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# Down Syndrome: A Complex and Interactive Genetic Disorder

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## 1. Introduction

### 1.1 Trisomy 21 causes phenotypes associated with Down syndrome

Down syndrome (DS) occurs in approximately 1 out of 700 live births and most commonly results from three copies of human chromosome 21 (Hsa21) (Christianson, 2006). DS is a multifaceted disorder with over 80 clinically defined phenotypes including those affecting the central nervous system, heart, gastrointestinal tract, skeleton and immune system (Epstein, 2001; Van Cleve and Cohen, 2006). Phenotypes associated with individuals with DS vary in both incidence and severity, leading to a vast array of phenotypic combinations among those with Trisomy 21 (Ts21) (Deutsch et al., 2005; Epstein, 2001; Van Cleve et al., 2006; Van Cleve and Cohen, 2006). For example, cognitive impairment, hypotonia and craniofacial features are universal phenotypes, whereas cardiac abnormalities only affect approximately half of individuals with DS. Individual phenotypes similar to those seen in DS have been documented in individuals without Ts21, but a general higher incidence and severity of these phenotypes in individuals with DS suggests that trisomy plays an important role in initiating or modifying these features (Epstein, 2001; Roper and Reeves, 2006). Although it has been suggested that common mechanisms may be involved in similar phenotypes seen in individuals with and without Ts21, common pathophysiology must be proven for each individual phenotype.

A chromosomal basis for DS was postulated as early as 1932 (Patterson and Costa, 2005). After Jerome Lejeune established that DS was caused by an extra Hsa21, he studied the metabolic pathways associated with Ts21 phenotypes in an attempt to cure DS (Neri and Opitz, 2009). Alternate causes of DS, including translocations and mosaicism of extra material from Hsa21, were established shortly after the chromosomal basis of DS had been discovered (Patterson and Costa, 2005). These early genetic findings set the stage for current paradigms and research surrounding the gene-phenotype relationships in DS.

Although ~95% of the incidence of DS is due to trisomy of the entire Hsa21, DS also results from extra genetic material from Hsa21 translocated to other chromosomes. Additionally, some individuals with DS phenotypes have Ts21 in only a portion of their cells (mosaicism). Differences in genetic and cellular composition of the trisomy may lead to the observed differences in DS phenotypic incidence and severity. Genotypic and phenotypic variations have also been used to hypothesize about trisomic genes or chromosomal regions that may cause or significantly alter DS phenotypes. Such genotype-phenotype correlations are important in defining the etiology of traits associated with DS as well as suggesting possible therapeutic mechanisms to overcome deficits seen in individuals with DS.

### 1.2 Hypotheses regarding genotype-phenotype correlation in DS

Early investigations into the genotype-phenotype relationship drew upon information from individuals with partial Ts21. By examining these individuals, determining the extra chromosomal material and corresponding phenotypes, an early causal hypothesis linked DS to trisomy of the distal end of Hsa21 (Patterson and Costa, 2005). A reductionist variation of this hypothesis later stated that individual phenotypes and features could be mapped to specific regions of the genome, and the addition of these regions with their respective phenotypes would cause the multitude of traits associated with DS (Neri and Opitz, 2009). Others had a more global or genomic view of the genotype-phenotype relationship and hypothesized that DS phenotypes were due to a general genomic imbalance initiated by Ts21. Many new ideas have extended or combined previous hypotheses as well as proposed novel views about the correlation between genes and phenotypes in DS, especially with an increased ability to precisely define the genotypes and phenotypes associated with DS. Yet, the exact mechanisms of how the triplication of genes on Hsa21, the smallest and least gene dense of the autosomes, causes one of the myriad phenotypes associated with DS have still largely not been established. One or more of the hypothesized mechanisms may be correct for a single phenotype associated with DS, yet it is unlikely that a single overriding mechanism would describe the etiology of all Ts21 phenotypes (Epstein, 2001).

#### 1.2.1 Gene dosage imbalance hypothesis

The "gene dosage imbalance" hypothesis suggests that an increased dosage or copy number of genes on Hsa21 would lead to an increase in gene expression and protein product in the individual (Antonarakis et al., 2004; Epstein, 2001; Pritchard and Kola, 1999). In essence, overexpression of the products of dosage sensitive genes on Hsa21 would lead to DS phenotypes. This hypothesis has been extended to include the possibility that specific genes or subsets of genes may control specific DS phenotypes (Patterson, 2007). Most studies have concluded that three copies of Hsa21 genes leads to an average 50% higher expression of trisomic genes as compared to euploid (normal chromosomal complement) individuals (Gardiner et al., 2010). As additional high throughput analyses are performed, however it is becoming increasingly clear that the gene expression changes may be specific to a group of genes in a particular tissue at a precise developmental or mature stage. Additionally, some trisomic gene expression changes may overlap with normal expression of these genes in the euploid chromosomal component (Ait Yahya-Graison et al., 2007; Prandini et al., 2007; Wiseman et al., 2009).

#### 1.2.2 Amplified developmental instability hypothesis

The "amplified developmental instability" hypothesis states that a non-specific dosage of a number of trisomic genes leads to a genetic imbalance that causes a large impact on the expression and regulation of many genes throughout the genome (Pritchard and Kola, 1999; Shapiro, 1983). This disruption in genetic homeostasis or more generalized change in gene expression throughout the genome would affect signaling pathways and lead to phenotypes associated with DS. Therefore, it has been hypothesized that traits in individuals with DS are not caused by the altered expression of a particular gene, but rather the general changes in expression throughout the genome caused by trisomy that lead to DS traits (Patterson, 2009). An additional premise of this hypothesis is that the larger the number of trisomic genes, the higher the incidence and severity of DS phenotypes due to increased genetic instability. An extension of this hypothesis is that some phenotypic changes associated with

DS may not be attributable to specific genetic changes but rather to changes in the expression of the entire chromosomal domain (Gardiner et al., 2010).

### 1.2.3 Critical region(s) hypothesis

Phenotypic analyses conducted on individuals with partial trisomy for Hsa21 led to a hypothesis that only one or a few small chromosomal regions, termed "Down syndrome critical regions" (DSCR), contain genes responsible for the majority of DS phenotypes (Delabar et al., 1993; Korenberg et al., 1990). It has been suggested that DS (including many of the major, most well defined phenotypes) was caused by one specific DSCR (Epstein, 2001). Even before the identification of specific genes on Hsa21, a region of 1.6-2.5 Mb on Hsa21was thought to be responsible and sufficient to cause most DS phenotypes (Dahmane et al., 1998; Ohira et al., 1996). The sequencing of Hsa21 proved to be an important factor in the progression of DS research (Hattori et al., 2000) and led to further insight into genotype-phenotype correlations associated with DS and precise characterizations of DSCR regions (Patterson, 2009). A region of 3.8-6.5 Mb on 21q21.22 containing approximately 30 genes has been traditionally identified as the DSCR, although recent studies have demonstrated that this DSCR is not sufficient for all major DS phenotypes, though its inclusive genes may be relevant for some individual phenotypes (Delabar et al., 1993; Lyle et al., 2009). A current hypothesis states that certain chromosomal regions may contain a significant gene or genes necessary for the development or maintenance of specific but not all DS phenotypes (Korbel et al., 2009; Lyle et al., 2009; Olson et al., 2007).

### 1.2.4 Other genetic, epigenetic and environmental considerations

The hypotheses of how genetic mechanisms control DS phenotypes are not mutually exclusive, may include a combination of different mechanisms and may be unique for each particular phenotype. It is now believed that contributions to phenotypes associated with DS are likely to come from a number of genes. Variability in genomes may also come from allelic heterogeneity or other differences in the genetic architecture (Antonarakis et al., 2004). There may be genes that have a larger effect on certain phenotypes (Epstein, 2001; Patterson, 2009), or genetic contributions may be additive, subtractive, or epistatic (Gardiner et al., 2010). Recently it has been shown that epigenetic modifications and allelic differences also play a significant role in the development of these phenotypes, not just the simple presence of the chromosomal imbalance (Belichenko et al., 2009; Elton et al., 2010). Other factors including environmental influences and stochastic events also add to the differential manifestation of phenotypes.

### 1.2.5 Dosage compensation

Extra copies of genes may be found as a result of a number of duplication events, yet many of the resultant trisomies may be difficult to detect or have subtle phenotypes. Though there may be an extra copy of a gene, and even alterations in gene expression with the resultant protein product, dosage imbalance for a specific gene may have no effect on a phenotype. Such genes have been "dosage compensated" by some mechanism (Antonarakis et al., 2004). Gene expression experiments of cells with Ts21 have shown that a high percentage of three copy genes may undergo dosage compensation (Ait Yahya-Graison et al., 2007). This compensation could be accomplished by gene regulatory networks or negative feedback

loops. Dosage compensation may be tissue or time dependent and may play an important role in the development and progression of DS phenotypes.

### 1.3 Mouse models of DS to understand genotype-phenotype correlation

In addition to being valuable tools for better understanding Ts21 and DS phenotypes, DS mouse models have also been utilized as predictive models of novel DS phenotypes (Baxter et al., 2000). Mice provide access to all tissues at all developmental stages, the ability to understand genetic and cellular mechanisms caused by trisomy, and a resource to understand potential treatments for phenotypes. Genes found on Hsa21 are highly conserved in order and homology on three different mouse chromosomes: mouse chromosome (Mmu)16, 17, and 10. Several DS mouse models have been created with segmental trisomy for different regions of the distal portion Mmu16, that contains nearly half the gene homologs found on Hsa21 (Hattori et al., 2000; Pletcher et al., 2001). The Ts65Dn mouse is the most commonly used DS model with segmental trisomy for the distal portion of Mmu16. Three copies of the region between *Mrpl39* and *Znf295* spanning 13.6 Mb on Mmu16 and containing over 100 Hsa21 gene homologs results in several DS-like phenotypes (Reeves et al., 1995). The Ts1Cje mouse contains segmental trisomy for approximately 78% (*Sod1-Znf295* excluding *Sod1*) of the triplicated genes in the Ts65Dn model (Olson et al., 2004b; Sago et al., 1998). Both Ts65Dn and Ts1Cje mice contain three copies of the putative DSCR region and exhibit a significant amount of phenotypic similarity to humans with DS. The Ts1Rhr/Ms1Rhr mouse models were created by generating a 3.9 Mb reciprocal duplication/deletion containing 33 genes (*Cbr1-Orf9*) homologous to those found in the most commonly defined human DSCR (Olson et al., 2004a). The transchromosomal Tc1 mouse model of DS has a copy of Hsa21 in its cells, though there are some deletions of Hsa21 regions and significant mosaicism in this model (O'Doherty et al., 2005). The Ts1Yey model contains the entire region of Hsa21 homology on Mmu16 from *D930038D03Rik-Znf295* duplicated on one Mmu16 and results in trisomy for this region in the mouse model (Li et al., 2007) (See Table 1).

More recently, mouse models of DS have been made that contain small regions of homology from chromosomes other than Mmu16. The Ts1Yah model contains a duplication of Mmu17 from *Abcg1-U2af1* with homology to Hsa21 (Pereira et al., 2009). The Ts2Yey model has a duplication of the entire region of Mmu10 homologous to Hsa21 (*Prmt2-Pdxk*). The Ts3Yey mouse has a duplication of the entire Mmu17 chromosomal region that corresponds to Hsa21 (*Abcg1-Rrp1b*) (Yu et al., 2010). Mice containing entire regions of homology to Hsa21 are being generated to examine DS phenotypes. Mouse models of DS may exhibit certain DS-like phenotypes with differing severity, but phenotypes may not be conserved across models, thus adding to the usefulness of the models for understanding the genotype-phenotype relationships associated with DS.

### 1.4 Using modern genetic and genomic tools to understand genotype-phenotype correlation in DS

Because DS and its resultant phenotypes are caused by trisomy, the gene-phenotype correlation (the genes responsible for the cellular or developmental changes) is of great importance in understanding DS. DS may be viewed as a chromosomal disorder due to three copies of Hsa21, a genetic disorder resulting from the altered expression of trisomic genes, or a disorder that results from alterations in gene expression and pathways

| Common Name | Genetic triplication | Number of triplicated genes |
|---|---|---|
| Ts65Dn | Ts($17^{16}$)65Dn | 104 (Mmu 16) |
| Ts1Rhr | Dp(16*Cbr1-Orf9*)1Rhr | 33 (Mmu 16) |
| Ts1Cje | Ts($12^{16}$C-tel)1Cje | 81 (Mmu16) |
| Tc1 | Tc(Hsa21)1TybEmcf | 131 (~92% of Hsa 21) |
| Ts1Yey | *Dp(*16 *D930038D03Rik-Znf295)1Yey* | Mmu 16 |
| Ts2Yey | *Dp(10Prmt2-Pdxk)2Yey* | Mmu 10 |
| Ts3Yey | *Dp(17Abcg1-Rrp1b)3Yey* | Mmu 17 |

Table 1. Common DS mouse models with defined trisomic regions.

throughout the entire genome. Several studies have documented differential gene expression in tissues originating from varying spatial and developmental environments in attempts to determine genetic mechanisms affecting DS phenotypes (Conti et al., 2007; Lintas et al., 2010; Lyle et al., 2004; Moldrich et al., 2007). High throughput analysis utilizing tools such as microarray analysis, qPCR (quantitative polymerase chain reaction) and SAGE (serial analysis of gene expression) not only allow for a better understanding of gene expression aberrations in a spatiotemporal sense, but also permit for a better understanding of gene interactions within the context of natural physiological conditions. Though disparities exist between tissues and developmental time points, such analyses have provided insight into genes that appear to be critical to the development of specific DS phenotypes. New genetic information, coupled with precisely defined cellular and molecular phenotypes, (often using mouse models), has allowed the testing of multiple hypotheses concerning the gene-phenotype relationship. Functional groupings of dysregulated genes may add additional mechanistic insight into the origin of DS phenotypes, and such groupings may transcend both time and tissue type. The intersection of genetic and functional information may lead to new insights into the gene-phenotype relationship and the mechanisms leading to the development of DS phenotypes. With increasing ability to assess large scale gene expression, the importance of both trisomic and non-trisomic genes and genetic pathways utilizing gene products from the entire genome has been investigated. Instead of a simple notion of trisomy for Hsa21 causing a disorder or trisomic expression of specific genes on Hsa21 causing a disorder, current technologies and techniques now suggest that DS, with its myriad phenotypes, is a complex disorder with many interactions on genetic and mechanistic levels.

## 2. Trisomic and non-trisomic genes

### 2.1 The importance of trisomic and non-trisomic genes

Numerous gene expression studies have been performed on an assortment of tissues from individuals with DS as well as mouse models of DS (Tables 2 and 3). Although these assays vary widely in technique and scope, they have generally focused on the altered expression of trisomic genes. A strict interpretation of the gene dosage hypothesis suggests that all trisomic genes theoretically exhibit a 1.5 fold upregulation in every tissue and cell when compared to normal subjects, and nearly all studies use this expected fold change as a standard for expression analysis (Giannone et al., 2004; Mao et al., 2003). Interestingly, in studies that have analyzed both trisomic and disomic (non-trisomic) gene expression in

trisomic compared to normal individuals, a significant number of non-trisomic genes have been found to be dysregulated in association with DS phenotypes (Table 4) (Lockstone et al., 2007; Rozovski et al., 2007; Slonim et al., 2009).

| Study | Strain | Age | Tissue | Platform |
|---|---|---|---|---|
| Chrast et al. 2000 | Ts65Dn | Adult | Brain | SAGE |
| Saran et al. 2003 | Ts65Dn | 3-4 months | Cerebellum | Microarray |
| Amano et al. 2004 | Ts1Cje | Postnatal 0 | Brain | Microarray |
| Lyle et al. 2004 | Ts65Dn | Adult | Brain, Heart, Kidney, Liver, Lung, Muscle | qPCR |
| Dauphinot et al. 2005 | Ts1Cje | Postnatal 0, 15, 30 | Cerebellum | Microarray |
| Kahlem et al 2007 | Ts65Dn | 3-4 months | Lung, Skeletal Muscle, Midbrain, Cerebellum, Cortex, Testis, Liver, Heart, Kidney | Microarray |
| Sultan et al. 2007 | Ts65Dn | 13-26 weeks | Cerebellum, Midbrain, Cortex | qPCR |
| Laffaire et al. 2009 | Ts1Cje | Postnatal 0, 3, 7, 10 | Cerebellum, Granule Cell Layer | Microarray |
| Moldrich et al. 2009 | Ts1Cje | E14 | Neural Progenitor Cells | Microarray |
| Hewitt et al. 2010 | Ts1Cje | Adult | Neural Stem Cells | Microarray, qPCR |

Table 2. Features of gene expression assays performed in mice.

| Study | Age | Tissue | Platform |
|---|---|---|---|
| Gross et al. 2002 | Gest. 16-24 weeks | Placenta | Microarray |
| FitzPatrick et al. 2002 | Fetal | Amniocytes | Microarray, qPCR |
| Mao et al. 2003 | Gest. 17-21 weeks | Cerebral cortex, astrocytes | Microarray |
| Giannone et al. 2004 | Adult | T lymphocytes | Microarray |
| Tang et al. 2004 | Adult | Blood | Microarray |
| Chung et al. 2005 | Fetal | Amniocytes | Microarray |
| Deutsch et al. 2005 | Adult | Lymphoblastoids | qPCR |
| Mao et al. 2005 | Fetal | Cerebellum, heart | Microarray |
| Li et al. 2006 | Gest. 15-23 weeks | Heart, fibroblasts | Microarray, qPCR |
| Ait Yahya-Graison et al. 2007 | Adult | Lymphoblasts | Microarray |
| Altug-Teber et al. 2007 | Fetal | Amniocytes, Chorionic Villus Cells | Microarray |
| Conti et al. 2007 | Gest. 18-22 weeks | Heart | Microarray |
| Lockstone et al. 2007 | 47-76 years | Brain | Microarray |
| Prandini et al. 2007 | 25 years | Lymphoblasts, Fibroblasts | qPCR |
| Rozovski et al. 2007 | Fetal | Placenta | Microarray, qPCR |
| Chou et al. 2008 | Gest. 16-21 weeks | Amniocytes | Microarray |
| Esposito et al. 2008 | Gest. 19-21 weeks | Frontal cortex | Microarray |
| Sommer et al. 2008 | 1-4 years | Lymphocytes | SAGE, qPCR |
| Slonim et al. 2009 | Fetal | Aminocytes | Microarray |

Table 3. Features of gene expression assays from human derived tissues.

The number and variety of genes found to be dysregulated in response to trisomy seems to vary greatly in a spatiotemporal dependent manner, and provide evidence against the idea of a specific 1.5 fold dysregulation of trisomic genes in all tissue types (Mao et al., 2005; Potier et al., 2006; Sultan et al., 2007). Additionally, much of the variation in both trisomic and disomic gene expression in subjects with trisomy overlaps with the variation observed among normal individuals, which may limit the number of trisomic genes found to be differentially expressed (Prandini et al., 2007), and contribute to the variable phenotypes associated with DS (Chou et al., 2008). We therefore hypothesize that both trisomic and non-trisomic genes are important for the origin and development of DS phenotypes.

### 2.2 Gene expression studies in humans and mouse models of DS

#### 2.2.1 Altered expression of trisomic genes and the gene dosage hypothesis

The gene dosage hypothesis and the proposed 1.5 fold increase in all trisomic gene expression have motivated much of the gene expression research of DS phenotypes. Assessment of gene expression in human fetal brain tissue and astrocyte cell lines taken at 17-20 weeks gestation revealed a general upregulation of trisomic gene expression, and seemed to agree with the 1.5 fold change hypothesis (Mao et al., 2003). Analysis of brain tissue isolated from 13-16 week old Ts65Dn mice also identified a global upregulation of trisomic genes in the cerebellum, cortex, and midbrain, and the average fold changes of trisomic gene expression were 1.44, 1.37, and 1.39, respectively, in Ts65Dn mice (Sultan et al., 2007).

Yet, other studies suggest the global upregulation of trisomic genes in agreement with the gene-dosage hypothesis is likely not occurring in DS. Many studies indicate that not all trisomic genes are dysregulated in all trisomic tissues. Furthermore, several studies on trisomic tissues have demonstrated expression levels significantly different from the expected 1.5 fold increase, indicating that having three copies of a gene does not necessarily confer a 1.5 fold upregulation. For example, expression analysis of 99 genes in 6 different tissues (brain, heart, lung, kidney, liver, and muscle) in P30 and 11 month old Ts65Dn mice revealed that 37% of the trisomic genes were expressed at a 1.5 fold increase, 45% were <1.5 increase, 18% >1.5 increase, and 9% were not significantly different from normal mice (Lyle et al., 2004). Additionally, only 15% and 29% of tested trisomic genes were found to be overexpressed in DS fetal hearts and cultured fibroblasts, respectively (Li et al., 2006). Of 134 differentially expressed genes assessed in lymphoblastoid cell lines (LCLs), 58/134 had overexpression greater than 1, (1.25-2.27), and 86/134 exhibited fold changes significantly different from 1.5 (9 were greater than 1.5 fold (1.64-2.27) and 77 were less than 1.5 (0.74-1.4)) (Ait Yahya-Graison et al., 2007). Of 117 trisomic genes in LCLs and 114 trisomic genes in fibroblasts, only 39% and 62% respectively were found to have significant expression differences from euploid cells (Prandini et al., 2007). Based on these data, four classes of genes in trisomic tissues have been suggested: those that exhibit expression significantly different from 1 but not different from 1.5 fold, amplified genes (expression significantly greater than 1.5 fold), compensated genes (expression significantly less than 1.5 fold), and the genes with expression differences not different from 1 or 1.5 fold (Ait Yahya-Graison et al., 2007; Prandini et al., 2007). These extensive studies indicate that a global 1.5 fold upregulation of all trisomic genes in DS tissues is unlikely and suggest that mechanisms in addition to trisomy are also affecting gene regulation. Although the expression levels of some trisomic genes fit within the theoretical 50% upregulation, trisomic genes in several tissues at different time points exhibit expression levels much different from the expected 1.5 fold increase.

| Study | Trisomic Genes | Disomic Genes | # Significant Trisomic Genes | # Significant Disomic Genes | # Genes Examined |
|---|---|---|---|---|---|
| Gross et al. 2002 | *C21ORF3* | *KRT8, ALDH7, KISS1* | 2 | 5 | 8976 |
| FitzPatrick et al. 2002 | *N/A* | *IGFBP3, RGS5, IGFBP5* | 39 | 1934 | 8020 |
| Mao et al. 2003 | *DSCR2, SOD1* | N/A | Global upregulation | N/A | ~15,000 |
| Giannone et al. 2004 | *SOD1,CSTB* | *HLA-DRB3, GABRG2, ACAT2* | 2 | 15 | 334 |
| Tang et al. 2004 | *DSCR2, CSTB* | *FHL1, ALOX12, RGS10* | ~20% | 6% | ~12,000 |
| Chung et al. 2005 | *COL6A1, PRSS7* | *AKT, CASP5, JUN* | 2 | 8 | 102 |
| Deutsch et al. 2005 | *APP* | N/A | 25 | 7 | 41 |
| Mao et al. 2005 | *SOD1, SON, DSCR3* | *ADAMTS1* | 26 | 14 | ~12,500 |
| Li et al. 2006 | *SH3BGR, MX1, GART* | *IFI27* | 17 | 41 | ~10,000 |
| Ait Yahya-Graison et al. 2007 | *SAMSN1, DYRK1A SNF1LK, MX1* | N/A | 29% | N/A | 359 |
| Altug-Teber et al. 2007 | *DSCR1* | N/A | 16 | N/A | ~12,500 |
| Conti et al. 2007 | *DSCR1, DYRK1A* | *NFATc* | 32 | 441 | ~6300 |
| Lockstone et al. 2007 | *APP, RCAN, BACE2* | *APOE, NOTCH2* | 40 (25%) | 4.4% dysregulated | ~12,500 |
| Prandini et al. 2007 | *GABAPA, PFKL, U2AF1* | | 39%, 62% | N/A | 123, 132 |
| Rozovski et al. 2007 | *APP* | *LOX, MEST, MAT2A* | 41 overexpressed | 709 overexpressed | ~12,500 |
| Chou et al. 2008 | N/A | N/A | ~1.28 fold | 17 with high variation | ~18,000 |
| Esposito et al. 2008 | *APP, S100B* | *AQP4* | ~1.5 fold in DSCR | Modest pathway-specific changes | ~12,500 |
| Sommer et al. 2008 | *N/A* | *2M, CD74, CD52* | 7 | ~250 | N/A |
| Slonim et al. 2009 | *CLIC6, ITGB2, RUNX1* | N/A | 5 | 409 | ~12,500 |
| Chrast et al. 2000 | *Ifnar2, Ifngr2, Cbr* | N/A | 3 | 330 | N/A |
| Saran et al. 2003 | N/A | N/A | ~1.45 fold average | ~1.1 fold average | ~12,500 |
| Amano et al. 2004 | N/A | N/A | ~1.5 fold average | ~1.0 fold average | ~11,300 |
| Lyle et al. 2004 | *Adamts1, Mx1* | N/A | ~1.5 fold average | N/A | 99 |
| Dauphinot et al. 2005 | *Dscr3, Hmgp14, Donson* | *HoxA5, Dlx1* | ~1.5 fold average | ~1.0 fold average | ~12,500 |
| Kahlem et al 2007 | *Bace2, App, Mx1* | N/A | ~1.5 fold average | N/A | 136 |
| Sultan et al. 2007 | *Bace2, Kcne2, App, Cbr1* | N/A | ~1.5 fold average | N/A | 50 |
| Laffaire et al. 2009 | *Olig1, Dscam, Girk2, Son* | N/A | 11-13 in cerebellum<br>23 in GCL | 372-1164 in cerebellum<br>9 in GCL | ~15,600 |
| Moldrich et al. 2009 | *Olig1, Olig2, Dscam, Dscr3* | *Sox21, Calcoco1* | 29 of 54 | 1847 | ~25,000 |
| Hewitt et al. 2010 | *Itsn1* | *Mcm7, Brca2,Prim1* | ~1.5 fold average | 6.5% of probes | ~14,000 |

Table 4. Significant trisomic and disomic genes highlighted by gene expression studies. Common genes of interest were selected from studies that stated more than three genes. N/A= not applicable or available.

### 2.2.2 Altered expression of non-trisomic genes

While many gene expression studies have focused on expression levels of genes in three copies, increasingly the expression of non-trisomic genes has also been analyzed. Two early microarray studies using amniotic fluid and placenta found extreme variation associated with the genes on Hsa21, however both of these studies showed that disomic genes also contained high variation in expression in trisomic tissue (Chung et al., 2005; Deutsch et al., 2005). It was observed that two-copy genes were downregulated implying that Ts21 was affecting genes on other chromosomes (Chung et al., 2005). An additional microarray analysis on cells derived from DS fetal placenta found 750 genes were overexpressed with the majority located on chromosomes other than 21, implicating non-trisomic genes as significant contributors to DS phenotypes (Rozovski et al., 2007). Adult human DS brains exhibited 400 differentially expressed genes when compared to normal individuals and approximately 350 of these were not found on Hsa21 (Lockstone et al., 2007). Expression analyses conducted on uncultured DS amniotic fluid cells identified a total of 414 dysregulated genes with only five located on Hsa21 (Slonim et al., 2009). Two recent analyses of cells from developing Ts1Cje brains have also indicated a large number of dysregulated non-trisomic genes in addition to significantly dysregulated trisomic genes (Laffaire et al., 2009; Moldrich et al., 2009). Additionally, 6.5% of all disomic genes were found to be dysregulated in the primary neural progenitor cells isolated from the brains of adult DS mice (Hewitt et al., 2010). Based on these findings, it has been suggested that trisomy causes a disruption in gene regulation throughout the entire genome and not just of the genes in three copies. Despite these findings, the phenotypic effects of altered disomic gene expression in DS is highly debated and not well understood (Lyle et al., 2004; Mao et al., 2005; Saran et al., 2003).

## 2.3 Measuring gene regulation with tissue and age specificity

The most tissue-specific developmental microarray research in mouse models of DS has focused on the developing cerebellum, which continues to develop after birth. Extensive microarray analyses of the hypocellular cerebellum in adult and early postnatal trisomic mice have revealed dysregulation of both trisomic and non-trisomic genes. For example, microarray analyses were performed on the entire cerebellum during postnatal days 0, 15, and 30 (P0, P15, P30) in the Ts1Cje model of DS. Though genes present in three copies had an average relative expression of about 1.5 fold, only five (P0), nine (P15), and seven (P30) three copy genes of the 63 trisomic genes tested were expressed at levels >2 or <1.2. Additionally, 406 of 8250 two copy (non-trisomic) genes examined had ratios comparing trisomic and euploid expression that were significantly different from 1 (Dauphinot et al., 2005). Three trisomic genes were dysregulated at all developmental time points. Interestingly, changes in development influenced gene expression differences between stages more than between trisomy and euploid animals (Dauphinot et al., 2005; Potier et al., 2006). As these studies showed, gene expression can vary greatly in one tissue from early in development to adulthood. Because individual genes are shown to have variation in expression throughout development, certain genes may be relevant to a particular phenotype at one time point and unrelated at another.

## 2.4 Gene expression variation in response to Trisomy 21

In addition to the importance of understanding the temporal and spatial relationships associated with trisomic gene expression, variation in gene expression of both disomic and

trisomic genes may contribute to DS phenotypes. Expression studies conducted on fibroblasts and LCLs identified large gene expression variation between cell lines as well as between individual genes (Ait Yahya-Graison et al., 2007; Deutsch et al., 2005; Prandini et al., 2007). It was suggested that high levels of gene expression variation could explain why the majority of Hsa21 genes were not significantly upregulated and the variability in DS phenotypes. The latter is explained by the level of overlap between gene expression in trisomic and euploid individuals. Genes with distinct expression profiles may be candidates for the constant features of DS, whereas the genes that exhibit overlapping expression profiles may be responsible for the variable DS phenotypes (Prandini et al., 2007).

Studies conducted in Ts65Dn mice also exhibited differing levels of variable gene expression. Sultan et al. showed that 31 out of 33 trisomic genes were upregulated in the Ts65Dn brain. Of these 31 genes, 24 genes exhibited a small amount of variation (coefficient of variation of <0.2) and seven genes exhibited high variation. These authors suggested that the level of variation in gene expression indicated how tightly the genes were being regulated in the brain tissue of Ts65Dn animals.

Understanding gene expression variation in trisomic tissues is a key component in the study of DS gene-phenotype relationships. This expression variation may influence the results of microarray and qPCR studies and lead to the false identification/exclusion of candidate genes for specific phenotypes. Additionally the phenotypic variability observed in individuals with DS may be attributed to trisomic gene expression variation in different tissues, and it is likely that the level of variation within an individual gene is directly related to how tightly that particular gene is regulated. Further research is needed to identify the exact role expression variation plays in the development of DS phenotypes.

### 2.5 Summary

Gene expression assays in DS are an essential tool for understanding the mechanisms behind Ts21. While the gene dosage hypothesis may be applicable to specific genes with spatiotemporal specific expression, gene expression analyses suggest that not all trisomic genes are dysregulated at a 1.5 fold ratio, the expression of some trisomic genes is similar to euploid, and variation in gene expression plays a significant role in influencing phenotypic development. Differences in developmental stages as well as particular tissue types may contribute to some of the variable findings across studies. Additionally, the level of gene expression variation differs between individuals of the same genotype, adding to the complexity of understanding expression analyses. Though trisomic genes are important and are the likely initiators of DS phenotypes, disomic genes are also dysregulated suggesting that both trisomic and disomic genes can influence the DS phenotype.

## 3. Complex genotype-phenotype relationships

Characterization of certain DS phenotypes along with the genetic analysis of individuals with partial trisomies led to a hypothesis of the "Down syndrome critical or chromosomal region" (DSCR) believed to contain the genes necessary for the development of the most common phenotypes observed in individuals with DS (Delabar et al., 1993; Korenberg et al., 1990). The sequencing of Hsa21 and mapping of specific genes in the putative DSCR further defined the genetic content of the region and allowed for a more detailed trisomic gene-phenotype correlation. More recent analyses of critical regions using mouse models of DS as well as samples from individuals with partial trisomies have questioned the validity of the

original DSCR hypothesis of a single region or gene important for most of the common DS traits (Korbel et al., 2009; Lyle et al., 2009; Olson et al., 2004a). Current data suggest a single critical region or critical gene is not sufficient to cause multiple characteristic DS phenotypes. Rather, specific genes within susceptibility regions may play important roles in the establishment and maintenance of specific, but not all, DS phenotypes.

### 3.1 Origins of the Down syndrome critical region hypothesis

The DSCR was originally hypothesized based on the assessment of a family with partial Hsa21 trisomy and the variable expression of several of the characteristic physical features of DS. A "critical region" within 21q22 was believed to be responsible for several DS phenotypes including craniofacial abnormalities, congenital heart defects of the endocardial cushions, clinodactyly of the fifth finger and mental retardation (Niebuhr, 1974). Soon after the DSCR hypothesis, genetic analyses of the Hsa21 DNA revealed several gene-rich segments located within 21q22 and placed further emphasis on this region as critical to the development of DS phenotypes (Brahe et al., 1990).

The hypothesis of a DSCR shifted the focus of many in the DS research community toward understanding how these genomic regions could correlate with the development of DS phenotypes. Regions linked to congenital heart defects (CHD), duodenal stenosis and craniofacial abnormalities in individuals with DS were identified through further analyses (Korenberg et al., 1992). An analysis of ten patients with partial trisomy for different segments of Hsa21 led to the mapping of 24 DS phenotypes to six chromosomal regions. Of these 24 phenotypes, 13 were mapped to 21g22.2 – proximal 21q22.3 region and six were linked to the *D21S55-MX1* region (Delabar et al., 1993). CHD was not included in the phenotypes associated with the D21S55-*MX1* region, and a later assessment of 19 individuals led to the mapping of CHD to a region outside of the D21S55-*MX1* region on 21q22.3 including *DSCAM,* a gene known to be involved in cell adhesion and expressed in the heart during development (Barlow et al., 2001a). A similar experiment comparing gene overlap regions with the phenotypic traits of 16 individuals with partial Ts21 along with the phenotypic variability observed in DS led to the hypothesis that factors in addition to trisomic genes both within and outside the DSCR likely contribute to most DS phenotypes, as opposed to a single critical region (Korenberg et al., 1994). Several additional genetic aspects including allelic heterogeneity, epistatic interactions, imprinting effects, uncharacterized epigenetic modifications and environmental events as well as the general variability observed in non-affected individuals were also suggested to have an impact on phenotypic variability (Korenberg et al., 1994).

The DSCR hypothesis, derived from analyses of the shortest region of overlap in individuals with partial Ts21, paved the way for the establishment of gene-phenotype relationships associated with DS. However, the high variability in phenotypic expression caused by an array of trisomic genetic consequences severely restricts the resolution of the gene-phenotype relationships associated with DS. The sequencing of Hsa21 as well as the development of several mouse models of DS provided further insight into the DSCR hypothesis.

### 3.2 Disproving the DSCR with mouse models

Unlike the analyses of partial trisomy in humans, comparisons of DS mouse models with differing segmental aneuploidies (both on their own and crossed between each other) has allowed for a systematic and meticulous analysis of the relationship between certain

chromosomal regions and DS phenotypes. The Ts1Rhr/Ms1Rhr mouse model was created by generating a 3.9 Mb reciprocal duplication/deletion containing 33 genes (*Cbr1-Orf9*) homologous to those found in the most commonly recognized putative human DSCR (Olson et al., 2004a). Human DS-like phenotypes documented in Ts65Dn and Ts1Cje mice, which contain larger segmental trisomies, and the creation of Ts1Rhr and Ms1Rhr mice, established a system suitable to test the DSCR hypothesis in mice. Ts65Dn mice are smaller in size with shorter femurs and exhibit craniofacial abnormalities including smaller skulls and mandible bones, as well as brachycephaly when compared to euploid littermates—defects also found in humans with DS and previously attributed to the DSCR (Delabar et al., 1993; Richtsmeier et al., 2000). Analysis of Ts1Rhr mice revealed significant increases in overall size, length of femur, and mandible size when compared to euploid littermates and an absence of brachycephaly, indicating major differences between Ts1Rhr and Ts65Dn or Ts1Cje phenotypes (Olson et al., 2004a). Mice generated from a cross between Ts65Dn and Ms1Rhr mice (removing triplication of the DSCR in the Ts65Dn mouse) exhibited craniofacial phenotypes similar to those observed in Ts65Dn mice (Olson et al., 2004a). Comparison of the craniofacial phenotypes observed in Ts1Rhr and Ts65Dn/Ms1Rhr mice indicated that the DSCR is not sufficient to produce these phenotypes in DS mouse models, but may still contribute in the genetic context of the phenotypes observed in Ts65Dn mice through genetic interactions.

In addition to the studies conducted on craniofacial skeletal abnormalities, DS-associated brain phenotypes have also been compared in DS mouse models. Ts65Dn mice exhibit a similar sized brain and hippocampus but smaller cerebellum and reductions in granule and Purkinje cell density when compared to euploid littermates (Baxter et al., 2000). Ts1Cje and Ms1Cje/Ts65Dn mice exhibit similar but less severe defects to those observed in Ts65Dn mice (Olson et al., 2004b). Analysis of Ts1Rhr mice revealed a similar sized brain and hippocampus when compared to euploid littermates, analogous to what was observed in Ts65Dn and Ts1Cje mouse models. The cerebellum of Ts1Rhr mice exhibited a small but significant reduction in size compared to euploid mice, but no differences were found in granule or Purkinje cell density, suggesting that trisomy for the DSCR is not sufficient to produce some brain phenotypes associated with DS (Olson et al., 2007).

Furthermore, studies have shown that Ts65Dn mice exhibit impairment of hippocampal function similar to that observed in individuals with DS (Holtzman et al., 1996; Reeves et al., 1995). Based on the DSCR hypothesis, genes within this region control the cognitive impairment phenotype associated with DS. If this were true, Ts1Rhr mice should exhibit similar deficits to Ts65Dn mice in hippocampal function. In contrast to Ts65Dn mice, Ts1Rhr exhibited normal hippocampal function in the Morris water maze test and normal induction of long term potentiation (LTP) in the CA1 suggesting the DSCR is not sufficient to cause the cognitive impairment associated with DS (Aldridge et al., 2007; Olson et al., 2007). Interestingly, when three copies of the DSCR were reduced to two, Ts65Dn/Ms1Rhr mice performed similarly to euploid animals in the water maze test suggesting that although the DSCR is not sufficient on its own to cause cognitive impairment, it is necessary for the phenotype to occur (Olson et al., 2007).

It is important to note that there are background differences between Ts65Dn, Ts1Cje, Ms1Rhr, and Ts1Rhr mice utilized in the studies described above. Inbreeding strategies for Ts65Dn and Ms1Rhr mice have not been successful, and these mice are maintained on an ~50% B6 and 50% C3H advanced intercross genetic background. The majority of studies previously

documented in Ts1Rhr mice used a mixed background of approximately 50% B6, 25% 129 and 25% C3H (except for the Morris water maze and LTP study where Ts1Rhr were on a B6 background) and it is possible that background differences may cloud direct comparisons between these mouse models. Behavioral and neurophysiologic phenotypes were assessed in Ts1Rhr mice maintained on a ~50% B6 and 50% C3H background to mimic the same genetic background as Ts65Dn mice. In contrast to what was observed in Ts1Rhr mice on other backgrounds, these mice exhibited several (20 of 48) similar neurologic phenotypes as Ts65Dn mice suggesting that the DSCR may be sufficient to confer some characteristic DS cognitive and brain abnormalities (Belichenko et al., 2009). The differences in neurological phenotypes in Ts1Rhr mice on different genetic backgrounds suggest the importance of understanding the complex genetic interactions associated with DS. Although evidence in mouse models implies that there is no single region critical to the majority of DS phenotypes, it is apparent that a gene or genes within this region are important in certain phenotypes and these phenotypes may be dependent on genetic background. Either allelic differences associated with trisomic genes or other two-copy genes in the genome may be responsible for the differences observed between trisomies with different genetic backgrounds.

### 3.3 New views on the DSCR and additional analyses in humans

Advancements in DNA technology as well as additional individuals identified with partial trisomy have led to more information on the hypothesized DSCR and gene-phenotype relationships in individuals with DS. A genotype-phenotype correlation analysis conducted on 30 individuals with either partial trisomy or partial monosomy for Hsa21 using array comparative genome hybridization found that four individuals with partial trisomy for the proximal portion of Hsa21 (not including the putative DSCR) exhibited several DS phenotypes (Lyle et al., 2009). Additionally, it was shown that there are multiple regions necessary to produce the cognitive impairment and hypotonia associated with DS, as well as a region previously identified as a candidate for CHD (*D21S3-PFKL*) (Barlow et al., 2001a). These new results suggest that the concept of one chromosomal region important for the majority of DS phenotypes does not exist due to multiple genetic regions critical for DS phenotypes. Due to the identification of multiple regions contributing to the same phenotypes, these regions may be more aptly termed "susceptibility" regions and may correspond to one or a limited number of phenotypes (Lyle et al., 2009). Individuals with triplication of these genes are more likely to exhibit the DS phenotypes associated with these regions.

High-resolution genetic mapping to determine gene involvement of eight specific DS phenotypes using 30 individuals with segmental trisomy for Hsa21 also concluded that specific genetic regions may be important for certain DS phenotypes. Genomic analysis revealed that the interaction of *DYRK1A* and *RCAN1*, thought to be involved in many DS phenotypes (Arron et al., 2006), were not essential in the development of CHD and mental retardation because individuals without trisomy for these genes exhibited severe abnormalities (Korbel et al., 2009). Additionally, several DS phenotypes, including transient myeloproliferative disorder, AMKL, and cognitive impairment, require triplication of multiple Hsa21 regions and genes (Korbel et al., 2009). A combination of data from this work and that in mouse models led to the specification of a CHD-causing region smaller than but included in a previously defined region for CHD (Barlow et al., 2001a). The newly defined region contains only 10 genes including *DSCAM*, a cell adhesion molecule highly expressed in the developing heart, also believed to contribute to the high levels of Hirschprung disease associated with DS (Korbel et al., 2009; Korbel et al., 2007).

Additional evidence against the synergy of *DYRK1A* and *RCAN1* in causing craniofacial and cardiac abnormalities associated with DS (Arron et al., 2006; Richtsmeier et al., 2000) came from a family with a 4.3 Mb duplication of chromosome 21q22 (including *DYRK1A* but not *RCAN1* or *DSCAM*). Individuals with the duplication presented with severe DS-like craniofacial abnormalities, but other malformations including cardiac defects were not observed. Based on these observations, the authors suggested two distinct regions important for these DS phenotypes with the distal region containing the genes including *DYRK1A* associated with craniofacial abnormalities and the proximal region (including *RCAN1* and *DSCAM*) associated with cardiac abnormalities (Ronan et al., 2007).

Interestingly, studies of individuals without DS provide evidence against the theory of only these distal and proximal specific regions associated with DS phenotypes. A patient with Silver-Russell syndrome, which has little similarity with DS, and his healthy father displayed a 0.46 Mb duplication of 21q22 including the *RCAN1* gene. No DS-associated phenotypes were documented in either individual suggesting that *RCAN1* alone is not sufficient to produce DS phenotypes (Eggermann et al., 2010). Furthermore, a child with a non-mosaic ring chromosome 21 duplication containing most of the long arm of Hsa21 including the hypothesized DSCR was found to have several characteristic DS phenotypes including cardiac and gastrointestinal defects but lacked the usual facial features associated with DS (Crombez et al., 2005).

Taken together, these studies provide evidence against the original DSCR hypothesis. It has been shown that genes and regions of genes located both within and outside of the hypothesized DSCR are critical to the initiation and severity of specific DS phenotypes. Studies conducted on humans with DS, DS mouse models, and other transgenic models have uncovered that several genes found within the putative DSCR are known to have a major effect on DS phenotypes. Thus, although the DSCR concept as traditionally defined does not seem to be correct, there are critical genes within this region that have a major impact on specific DS phenotypes.

### 3.4 Individual genes associated with the DSCR

Although it is evident the DSCR does not contribute to all of the phenotypes associated with DS, because the DSCR has been heavily studied, several genes found within this region have been implicated as candidate genes for individual DS phenotypes. The most extensively studied genes found within this region are Regulator of calcineurin1 (*RCAN1/DSCR1*) and dual specificity tyrosine-phosphorylation kinase 1a (*DYRK1A*), which are hypothesized to play important roles in several developmental pathways, including CNS, craniofacial skeletal and cardiac (Arron et al., 2006; Park et al., 2009; Richtsmeier et al., 2000). *DYRK1A* and *RCAN1* are both involved in the regulation of NFAT, a critical transcription factor necessary for the processes of vertebrate development and organogenesis (Graef et al., 2001). Transgenic *Nfatc* mutant mice exhibit several characteristics similar to DS mouse models as well as humans with DS including cognitive impairment and craniofacial and cardiac abnormalities. Mice with overexpression of *Dyrk1a* and *Rcan1* exhibited similar phenotypes to *Nfatc* mutants, suggesting that these genes are likely playing a part in the development of DS phenotypes (Arron et al., 2006).

In addition to *DYRK1A* and *RCAN1*, Down syndrome cell adhesion molecule (*DSCAM*) has been suggested to play a critical role in the developing brain and has also been identified as a candidate gene for the increased levels of CHD observed in DS individuals (Alves-Sampaio et al., 2010; Barlow et al., 2001a; Barlow et al., 2002). DSCAM is a critical factor in

neural differentiation, axon guidance, and the establishment of neural networks and it has been suggested that the disruption of these processes contributes to the DS neurocognitive phenotype (Agarwala et al., 2001; Barlow et al., 2001b; Yamakawa et al., 1998). A recent study found that *Dscam* was overexpressed in hippocampal neurons of the Ts1Cje mouse model. Overexpression of *Dscam* impaired dendritic branching leading to an inhibitory effect on synaptogenesis and neurite outgrowth, further implicating the role of *Dscam* in the development of the DS brain phenotype (Alves-Sampaio et al., 2010).

Also found within the DSCR, *ETS2* is a transcription factor known to be involved in the regulation of cellular proliferation, differentiation, transformation and apoptosis (Seth and Watson, 2005). Extensive studies on *Ets2* in transgenic and DS mouse models have revealed that the gene may play a role in the neuronal, tumor suppressive, and craniofacial phenotypes associated with DS (Hill et al., 2009; Sussan et al., 2008; Wolvetang et al., 2003). Additionally, *KCNJ6/GIRK2* overexpression in the hippocampus of Ts65Dn mice has been shown to cause an abnormal balance between inhibitory and excitatory synapses, implicating the gene in the DS-brain phenotype (Best et al., 2007). Additionally, this overexpression was also implicated in the reduced cerebellar size and alterations in granule cell neuron differentiation observed in the weaver mouse (Patil et al., 1995).

### 3.5 Gene(s)-phenotype relationships

The establishment of a specific phenotype requires the successful coordination of a number of genetic interactions. In many human disorders a single gene or gene network is responsible for causing the associated phenotypes. However, evidence from both humans and mouse models of DS suggests that specific DS phenotypes are influenced by genetic aberrations in multiple genes as opposed to a single gene. The most glaring case of multiple genes affecting a phenotype comes from the study of cognitive impairment through the analysis of human and mouse DS brains. *DYRK1A, RCAN1,* and *DSCAM* have all been shown to regulate the stages of neuronal cell maturation (proliferation, differentiation, and apoptosis) in the developing brain (Agarwala et al., 2001; Barlow et al., 2001b; Park et al., 2010; Sun et al., 2011). Triplication of *Olig1*, *Olig2*, and *Kcnj6,* leads to significant changes in the ratio of inhibitory to excitatory neurons in the Ts65Dn forebrain (Best et al., 2007; Chakrabarti et al., 2010) and *DSCAM* regulates dendritic branching and neuronal network establishment (Alves-Sampaio et al., 2010), suggesting that increased inhibitory activity and the inability to form neuronal networks are also contributing to the cognitive impairment phenotype. In addition to cognitive impairment, an AD-like phenotype is apparent in most adult individuals with DS. Dysregulation of *DYRK1A, RCAN1,* and/or *Ets2* lead to an increase in the number of neurofibrillary tangles and β-amyloid plaques in the brains of humans and mice (Ermak et al., 2001; Ryoo et al., 2008; Sun et al., 2011), respectively, indicating that multiple genes are contributing to the DS-AD phenotype.

### 3.6 Summary

Based on thorough analyses of studies on humans and DS mouse models, it is evident that there is not a single critical region of genes sufficient to cause all DS phenotypes. Alternatively, it is likely that there are multiple critical regions or critical genes contributing to a respective phenotype or group of phenotypes associated with DS (Lyle et al., 2009). Although studies conducted regarding the DSCR have provided a wealth of evidence refuting the idea, it is important to understand that several genes within this region have

been identified as key contributors to more specific DS phenotypes. Furthermore, both non-trisomic genes and trisomic genes located outside of the hypothesized DSCR have also been implicated in the development of specific DS phenotypes and in some cases may be linked with DSCR associated genes. It is evident that DS phenotypes are influenced by a multitude of complex genetic interactions and it seems likely that multiple genes and gene networks will be involved in the development of most DS phenotypes. Important genes or regions of genes contributing to specific DS phenotypes should be defined as susceptibility genes or regions, as opposed to defining a single Down syndrome critical region or single gene-phenotype relationship (Lyle et al., 2009).

## 4. Functional analysis of genes

### 4.1 Introduction

Although hypotheses have been developed concerning cellular and developmental mechanisms relating to DS phenotypes, no conclusive evidence exists for a single mechanism likely responsible for the majority of DS phenotypes. Instead, multiple common mechanisms may be responsible for individual phenotypes in specific tissues or at precise developmental stages and groups of genes with related functions may also be dysregulated in DS pathology. Functional databases provide the foundation for elucidating gene-phenotype relationships by clustering dysregulated genes with common functions. These clusters identify potential cellular, developmental and biological functions, as well as the number of genes in each cluster and significance of those categories. Depending on the functional annotation tool, relevant pathways, molecules for potential pharmacological interventions or insight into genetic mechanisms may be suggested. The use of functional databases extends the value of high throughput arrays and tests hypotheses that specific groups of genes with related function may be dysregulated in DS. Rather than a simple dosage increase of Hsa21 genes, it has been hypothesized that trisomy has a more global dysregulational effect on the genome, though developmental changes and tissue type do maintain a significant role (Altug-Teber et al., 2007; Dauphinot et al., 2005; Sommer et al., 2008). We hypothesize that the interaction between trisomic and disomic genes have a significant effect on the way DS phenotypes arise, manifest and progress.

Several studies have documented differential gene expression in unique spatial and developmental environments to determine mechanisms affecting DS phenotypes (Conti et al., 2007; Lyle et al., 2004; Moldrich et al., 2007). Other studies often concentrate on the cellular or developmental mechanisms as a causative factor of DS phenotypes (Chakrabarti et al., 2007; Contestabile et al., 2009; Cooper et al., 2001; Roper et al., 2006; Roper et al., 2009). High throughput analyses are important for investigating gene-phenotype relationships in analyses of various tissue and cell types from individuals with DS, mouse models of DS, and cell lines previously derived from individuals with DS or engineered to contain this extra genetic material. We hypothesize that a single generalized pathway or mechanism does not underlie the phenotypes of DS, but rather several pathways and mechanisms contribute to the phenotypes of DS, though some functional groups may cluster together in certain tissues or within phenotypes. Assessment of the gene-phenotype relationships in high throughput meta-analysis provides novel information regarding the importance of developmental processes in the DS pathophysiology.

### 4.2 Previously established functional analyses

Studies of DS have utilized high throughput analysis on several tissues including cultured DS neural progenitor cells and tissue samples from DS amniocytes, hearts, cerebra and cerebella and cultured Ts1Cje neural progenitor cells (NPCs). Though differences are present between developmental time points and tissues, genes involved with cellular cycling, cell adhesion, signal transduction, DNA and RNA metabolism and binding, gene expression regulation and transcription, mitochondrial function and oxidative phosphorylation, kinase activity, and ECM production and maintenance were the most highly dysregulated and common categories observed, as well as maintain the ability to transcend time and tissue differences (Table 5). More specifically, certain cell types of representative models of DS or from individuals with DS share multiple common annotation results. We suggest a similar phenomenon may regulate multiple DS phenotypes in which dysregulated mechanisms affect the same tissue in order to produce a phenotype (Figure 1).

#### 4.2.1 Cell cycle alterations contribute to neurological phenotypes

NPCs display altered gene expression related to cell cycling, proliferation, signaling, transcription and metabolism of chromosomal material. It is well established that deficits in proliferation and mitotic activity of specific cellular populations in the DS brain exist, including areas of the cerebellum and multiple areas of the cerebrum (Baxter et al., 2000; Chakrabarti et al., 2007; Gardiner et al., 2010; Roper et al., 2006). Additionally, impairment of proliferation in the cerebellum of fetuses with DS has also been reported (Guidi et al., 2010) as well as in the Ts65Dn neonate peripheral tissues and fibroblasts, suggesting a general deficit in proliferation as a mechanism for multiple DS abnormalities (Contestabile et al., 2009). Individuals born with DS have reduced brain weights coupled with a smaller, dysmorphic skull and multiple cellular abnormalities within the brain including reduction in the number of neurons in the cerebral cortex as well as cellular deficiencies in multiple other structures (Aylward et al., 1997; Fink et al., 1975; Wisniewski, 1990). Therefore, these phenotypic deficits may be caused by a similar mechanism. However, downregulation of genes with proliferation-promoting function or upregulation of genes involved in the arrest of proliferation could equally, if not synergistically, contribute to the general proliferation deficit hypothesized to occur in several DS phenotypes. Overexpression of *Dyrk1a* in mouse NPCs was recently found to inhibit proliferation and stimulate precocious neuronal differentiation (Park et al., 2010; Yabut et al., 2010). *DYRK1A* has previously been implicated in the physiopathology of the cognitive impairment observed in individuals with DS (Altafaj et al., 2001; Smith et al., 1997) and is overexpressed approximately 1.5 fold in the DS and Ts65Dn brain (Dowjat et al., 2007; Guimera et al., 1999), suggesting upregulation of *DYRK1A* in concert with other Hsa21 and disomic genes in the DS brain may lead to deficits that underlie both cognitive phenotypes and other cellular phenotypes of DS.

#### 4.2.2 Changes in cell adhesion contribute to DS phenotypes

In addition to brain, other tissues appear to be affected by alterations in cell homeostasis. Because cell cycling, cell adhesion, signal transduction, and ECM production all display dysregulation in DS fetal amniocytes, heart, cerebra and cerebella, one cannot conclude that a simple deficit in proliferation, such as that observed in DS skin fibroblasts (Kimura et al., 2005), is sufficient to result in the DS phenotypes observed. While the DS brain is reduced in

size overall, it is also dysmorphic, pointing to both a decrease in proliferation, but perhaps also altered cell cycling, changes in the migratory pattern of progenitor cells and even the decreased founder population of these cells due to decreases in neurogenesis, implicating a complex interaction of mechanisms in the DS neurological phenotype (Bhattacharyya et al., 2009; Guidi et al., 2010).

| Study | Tissue | Time point | Analysis Tool | RNA/DNA metabolism | Cell cycling | Cell adhesion | Cell fate | Transcription | Oxidative phosphorylation | Mitochondria | Cell Proliferation | Apoptosis | Regulation of gene expression | RNA/DNA binding | Signal Transduction | Cytoskeleton organization | Monovalent cation transport | Collagen | Integral to plasma membrane | Other annotations of interest |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (Mao et al., 2005) | DS cerebrum | | G | | | | | + | | + | | | | + | | | + | | | Monovalent cation transport |
| (Mao et al., 2005) | DS cerebellum | | G | + | | | | | | | | | | + | | | | | | Nerve impulses |
| (Mao et al., 2005) | DS astrocytes | | G | + | | + | | | | | | | | | | | | + | + | |
| (Mao et al., 2005) | DS heart | | G | + | | | | | | + | | | | | | | + | | + | Cell-cell signalling |
| (Conti et al., 2007) | DS heart | 18-22 wks | G | | | 22 | | | 9 | 48 | | | | | | | | 8 | | ATP synthesis, ECM, phosphate transport |
| (Lockstone et al., 2007) | DS dorsolateral prefrontal cortex | | O | | 16 | 3 | 3 | 5 | | | 9 | 7 | 6 | | | 6 | | | | Immune system, Notch and tryrosine signalling, cell migration, endocytosis |
| (Rozovski et al., 2007) | DS trophoblasts | Fetal | N | 4 | | | | | 6 | | | | | | | | | | | Ubiquitine cycle, purine biosynthesis |
| (Chou et al., 2008) | DS amniocytes | 16-22 wks | G | | | + | | + | | | | | | | + | | | | | |
| (Esposito et al., 2008) | DS NPCs | 19-21 wks | I | | 25 | | | | | | | | 20 | | 25 | | | | | Molecular transport |
| (Sommer et al., 2008) | DS lymphocytes | 1-4 yrs | D | 12 | 5 | | | | 14 | | | | | | | 12 | | | | Protein synthesis, kinase binding, antigen presentation |
| (Bhattacharyya et al., 2009) | DS NPCs | | G | | 131 | | 131 | | | | 131 | 81 | | | | | | | | Neurogenesis |
| (Moldrich et al., 2009) | Ts1Cje NPCs | E14.5 | G | | | | 75 | | | | | | | | | | | | | Development, homeostasis |

Table 5. Published functional analysis data for phenotypes of DS. Functional annotations were selected by lowest p-values for the categories established by the investigators and of specific interest to the authors. Studies are listed by tissue type and annotations are listed by most common to least common categories horizontally. Analysis tool abbreviations: G: GeneOntology (www.geneontology.org), I: Ingenuity Pathway Analysis (www.ingenuity.com), O: OntoExpress (vortex.cs.wayne.edu/projects.htm), N: NetAffex (www.affymetrix.com/analysis/index.affx), D: DAVID (david.abcc.ncifcrf.gov).

In addition, approximately 50% of newborns with DS display a congenital heart defect, most of which involve septal and canal defects (Pediatrics, 2001). Previous studies have

established a link between DS atrioventricular septal defects (AVSDs) and the importance of collagen VI in developing endocardial cushions (Baptista et al., 2000; Davies et al., 1995). Characterization of the involvement of collagen VI in cardiac development was later established when investigators observed altered collagen VI expression in AV endocardial cushions in the DS heart (Gittenberger-de Groot et al., 2003). Interestingly, genes encoding collagen VI are found on Hsa21 and its role as an extracellular matrix (ECM) component makes its presence ubiquitous throughout the body (Aumailley et al., 1991). A similar idea has recently been proposed in which atrioventricular canal (AVC) defects, which comprise the majority of congenital heart defects observed in DS, are a result of increased cell adhesion. Deficits in cell adhesion may lead to decreased cell migration to the ventricular canal because of changes in the ECM, leading to alterations in epithelial-mesenchymal transformations (Delom et al., 2009). Using a transchromosomic model of DS, investigators found both decreased cell migration and an increased affinity for adhesion to collagen VI (Delom et al., 2009). Importantly, differences in Hsa21 genes or groups of genes causing aberrations in both cellular properties may act in concert to lead to the development of AVC defects. It is therefore plausible that cell adhesion complexes coupled with ECM component alterations significantly contribute to congenital heart abnormalities in DS.

In addition to congenital heart defects, individuals with DS are at an increased risk for dermatological disorders such as atopic dermatitis, causing susceptibility toward opportunistic infections (Madan et al., 2006). Studies utilizing DS skin fibroblasts have shown that these cells display an increased adhesion to collagen VI, likely leading to aberrant migration of these cells to target areas of the body (Jongewaard et al., 2002). This decreased migration would leave a smaller population of cells to make up an epidermal barrier and thus would compromise the integrity of the skin. Dysregulation of genes involved in cell adhesion may also lead to changes in junctional complexes that are found to be altered in functional analyses, and these changes may contribute to atypical migration of the cells with which they interact.

### 4.2.3 Changes in DNA and RNA metabolism may lead to transcription and translation dysregulation

Genes involved with the metabolism and expression of nucleic acids are highly dysregulated in multiple DS tissue types (brain, heart, trophoblasts) and time points in multiple functional analyses. The observed dysregulation of the genome due to the presence of the extra chromosomal material and its interaction with the rest of the genome is likely directed by multiple types of regulation. It may be that fewer transcripts are formed given a higher rate of metabolism of genetic material. The reduced transcripts directing cellular proliferation, migration or an anti-apoptotic state could lead to the smaller or altered structures observed in DS.

In particular, changes in metabolism of genetic transcripts have been documented in the expression of multiple genes in both DS-related and unrelated tissues. Several studies sampling multiple DS-derived tissues have described the transcriptional alterations of Hsa21 genes, including two genes involved in nucleic acid metabolism: GART (purine metabolism) and ZNF294 (transcriptional regulation) (Lintas et al., 2010). DNA and RNA binding and metabolism may also be related to transcription factor activity, dysregulating genes downstream of these alterations. Alterations of transcription factors such as NFAT, as a result of dysregulation of *DYRK1A* and *DSCR1*, have the potential to lead to DS developmental phenotypes (Arron et al., 2006). The dysregulation of NFAT may initiate a

cascade of events affecting multiple downstream targets. Many of these genes may also be involved with cell cycling and maintenance as well as DNA and RNA binding and metabolism. Such studies, however, remain to be performed.

#### 4.2.4 Mitochondrial changes penetrate neurological and metabolic phenotypes

The high incidence of mitochondria-related dysregulation in DS hearts, lymphocytes, and trophoblasts coupled with the increased prevalence of biomarkers of reactive oxygen species (ROS) in individuals with DS (Jovanovic et al., 1998) is an interesting and well-studied paradigm. Mitochondrial impairment in fetal hearts suggested by the downregulation of genes from five mitochondrial complexes, as well as the cerebellum and other brain regions, platelets, astrocytes and cultured fibroblasts suggests pervasive impairment of mitochondria in individuals with DS (Arbuzova et al., 2002; Busciglio et al., 2002; Conti et al., 2007; Kim et al., 2001; Kim et al., 2000; Prince et al., 1994). Interestingly, this mitochondrial impairment may affect other DS pathophysiology. Dysregulation of the mitochondria has the ability to cause alterations in ATP synthesis, electron transport, monovalent cation transporter activity, oxidative phosphorylation, and general changes in physical components (Conti et al., 2007) (all found as dysregulated functional groups in Table 5). Furthermore, changes in membrane potential caused by this dysregulation may lead to increased mitochondrial susceptibility to other insults, leading to a constant feedback in the balance of genetic regulation (Roat et al., 2007). Two genes in particular located on Hsa21, *SOD1* and *BACH1*, have been implicated the generation of ROS due to elevated $H_2O_2$ levels and decreased transcriptional activity, respectively. Elevated levels of *SOD1* have been implicated in deposition of β amyloid and *BACH1* downregulation has been proposed as a contributing mechanism to the development of Alzheimer disease neuropathology (Percy et al., 1990; Shim et al., 2003). High levels of oxidative stress in the brains of individuals with DS may indicate that dysregulation of normal processes in the mitochondria lead to anomalies including Alzheimer pathology (de Haan et al., 1997; Lockstone et al., 2007). A disproportionate number of ROS, caused by mitochondrial and other changes, combined with altered metabolism and feedback mechanisms suggest the complexity of the interacting mechanisms involved in DS phenotypes affecting the brain, heart and skin (Busciglio and Yankner, 1995; Li et al., 2006; Sinha, 2005).

### 4.3 DAVID analysis of trisomic genes in humans with DS and DS mouse models

DS mouse models have the ability to predict phenotypes not previously observed in individuals with DS (Baxter et al., 2000; Pennington et al., 2003). We hypothesized that by analyzing the genes trisomic genes on Hsa21 as well as their homologs in two DS mouse models, we would observe common mechanisms as found by functional analysis of altered gene expression. Using DAVID (http://david.abcc.ncifcrf.gov/) we analyzed trisomic genes from Hsa21 and the Ts1Rhr and Ts65Dn mouse models (http://chr21.molgen.mpg.de/HSA21db.html). Functional annotations were obtained using the protocol described by Huang et al. 2009.

Our analyses of Hsa21 genes revealed dysregulation of functional annotations including keratin, intermediate filament/cytoskeleton digestion, protein dimerization, interferon receptor activity, cytokine receptor activity, multiple junctions, cell adhesion, ionic channels and ECM (Table 6). Analysis of the genes triplicated in Ts65Dn mice revealed dysregulation of genes involved in ion transport, voltage-gated channels, ionic channels, monovalent inorganic cation transport, tight junctions, calcium dependent cell-cell adhesion, regulation

of transcription, DNA binding, behavior, neuron development, neuron differentiation and synaptic transmission and cell-cell signaling. Analysis of genes in three copies from the Ts1Rhr mouse model identified functions integral to binding and related to regulation of transcription factor activity, transcription and DNA binding as the most enriched categories with the lowest p-values.

As predicted, some, but not all functional groups found in previous analyses were also seen in our DAVID analysis. Data compiled using DAVID analysis of Hsa21 and two DS mouse models provide insight into what mechanisms appear to be generally dysregulated when trisomy for these regions occurs. Ts65Dn mice display many similarities to individuals with DS with regard to general brain, behavior, stature, heart and craniofacial phenotypes. Interestingly, a high level of overlap in functional categories also exists between the two analyses. For example, our analysis of Hsa21 and Ts65Dn revealed dysregulation of genes involved in cell adhesion, tight junctions and ionic transport. Ts65Dn mice replicate some of the heart defects observed in DS, and aberrant cell adhesion and migration have been implicated in these defects (Delom et al., 2009; Moore, 2006; Williams et al., 2008). Ts1Rhr and Ts65Dn triplicated genes led to functional hits in transcription, transcription factor activity, and RNA/DNA binding. While Ts1Rhr and Ts65Dn mice display some similar phenotypes, Ts1Rhr mice exhibit a number of phenotypes contrary to those seen in DS. It is noteworthy, though, that though these functional groups are dysregulated, they may be regulated in a manner divergent from one another, such as higher levels of transcription and RNA/DNA binding in Ts1Rhr than Ts65Dn, leading to the exaggerated phenotypes which Ts1Rhr mice display. Interestingly, no functional groups overlapped between the Hsa21 and Ts1Rhr analyses and this result provides additional evidence for the different phenotypes observed between humans with DS and this mouse model. Clearly, the involvement of other trisomic genes as well as disomic genes plays an important role in the identification of functional categories. Given that our analyses only included trisomic genes, it seems plausible that significant overlap between the past and current studies was not found.

| Study | RNA/DNA binding | Cell adhesion | Transcription | Transcription factor activity | Tight Junctions | Ionic transport | Voltage-gated channel | Cell junctions | Cell signaling | Apical Junction Complex | Intermediate Filaments | Protein dimerization activity | ECM | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hsa21 Genes | | 13 | | | 6 | 8 | | 7 | | 6 | 27 | 11 | 6 | Keratin, digestion, interferon receptor activity, cytokine receptor activity |
| Ts65Dn mouse model | 7 | 4 | 7 | 6 | 5 | 8 | 5 | | 3 | | | | | Behavior, neuron development, neuron differentiation, synaptic transmission |
| Ts1Rhr mouse model | 3 | | 4 | 3 | | | | | | | | | | Integral to membrane |

Table 6. DAVID analysis of trisomic genes from Hsa21 and Ts65Dn and Ts1Rhr mice. Functional annotations are listed in columns by most common dysregulated annotations, then by highest to lowest enrichment score and smallest to largest p-value in succession for each category.

### 4.4 Summary

Functional analyses of genetic information for DS have the potential to revolutionize our understanding about the gene-phenotype relationships in DS (Ait Yahya-Graison et al., 2007; Gardiner, 2010). Our meta-analysis of current functional analyses has indicated that cell cycling, maintenance, function, and adhesion, as well as DNA and RNA metabolism and binding and mitochondrial function are leading common mechanisms underlying DS phenotypes. The combination of multiple genes to produce specific disorder-related phenotypes appears to be a common theme in DS phenotypes. Groups of genes may act in concert to produce one or more phenotypes, or contribute a key factor in the development or maintenance of a phenotype. In many cases, functional groups and mechanisms overlap between tissues of different origins, but no general mechanism appears to be ubiquitously dysregulated amongst the tissues studied (Figure 1).

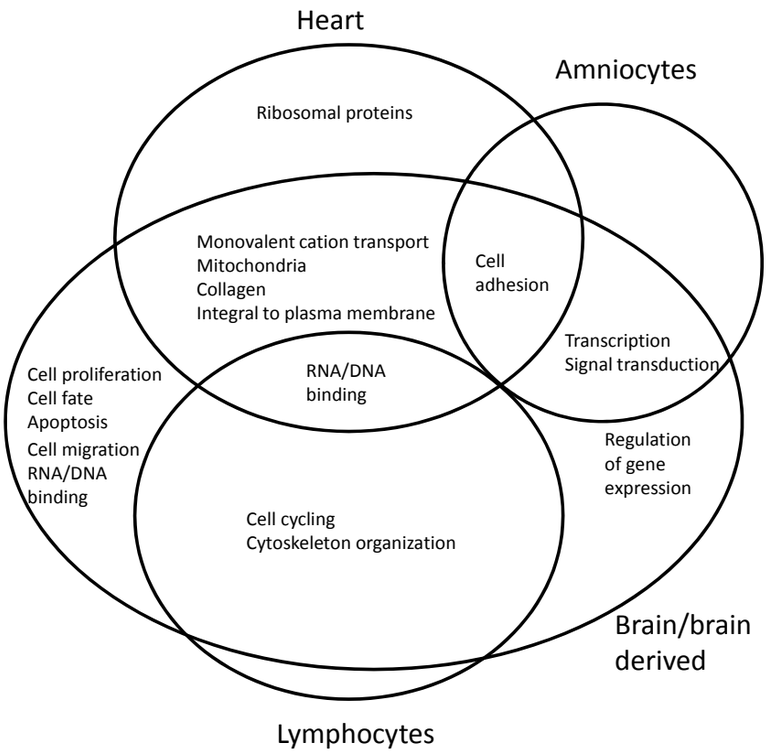


Fig. 1. Overlap of dysregulated functional groups occurs between multiple tissues, but no single mechanism appears to be dysregulated among all tissues studied.

## 5. Conclusions

Although the initial genetic insult is known and the phenotypes related to DS have been characterized, the relationship between genes and phenotypes has not been well distinguished for most DS phenotypes. Though phenotypes associated with DS must be caused by Ts21, high throughput gene expression analyses demonstrate the dysregulation of both trisomic and non-trisomic genes in tissues that are important for traits associated with Ts21. Hypotheses concerning critical regions and genes, thought to be important in most of

the major phenotypes associated with DS, have been disproved. Instead, it is postulated that a region of Hsa21 may be critical for a specific phenotype and a genomic region may contain a gene that is important (but not exclusive) for the causation of the trait. Moreover, there may be singular genes that are important in many, but not all, phenotypes associated with DS. Functional analyses of differentially expressed genes and genes in three copies may be used to further understand the relationships between genes and phenotypes. A number of differentially expressed genes may be tied into a mechanism and there may be mechanisms that are critical to a number of phenotypes. Thus, more accurate genotyping, large scale gene expression meta-analysis and functional mechanism investigations are helping to define gene-phenotype relationships in this complex and interactive disorder.

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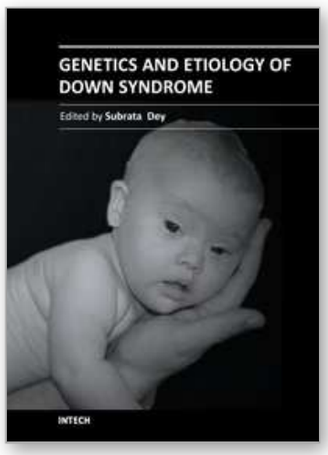


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This book provides a concise yet comprehensive source of current information on Down syndrome. Research workers, scientists, medical graduates and paediatricians will find it an excellent source for reference and review. This book has been divided into four sections, beginning with the Genetics and Etiology and ending with Prenatal Diagnosis and Screening. Inside, you will find state-of-the-art information on: 1. Genetics and Etiology 2. Down syndrome Model 3. Neurologic, Urologic, Dental & Allergic disorders 4. Prenatal Diagnosis and Screening Whilst aimed primarily at research workers on Down syndrome, we hope that the appeal of this book will extend beyond the narrow confines of academic interest and be of interest to a wider audience, especially parents and relatives of Down syndrome patients.

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