pleiotropic Signaling Receptor in the Gastrointestinal System

In addition to the neurobiological and genetic findings discussed above, MET signaling is also important in the development and functioning of cells and tissues outside of the nervous system. In the mouse, Met signaling has been implicated in cell proliferation, migration, differentiation and survival (Beilmann, et al., 2000; Birchmeier, Birchmeier, Gherardi, & Vande Woude, 2003; Bladt, Riethmacher, Isenmann, Aguzzi, & Birchmeier, 1995; Dignass, Lynch-Devaney, & Podolsky, 1994; Giacobini et al., 2007). Of particular relevance to this thesis, Met signaling is also implicated in the development and function of the gastrointestinal system. Met expression in mouse gastrointestinal tissue has been reported during development and in the adult, but these studies generally have lacked adequate resolution to determine cell specificity. Expression at embryonic day 9 (E9) is detected in the hindgut (the anatomical precursor of the distal third of the colon and rectum) (Andermarcher, Surani, & Gherardi, 1996), and later at E15 and E17 in the immature intestines (Sonnenberg, Meyer, Weidner, & Birchmeier, 1993). Met expression is detectable in the adult small and large intestine, although at a

low basal level (Boon et al., 2006). Rodent studies are paralleled by human expression studies, showing MET to be expressed in various gastrointestinal tissues along the developing alimentary canal throughout development (Kermorgant et al., 1997) and in the adult colon (Crepaldi et al., 1994). Presuming the expression is not superfluous, these studies suggest that the expression of Met/MET is functional, playing a role in gastrointestinal development and physiology. Some insight into the role of MET in the large intestine may be gleaned from a series of studies which report that administration of exogenous hepatocyte growth factor (HGF), Met's endogenous ligand, can augment epithelial repair processes in acute gastrointestinal injury paradigms. These findings have been demonstrated in mice (Oh et al., 2005) and rats (Numata, et al., 2005; Tahara, et al., 2003), in two different injury paradigms and methods of delivering HGF, by independent research groups, strongly supporting the hypothesis that Met signaling is important in physiological repair processes in the rodent large intestine. These findings are consistent with a study of MET expression in human colonic tissue which showed increased expression in inflamed tissue of patients with ulcerative colitis, compared to control tissue (Kitamura et al., 2000).

These additional biological roles played by MET in the gastrointestinal system demonstrate the pleiotropic biology of MET, a concept which served as the intellectual framework for a follow-up study of the reported associations between *MET* and risk for ASDs. A subpopulation of individuals with ASDs also have co-

occurring gastrointestinal dysfunction (discussed in the following section). It was therefore hypothesized that those with co-occurring GID and ASDs represented a more etiologically-homogenous subgroup of individuals compared to a phenotypically-diverse population of cases of idiopathic ASD, and that this subgroup may share a common biology which places them at parallel risk for both gastrointestinal and brain dysfunction (Campbell, et al., 2009). Campbell and colleagues found the rs1858830 *C* allele — a common variant previously associated with risk for ASDs — to be significantly overtransmitted in individuals with co-occurring GID and ASDs, compared to ASDs-only (Campbell, et al., 2009). The field of genetics of ASDs has generally shifted its focus away from study of common variants to that of rare variants as prime suspects for risk and pathogenesis, with some claiming that common variants play no role at all (Levy, et al., 2011). However, the evidence in support of the common variant rs1858830 (and thereby altered MET signaling in general) as a risk factor for ASDs is substantial and makes for a compelling rationale to further study this fascinating intersection of biology and disease.

Co-Occurring Gastrointestinal Dysfunction in Autism Spectrum Disorders

As part of the heterogeneity of ASDs, a subgroup of individuals with ASDs present with co-occurring gastrointestinal dysfunction (Buie et al., 2010a; Buie et al., 2010b). Although reports on the prevalence of GID in the ASDs vary widely, from 9% (Black, Kaye, & Jick, 2002) to 24% (Molloy & Manning-Courtney, 2003)

to 54% (Levy et al., 2007) to 70% (Valicenti-McDermott et al., 2006), it is important to note that overall prevalence of GID in the ASDs is irrelevant to the biological study of specifically those individuals with co-occurring GID and ASDs. The lack of replication or agreement regarding prevalence estimates suggests the true rate of GID in the ASDs has not yet been accurately determined, and a critical review of epidemiological studies of GID in ASDs concluded that they are hindered by serious flaws, including small sample sizes, reporting biases, and lack of proper comparison groups (Erickson et al., 2005). However, whatever the prevalence might be, it is a truism that there is a subpopulation of individuals with GID. For these individuals, in addition to the difficulties inherent in their overall ASD, the presence of co-occurring GID may have implications for their clinical care, including delayed diagnosis (Levy, et al., 2010) or negatively-impacted behavioral interventions and management (Bauman, 2010a). A recent consensus report by clinicians and investigators noted that the consistent theme among reports on GID in ASDs is a lack of quantity and quality — that what is needed most in this field is methodological rigor, adequate sample sizes, and cross-disciplinary integration (Buie, et al., 2010a).

Several mechanisms have been proposed to underlie co-occurring GID in ASD. There may be distinct genetic and biological differences between individuals with or without co-occurring GID (Campbell, et al., 2009). Behavioral rigidity may lead individuals with ASDs to choose a restricted diet which induces GID (Ibrahim, Voigt, Katusic, Weaver, & Barbaresi, 2009). Medications commonly

prescribed to individuals with ASDs may induce weight gain or suppress appetite in general, thus leading to GID (Ibrahim, et al., 2009). Psychotropic medications with adverse side effects on the gastrointestinal system may induce gastrointestinal symptoms directly (Ibrahim, et al., 2009). Finally, the "leaky gut" hypothesis suggested by Andrew Wakefield and colleagues, proposed that the measles-mumps-rubella (MMR) vaccine induced a chronic colitis in children, which increased intestinal permeability and thereby allowed increased absorption of neurotoxic compounds which ultimately caused the behaviors of ASD (Wakefield et al., 1998).

A full examination of research on GID in ASDs must address the historical legacy of Wakefield's report. In 1998, Wakefield and colleagues proposed a link between autism, the MMR vaccine, and gastrointestinal abnormalities (including lymphoid-nodular hyperplasia and colitis) (Wakefield, et al., 1998). The majority of the original authors subsequently retracted their interpretation and conclusions (Murch et al., 2004). It is now clear the extent to which Wakefield egregiously violated scientific and ethical principles (Deer, 2011), and the damage his claims have inflicted on public health vaccination programs (Freed, Clark, Butchart, Singer, & Davis, 2010; Jansen et al., 2003; Parker et al., 2006; Smith, Ellenberg, Bell, & Rubin, 2008). The current consensus among professionals, and current data, do not support the "leaky gut" hypothesis (Buie, et al., 2010a; Hornig et al., 2008). Importantly and without any ambiguity, the present thesis is biologically and intellectually unrelated to Wakefield's line of investigation. Moreover, another

important consequence of Wakefield's work is a deep stigma has been attached to research on GID in ASDs, deterring investigators and prompting suspicion of those who do study GID. Anecdotally, many clinicians and researchers view GID in the ASDs with skepticism, which is in contrast to what parents report, often resulting in a tension between parents and providers involved in the clinical care of children with ASDs. Without high quality studies of GID, the field is left with many unexplored clinical and biological issues regarding GID in ASDs (Buie, et al., 2010a).

This thesis sought to address some of the unanswered questions regarding GID in ASDs. Building on work previously reported by Campbell and colleagues (Campbell, et al., 2009), the biological framework for this thesis is pleiotropic consequences of altered MET signaling and thus shared genetic risk for co-occurring GID in ASDs. The aims of this thesis were as follows: 1) to describe the specific clinical and behavioral phenotype of children with co-occurring GID and ASDs, examining relationships between proposed causes of GID and GID outcomes, comparing parent versus clinician evaluations of GID, and exploring the functional implications of GID in ASDs (Chapter II); 2) to replicate previous genetic findings in an independent cohort with higher quality phenotype data and extend these findings to additional biomarkers (Chapter III); and 3) to elucidate the specific contribution of Met signaling to gastrointestinal biology in a conditional knockout mouse model (Chapter IV). Through these aims, the larger goals of this thesis were to improve clinical care, and possibly gain insight into

ASD risk and pathogenesis, for a subgroup of individuals with co-occurring GID and ASDs.

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CHAPTER II

Gastrointestinal Dysfunction in Autism: Parental Report, Clinical Evaluation, & Associated Factors

Introduction

Autism spectrum disorder (ASD) is defined by deficits in social interaction and communication, and a restricted repertoire of activities and interests (Volkmar, 2005). ASD is heterogeneous and complex, often presenting with behavioral and medical comorbidities, including mood disorders, sleep abnormalities, and gastrointestinal dysfunction (GID) (Geschwind, 2009). However, a recent consensus report highlighted many unexplored issues in GID and ASD that have important implications for clinical care (Buie et al., 2010).

The absence of rigorous, prospective clinical phenotyping in children with ASD and parent-suspected GID has impeded understanding potential relationships between GID and ASD. Research on GID in ASD has largely been limited to studies relying on parental reports (Smith, Farnworth, Wright, & Allgar, 2009; Valicenti-McDermott et al., 2006) or retrospective review of records (Black, Kaye, & Jick, 2002; Xue, Brimacombe, Chaaban, Zimmerman-Bier, & Wagner, 2008), lacking comparison groups (Molloy & Manning-Courtney, 2003; Nikolov et al., 2009), or focused on the prevalence of GID in ASD compared to non-ASD populations (Ibrahim, Voigt, Katusic, Weaver, & Barbaresi, 2009; Mouridsen,

Rich, & Isager, 2010). It has been suggested that GID in children with ASD does not have a biological basis but rather results from behavior, medications or dietary habits (Ibrahim, et al., 2009).

Our laboratory has previously identified genetic differences between individuals with ASD with and without comorbid GID (Campbell et al., 2009), reframing questions of GID in a biologically-reasoned context in which subgroups of individuals with ASD may be at greater risk for GID because of pleiotropic expression of risk-associated genes in both the brain and gastrointestinal system. Here we report findings of an ongoing study that further explores this hypothesis. Our objectives were to characterize GID in children with ASD, to compare parental reports of GID symptoms on a validated instrument to results of clinical evaluations by a pediatric gastroenterologist, and to evaluate the relationships of dietary habits and medication status to GID in ASD.

Methods

Study Procedures

Children were recruited into three groups: co-occurring ASD and GID (ASD-GID); ASD without any GID (ASD-only); and GID without any ASD (GID-only). At enrollment, parents of children with ASD were queried in a structured interview to assess for ongoing GI complaints and assign GID group status. The ASD-GID and GID-only groups were evaluated by a pediatric gastroenterologist. The ASD-

GID and ASD-only groups were assessed with the Autism Diagnostic Observation Schedule (ADOS). Parents in all groups completed questionnaires about their child's behavior and GI symptoms, and a dietary journal. The research protocol was approved by the Vanderbilt University Institutional Review Board, and written informed consent was obtained from parents of participants.

Participants

Children were recruited at Vanderbilt University in Nashville, Tennessee, between 2009 and 2011. Children in both ASD groups were recruited primarily through the hospital's ASD medical clinic, although some families self-referred after seeing study flyers in other locations, such as family resource events. Children in the GID-only group were recruited through the pediatric gastroenterology outpatient clinic. Exclusion criteria included severe sensory or motor impairment, neurodevelopmental disorders of known etiology (e.g. Fragile X Syndrome), gestational age less than 36 or greater than 42 weeks, and birth weight less than 2500 grams. Inclusion criteria included age between 5 and 18 years, meeting ASD criteria on the ADOS (for the ASD groups), and GI symptoms that had lasted more than a month (for the GID groups).

Measures

Clinical evaluation: Children in both GID groups were evaluated by one of six pediatric gastroenterologists; one of those six (K.C.W.) evaluated 78.9% of

participants. The evaluation included a medical history, gastrointestinal symptoms review, and physical examination; laboratory tests and procedures were pursued at the physician's discretion when non-functional GI disease was suspected. Because of pronounced behavioral manifestations, blinding the gastroenterologist to ASD status was not possible.

ADOS: Children in both ASD groups had ASD clinical diagnoses at enrollment, that were confirmed by assessment with the ADOS, a standardized instrument for diagnosing ASD (Lord, Rutter, DiLavore, & Risi, 1999). ADOSs were performed within two years of the study by a research-certified assessor who was blinded to GID status, and the revised scoring algorithm was used (Gotham, Risi, Pickles, & Lord, 2007). Item scores were not available for one participant, and one participant was assessed with a Module 4, which does not have a revised scoring algorithm; for these two participants, the original scoring algorithm was used.

Expressive language: Item A1 on Module 1 of the ADOS rates non-echoed language. A score of either 3 or 8 was considered nonverbal for analyses.

Social Responsiveness Scale (SRS): The SRS is a 65-item, parent-report instrument that provides a validated continuous measure of social impairment (Constantino & Gruber, 2005). SRS data was not available for one participant.

Questionnaire on Pediatric Gastrointestinal Symptoms - Rome III Version (QPGS): The QPGS is a 71-item parent-report instrument that assesses GI

symptoms, classifies functional GI disorders (FGIDs) according to Rome-III criteria (Drossman, 2006; Walker, Caplan-Dover, & Rasquin-Weber, 2000), and is widely used in research on pediatric GID (Baber, Anderson, Puzanovova, & Walker, 2008; Caplan, Walker, & Rasquin, 2005a, 2005b). While organic GI disorders result from a clear anatomic, metabolic or pathologic process that is readily identified clinically, FGIDs instead have no overt pathology which can be identified. For analysis, missing responses on the QPGS were interpreted as a lack of evidence for the assessed symptom. If eight or more items were missing, the QPGS was omitted from analysis; instrument data from two participants were excluded by this criterion. Three participants who were initially enrolled in the ASD-only group subsequently met criteria for one or more FGID classifications; these participants were moved to the ASD-GID group and evaluated by a pediatric gastroenterologist. Four participants who were enrolled in the ASD-only group subsequently met criteria for fecal incontinence on the QPGS, but no other FGID; we interpreted this to be a toilet-training issue, rather than indicative of GID, and thus these individuals remained in the ASD-only group. The instrument is available online at romecriteria.org.

Dietary journal: Parents were asked to record all food eaten by the participating child for seven days, into 11 categories but without serving size information. For analysis, we required complete data from at least five days. Two journals were excluded based on this criterion, and 20 parents did not complete the journal.

Medication classification: Medications were queried in the Micromedex database (Thomson Reuters, New York, NY) and classified as having potential GI side effects if adverse effects of abdominal pain, constipation, indigestion, nausea, vomiting, or diarrhea were reported in greater than 10% of patients. Supplemental Table 1 lists medications and corresponding classifications.

Data Analysis

Study data were managed using REDCap, a secure, research-oriented, web-based application (Harris et al., 2009). Statistical analyses were computed using SPSS version 18.0.3 (IBM, Somers, NY). Participant characteristics were described by mean and standard deviation for continuous measures (age and BMI percentile) and by percent for nonparametric measures (sex, ethnicity, race, ADOS classification, and nonverbal status). ADOS classification and nonverbal status were compared using χ^2 tests; BMI percentiles were compared by a one-way analysis of variance (ANOVA). Prevalences of GI diagnoses were reported as percentages. Kappa coefficients (κ) were calculated to assess parent-physician agreement for a diagnosis of constipation, and parent-physician percent agreement for presence of any GID was compared with a χ^2 test. SRS T-scores were plotted as boxplots and compared with an ANOVA; T-scores of nonverbal versus verbal subgroups were compared with a two-tailed t-test. Dietary habits were plotted as group means of percent of food category eaten with 95% confidence intervals (CI), and compared with an ANOVA. The

proportion of subgroups taking medications with potential GI side effects was compared with a χ^2 test. To examine factors associated with the outcome of constipation, an exploratory logistic regression model was developed to test the additive effect of factors with potential clinical relevance (SRS T-score, nonverbal status, BMI percentile, and medication status). After univariate logistic regression models were tested, factors with significant unadjusted odds ratios (ORs) were retained and then entered into a final model, with sex and age as covariates. Adjusted ORs for constipation are reported. For all statistical tests, a *P* value of less than 0.05 was considered significant. When applicable, Tukey's HSD post hoc test was computed.

Results

Participant Characteristics

121 children were enrolled into three groups (ASD-GID = 40; ASD-only = 45; GID-only 36; Table 1). Participants were predominantly male (range: 63.9 to 86.7%) and of non-Hispanic white racial and ethnic origin (range: 77.5 to 88.9%), and ranged in age from 5.1 to 17.9 years. There was no difference in ADOS classification between the ASD groups (proportion who met criteria for autism: ASD-GID 95.0%; ASD-only 91.1%; $P = 0.48$).

Table 1. Characteristics of Study Participants

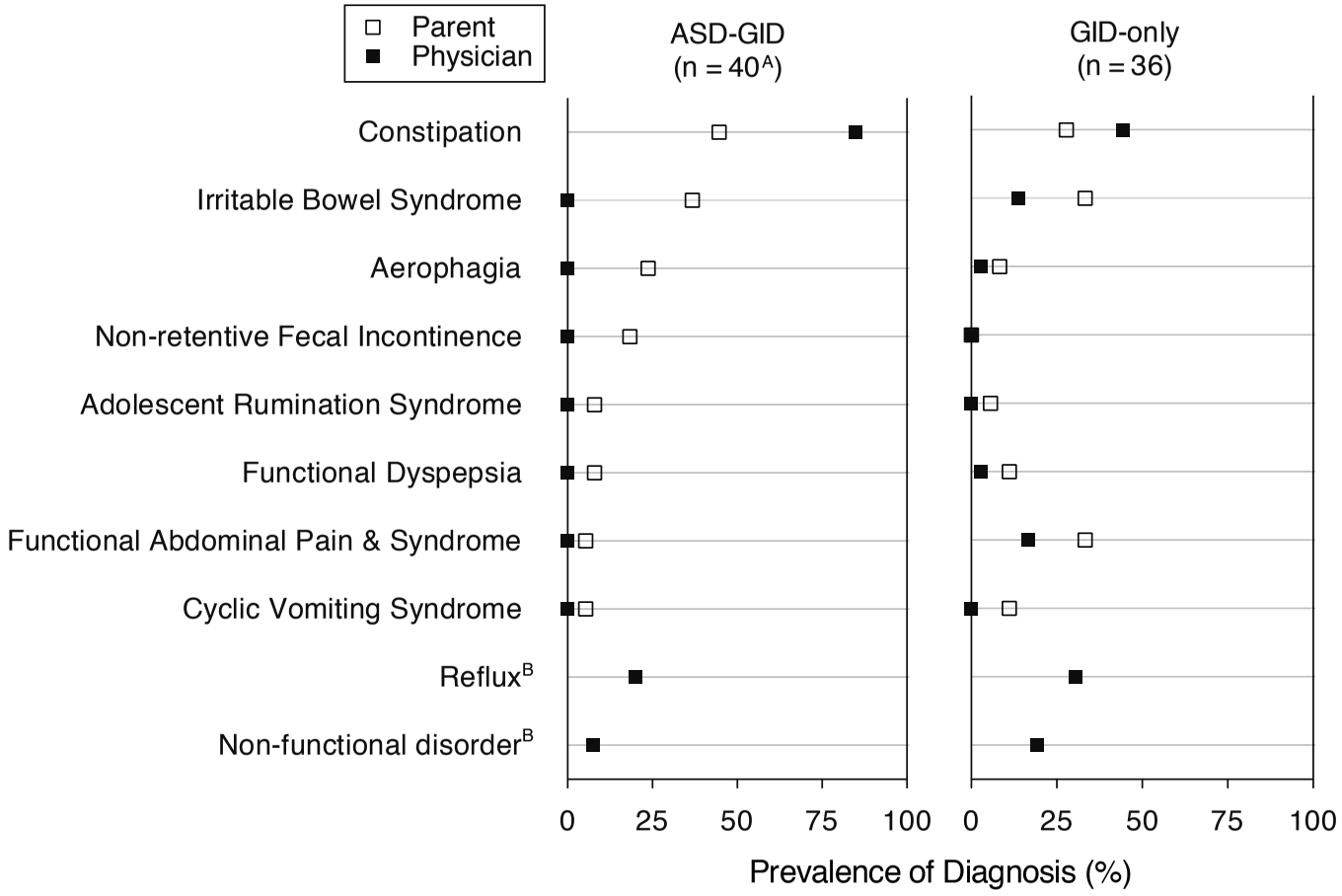
	ASD-GID (n = 40)	ASD-only (n = 45)	GID-only (n = 36)
Age in years, mean (SD)	10.8 (3.7)	12.4 (3.4)	11.0 (3.4)
Male sex, % (n)	72.5 (29)	86.7 (39)	63.9 (23)
Ethnicity and race, % (n)			
Hispanic	12.5 (5)	0 (0)	2.8 (1)
Non-Hispanic white	77.5 (31)	86.7 (39)	88.9 (32)
Non-Hispanic black	7.5 (3)	8.9 (4)	8.3 (3)
Non-Hispanic other	2.5 (1)	4.4 (2)	0 (0)
ADOS Classification, % (n)			
Autism	95.0 (38)	91.1 (41)	n/a
Autism Spectrum	5.0 (2)	8.9 (4)	n/a
Nonverbal, % (n)[A]	30.0 (12)	6.7 (3)	0 (0)
BMI-for-age percentile, mean (SD)[B]	76.2 (30.2)	68.9 (31.0)	57.2 (36.3)

[A] ASD-GID versus other groups, $P < 0.01$
[B] ASD-GID versus GID-only, $P = 0.03$

Gastrointestinal Diagnoses

Functional constipation (FC) was the most common diagnosis of GID in ASD (85.0%; Figure 1) when evaluated by a pediatric gastroenterologist. Although FC was also common in the GID-only group (44.4%), a comparison of prevalence of FC in ASD versus non-ASD was not appropriate, as this study was not designed to be epidemiologically representative. Instead, the diagnoses in the GID-only group show that this group had primarily FGIDs and was thus similar to the ASD-GID group in nature of GID, to enable subsequent comparisons across groups for other factors. Reflux was the next most common diagnosis in the ASD-GID group (20.0%), and three children were found to have non-functional, organic disease underlying their GI symptoms (one case each of eosinophilic esophagitis, *H. pylori*, and celiac disease).

FC by the parent-reported QPGS was also the most common diagnosis in the ASD-GID group (44.7%). Parent-physician agreement for a diagnosis of FC was fair for the ASD-GID group ($\kappa = 0.26$) and moderate for the GID-only group ($\kappa = 0.42$) (Landis & Koch, 1977). However, percent agreement between a physician's diagnosis for any GID and a parent's report yielding any diagnosis on the QPGS was 92.1% in the ASD-GID group, compared to 88.9% in the GID-only group ($P = 0.64$).



A Parent data were missing for two individuals, thus n = 38 for ASD-GID parental report; both individuals were evaluated by a physician

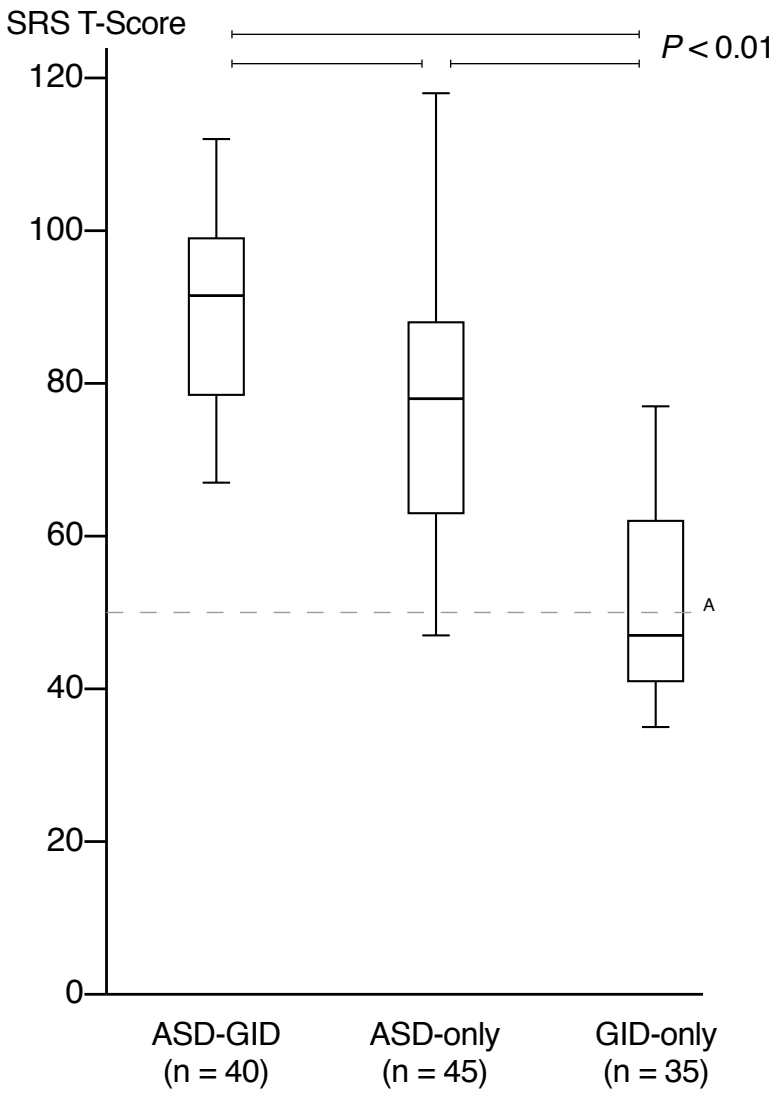
B Parent-report instrument does not collect data on reflux or non-functional disorders

Figure 1. Gastrointestinal Dysfunction in Children With and Without ASD, by Physician's Evaluation and Parent's Report. Constipation was the most prevalent diagnosis, and concordance between parents and clinicians was fair to moderate for a specific diagnosis of constipation, but high when considering presence versus absence of any gastrointestinal dysfunction

Language & Social Impairments

To explore the functional significance of comorbid GID, we investigated group differences in language and social function. The percentage of nonverbal children was larger in the ASD-GID group, compared to the ASD-only group (Table 1; ASD-GID 30.0%; ASD-only 6.7%; $P < 0.01$).

Children in the GID-only group did not show an elevated SRS T-score above that in typically-developing children (normed with mean 50 and SD 10 (Constantino & Gruber, 2005); GID-only mean 51.3; Figure 2). As expected (Constantino et al., 2003), children in the ASD-only group showed an elevated T-score compared to the GID-only group (ASD-only mean 77.7; GID-only mean 51.3; $P < 0.001$). Interestingly, children in the ASD-GID group showed an elevated T-score compared to children in both ASD-only and GID-only groups, indicating the most severe social impairment in the co-occurring group (ASD-GID mean 89.2; ASD-only 77.7; $P = 0.001$). The elevated SRS T-score in the ASD-GID group was not associated with impaired language, as scores of nonverbal and verbal children in this group were not different (nonverbal mean 86.2; verbal mean 90.4; $P = 0.35$).



[A] Dashed line indicates normative data in typically-developing children

Figure 2. Social Impairment Measured by T-Scores on the Social Responsiveness Scale. Social impairment was significantly greater in both ASD groups, compared to the GID-only group; the most social impairment was seen in the ASD-GID group.

Indistinguishable Diets

To explore whether GID in ASD was associated with a restricted or limited diet, parents were asked to record 11 categories of food that their child ate during the course of seven days (Figure 3). Comparison of the relative distributions of food categories showed no significant differences across groups.

Factors Associated with Constipation

Logistic regression was used to determine what factors were associated with functional constipation, the most prevalent GI diagnosis in our sample. Elevated BMI has been associated with FGIDs in typically-developing children (Pashankar & Loening-Baucke, 2005; Teitelbaum, Sinha, Micale, Yeung, & Jaeger, 2009), and obesity prevalence in ASD has been reported to be 30% (Curtin, Anderson, Must, & Bandini, 2010). Mean BMI-for-age percentile (Kuczmarski et al., 2002) was not statistically different between ASD-GID and ASD-only children (Table 1), and in univariate logistic regression, BMI was not significantly associated with a diagnosis of constipation in ASD (OR 1.01, 95% CI 0.99 – 1.02). There were no differences among ASD subgroups in the proportion taking medications with potential constipating side effects (67.6% of ASD-GID with constipation; 83.3% of ASD-GID with GID other than constipation; 64.4% of ASD-only; $P = 0.65$). Table 2 lists medications and corresponding classifications. In univariate logistic regression, potentially constipating medications were not associated with

Figure 3. Percent of food categories eaten during seven days. Parents recorded foods eaten by their children for seven days, in 11 different food categories. Relative distributions of food categories eaten did not differ significantly across any of the three groups.

Table 2. Classification of Medications by Potential Gastrointestinal Side Effects

Without potential GI side effects
amitriptyline, atenolol, benztropine, chlorpromazine, clonazepam, clonidine, diazepam, doxazosin, eszopiclone, haloperidol, hydroxyzine, levothyroxine, lithium, lorazepam, melatonin, zonisamide
With potential GI side effects, except constipation
albuterol, amphetamine-dextroamphetamine, atomoxetine, baclofen, desmopressin, dexmethylphenidate, escitalopram, fluoxetine, lamotrigine, levetiracetam, lisdexamfetamine, methylphenidate, oxcarbazepine, sertraline, somatropin, topiramate, trazodone, valproic acid-divalproex sodium, ziprasidone
With potential GI side effects, including constipation
aripiprazole, citalopram, clomipramine, clozapine, fluvoxamine, guanfacine, olanzapine, oxybutynin, paroxetine, pregabalin, quetiapine, risperidone

Table 3. Factors Associated With a Diagnosis of Constipation in Children With ASD (n = 85)

	Adjusted Odds Ratio	95% CI	*P* Value
Sex	0.79	0.20 - 3.11	NS
Age	0.81	0.69 - 0.94	0.01
SRS T-score	1.05	1.01 - 1.09	0.02
Nonverbal	11.98	2.54 - 56.57	0.002

constipation in ASD (OR 1.04, 95% CI 0.42 – 2.64). Thus, in our final multivariate logistic regression model, we adjusted for age and sex (due to the large developmental age range and preponderance of males in our sample), and included SRS T-score and nonverbal status, as those were different between ASD-GID and ASD-only groups. Table 3 reports adjusted ORs, showing that younger (OR 0.81, 95% CI 0.69 – 0.94; $P = 0.01$), more socially impaired (OR 1.05, 95% CI 1.01 – 1.09; $P = 0.02$), and nonverbal (OR 11.98, 95% CI 2.54 – 56.57; $P = 0.002$) children with ASD had increased odds for functional constipation.

Discussion

For the first time, data obtained from clinical specialists and parents were integrated in the same study to address gaps in understanding GID in ASD (Buie, et al., 2010). A number of unique findings address what we suggest are misconceptions of GID in ASD. The majority of children in the ASD-GID group had functional, rather than organic, GID; children with ASD had the same types of GID as children without ASD. For constipation, the most common diagnosis in the ASD-GID group, we saw fair parent-physician agreement ($\kappa = 0.26$), which is similar to reported agreement among pediatric gastroenterologists ($\kappa = 0.36$) (Saps, Chogle, Sztainberg, Dhroove, & Di Lorenzo, 2010). Percent agreement between any type of parent-reported GID and any physician diagnosis, however, was high (92.1% in ASD-GID), and no different than that in the GID-only group.

This suggests that parents of children with ASD are similar to parents of children without ASD in reporting GID, and parents from both groups are limited in their ability to discriminate subtypes of GID. Moreover, parental report of GI symptoms in a child requires inference that may by limited by communication deficits in ASD. The parental report data in Figure 1 suggest a variety of FGIDs are present in ASD, whereas by a physician's evaluation, most children were given a working diagnosis of functional constipation. This suggests that in children with ASD the manifestations of functional constipation can be variable and the expertise of a gastroenterologist is needed to determine the nature of the GID.

Interestingly, some children were initially enrolled into the ASD-only group, as parents did not report ongoing GID. However, upon completing the QPGS, 19 children met criteria for one or more FGIDs. To properly re-assign them to the ASD-GID group, we requested that the children be evaluated by a pediatric gastroenterologist. Several families could no longer be contacted and other families chose not to be evaluated; thus, no data from these 19 children are included in this report. This finding, combined with high parent-physician concordance of GID presence, suggests that parents of children with ASD do not over-report GID, and in fact may under-report GID.

We suggest that comorbid GID has implications beyond medical status for children with ASD. Our data show a large portion of children with co-occurring ASD and GID lack expressive language; as a novel finding, this association warrants further study. This study did not assess for behavioral problems, such

as aggression or self-injurious acts. However, it will be important to clarify the relationship between externalizing behaviors, language level, and GI disorders, in a rigorous manner. Children in the ASD-GID group also showed increased social impairment on the SRS, compared to the ASD-only group. Because GID-only children did not show increased SRS scores, this suggests an interaction between ASD and GID statuses. Additional insight may derive from monitoring SRS scores or other behaviors during GID treatment, to determine whether improved medical status can enhance child responsiveness to established ASD behavioral treatments or decrease problematic behaviors (Bauman, 2010). In addition, observation of parent-child communication regarding the toileting needs of nonverbal children with ASD will clarify the extent to which lack of expressive language may itself contribute to constipation by limiting appropriate toileting behavior.

We were surprised to find no differences in dietary habits between the three groups. Previous studies have reported increased food selectivity in younger children with ASD (Bandini et al., 2010; Emond, Emmett, Steer, & Golding, 2010). The data reported here focus on older children, and do not show evidence of food selectivity beyond that seen in a non-ASD population. We suggest that it is unlikely that dietary habits play a large causal role in GID status in our study population.

Potential medication side effects and BMI did not contribute to a diagnosis of constipation. Although it has been suggested that constipation in ASD is caused

by such factors (Ibrahim, et al., 2009), our data do not support this conclusion. For any given child, medications may contribute to GID. However, group-level analysis did not detect an association between potential medication side effects and GID in ASD. Associations between impaired language and social function remained after adjustment for age and sex. Although the OR for the SRS is modest (1.05), this indicates a 5% increase in odds of constipation for each point increase in the SRS T-score. The OR for nonverbal status is large (11.98), and has important implications for those involved in the clinical care of children with ASD who lack traditional communication abilities. Consistent with the recent consensus report (Buie, et al., 2010), the data presented here affirm the need for healthcare providers to evaluate their patients for latent constipation, and to consider empiric treatment of constipation in nonverbal children.

An important limitation of this study was that the physician's evaluation was often limited to a single encounter. Tests and procedures were ordered as needed to determine if organic disease was underlying a child's GID, thus it is likely that the diagnoses presented here are functional. As FGIDs are symptom-based diagnoses of exclusion, working through the differential diagnosis may require multiple visits to determine the specific underlying FGID (for example, determining if intermittent abdominal pain is due to functional constipation or functional abdominal pain). The diagnoses presented here are the most likely diagnoses given the child's clinical presentation at study enrollment. Future

studies will benefit from follow-up evaluations of participants to determine whether original diagnoses are confirmed after subsequent clinical evaluations.

An additional potential limitation of this study is possible ascertainment bias. Although some study participants self-referred to the study by responding to community flyers, the majority of participants were recruited from medical clinics at a large academic medical center. The data presented here may not generalize to a diverse community-based sample, as the participants in this study may be more severely impaired than the average child in the community. However, with this potential limitation taken into consideration, the data presented here still provide insight into the population studied.

Conclusion

This report characterized GID in children with ASD, evaluated by pediatric gastroenterologists and compared to parental report of GI symptoms, for the first time in comparison to ASD-only and GID-only groups. Parental report of presence of any GID in ASD was highly concordant with a physician's diagnosis of any GID, validating parental concerns for GID in children with ASD. Parents were sensitive to the existence, although not necessarily the nature, of GID. Functional constipation was the most prevalent GID in ASD. Odds for constipation were significantly associated with younger age, increased social impairment, and lack of expressive language. Factors previously suggested as

causes of GID in ASD, diet and medications, were not associated with GID. These data support our evolving hypothesis (Campbell, et al., 2009) that GID in ASD represents pleiotropic expression of genetic risk factors, and thus a fundamental difference in biology between individuals with ASD with and without comorbid GID.

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CHAPTER III

Biological Stratification Of Individuals With ASD Shows Subpopulation Enrichment Of 15-F_{2t}-Isoprostane

Introduction

Progress in understanding the etiology and prognosis of individuals with autism spectrum disorders (ASDs) has been, to date, modest. It has been proposed that profound heterogeneity across individuals — both in underlying pathogenic mechanism and in phenotypic manifestation of the disorder — has been a significant source of this limited progress (Campbell et al., 2009). However, viewed through an optimistic lens, this heterogeneity is an opportunity to stratify affected individuals, thereby leveraging complexity to yield subpopulations with greater intra-group homogeneity and potentially enriching for a disease-specific signal within a subpopulation. Consistent with this view, a recent report demonstrated that, using genome-wide association (GWA) data, a variant association with ASD surpassed the Bonferroni-corrected GWA threshold of significance only when sex of cases was incorporated as a modifying factor; without sex included, the association did not meet criteria for significance (Lu & Cantor, 2010).

Another example of the power of stratifying cases to enrich for signal detection was reported recently in individuals with ASDs and co-occurring gastrointestinal dysfunction (ASD-GID) compared to those without co-occurring

GID (ASD-only) (Campbell, et al., 2009). As discussed in Chapters I and II, GID is a co-occurring condition for a subpopulation of individuals with ASDs. Campbell and colleagues hypothesized that given the expression of the MET receptor tyrosine kinase in multiple organ systems, including the brain and gastrointestinal (GI) system, that pleiotropic effects of a previously-described ASD-associated functional risk variant in the gene, rs1858830, could mediate shared risk for dysfunction in parallel organ systems. To this end, they reported data demonstrating that the risk allele of rs1858830 was over-transmitted to individuals with ASD-GID, compared to ASD-only. Allelic frequency for the risk variant was enriched in the co-occurring group, consistent with the hypothesis that stratified populations are an important strategy for addressing the difficulties imposed by disease heterogeneity.

The genetic signal enrichment in a GID-stratified population reported by Campbell and colleagues suggested two important future studies to undertake. First, a previous report had demonstrated significantly decreased MET protein expression in postmortem temporal cortex of ASD cases versus matched controls (Campbell et al., 2007). Given the genetic signal enrichment in individuals with ASD-GID (Campbell, et al., 2009), a subsequent hypothesis was that protein expression in peripheral blood would show similar differences as seen in postmortem brain. Second, it was hypothesized that genetic differences between ASD-GID and ASD-only groups may coincide with other biological

differences between groups, independent of MET signaling system biology, per se.

Metabolic dysfunction is one potential biological abnormality in ASD that has recently been investigated by a number of investigators. One report concluded that 7.2% of individuals with ASDs have mitochondrial dysfunction (Oliveira et al., 2005). More recently, an examination of 10 cases with ASD compared to 10 controls showed decreased mitochondrial activity levels and elevated pyruvate levels in ASD cases (Giulivi et al., 2010). Variants in several genes involved in the pyruvate metabolic pathway have been associated with ASD (Anney et al., 2011). Dysfunctional mitochondrial metabolism can produce free radicals and reactive oxygen species. Normally, cellular homeostatic and antioxidant systems (such as glutathione metabolism) accommodate free radical production. Variants in genes of the glutathione metabolic pathway and altered blood levels of glutathione have been reported in ASD (Bowers et al., 2011; James et al., 2006; Melnyk et al., 2011). Under conditions of oxidative stress, however, abundant free radicals can mediate damage to cellular components, including DNA, proteins and lipids (Milne, Sanchez, Musiek, & Morrow, 2007).

Of the diverse strategies for quantifying oxidative stress and metabolic dysfunction, some of which were used in the studies described above, the measurement of urinary or plasma 15-F_{2t}-isoprostane (isoP; also known as 8-iso-$PGF_{2\alpha}$) is widely considered to be the gold standard measure of oxidative stress (Milne, et al., 2007). The NIH-commissioned Biomarkers of Oxidative Stress

Study found isoP measurement to be the most sensitive and specific metric of oxidative stress (Kadiiska et al., 2005a; Kadiiska et al., 2005b). F2-isoprostanes are formed by the non-enzymatic free radical-catalyzed oxidation of arachidonic acid, which is present in all fatty acid membranes (Milne, et al., 2007). Increased isoprostane levels, resulting from increased free radicals, have been associated with many diseases, including Crohn's disease (Cracowski et al., 2002), kidney disease (Oberg et al., 2004), and stroke severity (Zeiger et al., 2009), suggesting elevated isoprostanes represent a high-level measure of systemic oxidative stress that is not specific to a particular disease. Two previous studies, with modest sample sizes, have reported increased urinary isoP levels in children with ASD compared to controls (Ming et al., 2005; Yao, Walsh, McGinnis, & Praticò, 2006).

Building on the previous work demonstrating enrichment of a genetic signal (Campbell, et al., 2009), the objectives of this study were threefold. The first objective was to replicate the previous genetic findings, using more detailed phenotype information. The second objective was to extend the genetic findings to peripheral blood expression of *MET* mRNA. The final objective was to examine differences in isoP levels between groups, as a potential biomarker parallel to the genetic enrichment findings.

Methods

Participants and Study Procedures

Children were recruited at Vanderbilt University in Nashville, Tennessee into three groups: co-occurring ASD and GID (ASD-GID); ASD without any GID (ASD-only); and GID without any ASD (GID-only). All children in the ASD groups were assessed with the Autism Diagnostic Observation Schedule (ADOS). Children in the GID groups were assessed by either parental report on a research-validated instrument that categorizes GI complaints into functional gastrointestinal disorders according to Rome-III criteria (the Questionnaire on Pediatric Gastrointestinal Symptoms, or QPGS; (Drossman, 2006; Walker, Caplan-Dover, & Rasquin-Weber, 2000)), or by a clinician who specializes in caring for children with GID. Details of these GID evaluations are articulated in the Methods section of Chapter II, with the only difference being that participants included here in Chapter III were not required to have data from both parental report and clinical evaluation. The concordance for presence of any type of functional GID in children when reported by parents on the QPGS compared to physician examination exceeds 90% (see Chapter II). Additionally, parents in the ASD-only group completed the same GID parental report instrument as the other two groups, to ensure the ASD-only group was devoid of any latent GID that was not identified at study enrollment. All children donated a blood or saliva sample. Exclusion criteria included severe sensory or motor impairment,

neurodevelopmental disorders of known etiology (Rett Syndrome, Tuberous Sclerosis, Down Syndrome, Phenylketonuria, 22Q Deletion Syndrome, Fragile X Syndrome and Neurofibromatosis), gestational age less than 36 or greater than 42 weeks, and birth weight less than 2500 grams. Inclusion criteria included age between 5 and 18 years at enrollment, meeting ASD criteria on the ADOS (for the ASD groups), and GI symptoms that had lasted more than a month (for the GID groups). The research protocol was approved by the Vanderbilt University Institutional Review Board, and written informed consent was obtained from parents of participants.

Specimen Collection and Nucleic Acid Extraction

Blood or saliva was collected from study participants. Blood was collected into both EDTA-coated (for DNA) and PAXgene Blood (for RNA) tubes (both from BD, Franklin Lakes, NJ). For participants who opted not to donate blood, saliva was collected into Oragene-DNA collection kits (DNA Genotek, Kanata, Ontario, Canada). Genomic DNA was extracted using the QIAamp DNA Kit (Qiagen, Valencia, CA). Total RNA was isolated from whole blood collected into PAXgene tubes using the PAXgene 96 Blood RNA Kit (BD) according to the manufacturer's protocol, which included a DNase I treatment. RNA was assessed for purity with a ND-1000 Nanodrop (Thermo Scientific, Wilmington, DE). Nearly all RNA samples had A260/A280 ratios of approximately 2.0; two samples had absorbance ratios below 1.90 and were thus excluded. Multiple RNA samples

were randomly chosen for integrity assessment using RNA 6000 Nano chips on an Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA). RNA Integrity Number was consistently greater than 9.0 across samples.

Genotypes of the ASD-Associated MET *Variant rs1858830*

Genotypes at rs1858830 were determined by direct sequencing, as described elsewhere ((Campbell, et al., 2007)). In brief, genomic DNA was extracted from blood or saliva specimens, and 20 ng was used as template for a polymerase chain reaction (PCR) amplification of a 652 bp amplicon which includes rs1858830, using the KOD Xtreme Hot Start DNA Polymerase kit (EMD Chemicals, Gibbstown, NJ) by the manufacturer's protocol. Primer sequences were: forward primer, GATTTCCCTCTGGGTGGTG; reverse primer, CAAGCCCCATTCTAGTTTCG. Thermal cycling conditions were as follows: 94º for 2 m, followed by 35 cycles of 98º for 10 s then 58º for 30 s then 68º for 1 m, and a final extension at 68º for 7 m. Agarose gel electrophoresis of the PCR product from a random sampling of reactions confirmed specific amplification of the 652 bp amplicon. A 2 µl aliquot of PCR product was diluted 1:10 in water, and direct sequencing off the reverse primer was performed using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were aligned to the reference sequence of the plus strand of chromosome 7 and chromatograms were visually inspected to determine genotypes using Sequencher version 4.10.1 (Gene Codes, Ann Arbor, MI). Genotypes were called while blinded to sample

identity and case status, and heterozygote calls were required to have a secondary peak on the chromatogram with at least 50% the height of the primary peak. Observed genotypes at rs1858830 were in Hardy–Weinberg equilibrium (χ^2; $P = 0.97$).

Measurement of MET mRNA Expression in Blood

For each sample, complementary DNA (cDNA) was reverse transcribed from 8 µl of input DNase-treated total RNA, using the SuperScript III First-Strand Synthesis kit primed with random hexamers in a 20 µl reaction, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Quantitative reverse transcriptase-polymerase chain reaction (qPCR) was performed using 2 µl of input cDNA in a 20 µl reaction, with TaqMan Gene Expression Master Mix and TaqMan Gene Expression assays, according to the manufacturer's protocol (both from Applied Biosystems). Assay IDs were as follows: *GAPDH*, Hs99999905_m1; *MET*, Hs01565584_m1; *NOD2*, Hs00223394_m1; *POLR2A*, Hs00172187_m1. Assays were manufacturer-validated, highly efficient, and amplified regions spanning exon-exon junctions. Each sample was run in triplicate and average values were used for analysis, with all four assays run for a given sample on the same 96-well plate. Each plate contained multiple no-template negative control wells and reactions were run on a CFX96 real-time system (Bio-Rad, Hercules, CA). Thermal cycling conditions were as follows: 50ºC for 2 m, 95ºC for 10 m, and 50 cycles of 95ºC for 15 s and 60ºC for 1 m. For

data analysis, raw amplification plots were visually inspected for wells that failed amplification and were subsequently manually excluded. Threshold cycle number (Ct) values were then exported for further analysis. Individual triplicate Ct values were compared for deviation from the replicate series mean, and if any replicate varied from the mean by more than 2 Ct values, it was excluded and the mean of the remaining duplicates was used as the average Ct value for that sample. After these exclusions, each sample had at least duplicates remaining for each target gene. Using the ΔCt method, *MET* Ct expression values were normalized to *POLR2A*, *GAPDH*, and *NOD2* Ct values separately.

Measurement of 15-F_{2t}-Isoprostane in Plasma

Whole blood was collected into EDTA-coated blood tubes (BD) and centrifuged at 1000 *g* for 10 m at 4ºC. Plasma was drawn off the sample and aliquoted before freezing at –80ºC. If blood was not centrifuged immediately, it was placed on ice within 15 m of blood draw. Plasma was frozen within 1 h of blood draw, and kept at –80ºC until further processing for measurement of isoP. IsoP levels were measured by gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NICI-MS) at the Vanderbilt University Medical Center Eicosanoid Core (Milne, et al., 2007). Samples were processed in batches of 21, with each batch containing an aliquot of an inter-batch normalizing standard. For analysis, samples within a batch were normalized to the inter-batch standard; normalized data are presented here. Additionally, 10 unaffected control

plasma samples were contributed by the laboratory of Dr. BethAnn McLaughlin (Vanderbilt University). These control samples came from male subjects who were all of non-Hispanic white ethnicity and race, and between 5 and 15 years old, making them similar to the experimental groups. Control participants were all assessed for the absence of seizures, neurodevelopmental delay, and GID.

Analyses

Study data were managed using REDCap, a secure, research-oriented, web-based application (Harris et al., 2009). Statistical analyses were computed using SPSS version 18.0.3 (IBM, Somers, NY) and Prism version 5.0d (GraphPad, La Jolla, CA). Prism was also used for generating plots. Categorical values (genotype and allele frequencies at rs1858830) were compared with a χ^2 test. Continuous measures (average ΔCt and isoP levels) were compared with a one-way ANOVA with Tukey post-hoc tests. IsoP levels were plotted as mean and 95% confidence intervals (CIs). For all statistical tests, a *P* value of less than 0.05 was considered significant.

Results

Characteristics of Study Participants

162 children were recruited for this study (Table 1; group sizes: ASD-GID = 74, ASD-only = 40, GID-only = 48), with characteristics similar to the cohort

Table 1. Characteristics of Study Participants

	ASD-GID (n = 74)	ASD-only (n = 40)	GID-only (n = 48)
Present in Chapter II cohort, % (n)	54.1 (40)	97.5 (39)	72.9 (35)
Age in years, mean (SD)	11.2 (3.5)	12.8 (3.2)	11.2 (3.3)
Male sex, % (n)	75.7 (56)	87.5 (35)	62.5 (30)
Ethnicity and race, % (n)			
Hispanic	8.1 (6)	2.5 (1)	6.2 (3)
Non-Hispanic white	78.4 (58)	82.5 (33)	89.6 (43)
Non-Hispanic black	10.8 (8)	10.0 (4)	4.2 (2)
Non-Hispanic other	2.7 (2)	5.0 (2)	0 (0)
ADOS classification, % (n)			
Autism	95.9 (71)	92.5 (37)	n/a
Autism Spectrum	4.1 (3)	7.5 (3)	n/a
Specimen availability, % (n)			
DNA	100 (74)	100 (40)	100 (48)
RNA	71.6 (53)	75.0 (30)	68.8 (33)
Plasma	40.5 (30)	72.5 (29)	41.7 (20)

described in Chapter II. On average, children were young adolescents (range of mean ages among groups: 11.2 – 12.8 y). The majority of participants were male (range among groups: 62.5 – 75.7%) and of non-Hispanic white self-reported ethnicity and race (range among groups: 78.4 – 89.6%). The vast majority of children in both ASD groups met ADOS criteria for autism (ASD-GID: 95.9%; ASD-only: 92.5%), with the remaining children in each group meeting criteria for autism spectrum. 100% of participants had DNA available for genetic analyses; RNA and plasma availability was incomplete for all groups, ranging from 40.5% to 75% available within a group. There was substantial overlap between children included in this study, and those included in Chapter II (ranging from 54.1% for ASD-GID to 97.5% for ASD-only). Nineteen children included in the current study were excluded from the ASD-GID group in Chapter II because they had a complete GI parent report, but did not have a clinical GI evaluation. The high concordance seen in Chapter II between parent report and clinical evaluation of presence of GI complaints (92% agreement) justified inclusion of these 19 children in the ASD-GID group for this study. Children in the ASD-only group were required to have a complete parent report of GI complaints, and subsequently not meet criteria for any GID classification (except fecal incontinence, which as discussed in Chapter II, is confounded by toilet training; four children met criteria for incontinence, but were still included in the ASD-only group), to ensure no latent or undiagnosed GID was present in the ASD-only group. Finally, gastrointestinal dysfunction, for this study, is treated as a binary

variable (yes or no), without distinction for type of GID. There is, however, substantial homogeneity of type of GID within this group (i.e., 85% constipation seen in Chapter II).

Genotypes at MET *rs1858830*

All samples were genotyped at the ASD-associated *MET* variant rs1858830 (Table 2). No significant differences were seen between ASD-GID and ASD-only groups for either genotypic or allelic frequencies. With an increased prevalence of *C/C* homozygotes, genotypic frequencies in the ASD-GID group were significantly different from those in the GID-only group ($P = 0.007$). This difference was not found for allelic frequencies ($P = 0.111$), however.

MET *Expression in Peripheral Blood*

RNA was available for a subset of participants in each group, and used to measure *MET* expression in peripheral blood by qPCR. Using the ΔCt method of relative quantification, *MET* mRNA expression was normalized to three different reference genes. The genes encoding housekeeping proteins GAPDH and POLR2A are standard normalizing genes. Peripheral blood expression of *MET* is primarily in monocytes (Beilmann, Vande Woude, Dienes, & Schirmacher, 2000). Thus, *NOD2*, which is a specific marker for monocytes, was also used for normalization (Ogura et al., 2001). Tables 3 and 4 show average ΔCt values, relative to these three normalization genes. Table 3 shows *MET* expression

Table 2. Genotype and Allele Frequencies at *MET* rs1858830

		MET Genotype				*MET* Allele		
	n	C/C	C/G	G/G	*P*	C	G	*P*
ASD-GID	74	0.432	0.392	0.176	Ref.	0.628	0.372	Ref.
ASD-only	40	0.500	0.400	0.100	0.532	0.700	0.300	0.309
GID-only	48	0.188	0.667	0.146	0.007	0.521	0.479	0.111

Table 3. Comparison of MET Expression in Peripheral Blood Measured by qPCR, Stratified by Study Group

	ASD-GID (n = 53)	ASD-only (n = 30)	GID-only (n = 33)	*P*
MET ΔCt, mean (SD)				
relative to POLR2A	10.6 (1.0)	11.0 (0.9)	10.7 (0.9)	NS
relative to GAPDH	14.8 (0.9)	15.2 (1.0)	14.8 (0.9)	NS
relative to NOD2	9.6 (0.9)	9.6 (1.0)	9.3 (1.0)	NS

Table 4. Comparison of MET Expression in Peripheral Blood Measured by qPCR, Stratified by rs1858830 Genotype

	C/C (n = 42)	C/G (n = 56)	G/G (n = 18)	*P*
MET ΔCt, mean (SD)				
relative to POLR2A	10.9 (0.9)	10.5 (1.0)	11.1 (0.8)	NS
relative to GAPDH	14.8 (0.9)	14.9 (1.0)	15.1 (0.9)	NS
relative to NOD2	9.6 (1.0)	9.4 (1.0)	9.9 (0.8)	NS

stratified by study group, whereas Table 4 shows expression stratified by genotype at rs1858830. No significant differences were seen in *MET* expression among study groups or genotypes, for any of the three normalizations.

IsoP Levels in Peripheral Blood Plasma

Plasma was available for a subset of participants and was used to measure isoP (Figure 1). Interestingly, the ASD-GID group mean was significantly elevated above the ASD-only group mean (ASD-GID mean 0.173 ng/ml, 95% CI 0.145 – 0.202; ASD-only mean 0.122 ng/ml, 95% CI 0.105 – 0.139). The ASD-only and GID-only group means were not significantly different (GID-only mean 0.135 ng/ml, 95% CI 0.111 – 0.160). ASD-GID, ASD-only, and GID-only all had significantly elevated group mean isoP levels compared to unaffected control children (Unaffected mean 0.056 ng/ml, 95% CI 0.045 - 0.067).

Discussion

Three distinct biomarkers were examined in the current study of stratified ASD participants. The most significant finding of this study is the elevation of plasma isoP levels in the ASD-GID group. The isoP elevation in ASD-only compared to unaffected controls is consistent with two previous reports, which showed a similar elevation in urinary isoP relative to controls (Ming, et al., 2005; Yao, et al., 2006). However, neither study reported isoP levels stratified by GID. Ming and colleagues did in fact report on presence of GID in their cohort, so

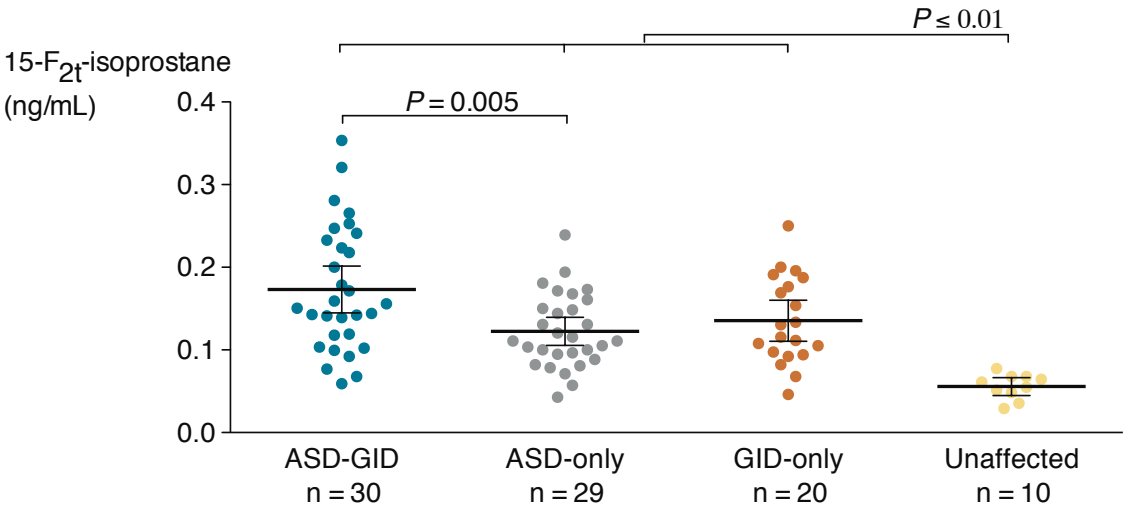

Figure 1. Plasma 15-F_{2t}-isoprostane levels among four study groups. Plasma was available for a subset of participants and IsoP was measured. ASD-GID group mean was significantly elevated above the ASD-only group mean (ASD-GID mean 0.173, 95% CI 0.145 - 0.202; ASD-only mean 0.122, 95% CI 0.105 - 0.139). ASD-only and GID-only group means were not significantly different (GID-only mean 0.135, 95% CI 0.111 - 0.160). All three experimental groups were significantly different from the unaffected group (Unaffected mean 0.056, 95% CI 0.045 - 0.067). Data are plotted as mean and 95% CI around the mean.

stratification would be possible in a re-analysis of their data. Yao and colleagues do not mention assessing for GID in their population, but, interestingly, their data show a subpopulation of ASD-diagnosed individuals with much higher isoP levels. In the context of the data reported here, assessing individuals in the Yao et al dataset for GID seems warranted. Given the findings reported in Chapter II (namely, high prevalence of nonverbal children with GID and possible under-reporting of GID by parents), it is likely that some fraction of the Yao cohort has undiagnosed, or diagnosed but unreported, GID. Moreover, elevation of isoP levels in the ASD-GID group, above that in both GID-only and ASD-only groups, is consistent with a hypothesis that dysfunctional biology in parallel organ systems (in this case, oxidative stress in the brain and gastrointestinal system, both of which have high metabolic activity) will have additive effects apparent in biomarker measures of that dysfunctional biology.

An additional primary objective of this study was to replicate the previously-reported enrichment of rs1858830 signal in an ASD-GID population (Campbell, et al., 2009). While the data reported here are exploratory and limited by sample size, the trends in rs1858830 allele frequency are consistent with what was hypothesized and with previous reports (Campbell, et al., 2009; Campbell et al., 2006). The ASD-associated *C* allele of rs1858830 was present at increased frequencies in both ASD groups (63 and 70%, which are even higher than that reported by Campbell et al (Campbell, et al., 2009)) relative to the GID-only group (52%), although this difference lacked statistical significance. Genotypic

frequencies, however, were significantly different between ASD-GID and GID-only. While it is surprising to see a relatively high *C* allele frequency in the ASD-only group, which would be inconsistent with the Campbell et al study (Campbell, et al., 2009), these data are inconclusive given the small sample size. Additionally, although case-control association studies can be confounded by population stratification, the cohort in this study was mostly self-reported non-Hispanic white (and self-report correlates highly with genetically-defined ethnicity and race (Tang et al., 2005)), making it unlikely that these data are confounded by racial differences. To further understand and formally test for replication of rs1858830 enrichment, a larger cohort will need to be studied in the future.

Given previous findings of MET expression differences in postmortem brain tissue of individuals with ASD (Campbell, et al., 2007), it was hypothesized that peripheral blood expression of MET would be different in cases versus controls, and potentially different within ASD cases when stratified by presence of GID. Postmortem brain MET protein expression differences were seen between ASD cases compared to controls, as well as differences within controls when stratified by rs1858830 genotype. There were no significant differences found in this study for *MET* mRNA expression in peripheral blood, when the cohort was stratified by group membership or by rs1858830 genotype. *MET* expression was normalized to both standard housekeeping genes, and a monocyte-specific marker, as *MET* expression in blood is primarily in monocytes, a cell population which could vary substantially between participants depending on immune status. The lack of

differences between groups reported here was initially surprising. Recent unpublished data, however, provides a useful context for interpreting these findings. In an exploratory study of over 120 samples from ASD, developmentally-delayed, and typically-developing children, measurement of MET protein expression in peripheral blood also showed no differences, by affected status or rs1858830 genotype (S. Spence, P. Gorrindo, D. Campbell, P. Levitt, and S. Swedo, unpublished observations). Replication of lack of expression differences in an independent cohort, and at the protein level, corroborates the mRNA findings reported here. However, in a cohort of over 70 samples in which peripheral blood mononuclear cells (PBMCs) isolated from fresh blood were stimulated with lipopolysaccharides (LPS) to induce MET expression, rs1858830 genotype did correlate significantly with MET protein expression differences (D. Campbell, personal communication). Integrating these unpublished findings and the data reported here, one possible interpretation of our findings is that MET expression in peripheral blood is very low at basal levels, but when MET expression is induced, the functional variant rs1858830 significantly affects transcriptional level of the gene. Thus, induced MET expression in PBMCs, altered by rs1858830 genotype, is consistent with the previously published postmortem brain expression findings (Campbell, et al., 2007).

These studies have a number of limitations, which are opportunities for improvement and subsequent studies. The genetic study would benefit from a

much larger sample. Additionally, family status (simplex versus multiplex) was not rigorously assessed in this cohort. Previous data on the rs1858830 ASD association demonstrates specific signal for multiplex, rather than simplex, families (Campbell, et al., 2006). Thus, a larger sample with additional recruiting resources and time may benefit from specifically recruiting multiplex families. The lack of MET expression differences, now clarified in the context of unpublished studies, was surprising. Future studies could test for MET expression differences in response to LPS stimulation. However, it should be noted that this is a nontrivial endeavor, as PBMC isolation and stimulation requires significant technical skills. Finally, while the isoP findings are exciting, characterizing lipid status (including low-density lipoprotein, LDL) in these samples will be required for a more complete understanding of the data. Oxidation of LDL can lead to isoP formation (Lynch, Morrow, Roberts, & Frei, 1994), and given the elevated mean body-mass index (BMI) seen in the ASD groups (Chapter II), it is possible that elevated LDL levels contributed to elevated isoP levels. Importantly, BMI was elevated in both ASD groups, with no significant difference between them, so it is unlikely that there is a ASD-GID group specific effect of LDL levels, however this remains to be formally tested in future studies.

Conclusion

The studies reported here were grounded in an original finding of genetic signal enrichment in a biologically-stratified subpopulation of individuals with

ASDs (Campbell, et al., 2009). The overall aim of these studies was to test the validity of biological stratification as a viable and powerful strategy to address heterogeneity within the ASDs. While the genetic and transcript expression data were inconclusive, the isoP findings provide strong support for stratification as a useful approach for studies of ASD. Future studies may benefit from analogous stratification on other readily assessed clinical and biological aspects of the ASDs, such as presence of seizures or immune dysfunction. Moreover, the elevated isoP levels in the ASD-GID group parallel clinical and behavioral differences in this group (increased language and social impairment reported in Chapter II). It remains to be tested if there is a direct relationship between these behavioral findings and isoP levels. Finally, changes in isoP levels can change rapidly within an individual in response to stimuli, which offers the possibility of isoP level as a biomarker sensitive to treatments for GID.

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CHAPTER IV

Met Signaling Is Involved In Gastrointestinal Repair After Acute Epithelial Injury

Introduction

The Met receptor tyrosine kinase, through activation by its endogenous ligand hepatocyte growth factor (HGF; also known as Scatter Factor), provides the molecular initiation signal for downstream intracellular pathways that underlie a variety of biological processes (Trusolino, Bertotti, & Comoglio, 2010). Met signaling is implicated in the development of multiple tissues and organ systems, including the placenta (Uehara et al., 1995), liver (Schmidt et al., 1995), and nervous system (Akimoto et al., 2004; Judson, Amaral, & Levitt, 2010a; Judson, Bergman, Campbell, Eagleson, & Levitt, 2009; Judson, Eagleson, Wang, & Levitt, 2010b; Tyndall & Walikonis, 2006). MET was originally discovered as a proto-oncogene (Cooper et al., 1984), and the data supporting MET's involvement in cancer pathology is vast (Birchmeier, Birchmeier, Gherardi, & Vande Woude, 2003; Trusolino, et al., 2010). Acute injury paradigms have also demonstrated the importance of Met signaling for tissue repair and regeneration in multiple organs, including the liver (Borowiak et al., 2004; Huh et al., 2004) and skin (Chmielowiec et al., 2007). Of particular relevance to the studies described here, multiple reports have also implicated Met signaling in repair of the gastrointestinal (GI) epithelium after acute injury, as administration of exogenous

HGF bolsters repair of the damaged epithelium (Numata et al., 2005; Oh et al., 2005; Tahara et al., 2003).

Recent studies have implicated MET signaling in developmental neurobiology. As an increasing number of neurodevelopmental roles played by Met were reported (Akimoto, et al., 2004; Tyndall & Walikonis, 2006), and because the *MET* gene resides under a linkage peak on chromosome 7q that has been reported multiple times in studies of autism spectrum disorders (ASDs) (International Molecular Genetic Study of Autism Consortium, 1998, 2001; Lamb et al., 2005; Philippe et al., 1999; Schellenberg et al., 2006), *MET* was examined as a candidate risk gene for ASDs (Campbell et al., 2006). Multiple studies by independent investigators and using distinct cohorts have shown association between genetic variants in *MET* and ASD diagnosis (Campbell, Li, Sutcliffe, Persico, & Levitt, 2008; Campbell, et al., 2006; Jackson et al., 2009; Sousa et al., 2009; Thanseem et al., 2010). As individuals with ASDs can have co-occurring gastrointestinal problems (Geschwind, 2009), and given the pleiotropic activity of Met signaling in both the nervous and GI systems, a subsequent study showed significant enrichment of a genetic variant in *MET* specifically in individuals with co-occurring ASDs and GI problems, compared to those with only ASDs (Campbell et al., 2009). This clinical context provides a compelling rationale for studying the specific roles played by Met signaling in the functioning of the GI system.

The objectives of the present study were threefold. The first objective was to study the developmental expression patterns of Met protein in the rodent GI system. These expression data informed the selection of a tissue-specific Cre recombinase line to specifically delete *Met* from the GI epithelium. Conditional null mice, in which a tissue-specific Cre line is leveraged to only delete a gene of interest in a subpopulation of cells, enable studies which are not subject to confounds of whole organism genetic deletion. In the case of *Met*, full knockout mice are embryonically lethal (Bladt, Riethmacher, Isenmann, Aguzzi, & Birchmeier, 1995) and thus a conditional deletion strategy is required for studying adult phenotypes. Thus, the second objective was to assess the consequences of loss of Met signaling in the GI epithelium in conditional null mice. Finally, the third objective was to assess conditional null mice for their susceptibility to acute GI injury and capacity to repair.

Methods

Animals

Mice used in these studies were on the C57Bl/6 background and housed in a vivarium on a 12 h light/dark cycle. Water and chow were provided *ad libitum*. Gastrointestinal-specific *Met* conditional null mice ($Villin^{cre/+}; Met^{fx/fx}$) were generated by mating mice homozygous for a *Met* allele, in which exon 16 is flanked by *loxP* sites (Huh, et al., 2004; $Met^{fx/fx}$, courtesy of Dr. Snorri

Thorgeirsson, NIH/Center for Cancer Research, Bethesda, MD) to *Villin*^{cre} transgenic mice (El Marjou et al., 2004; courtesy of Dr. Brent Polk, Children's Hospital Los Angeles, Los Angeles, CA) that were also heterozygous for the floxed *Met* allele (*Villin*$^{cre/+}$*; Met*$^{fx/+}$). Animals were genotyped by polymerase chain reaction (PCR) as previously described (Judson, et al., 2009). In brief, *Villin*^{cre} primers were: forward 5'-TTCGGCTATACGTAACAGGG-3', reverse 5'-TGCATGCAACGAGTGATGAG-3'. *Met*^{fx} primers were: forward 5'-GCAACTGTCTTTTGATCCCTGC-3', reverse 5'-TGTCCAGCAAAGTCCCATGATAG-3'. PCR thermal cycling conditions were as follows: *Villin*^{cre}, 5 m at 94°C, 35 cycles of 94°C for 45 s, 55°C for 30 s, 72°C for 1 m, and a final 7 minutes at 72°C; *Met*^{fx}, 5 m at 94°C, 35 cycles of 94°C for 1 m, 60°C for 1 m, 72°C for 2 m, and a final 5 minutes at 72°C. For the studies reported here, the term "wildtype" refers to both *Villin*$^{+/+}$*; Met*$^{fx/fx}$ homozygous and *Villin*$^{+/+}$*; Met*$^{fx/+}$ heterozygous control animals, as they are both considered equivalently unaffected at the *Met* locus. Developmental weight growth of conditional null and wildtype control animals was measured by weighing pups on the day of birth (postnatal day 0, P0), P4, P7, P14, and day of weaning on P21. Adult male animals ranged in age from P60 to P125. Studies were completed according to experimental protocols approved by Institutional Animal Care and Use Committee at the University of Southern California. Animal suffering and number of animals used in experiments were minimized.

LoxP Recombination Assay

As previously described (Eagleson, Campbell, Thompson, Bergman, & Levitt, 2010), genetic recombination of the floxed *Met* allele was confirmed in a PCR-based assay on genomic DNA isolated from colon and brain tissue. Cre-mediated excision of floxed *Met* exon 16 generates a unique DNA sequence which acts as template for amplification of a 650 bp amplicon using 5'-CCAGGTGGCTTCAAATTCTAAGG-3' and 5'-CAGCCGTCAGACAATTGGCAC-3' primers. Non-excised floxed exon 16 acts as template for amplification of a 313 bp amplicon using 5'-CCAGGTGGCTTCAAATTCTAAGG-3' and 5'-TTAGGCAATGAGGTGTCCCAC-3' primers.

Histology

Mice were sacrificed by cervical dislocation under anesthesia (isoflurane), and tissue was harvested and immersion-fixed in 0.1 M sodium phosphate-buffered 4% paraformaldehyde (PFA) overnight at 4°C. Embryonic tissue was fixed intact and adult tissue was flushed with ice-cold PBS and then prepared as Swiss rolls prior to fixation (Moolenbeek & Ruitenberg, 1981). Tissue was cryoprotected by serial incubations in 10%, 20% and 30% graded sucrose solutions (% w/v in 0.1 M sodium phosphate buffered 0.9% saline, PBS) for approximately 12 hours each, and then frozen over liquid nitrogen in TFM embedding medium (Triangle Biomedical Sciences, Durham, NC). Tissue was stored at –80°C until sectioning

on a cryostat at 14 to 25 µm. Slides were processed for staining with either hematoxylin and eosin (HE) or anti-Met immunohistochemistry (described below).

Mucosal thickness was measured in HE stained slides at the proximal and distal ends of the Swiss roll, measuring from the basal extent of the muscularis mucosae to the apical extent of the epithelium. For each animal, four measurements were made (two per end of the roll) and averaged.

Immunohistochemistry

Immunohistochemical staining of Met protein expression was performed as described previously (Judson, et al., 2009), with minor modifications noted below. Sections on slides were dried overnight at room temperature, fixed for 15 m with 4% PFA and rinsed with PBS. Slides were incubated for 5 m in 0.3% H_2O_2 in methanol, rinsed in 0.2% Triton-X-100 in PBS (PBS-0.2Tx), incubated in 0.1 M Tris-glycine (pH 7.4), rinsed again in PBS, and then incubated in Blotto-Tx (4% Carnation dried milk in PBS-0.2Tx) for at least 1 h. Sections were subsequently incubated in polyclonal goat anti-Met primary antibody (HGFR, catalog #AF527, R&D Systems, Minneapolis, MN), diluted 1:250 in 0.1% Triton-X-100 in PBS (PBS-0.1Tx), for 2 h at room temperature. Sections were then washed several times in 0.2% Tween-20 in PBS (PBS-0.2Tw), and incubated with Biotin-SP-conjugated donkey anti-goat IgG secondary antibody (Jackson Immunoresearch), diluted 1:500 in PBS-0.1Tx, for 1 h at room temperature. Sections were then washed in PBS-0.2Tw before processing with the ABC Elite

kit (Vector, Burlingame, CA), followed by a 5 m incubation in 0.5% 3,3'-diaminobenzidine with 0.015% H_20_2. Finally, slides were dehydrated in ethanol, cleared in d-limonene (VWR, Radnor, PA), and cover-slipped in DPX mounting medium (EMS, Hatfield, PA).

Dextran Sulfate Sodium Treatment

Dextran Sulfate Sodium (DSS) was administered to male adult null and wildtype animals to induce acute gastrointestinal epithelial injury (Kimura et al., 1993; Whittem, Williams, & Williams, 2010). Several days before DSS treatment, adult (P60-125) male animals were separated to individual cages in which Lixit valves were removed and water bottles were the only source of water in the cage. At approximately 12 pm on day 0, the water bottle was replaced with an equivalent bottle containing 200 mL of 0.45 μm cellulose acetate-filtered 3% DSS (w/v in drinking water; molecular weight 36 – 50 kilodaltons; MP Biomedicals, Solon, OH). Chow weight was also recorded on day 0. DSS volume and chow weight were recorded at the end of the treatment period on day 8, when animals were sacrificed and tissue was collected. Colon tissue was collected as a single specimen from cecum to rectum, flushed, weighed and measured, filleted and prepared as a Swiss roll. During the experiment, animals were weighed and stool samples were collected daily to calculate the Disease Activity Index (DAI) (Cooper, Murthy, Shah, & Sedergran, 1993) for each day of treatment. Fecal occult blood was detected by guaiac-based Coloscreen-ES tests (Helena

Laboratories, Beaumont, TX). During the DSS administration, food and DSS solution were available to animals *ad libitum*. DSS was administered in two paradigms, as follows: chronically for 8 days; and for 4 days followed by 4 days of recovery in which fresh, non-DSS water replaced DSS in the cage. Additionally, water-only control experiments were performed, with tissue harvested in the same manner for DSS-treated tissue. For the duration of the experiments, from initial cage separation, through harvesting, tissue processing, and analysis, the investigator was blinded to animal genotype.

Histologic Colitis Scoring

HE stained sections were scored by an expert pathologist who was blinded to genotype and DSS condition (chronic, recovery, water-only control), using a previously-described and commonly-used histologic colitis score (Dieleman et al., 1998). Using this scoring system, and with the investigator blind to treatment category and genotype, 3 individual scores were generated independently: an inflammation score (from 0/none to 3/severe); an inflammation extent score (from 0/none to 3/transmural); and a crypt damage score (from 0/none to 4/entire crypt and surface epithelium lost). Percent involvement, for inflammation and crypt damage, was also assessed (from 1/less than 25% to 4/greater than 75%). A final score of colitis extent and severity was calculated by multiplying the percent involvement of inflammation times the inflammation scores, and the percent

involvement of crypt damage times the damage score, and summing the results for a total injury and inflammation score, which is reported here.

Microscopy

An Axioplan II microscope and AxioCam HRc and MR3 cameras (Zeiss, Jena, Germany) were used for micrograph acquisition. Image annotation, resizing, de-skewing and distance measurements were completed using Axiovision software version 4.1 (Zeiss).

Data Analysis

Study data were managed using REDCap, a secure, research-oriented, web-based application (Harris et al., 2009). Statistical analyses were computed using SPSS version 18.0.3 (IBM, Somers, NY) and Prism version 5.0d (GraphPad, La Jolla, CA). Prism was also used for generating plots. Continuous measures (developmental weight, mucosa thickness, colon length ratio, histological colitis score) were plotted as mean and 95% confidence intervals (CIs) and were compared between wildtype and conditional nulls with two-tailed t-tests. Weight loss and DAI during the course of DSS experiments were compared with a repeated measures two-way ANOVA for time and genotype. For all statistical tests, a *P* value of less than 0.05 was considered significant.

Results

Normative Met Gastrointestinal Expression

Met protein expression in the GI system was mapped in wildtype animals using immunohistochemistry. Three ages were selected for study: embryonic day 15 (E15), P21, and adulthood (Figure 1). E15 is a time of rapid GI epithelium development, and Met expression was seen primarily in cells lining the lumen, precursors to the mature mucosa. By P21, colonic crypts are near maturity, and Met expression is still apparent. In the adult, Met expression was still apparent in epithelial cells, with a preponderance of staining in the lower portion of the colonic crypts, a site of continual cellular proliferation in the adult.

Genetic Recombination in Conditional Nulls

Villin^{cre} mice express Cre recombinase protein specifically in the GI epithelium, from early embryogenesis through adulthood (El Marjou, et al., 2004). The expression studies in Figure 1 suggested that the *Villin*^{cre} line would be an appropriate tissue-specific Cre line for *Met* conditional deletion from the GI epithelium. *Villin*^{cre}-mediated recombination of the floxed allele of *Met* exon 16 in colons of conditional null mice was confirmed by a PCR-based assay (Figure 2). Genomic DNA from colons and brains of wildtype and null animals was extracted. DNA from both tissues and both genotypes amplified a control amplicon, the template for which is the non-recombined floxed *Met* allele. The band for the

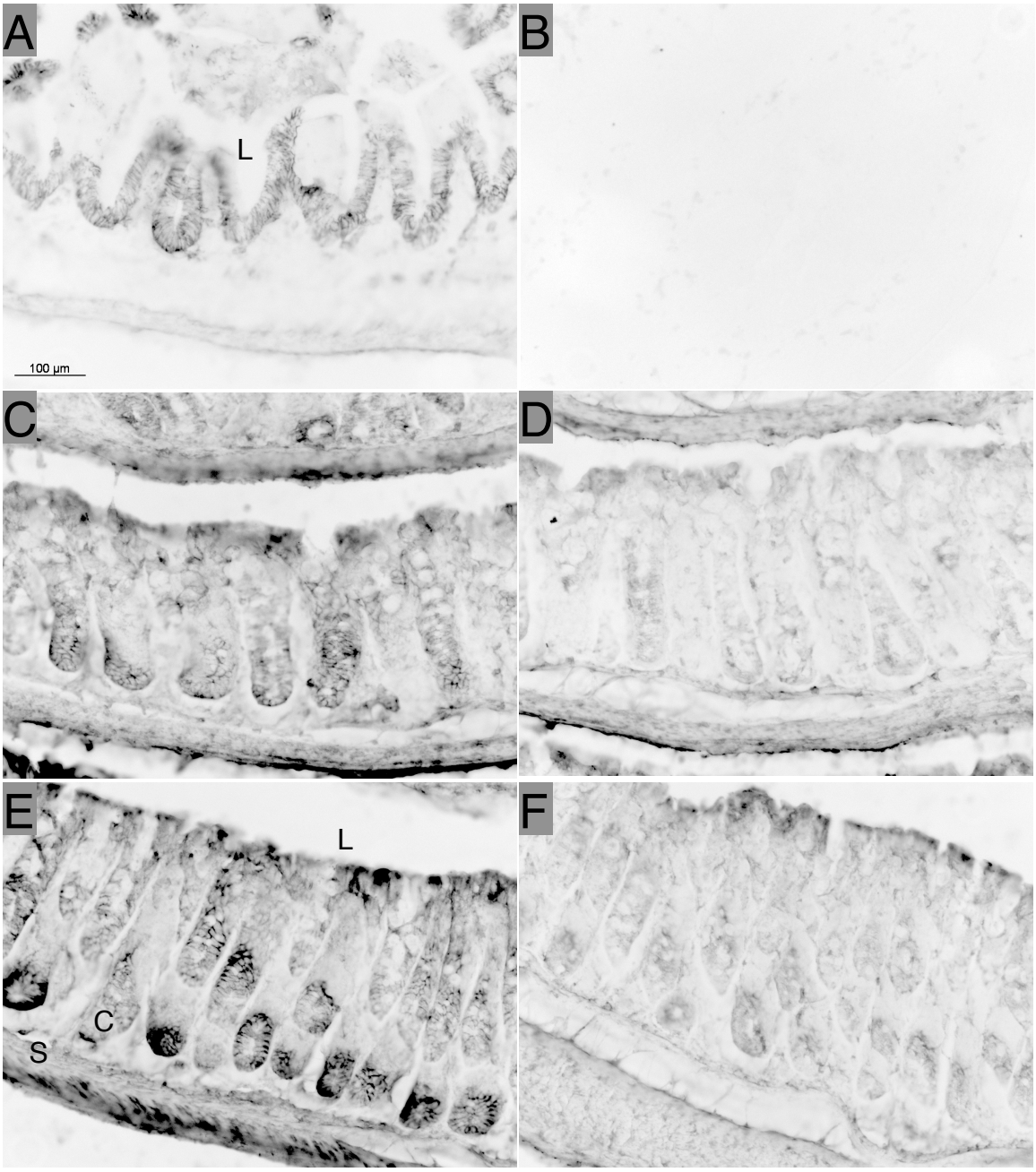

Figure 1. Immunohistochemical stain for Met expression in the mouse gastrointestinal system. *A*, E15; *B*, E15 without primary antibody. Met expression is seen in the developing epithelium, preceding the development of mature crypts. *C*, P21; *D*, P21 without primary. At P21, colonic crypts are nearly mature, and Met signal is still apparent in epithelial cells lining the crypts. *E*, adult; *F*, adult without primary. Met expression persists into adulthood in crypt epithelial cells. Scale bar, 100 µm. Abbreviations: L, lumen; S, submucosa; C, crypt.

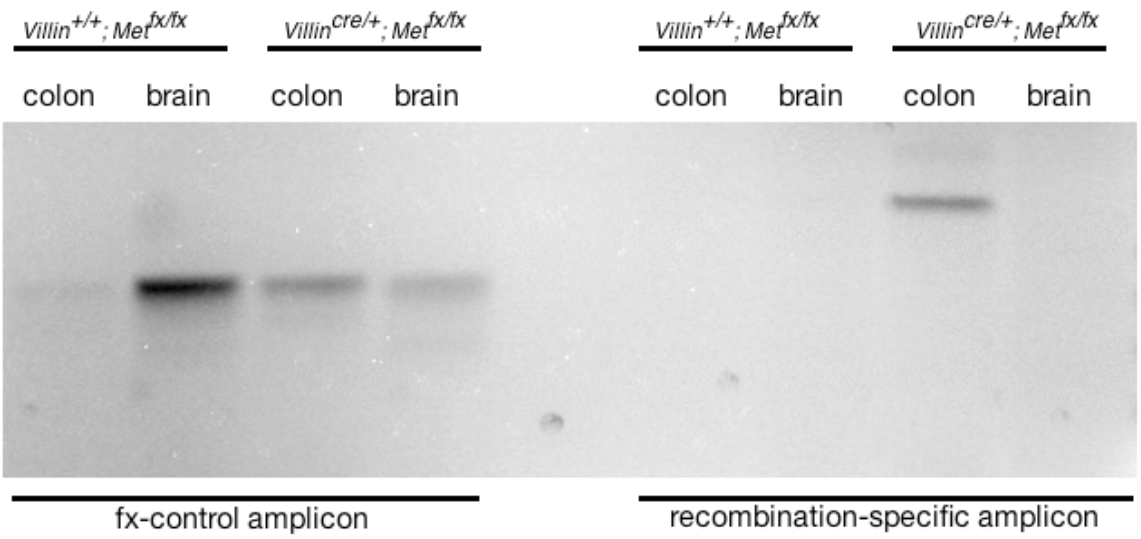

Figure 2. Genetic recombination in *Villin$^{cre/+}$; Met$^{fx/fx}$* animals, specifically in the colon. Genomic DNA was extracted from colon and brain tissue from *Villin$^{+/+}$; Met$^{fx/fx}$* wildtype and *Villin$^{cre/+}$; Met$^{fx/fx}$* conditional null animals and assessed with a PCR-based recombination assay. At left, all tissues show amplification of the fx-control amplicon. At right, only colon in *Villin$^{cre/+}$; Met$^{fx/fx}$* null animals shows a specific band indicating recombination.

Villin$^{+/+}$*; Met*$^{fx/fx}$ wildtype (lane 1) is faint, but present. Cre protein in *Villin*^{cre} mice is expressed specifically in GI epithelial cells, leaving other GI cell types (such as smooth muscle, immune, enteric nervous) untargeted for recombination. As such, non-recombined floxed *Met* DNA persists in DNA extracted from a heterogenous GI cell population (such as that from whole colon); the control amplicon is also generated from *Villin*$^{cre/+}$*; Met*$^{fx/fx}$ colon tissue (lane 3). Importantly, the recombination-specific amplicon is only generated from colon DNA of *Villin*$^{cre/+}$*; Met*$^{fx/fx}$ animals, demonstrating specific Villin^{cre}-mediated recombination of the floxed *Met* allele only in colon tissue. As previously reported, genetic excision of this floxed *Met* allele is sufficient to preclude Met receptor phosphorylation in response to HGF stimulation, indicating that recombination successfully incapacitates Met receptor signaling function (Judson, et al., 2010b).

Developmental Growth in Conditional Nulls

As the GI epithelium is critical for nutrient absorption, the developmental weight gain of conditional null pups was measured (Figure 3). Five to 10 animals at each age, for each genotype and sex, were weighed on day of birth (P0), P4, P7, P14 and day of weaning (P21). Comparing wildtype versus nulls in pre-weaning males and females, as well as adult males, showed no significant differences in weight.

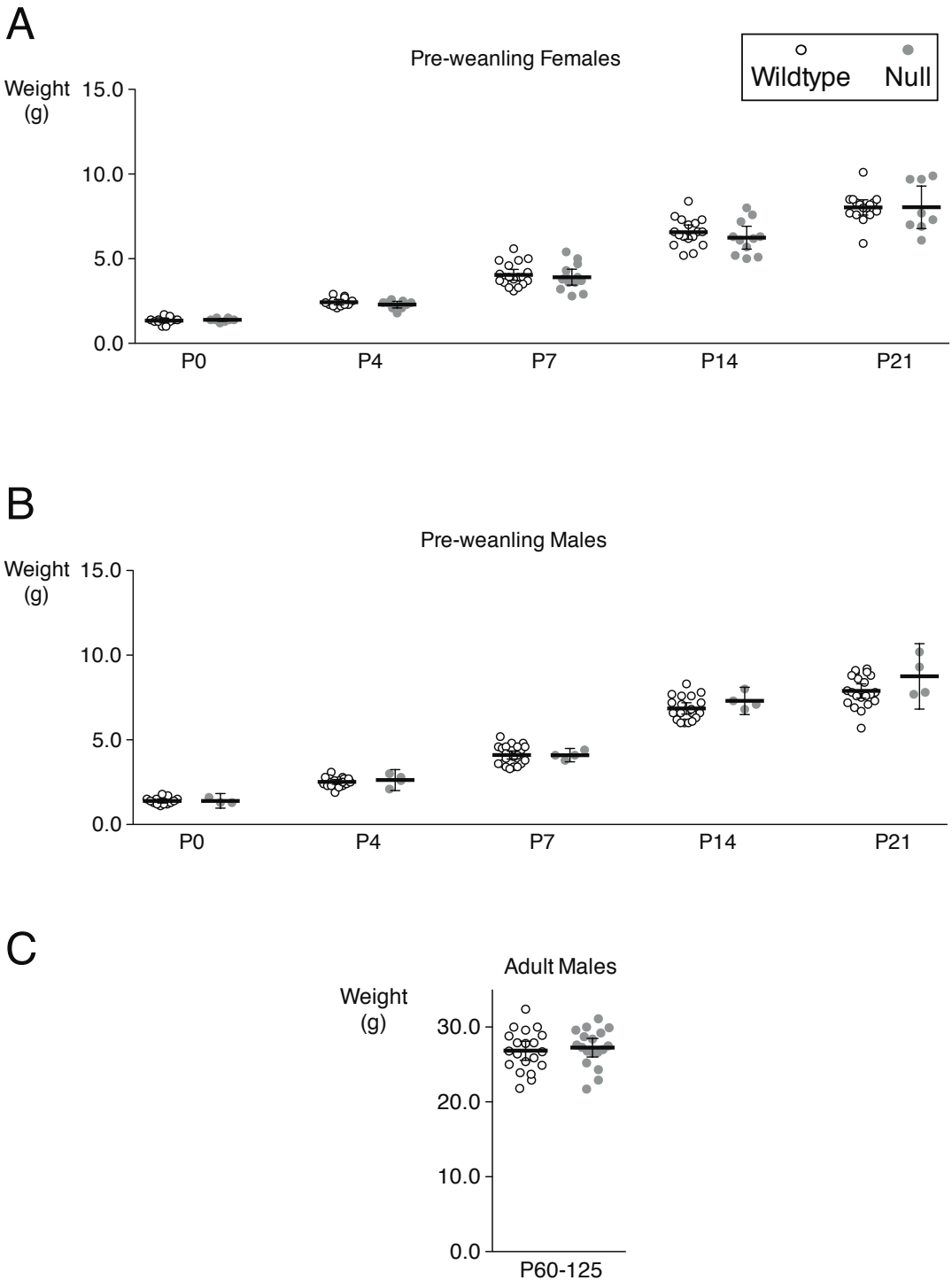

Figure 3. Comparing wildtype versus conditional null animals for weight gain during adolescence and in adulthood. *A*, pre-weanling females; *B*, pre-weanling males; *C*, adult males. No significant differences were found between genotypes, for either sex, at any age. Horizontal bars indicate means; vertical bars are 95% CIs.

Histology in Conditional Nulls

As Met signaling is important in the development of other organs (Schmidt, et al., 1995; Uehara, et al., 1995), it was hypothesized that loss of Met signaling in GI epithelial cells could alter development of the colonic mucosa. Qualitative histological examination of Swiss roll preparations of mouse colon (wildtype n = 5, null n = 6) showed no gross differences between genotypes (Figure 4). Quantitative analysis of mucosal thickness showed no difference between genotypes (Figure 5; mean thickness in μm ± SD: wildtype 251.8 ± 18.4; null 242.3 ± 18.1).

Systemic Effects of DSS Treatment

Although conditional null animals did not have a measurable histological difference in mucosal thickness (Figure 5), because Met signaling is involved in repair and regeneration of other tissues (Borowiak, et al., 2004; Chmielowiec, et al., 2007; Huh, et al., 2004) and HGF administration augments GI epithelial repair (Numata, et al., 2005; Oh, et al., 2005; Tahara, et al., 2003), *Met* conditional null animals were subjected to acute injury experiments to directly assess the response of null animals to GI injury. DSS is a commonly used model for acute GI epithelial injury and repair, and was the paradigm used in these studies. As is well known, DSS treatment causes colonic shortening, due to inflammation and edema (Figure 6A). For both wildtype (n = 14) and null (n = 11) animals, colon length (relative to the animal's total body weight) was significantly decreased with

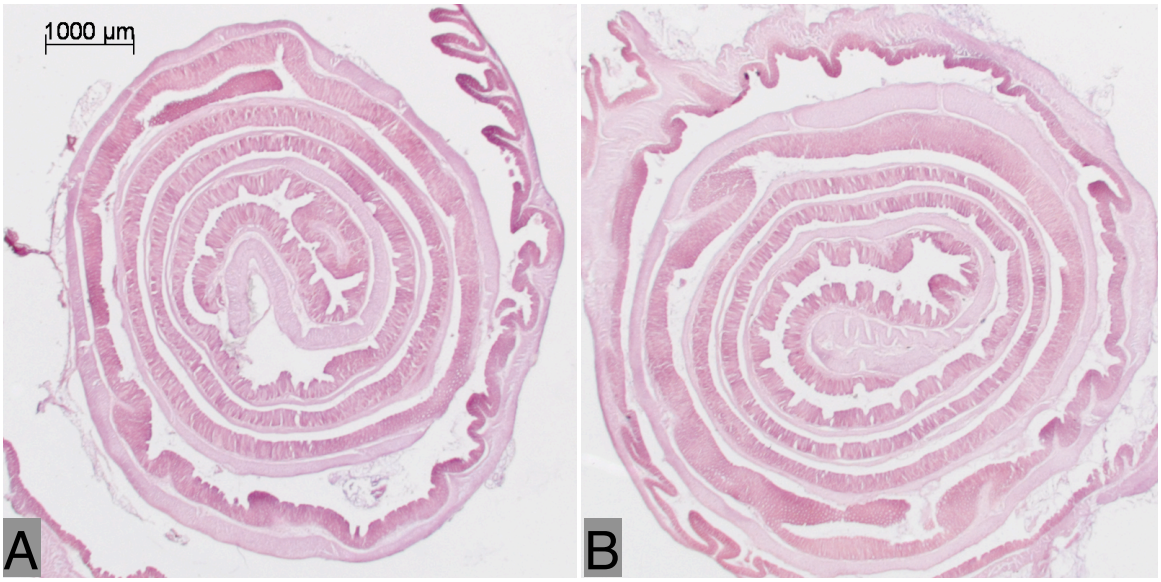

Figure 4. Swiss roll preparation of mouse colon, comparing wildtype and null mice. *A*, *Villin*$^{+/+}$*; Met*$^{fx/fx}$ wildtype. *B*, *Villin*$^{cre/+}$*; Met*$^{fx/fx}$ conditional null. In both panels, the rectum is located at the center of the roll, and sections are HE stained.

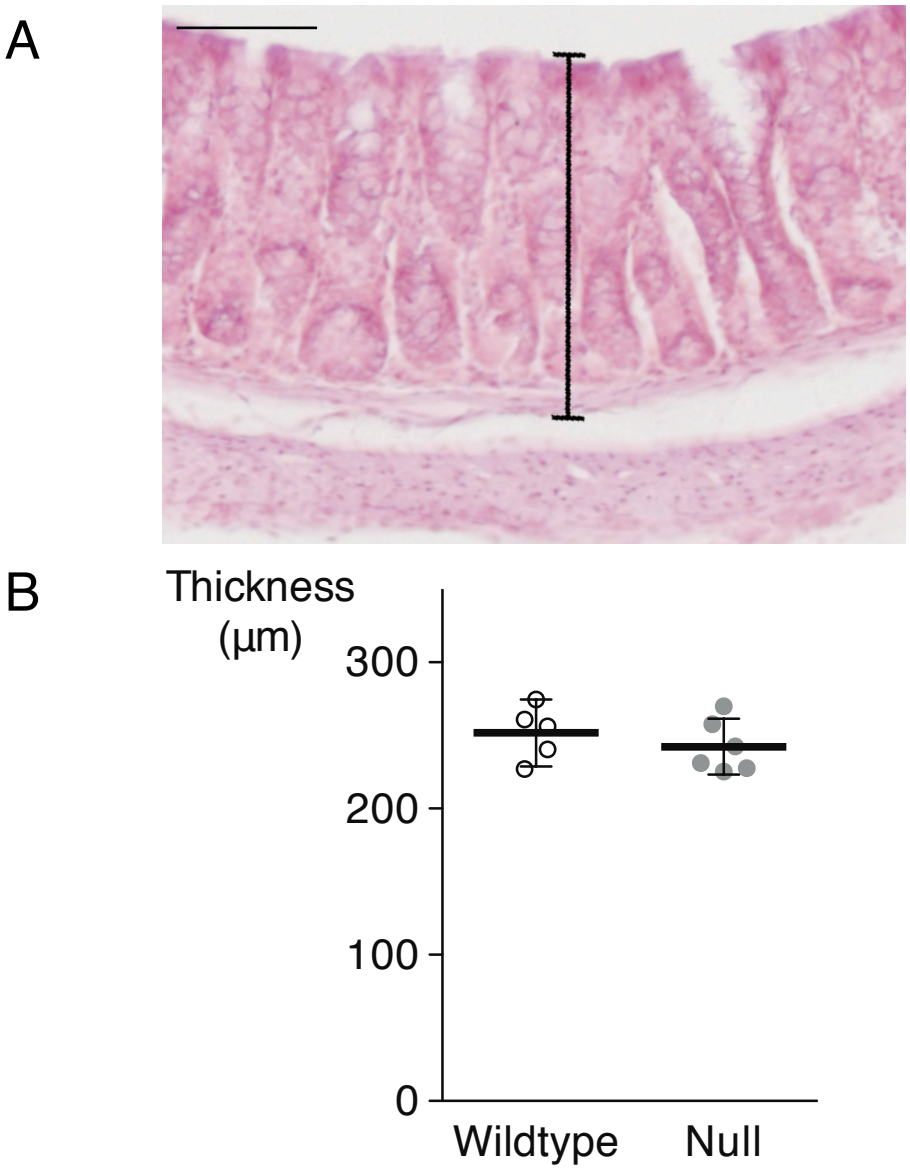

Figure 5. Mucosa thickness in conditional null mice. *A*, Micrograph of wildtype tissue showing an example measurement of mucosa thickness, 265 µm. Scale bar 100 µm. *B*, No significant difference in mucosa thickness between genotypes (wildtype mean 251.8 µm, SD 18.4; null mean 242.3 µm, SD 18.1).

DSS treatment relative to water-only control treatment (Figure 6B; $P \leq 0.01$). Within DSS and water-only treatment groups, no difference was seen between genotypes (group mean ratios in cm/g ± SD were as follows: wildtype, water 0.368 ± 0.024, DSS 0.312 ± 0.040; null, water 0.375 ± 0.044, DSS 0.288 ± 0.025). During the course of DSS treatment over 8 days, animal body weight was measured daily (Figure 7A). On day 5 of treatment, animals of both genotypes begin rapidly losing body weight (Figure 7B, which has an enlarged y-axis of data in Panel A). No significant differences in total weight lost were seen between genotypes (mean in percent of day 0 weight lost ± SD: wildtype 12.7 ± 6.3; null 11.5 ± 2.7). DAI (Cooper, et al., 1993) was also calculated for each day of DSS treatment. DAI is a composite score of weight loss, fecal occult blood, and stool consistency, with higher numbers indicating increased disease activity. No significant differences in DAI were seen between genotypes (Figure 7C). No significant differences were seen between genotypes for chow (mean in g ± SD: wildtype 30.2 ± 4.5; null 29.6 ± 4.5) or DSS (mean in mL ± SD: wildtype 52.4 ± 22.7; null 48.0 ± 4.9) consumed over the 8 days of DSS treatment.

Histologic Colitis Score After Chronic DSS

Pathological effects of DSS-induced acute injury are commonly scored using a composite histologic colitis score, which rates extent and severity of inflammation and extent of crypt damage (Dieleman, et al., 1998). In this metric, higher scores indicate worse injury and inflammation, and the maximum score is

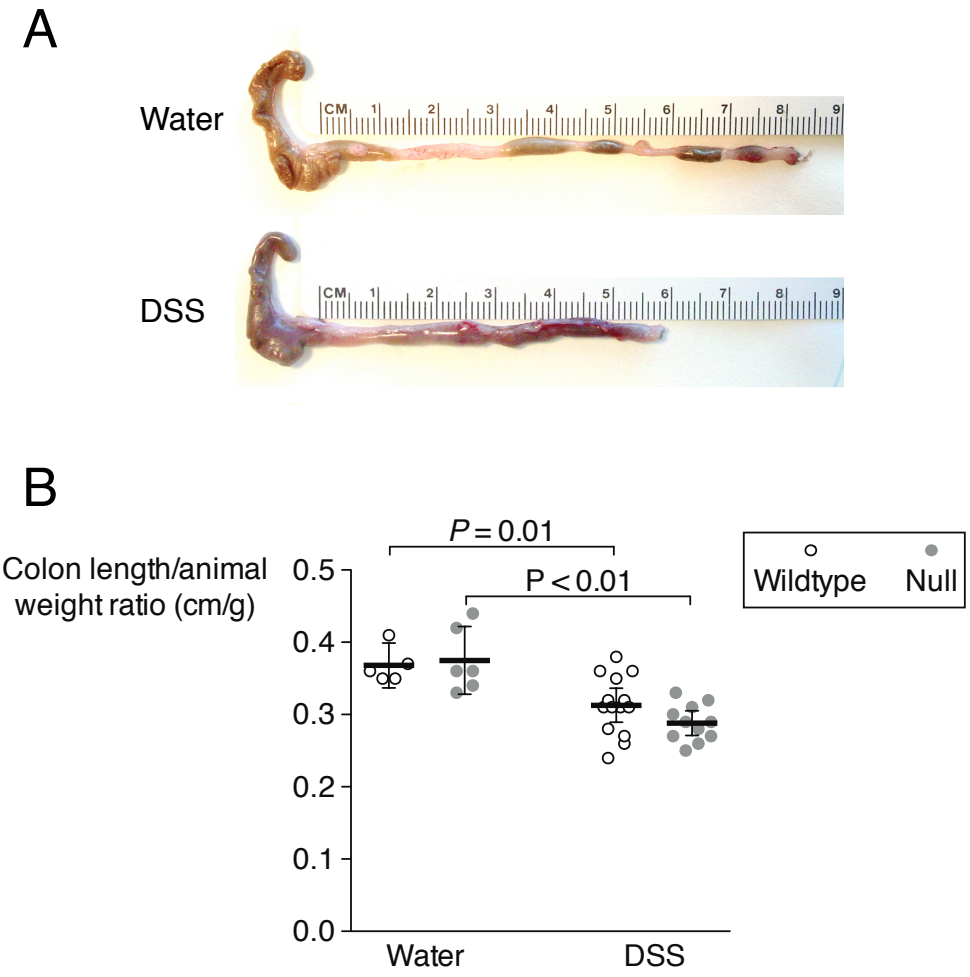

Figure 6. DSS treatment causes colonic shortening. *A*, Examples of harvested colons from wildtype animals, comparing animals fed drinking water versus 3% DSS for 8 days. *B*, Group data for wildtype and null animals on water versus DSS. No differences between genotypes in either water or DSS treatment groups. Both genotypes showed significant shortening with DSS treatment. Group mean ratios in cm/g ± SD were as follows: wildtype, water 0.368 ± 0.024, DSS 0.312 ± 0.040; null, water 0.375 ± 0.044, DSS 0.288 ± 0.025. Horizontal bars indicate means; vertical bars are 95% CIs.

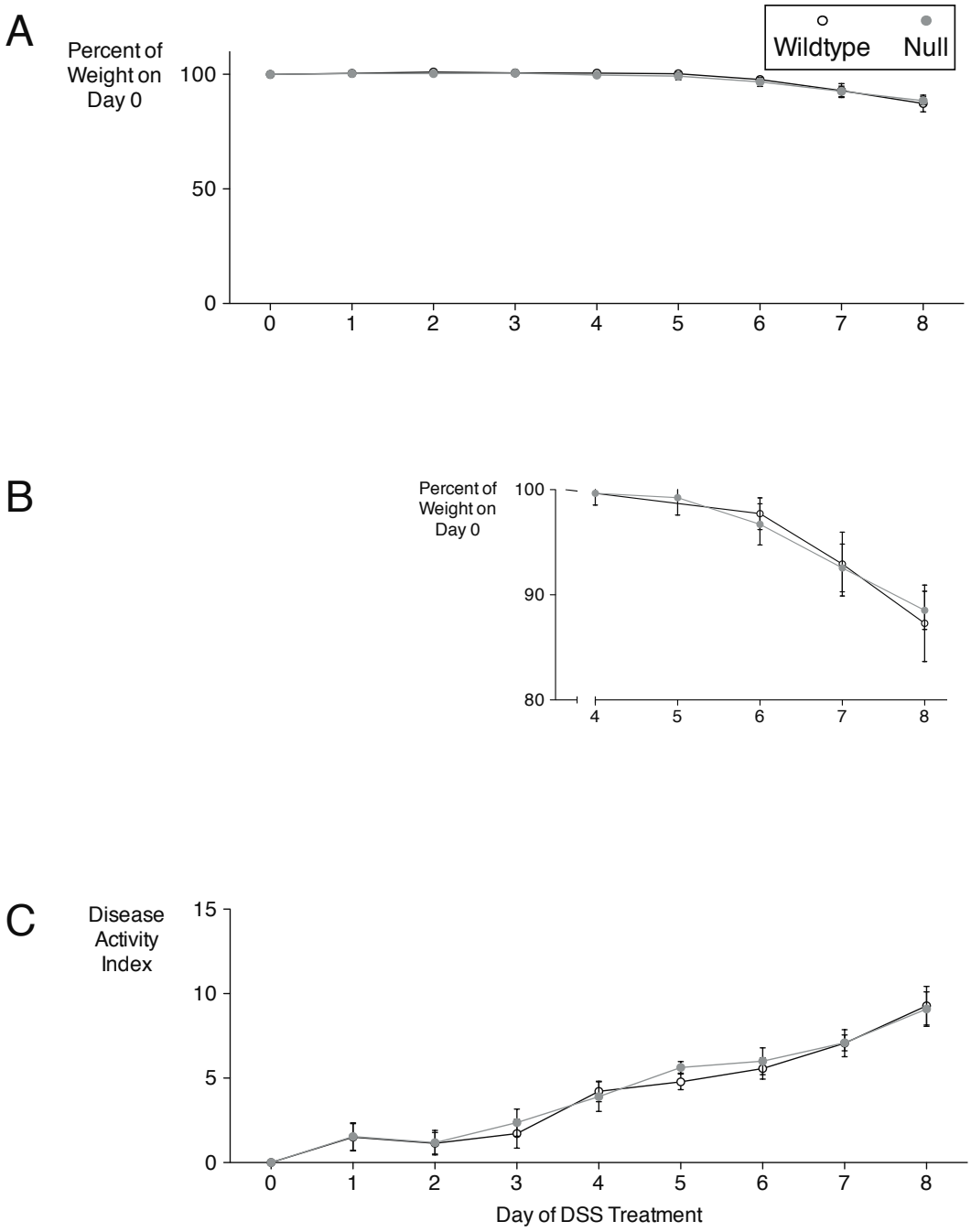

Figure 7. Systemic effects of DSS treatment, comparing wildtype versus null animals. *A*, Percent of weight relative to Day 0 of 3% DSS treatment for 8 days. *B*, Focused view of weight loss from *A* showing days 4 to 8 of DSS treatment on a enlarged vertical scale. *C*, Disease Activity Index over DSS treatment period. No significant differences between genotypes for weight loss or Disease Activity Index.

40 for a single animal. An expert pathologist (Dr. Kay Washington, Vanderbilt University Medical Center), blinded to genotype and DSS versus water-only treatment, scored tissue for colitis (Figure 8). At low power magnification, no gross differences were readily apparent between genotypes (Figure 8A-B). Higher power magnification showed dense infiltration of basophilic inflammatory cells and obliteration of the normal crypt axis and architecture, although with no apparent qualitative differences between genotypes (Figure 8C-D). Statistical comparison of total histologic scores between genotypes showed no difference after DSS treatment (Figure 8E; group mean score ± SD: wildtype 29.4 ± 6.1; null 31.7 ± 7.3). Water-only controls showed no injury or inflammation, for either genotype (Figure 8E).

Histologic Colitis Score After Recovery From DSS

Because Met signaling has been implicated in tissue repair and regeneration, *Met* conditional null animals were tested for repair capacity in response to acute DSS-induced injury. In this recovery paradigm, rather than a chronic 8-day administration of DSS, animals were exposed to DSS for 4 days and then allowed to recover for 4 days on water. Similar to chronic DSS administration, no gross histological differences were seen between genotypes at low magnification (wildtype n = 6; null n = 6; Figure 9A-B). At higher magnification, tissue repair is readily apparent with normal crypt architecture reappearing in both genotypes (Figure 9C-D). Infiltrating cells are still seen, however. In contrast to the 8-day

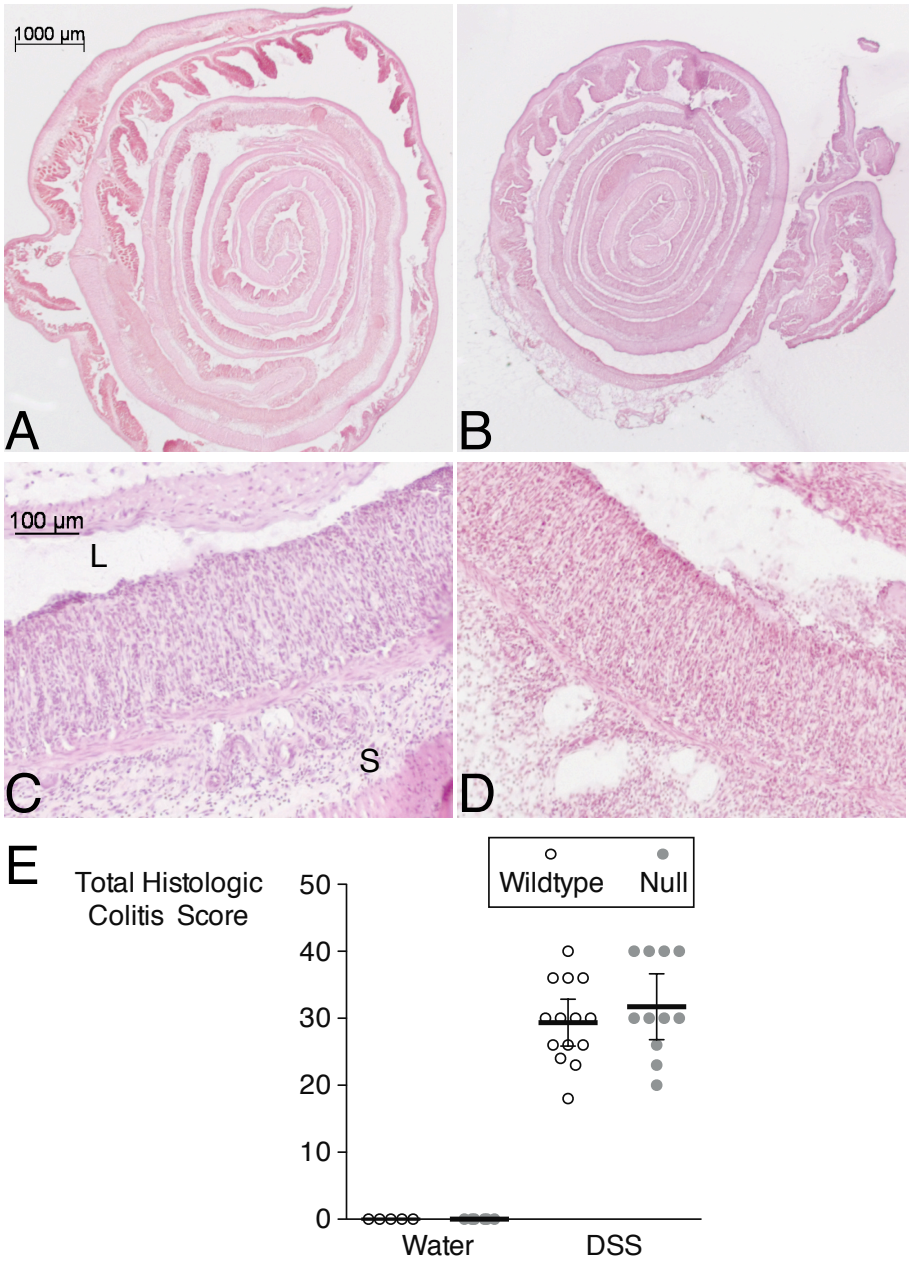

Figure 8. Colitis after DSS administration in wildtype compared to null animals. *A*, *Villin*$^{+/+}$*; Met*$^{fx/fx}$ wildtype and *B*, *Villin*$^{cre/+}$*; Met*$^{fx/fx}$ null low power micrographs of Swiss rolled-colon after 8 days of 3% DSS treatment, HE stained. *C*, wildtype and *D*, null high power micrographs of representative tissue with histologic colitis scores similar to group means seen in *E*. *E*, Total histologic colitis score, comparing wildtype and null animals in water-only control experiments and DSS experiments. No significant differences between genotypes in either treatment condition (for DSS, group mean score ± SD: wildtype 29.4 ± 6.1; null 31.7 ± 7.3). Total histologic colitis score includes inflammation severity and extent, crypt damage, and percent involvement. Abbreviations: L, lumen; S, submucosa.

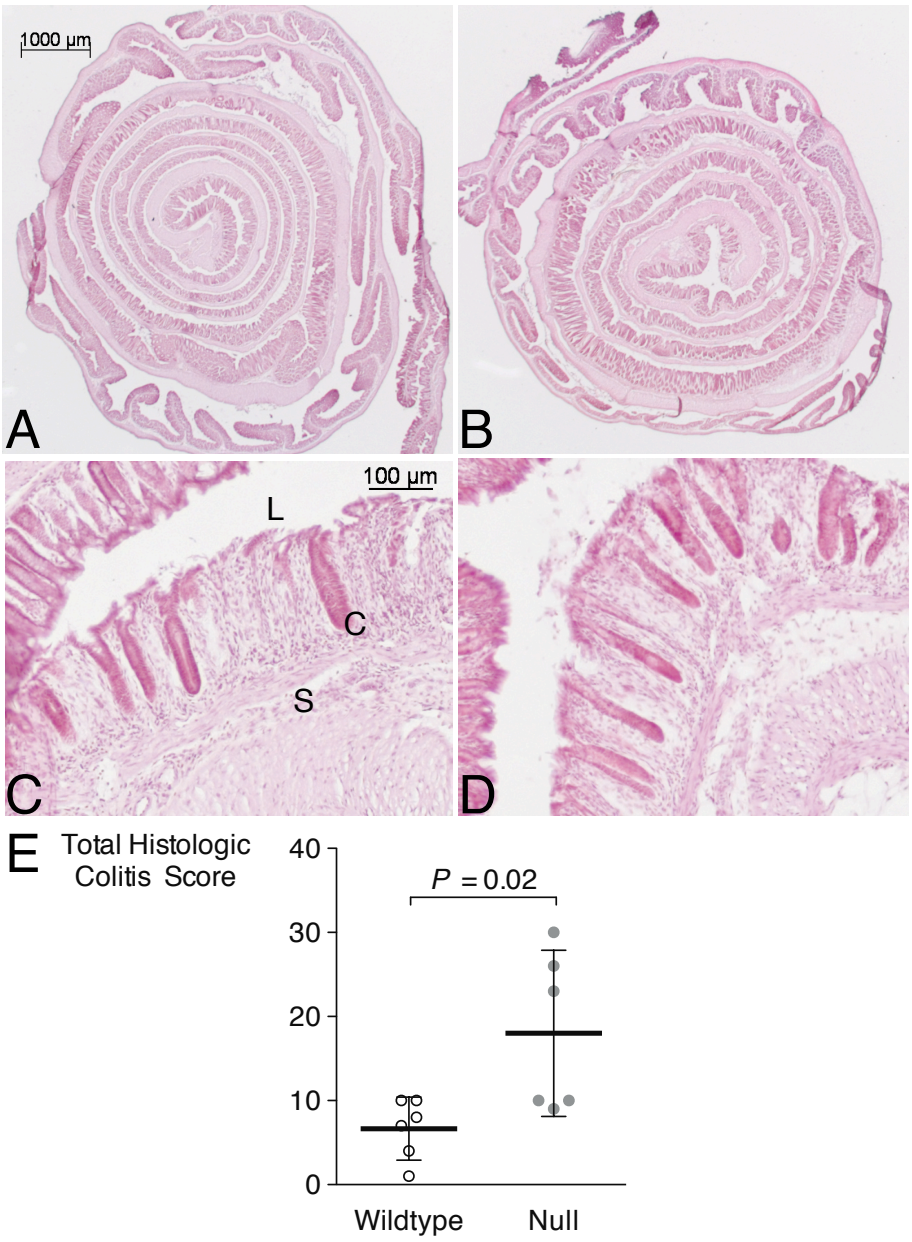

Figure 9. Colitis after DSS administration and subsequent recovery period in wildtype compared to null animals. *A*, *Villin*$^{+/+}$*; Met*$^{fx/fx}$ wildtype and *B*, *Villin*$^{cre/+}$*; Met*$^{fx/fx}$ null low power micrographs of Swiss rolled-colon after 4 days of 3% DSS treatment followed by 4 days of recovery on water, HE stained. *C*, wildtype and *D*, null high power micrographs of representative tissue with histologic colitis scores similar to group means seen in *E*. *E*, Total histologic colitis score, comparing wildtype and null animals after DSS treatment and recovery. Null animals maintain a significantly increased score after DSS recovery (group mean score ± SD: wildtype 6.7 ± 3.6; null 18.0 ± 9.4). Total histologic colitis score includes inflammation severity and extent, crypt damage, and percent involvement. Abbreviations: L, lumen; S, submucosa; C, crypt.

treatment results, quantitative analysis of total histologic score in the recovery experiment showed a significant difference between wildtype and null animals (Figure 9E, $P = 0.02$; group mean score ± SD: wildtype 6.7 ± 3.6; null 18.0 ± 9.4). Null animals had higher scores in this recovery paradigm, indicating increased inflammation and injury, compared to wildtype animals. The high power exemplary micrographs (Figure 9C-D) depict tissue with scores approximating group mean scores. However, due to the apparent bimodal distribution of scores in null animals, a single animal with a score approximating the null mean score was not available. Additionally, one null animal had a total score of 0, which was greater than two standard deviations from the group mean, and was thus excluded as an outlier.

Met Expression After DSS

Met protein expression in wildtype animals after DSS treatment was studied by immunohistochemistry. Compared to water-only control experiments in which Met is expressed at modest levels in the lower compartment of crypts (Figure 10A), Met expression was dramatically increased after both chronic DSS treatment (Figure 10C) and DSS treatment followed by recovery (Figure 10D). Met expression was increased in both the lower portion of crypts where cellular proliferation occurs, as well as in mature enterocytes lining the lumen.

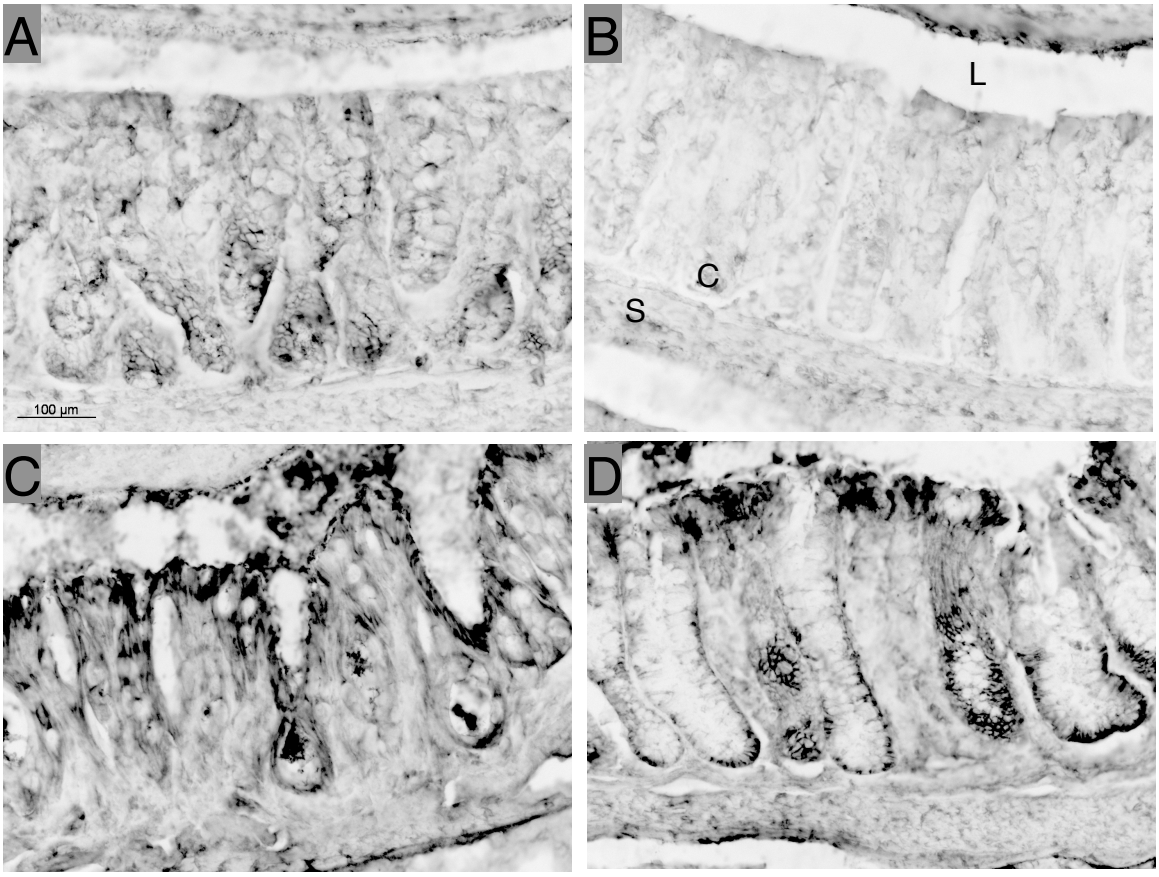

Figure 10. Immunohistochemical stain for Met expression in wildtype animals after water compared to DSS treatments. *A*, Water. *B*, Water, stained without primary antibody. *C*, 3% DSS treatment for 8 days. *D*, DSS treatment for 4 days, followed by 4 days of recovery on water. Met expression dramatically increased after DSS treatment. Scale bar, 100 µm. Abbreviations: L, lumen; S, submucosa; C, crypt.

Discussion

The results of the studies reported here provide the first data from a genetic model implicating Met signaling in gastrointestinal physiology. The findings are consistent with existing reports in the literature implicating HGF treatment for enhancing gut epithelial repair after experimental injury. Our protein expression studies corroborate what has been previously reported, and contribute additional details regarding cellular specificity. Met protein was detected in the E15 intestine primarily in the immature epithelium, which is consistent with a previous report that demonstrated *Met* mRNA expression at E15 in the developing intestine, although without cellular resolution (Sonnenberg, Meyer, Weidner, & Birchmeier, 1993). Similarly, modest protein expression was detected in adult GI epithelial cells, similar to what has been previously reported (Boon et al., 2006). The relative decrease in Met GI expression during development, from E15 to P21, is reminiscent of the temporal expression profile reported in the nervous system (Judson, et al., 2009), suggesting that Met signaling is most likely active during specified periods of development but then is down-regulated in a mature, uninjured epithelium.

After mapping expression of Met protein in the GI epithelium, the *Villin*^{cre} line was chosen to conditionally delete floxed *Met* specifically in GI epithelial cells. Conditional null animals displayed normal development, as weight gain was unaffected and adult mucosal thickness was not different relative to wildtype animals. As Met was expressed robustly during GI development, this absence of

a phenotype in conditional null animals was surprising. Other knockout mice lacking genes of interest in the GI system have shown basal phenotypes. For example, mice lacking secretory mucin (*Muc2*$^{-/-}$), the structural component of the mucus layer lining the GI lumen, display growth retardation and spontaneously develop colitis (Van der Sluis et al., 2006). However, the absence of a phenotype in the null animals reported here is not unprecedented. Mice lacking intestinal trefoil factor (*Itf*$^{-/-}$), a protein specifically and abundantly expressed in the GI tract, were reported to develop normally and be indistinguishable from wildtype animals, with normal growth and absence of fecal occult blood (Mashimo, Wu, Podolsky, & Fishman, 1996). *Itf*$^{-/-}$ mice did, however, display increased susceptibility to and impaired healing after acute injury induced by DSS. It is possible that any altered phenotype due to deletion of Met signaling may be far more subtle than the general measures used here were able to detect. This is reminiscent of the subtle changes in dendritic and spine structure of neurons that normally express Met, which was reported in the mouse brain (Judson, et al., 2010b). There are, however, far more impactful physiological consequences in the brain due to Met signaling disruption (Qiu, Anderson, Levitt, & Shepherd, 2011). This is consistent with the functional findings here.

As Met signaling is implicated in injury repair in other tissues, *Villin*$^{cre/+}$*; Met*$^{fx/fx}$ conditional null animals were assessed for susceptibility to DSS-induced acute GI injury. Chronic DSS treatment showed no difference in susceptibility to injury between wildtype and null animals, as measured by total histologic colitis score.

This finding was surprising, as it was hypothesized that if Met signaling was involved in mounting a repair response to epithelial injury, then loss of Met signaling would increase an animal's susceptibility to injury as it would be limited in its capacity to respond to the ongoing DSS injury. A possible explanation for this lack of increased susceptibility between null and wildtype animals is that the 8-day DSS paradigm used in these studies may have a ceiling effect. The histologic score has a maximum of 40, and both genotype group means were approaching this maximum (approximately 30 in both groups). Lower concentration of DSS administration, or for a shorter duration, may in future studies enable differentiation of susceptibility between genotypes with a less robust induced injury.

We addressed the potential ceiling effect of the chronic treatment by using a classic injury/repair paradigm. *Met* conditional null animals were significantly less able to repair in the DSS recovery paradigm, compared to wildtype mice, measured by histologic colitis score. This finding is consistent with previous studies demonstrating Met signaling system involvement in repair of skin wounds (Chmielowiec, et al., 2007) and liver regeneration after injury (Borowiak, et al., 2004; Huh, et al., 2004). In the context of Met protein expression data, which showed up-regulation of Met after DSS administration, an interpretation of these findings would be that loss of functional Met signaling, which in wildtype animals is involved in epithelial repair, causes null animals to have an inadequate repair response capacity.

This report has several limitations and opportunities for future studies. The sample size for the recovery experiments needs to be enlarged to clarify if the conditional null histological colitis scores truly are bimodal, or instead merely represent natural biological variation and heterogeneity. The colitis scoring system, while common in the literature, is only semi-quantitative and restricted to discrete scores. A continuous measure of damage, for example by staining intact actin in enterocytes with phalloidin, would be more sensitive for detecting modest effects in null animals. Quantitative analysis of specific classes of infiltrated immune-competent cells would also provide additional evidence for differing pathology beyond the colitis scores. For example, cell counts for macrophages (by F4/80 staining) and $CD4^+$ T cells, and granulocyte infiltration (by myeloperoxidase activity assays) would all be important future studies. Moreover, the immunohistochemical staining data for Met expression is qualitative in nature, and should be augmented by quantitative Western blot studies. Co-labeling studies with markers for specific crypt cell types will also be important, to better understand the particular role played by Met signaling in response to injury. For example, Musashi-1 is a putative marker of the colonic stem cell (Kayahara et al., 2003; Potten et al., 2003), and co-staining may help to clarify if Met's role is primarily in proliferation and differentiation of immature enterocytes.

Conclusion

Met signaling is involved in diverse biological processes, by initiating multiple signal cascades that possess a degree of overlap and crosstalk (Trusolino, et al., 2010). The data reported here showing absence of a basal phenotype in *Met* conditional null animals may be explained by this signaling redundancy in the context of formidable homeostatic mechanisms, or that Met signaling is not critically involved in normal development and function of the GI epithelium. Data reported here do, however, demonstrate a critical role for Met in GI epithelial repair after acute injury. The data reported here are unable to clarify what specific cellular processes are deficient and responsible for impaired responsiveness in null animals. Three possible processes are epithelial motility and proliferation (both of which Met signaling is implicated in (Dignass, Lynch-Devaney, & Podolsky, 1994)), and regulation of infiltrated cells (in which Met signaling is involved in both paracrine and autocrine signaling for macrophage functioning (Galimi et al., 2001). Future studies may elucidate the relative contributions of these processes to the impaired repair capacity of *Met* null animals.

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CHAPTER V

FUTURE DIRECTIONS

Translational in nature, this thesis sought to use a tripartite approach to understanding complexity in autism spectrum disorders (ASDs) through the lens of co-occurring gastrointestinal dysfunction (GID) in children. With the wealth of data analyzed (over 5,000 lines of custom computer code were written for the project), rich phenotypes apparent in ASD and GID populations were described. Unique behavioral features of co-occurring ASD-GID children (increased language and social impairment) were paralleled by elevated levels of 15-F_{2t}-isoprostane (isoP), a marker of oxidative stress, in a subgroup of ASD-GID children. In a mouse model lacking functional signaling of the Met receptor tyrosine kinase, a well-established ASD-risk gene, a deficit in epithelial repair was described, providing data that will contribute to understanding gastrointestinal biology. The findings of this thesis will add to the developing literature regarding, and clinical care of, children with ASD and GID.

In a larger context, the broad goal of this thesis was to use GID as a model for how heterogeneity can be leveraged to the investigator's advantage, by appreciating pleiotropy in the context of whole-body physiology, and stratifying based on a readily-assessed comorbid condition such as GID. We hope that the combined findings reported in this thesis will serve as an inspirational model for

future studies, highlighting the advantages of a translational approach and stratification to address complexity. Building on this thesis, a number of interesting avenues of future study are discussed below.

Characteristics of a Latino Population and Cross-Cultural Comparisons

Prevalence of ASDs in Latino populations has been reported to be significantly lower than in non-Latino white populations, with some suggesting that socioeconomic status, access to services, or cultural factors affect the likelihood of diagnosis in Latino populations (Liptak et al., 2008; Mandell et al., 2008). Cross-cultural comparisons that explore similarities and differences between Latino and non-Latino populations may thus provide insight into the natural history and ecology of ASDs within a Latino context. For logistical reasons, this thesis did not include data from the second recruitment site, Children's Hospital of Los Angeles (CHLA). However, similar data to that collected at Vanderbilt (Chapters II and III) has been collected from participants in Los Angeles. Corresponding to the high prevalence of Latino patients in the general patient population of CHLA, approximately 40% of the participants in the CHLA cohort recruited for this study are of self-identified Latino ethnicity. We are not aware of any studies that have looked rigorously in Latino populations at issues involving co-occurring GID and ASD, making data from the CHLA cohort a potentially rich opportunity for future analysis. For example, in an exploratory analysis of the utilization of gluten-free/casein-free (GFCF) diets in 51 children at

CHLA, significant differences exist between Latino and non-Latino white populations (M. Miller, P. Gorrindo and P. Levitt, unpublished observations). Comparing ethnicities in those 51 children, significantly more white (11 of 31) versus Latino (1 of 20) children have tried a GFCF diet (χ^2, $P = 0.01$). Comparing ASD and GID status, 10 of 30 ASD-GID children have tried a GFCF diet, whereas only 1 of 7 ASD-only and 1 of 14 GID-only children have. Focusing only on the 30 ASD-GID children, 52.9% of white children (9 of 17), but only 7.7% of Latino children (1 of 13) have tried GFCF diets (χ^2, $P = 0.009$). While these data are limited by a small sample size, they do offer a glimpse into the possibilities of describing unique differences between Latino and white populations with ASD and GID.

Regression Modeling and Potential Predictive Utility of Elevated IsoP Levels in ASD-GID Children

The elevated isoP levels in a subgroup of children with ASD-GID was surprising to us, and offers an intriguing line of future research. Active and ongoing work seeks to apply advanced analytic techniques, including regression modeling, to better characterize the subgroup of individuals with elevated isoP levels. The extent to which, if any, isoP levels correlate with behavioral findings in the ASD-GID group, such as increased language and social impairment, will be interesting. Within the ASD-GID group, determining what, if anything is similar amongst the subgroup of individuals with elevated levels, and, related, what is

different between that cluster and other children in the ASD-GID group may provide insight into the biology of ASD. Additionally, as discussed below, integrating isoP measurements in studies of treatment response may demonstrate a biomarker sensitive to disease state or prognosis. With a better understanding of isoP-phenotype relationships, if a regression model with high sensitivity and specificity can be built, we possess the unique opportunity to then test the predictive capacity of such a model in the CHLA cohort. Prediction and replication of the isoP findings in an independent cohort will provide substantial support to the initial findings reported in Chapter III.

Nonverbal Children: A Conspicuous Phenotype with Potential Clinical Implications

The report in Chapter II of a significant portion of nonverbal children in the ASD-GID group (30%) was striking and unexpected. The complete absence of expressive language capacity is a robust and salient phenotype that warrants deeper investigation. Future work may gather a comprehensive characterization of language and communication abilities, including a history of language development and nonverbal communication strategies. Use of specific, research-quality instruments (Vineland Adaptive Behavior Scales-II; Kaufman Brief Intelligence Test-2) to evaluate communication skills should be incorporated into all future studies of the ASD-GID population.

Additionally, it will be important to interrogate the relationship between GID status and language capacity. One hypothesis is that the co-occurrence of nonverbal status with GID and ASD represents a specific syndrome, a complex and pleiotropic manifestation of disrupted biology. Human genetic studies of larger cohorts stratified by language impairment, in addition to GID status, may provide useful data to this end. An alternative hypothesis is that nonverbal children, lacking conventional communication abilities, are unable to communicate internal states to caregivers and thus are unable to impose any control on behavioral or environmental factors that modify GID state. One approach to this question is to assess physiological states of children, independent of their communication abilities, and correlate changes in state with coincident environmental changes. An exploratory collaboration seeks to refine a multimodal real-time physiological monitor. In its current incarnation, the non-invasive device is worn by a study participant for a number of days, enabling the device to capture longitudinal data on galvanic skin response (GSR), heart rate, and general locomotive and activity level — all independent of the participant's communication ability. Parental reports of time of eating and going to the bathroom, for example, can then be combined with physiological measures of internal state to specifically test for correlations between acute events and heightened arousal.

Finally, an important clinical implication of the nonverbal findings is that, lacking traditional communication abilities, some children may have ongoing,

latent GID that has not yet been detected by their caregivers or clinical care providers. To this end, an important future area of work would be the development of a highly sensitive GID screening tool, independent of verbal capacity of the child, which could be used by parents or primary care clinicians to determine if evaluation by a pediatric gastroenterologist is warranted (Buie et al., 2010).

Deeper Characterization of GID: Focus on Constipation

The relative homogeneity of type of GID in ASD-GID children was somewhat surprising (85% constipation, Chapter II). Future studies may benefit from focusing specifically on constipation, to develop a richer understanding of GID in ASD. The potential causes of constipation in children are myriad, ranging from toilet phobia, depression, or dehydration, to anatomical malformations, electrolyte imbalances, or connective tissue disorders (Constipation Guideline Committee of the North American Society for Pediatric Gastroenterology Hepatology and Nutrition, 2006). Some possible causes are rare, and multiple factors can combine in complex ways to influence outcome. While many of these possible causes were assessed in the study described in Chapter II, future studies would benefit from a systematic and comprehensive assessment by pediatric gastroenterologists of each potential cause to clarify the relative contributions of functional, anatomic, metabolic, infectious, inflammatory, neoplastic, dietary, and pharmacological factors to GID status.

Moreover, the GID described in Chapter II is not acute, as a study inclusion requirement was duration of GID greater than one month. A close examination of the natural history and developmental time-course of chronic GID presentation may offer insight into etiology and prognosis. As ASDs are neurodevelopmental disorders, with a changing biology interacting with a dynamic environment over the course of development, some have proposed that biology-behavior-environment interactions funnel an individual into an increasingly atypical social environment (Jones & Klin, 2009). It is possible then, by extension, that GID status funnels an individual into a similarly abnormal "gastrointestinal environment" — a parallel concept to the social environment, which involves the various possible causes and outcomes of GID discussed above.

Functional Implications of Co-occurring GID in ASD

The language and social impairments reported in a portion of ASD-GID children suggested that GID has important implications for the overall functional abilities of an individual, with synergistic effects of ASD and GID statuses when co-occurring. These findings are corroborated by exploratory analysis of an additional dataset. For a subpopulation of children described in Chapter II, we serendipitously were able to assess children for functional behaviors on the Child Behavior Checklist (CBCL; (Achenbach & Rescorla, 2001). For 17 ASD-GID children and 9 ASD-only children, we see significantly higher scores on functional impairment metrics in the ASD-GID group compared to ASD-only, including

attention problems, rule-breaking behaviors, aggressive behaviors, and anxiety problems (P. Gorrindo and P. Levitt, unpublished observations). These findings, although in a small cohort, suggest GID status correlates with additional problem behaviors that have functional implications on daily living for children (Buie, et al., 2010). Moreover, it has been reported that family quality of life also is negatively impacted by more severely affected children (Herring et al., 2006) which can negatively impact family-based intervention strategies (Osborne, McHugh, Saunders, & Reed, 2007).

To further explore the relationship between GID, functional impairment in affected children, and parental quality of life, a longitudinal study before and after GID treatment is proposed here. On study enrollment, all ASD participants would be screened for possible constipation using Rome-III criteria (Drossman, 2006; Walker, Caplan-Dover, & Rasquin-Weber, 2000). Standard of care for constipation is parental counseling and advised use of over-the-counter stool softeners and laxatives (SSL). Participants who screen for high likelihood of constipation would be randomized to one of two study arms: (a) treatment with SSL beginning immediately, after evaluation by a pediatric gastroenterologist or (b) wait-list control, with a one month delay in evaluation by gastroenterologist and initiating SSL treatment. Baseline measures of problem behaviors, social impairment, and parental quality of life would be assessed at study enrollment for both arms. As treatment with SSL would be administered by one parent, during this study a requirement would be that a second parent be available to act as an

informant blinded to treatment status of the child, completing the CBCL (assessing for functional ability and problem behaviors) and SRS (assessing for social impairment) regarding the child in the study, and the Questionnaire on Resources and Stress (QRS; (Osborne, et al., 2007), an instrument assessing quality of life issues for the SSL-administering, non-blinded parent. Additionally, blood would be drawn at baseline, to measure isoP levels. These measures would be repeated at one month after enrollment. For the treatment arm, at that time they would then have completed one month of treatment. For the wait-list control arm, they would have not yet received treatment, but would subsequently begin treatment at that time. The measures would be repeated again at two months after enrollment, which would be two months of treatment for the treatment arm, and one month of treatment for the wait-list arm. During the course of the study, weekly phone calls with participating parents would assess for treatment compliance by ascertaining frequency of use of SSL and stooling history during the preceding week. Primary analytic outcomes would be, comparing means between study arms, the following: change in isoP levels, as well as CBCL, SRS, and QRS scores at one and two months from baseline. It is hypothesized that the treatment arm will see significant improvements (lower scores on the instruments, and isoP levels) after one month, whereas the wait-list arm will not see significant change during the first month, but will see indistinguishable improvements during the second month compared to improvements seen in the first month of the treatment arm. These findings would

be able to objectively and quantitatively address the suggestion that appropriate treatment of co-occurring medical conditions in children with ASDs can positively impact both the developmental course of the affected child, and the quality of life of the family as a whole (Bauman, 2010).

Methodological Refinements To Detect A Subtle Repair Phenotype in Conditional Null Mice

As reported in Chapter IV, *Villin*$^{cre/+}$*; Met*$^{fx/fx}$ conditional null animals have a deficit in repair capacity in response to an induced acute gastrointestinal (GI) epithelial injury. There are two points worth noting regarding these findings. First, no phenotype was seen in 8-day chronic administration of dextran sulfate sodium (DSS). Second, in a 4-day on/4-day off recovery paradigm of DSS administration, while a group effect was seen using a histologic colitis score, there was substantial variability within the conditional null group using this metric. While one possible explanation of these findings is that they faithfully represent the underlying biology of Met in the gastrointestinal system, another possibility is that Met signaling has modest effects on cellular biology and the analytic approaches employed were of insufficient resolution to detect such differences. For example, relatively modest changes in spine and dendritic structure were seen in neurons lacking Met (Judson, Eagleson, Wang, & Levitt, 2010).

To improve our detection ability of modest changes in the GI epithelium, two major approaches are possible. First, a less dramatic injury may unmask

differences that in the existing paradigm are indistinguishable. A lower concentration or shorter duration of administration of DSS may prove useful. Additionally, combined with an attenuated DSS treatment, it would be interesting to explore additive effects of psychosocial stressors. It has been reported that DSS-induced injury can be modulated by environmental stressors, such as cage overcrowding (Reber et al., 2006; Veenema, Reber, Selch, Obermeier, & Neumann, 2008). One potential future study could be low-dose (1%, instead of 3%) DSS, with and without a socially-stressful environmental factor to see if psychosocial stress can, in the context of low-dose DSS, elicit a phenotype in nulls but not wildtype animals.

Second, more sensitive and quantitative measurements may be better able to detect subtle epithelial phenotypes. DSS studies commonly measure myeloperoxidase (MPO) activity, as an index of granulocyte infiltration (Beck et al., 2010). The tissue harvest protocol for the DSS studies in Chapter IV included filleting a narrow longitudinal piece of colon tissue prior to immersion fixation, and flash-freezing the sample in liquid nitrogen. These frozen samples could thus be used for both MPO activity assays, and Western blots for Met protein levels. Additionally, a novel metric of epithelial integrity is proposed here, using phalloidin staining of enterocyte actin filaments. Figure 1 shows proof-of-principle staining of actin using Alexa 488-conjugated phalloidin (Invitrogen, Carlsbad, CA). Panel A shows wildtype tissue exposed to water only, with a bright and continuous band of phalloidin signal lining the lumen, as expected. Panel B

shows wildtype tissue exposed to 8-days of chronic DSS administration, with discontinuous patches of staining signal (arrow), representing patches of intact epithelium, interspersed among denuded epithelium which does not stain with phalloidin (arrowhead). We propose using an automated image acquisition and processing strategy, in which a strong phalloidin signal is distinguished from background by signal intensity thresholding, to gather a section-level and continuous index of epithelial integrity. Additionally, in response to DSS injury, colonic restitution includes up-regulated cellular proliferation (Edelblum et al., 2008). Figure 2 shows preliminary staining for Ki67, a marker of proliferating cells, in wildtype tissue treated with water (Panel A) compared to 8-day DSS (Panel B). As seen in the figure, response to DSS includes a dramatic increase in proliferating cells; cell counts of Ki67-positive cells may evince a modest genotype effect in Met conditional null animals. Finally, staining for markers of specific classes of immune-competent infiltrating cells in response to DSS may reveal differences in null animals. Two important classes of cells in the DSS paradigm include macrophages and $CD4^+$ T cells. Figure 3 shows preliminary staining for both cell types, in wildtype tissue treated with water (Panels A and C), and dramatically increased staining signal in wildtype tissue after 4-day on/4-day off DSS administration (Panels B and D). Ongoing work seeks to refine the immunohistochemical protocol for these markers, after which rigorous cell count studies may be undertaken to corroborate the findings reported in Chapter IV.

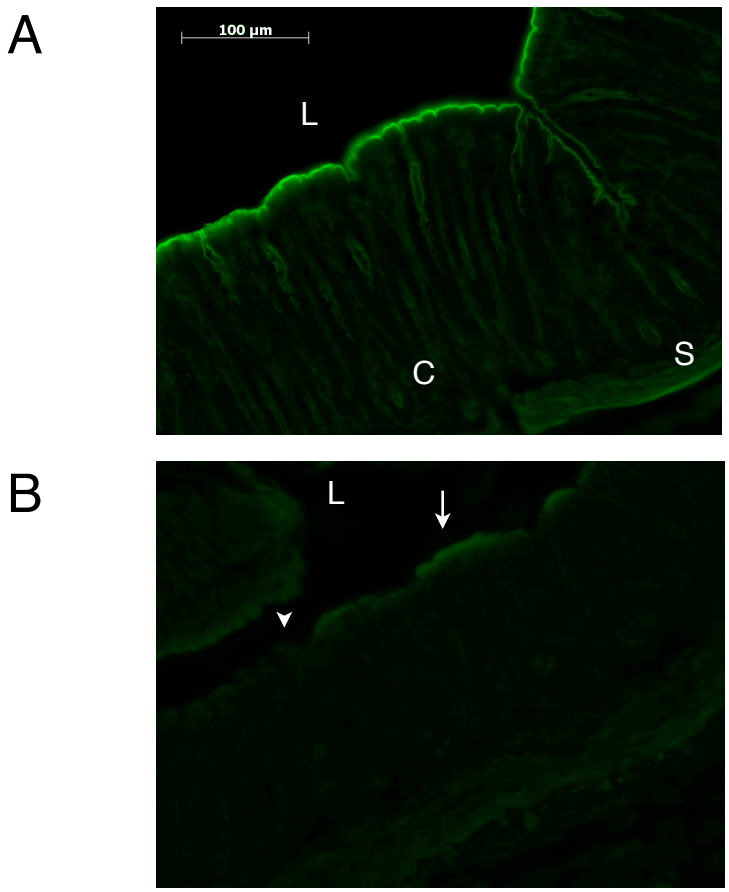

Figure 1. Apical actin filaments in enterocytes lining the gastrointestinal lumen stained with phalloidin. *A*, Wildtype tissue after water-only control treatment, with intense phalloidin-staining signal apparent in cells lining the lumen. *B*, Wildtype tissue after 8-day 3% DSS treatment, resulting in significant epithelial and crypt damage. Patches of intact staining, representing presumably intact epithelium, are apparent (arrow), compared to stretches of denuded epithelium lacking any phalloidin staining (arrowhead). Abbreviations: L, lumen; S, submucosa; C, crypt.

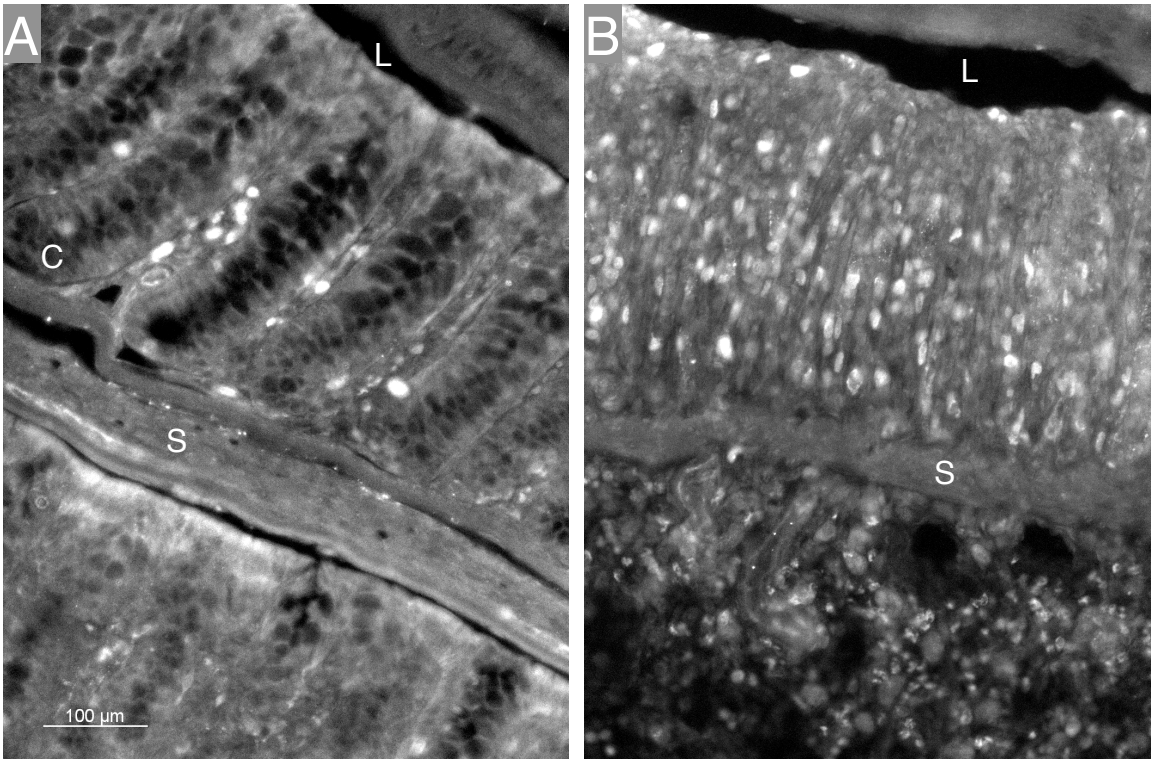

Figure 2. Immunohistochemical stain for Ki67 as a measure of active cellular proliferation in colon tissue. *A*, Low basal signal for Ki67 in wildtype tissue on water. *B*, Although the crypt architecture is significantly damaged, there is a robust increase in Ki67 staining in wildtype tissue after 3% DSS treatment for 8 days. Scale bar, 100 µm. Abbreviations: L, lumen; S, submucosa; C, crypt.

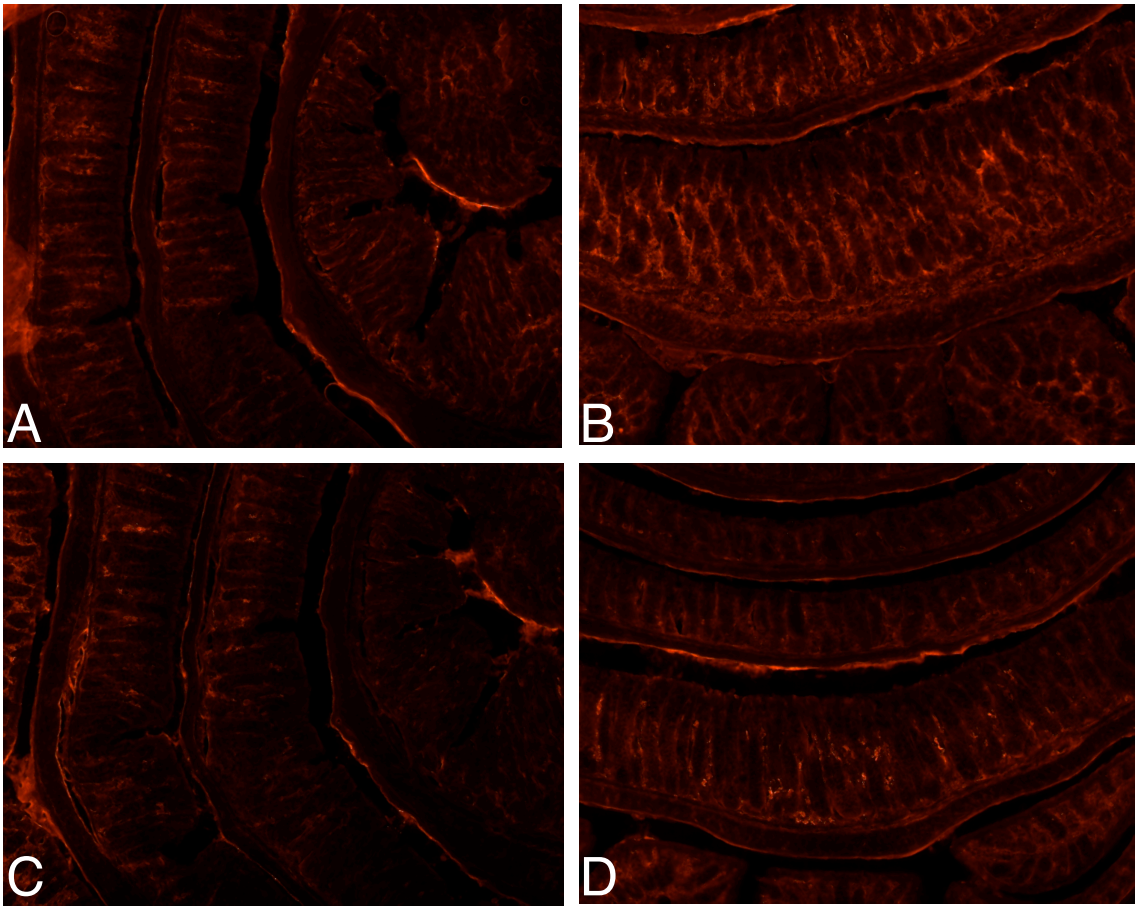

Figure 3. Immunohistochemical staining for infiltrated immune-competent cells in response to DSS administration. *A* and *B*, F4/80 staining for macrophages. *C* and *D*, CD4 staining for CD4+ T cells. *A* and *C*, wildtype tissue treated with water. *B* and *D*, wildtype tissue after 4-day on/4-day off DSS in the recovery paradigm. Dramatic increases in staining for both markers are apparent after DSS administration (*B* and *D*).

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