

Too much of a good thing: mechanisms of gene action in Down syndrome

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The molecular mechanisms underlying the specific traits in individuals with Down syndrome (DS) have been postulated to derive either from nonspecific perturbation of balanced genetic programs, or from the simple, mendelian-like influence of a small subset of genes on chromosome 21. However, these models do not provide a comprehensive explanation for experimental or clinical observations of the effects of trisomy 21. DS is best viewed as a complex genetic disorder, where the specific phenotypic manifestations in a given individual are products of genetic, environmental and stochastic influences. Mouse models that recapitulate both the genetic basis for and the phenotypic consequences of trisomy provide an experimental system to define these contributions.

It has been more than 40 years since Lejeune and colleagues demonstrated that DS is caused by trisomy of a G group chromosome¹ now identified as chromosome 21 (Chr 21). Trisomy 21 results in a constellation of more than 80 clinical traits, a subset of which is observed in any given individual with DS (Ref. 2). A working assumption about gene dosage is that the extra copies of Chr 21 genes result in increased levels of transcript and protein, on the order of 1.5- to 3-fold³. Although the cause of DS has been known for decades, the mechanisms by which the resulting gene-dosage imbalance disrupts development to produce the specific features of DS are not well understood.

Clinical features of DS

Trisomy 21 is the most common autosomal aneuploidy compatible with postnatal survival. It occurs in 1 out of 700 live births in all ethnic groups. Trisomy 21 usually results from nondisjunction in meiosis, most frequently in female meiosis I (Ref. 4). There is a pronounced maternal-age effect on the occurrence of trisomy 21, with increased risk when maternal age passes 35 years. This has been attributed to the lack of a meiotic checkpoint in the oocyte⁵. Extensive independent studies failed to substantiate any heritable predisposition to this or other trisomies in the past. A recent study associates increased risk of DS in younger mothers with specific mutations in two enzymes of folate metabolism⁶, seeming to run counter to this observation. However, further explanation is required from analysis in larger and more diverse populations.

Trisomy 21 can affect many aspects of development, producing a wide and variable set of clinical features in a given individual^{2,7}. This

aneuploidy is the leading cause of congenital heart disease (CHD) and the most frequent genetic cause of mental retardation. It is a significant risk factor for Hirschsprung's disease (absence of enteric ganglia along a variable length of the intestine, also known as aganglionic megacolon) and also for childhood leukemia. Features that are always apparent include some degree of cognitive impairment, the characteristic appearance of the face, reduced size and altered morphology of the brain (including reduced cortical size, complexity and neuronal density, and a disproportionately small cerebellum), loss of cholinergic neurons in the basal forebrain and the occurrence of Alzheimer-like histopathology at an early age. A large percentage of DS individuals exhibit some hearing loss, which is usually not neurosensory but conductive and correlates with defects of the inner ear and pinna. Other clinical features include effects in the heart, gut and immune system. Few of the many features of DS have been assessed quantitatively.

Variability in DS

The majority of features that occur in DS are not present in every individual with trisomy 21, and those features that are present can vary considerably in severity². For variable features, trisomy 21 is more accurately viewed as a predisposing factor than as the causative factor. For example, Hirschsprung's disease occurs in one out of 20–30 individuals with trisomy 21, but only in 1 out of 5000 in the population at large. Trisomy 21 is thus a significant risk factor for Hirschsprung's disease, but is not sufficient to cause it because >95% of those with trisomy 21 do not have this condition. Similar arguments can be made for trisomy 21 as a risk factor for CHD, deafness, improper articulation of the first two cervical vertebrae (atlanto-axial instability) and many other features of DS.

Several genetic factors are likely to contribute to individual variability in DS. First, different allele combinations of Chr 21 genes might have different effects when present in three copies. Variants in and around the *collagen 6A1* and *6A2* genes on distal Chr 21 have been correlated with the occurrence of CHD in DS (Ref. 8), although a direct role of these genes in heart disease has not been demonstrated. Second, the genetic background of the individual in whom

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trisomy 21 occurs is an obvious source of phenotypic variation. Even classical mendelian mutations that have been mapped, cloned and characterized as discrete monogenic traits, have variable clinical outcomes (e.g. see Refs 9,10). The genetic contribution to this variation should be amenable to mapping as with other genes contributing to complex traits. Third, many traits are sensitive to environmental influences. In euploid individuals, allelic variation of primary and background genes is proposed to determine the level of resistance or sensitivity to these effects, determining the degree and nature of variation in the phenotype. Trisomic individuals have an additional, unique genetic influence in the hundreds of genes expressed at inappropriate levels in diverse genetic pathways¹¹. It is not unreasonable to postulate that the resultant destabilizing effect contributes to the elevated individual variability observed in DS. The variable features of DS whose presence or absence depends on genetic background and environmental influences might be those most tractable and amenable to interventions that ameliorate the phenotypic consequences of trisomy 21.

Recent developments support experimental approaches to this complex explanation of the variable occurrence of DS features in trisomy 21. The gene catalog derived from the complete Chr 21 sequence¹² provides a template for determining sequence variation in the entire set of candidate genes that primarily affect developmental processes in DS. Advances in the analysis of quantitative trait loci now permit more sophisticated approaches to define the factors of genetic background that contribute to the occurrence (or not) of DS features and the increased individual variability seen among individuals with three, rather than two, copies of Chr 21. Mice with segmental trisomy have exact parallels of quantitative developmental phenotypes of DS, providing a system for the study of development at all levels.

Mechanisms of gene action in DS

By the 1980s, two distinct schools of thought had emerged to explain why the inheritance of three copies of Chr 21 genes results in disruption of normal patterns of development. The 'developmental instability' hypothesis holds that the correct balance of gene expression in pathways regulating development is disrupted by dosage imbalance of the hundreds of genes on Chr 21 (Refs 11,13). Support for this idea includes first, that features seen in DS also occur in other trisomies and in the population at large (albeit at much lower frequency); and second, that there is significantly increased individual variability among those with trisomy 21 as compared with euploid individuals. Although proponents continue to argue that 'more commonalities exist among the autosomal trisomy syndromes than differences', this hypothesis has been expanded to acknowledge that 'ultimately, the ability to distinguish different

trisomies clinically must be referable to interactions of products of those chromosomes'¹⁴. This has been criticized as being more a truism than a testable hypothesis³.

The 'gene-dosage effects' hypothesis holds that dosage imbalance of a specific individual gene or small group of genes from Chr 21 is responsible for specific individual DS traits, in contrast to the less specific relationship between genes and DS phenotypes in the developmental instability hypothesis¹⁵. Observations of a small percentage of DS individuals in whom a cytogenetic rearrangement results in triplication for a subset of the chromosome (segmental trisomy 21) provided the initial impetus for correlating individual genes with specific DS traits¹⁶. Comparison of minimally overlapping segments of Chr 21 occurring in individuals who show the same specific DS feature has been used to create phenotype maps of the chromosome (Fig. 1) (Refs 15, 17–20). However, there is no direct support for these maps, because there are no human beings that have three copies of just the minimal region of overlap that is assigned to a given phenotype. Moreover, analysis of phenotypes is complicated because most of the individuals with segmental trisomy also have further chromosomal rearrangements producing segmental trisomy or monosomy for additional regions of the genome. An implicit assumption of this hypothesis, which is not supported currently by any clinical or experimental observation, is that three copies of one or a few 'critical' genes on an otherwise diploid background would produce the relevant feature of DS with the same degree of severity as seen in trisomy 21.

A specific point of contention with the gene-dosage effects hypothesis is the concept of the DS 'critical' or 'chromosome' region (DSCR). Phenotype maps identify the region in common among individuals with segmental trisomy 21 who have the same DS trait (Fig. 1). In a few cases, the genetic boundaries and the phenotypes associated with these DSCRs are well defined¹⁹. However, both of these properties change with each new Chr 21 breakpoint identified within a DSCR, making the term uninterpretable without redefinition at each use. Because most phenotypes of DS occur in only a subset of individuals with trisomy 21, those individuals who do not express the trait are not considered, even though they might have three copies of the DSCR. This introduces an ascertainment bias and limits the already small sample size for these analyses.

Although the merits, and especially the deficiencies, of both the gene-dosage effect and developmental instability hypotheses are actively debated^{14,21}, essential elements of these ideas are not mutually exclusive. Components of both can be integrated by focusing on the mechanism of gene action in DS to provide a conceptual structure for understanding the etiology of this complex disorder. However, attempts directly to connect unquantified,

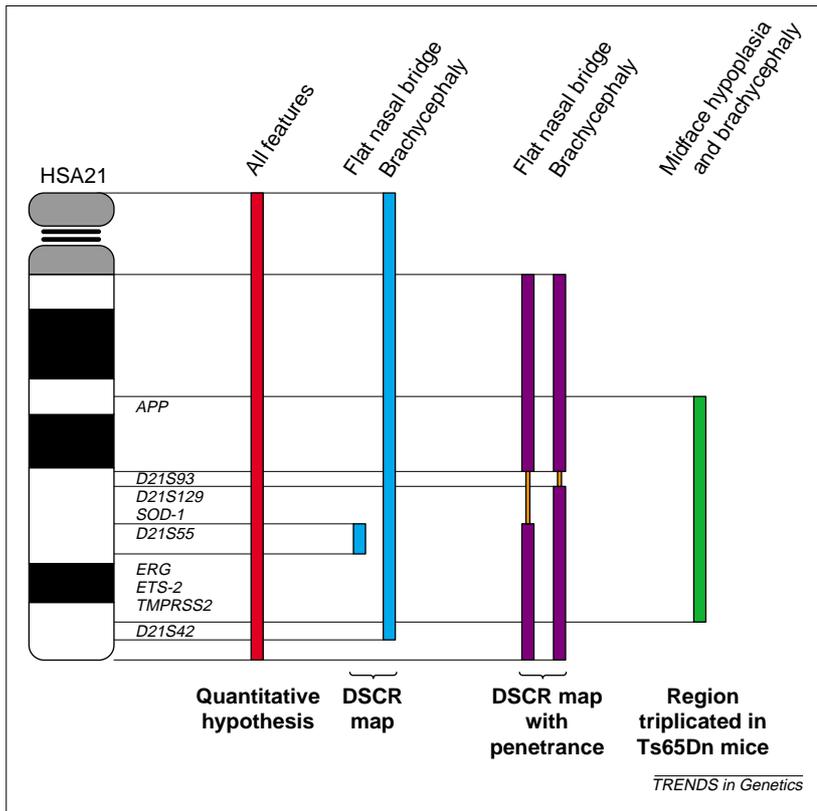


Fig. 1. Phenotypic maps localizing genes that affect development of the craniofacial skeleton in DS. The developmental instability hypothesis maintains that small nonspecific effects by the hundreds of genes on Chr 21 disrupt the evolved genetic balance that regulates development, resulting in all the features of DS, as represented by the red bar extending the length of Chr 21 (Ref. 13). Phenotypic mapping in individuals with segmental trisomy 21 has been used to produce maps of Down syndrome critical regions (DSCRs). A DSCR map for flat nasal bridge predicts that a gene(s) in a small chromosome segment around marker *D21S55* is responsible, whereas a gene whose dosage imbalance produces a short, broad skull (brachycephaly) is not well-localized (blue) (Ref. 19). Korenberg *et al.*³⁸ studied these traits in a similar set of individuals, but considered effects of penetrance when making a phenotype map (DSCR with penetrance) to localize the effects. They identified small segments that made a weak contribution to the flat nasal bridge and brachycephaly (narrow yellow lines), but included strong effects from most of the chromosome (purple). Ts65Dn mice show effects on the development of the craniofacial skeleton directly comparable to those in DS, providing independent confirmation of the contribution of genes in this region (green)³⁶.

highly variable phenotypes in trisomic adults with individual genes are likely to be simplistic. Rather, the initial genetic insult is expected to have had an effect in the development of a specific cell or cells, and this alteration might have a cascade effect as interactions occur with other cells further down a developmental pathway. In other words, a trisomy-induced alteration of the phenotype in one cell can affect how it interacts with other cells, causing concomitant changes in gene expression in these downstream cells. The changes in secondarily affected cells are undoubtedly a result of aneuploidy, but could represent a perfectly normal response, in terms of gene expression, to the signals received from the first, directly affected cell. A comprehensive explanation of the etiology of DS features should consider developmental consequences of aneuploidy and not only the direct overexpression of the triplicated genes. Such an analysis cannot be completed successfully by studying humans.

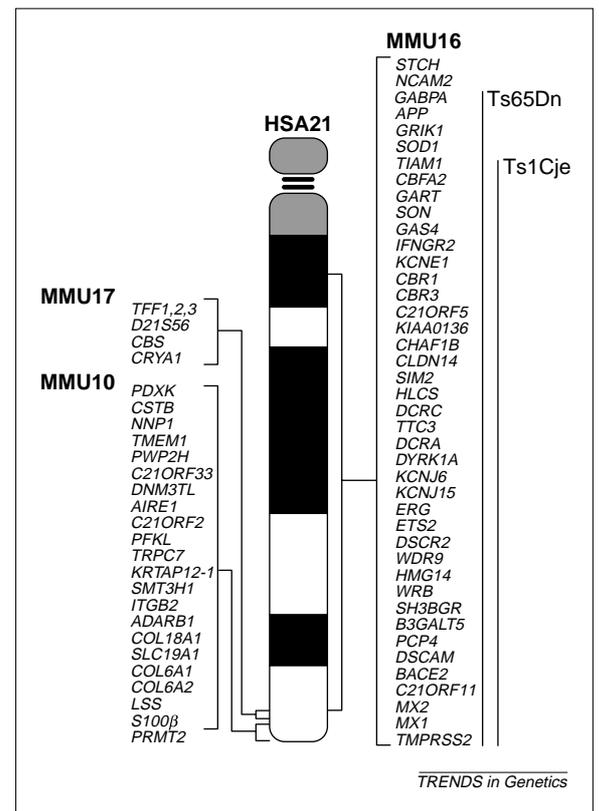


Fig. 2. Comparative genetic maps demonstrate a high degree of conservation between Chr 21 and mouse Chr 16, 17 and 10. Sixty-eight Chr 21 genes have been mapped to corresponding positions in these three regions of the mouse genome, or vice versa, and no discrepancies of content or order have been shown (Refs 39, 40; and R.H. Reeves, unpublished). The genes from segments of Chr 16 triplicated in Ts65Dn or Ts1Cje mice are indicated (not to scale).

Transgenic mice

A basic assumption of using animal models to study DS is that although the phenotypes will vary in ways that reflect species differences, the genetic processes disrupted by gene-dosage imbalance will frequently be conserved. Chr 21 gene order and content are highly conserved in mouse, facilitating development of genetic models (Fig. 2). The gene-dosage effects hypothesis lends itself to direct testing by transgenesis, and a large number of genetically modified mice have been reported (see Ref. 22 for a recent review).

Transgenic studies can be divided broadly on the basis of the genetic regulation of the transgene. Genes under control of their natural promoters are expected to recapitulate the cell-type- and stage-specific expression of the endogenous genes, reflecting the genetic situation in DS. An alternative approach uses promoters that result in overexpression of genes at very high levels, in ectopic locations and/or at inappropriate times during development. Such analyses can provide important information about the immediate functions of a gene, but abnormal expression patterns are likely to affect cells that would normally never express the gene at any level. Subsequent downstream interactions of

these cells could have consequences that are not directly a product of the transgene, complicating the extrapolation of the resulting phenotypes to those that arise from expression of one extra copy of a temporally and spatially regulated gene, as occurs in DS.

Transgenic mice expressing elevated levels of the human gene encoding Cu/Zn superoxide dismutase, *SOD1*, provide one of the best-studied models of DS (Ref. 23). These mice demonstrate more than a dozen wide-ranging effects, from increased resistance to diabetes-associated embryopathy to increased susceptibility to malaria. Several features of these mice have specific parallels in DS; for example, abnormal neuromuscular junctions of the tongue, impaired serotonin uptake by platelets *in vitro* and diminished prostaglandin synthesis in cultured fibroblasts^{24–26}. The early success in recapitulating DS characters in mice overexpressing *SOD1* encouraged the transgenic mouse approach, and especially the approach using genes under control of their natural promoters.

However, proper gene regulation can be difficult to obtain in conventional transgenesis because the full extent of normal regulatory elements of a gene are usually not defined. Large insert transgenic mice containing yeast artificial chromosomes (YACs) or bacterial artificial chromosomes (BACs) have been used to obtain appropriate gene regulation by supplying tens of kilobases of flanking sequence, presumably including most or all regulatory sequences. This assumption appears justified by results such as those obtained with the gene encoding the amyloid precursor protein, *APP* (Ref. 27). A transgenic mouse carrying the human-derived YAC not only showed tissue-specific expression of this large gene, which spans more than 400 kb, but also demonstrated tissue-specific splicing to produce alternative mRNAs in the same pattern as human.

The YAC transgenic approach was expanded by Smith *et al.*²⁸, who created a set of transgenic mice, each with all or part of a YAC from a set of four clones spanning more than 2 Mb of Chr 21. From this set, a behavioral phenotype was reported for a YAC that included the gene, *Dyrk1a*. *Dyrk1a* is a mammalian homolog of a *Drosophila* gene, *minibrain*, which, when mutated, leads to reduced production of neurons in the developing fly brain. YAC transgenic mice carrying this gene displayed a subtle deficit in the Morris water maze test, whereas other animals in the set were not different from controls. This deficit is not equivalent to the Morris maze deficit seen in mice with segmental trisomy 16 (Ts65Dn and TsCje1 mice, see below) as sometimes assumed. In fact, segmental trisomy causes a robust deficit beginning at the top levels of the hierarchical tests that together comprise the Morris maze analysis, and the deficit has been independently verified in a number of laboratories. *Dyrk1a* YAC transgenic mice have an extremely

subtle defect at the lowest level of this test.

Discounting concerns about the reproducibility of subtle behavioral phenotypes in different laboratories²⁹, the deficiency reported for the YAC transgenic mice is very different from that in mice with segmental trisomy, which are at dosage imbalance for a much larger segment that includes *Dyrk1a* and the entire 2 Mb represented in the mice comprising the YAC 'in vivo library'.

Transgenic mice provide important information about the roles of individual genes in DS. However, no transgenic mice with appropriately regulated single genes have been reported to recapitulate a major diagnostic feature of DS. Combining all features reported for transgenic mice which express elevated levels of Chr 21 genes does not provide a profile suggestive of DS. If, as the dosage effects hypothesis states, major effects of DS are caused by a few critical genes and can be recapitulated in mice, the responsible genes have not yet been tested using the transgenic approach. Testing the developmental instability hypothesis would require combining dozens or hundreds of transgenes.

Genetic models of DS in segmentally aneuploid mice

Evaluating the effects of single genes in mouse models is complicated by not knowing what phenotypic consequences of gene-dosage imbalance *can* occur in trisomic mice. The creation by Davisson *et al.* of 'Ts65Dn' mice³⁰ that have segmental trisomy for the distal end of mouse Chr 16 (Fig. 2) provides a system to address this problem. The distal Chr 16 segment in Ts65Dn mice corresponds to a portion of Chr 21 that spans 15.6 Mb and contains 108 of the 225 genes in the Chr 21 gene catalog¹². Thus, the genetic insult in these mice corresponds closely to that of segmental trisomy 21 in humans³¹. A second segmental trisomy 16 model, Ts1Cje, arose as a fortuitous translocation of Chr 16 in a transgenic mouse line³². These mice are at dosage imbalance for a subset of the segment triplicated in Ts65Dn, corresponding to a human Chr 21 region that spans 9.8 Mb and contains 79 genes. Chimeric mice in which a large percentage of cells contain all or part of human Chr 21 have also been reported³³. Mice with segmental trisomy provide the experimental basis to investigate corresponding developmental processes disrupted by the analogous trisomy in mouse and human.

Efforts to create mice with three copies of a defined chromosome segment using chromosome engineering³⁴ are being pursued currently in a number of laboratories and will provide refined genetic models for assessment of hypotheses concerning critical genes versus destabilizing effects of trisomy. For example, if a quantitative phenotype is expressed to the same degree in Ts65Dn and Ts1Cje mice, the gene or genes responsible can be excluded from the set of 39 genes not represented in both, and further localized in mice with smaller segmental trisomies that express the trisomic



Fig. 3. Dysmorphology of the craniofacial skeleton in Ts65Dn mice parallels that in DS. Euclidean distance matrix analysis was used to compare form in euploid and Ts65Dn mice. Bones that were most profoundly affected in Ts65Dn mice (left) and corresponding human bones (right) are colored. Lines on the mouse skull indicate some of the distances that are significantly reduced as a result of segmental trisomy when compared statistically with normal littermates. The ability to study phenotypes in a viable mouse model allows verification of phenotypic parallels with DS to provide a catalog of traits that can be compared across species³⁴. The affected craniofacial bones include: nasal, dark green; maxillae and premaxillae (fused into one element in humans, separate bones in mice), light green and lavender; jugal/zygomatic, pink; frontal, orange; mandible, purple; occiput, red.

phenotype to the same degree. If one gene is completely responsible for the phenotype, then transgenic mice that overexpress that critical gene at appropriate levels and in the proper temporal and spatial patterns should produce the same effect as seen in the segmental trisomies according to the gene-dosage effects phenotype hypothesis. Accurate genetic models of trisomy 21 provide the substrate for identifying corresponding consequences of gene-dosage imbalance in mouse and human.

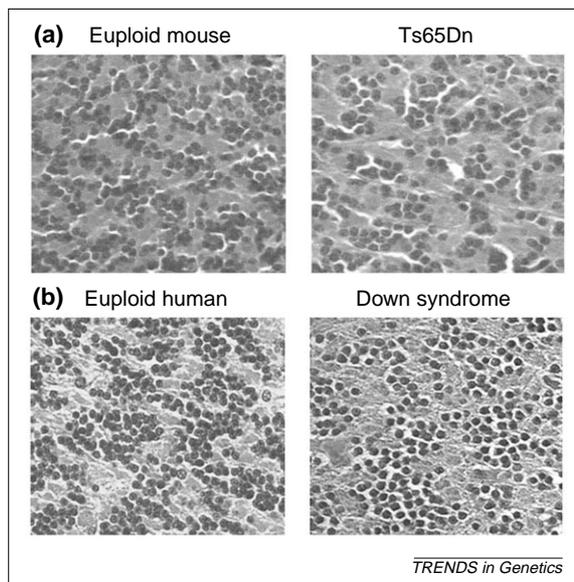


Fig. 4. Perturbation of development in the cerebellum of Ts65Dn mice correctly predicted lower granule-cell density in the internal granule layer (IGL) in trisomy 21, a previously unrecognized feature of DS (Ref. 37). (a) Darkly staining granule cells are present at lower density in the IGL of Ts65Dn as compared to euploid mice. (b) Analysis of the IGL in individuals with two or three copies of Chr 21 demonstrates the same reduced granule-density in DS as in Ts65Dn.

Phenotypes of DS in mouse models

Regulation of orthologous genes in development is likely to be similar among mammals, and therefore the primary effects of gene dosage imbalance on conserved genetic pathways are expected to be analogous in mice and humans. However, the end results are necessarily different; DS is a (variable) clinical endpoint in human development. Species differences complicate interpretation of some complex characters. Phenotypes of DS that are most likely to be recapitulated directly in mouse models include characters that occur universally in DS and that represent the end result of developmental pathways that are highly conserved across vertebrate evolution. Those phenotypes that have been assessed quantitatively in DS individuals provide a more precise basis for comparison in the different species. Two such phenotypes have been analyzed recently in Ts65Dn mice.

The characteristic flattened faces of DS patients reflect, in part, the reduced development of the underlying craniofacial skeleton (Fig. 3). Measurements of the skull in DS quantify local contributions to mid-face hypoplasia, reduced facial height and reduced bizygomatic breadth due to reduced dimensions of the maxilla, zygomatic, nasal and frontal bones³⁵. The mandible is also reduced in size, and most dimensions are observed to be more variable among those with three copies of Chr 21. These characteristics are recapitulated in the Ts65Dn mouse, as demonstrated by three-dimensional morphometric methods³⁶. Conservation of the genetic programs regulating skull development is confirmed by directly analogous effects produced by the same genetic insult in mouse and human.

A second study detected an exact parallel in maldevelopment of the cerebellum in DS and Ts65Dn mice³⁷. As in DS, the Ts65Dn cerebellum is reduced relative to the volume of the total brain. Analysis of the cellularity of different regions detected a reduction not only in size, but also in granule-cell density in the internal granule layer (IGL) in Ts65Dn mice (Fig. 4). This trait had not been examined in DS previously. When eight brains from individuals with trisomy 21 were compared with brains of age-matched euploid individuals, the same reduced granule-cell density was observed³⁷. The ability to predict quantitative endpoints of development in DS using the Ts65Dn mouse meets the most stringent requirement for an animal model. This is especially important in a model of trisomy 21, because many DS phenotypes are established during prenatal development and cannot be systematically analyzed in humans.

Conclusion

Quantitative target phenotypes that are directly comparable in effect and magnitude in humans and mice, and that result from the corresponding genetic insult, provide an experimental basis for identifying

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the Chr 21/ mouse Chr 16 genes that cause abnormal development in trisomy. Both genetic and phenotypic characterizations are necessary to determine how the initial increase in transcription causes cellular, and subsequently, systems-level changes that disrupt development. Genetic contributions can be determined when phenotypes are assessed in trisomic mice with smaller (or larger) regions that are orthologous to human Chr 21. Individual gene effects can be 'subtracted' from the trisomic background by crossing segmental trisomies with

gene-targeted mice, leaving just two copies of the tested gene. The availability of inbred mice provides a further tool for dissecting the contribution of genetic background to phenotypic variability, when the consequences of trisomy can be assessed quantitatively in different strains and the different allele combinations are at dosage imbalance. Consideration of DS as a complex disorder of development provides a complicated but realistic approach for determining the effects of gene dosage imbalance on the DS phenotype.

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