Chapter 8

Molecular Pathways of Down Syndrome Critical Region Genes

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Identification and annotation of the DSCR

Down syndrome (DS) is a very complex disorder that requires, even more than other human genetics diseases, a "system level" understanding [1,2], both under the clinical and under the molecular genetics perspectives. Under the clinical point of view, all individuals affected by Down syndrome are characterized by learning disabilities, distinctive facial features, and low muscle tone (hypotonia) in early infancy. However, in most cases the clinical picture is complicated by additional problems, such as heart defects, leukemia, and early-onset Alzheimer's disease [3,4]. The degree to which an individual is affected by these characteristics varies from mild to severe. After the pioneering description by J.L. Down in 1866, almost one century was needed to decipher the etiology of the syndrome. The work of Lejeune proved that DS was caused by an extra copy of chromosome 21 (HSA21) [5], thus providing the first evidence for a genetic basis of intellectual disability. The main implication of this seminal discovery is that the complex phenotype seen in DS patients [6] must be caused by overdosage of HSA21 genes. However, it also raised the outstanding questions of whether one or few HSA21 genes may play a dominant role in the syndrome and whether specific HSA21 genes could contribute to specific phenotypic tracts. Answering these questions is still of paramount importance, because the identification of one or few 'dominant' molecular players could pave the road for the development of targeted therapeutic approaches. The development of molecular karyotyping has provided strong support to the view that a restricted region of HSA21, commonly referred to as Down Syndrome Crtitical Region (DSCR) might be responsible for the different phenotypes that characterize DS. In 1976 Poissonnier and coworkers, by using chromosome staining methods, found that one DS patient not possessing an extra HSA21 had only a partial trisomy, involving 21q22.1 and 21q22.2 bands [7]. Afterwards, it turned out

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that partial trisomies are responsible for approximately 1% of DS cases [8,9]. These patients show variable phenotypes, depending on the extension of the triplicated region. Therefore, partial trisomies of genes carried by chromosome 21 have been extremely valuable in investigating the involvement in DS. The analysis of 10 partial trisomy patients, [10] suggested that two regions of chromosome 21 were linked to most of the Jackson signs [3], including cognitive disorders. These regions, referred to has DCR-1 and DCR-2, respectively, encompassed the 21q22.2 band and were located around the D21S55 Site Targeted Sequence (STS) and between D21S55 and the MX1 gene, respectively. Korenberg and coworkers studied a different population and observed that the proximal and distal regions of the 21q arm were also associated with the full DS phenotype [11]. Although these studies confirmed the strong association of DS phenotypes with the DCR-1 region, they also suggested that DS is a contiguous gene syndrome, arguing against a single DS chromosomal region responsible for most of the DS phenotypic features [11]. More recently, an additional causal link of the region located between D21S17 and ETS2 to clinical features of DS was confirmed through lattice analysis [12]. Although the notion of a DSCR has gained wide acceptance in DS research, it must be underscored that some of the data that support it remain controversial and that its existence has recently come under considerable question. Indeed, a detailed study of segmental trisomy 21 in DS subjects, performed by using array comparative genome hybridization (GCH), excludes the implication of a single but rather suggest that multiple regions of HSA21 contribute to many of the phenotypes of DS, including intellectual disability DSCR [13]. Despite these apparent inconsistencies, we think that, in practical terms, the crucial point is not to prove whether one or more "critical region" exist, but rather to understand which dosage-sensitive genes contribute to specific DS phenotypes. Indeed, it is quite clear that the classical "reductionist" approach of identifying one or few master genes, which has been very successful in the case of Mendelian disorders, is not appropriate to unravel the extremely more complicated case of DS. In this case, the overall phenotype is certainly produced by the combined action of several genes, causing complex rearrangements of different molecular networks [14]. The relevance of the mentioned studies has been to restrict the list of HSA21 genes that may contribute more significantly to the clinical manifestations.

For these motivations, in Tables 1 and 2 we adopt an inclusive definition of the DSCR, which extends from the RCAN1 gene to the MX1 gene. This definition takes into account not only the putative borders that have been identified in the mentioned studies, but also the fact that the RCAN1 gene as been commonly considered as part of the DSCR, even though a precise mapping on the current release of the human genome sequence (HG19) would locate it outside the centromeric border defined by [12]. Obviously, the usefulness of this information will strongly depend on the degree of functional characterization of the genes comprised in the interval. Under this respect, as it is generally true for the human genome, it must be recognized that our knowledge is still quite limited.

HSA21 was one of the first human chromosomes to be fully sequenced [15]. Nevertheless, the list of the possible functional sequences located in the DSCR has progressively changed, not only for the uncertainty of defining precise borders, but especially for the changes in the current view of what a human gene is. Obviously, the initial emphasis has been to identify the protein-

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coding sequences, whose number is approximately of 40, on the basis of a comprehensive definition of the DSCR and of the present annotation of the human genome (Table 1). However, systematic studies performed in the last few years revealed that many genomic sequences that have been initially considered as "junk DNA", are endowed with extremely relevant functional potential [16]. Indeed, genome-wide interrogations have revealed that a large majority of the human genome is transcribed and that a significant proportion of transcripts appears to be non-protein coding (ncRNA). Although it is well recognized that some ncRNAs play essential enzymatic activities in translation, splicing and ribosome biogenesis, the functions of most ncRNAs are still unknown. It is now believed that they could participate in complex regulatory circuits responsible for the fine-tuning of gene expression at both the transcriptional and post-transcriptional levels [16]. The best known ncRNAs are miRNAs, ~22 nucleotide-long molecules that mediate post-transcriptional gene silencing by binding complementary sequences located in the 3′ UTR of the mRNAs. Long intergenic ncRNAs (lincRNA) represent a less characterized but more abundant and heterogeneous class, and comprise transcripts longer than 200 nt involved in many biological processes, including transcriptional control, epigenetic modification and post-transcriptional control on mRNAs [16]. A recent discovery demonstrated that both mRNAs and ncRNAs can deploy their functions by contributing to an extensive RNA-RNA interaction network, based on the competition of these molecules for the binding of shared miRNAs (the ceRNA hypothesis) [17-20]. Importantly, transcribed pseudogenes could also be involved in these complex regulatory interactions [21]. In light of this growing complexity, we think that the presence of many 'non conventional' sequences within the DSCR should be taken into consideration when exploring the molecular consequences of an increased dosage of this region. We provide an updated list of them in Table 2.

DCR	Gene Name	Entrez Gene ID	Main molecular function	Essential references	Expression in adult brain
1	RCAN1	1827	CaN inhibitor	See main text	Yes
1	CLIC6	54102	Channel	See main text	Yes
1	RUNX1	861	Transcription factor	See main text	Yes
1	SETD4	54093	Unknown	No information	Yes
1	CBR1	873	Enzyme	[165]	Yes
1	CBR3	874	Enzyme	[165]	
1	DOPEY2	9980	Unknown	[166]	Yes
1	MORC3	23515	RNA-binding	[167]	
1	CHAF1B	8208	Chromatin assembly	[168]	Yes
1	CLDN14	23562	Tight junctions component	[169]	
1	SIM2	6493	Transcription factor	See main text	Yes
1	HLCS	3141	Enzyme	[170]	Yes

DCR	Gene Name	Entrez Gene ID	Main molecular function	Essential references	Expression in adult brain
1	DSCR6	53820	Unknown	[171]	Yes
1	PIGP	51227	Enzyme	[172]	Yes
1	TTC3	7267	E3 ligase	See main text	Yes
1	DSCR3	10311	Unknown	[173]	Yes
1	DYRK1A	1859	Protein kinase	See main text	Yes
1-2	KCNJ6	3763	Channel	See main text	
1-2	DSCR4	10281	Unknown	[174]	
1-2	DSCR8	84677	Unknown	[175]	
1-2	KCNJ15	3772	Channel	[176]	
1-2	ERG	2078	Transcription factor	See main text	Yes
1-2	ETS2	2114	Transcription factor	See main text	Yes
2	PSMG1	8624	Chaperone	[177]	Yes
2	BRWD1	54014	Transcription factor	See main text	Yes
2	HMGN1	3150	Transcription factor	See main text	Yes
2	WRB	7485	Protein trafficking	[178]	Yes
2	LCA5L	150082	Ciliary protein	[179]	
2	SH3BGR	6450	Unknown	No information	Yes
2	B3GALT5	10317	Enzyme	[180]	
2	C21orf88	114041	Unknown	No information	Yes
2	IGSF5	150084	Adhesion molecule	[181]	
2	PCP4	5121	Unknown	[182]	Yes
2	DSCAM	1826	Adhesion molecule	[183]	
2	BACE2	25825	Protease	See main text	Yes
2	FAM3B	54097	Cytokine	[184]	
2	MX2	4600	Unknown	[185]	
2	MX1	4599	Unknown	[185]	Yes

Table 1. Summary of the protein-coding genes contained by the DSCR. The first column indicates whether the genes belong to the DCR-1, to the DCR-2 or to the overlap region. The evidence for expression in adult brain is derived from the EVOC data [186] contained in the Ensembl genome browser. Genes are given in their physical order, starting from the more centromeric sequence.

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DCR	Gene Name	Ensembl ID	Entrez Gene ID	HSA21 coordinates	Gene Biotype	Evidence of expression (EST)
1	LINC00160	ENSG00000230978	54064	36096105 - 36109478	lincRNA	
1	AP000330.8	ENSG00000234380	100506385	36118054 - 36157183	Antisense	
1	AF015262.2	ENSG00000234703		36508935 - 36511519	lincRNA	+
1	RPL34P3	ENSG00000223671	54026	36844395 - 36844730	Pseudogene	+
1	EZH2P1	ENSG00000231300	266693	36972030 - 36972320	Pseudogene	
1	AF015720.3	ENSG00000230794		37085437 - 37105240	processed transcript	+
1	MIR802	ENSG00000211590	768219	37093013 - 37093106	miRNA	
1	RPS20P1	ENSG00000229761	54025	37097045 - 37097398	Pseudogene	
1	PPP1R2P2	ENSG00000234008	54036	37259493 - 37260105	Pseudogene	
1	AP000688.8	ENSG00000231106		37377636 - 37379899	lincRNA	+
1	RPL23AP3	ENSG00000214914	8489	37388377 - 37388844	Pseudogene	++
1	RIMKLBP1	ENSG00000189089	54031	37422512 - 37423675	Pseudogene	
1	AP000688.11	ENSG00000236677		37432730 - 37436706	Antisense	+
1	U6	ENSG00000200213	1497008	37438843 - 37438950	snRNA	
1	AP000688.14	ENSG00000230212	100133286	37441940 - 37498938	sense intronic	
1	AP000688.15	ENSG00000236119		37455157 - 37462712	lincRNA	+
1	AP000688.29	ENSG00000233393		37477179 - 37481988	lincRNA	+
1	MEMO1P1	ENSG00000226054	728556	37502669 - 37504208	Pseudogene	
1	CBR3-AS1	ENSG00000236830	100506428	37504065 - 37528605	lincRNA	
1	RPS9P1	ENSG00000214889	8410	37504748 - 37505330	Pseudogene	
1	RPL3P1	ENSG00000228149	8488	37541268 - 37542478	Pseudogene	
1	Metazoa_SRP	ENSG00000265882		37585858 - 37586136	miscellaneous RNA	
1	snoU13	ENSG00000238851		37630724 - 37630829	snoRNA	
1	SRSF9P1	ENSG00000214867	54021	37667471 - 37668000	Pseudogene	
1	AP000692.9	ENSG00000228107		37732928 - 37734338	processed transcript	+
1	ATP5J2LP	ENSG00000224421	54100	37761176 - 37761410	Pseudogene	
1	AP000695.6	ENSG00000230479		37802658 - 37853368	Antisense	+
1	AP000695.4	ENSG00000233818		37818029 - 37904706	Antisense	
1	PSMD4P1	ENSG00000223741	54035	37858281 - 37859709	Pseudogene	+
1	AP000696.2	ENSG00000231324		38004979 - 38009331	lincRNA	++
1	AP000697.6	ENSG00000224269		38071073 - 38073864	Antisense	+
1	HLCS-IT1	ENSG00000237646	100874294	38176285 - 38178585	sense intronic	++
1	RN5S491	ENSG00000199806	100873733	38224211 - 38224328	rRNA	
1	AP000704.5	ENSG00000224790		38338812 - 38344128	lincRNA	++
1	Y_RNA	ENSG00000207416		38359039 - 38359151	miscellaneous RNA	
1	MRPL20P1	ENSG00000215734	359737	38366943 - 38367375	Pseudogene	
1	U6	ENSG00000212136	1497008	38417830 - 38417936	snRNA	
1	TTC3-AS1	ENSG00000228677	100874006	38559967 - 38566227	Antisense	++
1	DSCR9	ENSG00000230366	257203	38580804 - 38594037	lincRNA	

DCR	Gene Name	Ensembl ID	Entrez Gene ID	HSA21 coordinates	Gene Biotype	Evidence of expression (EST)
1	Metazoa_SRP	ENSG00000263969		38587906 - 38588202	miscellaneous RNA	
1	AP001432.14	ENSG00000242553		38593720 - 38610045	lincRNA	+
1-2	KCNJ6-IT1	ENSG00000233213	100874329	39089405 - 39091872	sense intronic	+
1-2	AP001427.1	ENSG00000264691		39334968 - 39335068	miRNA	+
1-2	DSCR4-IT1	ENSG00000223608	100874327	39378846 - 39382920	sense intronic	+
1-2	snoU13	ENSG00000238581		39559551 - 39559656	snoRNA	
1-2	DSCR10	ENSG00000233316	259234	39578250 - 39580738	lincRNA	
1-2	AP001434.2	ENSG00000226012		39609139 - 39610123	lincRNA	+
1-2	SPATA20P1	ENSG00000231123	100874060	39610149 - 39610586	Pseudogene	
1-2	AP001422.3	ENSG00000231231		39695557 - 39705343	lincRNA	++
1-2	SNRPGP13	ENSG00000231480	100874428	39874369 - 39874545	Pseudogene	
1-2	LINC00114	ENSG00000223806	400866	40110825 - 40140898	lincRNA	
2	AP001042.1	ENSG00000229986		40218171 - 40220568	lincRNA	
2	AF064858.6	ENSG00000205622	400867	40249215 - 40328392	lincRNA	
2	AP001043.1	ENSG00000229925		40260696 - 40275829	processed transcript	+
2	SNORA62	ENSG00000252384		40266709 - 40266791	snoRNA	
2	RPSAP64	ENSG00000227721		40266841 - 40267176	Pseudogene	
2	AP001044.2	ENSG00000234035		40285093 - 40287072	lincRNA	+
2	AF064858.7	ENSG00000232837		40346355 - 40349700	lincRNA	+
2	AF064858.8	ENSG00000235888		40360633 - 40378079	lincRNA	+
2	AF064858.11	ENSG00000237721		40378574 - 40383255	lincRNA	+
2	AF064858.10	ENSG00000237609		40400461 - 40401053	lincRNA	+
2	RPL23AP12	ENSG00000228861	391282	40499494 - 40499966	Pseudogene	+
2	PCBP2P1	ENSG00000235701	54040	40543056 - 40544032	Pseudogene	
2	TIMM9P2	ENSG00000232608	100862727	40588550 - 40589432	Pseudogene	
2	BRWD1-IT1	ENSG00000237373		40589019 - 40591731	processed transcript	+
2	METTL21AP1	ENSG00000229623	100421629	40607312 - 40607946	Pseudogene	
2	BRWD1-AS1	ENSG00000238141	100874093	40687633 - 40695144	Antisense	+
2	Y_RNA	ENSG00000252915		40716463 - 40716554	miscellaneous RNA	
2	snoU13	ENSG00000238556		40717300 - 40717383	snoRNA	
2	RNF6P1	ENSG00000227406	100420924	40745689 - 40748992	Pseudogene	
2	MYL6P2	ENSG00000235808	100431168	40860253 - 40860686	Pseudogene	++
2	RPS26P4	ENSG00000228349	692146	40863470 - 40863824	Pseudogene	+
2	AF121897.4	ENSG00000235012		40897510 - 40901782	Pseudogene	
2	AF064860.5	ENSG00000225330		41002198 - 41098012	processed transcript	+
2	AF064860.7	ENSG00000231713		41099682 - 41102607	lincRNA	+
2	MIR4760	ENSG00000263973	100616148	41584279 - 41584358	miRNA	
2	DSCAM-AS1	ENSG00000235123	100506492	41755010 - 41757285	Antisense	
2	SNORA51	ENSG00000207147		41885071 - 41885206	snoRNA	

DCR	Gene Name	Ensembl ID	Entrez Gene ID	HSA21 coordinates	Gene Biotype	Evidence of expression (EST)
2	AF064863.1	ENSG00000221396		41949429 - 41949538	miRNA	+
2	DSCAM-IT1	ENSG00000233756	100874326	41987304 - 42002693	sense intronic	++
2	YRDCP3	ENSG00000230859	100861429	42235920 - 42236399	Pseudogene	
2	LINC00323	ENSG00000226496	284835	42513427 - 42520060	Antisense	
2	MIR3197	ENSG00000263681	100423023	42539484 - 42539556	miRNA	
2	AL773572.7	ENSG00000225745		42548249 - 42558715	processed transcript	++
2	BACE2-IT1	ENSG00000224388	282569	42552024 - 42552553	Antisense	+
2	AP001610.5	ENSG00000228318		42813321 - 42814669	Antisense	+

Table 2. Summary of the non-protein-coding elements contained by the DSCR. The first column indicates whether the genes belong to the DCR-1, to the DCR-2 or to the overlap region. Elements are given in their physical order, starting from the more centromeric sequence. Genomic coordinates refer to the HG19 version of the human genome sequence. The evidence for expression is derived from the ESTs linked to the Ensembl genome browser. + = at least one EST sequence supporting the Ensemble prediction. ++ prediction supported by several EST sequences.

2. Functional analysis of the DSCR through mouse models

Animal models are essential to understand the molecular pathogenesis of DS. Moreover, although none of them can faithfully mimic the human situation, they are crucial for the preclinical development of new therapeutic strategies. The availability of sophisticated tools for mouse genetics and the conserved synteny between mouse chromosome 16 (MMU16) and HSA21 have provided the basis for the development of many mouse models of DS, allowing to test the critical region concept and to perform a genetic dissection of the complex DS phenotype.

The first mouse models have been obtained by studying the effects of partial trisomies of MMU16 derived from Robertsonian translocations. These mice live until adulthood and show many clinical phenotypes similar to DS patients, in particular the neuropathological and neurobiological alterations, including learning and behavioral abnormalities [22-25]. The most studied mouse model for DS is theTs65Dn mouse, which possesses an extra copy of the distal 13 Mbp part of MMU16, including ~ 104 mouse genes orthologous to those on HSA21 [23]. These mice show a number of developmental and functional parallels with DS, including craniofacial abnormalities and behavioural changes [26-32]. Moreover, they show alterations in the structure of dendritic spines in cortex and hippocampus [33] and reduced long-term potentiation (LTP) in the hippocampus and fascia dentata (FD) [34-36].

Ts1Cje mice, which are trisomic for a shorter but fully overlapping segment of MMU16 (~81 genes), show similar changes, usually to a lesser degree [24,25,37,38]. Comparison of the behavioral performances of the Ts1Cje and Ts65Dn showed that the learning deficits of Ts1Cje mice are similar to those of Ts65Dn. The data obtained from these models strongly supported the concept of DSCR, because they indicated that conserved genes are capable to influence

cognition through their dosage lie in a region spanning from Sod1 to Mx1, which contains the mouse counterpart of the human DCR-1.

Probably, the most elegant studies that have addressed the role of the mouse genome region syntenic to the human DSCR are those undertaken by Roger H. Reeves and coworkers. Using chromosome engineering, this group has generated a mouse line referred to as Ts1Rhr, trisomic for a segment closely corresponding to the DCR-1 region, as defined by [10] and [11] and including 33 genes [39]. Moreover, they obtained the corresponding deletion, resulting in the monosomic line Ms1Rhr. Interestingly, the first results produced by the analysis of these models did not confirm strongly the DSCR hypothesis. Indeed, the craniofacial dysmorphologies of Ts1Rhr are less marked and distinct from those detected in Ts65Dn and Ts1Cje mice [39]. Furthermore, no differences were initially detected between Ts1Rhr and normal controls in the Morris water maze, in the induction of LTP in the hippocampal CA1 Region and in the hippocampal and in cerebellum volume [39-41]. These results seemed to suggest that triplication of the Ts1Rhr segment is not sufficient to produce these correlates of DS phenotypes. However, the intercross of the monosomic line Ms1Rhr with the Ds65Dn line, which restored in a disomic condition for DCR-1 genes, generated mice showing normal performances in the Morris water maze, indicating that trisomy of DCR-1 is necessary for these cognitive phenotypes [41]. Importantly, a more recent report established that, if the Ts1Rhr mutation is analyzed on the same genetic background of the Ts65Dn and Ts1Cje mice and with more stringent tests, important cognitive and synaptic neurobiological phenotypes can be detected [42]. In particular, 20 of 48 phenotypes, many of which are shared with Ts65Dn mice, distinguished Ts1Rhr animals from their 2N controls. In addition to the genetic background difference, it must be noticed that the task used in this work was less stressful and more sensitive than the water maze, which may further account for the initial discrepancy [42]. These phenotypes were correlated with changes in synaptic density and in dendritic spine morphology, further indicating that DCR-1 genes strongly contribute to these abnormalities [42]. In conclusion, taken together, these results provide strong support to the view that increased dosage of DCR1 genes is necessary and sufficient to confer to mice some of the neurobiological phenotypes characteristic of DS.

The use of mouse genetic tools has allowed the production of even more restricted models, addressing the role of specific subregions of the human or mouse DSCR, or even the role of single DSCR genes. For instance, the isolation from the DSCR of huge genomic clones maintained as Yeast Artificial Chromosomes (YAC) or as Bacterial Artificial Chromosomes (BAC) and their microinjection in mouse oocytes has allowed the generation of transgenic lines covering the entire length of the human DSCR [43-45]. The characterization of these mice has shown that the approach can be very useful to study the function of specific genes. However, it became also clear that this strategy is of limited usefulness to establish genes contribution to the phenotype. For instance, BAC transgenesis allowed the production of a mouse line carrying a single extra copy of the DYRK1A gene [46]. Interestingly, these mice showed impaired cognitive behaviours, but they were characterized by increased hippocampal LTP, while all the models discussed above show depressed hippocampal LTP [46]. The same conclusion applies even better to the models obtained through classical transgenesis ap-

proaches, in which a single human or mouse gene is inserted in the mouse genome in the form of a cDNA driven by a non-physiological promoter [47].

On the other hand, the combination of gene targeting technologies with the "classical" DS model discussed above allows a subtractive strategy, providing the most stringent test to address the relevance of single genes for the overall phenotype. Indeed, once a null allele for a DSCR gene is available, a compound mutant can be generated, carrying the specific mutation in a trisomic background. The subtractive approach allowed to detect a significant rescue of the phenotype in the case of some DS-related genes, belonging to the DSCR as in the case of DSCR1[48], Olig1 and Olig2 [49], or even external to it, as in the case of APP [50,51].

3. Functional role of DSCR genes in DS intellectual disability: Towards the identification of drugable pathways

In the following section we will summarize the most relevant functional information available on DSCR genes, trying to especially underscore their implication in molecular networks relevant to intellectual disability. As it is obvious from the previous sections, this discussion will involve not only genes that strictly belong to the DSCR, but also their interactions with other HSC21 genes, whose functional involvement is supported by abundant literature. In particular, we will try to discuss as much as possible the single DSCR genes on the basis of their common features. The essential information about genes not included in this section is reported in Tables 1 and 2. While deploying this summary, we will also provide a perspective of how this information can be useful for progressing towards the development of new therapeutic strategies that may take into account the complex nature of DS.

3.1. Pathogenesis of intellectual disability in DS

In order to evaluate the possible degree of functional involvement for specific genes, it is very important to briefly analyze the principal biological processes that have been to cognitive impairment in the DS. To this regard, studies performed both in humans and in animal models have shown that trisomy 21 leads to an unbalance of key cellular events, such as neuronal cell proliferation and differentiation, which can be detected during development and post-natal life using morphological methods [52,53]. Importantly, these defects may coexist with or may be causally related to functional deficits, that can be revealed using sophisticated physiological methods [52,53]. Reduced neurons number is found in cortex, hippocampus and cerebellum of DS brain and are accompanied by impaired neuronal function. Brain hypocellularity is acquired during early developmental stages and is paralleled by impaired cognitive development leading to intellectual disabilities. Further deterioration of cognitive abilities occurs in adolescence and adulthood, possibly due to degenerative mechanisms [28]. Although the syndrome invariably results in AD-like neuropathology, the actual onset of dementia is quite variable. The availability of genetic models of trisomy 21 has been instrumental in gaining insights into the pathogenic mechanisms leading to DS cognitive disability. Morphological abnormalities of neuronal dendritic com-

partment are paralleled by functional electrophysiological deficits and impairment of learning and memory, pointing to the existence of defective neural network connectivity and faulty neuronal communication as primary determinants of DS cognitive disabilities [34-38,42,54]. Such pathological scenario arises from a combination of neurodevelopmental abnormalities and neurodegenerative processes. Addressing which processes are irreversible and which ones can be prevented or reverted by manipulating genes and pathways is of paramount importance for the development of new therapeutic strategies. Although the crossover between neurogenesis dysfunction and neurodegeneration is still poorly understood, it is likely that common pathways differentially affect various cellular functions during development and aging. Thus, the developmental aspects are fundamental in defining the most important functional consequences of the genetic imbalance in DS at the cognitive level. However, the IQ of DS patients decreases in the first decade of life, indicating that the maturation of central nervous system is compromised [8]. Indeed, on one side, different observations suggest that neurogenesis impairment starting from the earliest stages of development may underlie the widespread brain atrophy of DS, the delayed and disorganized lamination in the DS fetal cortex [55] and hippocampal hypoplasia [56]. On the other, postmortem studies show that DS patients start their lives with an apparently normal neuronal architecture that progressively degenerates. During the peak period of dendritic growth and differentiation (2.5 months old infants), no significant differences were detected in dendritic differentiation between euploid and DS cases in pyramidal neurons of prefrontal cortex [57]. Similarly, DS infants younger than 6 months showed greater dendritic branching and length than normal infants [58] [59] in contrast to the reduced number of dendrites and degenerative changes in DS children older than two years [60].

3.2. Transcription factors and co-factors encoded by the DSCR

The DSCR contains 6 genes encoding for transcription factors (Table 1), which are likely to play crucial roles in determining DS phenotypes, considering their potential to affect many cellular networks. Two of them, ERG and ETS2 belong to the erythroblast transformation-specific (ETS) family. Members of this family are key regulators of embryonic development, cell proliferation, differentiation, angiogenesis, inflammation, and apoptosis [61]. ERG is required for vascular cell remodeling and hematopoesis [62,63], while ETS2 has been linked to thymocytes development and apoptosis [64]. Together with RUNX1 [65], these proteins are very likely to contribute to the hematological abnormalities that characterize DS, but not to contribute significantly to ID. In contrast, BRWD1 and HGMN1 are two proteins highly expressed in brain that is involved in chromatin-remodeling [66,67]. Importantly, HGMN1 has been found to regulate the expression of the ID gene MeCP2 [67]. Under the same perspective, another interesting candidate is the bHLH factor SIM2 that together with its paralog SIM1 is the homolog of *Drosophila* single-minded (sim) gene. The *Drosophila* sim gene encodes a transcription factor that is a master regulator of fruit fly neurogenesis [68], raising the possibility that SIM2 could perform a similar function in mammals. However, a role of SIM2 in mammalian neurogenesis has not been so far confirmed, while this gene has been shown to repress myogenesis in mouse [69]. Besides to directly regulating transcription, DSCR genes could strongly modulate the activity of transcription factors encoded by other loci. The best

characterized example is RCAN1, which was initially named DSCR1 [70]. The gene name was then changed after realizing that the encoded protein inhibits calcineurin-dependent transcriptional responses by binding to the catalytic domain of calcineurin A and interfering with the phosphorylation of the NFAT transcription factor [71,72]. RCAN1 is overexpressed in DS brain [14,73] and seems to play a key role in the regulation of mitochondrial function and oxidative stress. Indeed, the *Drosophila* homolog of RCAN1 especially affects the activity of the mitochondrial ADP/ATP translocator [74]. Moreover, it has been shown that, when RCAN1 is overexpressed in PC12 cells, it induces the expression of superoxide dismutase type 1 (SOD1) [75], which is encoded by another HSA21 gene [15] and is upregulated in DS brain [76]. Importantly, RCAN1 acts as a stress response element: its acute overexpression protects cells from oxidative stress [77]. Indeed, RCAN1 overexpression may have beneficial effects by counteracting the oxidative damage associated with DS. Elevated levels of DNA damage, lipid peroxidation [78] and pro-oxidant state develop early in life in DS subjects [79]. Nevertheless, it is very likely that the benefits arising from these actions on oxidative stress may be overcome by the long-term detrimental effects on synaptic functions and neuronal survival due to the chronic RCAN1 overexpression, which will be discussed in sections 3.4 and 3.5.

3.3. Signaling proteins encoded by the DSCR

Modifications of the cellular cytoskeleton in response to extracellular stimuli, such as growth factor engagement and cell-cell contacts are essential for neuronal proliferation, for the formation of axons and Dendrites, for the differentiation and for the establishment, maintenance and remodeling of neuronal connections. Many of the well-characterized DSCR genes, such as DSCAM, CLDN14, PIGP, LCA5L, IGSF5 and FAM3B are implicated in these processes. However, the best characterized proteins belonging to this category are DYRK1A and TTC3.

3.3.1. DYRK1A

DYRK1A, dual-specificity tyrosine-phosphorilation-regulated kinase1A, encodes a protein kinase capable to phosphorylate serine, threonine and tyrosine residues, highly conserved at the aminoacidic level across vertebrates and invertebrates [80]. The orthologus *Drosophila* gene is involved in neuroblast proliferation and it is named *minibrain* (MNB), because null mutations affect post-embrionic neurogenesis, resulting in reduced brain size [81]. The highly conserved structure of this kinase and its mapping to the DSCR prompted extensive studies on its vertebrate homologues [82]. These studies have revealed that the dosage of DYRK1A is extremely important to normal brain development. Indeed, mice homozygous for a null mutation of DYRK1A die early in development and even heterozygous mice display reduced viability and a smaller brain, characterized by reduction of neuronal counts in specific regions [83]. Accordingly, truncation of the human MNB⁄DYRK1A gene has been reported to cause microcephaly [84,27]. Furthermore transgenic mice overexpressing DYRK1A show severe impairment in spatial learning and memory in the Morris water maze tests, indicating hippocampal and prefrontal cortical function alteration [45,85]. Moreover, these transgenic mice show abnormal LTP and LTD, indicating synaptic plasticity alterations [46]. These defects

are similar to those found in murine models of DS with trisomy of chromosome 16, suggesting a causative role of DYRK1A in cognitive disorders present in DS patients. DYRK1A is expressed in the cortex, in the hippocampus and in the cerebellum [86,18] and is overexpressed in the mouse trisomic model Ts65Dn [87], in DS fetal brain and other trisomic tissues [88]. These data obtained from different experimental systems have revealed various possible functions of DYRK1A in central nervous system (CNS) development, including its influence on proliferation, neurogenesis, neuronal differentiation, cell death and synaptic plasticity [46, 89-92]. These multiple biological functions of DYRK1A are due to its interactions with numerous cytoskeletal, synaptic and nuclear proteins, including transcription and splicing factors [93]. Together with other studies [85,94-96], these data strongly support the involvement of Dyrk1A in several neuropathological phenotypes and in the cognitive deficits that characterize Down syndrome. More recently, the observation that DYRK1A is overexpressed in the adult DS brain [97] implicated this protein also in the DS neurodegenerative phenotype. In particular, DYRK1A overexpression appears to be the cause of gene dosage-dependent modifications of several mechanisms that may contribute to the early onset of neurofibrillary degeneration. In fact, it has been demonstrated that Dyrk1A phosphorylates tau at several sites *in vitro* [98] and such sites are phosphorylated in DS brain [99]. Dyrk1A-induced tau phosphorylation inhibits the biological activity of tau, primes it for further phosphorylation by glycogen synthetase-3β (GSK- 3β) and promotes its self-aggregation into neurofibrillary tangles (NFTs) [99]. Interestingly, besides to phosphorylating protein, DYRK1A also colocalizes with NFTs [100]. In addition, neuropathological and molecular studies indicate that overexpressed nuclear DYRK1A contributes to the modification of the alternative splicing of Tau leading to neurofibrillary degeneration [101,102]. Neurofibrillary degeneration is the leading cause of neuronal death and dementia in Alzheimer's disease (AD) and in DS⁄AD. The multi-pathway involvement of DYRK1A in neurofibrillary degeneration indicates that therapeutic inhibition of the activity of overexpressed DYRK1A may delay the age of onset and inhibit the progression of neurodegeneration in DS. To this regard, the studies recently performed by the group of Delabar [103] represent, arguably, the best example of how the functional knowledge about DSCR genes can be translated into new potential therapeutic strategy. Indeed, this research group has found that Epigallocatechin gallate (EGCG) - a member of a natural polyphenols family, found in great amount in green tea leaves - is a specific and safe DYRK1A inhibitor and that its administration can revert the brain defects induced by overexpression of DYRK1A [103]. Together with a previous report showing that EGCG administration may beneficially affect the LTP abnormalities detected in Ts65Dn mice [104], this study paved the way for the promotion of clinical trials, which are already in Phase 2 (see for instance http://clinicaltrials.gov/ct2/show/NCT01394796).

3.3.2. TTC3

Since its discovery in 1996, the TTC3 gene has been considered an important candidate for the CNS-related phenotypes that characterize DS, because of its mapping within the DSCR [105,106]. This hypothesis was further supported by the analysis of TTC3 expression during normal development. Indeed, during mouse and human brain embryogenesis, TTC3 expression shows regional and cellular specificities well correlated with the anatomical defects

observed in DS patients [55,107]. In particular, TTC3 is expressed at highest levels in the post-mitotic areas of central nervous system (CNS), suggesting a role in neuronal cell differentiation [108,109]. Moreover, it has been reported that the expression of TTC3 is increased in tissues and in cells derived from DS experimental models [110] and from DS individuals [111,112]. In 2007, on the basis of both overexpression and knockdown experiments performed in PC12 neuroblastoma cells, we demonstrated that the TTC3 protein may play a pivotal role in regulating the differentiation program of neuronal cells, starting from the earliest stages [113]. More specifically, increased TTC3 function strongly prevents the neurite sprouting normally elicited by NGF-treatment, while TTC3 knockdown increases neurite length [113]. Importantly, TTC3 may affect not only the generation of neuronal processes, but also their maintenance (Berto et al., unpublished)., and its effects on neuronal differentiation are mediated by the activation of a specific pathway comprising the master cytoskeletal regulator RhoA and its effettor proteins, namely Citron-isoforms [113] Rho kinases (ROCKs) and LIM-kinase (Berto et al., in preparation), which have been implicated in all the different aspects of the neuronal differentiation program [114] and in different aspect of cognitive disorders [115]. Importantly, specific inhibitors of ROCKs, such as Fasudil, have been already approved by FDA, and therefore represent ideal candidates for testing in the experimental models [116]. In addition, a recent report by the group of Dr. M. Noguchi has shown that TTC3 can down-modulate the activity of the Akt kinases (AKTs), by promoting their ubiquitination and degradation [111]. This observation is particularly important, not only because AKTs have been shown to regulate neuronal survival [117], axonogenesis [118], dendritogenesis and synaptogenesis [119], but especially because these proteins are effectors of the PI3K pathway, which is the subject of extensive pharmacological investigation, in light of its centrality in cancer and inflammation research [120,121].

3.4. Gene networks affecting the excitatory-inhibitory balance in DS

The majority of forebrain is comprised of excitatory glutamatergic projection neurons and approximately 10% inhibitory γ-amminobutyric acid (GABA) interneurons. The normal functioning of the neural networks underlying cognitive functions depend on a finely-tuned balance of excitatory and inhibitory activities [122]. Accordingly, different reports have supported the possibility that cognitive impairment in DS models can be related to specific alterations of the excitatory/inhibitory balance, which may result from the direct action of DSCR genes or from more indirect mechanisms. For instance, it has been hypothesized that the increased dosage of HSA21 gene could favor the excitatory inputs in the hippocampus by increasing the activity of N-methyl-D-aspartate (NMDA) receptor (NMDAR), with potential effects on synaptic plasticity and neuron survival [123]. This theory was based on the observation that that several HSA21 genes, such as APP, SOD1, RCAN1 and DYRK1A, directly interact or indirectly affect the activity of the NMDARs. The best characterized pathway is that involving RCAN1, which regulates NMDARs by directly binding and inhibiting the calcineurin protein phosphatase (CaN) [71,77,124]. NMDARs are CaN targets [125] [126] and CaN inhibition leads to increased NMDARs [127] activity, by decreasing channel open probability and mean time [127]. On this basis Costa and co-workers hypothesized that the noncompetitive NMDA antagonist memantine, which acts as open channel blocker and is currently approved

for AD therapy, could mimic the actions of CaN and restore normal NMDARs function, possibly improving learning and memory [123]. Indeed, memantine ameliorates contextual fear conditioning learning in 4–6- and 10–14-month old Ts65Dn mice when administered at 5 mg/kg by acute intraperitoneal injection before context exposure. Despite these studies, a recently published clinical trial reported that memantine is not an effective pharmacological treatment for cognitive decline or dementia in DS patients who are above 40 years old [128]. This suggests that therapies that are effective in DS models and in AD patients may not necessarily confer benefits in DS.

More consistent reports have shown that the LTP phenotypes and the reduced performance in cognitive tests observed in mouse models could be the result of excessive GABA-ergic responses, producing a net decrease of synaptic output [36,37,129]. This phenomenon could be a direct effect of the overexpression of at least three proteins encoded by the DSCR, namely the chloride channel CLIC6 and the rectifying potassium channels KCNJ6 and KCNJ15. Accordingly, primary hippocampal neurons derived from Ts65Dn mice display a significant increase in GABA-mediated GIRK currents, consistent with the increased expression of KCNJ6/GIRK2 [130]. However, some of the data are also consistent with an increased presynaptic availability of GABA [129], produced by undefined and probably indirect mechanisms. On this basis, several pharmacological interventions have been proposed to restore the excitatory-inhibitory imbalance by decreasing the excessive inhibition of GABAergic neurotransmission prevalent in DS mouse models [131]. In particular, Ts65Dn mice have been treated with non-competitive $GABA_A$ antagonists, pentylenetetrazol (PTZ) and picrotoxin (PTX), which inhibit $GABA_A$ receptors. Chronic treatment with PTZ reversed the deficits seen in the novel object recognition task (NORT) and spontaneous alternation tasks in Ts65Dn mice [129,132]. Surprisingly, the improvement in cognition and LTP was sustained for up to 2 months after initial treatment, suggesting a long-lasting effect on neuronal circuit modification. Chronic treatment with PTZ for 8 weeks in Ts65Dn mice did not modify sensorimotor abilities and locomotor activity in home cages. However it did rescue learning and memory performance in the Morris water maze (MWM) task [133]. Recently, chronic treatment in Ts65Dn mice with an inverse agonist selective for the α5 subunit of the $GABA_A$ benzodiazepine receptor (α5IA) improved cognitive deficits in the MWM and normalized Sod1 overexpression with an enhancement in learning-evoked immediate early genes expression levels [134]. Encouraged by this body of evidence, Roche, a healthcare company, recently announced the commencement of a trial to examine the cognitive impact of reducing GABA-ergic neurotransmission in the hippocampus using a drug selective for the α5 subunit of $GABA_A$ receptors (http://www.roche-trials.com).

Finally, the imbalance in excitatory/inhibitory ratio could be the result of abnormal neurogenesis. Indeed, reduced cell numbers in the DS hippocampus could be caused by impaired adult neurogenesis, which has been observed in Ts65Dn [135] [136] and Ts1Cje mice [137]. Therefore, approaches targeting neurogenesis seem very promising for DS therapy. Interestingly, a fascinating connection has been documented between the DSCR gene KCNJ6 and adult neurogenesis, mediated by serotonin signaling. DS has long been associated with defects in the serotonergic system [138]. In particular, the serotonin 5-HT1A receptor expression peaks

earlier in developing DS brains and decreases to below normal levels by birth [139]. Moreover reduced 5-HT levels are present in adults with DS [140]. Since 5-HT depletion causes a permanent reduction in neuron number in the adult brain [138], it is conceivable that alterations in the serotonergic systems during early life stages may contribute to the reduced neurogenesis of the DS brain. Activity of the serotonin receptor 1A (5HTR1A) is required for adult neurogenesis in the hippocampus [141] and is mediated by the potassium channel KCNJ6. Overexpression of KCNJ6, as in the Ts65Dn, may over-inhibit presynaptic 5HTR1A, causing reduced levels of serotonin. Fluoxetine, an antidepressant that inhibits serotonin (5-HT) reuptake, inhibits KCNJ6 and increases presynaptic levels of serotonin. Consistent with this, it has been already demonstrated that fluoxetine is able to rescue neurogenesis in the adult Ts65Dn [135]. Recently, treatment during the early postnatal period restored neurogenesis and the total number of neurons in the dentate gyrus. This effect was accompanied by the full recovery of a cognitive task [142]. The releance of these data is even greater if considering that fluoxetin is an antidepressant widely used by adults and prescribed in children and adolescents [143] and that it does not seem to have negative effects on post-natal development [144].

3.5. The DSCR and Alzheimer-related molecular networks

Most DS patients experience a decline in cognition during adulthood, followed by the development of classical Alzheimer's disease (AD) neuropathology, characterized by the accumulation of amyloid plaques containing high levels of the A-beta fragments of the APP protein, by neurofibrillary tangles containing high levels of hyperphosphorylated Tau protein and by massive neurodegeneration [145]. Increased dosage of the APP gene, which is located outside the DSCR, is very likely the most important factor that underlies this phenomenon [146]. Indeed, increased dosage of APP is sufficient to strongly increase the risk of AD, since APP gene duplication has been detected as the mutation responsible for some early-onset familial cases of AD [147]. The link between AD and the APP gene has been further strengthened by the finding that an extra copy of APP seems to be necessary for the development of AD in DS. Indeed, it has been reported the case of an old patient affected by DS but not showing any signs of dementia [148]. At autopsy, plaques and tangles were absent in the brain of this individual. The patient had a segmental trisomy HSA21, not including the APP gene [148]. These data strongly support that the early onset of AD pathology in DS is in part due to overexpression of the APP gene. The data obtained from experimental models further support the crucial role of APP in DS [51]. Indeed, it has been shown that APP overexpression in Ts65Dn impairs the retrograde transport of nerve growth factor (NGF) from the hippocampus to the basal forebrain, causing the degeneration of BFCN [51], which significantly degenerates in Ts65Dn. Importantly, APP is one of the few genes for which a successful subtractive genetic approach has been reported, since restoring APP gene dosage to two copies in the Ts65Dn model corrected the water maze phenotype and prevented BFCN degeneration [50,51]. Finally, APP-mediated pathological mechanism may also contribute to the developmental abnormalities detected in mouse models, since it has been suggested that APP overexpression can result in increased Notch signaling pathway, which is crucial for neuronal and glial differentiation [149]. However, it is conceivable that also some of the DSCR genes may cooperate with APP in accelerating the AD-related neuropathological phenotypes observed in DS patients. In

particular BACE2 could promote the beta-cleavage of APP, further increasing the amount of generated A-beta peptides [150-152]. DYRK1A can also play an important role, because it can stimulate the phosphorylation of APP and Tau, resulting in increased cleavage and aggregation, respectively [98,153]. Finally, Tau hyperphosphorylation can be stimulated by increased expression of RCAN1, since phosphorylated Tau is one of the substrates of calcineurin [154]. Moreover, it has been shown that this activity of RCAN1 can be modulated by DYRK1A [155] Therefore it is very likely that the development of new approaches aimed at targeting these proteins could turn out to be beneficial both for AD and for DS management.

3.6. DSCR-dependent RNA-networks

As it is generally the case for the human genome, besides to protein coding genes, the DSCR contains many sequences that have been so far almost completely neglected, because they are not predicted to encode for proteins [16]. However, as we show in Table 2, on the basis of the current knowledge, many of these loci display features indicating that they could be functionally relevant and could contribute to the pathogenesis of DS phenotypes. Indeed, besides to the two copies of snRNAs and five copies of snoRNAs associated to splicing factors, the DSCR contains many regions that are transcribed to produce processed transcripts, devoid of coding potential. Some of these sequences, such as antisense transcripts, processed pseudogenes and sequences located in proximity of promoters, are closely associated to functioning genes, and could be involved in their regulation, as it has been shown in many other cases [156-158]. In many other cases, the genes appear to produce llincRNAs, that could act in cis to modify chromatin structure, or in trans to modify gene expression at the transcriptional and post transcriptional level, as it has been shown in the cases of HOTAIR [159] and of LincRNA-p21 [160,161]. Although the function of these molecules is at the moment completely unknown, their study could be extremely interesting. Indeed many of these sequences have been implicated in the epigenetic and in the post-transcriptional control of gene expression. Moreover, since these sequences diverge much more rapidly than the sequences of protein-coding genes, it is very likely that they could be strongly implicated in the control of human-specific features and phenotypes. Therefore, it seems reasonable to anticipate that the functional study of lincRNA-encoding genes in DS models and the study of their variation in humans will be a fertile ground for future research. Finally, the DSCR contains at least three genes encoding miRNA precursors (probably five, if considering also those that have only been predicted). Interestingly, mir-802, which is encoded by the DSCR, and mir-155, which is located on HSA21 in a more centromeric position, have been shown to repress the expression of MeCP2 [162], whose inactivation is the cause of Rett syndrome. Since MeCP2 is also repressed by HMGN1, this study further underscore the potential relevance of MeCP2 repression in DS and provides a very interesting example of how the intertwining of transcription and post-transcriptional regulatory networks dependent on DSCR genes can produce intellectual disability. Considering the reported reversibility of MeCP2 downregulation phenotypes [163] and the great efforts that are being dedicated to identify drugable pathways downstream of MeCP2 [164], it is conceivable that the functional exploration of these networks in DS could be also relevant for the development of future therapies.

4. Concluding remarks

Functional information on HSA21 genes is still quite partial and mostly limited to a subset of protein-coding genes. However, the recent success in DS models of therapeutic strategies targeted either on specific DSCR genes, or even on much broader mechanisms, justifies to our opinion an optimistic view of the future. In particular, we think that it will be reasonable to expect that a high level of understanding of the complex networks implicating DSCR genes through systems biology approaches will provide very useful insight, which could be translated into new therapies that could turn out to be useful not only for DS, but also for other disorders such as Alzheimer's disease and Rett syndrome.

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