DYRK1A interacts with the REST/NRSF-SWI/SNF chromatin remodelling complex to deregulate gene clusters involved in the neuronal phenotypic traits of Down syndrome

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The molecular mechanisms that lead to the cognitive defects characteristic of Down syndrome (DS), the most frequent cause of mental retardation, have remained elusive. Here we use a transgenic DS mouse model (152F7 line) to show that DYRK1A gene dosage imbalance deregulates chromosomal clusters of genes located near neuron-restrictive silencer factor (REST/NRSF) binding sites. We found that Dyrk1a binds the SWI/SNF complex known to interact with REST/NRSF. The mutation of a REST/NRSF binding site in the promoter of the REST/NRSF target gene L1cam modifies the transcriptional effect of Dyrk1a-dosage imbalance on L1cam.

Dyrk1a dosage imbalance perturbs Rest/Nrsf levels with decreased Rest/Nrsf expression in embryonic neurons and increased expression in adult neurons. Using transcriptome analysis of embryonic brain subregions of transgenic 152F7 mouse line, we identified a coordinated deregulation of multiple genes that are responsible for dendritic growth impairment present in DS. Similarly, Dyrk1a overexpression in primary mouse cortical neurons induced severe reduction of the dendritic growth and dendritic complexity. We propose that DYRK1A overexpression-related neuronal gene deregulation via disturbance of REST/NRSF levels, and the REST/NRSF–SWI/SNF chromatin remodelling complex, significantly contributes to the neural phenotypic changes that characterize DS.

INTRODUCTION

Down syndrome (DS) (OMIN 190685) is characterized by a variety of phenotypic traits including cognitive defects (1,2). Abnormal cell-cycle kinetics during neurogenesis leading to microcephaly (3), dendritic anomalies (4) and abnormal synaptic plasticity (5) has been documented both in mouse DS models and in human DS. DYRK1A, located in the DS
critical region, is a candidate gene responsible for learning and memory impairment (6). It encodes a protein kinase whose unique genetic and biochemical properties have been evolutionarily conserved from insects to humans (7). Functional screening of 2 Mb of human chromosome 21q22.2 in transgenic mice implicated DYRK1A in the production of learning defects associated with DS (6). Cultured fetal DS neurospheres were found to have decreased transcript levels of the REST/neuron-restrictive silencer factor (NRSF) gene and downstream targets such as SCG10 (8). Furthermore, a recent study reported that DYRK1A-dosage imbalance perturbs REST/NRSF levels (9). However, the molecular mechanisms linking DYRK1A and REST/NRSF levels remain elusive. In the present study, we characterize the molecular changes induced by in vivo DYRK1A overexpression in 152F7 transgenic mice that contained a 570 kb human YAC, including the DYRK1A gene (6). We show here that DYRK1A gene dosage imbalance deregulates chromosomal clusters of genes that are located near REST/NRSF binding sites (10,11,12,13). Furthermore, we demonstrate that Dyrk1a binds the SWI/SNF complex known to interact with REST/NRSF. Using an L1camLacZ construct, we show that Dyrk1a-dosage imbalance perturbs a REST/NRSF target gene, depending on the presence or the absence of repressor
element 1 or neuron-restrictive silencer element (RE1/NRSE) site in the promoter of the gene. We also present evidence for a coordinated deregulation of multiple genes that are responsible for a key neuronal phenotypic trait that is present in DS: dendritic growth impairment during pre-natal cortex development. Manipulating Dyrk1a overexpression by adeno-viral transduction in primary mouse cortical neurons induces an increase in Rest/Nrsf transcript levels and a decrease of dendritic growth and complexity.

RESULTS

DYRK1A dosage imbalance deregulates chromosomal clusters of genes located near REST/NRSF binding sites

Transcriptome profiling was carried out on microdissected subregions of the brain (telencephalon and diencephalon–mesencephalon) from embryonic stages E11.5, E12.5 and E13.5 (Fig. 1A). We identified a total of 170 differentially expressed genes using either microarrays composed of a sub-ensemble of genes or pangenomic microarrays (Table 1; Supplementary Material, Table S1A and B). These genes are organized in chromosomal clusters and code for proteins that are involved in cell-cycle progression such as histones (14), SWI/SNF components (15,16) and for proteins that are involved in neuritic outgrowth (17), including L1cam that is encoded by a bona fide REST/NRSF gene (18) (Fig. 1B). The REST/NRSF transcription factor binds to RE1/NRSE sites that include a conserved 21 bp motif (19) (Fig. 2A). We identified 9580 putative RE1/NRSE sites conserved in human and mouse species of a total of 27 706 Ensembl mouse genes analysed using stringent phylogenetic criteria (see Supplementary Material, Table S2 and text). This number can be compared with only 186 conserved sites that were recently generated by a computer (see Supplementary Material). Sixty three of the 170 differentially expressed genes had a putative RE1/NRSE site within a 70 kb window. Furthermore, 136 of the 170 genes (76%) were located at <500 kb from a putative RE1/NRSE site. Genes that were differentially expressed in 152F7 mice appeared to be closer to putative RE1/NRSE sites than those of the whole Ensembl mouse genome (Fig. 2B). We identified 239 genes deregulated by Dyrk1a overexpression in a mouse neuroblastoma N18 cell line. Out of these 239 genes, 109 had putative RE1/NRSE sites (Supplementary Material, Table S3). They were also organized in chromosomal clusters and in the vicinity of RE1/NRSE sites when compared with randomly selected genes with 184 of 239 located at <500 kb from a putative RE1/NRSE site.

DYRK1A dosage imbalance perturbs rest levels

It is well documented that REST can act as a hub for the recruitment of multiple chromatin-modifying enzymes (20). Thus, a precise stoichiometry of Dyrk1a is expected to be required for normal Rest/Nrsf gene expression (21). Furthermore, the Rest/Nrsf gene expression depends on the RA receptor complex that includes Rest/Nrsf interactors (11). To test the hypothesis that Dyrk1a gene imbalance modifies Rest/Nrsf expression, we measured Rest/Nrsf transcript levels in brain subregions of 152F7 transgenic embryos and found an increased expression in 152F7 samples compared with controls (Fig. 3A). Similarly, overexpression of Dyrk1a in both primary embryonic cortical neurons and N18 cell line neurons increased Rest/Nrsf expression (Fig. 3B). In contrast, we found a decrease in Rest/Nrsf expression compared with controls for post-natal subregions of hippocampus (dentate gyrus, CA1 and CA3) isolated by laser-assisted microdissection (Fig. 3C and D).

DYRK1A binds the SWI/SNF complex

Based on these data, suggesting that the genes that are deregulated occur in chromosomal clusters, we investigated a possible link between DYRK1A and chromatin remodeling. Minibrain, the Drosophila orthologue of DYRK1A, was recently reported to interact with Snr1 (22), the INI1 (23) orthologue, which is a member of the SWI/SNF complex. We, therefore, studied the potential interaction of DYRK1A with the SWI/SNF complex. Using overexpression of a tagged form of Dyrk1a, we analysed immunoprecipitates and found that Dyrk1a binds the SWI/SNF complex as
evidenced by the immunodetection of two components of the core complex, Brg1 and Ini1 (Fig. 4A). REST/NRSF target genes may be deregulated in the presence of a \textit{DYRK1A} gene dosage imbalance. By comparing the expression levels of \textit{L1cam} reporter genes with and without an RE1/NRSE site (18) (Fig. 4B), we show that when \textit{Dyrk1a} was overexpressed, the level of expression of the reporter gene in the system lacking the RE1/NRSE site was significantly less than in that harbouring the RE1/NRSE site. Thus, \textit{DYRK1A} overexpression affects the transcription from the \textit{L1cam} promoter, and at least part of this effect is dependent on Rest/Nrsf.

\textbf{DYRK1A overexpression induces reduction in the dendritic growth and complexity}

As dendritic abnormalities have been reported in DS patients (6), we next studied whether \textit{Dyrk1a} overexpression may modify dendritic parameters. Therefore, we overexpressed the \textit{Dyrk1a}-enhanced green fluorescent protein (EGFP) adenoviral construct alone in primary cultures of embryonic neocortical neurons in order to demonstrate whether \textit{Dyrk1a}-dosage imbalance was sufficient to generate an abnormal dendritic phenotype. Overexpression of \textit{Dyrk1a}-EGFP induces a severe reduction of the neurite growth and complexity (Fig. 5A–D). No axonal specific staining was performed in this study. However, taking into account that neurons are polarized cells with multiple dendrites and a single axon, these results suggest that \textit{Dyrk1a} overexpression induces a decrease of dendritic growth and dendritic complexity. Moreover, we cultured pre-natal neocortical neurons from 152F7 mice \textit{ex vivo} and compared transgenic and wild-type littermate embryos on day E13.5. The same specific subcellular phenotype of reduced dendritic outgrowth and complexity was also observed in this case (Fig. 5E–G). These observations demonstrate that \textit{Dyrk1a} gene dosage imbalance is sufficient to induce the dendritic abnormalities in the DS model. Among the genes deregulated in 152F7 embryos, we identified three genes involved in axonal outgrowth (\textit{L1cam} and \textit{Dpysl3}) (18,24) and in dendrite outgrowth regulation (\textit{Elmo2}) (25), respectively (Fig. 5H). These three genes have putative RE1/NRSE sites, as indicated in Figure 5H. The functional relationship between the transcript levels for these genes and REST/NRSF expression was assessed by Q-RT-PCR. In wild-type

\begin{figure}[h]
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\caption{Differentially expressed genes are located closer to putative RE1/NRSE sites than randomly chosen genes. (A) RE1/NRSE matrices and a schematic representation of the criteria used to identify conserved human-mouse RE1/NRSE sites. (B) Positions of the 170 (152F7 versus control) and 245 differentially expressed genes (\textit{Dyrk1a} overexpression versus control) related to putative RE1/NRSE sites when compared with randomly selected genes, expressed as a cumulative probability. The statistical significance is calculated using unilateral binomial analysis and is shown for 152F7 and \textit{Dyrk1a} differentially expressed genes (the blue line indicates a probability value of 0.95).}
\end{figure}
Figure 3. Perturbation of rest levels by Dyrk1a dosage imbalance (A). Q-RT-PCR analysis shows that Dyrk1a overexpression is associated with Rest/Nrsf up-regulation in embryonic 152F7 brain subregions (T, telecephalon; C, cortex; M, mesencephalon) at E11.5, E12.5 and E13.5 (\(P < 0.01\); ***\(P < 0.0001\)). (B) Q-RT-PCR analysis shows that Dyrk1a overexpression is associated with Rest/Nrsf upregulation in Dyrk1a overexpressing primary cortical neurons and N18 (\(P < 0.01\); ***\(P < 0.0001\)). (C) Q-RT-PCR analysis shows that Dyrk1a overexpression is associated with Rest/Nrsf deregulation in the three hippocampal subregions (DG, dentate gyrus; CA3, CA1) of 152F7 (red) and control P21 (grey) mice. (\(P < 0.01\); **\(P < 0.001\)). (D) Quantitative ISH showed significant differences (***\(P < 0.0001\)) of Dyrk1a ISH intensities in the hippocampus dentate gyrus (DG), CA3 and CA1 subregions in wild-type (WT) and transgenic (152F7) P21 mouse hippocampus. Half-brain coronal 15 \(\mu\)m sections were used. The bars indicate SEM.
the level of expression of these genes, similar to that of bona fide downstream target of REST/NRSF such as Bdnf, decreased markedly with increasing NRSF transcript levels. In contrast, the inverse linear correlations between the levels of expression of REST/NRSF target genes and REST/NRSF transcripts were lost in transgenic mice.

Altogether, these results suggest that an increase of Dyrk1a is followed by an increase in Rest/Nrsf in embryonic neurons (Fig. 3A and B) and that Rest/Nrsf increase induced neurite outgrowth defects. These results are in full agreement with a previous study in which we analysed the reverse condition; the silencing of Rest/Nrsf that induced an increase in the neuritic length (26). We showed that partial silencing of Rest/Nrsf decreased the dose of Elmo2, whereas (Fig. 5H) we show here that the doses of Rest/Nrsf and Elmo2 in the mouse brains are inversely proportional. Differential regulation of the Elmo2 gene probably lies in the duality of the Rest/Nrsf function: depending on the spatio-temporal context, Rest/Nrsf can act either as a repressor or as a driver of the transcription of a particular target gene. The importance of the spatio-temporal context was fully demonstrated by analysing the L1cam-LacZ expression in transgenic lines (18).

**DISCUSSION**

A deregulation of REST/NRSF and of some REST/NRSF target genes was previously reported in neurospheres derived from DS brain (8). Furthermore, it was recently found that DYRK1A-dosage imbalance perturbs REST/NRSF levels from undifferentiated embryonic stem cells to adult brain across several DS models (9). Interestingly, both overexpression and inhibition of Dyrk1a have the same consequence of reducing Rest/Nrsf transcript levels, suggesting the deregulation of a protein complex characterized by a strict stoechiometry (9). In the present study, we show, to the best of our knowledge for the first time, that DYRK1A interacts with the REST/NRSF–SWI/SNF chromatin remodelling complex. Various studies have shown that REST/NRSF can act as a hub for the recruitment of multiple

**Figure 4.** SWI/SNF and Rest/Nrsf interactions and the regulation of L1cam (a REST/NRSF target gene) via Dyrk1a. (A) Dyrk1a interacts with a complex containing Brg1 and In1. N18 cells were transiently transfected with Dyrk1a-EGFP or control p-EGFP. N18 cells were immunoprecipitated (IP) using anti-Brg1 antibody. IP was performed with the anti-Brg1 antibody. The precipitated fractions and supernatants were then resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and analysed by western blot using anti-Brg1, anti-In1 or anti-EGFP antibody. The same IP extracts were blotted on two distinct SDS–PAGE in order to detect proteins of 80–200 and 15–80 kDa, respectively. Note the band of 115 kDa expected for the Dyrk1a-EGFP fusion protein and the 25 kDa band expected for the EGFP. Note that no cross-reaction was found with the EGFP protein. (B) The mutation of the RE1/NRSE site modifies the gene expression induced by Dyrk1a-EGFP overexpression. Relative differences are shown in Lac-Z expression levels in (i) N18 cells co-transfected with the Dyrk1a-EGFP expression vector and the L1LlacZ vector with respect to N18 cells co-transfected with the EGFP control vector and the L1LlacZ vector and (ii) in N18 cells co-transfected with the Dyrk1a-EGFP expression vector and the L1LlacZΔN vector with respect to N18 cells co-transfected with the EGFP control vector and the L1LlacZΔN vector. The significant decrease (**P < 0.0001) in the LacZ expression is consistent with an interaction between Dyrk1a and Rest/Nrsf with RE1/NRSE sites. SEM is indicated by the bars.

**Figure 5.** Defective dendritic outgrowth in pre-natal neocortical neurons overexpressing Dyrk1a and in pre-natal 152F7 neurons. Dissociated neocortical neurons from E13.5 embryos were cultured for 1 day in vitro, infected on day 1 and fixed on day 2. (A) Schematic representation of the adenoviral construct overexpressing Dyrk1a. (B and C) Neurite length and branching number are smaller in Dyrk1a-EGFP-transduced primary neurons when compared with controls (EGFP-transduced neurons). Confocal images of cultured neurons infected with adenoviral GEF control vector (B) or adenoviral Dyrk1a-EGFP vector (C1–C3) visualized by GFP fluorescence (green) and stained with DAPI (blue) to localize nuclei. Scale bar = 10 μm. (D) Quantitative analysis of GFP-labelling for mean neurite number, mean neurite length and mean branch number in GFP control and Dyrk1a-EGFP transductions. The mean neurite number was not modified by Dyrk1a overexpression, whereas mean neurite length and mean branch number were significantly lower (**P < 0.001 and ***P < 0.0001), with Dyrk1a gene dosage imbalance. The bars indicate the SEM from 25 neurons in three independent experiments. (E and F) Dissociated cultures of embryonic day 13.5 neurons from wild-type (E: +/+ ) and transgenic (F: 152F7/+) mice. MAP2 immunofluorescence (green) is overlaid with DAPI nuclear counter-staining (blue). (G) Mean branching length and mean branch number are decreased in transgenic cortical neuron cultures. Quantitative analysis of MAP2-labelled dendrites shows a significant difference in mean dendrite length and mean branch number in wild-type (WT) and 152F7 transgenic neurons (**P < 0.0001). The bars indicate the SEM of 25 neurons in three independent experiments. Scale bar = 10 μm. (H) Gene transcript levels of L1cam, Dpy30, Elmo2 and Bdnf are correlated with Nrsf transcript levels under control conditions. This correlation is lost in transgenic samples. Top: a schematic representation of the L1cam, Dpy30, Elmo2 and Bdnf genes with RE1/NRSE site positions (black bars), exons (red), untranslated region (dark blue), the 5’ and 3’ regions (light blue) and promoters (orange). Bottom: the correlation of the gene transcript level with the Nrsf transcript level is shown. Circles, triangles and squares indicate E12.5, E13.5 and E11.5, respectively (red for controls and dark blue for 152F7).
chromatin-modifying enzymes that interact with the SWI/SNF chromatin remodelling complex (20). This complex contains a single molecule of either Brm or BRG1 as the ATPase catalytic subunit with approximately 10 protein components. The levels of expression of the core SWI/SNF subunits, including BRG1/Brm, BAF155, BAF170, BAF60, hSNF5/Ini1 and BAF57, are stoichiometric, with few to no unbound molecules in the cell (21). Furthermore, the SWI/SNF complex forms a larger complex with neuron-restrictive silencer factor (NRSF) and its corepressors, mSin3A and CoREST (16). We demonstrate here that REST/NRSF binds the SWI/SNF complex as evidenced by the immunodetection of Brg1 and Ini1 which are two components of the SWI/SNF core complex. The SWI/SNF protein that directly interacts with Dyrk1a was not determined in this study. However, as Minibrain, the drosophila ortholog of DYRK1A, was recently reported to interact with Snr1 (27), the INI1 (23) ortholog, one can speculate that Ini1 is the SWI/SNF direct interactor of Dyrk1a.

We also demonstrate in this study that the genes that are found to be deregulated in the brains of 152F7 mice are located in clusters near REST/NRSF binding sites. This result is in full agreement with the report of Lunyak et al. (2002) that REST/NRSF regulates gene expression via an associated corepressor, CoREST, that serves as a functional molecular beacon for the recruitment of molecular machinery and imposes silencing across a chromosomal interval, including transcriptional units that do not themselves contain REST/NRSF response elements (27). Furthermore, we demonstrate that Dyrk1a dosage imbalance and REST/NRSF interact to affect gene expression via a bona fide downstream target of the REST/NRSF, L1-Cam transgene.

The multiprotein complex of SWI/SNF requires strict stoichiometry to function properly (21). The SWI/SNF complex forms a larger complex with REST/NRSF and its corepressors, mSin3A and CoREST (16). Furthermore, it was recently reported that DYRK1A locus is the strongest expression quantitative trait locus for the REST/NRSF level in the human genome and that both over- and under-expressions of DYRK1A affect REST/NRSF levels (9). Putting these data together, it is tempting to speculate that the participation of DYRK1A in the complex dependent on strict stoichiometry, such as the REST/NRSF–SWI/SNF complex, might explain this phenomenon.

Both in 152F7 brain subregions and in Dyrk1a overexpressing cortical neurons and N18 cell line neurons, we found that transgenic neurons have an increased expression of Rest/Nrsf when compared with controls. In contrast, we observed a decreased expression of Rest/Nrsf in hippocampal subregions of 152F7 juvenile mice compared with controls. From these data, we can speculate that it is the chromatin status (which is expected to be different in embryonic stem cells, embryonic and adult neurons) (11) that determines the changes in the Rest/Nrsf expression. Therefore, only a precise Dyrk1a dosage allows normal Rest/Nrsf gene expression.

In both triallelic Dyrk1a mice (28) and Dyrk1a+/− mutants (29), the pyramidal cells that constitute 70% of the neurons in the cerebral cortex have smaller dendritic arbors, shorter basal dendrites and fewer dendritic spines than normal. These studies indicate that these dendritic parameters are sensitive to Dyrk1a gene dosage. We show here that cultured cortical neurons from the 152F7 model have smaller neurite length and less dendritic complexity. Overexpression of Dyrk1a in primary mouse cortical neurons is sufficient to induce similar changes, indicating that Dyrk1a dosage imbalance is sufficient to impair dendritic length and complexity. Taking into account that DYRK1A interacts with the REST/NRSF–SWI/SNF chromatin remodelling complex, as demonstrated in this study, this dendritic phenotype can be induced by transcriptional changes of the genes involved in the REST/NRSF–SWI/SNF complex and in REST/NRSF-dependent genes that encode proteins involved in neuritic length and dendritic spine functions. Modification of dendritic length was reported in Drosophila by changes in the dosage of genes involved in the SWI/SNF complex (30). Furthermore, components of the SWI/SNF complex are encoded by bona fide downstream targets of REST/NRSF, as demonstrated in the case of SMARCE1 (27). In the present study, we also found that dosage imbalance of genes involved in neuritic growth such as L1-Cam, Dpysl3, Elmo2 and Bdnf induces abnormal neuritic growth of mouse cortical neurons. Further work will be required to identify the respective contributions of REST/NRSF target genes encoding SWI/SNF components and REST/NRSF target genes encoding proteins involved in the neuritic growth in this key phenotype.

Altogether, our data indicate that DYRK1A gene dosage imbalance induces an SWI/SNF-linked deregulation of gene clusters involved in the neuronal phenotypic traits of DS and points to possible new therapeutic approaches in DS patients.

MATERIALS AND METHODS

Animals and genotyping

We used wild-type mice of the FVB strain and the 152F7 transgenic line (6). Genotypes were determined using genomic DNA extracted from skeletal muscle fragments and the PCR protocol and primers as described previously (6).

Dissection and laser-assisted microdissection

Embryonic brain subregions were dissected as shown in Figure 1A. The left and right hippocampus was microdissected from genotyped P21 mouse brains using a laser-assisted capture microscope (Leica ASLMD instrument) with Leica polyethylene naphthalate membrane slides. See Supplementary Material.

Total RNA preparation, transcriptome profiling and quantitative real-time PCR (Q-RT-PCR) analysis

RNA preparation, transcriptome profiling using either microarrays containing a subensemble of genes or Agilent mouse pangenomic microrrays and Q-RT-PCR are described in Supplementary Material. Q-RT-PCR results are expressed in arbitrary units.
Identification of putative REST/NRSF-dependent genes

REST analysis was carried out with programs from the Genomatix Suite available online. GemLauncher and MatInspector software programs were used with a specific matrix in which values ≥0.60 were used for core similarity and ≥0.77 for matrix similarity. We used matrices of the ‘NRSF’ family. The NRSE site had to be conserved in two species (human and mouse) over 10 kb. A genomic region consisting of the gene, 70 kb upstream of the transcription start site and 70 kb downstream of the stop codon, was analysed. One hundred thousand randomly generated 5 kb mouse and human sequences yielded 186 conserved RE1/NRSE sites using the criteria described earlier.

NCBI and ENSEMBL databases were used to identify the function of genes and to map RE1/NRSE sites in genomic sequences.

Primary cell culture, cell lines, transfection and neurite analysis

E13.5 mouse telencephalic neurons were dissociated enzymatically, cultured, transfected after 1 day in culture and analysed on day 2. Primary neurons were transfected with constructs using LipofectAMINE (Invitrogen), and the N18 cell line was infected with a Dyrk1a adenovirus construct as described in Supplementary Material.

In situ hybridization (ISH) and quantitative analysis

We used Dyrk1a probes for quantitative ISH as described in Supplementary Material.

Immunocytochemistry and western blot analysis

These approaches are described in Supplementary Material.

Statistical methods

The expression ratio was computed as the ratio of the median intensity values for each spot. Data were then normalized using a lowess correction (31). Significantly differentially expressed genes were defined on the basis of the fold changes, the mean log intensities and the associated P-values (see Supplementary Material).

Regulatory network exploration

Networks of genes were defined as in Calvano et al. (32) using Ingenuity pathways analysis [http://www.ingenuity.com].

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Conflict of Interest statement. None declared.

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ACCESSION NUMBERS

Microarray data are deposited in GEO database (GSE 14021; GSE 14030; superseries GSE 14105; GSE 14072).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.


