

Mouse models of Down syndrome: gene content and consequences

Meenal Gupta¹ · A. Ranjitha Dhanasekaran¹ · Katherine J. Gardiner^{1,2,3}

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Abstract Down syndrome (DS), trisomy of human chromosome 21 (Hsa21), is challenging to model in mice. Not only is it a contiguous gene syndrome spanning 35 Mb of the long arm of Hsa21, but orthologs of Hsa21 genes map to segments of three mouse chromosomes, Mmu16, Mmu17, and Mmu10. The Ts65Dn was the first viable segmental trisomy mouse model for DS; it is a partial trisomy currently popular in preclinical evaluations of drugs for cognition in DS. Limitations of the Ts65Dn are as follows: (i) it is trisomic for 125 human protein-coding orthologs, but only 90 of these are Hsa21 orthologs and (ii) it lacks trisomy for ~75 Hsa21 orthologs. In recent years, several additional mouse models of DS have been generated, each trisomic for a different subset of Hsa21 genes or their orthologs. To best exploit these models and interpret the results obtained with them, prior to proposing clinical trials, an understanding of their trisomic gene content, relative to full trisomy 21, is necessary. Here we first review the functional information on Hsa21 protein-coding

genes and the more recent annotation of a large number of functional RNA genes. We then discuss the conservation and genomic distribution of Hsa21 orthologs in the mouse genome and the distribution of mouse-specific genes. Lastly, we consider the strengths and weaknesses of mouse models of DS based on the number and nature of the Hsa21 orthologs that are, and are not, trisomic in each, and discuss their validity for use in preclinical evaluations of drug responses.

Introduction

Down syndrome (DS) is caused by trisomy of human chromosome 21 (Hsa21) and the increased expression, due to dosage, of some subset of the encoded genes. The phenotype of DS includes variable severity of developmental perturbations that affect most organs and organ systems (Capone 2001; Antonarakis et al. 2004). DS is the most common genetic cause of intellectual disability (ID), and although ID can be mild, the average IQ is in the range of 40–50 (Canfield et al. 2006; Irving et al. 2008; Chapman and Hesketh 2000). DS is also the most common genetic cause of Alzheimer's Disease (AD): all individuals with DS develop the neuropathology of AD by the age of 30–40, and half will progress to an AD-like dementia by the age of 60 (Head et al. 2016). Because of its frequency, at one in approximately 1000 live births worldwide (Canfield et al. 2006; Irving et al. 2008), DS is a focus of much research.

As with the study of most human diseases, mouse has played a prominent role among model systems. DS, however, is more complicated to model adequately in mouse than many human conditions. DS is a contiguous gene syndrome that spans the entire ~35 Mb of the long arm of

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✉ Katherine J. Gardiner
Katherine.gardiner@ucdenver.edu

¹ Department of Pediatrics, Linda Crnic Institute for Down Syndrome, University of Colorado Denver School of Medicine, Aurora, CO, USA

² Department of Biochemistry and Molecular Biology, University of Colorado Denver School of Medicine, Aurora, CO, USA

³ Human Medical Genetics and Genomics, and Neuroscience Programs, University of Colorado Denver School of Medicine, 12700 E 19th Avenue, Mail Stop 8608, Aurora, CO 80045, USA

Hsa21. The concept of a “Down Syndrome Critical Region” (DSCR), defined as the minimal region of overlap among a small number of people with DS due to partial trisomy Hsa21 (Rahmani et al. 1990), was introduced as a region of Hsa21 containing genes essential for the DS phenotype. It was controversial when proposed (Korenberg et al. 1994) and has been discredited: trisomy of other segments of Hsa21 not overlapping with the DSCR is associated with a diagnosis of DS, including ID (Korenberg et al. 1994; Nelson and Gibbs 2004; Korbelt et al. 2009). Thus, genes distributed throughout Hsa21q remain of potential relevance to DS features: it is the function of a Hsa21 gene and its elevated expression in DS that dictates its potential relevance, not its location within Hsa21. For a small number of Hsa21 genes, detailed functional analyses strongly suggest their contributions to the DS phenotype (reviewed in Sturgeon et al. 2012). However, for the majority, functional information is limited or non-existent and, as a result, no gene and no segment of the long arm of Hsa21 (Hsa21q) can be excluded from possible contributions.

Regions of Hsa21q synteny in mouse involve segments of three chromosomes, the ~28 Mb of the telomeric region of mouse chromosome (Mmu) 16, an internal ~1.5 Mb segment of Mmu17, and an internal ~3 Mb segment of Mmu10 (Davisson et al. 2001). From a genetic perspective, a complete mouse model of DS would be trisomic for all three segments. However, while many mouse models have been constructed, none is trisomic for all Hsa21 orthologs, and breeding to obtain a complete trisomy is complicated. The oldest and, as a result, the most popular mouse model of DS is the Ts65Dn (Davisson et al. 1990, 1993; Reeves et al. 1995). It is trisomic for ~55 % of Hsa21 orthologous protein-coding genes and is also trisomic for a large number of genes that are not orthologs of Hsa21 proteins (Duchon et al. 2011); they map instead to Hsa6 and thus are not relevant to DS and may confound phenotypic consequences. The Ts65Dn does, however, indeed display many features relevant to DS, including abnormalities in neuronal number and morphology in some brain regions and deficits in learning and memory (reviewed in Rueda et al. 2012). Over the last few years, numerous drugs/small molecules have been shown to rescue one or more abnormalities in the Ts65Dn (reviewed in Gardiner 2014) and these successes have led to considerable enthusiasm for clinical trials. The Ts65Dn is, however, the only DS model so far used in preclinical assessments of drugs for cognition in DS. This leaves untested the effect on phenotype, and drug responses, of trisomy of the remaining Hsa21 genes.

There has been much discussion in the scientific literature recently regarding the failure rate (>80 %) of clinical trials that are based on preclinical evaluations in mouse

models (Perrin 2014; McGonigle and Ruggeri 2014). This is true in a broad range of diseases, e.g., cancer, disorders of the central nervous system, inflammation, Fragile X, etc. (Begley and Ellis 2012; McGonigle and Ruggeri 2014, McGonigle 2014). Clinical trials are expensive and not without a toll on participants. There should, therefore, be reasoned concern regarding clinical trials for cognition in DS and this concern should include critical consideration of the genetic validity of the mouse models used in pre-clinical studies.

The goal of this work is to review the gene content of Hsa21 and clarify the number, nature, and distribution of Hsa21 orthologs in the mouse genome. To aid in the design, and the critical interpretation of results, of pre-clinical experiments in DS mouse models, we first discuss the current information on Hsa21 classical protein-coding genes and new facts regarding long non-coding RNA genes. We then discuss the conservation of these genes in mouse, species-specific genes and the trisomic gene content of several mouse models. The goal is to provide the interested researcher with sufficient information to judge the appropriate use, and the abuse, of data obtained with each model. We start, however, with a review of the basic elements of mammalian genomic sequence annotation for gene content, how these data are curated and where they are available, to make clear how genes are identified and the confidence of these identifications.

Overview of genomic sequence annotation for gene content

Gene identification is still strongly based on experimental observation, i.e., detection of transcripts, by sequencing of cDNA libraries or RNAseq, and/or by targeted approaches of RT-PCR and RACE. Extensive contributions to mammalian transcript catalogs came first from the database of expressed sequence tags (dbEST), and then from the Functional Annotation of the Mammalian Genome (FANTOM) and the Encyclopedia of DNA Elements (ENCODE) projects (reviewed in de Hoon et al. 2015; and Harrow et al. 2012, Derrien et al. 2012). Both FANTOM and ENCODE involve not only large-scale experimental efforts, but also extensive manual curation, and together have produced annotations of gene structures, including experimentally verified 5' and 3' ends, alternative splice variants, and predicted coding capacities, for both human and mouse. Additional information includes identification of patterns in transcription factor binding and DNase hypersensitivity sites, histone modifications, and CpG islands.

Determination of the gene content of any human genomic segment is now based on integration and graphical

representation of comprehensive experimental datasets that include dbEST, FANTOM, and GENCODE, among other datasets, and application of well-established bioinformatics tools. Two popular public annotation databases are the Genome Browser at the University of California Santa Cruz (UCSC; <http://genome.ucsc.edu/>; Rosenbloom et al. 2013) and the Ensembl Genome Browser (<http://www.ensembl.org/index.html>; Flicek et al. 2014) supported by the European Bioinformatics Institute (EBI) at the Sanger Institute. Both annotate the same genomic sequences and the automated annotation tools provide similar functions. Although the formats differ, both browsers provide graphical displays of largely the same comprehensive information regarding gene structures and organization and both provide gene information for download.

The Entrez Gene database at the National Center for Biotechnology Information (NCBI) in the US provides, in part, Reference Sequences (RefSeq) for mRNA, protein, and functional RNAs (O'Leary et al. 2016). These consist of gene products for which supporting evidence has been manually reviewed and are designed, as the name implies, to serve as reference sequences for each annotated gene and its splice variants. The Vertebrate Genome Annotation (VEGA) database (<http://vega.sanger.ac.uk>) provides manually reviewed and curated gene structures for whole chromosomes/chromosomal regions, calling protein-coding, non-coding RNA transcripts, and pseudogenes; it is uniquely updated through the Human and Vertebrate Analysis and Annotation (HAVANA) (Harrow et al. 2014). The HUGO Gene Nomenclature Committee (HGNC) at EBI assigns standardized names and descriptions to human genes, both protein-coding and functional RNA, that have passed specific validation tests (Gray et al. 2015). UCSC, RefSeq, Ensembl, VEGA, and HGNC gene annotations all can be found in both the UCSC and the Ensembl browsers.

As the GENCODE and FANTOM projects matured, an important new genomic feature became evident: the presence of large numbers of transcripts lacking obvious protein-coding characteristics (Mattick 2003; Mattick and Makunin 2006). These transcripts were found to encode no ORFs or ORFs less than 100 amino acids in length, frequently lacking evolutionary conservation, and typically showing low levels of expression often restricted in time and place. There was considerable debate about the functional significance of these transcripts and suggestions that they were transcriptional noise (Louro et al. 2009; Hüttenhofer et al. 2005). However, intense study over the last several years has revealed a remarkable repertoire of biological functions among individual long non-coding RNAs (lncRNAs). The versatility in function arises in part because, as RNA molecules, the primary nucleotide sequences and/or associated secondary or tertiary structures can allow lncRNAs to recognize and bind to specific DNA

or RNA sequences and/or to individual proteins or protein complexes (Bergmann and Spector 2014). Some lncRNAs have been shown to regulate transcription of genes either neighboring their site of synthesis or at distal locations through interactions with chromatin (Vance and Ponting 2014). In stem cell biology, lncRNAs have been shown to regulate genes involved in neurogenesis, epidermal differentiation, and myoblast progenitors (Ng et al. 2013; Ramos et al. 2015; Lopez-Pajares et al. 2015; Ballarino et al. 2015), and to participate directly in the regulation of the pluripotent state in embryonic stem cells (Flynn and Chang 2014). Some lncRNAs function as tumor suppressors, others as oncogenes, and some alter the stability of mRNAs or the efficiency of translation (Zhou et al. 2012; Gupta et al. 2010; Mercer et al. 2009). On the whole organism scale, critical functions of several lncRNAs were demonstrated in individual knockout mice, where phenotypes included embryonic lethality (Sauvageau et al. 2013; Li and Chang 2014; Goff et al. 2015).

The proportion of lncRNA genes with assigned function remains small because critical sequence features are unknown and analysis is not so far amenable to high-throughput techniques. In addition, even predicting a gene as a functional non-coding versus a protein-coding RNA remains challenging and lacks absolute criteria. Short ORF proteins exist; several hundred human proteins less than 100 amino acids in length are annotated in SwissProt, among them the Hsa21-encoded proteins, SMIM11 and PCP4 (58 and 62 amino acids, respectively), and S100B, CSTB, and SUMO3 (each 92–98 amino acids). Larger scale identification of functional peptides translated from short ORFs has been reported (reviewed in Andrews and Rothnagel 2014; Saghatelian and Couso 2015), although criteria for mass spectrometric validation of short ORFs are still evolving (Bruford et al. 2015). In spite of the challenges in identification and functional determination, it is now generally accepted that the human genome contains large numbers of lncRNAs. Indeed, Derrien et al. (2012) reported >9000 lncRNA genes in their review of the GENCODE project within ENCODE.

With these advances in transcript identification and characterization, the number of Hsa21 genes has grown accordingly. When the genomic sequence of Hsa21 was first reported in 2000 (Hattori et al. 2000), 225 genes/gene models were annotated on 21q. Over the next ten years, the number of gene structures grew to >500 (Sturgeon and Gardiner 2011) and now stands at >550 (12-2015) in VEGA. The increases are in genes lacking sequence similarities to genes of known functions, encoding ambiguous and short ORFs, and classified as lncRNA genes. Given these increases, it is not surprising that, in spite of genomic resources and numerous databases, it has become time consuming for a DS researcher to understand the criteria

for gene annotation and to evaluate accurately the resulting gene content of Hsa21 and of mouse models of DS. In the following sections, we discuss protein-coding gene content and then lncRNA genes within Hsa21.

Protein-coding gene content of Hsa21

VEGA currently annotates 218 protein-coding genes on the long arm of Hsa21 (21q) and 15 on the short arm (21p) (Table 1). For considering possible contributions to phenotypic features in DS and in partially trisomic mouse models, we confine the discussion to genes on 21q. It is helpful to classify these proteins based on functional properties and by distribution within Hsa21. Forty-nine genes within 21q encode members of the large family of keratin-associated proteins, KRTAPs. These genes are present in two clusters, one of 33 genes within 21q22.11 and the other of 16 within 21q22.3. The majority of these genes are intronless and a number of KRTAP pseudogenes are present within both clusters. Other KRTAP gene clusters are present on human chromosomes 11 and 17. KRTAPs are restricted in expression and in function, with their role to provide structure and stability to hair fibers. It seems unlikely that these genes are driving important phenotypic features in DS (Rogers et al. 2002, 2006). Therefore, if the goal is to understand how trisomy of Hsa21 genes contributes to features of the DS phenotype, then including KRTAPs without qualification in a gene count artificially inflates the number of genes requiring thoughtful analysis. We, therefore, confine further discussion of protein-coding genes to non-KRTAP genes.

Of the remaining 169 protein-coding genes within 21q, five are classified as novel proteins; the amino acid sequences of these proteins show no similarities to any non-primate proteins and no domains with functional

Table 1 Distribution within Hsa21 of protein-coding and non-protein-coding genes

Hsa21 genes	21q	21p	Total
“Known” protein coding	164	3 (+11) ^a	167
Novel protein coding	5	1	6
Keratin-associated proteins (KRTAPs)	49	0	49
Total protein coding	218	4	222
Non-protein coding	280	45	325
Total	498	49	547

Data were retrieved from VEGA (http://vega.sanger.ac.uk/Homo_sapiens/Location/Chromosome?r=21) (December 14, 2015). KRTAP genes were identified from gene descriptions

^a 11 protein-coding genes mapped to Hsa21p in Vega were identified by BLASTN to be copies/duplications of known protein-coding genes within Hsa21q22.3

annotation or recognizable amino acid patterns. This leaves 164 protein-coding genes with some level of functional annotation. It is noteworthy that the large majority of these genes were included in the original annotation of Hsa21 (Hattori et al. 2000; Sturgeon and Gardiner 2011).

As one would expect, these 164 genes are diverse in their functional properties (Supplemental Table S1). Among them are 17 transcription factors/modulators, eight proteins functioning in the ubiquitin/proteasome pathway, seven involved in RNA processing, and eight relevant to Alzheimer’s Disease, including the amyloid precursor protein, APP, and seven proteins that modulate APP processing or trafficking (Fig. 1). Thirty-four 21q proteins are annotated in OMIM for mutations causing Mendelian disorders in the human population, including five that cause intellectual disability (Supplemental Table S2).

Figure 1 shows the distribution of protein-coding genes within Hsa21q, and the distribution of some specific functional categories, with respect to the regions of synteny within Mmu16, Mmu17, and Mmu10. Two features of the distribution of Hsa21 protein-coding genes are important to note: (i) in spite of the relatively large size of the Mmu16 syntenic region, spanning ~30 of the 35 Mb of Hsa21q, it contains only 65 % of these protein-coding genes, with the other 35 % contained within the ~4 Mb of the Mmu17 and Mmu10 syntenic regions, and (ii) proteins from each of the illustrative functional categories are found within each of the three mouse syntenic regions.

Of the 15 protein-coding genes on 21p, 11 are identical in nucleotide sequence and structure to genes established to map to 21q22. These include contigs of 4 genes (CBS

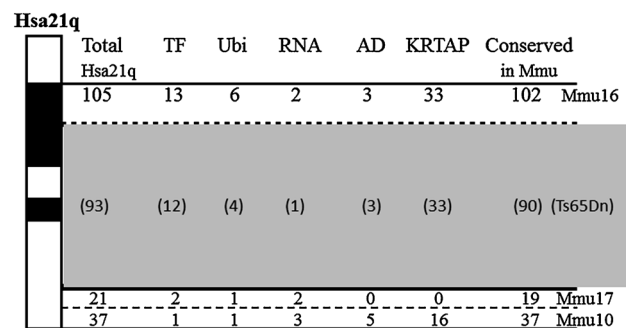


Fig. 1 Distribution of protein-coding genes within 21q. A schematic of a Giemsa-banded Hsa21q is shown on the left. The total number of (non-KRTAP) protein-coding genes and numbers within several functional classes are provided for syntenic regions on mouse chromosome 16 (Mmu16), Mmu17, and Mmu10. Syntenic regions are demarcated by horizontal solid or dashed lines; similar numbers for the subregion of Mmu16 trisomic in the Ts65Dn are shown shaded. TF transcription factors, Ubi members of the ubiquitin pathway, RNA proteins involved in RNA processing, AD proteins directly relevant to Alzheimer’s Disease or involved in processing or trafficking of the Hsa21-encoded protein APP (amyloid precursor protein). For functional annotations, see Supplementary Table S1

through CRYAA) and 5 genes (TRAPPC10 through DNMT3L) that map within 21q22.3 and adjacent genes SMIM11 and KCNE1 within 21q22.1. Further investigation is required to determine if the copies of these genes mapped to 21p are bona fide duplications or (more likely) arise from clone or mapping artifacts.

Non-coding gene content of Hsa21q

Among the >9000 human lncRNA genes reported by Derrien et al. (2012), 225 mapped to Hsa21. VEGA currently annotates 280 non-coding genes within 21q. These include 47 that are antisense to protein-coding genes, 5 microRNAs, and 228 other lncRNAs. The RFAM database annotates a further 24 putative microRNA genes. (VEGA annotates 45 lncRNA genes within 21p; several map with ~100 % identity to the same regions of 21q as the 11 protein-coding genes duplicated on 21p. We do not discuss 21p lncRNA genes further).

Experimental demonstration of the functions of Hsa21 non-coding genes is decidedly limited but nevertheless serves to indicate the potential importance of such genes to the DS phenotype. The MIR155 (originally, BIC) gene has been studied for roles in lymphoma (reviewed in Lawrie 2013; Faraoni et al. 2009), in mitochondrial function, in AD and inflammation, and in regulation of a number of neurologically relevant target genes (Quiñones-Lombraña and Blanco 2015; Song and Lee 2015; Bofill-De Ros et al. 2015). The MIR99AHG, or MicroRNA 99A host gene, contains three microRNA genes within its introns: mir99a, let7c, and 125b2. Each of these microRNA genes has been observed to be upregulated in a diverse set of malignancies. MIR99A has been shown to repress metastasis in several cancer types and to inhibit MTOR pathway signaling (Wu et al. 2015; Yu et al. 2015; Yang et al. 2014; Warth et al. 2015). MIRLET7c has been reported to suppress cell growth, migration, and invasion in nasopharyngeal and non-small-cell lung cancer (Liu et al. 2014; Zhao et al. 2014). Data on MIR125b2 are less clear because reports often do not discriminate between 125b1 and 125b2; however, MIR125b has been reported to promote glioblastoma cell growth but to suppress breast cancer growth (Wu et al. 2013; Feliciano et al. 2013). Although additional functions likely will be discovered for each of these microRNAs, it is already clear that they contribute to normal processes; contributions to aspects of the DS phenotype, when they are over expressed, cannot be discounted.

The mature spliced transcript of the host MIR99AHG gene also requires consideration. Originally named C21ORF34/35, it was shown to be composed of >11 exons, spanning >500 kb, and subject to considerable alternative

splicing, although no obvious ORF was found except in the 5'-most exons (Gardiner et al. 2002). Recently, a role for this transcript, alias MONC, in mediating acute megakaryoblastic leukemia (AMKL) was demonstrated (Emmrich et al. 2014). This is of interest because of the increased incidence of AMKL in DS, where MONC is elevated in expression. The organization of MONC/Mir99AHG as a functional lncRNA and not just a host gene for intronic small RNA genes is not unique in the human genome. The Growth Arrest-specific 5 (GAS5) gene that maps to Hsa1 is also a spliced transcript that contains several small RNA genes (small nucleolar RNA, snoRNA) within its introns (Smith and Steitz 1998). Within the mature, spliced host GAS5 transcript, a region of secondary structure binds to and represses the activity of the glucocorticoid receptor, thus regulating metabolic levels and cell survival. More recently, the GAS5 spliced transcript has been shown to function as a tumor suppressor and to have diagnostic potential in multiple malignancy types (Yu and Li 2015). Given such examples, it is conceivable that some of the many other Hsa21 lncRNA genes also have complex functional properties.

One last example of an RNA gene from Hsa21 involves an enhancer RNA (eRNA) associated with the nuclear hormone repressor interacting protein, NRIP1, that maps in proximal 21q21. Treatment of a breast cancer cell line with estradiol (E2) was shown to induce expression of a number of protein-coding genes and their associated upstream enhancer eRNAs. Among those chosen for detailed analysis was the NRIP1 eRNA that was shown to promote an intra-Hsa21 chromatin interaction between the NRIP1 locus in proximal 21q21 and that of the trefoil factor gene, TFF1, located within 21q22.3. Inhibition of eNRIP1 perturbed expression responses of both NRIP1 and TFF1 to estrogen (Li et al. 2013).

Expression patterns of lncRNAs are robustly reproducible and the current Hsa21 gene annotation is likely to be substantially correct. Hsa21 therefore encodes more RNA genes than protein-coding genes and it remains quite incorrect to state, without qualification, the gene content of Hsa21 by referring only to 222 protein-coding genes. Although data are limited, examples of functions of non-coding genes (on Hsa21 or elsewhere) indicate that much of the importance to understanding the development of the DS phenotype may lie within the overexpression of some of the hundreds of lncRNA genes encoded by Hsa21.

Number of genes in Hsa21 mouse syntenic regions

Conservation in mouse is not a prerequisite in the VEGA annotation system for calling a human gene structure. VEGA annotation for the mouse genome is not complete

and does not include the entireties of the Hsa21 syntenic regions. Accordingly, for this discussion, gene content of mouse Hsa21 regions of synteny has been determined from data present in the UCSC Genome Browser within RefSeq and Encode/Gencode tracks, plus manual assessments.

Among protein-coding genes, the two clusters of KRTAP genes seen on Hsa21 are present in mouse, one cluster on Mmu16 and the other on Mmu10. Further experiments and curation are needed for an accurate count of members in each cluster, but at 37 and 13, respectively, the numbers are similar to those on Hsa21q. Excluding KRTAPs, an additional 166 diverse protein-coding genes are annotated within the mouse regions and, of these, 158 have orthologs on Hsa21, with the distribution shown in Fig. 1: 102, 19, and 37 on Mmu16, Mmu17, and Mmu10, respectively. Mouse-specific protein-coding genes are the integrin beta 2-like gene, ITGB2L, and three transcripts encoding uncharacterized proteins, all located within the Mmu16 region. Conversely, Hsa21 protein-coding genes lacking orthologous mouse genes expected within the Mmu16 syntenic region include POTED (prostate, ovary, testis, and placenta expressed ankyrin domain family member D; Bera et al. 2002; located centromere proximal to the Mmu16 syntenic region), the T-complex-like-10 (TCP10L), the DS Critical Region 4 (DSCR4; Nakamura et al. 1997) that is expressed in placenta, testis, and connective tissue, and PLAC4 that exhibits the highest but not exclusive expression in placenta (Kido et al. 1993), plus five novel genes.

Among non-coding genes, the five Hsa21 microRNAs, including the Mir155/Bic gene, are conserved in mouse at the orthologous genomic positions relative to protein-coding genes. The mouse Mir99ahg/C21ORF34 gene hosts the orthologous microRNA genes and resembles the human gene in complexity, spanning >500 kb and encompassing 10–12 exons that show considerable alternative splicing (Gardiner et al. 2002). Nucleotide sequences of some exons are conserved, although at lower levels than orthologous protein-coding genes.

lncRNA genes are equally prevalent in mouse, but few are recognizably conserved at the nucleotide level (Table 2, Supplementary Table S3; Sturgeon and Gardiner 2011). This does not mean that functions of these genes are not conserved. Functions could be dictated by conserved nucleotide segments that are much shorter and more subtle than those seen in classical protein-coding genes (Mercer et al. 2009), or by genomic positions conserved relative to coding or regulatory sequences of target genes. As illustrated, many gene structures on Hsa21 were identified as antisense to RefSeq protein-coding genes (Sturgeon and Gardiner 2011); mouse orthologs of many of these RefSeq genes are also associated with antisense transcripts. Alternatively, of course, lncRNA functions may be

governed by conserved secondary or tertiary structures. With the expanding information regarding lncRNAs, it is unreasonable to exclude genes from consideration simply based on the lack of obvious nucleotide conservation between human and mouse.

To reiterate an important message from these annotations: the number of genes annotated within any segment of Hsa21 is not necessarily the number of genes annotated in the syntenic mouse region, and it is certainly not the number of genes recognizably conserved in the orthologous mouse region. While it is easier for the DS researcher to focus studies on the 158 diverse protein-coding genes on Hsa21 that are conserved in syntenic mouse genomic regions, to do so may not lead to the necessary understanding of the molecular basis of DS phenotypic features, nor to a reliable determination of the validity of mouse models of DS.

Mouse models of DS and trisomic gene content

Figure 2 shows the regions of Hsa21 synteny that are trisomic in several DS models. The majority of these models are trisomic for subregions of the Mmu16 syntenic segment. This is not due to any knowledge that genes on Mmu16 individually or collectively are of any greater biological import for the DS phenotype than those on Mmu17 or Mmu10, but rather it is due to the chromosomal location and relative size of syntenic regions. On Mmu16, the syntenic segment is the telomere proximal region, while those on Mmu17 and Mmu10 are internal (Davisson et al. 2001). The Mmu16 telomeric location facilitated the creation of the Ts65Dn: testis of male mice were irradiated to fragment chromosomes; offspring of breeding these mice to unirradiated females were screened cytogenetically to identify those carrying chromosomal rearrangements that produced trisomy of the telomere of Mmu16 (Davisson et al. 1990). The resulting Ts65Dn contains a marker chromosome composed of the majority of the Mmu16 syntenic segment translocated to the pericentromeric region of Mmu17 (Davisson et al. 1990, 1993; Reeves et al. 1995). Application of the same technique would not have been practical for precision generation and identification of trisomies of the small internal Mmu17 and Mmu10 syntenic segments. Also, because variations in gene density were not well documented at that time (which was prior to availability of whole-genome sequence), the Mmu16 region, at ~25 Mb (vs. ~1–3.0 Mb for the Mmu17 and Mmu10 segments), was presumed to encompass the large majority of Hsa21 orthologs. In fact, the Ts65Dn is trisomic for only 90 of the 158 non-KRTAP Hsa21 protein-coding orthologs. In addition, the assumption that the Mmu17 pericentric region, syntenic to Hsa6 and trisomic in

Table 2 Gene content of DS mouse models

	Total Mb	Start–stop, Mb	Start–stop, protein coding	Protein coding		RNA genes		
				Total	conserved	Human	Mouse	conserved
21p	10.6	1–10.6	BAGE2-TPTE	2	0	45	0	
21q	33.7	13.0–46.7	POTED-PRTM2	164	158	280	115	75
Tc1	42	na	LIPI-PRMT2	129	125	243	91	61
Prox Mmu16	1.1	13.0–14.1	POTED	1	0	5	0	0
Dp16	27.9	14.1–42.0	LIPI-ZBTB21	105	102	200	91	61
Ts3Yah	11.8	14.3–26.1	SAMSN1-GABPA	13	13	75	38	24
Prox Ts65Dn	11.4	14.1–25.5	LIPI-> MIR155HG	12	12	74	36	24
Ts65Dn	16.4	25.6–42.0	MRPL39-ZBTB21	93	90	125	55	37
Ts1Cje	10.3	31.7–42.0	>SOD1-ZBTB21	74	71	92	43	28
Ts1Rhr	5.3	36.1–41.4	CBR1-FAM3B	31	29	44	14	10
Ts1Yah	0.7	42.3–43.0	TFF3-CBS	12	12	11	4	0
Dp17	1.7	42.1–43.8	UMODL1-RRP1B	21	19	19	6	0
Dp10	2.9	43.8–46.7	PDXK-PRMT2	37	37	56	18	14

Total Mb: genomic size of the region of Hsa21 trisomic in each model. Start–stop, Mb and protein coding: the orthologous region of Hsa21 spanned in each model. Protein-coding genes (non-KRTAPs): total number in the trisomic region, and the number conserved between human and mouse. RNA genes: number of lncRNA present in the human and mouse regions spanned in each model, and conserved between human and mouse (by nucleotide sequence similarity). Numbers were obtained for human from VEGA (December 14, 2015) and for mouse from Gencode/Encode (Human Genome Browser) and Sturgeon and Gardiner 2011

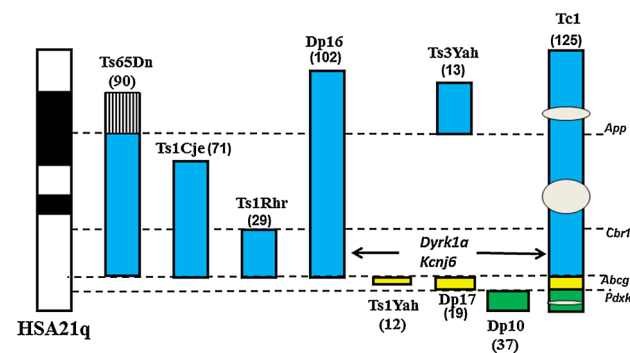


Fig. 2 Trisomic regions and number of non-KRTAP protein-coding genes trisomic in mouse models of DS. Blue, yellow, green Hsa21 syntenic regions on Mmu16, Mmu17, and Mmu10, respectively. Numbers in brackets indicate the number of conserved protein-coding genes that are trisomic. The Hsa6 syntenic region in the Ts65Dn is indicated by stripes and contains 35 protein-coding genes. Gray ovals within the Tc1 chromosome indicate major deletions. Arrows indicate the location of Dyrk1A and Kcnj6 within Mmu16

the Ts65Dn, could be ignored because its small size was also incorrect. In fact, this region was recently shown to encode 35 protein-coding and 15 lncRNA genes (prior reports of 60 genes included ten pseudogenes; Duchon et al. 2011), i.e., approximately one quarter of the protein-coding genes trisomic in the Ts65Dn are not Hsa21 orthologs. These genes are discussed further below.

The publication of the Ts65Dn provided the first viable segmental trisomic mouse model of DS and served to focus research on the Mmu16 region. Additional mouse models,

the Ts1Cje and the Ts1Rhr, are trisomic for subsets of genes trisomic in the Ts65Dn (Sago et al. 1998; Olson et al. 2004). The Ts1Rhr was the first DS model created using the Cre/lox technique of chromosomal engineering and was designed to replicate trisomy of the DSCR (Olson et al. 2004). It became of particular interest because it demonstrated that trisomy of this region was not sufficient to replicate all structural and functional abnormalities seen in the Ts65Dn, further discrediting the concept of the DSCR (Olson et al. 2004, 2007). Chromosomal engineering was next successfully used to create the Dp(16)1Yey (abbreviated Dp16) carrying a duplication of the entire Mmu16 syntenic segment (Li et al. 2007). Subsequently, models were generated with trisomy of partial and complete syntenic segments of Mmu17, respectively, the Ts1Yah (Pereira et al. 2009) and the Dp(17)1Yey (abbreviated Dp17; Yu et al. 2010b), the complete Mmu10 segment, the Dp(10)1Yey (abbreviated Dp10), and an additional subregion of Mmu16, the Ts3Yah (Brault et al. 2015). Unique among mouse models of DS is the Tc1, not trisomic for orthologous mouse regions, but instead carrying a human chromosome 21. While first reported to be an almost complete Hsa21q (O'Doherty et al. 2005), subsequent DNA sequencing revealed numerous deletions and rearrangements within the chromosome, caused by methods used in construction and leaving intact and functional only 125 of the 158 orthologs (plus KRTAP genes) (Gribble et al. 2013). In addition, being a chromosome with a human centromere in a mouse background, the Tc1 mice are

Table 3 Functional and phenotypic interactions among syntenic mouse regions

	MWM	YM	TM	NOR	CFC	LTP	References
1 Dyrk1a tg	Impaired	Impaired				Elevated	Ahn et al. (2006), Souchet et al. (2015)
2 Ts1Rhr	Normal		Impaired	Impaired			Olson et al. (2007), Belichenko et al. (2009)
3 Ts1Cje	Impaired		Impaired	Normal		Impaired	Siarey et al. (2005), Olson et al. (2007)
4 Dp16	Impaired		Impaired		Impaired	Impaired	Yu et al. (2010b), Zhang et al. (2014)
5 Dp16-Dyrk1a			Improved		Improved	Improved	Jiang et al. (2015)
6 Kcnj6 tg					Impaired	Normal	Cooper et al. (2012)
7 Dp16 – (Dyrk1a & Kcnj6)			Impaired		Impaired	Impaired	Jiang et al. (2015)
8 Dp16-Ts1Rhr	Normal		Improved		Improved	Improved	Zhang et al. (2014), Jiang et al. (2015)
9 Dp16 + Dp17 – Ts1Rhr	Impaired					Impaired	Zhang et al. (2014)
10 Ts1Yah	Enhanced	Impaired		Impaired		Enhanced	Pereira et al. (2009)
11 Dp17	Normal				Normal	Enhanced	Yu et al. (2010b)
12 Dp10	Normal				Normal	Normal	Yu et al. (2010b)
13 Dp10 + Dp17	Normal					Normal	Zhang et al. (2014)
14 Ts65Dn	Impaired	Impaired	Impaired	Impaired	Impaired	Impaired	Rueda et al. (2012)
15 Ts65Dn-Ts1Rhr	Normal					Normal	Olson et al. (2007)
16 Ts65Dn-Dyrk1a	Improved				Improved	Normal	García-Cerro et al. (2014)

MWM Morris water maze, *YM* Y-maze, *TM* T-maze, *NOR* novel object recognition, *CFC* context fear conditioning, *LTP* long-term potentiation. Normal, not different from wild-type littermate controls; improved, less impaired than Dp16 but not to the level of wild-type controls; enhanced, increased relative to controls

mosaics so that a variable number of cells in any tissue carry the Hsa21q.

Table 2 summarizes the conserved non-KRTAP protein-coding gene and RNA gene content of the major models shown in Fig. 2. Details on phenotypic features are available in recent reviews (Rueda et al. 2012; Choong et al. 2015).

Gene–phenotype correlations

Identifying specific genes that, when trisomic, cause individual DS phenotypic features has long been a goal of many researchers in the field. However, the rarity of individuals with DS due to partial trisomy Hsa21, and the lack of uniform comprehensive phenotypic determinations, has hampered progress. The availability of mouse models, segmental trisomies as shown in Fig. 2 and single-gene transgenics and knockouts, offers the potential to pursue this effort in mice. While the concept is simple, in practice, both the experiments and the interpretation of results are complex. To be relevant to DS, gene–phenotype correlations established in mouse models must be evaluated in the context of functional information for all genes that are trisomic in the model and all Hsa21 genes/orthologs that are not trisomic, plus data available from other DS models. It is important to recognize that gene–phenotype

correlations can depend on the genomic context: there are functional interactions among Hsa21 orthologs, some that are not in close physical proximity and/or not on the same mouse chromosome or trisomic in the same DS mouse model. Examples of context-specific trisomic gene consequences are listed in Table 3 and discussed here.

Effects of trisomy of Dyrk1a are context specific

The protein kinase, Dyrk1a, is the most popular Hsa21 gene in the study of DS (Becker et al. 2014). Among its known substrates are transcription factors, splicing factors, endocytic scaffolding proteins, and Hsa21-encoded proteins. Transgenic mice overexpressing Dyrk1a from human or mouse genomic constructs display, among other features, deficits in the Morris water maze (MWM) (Ahn et al. 2006; Souchet et al. 2014). However, the Ts1Rhr that is trisomic for Dyrk1a plus ~30 additional genes shows normal MWM performance (Olson et al. 2007). Thus, the effects of trisomy and elevated expression of Dyrk1A alone are modulated by the increased copy number of additional genes (or genetic background). This observation indicates that the consequences of trisomy of DYRK1A in DS may be different again from those in the single-gene transgenics and partial trisomy mice. These differences could also influence responses to drugs that inhibit DYRK1A activity (Becker et al. 2014).

Effects of trisomy of *Dyrk1a* and *Kcnj6* are not additive

The Dp16 mouse is impaired in the T-maze and context fear conditioning (CFC), and shows decreased long-term depression (LTP) (Li et al. 2007). Reducing *Dyrk1a* to two copies by crossing the Dp16 with a *Dyrk1a* knockout partially rescued these abnormalities, improving performance, but not to the levels of euploid controls (performance in the MWM was not tested) (Jiang et al. 2015). However, simultaneously reducing to two copies *Dyrk1a* and the adjacent potassium voltage-gated channel subunit gene, *Kcnj6*, failed to produce improvement in these same features. Current knowledge provides no mechanistic understanding of how normalization of *Kcnj6* levels in the context of the Dp16 trisomic segment abrogates the beneficial effects of normalization of *Dyrk1a* levels. One interpretation is that trisomy of *Kcnj6* provides some protection from more severe deficits due to trisomy of some subset of Dp16 trisomic genes. This protection must also be context specific because a mouse overexpressing *Kcnj6* alone also displays deficits in CFC (Cooper et al. 2012). An alternative explanation is suggested by inspection of the genomic sequence annotation: two lncRNAs are present, one antisense to *Kcnj6* and the other located between *Dyrk1a* and *Kcnj6*. It is possible that these genes have relevant unanticipated functions in the regulation of expression or activity of genes elsewhere in the Dp16 trisomic segment. These results, extended by Vidal et al. 2012, also emphasize the potential pitfalls of proposing individual Hsa21 genes or gene products (Jiang et al. 2015) as targets for therapeutics to rescue cognitive deficits.

Trisomy of the *Mmu17* segment influences the effects of *Mmu16* trisomy

The Ms1Rhr mouse carries a deletion created using chromosomal engineering and is monosomic for the 29 genes (*Cbr1-Fam3b*) trisomic in the Ts1Rhr (Olson et al. 2004). Crossing the Dp16 with Ms1Rhr reduced to disomy these 29 genes and resulted in the rescue of performance in the MWM, improvement in performance in the T-maze and CFC, and partial amelioration of LTP deficits (Zhang et al. 2014). However, in the presence of trisomy for the *Mmu17* region (i.e., by first crossing Dp16 mice with the Dp17), these phenotypic improvements were not seen (Zhang et al. 2014). This is in spite of the fact that the Dp17 themselves show no deficits in the same tasks and also show enhanced LTP (Yu et al. 2010b). Functions of Dp17 genes, therefore, must be influencing those of genes trisomic within the Dp16, either exacerbating the effects of those that cause the deficits or inhibiting the ameliorating effects of deletion of the *Cbr1-Fam3b* segment.

Interactions among *Mmu17* genes

The Ts1Yah mice that are trisomic for 12 of the 19 Hsa21 orthologs trisomic in the *Mmu17* region show enhanced performance in the MWM and enhanced LTP (Pereira et al. 2009). An attractive candidate in the *Mmu17* region is the trefoil factor 3 (*TFF3*) gene; it encodes a neuropeptide that enhances learning and memory (Shi et al. 2012). Interpretation is complicated, however, because in contrast to performance in the MWM, the Ts1Yah are impaired in both NOR and the Y-maze (Pereira et al. 2009). Furthermore, the Dp17 that are trisomic for the entire *Mmu17* syntenic region show neither enhanced MWM learning nor impaired NOR and Y-maze performance (Yu et al. 2010b). Thus, one or more of the 7 genes uniquely trisomic in the Dp17 must be providing protection from both the positive and the deleterious effects of genes trisomic in the Ts1Yah. Both the Ts1Yah and the Dp17 are also trisomic for the phosphodiesterase 9a (*PDE9A*) gene, a member of the family of 3'-5' cyclic nucleotide phosphodiesterase, PDEs. PDEs regulate intracellular signaling through degradation of cAMP and cGMP and play roles in mood and learning and memory. Inhibition of PDEs is suggested to be protective (Xu et al. 2011) and specific inhibition of *PDE9A* activity produces enhanced LTP (Hutson et al. 2011; Kroker et al. 2012). These observations are inconsistent with the phenotypes of the Ts1Yah and the Dp17. Current information on the additional seven genes trisomic in the Dp17 but not in the Ts1Yah does not suggest explanations for the phenotypic differences between the two trisomies.

Interactions between *Mmu17* and *Mmu10* genes

When Dp17 mice were crossed with Dp10, the double trisomics were reported to show normal LTP (Zhang et al. 2014). Therefore, trisomy of the *Mmu10* region modulated the elevated LTP observed in the Dp17 alone.

Interactions among *Dp10* genes

In vivo and in vitro studies with individual genes from the *Mmu10* orthologous region have revealed a number of functions relevant to both brain development and brain function (Table 4). Male Dp10 mice, however, have been reported to show no deficits in the MWM or CFC nor abnormalities in LTP at 2–4 months of age (Yu et al. 2010b). The beneficial or detrimental effects of trisomy of genes within the *Mmu10* region thus remain to be determined.

The data in Tables 3 and 4 were selected to illustrate the challenges in determining gene–phenotype correlations in trisomy. They emphasize the inherent limitations of focusing on individual Hsa21 genes as having unique

Table 4 Functions of selected Hsa21 proteins with orthologs trisomic in the Dp10

Protein	Functional description	Reference
PRMT2	Protein Arg methyltransferase. Substrates and consequences: activation of estrogen, progesterone, and androgen receptors; histone H3 and regulation of Wnt signaling, β -catenin signaling; STAT3 phosphorylation	Qi et al. (2002), Meyer et al. (2007), Blythe et al. (2010), Iwasaki et al. (2010), Su et al. (2014)
SUMO3	Small ubiquitin-like modifier; increased levels in aged mice correlate with LM. Overexpression affects A β production. Indirect modulation of estrogen, progesterone, and androgen receptors	Li et al. (2003), Zheng et al. (2006), Yang et al. (2012)
TRPM2	Ca-permeable cation channel; activation by oxidative stress confers susceptibility to cell death. Inhibition protects from ischemia through modulation of NMDA receptor subunit expression. Knockout protects males but not females from the effects of ischemia. Activation by A β contributes to vascular pathology in AD. Activity modulated by melatonin	Jia et al. (2011), Xie et al. (2011), Celik and Naziroglu (2012), Verma et al. (2012), Alim et al. (2013)
ADARB1	Adenosine deaminase (guanosine to inosine editing) of mRNAs and miRNAs. Substrates include Gli1 (hedgehog signaling), and serotonin 2C, GABAA3, and glutamate receptors. Knockout affects acoustic startle response in males but not females	Horsch et al. (2011), Tomaselli et al. (2013)
FTCD	Formimidoyltransferase cyclodeaminase. Mutations have been implicated in glutamate formiminotransferase deficiency, characterized by intellectual disability	Hilton et al. (2003)
S100B	Ca-binding protein. Neuroprotective or neurotoxic depending on concentration and localization. Synergistic function with APP to impair neurogenesis; exacerbates phenotype in AD mouse model; overexpression causes L/M deficits and neurodegeneration that are exacerbated by antioxidants	Winocur et al. (2001), Bialowas-McGoey et al. (2008), Donato et al. (2009), Mori et al. (2010), Lu et al. (2011)
COL18A1	Collagen 18A1 proteolytically cleaved to endostatin. Endostatin inhibits neurite outgrowth; required for cerebellar development. Accumulates in AD plaques	Deiningner et al. (2002), Al Ahmad et al. (2010)
CSTB	Lysosomal cysteine protease inhibitor; mutated in epilepsy; CSTB inhibition in AD mouse model improves A β clearance and prevents L/M deficits	Yang et al. (2011)
ITGB2	B2-integrin. Reduces A β accumulation; contributes to neuroinflammation in Tau-opathy	Choucair-Jaafar et al. (2011), Zenaro et al. (2015)

importance to the DS phenotype or as being effective drug targets. The phenotypic consequences of trisomy of Hsa21 genes are influenced by unknown and unpredictable functional interactions among trisomic genes. That such interactions exist is not a new concept (Olson et al. 2004) but is often ignored. When clinical trials are based on the results of preclinical evaluations carried out in a single, partially trisomic, mouse model, effects of genomic context may result in poor or non-significant outcomes.

Preclinical evaluations in the Ts65Dn and clinical trials for cognition in DS

Currently, the only mouse model used in preclinical evaluations of drugs for cognitive enhancement in DS is the Ts65Dn (reviewed in Gardiner 2014). Based on these evaluations, clinical trials have been conducted using antioxidants, memantine (a drug approved for treatment

of Alzheimer's Disease), and an extract of green tea (Lott et al. 2011; Hanney et al. 2012; Boada et al. 2012; De la Torre et al. 2014). Additional trials are in progress or planned, and now include discussions of prenatal treatments (Kuehn et al. 2016; Bartesaghi et al. 2015). Clinical trials so far have produced at best very marginally positive results. Trials in DS are not unique in their limited efficacy. Across therapeutic areas, it is estimated that, of clinical trials following from preclinical evidence in mice, >80 % fail (Perrin 2014). Proponents of DS trials argue that larger trials, longer trials, trials using younger participants (or fetuses), or trials supplemented with cognitive training are necessary to produce significant improvements in the functioning of a brain that has been perturbed throughout development. These are reasonable proposals but it is also necessary to optimize preclinical evaluations. Issues to be considered include inadequate sample size, use only of male mice, lack of genetic diversity in the models, failures to

randomize assignment to experimental groups, and failures to blind the experimenter to genotype and treatment (Perrin 2014; Macleod 2014; McGonigle and Ruggeri 2014), and, in the realm of cognition, use of too limited a number and range of learning/memory assessments. While improvements in these areas are important, for cognition in DS, it is also necessary to address the fact that the Ts65Dn does not represent the genetic perturbation that is DS.

Phenotypic features of the Ts65Dn mimic many of those seen in DS, e.g., in behavior, LM, and brain region and cellular abnormalities, and some (e.g., cerebellar cellular abnormalities; Baxter et al. 2000) even served to predict previously undocumented details of the DS phenotype (reviewed in Rueda et al. 2012). Thus, the Ts65Dn has been of inestimable value to DS research. It cannot be concluded, however, as many implicitly do in limiting their studies to only the Ts65Dn, that the trisomic genes causing a phenotypic feature in the Ts65Dn, and only those genes, cause the same feature in DS. To do so is a failure of disciplined reasoning and flies in the face of evidence in Tables 3 and 4. It makes two tacit, scientifically unsupported, assumptions: (i) Hsa21 genes not trisomic in the Ts65Dn make no contribution to the DS phenotype and do not influence drug responses, and conversely (ii) genes trisomic in the Ts65Dn that are not orthologs of Hsa21 genes, i.e., the Hsa6 orthologs, do not contribute to the Ts65Dn phenotype or to drug responses. Too much is known about these respective Hsa21 and Hsa6 genes to dismiss them.

Hsa21 orthologs not trisomic in the Ts65Dn

The collective evidence is very strong that Hsa21 orthologs not trisomic in the Ts65Dn influence the DS phenotype. For example, among the Hsa21 Mmu16 orthologs not trisomic are HSPA13, the Hsa21-encoded heat shock protein functioning in the ubiquitin pathway, that regulates intracellular pH in neurons (Bae et al. 2013); NRIP1, the nuclear hormone-interacting protein, that regulates transcriptional activity of glucocorticoid, estrogen, and other receptors and that participates in muscle metabolism and adipocyte function (White et al. 2008); and NCAM2, a neural cell adhesion molecule, that functions in axon and dendrite compartmentalization (Winther et al. 2012) and that results in neurodevelopmental disorders when regionally deleted (Petit et al. 2015). Other non-trisomic Hsa21 Mmu16 orthologs are the putative tumor suppressor gene, BTG3 (Winkler 2010), and the lncRNA/microRNA host gene Mir99ahg. Some of the Mmu17 and Mmu10 Hsa21 orthologs with potential contributions to DS features were discussed above (Table 4; see Block et al. 2014, 2015 for further details).

Non-Hsa21 orthologs trisomic in the Ts65Dn

Consideration of the functional features of the 35 Hsa6 protein-coding genes trisomic in the Ts65Dn is important because they are overexpressed in the Ts65Dn (Duchon et al. 2011). For example, the Sorting Nexin 9 (SNX9) gene functions in clathrin-mediated endocytosis; overexpression in cultured hippocampal neurons results in defects in synaptic vesicle endocytosis (Shin et al. 2007). SNX9, along with clathrin, also has a role in mitosis, based on observations that mutations in SNX9 cause abnormal chromosomal alignment and segregation (Ma et al. 2013). Furthermore, the *Drosophila* ortholog of SNX9 is found in a complex with the ortholog of the Hsa21 protein DSCAM, and it interacts with components of the actin polymerization machinery and with clathrin adaptor proteins, similar to observations in hippocampal neurons (Worby et al. 2001). A second example involves the dynein light chain genes (*Dynlt*), five of which are trisomic in the Ts65Dn. Cytosolic dynein is a molecular motor participating in retrograde axonal transport of signaling endosomes containing NGF, BDNF, and their receptors, TRKA and TRKB (Zhou et al. 2012). The dynein complex is composed of multiple proteins classified as heavy, intermediate, intermediate-light, and light chains. Overexpression of light chains alters the composition of signaling endosomes and decreases downstream signaling through MAPK (Duguay et al. 2011). Both SNX9 and *Dynlta-e* proteins are worth considering for contributions to abnormalities in endosomes and NGF retrograde transport observed in the Ts65Dn (Salehi et al. 2006).

Paralogs of several Hsa21/Mmu16 genes also map to the Ts65Dn Mmu17 segment. These include *Synj2*, a paralog of the Hsa21 phosphatidylinositol phosphatase, *SYNJ1* that is known to contribute to LM deficits; and *TIAM2*, a paralog of the Hsa21 guanine nucleotide exchange factor, *TIAM1*, that is involved in neurite outgrowth. *Scaf8* is a paralog of the Hsa21 serine-arginine-rich splicing factor *Scaf15*, and *claudin 20 (CLDN20)* is a paralog of the Hsa21 tight junction component genes *CLDN8*, *CLDN14*, and *CLDN17*. *Tcp10a* and *Tcp10b* are paralogs of the human-specific transcription factor T-complex 10-like, *Tcp10L*, that is involved in spermatogenesis (Yu et al. 2005). The *Pde10a* gene is a paralog of the Hsa21 gene *Pde9a*. Pairs of paralogous genes may differ in regulation and functional properties, but may also overlap in some features. Therefore, the addition of such genes to a trisomy may have phenotypic consequences.

It is unlikely that every DS-relevant phenotypic feature of the Ts65Dn is significantly impacted by the non-Hsa21 orthologous trisomic genes. Indeed, the comparative analysis of several DS models trisomic for segments of Mmu16 robustly supports the Mmu16 origin of the Ts65Dn

craniofacial abnormalities relevant to DS (Starbuck et al. 2014). However, as illustrated in Tables 3 and 4, the same conclusion is not currently possible for other phenotypic features and it is unscientific to fail to consider that non-Hsa21 orthologs influence either phenotypic features of the Ts65Dn or responses to drug treatments.

Additional limitations of the Ts65Dn

Another concern also rarely discussed is that male Ts65Dn are largely infertile and most commonly colonies are maintained by breeding trisomic females to euploid males (Moore et al. 2010). This means that Ts65Dn mice used in preclinical studies derive from gestation in trisomic dams, with unknown consequences for fetal development. This also means that rarely are female Ts65Dn used in such studies (Gardiner 2014), leaving undetected potential sex differences.

Alternatives to the Ts65Dn, and compromises, for preclinical drug evaluations

Mice trisomic for all Hsa21 orthologs can be obtained by crossing the Dp10 with the Dp17, and using the double trisomic offspring to cross with the Dp16 to obtain a “triple trisomy.” This breeding is time consuming and expensive. Two generations of breeding are required to obtain each mouse and the final yields are much lower than expected. While a cross between Dp10 and Dp17 produces the expected $\frac{1}{4}$ double trisomics, when these are crossed with the Dp16, there are losses in utero due to heart defects, as seen in the Ts65Dn and the Dp16 alone, and further decreases are caused by the deleterious effects of the complete trisomy (also seen in DS fetuses). For preclinical evaluations with behavioral testing or other analyses where it is desirable to have a dozen or more age- and sex-matched individuals, with and without drug treatment, generating sufficient numbers becomes impractical for most researchers, and certainly for extensive screening of potential drug treatments. This is likely reflected in the appearance of only two publications using the triple trisomic mice (Yu et al. 2010a; Belichenko et al. 2015), and none with preclinical evaluations.

It is inarguable that the Dp16 mouse, although also itself not ideal, is a better genetic model of DS than the Ts65Dn: it is trisomic for the entire Mmu16 syntenic region and is not trisomic for any non-Hsa21 orthologs, and both males and females are fertile, thus eliminating the need to breed with trisomic females. It remains to be more fully determined how different and similar the Dp16 vs. the Ts65Dn phenotypes are (Starbuck et al. 2014; Goodliffe et al. 2016). Where they differ from the

Ts65Dn, the potential reasons include the following: (i) the feature is modified or ameliorated by the additional Mmu16 Hsa21 orthologs trisomic in the Dp16 but not in the Ts65Dn; (ii) the feature was caused in whole or in part by genes in the Ts65Dn mapping to the centromeric, Hsa6 syntenic region of Mmu17; or (iii) the feature is affected by differences in genetic backgrounds or differences in the expression of trisomic genes present in an internal duplication vs. on a separate chromosome. These causes are not mutually exclusive and they also are not straightforward to determine. It is also argued that, because the majority of DS is due to an extra freely segregating Hsa21, the Ts65Dn is preferable to the Dp16 because the latter has an internal duplication and lacks an extra chromosome. While the Ts65Dn may be of interest for studies of chromosomal segregation, it is difficult to argue that for DS studies this feature outweighs the additional genetic limitations.

Drugs that have been successful in rescuing learning/memory in the Ts65Dn are diverse in their known targets and mechanisms of action (reviewed in Gardiner 2014). There is no understanding of how these drugs directly or indirectly influence the overexpression or elevated activities of trisomic genes to result in these rescues. As a result, it cannot be predicted that drug responses in the Ts65Dn, or the Dp16, will be replicated to any significant effect in full trisomy. It is, therefore, necessary to include trisomy of Mmu17 and Mmu10 genes in preclinical evaluations. Analysis of the individual, single trisomics Dp17 and Dp10 is uncomplicated and, at least, would ensure that all Hsa21 orthologs are interrogated. Analysis of double trisomics, i.e., offspring obtained by crossing two of the Dp16, Dp17, and Dp10, may be informative compromises for full trisomy. For example, based on data from Zhang et al. (2014), prior to clinical trials, it would be useful to know if a drug that rescued deficits in the Ts65Dn, not only rescued the same deficits in the Dp16, but also in the double trisomic Dp16/Dp17. The Dp16/Dp10 double trisomic would determine how Dp10 genes affect responses to antioxidants (S100B) and melatonin and memantine (TRPM2) (Table 4) that demonstrated efficacy in the Ts65Dn. Admittedly, it is expensive in time and resources to replicate in additional mouse models all the studies reported for the Ts65Dn, but it is also expensive to have clinical trials fail.

Conclusions

DS had long been considered by many to be too complicated a genetic condition to be amenable to productive study and certainly too complicated for successful drug therapy. Contrary to that pessimism, the last decade has

brought the development of several mouse models and the unprecedented demonstration that learning and memory deficits can be rescued in adult Ts65Dn mice with a large and diverse array of drugs. Effective therapy for cognition in DS now seems an attainable goal. The enthusiasm for the goal however needs to be tempered with critical evaluations of the limits of current knowledge and resources, and with rigorous consideration of design and interpretation of preclinical results obtained with partially trisomic mouse models.

For a small number of Hsa21 protein-coding genes, there is considerable functional and overexpression phenotypic information. For others, there are functional data from studies not directed at DS. For the large majority, however, there is little beyond sequence similarities. The cohort of lncRNA genes remains largely unexplored, but not without potential importance. These facts mean that predicting (or excluding) specific genes, particularly those in the Mmu17 and Mmu10 regions, as the causes of any DS phenotypic feature or as the mediators or modulators of a drug response is currently not possible.

It is being strongly argued in the recent literature that the first criterion for evaluating the usefulness of a mouse model of human disease must be its genetic validity, not mere phenotypic similarity (Perrin 2014; McGonigle and Ruggeri 2014; Macleod 2014). For DS, genetic validity is dictated by the presence and absence of trisomy of specific genes. The extent of the genetic limitations of the Ts65Dn has been made clear: 25 % of trisomic genes are not Hsa21 orthologs and 45 % of Hsa21 orthologs are not trisomic. As uniquely valuable as the Ts65Dn has been, it was never justifiable for use in preclinical evaluations of drug treatments. Its continued use needs to be driven by more than just history and popularity.

There is no simple, complete trisomy mouse model of DS. This does not mean that mouse models are not useful. Rather it means that multiple models, with differing complements of trisomic genes, need to be investigated. Understanding the value and the limitations of each of the partial trisomy models requires thoughtful consideration of the number and nature, not only of the Hsa21 orthologs that are trisomic, and therefore presumably driving the phenotype of the model, but also of the Hsa21 orthologs that are not trisomic. Their functions and possible interactions with trisomic genes and in drug responses that would differentially occur in people with DS and full trisomy Hsa21 need to be determined. Only then can phenotypic features, and their rescue by drug treatments, begin to be useful predictors for efficacy in clinical trials.

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