

Down syndrome and the genes of human chromosome 21: current knowledge and future potentials

Report on the Expert workshop on the biology of chromosome 21 genes: towards gene-phenotype correlations in Down syndrome. Washington D.C., September 28–October 1, 2007

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Abstract. Down syndrome (DS), trisomy of human chromosome 21, is the most common genetic cause of intellectual disability. With an incidence in some countries as high as one in approximately 700 live births, and a complex, extensive and variably severe phenotype, Down syndrome is a significant medical and social challenge. In recent years, there has been a rapid increase in information on the functions of the genes of human chromosome 21, as well as in techniques and resources for their analysis. A recent work-

shop brought together experts on the molecular biology of Down syndrome and chromosome 21 with interested researchers in other fields to discuss advances and potentials for generating gene-phenotype correlations. An additional goal of the workshop was to work towards identification of targets for therapeutics that will correct features of DS. A knowledge-based approach to therapeutics also requires the correlation of chromosome 21 gene function with phenotypic features.

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An overview of the phenotypic features of Down syndrome (DS) was presented by Mara Dierssen (Center for Genome Regulation, Barcelona, Spain). Important considerations include the tremendous variability among individuals with DS, both in the presence of specific features and in their severity. Of particular importance for the potential for therapeutic intervention is the observation that, although some aspects of DS are congenital, others appear in infancy and childhood (e.g. growth retardation and developmental delay, mental retardation, and decreased sensitivity to pain), perhaps as a result of premature maturation (e.g.

brachycephaly associated with premature closure of the coronal suture of the skull), and still others occur in adulthood or in the elderly (premature aging, dementia). The mature brain in DS exhibits gross abnormalities in morphology, including proportionally greater reductions in size of both cerebellum and hippocampus. The frontal lobe is smaller and cytoarchitectonic abnormalities include reductions in stellate cell populations in the cortex and alterations in the number, distribution, and structure of dendritic spines on pyramidal cells. While there are fewer functional columns in the cortex, the microcolumns present are of normal size, although wider in younger individuals and fewer in adults. Hippocampal dysfunction and temporal lobe changes specifically impair spatial representation abilities, memory integration of imitation, and spatial memory of visual constructs. Delayed recall difficulties implicate both hippocampus and cortex and may contribute to the cognitive inflexibility observed. IQ deteriorates progressively with

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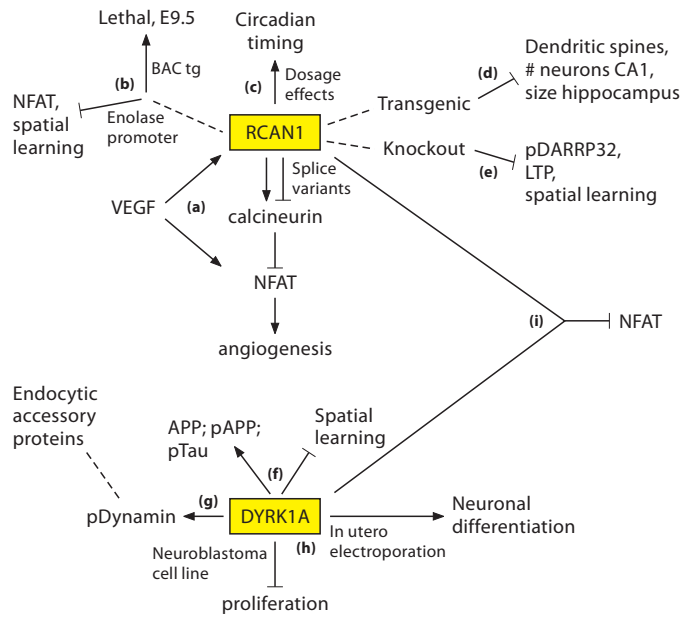


Fig. 1. Functional features of RCAN1 and DYRK1A revealed by different assays. Information derived from presentations, as described in the text, by (a) H. Zeng, (b) D. Crawford, (c) B. Rothermel, (d) D. Dubach, (e) E. Klann, (f) W. Song, (g) Y-W. Hwang, (h) O. Yabut, (i) I. Graef.

age. DS also affects motor function, morphologic syntax, verbal short term memory, explicit long term memory, and visuospatial short term memory.

An overview of the genes on chromosome 21 (chr21) was provided by Katheleen Gardiner (University of Colorado Denver). The most recent catalogue (<http://chr21db.cudenver.edu>) includes more than 500 annotated genes, of which 170 are protein coding and five are microRNAs that are highly conserved in mouse. An additional ~170 may encode proteins but show significantly less conservation, and a further ~185 genes cannot be classed unambiguously as either protein coding or functional RNA and do not appear to be conserved in orthologous mouse genomic regions. Considering only the 170 conserved protein coding genes, 112 map to mouse chr16 (Mmu16). Overlapping subsets of these are triplicated in the Mmu16 segmental trisomy models, Dp(16)1Yu (112 genes triplicated), Ts65Dn (94), Ts1Cje (70), and Ts1Rhr (33). In addition, 19 orthologs of chr21 protein coding genes map to mouse Mmu17 and 39 to Mmu10. Review of the known functions of chr21 protein coding genes makes clear that many interact directly, share overlapping sets of substrates or targets, and/or impact the same pathways, complexes or cellular processes. Therefore systems biologic approaches and network analyses are required to fully elucidate the relationship between trisomy of chr21 genes and the phenotypic features of DS.

Information is organized into the following topics: (1) individual chr21 gene functions ascertained through in vitro studies and single-gene mouse models, (2) pathways and cellular processes in which chr21 genes function, (3) pheno-

typic features of mouse models trisomic for large sets of genes, (4) methods for RNA and protein expression analysis, including measurement of natural variation in expression level and dosage effects in trisomy, and (5) results of treating mouse models with pharmacological agents.

Chr21 gene function: in vitro analysis and single gene mouse models

Several presentations described functions and interactions of the same chr21 proteins. However, because different assays were used, studies often revealed different functional features. Figure 1 summarizes data for Rcan1 and Dyrk1a, and Fig. 2 for App, Itsn1 and Tiam1.

RCAN1 (aka DSCR1, MCIPI, Adapt78)

Regulator of Calcineurin 1 protein, RCAN1, directly modulates the activity of the protein phosphatase, calcineurin. RCAN1 is upregulated by VEGF-A165 and in turn inhibits VEGFA-165 induced angiogenesis. Huiyan Zeng (Harvard Medical School, Boston, MA) discussed the expression patterns, tissue distribution and effects on angiogenesis of RCAN1 isoforms. RCAN1.1L protects against cell stress and RCAN1.4 inhibits cardiac and skeletal muscle hypertrophy. In cultured endothelial cells, RCAN1.1L promoted angiogenesis, even in the absence of VEGF-A165. RCAN1.4 inhibited angiogenesis by inactivating calcineurin, which in turn inactivated the transcription factor NFAT, while RCAN1.1 L activated NFAT and promoted angiogenesis (Fig. 1a). RCAN1.3 exerted no effects on angiogenesis.

Rcan1 function was investigated by Dana Crawford (Albany Medical College, NY) by creating transgenic mice overexpressing human RCAN1 under control of an enolase promoter (Fig. 1b). The transgene successfully inhibited NFAT dephosphorylation, and transgenic mice showed impairments in the Morris Water Maze, a test of spatial learning relevant to hippocampal deficits seen in DS. Lethality in RCAN1 BAC transgenic mice occurred at E9.5, just after RCAN1 overexpression begins. RCAN1 knockout mice demonstrated altered nitric oxide production and thus RCAN1 may alter vascular tone as one mechanism contributing to pathology.

Daphne Dubach (Monash University, Australia) described levels of dynamin, amyloid precursor protein (APP) and neurofilament, and calcineurin activity in both RCAN1 and Rcan1 null transgenic mice. She assessed retrograde transport with fluorogold and found no changes. In transgenic mice, she found hippocampal structures decreased in volume, and neurons in CA1 and the dentate gyrus decreased in size with fewer dendritic spines (20%) (Fig. 1d). Both overexpression and underexpression altered calcineurin activity and vesicular trafficking. Dynamin 1 levels decreased in RCAN1 transgenic mice. Although there was no overall change in Morris Water Maze performance, the transgenic animals took longer to find the platform.

In additional work with Rcan1, Eric Klann (New York University, NY) showed that knockout mice exhibit deficits

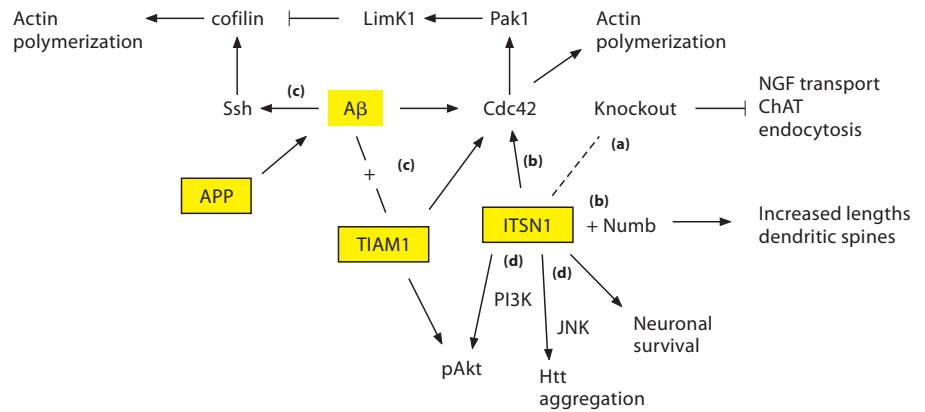


Fig. 2. Functional interactions of APP, TIAM1 and ITSN1 with the GEF Cdc42 and Numb. Information derived from the following presentations: (a) M. Pritchard, (b) K. Kaibuchi, (c) A. Mendoza-Naranjo, (d) J. O'Bryan.

in spatial learning and memory, reduced associative cue memory, and impaired late phase LTP, similar to transgenic mice overexpressing calcineurin (Fig. 1e). The mice have increased calcineurin activity, increased levels of cleaved calcineurin fragments, and decreased phosphorylation of the calcineurin substrate, DARPP32. He is now generating a conditional transgenic combining the *RCAN1* transgenic mouse with an alpha-CaMKII-cre line to target expression in forebrain and hippocampal CA1 neurons.

DYRK1A

Woojoo Song (Inje University, Korea) described mice that overexpress the serine-threonine protein kinase DYRK1A protein from a human BAC clone and showed they have increased levels of P-Thr688-APP, P-Ser202Tau, P-Thr212-Tau, and P-Ser404-Tau in brain. Further, the transgenic mice have elevated levels of beta-amyloid (25%) and exhibit learning deficits in the Morris Water Maze (Fig. 1f).

Odessa Yabut (Baylor College of Medicine, Houston, TX) showed that, in a neuroblastoma cell line, *Dyrk1a* inhibits proliferation by causing an arrest in G0/G1. In utero electroporation of *Dyrk1a* results, after 24 h, in *Dyrk1a*-expressing cells appearing in the subventricular/intermediate zone boundary, consistent with co-expression of postmitotic neuronal markers. Thus, *Dyrk1a* overexpression induces premature neuronal differentiation in a kinase-dependent manner and disrupts the expansion phase of neurogenesis (Fig. 1h).

Other genes

Melanie Pritchard (Monash University, Australia) discussed phenotypic features of mice with a null mutation in Intersectin 1 (*Itsn1*), a multidomain protein known to function in endocytosis and MAPK signaling pathways. Enlarged endosomes are one of the early signs of Alzheimer's disease (AD). Maintenance of basal forebrain cholinergic neurons (BFCN) depends on proper nerve growth factor (NGF) trafficking through endocytic processes. *Itsn1* knockout mice (Fig. 2a), who lack either the entire gene or the brain specific isoform, exhibit increased endosome area

and decreased levels of NGF and Choline Acetyl Transferase (ChAT) positive cells in their medial septum. Chromaffin cells of knockout mice exhibited significantly reduced numbers of endocytic events. Use of synaptoPHluorin enabled measurement of vesicle recycling and retrograde transport of fluorogold.

During nervous system development, commissural axons project towards and across the ventral midline, a process mediated by netrin-1 and the netrin-1 receptor, DCC. Elke Stein (Yale University, New Haven, CT) presented evidence that the chr21-encoded Down Syndrome Cell Adhesion Molecule, DSCAM, is also required for commissural axon guidance. The ectodomain of DSCAM can bind netrin-1 and block axon outgrowth. Thus, overexpression of DSCAM, by causing enhanced netrin-DSCAM interactions, may contribute to the axonal wiring defects seen in DS.

Synaptojanin 1 (*Synj1*) is a lipid phosphatase that dephosphorylates PtdIns(4,5)P₂, a lipid that regulates membrane transduction and membrane trafficking in the endocytic pathway. Gilbert DiPaolo (Columbia University, New York, NY) showed that both Ts65Dn and transgenic mice overexpressing *Synj1* show increased phosphatase activity and reduced mass of PtdIns(4,5)P₂. Restoring copy number rescues this phenotype. Tg(*Synj1*) mice exhibit learning deficits in the Morris Water Maze, although training improves performance.

The Autoimmune Regulator (AIRE) protein functions as both a transcriptional activator and an E3 ubiquitin ligase. Yoshitaka Yamaguchi (Keio University, Japan) has constructed three lines of thymic epithelial cells (TECs) expressing the SV40 antigen driven by the mouse *Aire* promoter. Their coculture with thymocytes led to adhesion and apoptosis.

Individuals with DS show increased incidence of syringomas, xerosis, and Alopecia areata and increased sensitivity to fungi and bacterial infections. Relevant to this, Jacqueline London (University of Paris) noted that mice transgenic for hSOD1, the human SuperOxide Dismutase gene, show a significant decrease in N-acetyltransferase 2 (NAT2), an enzyme involved in detoxification of aromatic xenobiot-

ics in the skin. In addition, transgenic mice overexpressing the human Amyloid Precursor Protein, hAPP, also show alterations in keratinocytes and cicatrization. Thus, overexpression of SOD1 and APP may contribute to the dermatologic disorders in DS. Study of Ts1Cje mice, which are not trisomic for either App or Sod1, will provide further information.

The Purkinje Cell Protein 4, PCP4, binds to calmodulin and modulates interactions with its targets, which include CaM KII and nNOS, by increasing rates of association and dissociation of Ca⁺⁺. *Pcp4* is expressed early in development in ectoderm and neural crest derivatives. Nicole Creau (University of Paris, France) created both a cell line containing a PAC with the entire murine *Pcp4* gene and ES cells containing the entire human *PCP4* gene. Induction of *PCP4* expression led to precocious differentiation in the cell line and to shorter and less branched dendrites in the resulting transgenic mice.

MicroRNAs

Five microRNAs localize to chr21: *Hsa-miR-99a*, *Hsa-let-7*, *Hsa-miR-125b(2)*, *Hsa-miR155* and *Hsa-miR-802*. Terry Elton (Ohio State University) showed that mature miR-155 and miR-802 levels are increased in isolated Down syndrome neurons and levels of all five are increased in Down syndrome heart, frontal cortex and hippocampus. All five map to Mmu16 and are triplicated in Dp(16)Yu1, while *miR155* and *miR802* are triplicated in Ts65Dn and Ts1Cje, and only *miR-802* is triplicated in Ts1Rhr. This distribution will allow some dissection of the contributions of each gene. Interestingly, *miR-99a*, *miR-let-7* and *miR-125b(2)* are 'hosted' in a terminal intron of the *C21orf34* gene, a gene of unknown function and undefined open reading frame.

Christelle Borel (University of Geneva Medical School, Switzerland) recapitulated published work on the role of *Hsa-miR-155* in the regulation of the Angiotensin receptor 1 gene (*AGTR1*). A target site for *miR-155*, located in the 3' UTR of *AGTR1* contains an SNP: *mir-155* can bind and down regulate the 1166A but not the 1166C, allele. The 1166C allele is associated with hypertension, suggesting that in DS, overexpression of *miR-155* may lower blood pressure through underexpression of *AGTR1*. Supporting this hypothesis is the observation that in fibroblasts from monozygotic twins discordant for Down syndrome, *AGTR1* protein levels are lower in the Down syndrome individual. These data emphasize the need to consider contributions of allelic variation in searching for chr21 gene-phenotypic correlations in DS.

Gene annotation

Alexandre Reymond (University of Lausanne, Switzerland) discussed new complexities in gene structure emerging from the ENCODE (ENCyclopedia of DNA Elements) project, which focuses on identification of all functional elements in 1% of the human genome (including approximately 1.5 Mb of chr21), plus GENCODE which focuses on all coding genes. Of genes examined, 81.5% have novel RACEfrags and half of those distal RACEfrags likely cor-

respond to bona fide novel exons. The 5' distal RACEfrags bind in the vicinity of transcriptional start sites in a statistically significant manner and the splice site strength of novel exons appears as high as that of GENCODE UTRs and CDSs. Novel exons isolated independently show some conservation in the mammalian lineage. Forty percent of tandem genes produce chimeric transcripts that may have novel functions. The functional implications of these abundant nontraditional transcripts, and their dosage effects in trisomy, remain to be determined. With only 1% of the genome analyzed in this way, it is not possible to predict novel ENCODE features elsewhere within chr21, but there is no reason to assume they will be less frequent.

Pathways and processes

RCAN1 and DYRK1A

Presentations in this category included further discussions of the roles of *Rcan1* and *Dyrk1a*, as well as chr21 proteins that affect the activity of the non-chr21 guanine nucleotide exchange factor (GEF), *Cdc42*, and potentially neurogenesis, plus dysregulation of mitochondrial function.

Circadian fluctuations in Ca⁺⁺ concentrations in specific cell populations help to reset their intrinsic clocks. Beverly Rothermel (University of Texas Southwestern, Dallas) showed that expression of *RCAN1.4* is circadian in heart, kidney, liver and suprachiasmatic nucleus (SCN).

Disruption of *RCAN1* leads to a longer innate circadian day and shifts expression of several key clock genes, suggesting possible perturbations in circadian rhythms in DS due to increased *RCAN1* expression (Fig. 1c). A *Cre* SCN-specific transgenic may help elucidate the role of calcineurin in this process in the SCN.

Dynamin 1, *SYNJ1* and amphiphysin 1 are among the substrates of *Dyrk1a*. Yu-Wen Hwang (College of Staten Island, New York, NY) showed that their phosphorylation modifies interactions with other endocytic accessory proteins and may regulate assembly of protein complexes. Phosphorylation of dynamin 1 at S857, in particular, alters its binding to amphiphysin 1, endophilin 1, *ITSN1*, *syn-dapin*, and other endocytic accessory proteins (Fig. 1g). Overexpression of *Dyrk1a* alters the kinetics of changes in dynamin phosphorylation rather than equilibrium levels. Because dynamin is dephosphorylated by calcineurin whose activity is inhibited by overexpression of *Rcan1*, phosphorylation patterns of endocytic proteins may be dysregulated in Down syndrome.

Transgenic mice harboring different mutations in the NFATc transcription factors exhibit phenotypes similar to features seen in DS. Because both *RCAN1* and *DYRK1A* impact NFAT activity, Gerald Crabtree's laboratory (Stanford University) constructed transgenic mice overexpressing cDNAs encoding *Rcan1* and *Dyrk1a*. Although the use of cDNA constructs eliminates much functional and regulatory complexity, nevertheless, as laboratory member Isabella Graef reported, these mice show reduced NFAT activity and altered gene expression (Fig. 1i).

Cdc42

The Numb protein has a well established role in determining neuronal cell fate and differentiation during development and later plays a role in axonal growth and has been implicated in clathrin-dependent endocytosis. Kozo Kaibuchi (Nagoya University, Japan) and colleagues localized Numb to dendritic spines (Nishimura et al., 2006) and showed that, when Numb expression in cultured neurons was reduced using siRNA, the number and length of dendritic spines was reduced without altering spine morphology. Pull-down assays with Numb from rat brain lysates identified chr21 ITSN1 as a binding partner and further work confirmed that ITSN1 co-localized with Numb on dendritic spines. Overexpression of either Numb or ITSN1 in hippocampal neurons caused an elongation of the spine neck while expression of ITSN1 mutants impaired spine development. Because actin dynamics are critical in dendritic spine morphogenesis and ITSN1 acts as a GEF for Cdc42, a Rho family member required for actin cytoskeletal reorganization, Kaibuchi and colleagues investigated the effects of Numb and ITSN1 on Cdc42 activity. They found that ITSN1 alone induces increased Cdc42-GTP levels and that co-expression with Numb induced even higher Cdc42 levels, indicating that Numb enhances ITSN1 GEF activity (Fig. 2b).

F-Actin, the polymerized form of actin, has a pathological role in AD. Fibrillar A β , derived from the chr21 protein, APP, stimulates actin polymerization in hippocampal neurons, and acts through the Rho GEFs, Cdc42 and Rac, with the latter activated by the chr21 protein TIAM1 (Mendoza-Naranjo et al., 2007). Mendoza-Naranjo (University College of London, England) described additional components of this pathway (Fig. 2c).

Cdc42 and Rac1 directly activate the p21-activated kinase (Pak1). When phosphorylated, Pak1, in turn, activates Limk-1, a serine protein kinase involved in the regulation of actin polymerization. Limk-1 phosphorylates and inactivates the actin depolymerizing factor cofilin. Cofilin is reactivated by Slingshot (Ssh)-catalyzed dephosphorylation. Mendoza-Naranjo reported that A β stimulates this pathway by activation of Pak1 and Limk-1. Intriguingly, instead of the expected inactivation of cofilin, cofilin activation was increased and still enhanced actin polymerization as demonstrated by an increase in F-actin. A β treatment also resulted in a calcium-dependent increase in Ssh activation, explaining the apparent paradox. Therefore it is likely that the balance of cofilin phosphorylation (inactivation) and dephosphorylation (reactivation) shifts in favor of dephosphorylation. F-actin levels increase because actin turnover requires cycles of cofilin activation and inactivation. Indeed, cofilin possesses both pointed-end depolymerization activity and F-actin severing activity which are critical for the constant supply of monomeric actin subunits and free barbed ends, respectively, for use as templates for actin polymerization. Therefore, the A β mediated activation of cofilin is not inconsistent with a shift towards actin polymerization.

John O'Bryan (University of Illinois at Chicago) has focused on defining the signaling pathways regulated by ITSN1. ITSN1 is a multi-domain protein that functions in the endocytic pathway by acting as a scaffold for the assembly of molecules involved in clathrin-mediated endocytosis. In addition to its role in actin reorganization (mentioned above), ITSN1 activates a JNK-dependent pathway that cooperates with various growth factors and Ras to activate transcription and promote cellular transformation. ITSN1 also associates with Cbl to regulate EGFR trafficking, signaling and degradation. O'Bryan reported on two further activities of ITSN1 (Fig. 2d). In the first, he showed that ITSN1 regulates a cell survival signaling pathway (Das et al., 2007). Reduction of ITSN1 expression by siRNA in either neuroblastoma cells forced to differentiate or in primary cortical neurons, increased apoptosis, suggesting that ITSN1 is necessary for the survival of differentiating neurons. ITSN1 silencing concomitantly decreased activation of the pro-survival kinase, AKT. A subsequent yeast-2-hybrid screen revealed PI3KC2 as an ITSN1 binding partner, a significant observation given the role of 3'-phosphoinositides in the activation of AKT. Further, ITSN1 enhanced the lipid kinase activity of PI3K-C2 inhibition of the PI3K-AKT pathway, producing a phenocopy of the loss of ITSN1 in the neuroblastoma cell line. Thus, ITSN1 has a role in regulating the survival of differentiating neurons through regulation of a PI3K-AKT pathway.

Huntington's disease results from the aggregation of a mutant form of Htt, a neuronal protein that contains an expanded polyglutamine (polyQ) repeat. ITSN1 interacts with both wild type and mutant forms of Htt and co-expression of ITSN1 with mutant Htt results in a significant increase in visible aggregates (Scappini et al., 2007). Because ITSN1 activates a JNK-dependent stress signaling pathway and stress responses augment Htt aggregation, O'Bryan and colleagues investigated whether ITSN1 stimulated Htt aggregation through JNK activation. They found that expression of a dominant-negative JNK inhibited aggregation of mutant Htt and blocked ITSN1 enhancement of this aggregation. Finally, they used a *Drosophila* model to measure polyglutamine protein-induced aggregation and found that ITSN1 enhanced polyQ-mediated neurotoxicity.

Neurogenesis

David Rowitch (University of California San Francisco) presented an overview of the bHLH transcription factors OLIG1 and OLIG2. Both genes are expressed in the developing and mature central nervous system. In development, OLIG transcription factors are required for oligodendrocyte lineage specification and, in the adult brain, are required for ongoing oligodendrogenesis in the progenitor cells of the subventricular zone. Oligodendrocytes, cells that produce myelin in the central nervous system, are required for maintenance of axonal integrity and participate in signaling networks with neurons (reviewed in Ligon et al., 2006).

In mice, *Olig1* is required for repair of the types of lesions that occur in patients with multiple sclerosis. Using *Olig1*

null mice, David Rowitch and colleagues (Arnett et al., 2004) demonstrated that *Olig1* was required for efficient remyelination of lysolecithin-induced lesions in the dorsal or ventrolateral funiculi of the spinal cord and of cuprizone-induced lesions in brain. In wild type controls extensive remyelination had occurred within 14 days of induction of the lesion whereas *Olig1* null mice showed little evidence of remyelination at this time point. Furthermore, in wild type mice, migration of oligodendrocyte progenitor cells initiated remyelination and led to the appearance of differentiated oligodendrocytes at the lesion site. However, virtually no mature oligodendrocytes were found within the *Olig1*^{-/-} lesions.

Olig2 function is also required for glioma formation. Rowitch and colleagues made use of a model of glioma in which neural stem cells, derived from *Cdkn2a* (*p16*^{INK4a}/*p19*^{Arf}) null mouse embryos and transformed with a constitutively active epidermal growth factor receptor (EGFRvIII), are cultured as neurospheres. When transplanted into the brains of immunocompromised (SCID) mice, these neurospheres form gliomas with 100% penetrance. Genetic removal of both *Olig1* and *Olig2* but not *Olig1* alone from this system prevented tumor formation. Moreover, reintroduction of *Olig2* reinstated glioma formation (Ligon et al., 2007). Rowitch also reported that, in neural progenitors, *Olig2* directly repressed the tumor suppressor and inhibitor of stem cell proliferation gene *CDKN1A* (*p21/WAF1/CIP1*) gliomas suggesting a potential mechanism.

The functional relevance of elevated *OLIG1* and *OLIG2* expression in Down syndrome (DS) has yet to be determined however the findings presented by Rowitch will direct investigations into the numbers and replication potential of neural progenitor cells in DS. It would be intriguing to examine the status of *CDKN1A* (*p21*) in DS, because, despite increased copy number of *OLIG2*, DS individuals do not present with a higher frequency of glioma.

Mitochondrial function

Consistent with the location on chr21 of multiple genes directly and indirectly involved in mitochondrial function and energy production, evidence continues to accumulate for contributions to the DS phenotype from oxidative stress and mitochondrial dysfunction. Douglas Wallace (University of California Irvine) discussed the hypothesis that suboptimal mitochondrial function increases levels of mitochondrial reactive oxygen species (ROS), increases the brain mtDNA mutation rate and leads to further impairment of mitochondrial function. The Wallace laboratory has reported increased somatic mtDNA rearrangement and base mutation. Evidence was presented here that individuals with both DS and AD also have increased levels of mitochondrial regulatory domain mutations. Thus, DS and AD may have a common mitochondrial pathophysiology.

Jorge Busciglio (University of California Irvine) described direct evidence for increased oxidative stress and decreased mitochondrial activity in DS, and discussed the hypothesis that these alterations may play a role in increased risk of AD and of type two diabetes. Dr. Busciglio presented

evidence of elevated levels of markers of oxidative stress, for example lipid peroxidation and free radicals, and reduced mitochondrial function in the absence of changes in total mitochondrial mass. Thus chronic oxidative stress may induce an adaptive metabolic response in DS. Further support for this hypothesis included increases in expression of oxidative stress-related genes and several genes mediating mitochondrial uncoupling, plus induction of similar gene expression profiles when normal diploid cells are subjected to chronic oxidative stress. These findings are consistent with the hypothesis that chronic oxidative stress may be a critical factor leading to metabolic abnormalities in DS.

Viral infections

Robert Finberg (University of Massachusetts Medical School, Worcester, MA) discussed chr21 genes potentially related to viral infection and reactivation, a topic relevant to the increased sensitivity to infection in DS. Interplay between virus receptor proteins and the genes defining the host response to infection is an important issue, and Dr. Finberg discussed evidence that the chromosome 21 genes, *USP25* (Ubiquitin Specific Protease 25), *C21orf34*, *CXADR* (Coxsackie Virus and Adeno Virus Receptor), *C21orf91* and *CHODL* are candidates for susceptibility to recurrent HSV infections. He discussed in particular the role of *CXADR* in host immune response.

Mouse models segmental trisomies

The most widely used and well-studied mouse model of DS is Ts65Dn which has a small translocation chromosome comprising the distal region of Mmu16 attached to the centromeric end of Mmu17 (Davisson et al., 1993; Reeves et al., 1995). Ts65Dn mice show DS-related phenotypes, including reduced birth weight, cognitive and behavioral impairments, craniofacial abnormalities, perinatal lethality, cardiovascular malformations, and neurological deficiencies (Escorihuela et al., 1995, 1998; Holtzman et al., 1996; Richtsmeier et al., 2000; Belichenko et al., 2004; Lorenzi and Reeves, 2006). Several groups described novel features of the Ts65Dn.

Clara Moore (Franklin and Marshall College, Lancaster, PA) and colleagues recently reported on the spectrum of cardiac abnormalities in Ts65Dn, including right aortic arch and intracardiac septal defects (Moore, 2006). The low incidence (17%), coupled with selective loss of trisomic neonates, makes such observations challenging. She reported additional features of the Ts65Dn, including abnormal branchial arch artery formation and reduced apoptosis in heart, reduced bone mineralization and resistance to stress that may lead to the kyphosis found in these mice, and contrary to the accepted view, fertility in some males plus the ability to transmit the trisomy.

Ts65Dn mice exhibit excessive synaptic inhibition in the dentate gyrus, a condition that could compromise synaptic plasticity and mnemonic processing. Zygmunt Galdzicki (Uniformed Services University of the Health Sciences,

Bethesda, MD) presented results consistent with increased levels of GABAB receptor-mediated GIRK channel current, which is in turn consistent with increased expression, due to gene dosage, of the chr21 encoded *Girk2* channel subunit (Siarey et al., 2006; Best et al., 2007). This overexpression would affect the balance between fast and slow inhibitory neural transmission in an input-specific manner. This directly links overexpression of GIRK2 to a GABAergic dysfunction and implicates the cortico-hippocampal circuit involving inhibitory temporoammonic terminals in cognitive dysfunction, opening new avenues for therapeutic interventions.

William Mobley (Stanford University) recapitulated the interesting report on the role of the *App* gene in Ts65Dn mice in both failed NGF trafficking and BFCN degeneration; reduction of *App* to disomy in Ts65Dn leads to a marked improvement of NGF transport and BFCN morphology.

Joan Richtsmeier (Pennsylvania State University, State College, PA) described skull shapes of five segmental trisomy models (Ts65Dn, Ts1Cje, Ts1Rhr, Tc1 and Ts65Dn reduced to disomy for *Ets2*) using 3-dimensional landmark data. She used principal coordinates analysis (PCOORD) to identify groups that cluster based on landmark features and the Euclidean Distance Matrix Analysis (EDMA) to summarize features that discriminate trisomic from euploid mice. Subtle but statistically significant differences were found between models and between models and euploid littermates.

To directly test whether trisomy results in a decreased incidence of solid tumors, Roger Reeves (Johns Hopkins University, Baltimore, MD) analyzed *Apc*^{Min}-mediated tumors in Ts65Dn, Ts1Rhr and Ms1Rhr mice. Both Ts65Dn *Apc*^{Min} mice and Ts1Rhr *Apc*^{Min} mice showed a significant reduction in tumor number compared to euploid controls. In contrast, Ms1Rhr mice, which are monosomic for the same 33 genes that are trisomic in Ts1Rhr, had a 52% increase in tumor number. Thus, trisomy for a small subset of chr21 genes is sufficient to provide protection from cancer of the small intestine in a dosage sensitive manner. This protective effect differs from tumor suppression, which requires normal gene function to prevent cellular transformation. *Ets2* was proposed as a candidate gene for the effect.

Although it has been informative, the Ts65Dn mouse is not a complete model for trisomy 21 and therefore efforts to create additional segmental trisomies remain important.

Yann Hérault (CNRS Institute for Transgenesis, Orleans, France) described his efforts to produce mice with triplication or monosomy of precise, annotated fragments of the chr21 orthologous regions of *Mmu17* and 10 (Besson et al., 2007). In theory, these new models will allow production of mice trisomic for two or more distinct genetic segments, thus more closely mimicking trisomy of a complete chr21. To date, he has obtained mice trisomic and monosomic for much of the *Mmu17* region, spanning *Abcg1* to *Pde9a*, and mice that are monosomic for the *Mmu10* region from *Prmt2* to *Col6a3l*. RNA expression analysis identified genes that are dosage sensitive, including *Pde9a*, *Ndufv3*, *Wdr4*, *Pknox*,

Cbs, *Lss*, *Mcm3ap*, *Pcnt* and *Prmt2*. Eugene Yu (Roswell Park Cancer Institute, Buffalo, NY) reported that the Dup(16)Yu1, which is trisomic for the entire segment of *Mmu16* orthologous to chr21, shows a 37% incidence of cardiovascular abnormalities; RNA overexpression of *Dscam*, *Ncam2*, *App* and *Itsn1* have been verified, and the mice have been shown to perform poorly in the Morris Water Maze.

Elizabeth Fisher (University College, London, England) updated features of the Tc1 mouse that carries a copy of chr21, constructed using the irradiation microcell-mediated chromosome transfer method (O'Doherty et al., 2005). Approximately 75% of Tc1 fetuses show cardiac abnormalities and microtomography revealed these mice have a significantly smaller mandible. They also show reduced density of cerebellar granule neurons with aging, decreased LTP in the dentate gyrus, and impaired performance in the novel-object recognition task, but not in the spontaneous alternation T-maze. The shortcomings of this model include a high degree of mosaicism within tissues, so that on average only one-third of brain cells carried chr21, plus internal deletions that include the important candidate genes *OLIG1*, *OLIG2*, *ITSN1* and *RCAN1*. In addition, the consequences of expression of human proteins in a mouse micro-environment must be taken into account in interpreting the phenotypes detected.

Jun Kudoh (Keio University, Japan) presented a novel method to produce mice harboring an extra copy of genes on a human artificial chromosome (HAC). HAC vectors are innovative tools that enabled the stable transfection of exogenous genes into mammalian cells without their incorporation into endogenous chromosomes. Kudoh and colleagues discussed preliminary work on transfecting chr21 HACs into ES cells.

Future functional genomic studies of brain and behavior will involve the full range of available transgenic methods and the incorporation of new technologies, including humanized mice. Elucidating the causal genes or genetic regions of DS using novel model animals is the principal goal of these models.

Expression and variation

RNA expression

The effects of gene dosage in DS and segmental trisomy mouse models continue to be investigated by oligonucleotide arrays. A new focus, however, is the analysis of natural variation in expression level among euploid controls as well as trisomic individuals.

Emilie Yahya-Graison (University of Paris, France) screened a chr21-specific oligonucleotide microarray containing probes for 339 genes, including 18 antisense transcripts, with RNA from euploid and DS-derived lymphoblastoid cell lines. She could separate genes into four classes based on trisomy vs. euploid expression ratio: for 30 genes the ratio was very close to 1.5; for 9, it was significantly >1.5; for 77, significantly <1.5, and for 5, it was highly variable among individuals. Dosage compensation occurred for

more than half of the genes. She suggested that consistently overexpressed genes likely affect phenotypic features common to all individuals with DS, while expression of highly variable genes may contribute to phenotypic variability. While gene-specific alternate transcripts showed similar changes in trisomy, effects sense and antisense transcripts were not necessarily correlated.

In related experiments, Stylios Antonarakis (University of Geneva Medical School, Switzerland) used Real Time RT-PCR to compare expression differences between 14 lymphoblast and 17 fibroblast cell lines derived from Down syndrome and euploid controls. Of approximately 100 chr21 genes, only 39 and 62 showed statistically significant increases in trisomic lymphoblast and fibroblast cell lines, respectively. When within-genotype variation was compared, trisomic genes again could be divided into groups. As in the previous talk, those where levels of expression in trisomy did not overlap with those in euploids were suggested to be relevant to the common phenotypic features of DS. Those where expression levels partially overlapped, most likely contribute to phenotypic variability and those with little expression difference between trisomic and euploids are least likely to participate in the pathologic phenotype.

Decreased numbers of granule and Purkinje cells and a resultant 15% reduction in cerebellar volume have been described previously in Ts1Cje mice (Olson et al., 2004). Julien Laffaire (CNRS, Paris) described studies of developmental regulation of cerebellar expression using samples from P0, P3, P7 and P10. Trisomic genes largely showed expression levels of 1.5 relative to euploid controls. Of genes whose expression changed during this time period, only *Girk2*, *Olig1* and *Dscam* were chr21 orthologs; non-chr21 orthologs included members of the *Sonic hedgehog* and *Notch* pathways. He observed a major gene dosage effect for *Girk2*, *Rcan1*, and *Dyrk1a*. He also reported a 30% decrease in granule cell proliferation seen at P0, but not at P3 and P7.

Marc Sultan (Max Planck Institute for Molecular Genetics, Berlin, Germany) discussed gene expression variation in the Ts65Dn mouse model. He found average gene expression ratios for trisomic vs. euploid were approximately 1.5 for 50 chr21 orthologs. However as with human cell lines, there was significant variation in expression levels among individual animals. Again three categories of genes were found. In cerebellum, cortex, and midbrain: nine, 17 and seven genes, respectively, showed consistently higher expression in trisomy; ten, nine and 14 genes showed trisomic expression levels partially overlapping with euploid levels, and 12, five and nine genes showed trisomic expression indistinguishable from euploid.

In similar studies, Jonathan Pevsner (Kennedy Krieger Institute, Baltimore, MD) previously reported that levels of chr21 gene expression in DS-derived fetal cerebrum, cerebellum, astrocytes, heart, fibroblasts, and lymphoblastoid cell lines differed significantly from euploid controls. However, in contrast to the previous presentations, they did not show differences among DS samples. Here, he reported that protein level measurements, of regulatory proteins and iTRAQ for over 1000 proteins, showed no evidence of gen-

eral increases in the levels for Hsa21 proteins, an observation at odds with literature reports from gene-specific analyses.

In a different approach, Thomas Lacroix (University of Colorado Denver) studied natural variation in expression levels of chr21 orthologs, using data from WebQTL derived from screening oligonucleotide arrays from each of the 78 RI lines derived from C57BL6/J × DBA/J with RNA from cerebellum, hippocampus, eye and liver. The arrays contained 185 of the 274 chr21 mouse orthologs. Of the 98 orthologs with detectable expression, the majority showed >2-fold variations among lines, raising questions regarding accurate ascertainment of natural variation. *Cis* or *trans* QTLs could be mapped for 15 of the 98 genes. Four orthologs, *C21orf59*, *C21orf66*, *Faspl*, and *Brd4* (*Hunk1*) are coordinately regulated, and expression of *C21orf51* highly correlates with 20 genes involved in mitochondrial function and NADH activity.

Two groups are surveying natural copy number variation by high density oligonucleotide arrays, with the intention to apply this technique to partial and complete trisomy 21 samples. Alexander Urban (Yale University, New Haven, CT) discussed an algorithm, *BreakPtr* used to process HR-CGH array data, allowing resolution to approximately 200 bps, or at the level of at least an exon, and recording of actual copy number of a given aberration. Jim Sikela (University of Colorado Denver) compared the Agilent 105K CGH custom chr21 arrays with a whole genome 244K CGH array with a 30-fold probe density for chr21. The 105K CGH array provided gene level resolution and should provide the foundation for the next generation of a phenotype/genotype map for Down syndrome.

Analysis of protein expression

Trisomy of protein coding genes and gene dosage-associated increases in RNA expression do not guarantee corresponding increases in protein expression. However, if a protein coding gene contributes to the DS phenotype, its effects are most likely to be at the protein level, making such measurements crucial. Unfortunately, this is challenging and not yet amenable to high throughput. Three investigators presented overviews of different approaches to analysis of protein expression in the brain.

Willard Freeman (Pennsylvania State College of Medicine, Hershey, PA) stressed the challenges of analysis of the brain proteome and attempts to combine these data with other forms of data, for example behavioral measures. Challenges include the complexity of the brain proteome and the presence of many low abundance proteins. He stressed the use of bioinformatic strategies to determine relevant changes in gene and protein expression using examples from microarray and 2-Dimensional Gel Electrophoresis (2-DIGE), plasma biomarker development and validation, and other methods. He also discussed the outlook for improvements in sensitivity of proteomic approaches, for example, protein fractionation.

Per Andren (Uppsala University, Sweden) described his use of imaging mass spectrometry (IMS) to locate specific

molecules and small proteins directly in frozen tissue sections. This elegant and sophisticated method can be used to produce spectra containing hundreds of peptide and protein signals from a tissue slice, which can then be analyzed to quantitate the levels of specific peptides or proteins in specific regions of tissue. In one example, IMS was used to show that the chr21 encoded protein, PCP4, which is a modulator of calmodulin, is predominantly located in mouse striatum. Administration of a drug, MPTP, which induces Parkinson's disease-like brain pathology and symptoms, produced a 30% reduction in PCP4 levels. Thus, this technique has the sensitivity and resolution necessary to identify and quantitate subtle changes in protein levels associated with trisomy.

Kevin Wang (University of Florida College of Medicine, Gainesville, FL) explored the use of cation-anion exchange chromatographic separation of proteins in tandem with 1-dimensional gel electrophoresis (CAX/1 D-PAGE) prior to mass spectroscopic identification of proteins. This method can identify a specific subset of differentially expressed brain proteins with increased or decreased abundance. Other approaches include high throughput immunoblotting. Protein changes caused by traumatic brain injury (TBI) were used to illustrate this approach.

These presentations indicated possible future directions that can be applied to analyze alterations in the brain proteome in DS model systems.

The potential for therapeutics

Two recent studies with Ts65Dn mice have created substantial interest both in the scientific community and among families and friends of those with DS. From the first study, Fabian Fernandez (Stanford University, Palo Alto, CA) described his work in Craig Garner's laboratory on the effects of the non-competitive GABAA antagonists, picrotoxin and pentylentetrazole (PTZ), on hippocampal based learning and memory tasks (Fernandez et al., 2007). An increase in inhibitory inputs known to exist in the hippocampus of Ts65Dn mice made testing of GABAA antagonists reasonable even if the direct connection to chr21 genes is not clear. Only ten days of drug treatment resulted in improved performance in the novel object recognition and the T-maze tasks, an improvement that persisted for several months after drug treatment ended. Partial normalization of LTP also indicated persistent improved hippocampal function. Because clinicians had previously used PTZ in clinical settings, although no longer FDA approved, appropriate dosage control may make it possible to move quickly into clinical trials in DS.

In the second study (Costa et al., 2007), Alberto Costa (University of Colorado Denver) treated Ts65Dn mice with the uncompetitive NMDA receptor antagonist, memantine. One target of memantine is the NMDA receptor, whose function is predicted to be perturbed by the integrated effects of increased expression of several chr21 genes, including *RCAN1*, *APP*, *TIAM 1*, *ITSN1* and *DYRK1A*, all of which

are intensively studied for relevance to DS. Two injections of memantine eliminated deficits in the hippocampal-based task, Contextual Fear Conditioning. Memantine has the added attraction of being FDA-approved for treatment of moderate to severe AD, thus facilitating its use in clinical trials in DS.

Jean Delabar (University of Paris, France) described studies of a single gene mouse model, a 'BAC transgenic' mouse that overexpresses *Dyrk1a*. This mouse has features similar to an earlier model overexpressing *Dyrk1a* plus five other genes from a YAC clone. He posited that a green tea component, ECGC, which inhibits *Dyrk1a*, will normalize its expression and improve learning/memory deficits. Dr. Cristina Fillat (Center for Genome Regulation, Barcelona, Spain) discussed another transgenic mouse model with elevated expression of *Dyrk1a* that displays hyperactive behavior and abnormal motor activity. She demonstrated that expression could be down-regulated by injection of an adenovirus vector containing shRNA and that specific phenotypic features were rescued by this treatment.

Ira Lott (University of California Irvine) summarized a substantial body of clinical work on AD in DS. He focused on oxidative damage in the DS brain, emphasizing themes from earlier presentations. Somatic mutations that suppress mitochondrial function and replication do occur in a substantial fraction of adults with DS who also have clinical features of AD; in contrast, they do not occur in brains of adults with DS who do not show dementia. Following on his studies of anti-oxidant treatments in animal models, he currently is conducting a large clinical trial to study effects of vitamin E, vitamin C and alpha-lipoic acid in individuals with DS with and without AD. Results of the study should be known early in 2009. A general discussion of implications for therapy in DS followed. Mary Lou Oster-Granite of NICHD discussed a draft report of the Trans-NIH DS Working Group and invited the audience to read and comment on the report located on the NICHD website.

Roger Reeves moderated discussion of the assumption that therapies for major features of Down syndrome – specifically cognitive deficits and dementia – are indeed plausible and deserve timely consideration. (For a review of some recent research developments that support this optimistic view, see Reeves and Garner, 2007). The promising results presented in the last year raise issues of the next steps necessary to move them toward use in patients. Four major points framed the discussion:

How do we move from experimental findings to clinical application?

What is required in terms of patient registries/repositories to support research?

What is required in terms of clinical trials/trial networks to support translation?

How do we, as a community, address the issue of 'human experimentation'?

Bench to clinic

A strong point was made regarding the need for a significantly better description of the phenotypic features of trisomy, i.e., fundamental knowledge of phenotypic details is lacking but required. There is a critical need for a dialog that brings clinical and basic researchers together and includes representation from the biotech/pharmaceutical industry. Here, discussants with experience in biotech emphasized that an insightful finding rather than a detailed presentation of mouse pathology/molecular biology is more likely to foster interest from biotech.

Patient registries, repositories

This issue is emerging but complex. First, here a Registry means a list of people with DS, including some description of individual specific features, who have given permission to be contacted for a study. Repository is a resource of banked DNAs, cell lines, databased phenotype information, etc. For several years, the CDC and NIH supported a substantial and highly successful infrastructure headed by Stephanie Sherman (Emory University) for recruitment across seven states, ascertainment of detailed phenotypic information and collection of samples from individuals with DS and their parents. However, the funding agencies recently abandoned support for this effort; in the context of this discussion, that decision was made at precisely the wrong moment. Much of that infrastructure still exists and could be revitalized.

Small consortia or individual labs currently recruit modest numbers of study participants. The literature is replete with grossly under-powered studies, both research-oriented and increasingly with clinical trials examining off-label uses of prescription drugs. The need for large-scale efforts and a national clinical trial network were emphasized.

The question of how to handle the logistics of registries/repositories was raised. Different interpretations by IRB committees and concerning HIPAA issues across institutions are currently a major confounding factor. If Institutional IRBs can be educated to a more uniform standard (e.g., to the 'streamlined' approach that has occurred at the federal level under Francis Collins at NHGRI), significant progress could be made. There are major issues regarding access to registries or repositories, as well. A recent meeting at the Waisman Center, University of Wisconsin (<http://www.waisman.wisc.edu/reg-meeting/>) addressed some of these concerns. Although more discussion is needed, this appears to be a looming roadblock, whose timely resolution is required.

Clinical trials

Challenges surrounding recruitment of an adequate number of patients are evident. A U.S. population of 350,000 people with DS limits the number available for trials that address a specific aspect of the phenotype present in a subset of those with DS. A national clinical trials network could facilitate accomplishing appropriately designed, well-powered studies for therapeutic treatments.

'Human experimentation'

This refers to treatment with off-label use of drugs or exotic therapies. Basic researchers must be cautious when presenting findings in this area. Parents frequently misinterpret as *possibility* the natural optimism expressed by researchers about *potential*, without consideration of the unknown risks. Investigators should use a conservative approach when reporting such research developments.

At the heart of this dilemma are parents who feel that a small window of opportunity exists early in life when treatments may positively impact their child (especially on cognitive abilities). The basic and clinical research communities respond slowly to rumors of effective treatments for cognitive deficits, although such rumors spread rapidly via internet interest groups. Parents appear able to obtain prescription drugs for off-label use with high success, although there are no figures on how widespread 'parental prescribing' is. Publication of 'clinical studies' of a few patients exacerbates this problem because the studies are often insufficiently powered to draw conclusions about safety or efficacy. There are advantages to conducting studies in a traditional clinical setting with the opportunity for closer physician scrutiny, however, the generation of more anecdotal information from underpowered studies seems likely to drive further parental experimentation. The mother of a child with DS and representative of a parents' group explained the peer pressure to give the latest drug to one's own child 'before it's too late.' She also indicated that, in her opinion, what parent groups want/demand now of federally funded researchers is the equivalent for DS of what Ritalin provided in ADHD (attention deficit hyperactivity disorder).

Clearly, a substantial gap exists in understanding among researchers, clinicians and parent groups about what is and is not possible and what is and is not a goal of research and treatment. Is the overall basic research portfolio focused on too many fundamental science questions as some active parents contend? Is there a translational research community that can move basic findings toward the clinic? (For DS, as for many other conditions, the answer is largely 'no'). Are clinical directors of DS clinics fully informed about the issues surrounding off-label use in children of a drug approved for adults that had an effect in a mouse behavior test? And do they have access to the expertise required to establish cause and effect in these 'mini-trials'? The community as a whole must develop a balanced perspective quickly to avoid hurting the children they seek to help.

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