

Two key genes closely implicated with the neuropathological characteristics in Down syndrome: *DYRK1A* and *RCAN1*

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The most common genetic disorder Down syndrome (DS) displays various developmental defects including mental retardation, learning and memory deficit, the early onset of Alzheimer's disease (AD), congenital heart disease, and craniofacial abnormalities. Those characteristics result from the extra-genes located in the specific region called 'Down syndrome critical region (DSCR)' in human chromosome 21. In this review, we summarized the recent findings of the *DYRK1A* and *RCAN1* genes, which are located on DSCR and thought to be closely associated with the typical features of DS patients, and their implication to the pathogenesis of neural defects in DS. *DYRK1A* phosphorylates several transcriptional factors, such as CREB and NFAT, endocytic complex proteins, and AD-linked gene products. Meanwhile, *RCAN1* is an endogenous inhibitor of calcineurin A, and its unbalanced activity is thought to cause major neuronal and/or non-neuronal malfunction in DS and AD. Interestingly, they both contribute to the learning and memory deficit, altered synaptic plasticity, impaired cell cycle regulation, and AD-like neuropathology in DS. By understanding their biochemical, functional and physiological roles, we hope to get important molecular basis of DS pathology, which would consequently lead to the basis to develop the possible therapeutic tools for the neural defects in DS. [BMB reports 2009; 42(1): 6-15]

Down syndrome and neural defects

Down syndrome (DS) was first described in 1866 by British doctor, John L. H. Down, as a disease showing mental retardation and characteristic facial appearance in the patients (1). Since then, for nearly 150 years, it has been one of the most frequent genetic disorders, occurring with an incidence rate of 1 per 700~800 live births. DS is caused by the presence of all or part of an extra human chromosome 21 (trisomy

21) (2, 3). Other phenotypic features of DS include cognitive impairment, learning and memory deficit, a high risk of leukemia, a decreased risk of solid tumors, congenital heart disease and hypotonia (3-5). Moreover, DS brains show additional neuropathological outcomes such as the arrest of neurogenesis and synaptogenesis, and neuronal differentiation defects (6, 7). They also exhibit a lower brain weight with reduced neuronal density, number, and volume regardless of region and age (6, 8, 9). Although the cause of these CNS hypoplasias in DS patients remains unclear, several reports using DS brains and cultured DS fibroblasts suggest that it may result from enhanced cell death and impaired cell proliferation (6, 7, 10, 11). It could be speculated that the reduction in neuronal density and number may give rise to the neurological impairments of DS, including mental retardation, cognitive abnormality, and learning and memory deficit (12-14). Furthermore, DS patients show signs of early onset of Alzheimer's disease (AD), characterized by the formation of amyloid senile plaques and neurofibrillary tangles (7, 15-17) and simultaneously with the typical neuropathological features.

Down syndrome critical region and mice models of DS

Trisomy 21 is divided into four categories according to the size of triplicated genomic region: complete trisomy, partial trisomy, microtrisomy, and single-gene duplication (3). The majority of DS patients (over 95%) have three complete copies of human chromosome 21, and others have mosaics or translocations (18). Several reports on partial trisomy 21 targeted a specific region of human chromosome 21 (part of 21q22.1 to 21q22.3) as a key suspect in causing the major phenotypic features of DS (19-21), and hence, named as 'Down syndrome critical region (DSCR)'. The human DSCR contains 33 presumed genes. While studying the biochemical properties and functional role of each DSCR gene product, many researchers have concentrated on investigating the gene-phenotype correlation and the effect of whole or partial triplication of DSCR in animal models. With this goal, several mice models of DS were developed and examined.

Among them, the Ts65Dn mice contain an extra segment of a distal ~17 Mb region of mouse chromosome 16, which corresponds to human chromosome 21 (22). Ts1Cje mice have an extra distal ~8.3 Mb region (23). Both models show many

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similar phenotypes to human DS patients, including impaired behavior and learning and memory deficit (22, 23). Ts65Dn mice exhibit the degeneration of septohippocampal cholinergic neurons, typical neuropathologic markers of AD (24) and altered neuronal proliferation in the hippocampi (11, 25), which may result in reduced neuronal density and number (25, 26). Despite these similarities, the models have several drawbacks. For example, mouse ortholog genes corresponding to the 231 genes of human chromosome 21 are randomly distributed throughout mouse chromosomes, such as 154 genes in a distal ~23.3 Mb region of chromosome 16, 58 genes in an internal ~2.2 Mb region of chromosome 10, and 23 genes in a centromere ~1.1 Mb region of chromosome 17 (3). In addition, they only contain a distal region of mouse chromosome 16, but not of chromosome 10 and 17. More importantly, mice with a complete trisomy of chromosome 16 are not viable after birth. To overcome these problems, O'Doherty *et al.* recently generated an aneuploid transchromosomal mouse line (Tc1) that stably transmits a freely segregating and nearly complete (~92%) human chromosome 21 (27). Tc1 mice exhibit altered behavior, impaired long-term potentiation in the dentate gyrus, hippocampus-dependent learning and memory deficit, and cardiac defect similar to DS patients (27).

Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A)

Roles of DYRK1A in various cellular events

Among DSCR genes, *DYRK1A* was proposed to be one of the potent candidate genes closely implicated in various DS phenotypes. *DYRK1A* is mapped to 21q22.13 of DSCR (28, 29), and encodes a proline-directed serine/threonine kinase (30). *DYRK1A* phosphorylates several transcription factors. First,

DYRK1A is involved in the neuronal differentiation of rat hippocampal progenitor cells through the phosphorylation of cyclic AMP response element-binding protein (CREB) at serine 133 residue (31). It also phosphorylates two transcriptional factors, nuclear factor of activated T cells (NFAT) and forkhead in rhabdomyosarcoma (FKHR), leading to the blockade of their nuclear translocation from the cytoplasm, thus functional inhibition (32-34). Second, *DYRK1A* targets many endocytic proteins, such as dynamin 1 and amphiphysin 1. By phosphorylation, *DYRK1A* regulates the assembly of endocytic complexes, which are composed of endophilin 1 and Grb2, suggesting that *DYRK1A* is closely associated with the endocytic pathway (35, 36). Moreover, *DYRK1A* phosphorylates eukaryotic protein synthesis initiation factor 2B epsilon (eIF2Bε) (37) and glycogen synthase (38), indicating that *DYRK1A* may affect the basic cellular metabolism. These overall findings suggest that *DYRK1A* is widely involved in various cellular events (Fig. 1).

DYRK1A transgenic mice

Three transgenic (TG) mouse lines were generated as an *in vivo* model to study the effect of *DYRK1A* overexpression. First, the TG mouse line (*DYRK1A* BAC TG mice) generated by Ahn *et al.* contains the genomic *DYRK1A* gene on a 777J19 bacterial artificial chromosome to express human *DYRK1A* (39). *DYRK1A* BAC TG mice showed a significant impairment in hippocampal-dependent memory tasks and shifts in both long-term potentiation and long-term depression (39). They also exhibited hyperphosphorylated microtubule-associated protein tau at serine 202, threonine 212, and serine 404 residues, which is a key component of neurofibrillary tangles (40). Furthermore, they displayed enhanced phosphorylation of amyloid precursor protein (APP) at the threonine 668 residue and β-amyloid production in hippocampus (41). These find-

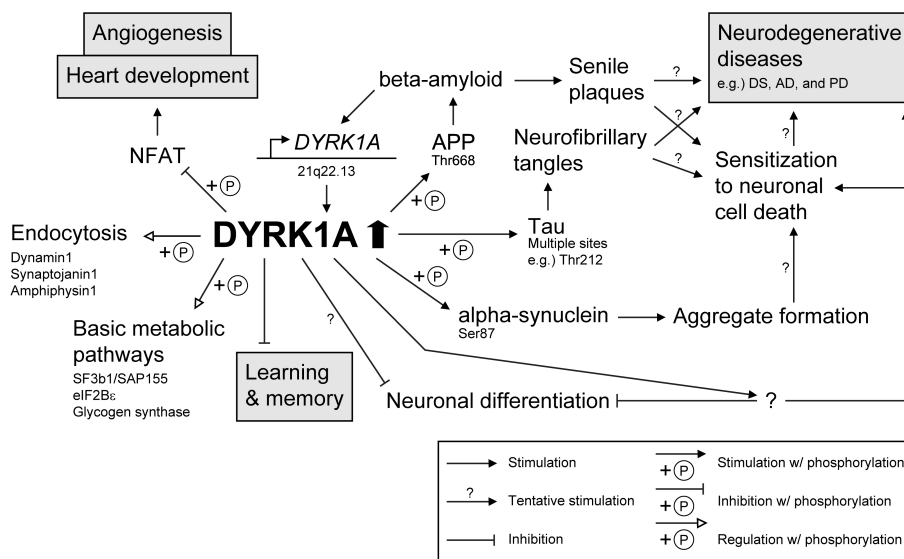


Fig. 1. Summary of the putative roles of *DYRK1A* in various cellular events and its association with the pathology of neurodegenerative diseases in DS.

ings suggest that DYRK1A phosphorylates two key target proteins in AD, and so its up-regulation may be closely associated with the early onset of AD in DS.

The second DYRK1A TG mice (TgDyrk1A) contain a 6.7 kb fragment from sMT-1a/Dyrk1A chimeric gene, and showed delayed craniocaudal maturation, altered motor skill acquisition, hyperactivity, and significant impairment in spatial learning and cognitive flexibility (42). A recent paper on TgDyrk1A mice demonstrated increased NR2A subunit expression and prolonged decay of NMDA-induced calcium transient in the cerebellum, suggesting that the phenotypes of TgDyrk1A mice may result from dysregulation of NMDA channels and altered calcium homeostasis (43).

The third DYRK1A TG mice (152F7 mice) bear an 152F7 fragment of yeast artificial chromosome containing DSCR genes, such as *DSCR5*, *TTC3*, *DSCR3*, and *DYRK1A* (44). They exhibited impaired passive avoidance, hyperactivity during development, brain enlargement with increased neuronal size, enhanced phosphorylation of FKHR and cyclin B1, and reduced phosphorylation of CREB (45). Moreover, 152F7 mice showed reduced neuronal density in the cerebral cortex, with learning and memory deficit (44).

In summary, these three types of DYRK1A TG mice consistently showed hippocampus-dependent learning and memory defect, hyperactivity, and altered synaptic plasticity. Therefore, it may be useful to study the cellular role of DYRK1A and its up-regulation effect on the basic cell metabolism and development.

DYRK1A and neurodegenerative diseases

In addition to DS, DYRK1A appears to be closely involved in neurodegenerative diseases, including AD, Parkinson's disease (PD), and Huntington disease (HD). As described above, DS patients show AD-like dementia earlier than normal individuals accompanied with the characteristic formation of amyloid senile plaques and neurofibrillary tangles (7, 15-17). In addition, α -synuclein-positive Lewy bodies (LBs) and neuritic processes, the pathological hallmarks of PD, frequently occur in DS brains with AD phenotypes (46). Furthermore, LB formation frequency in DS brains with AD is greater than in sporadic AD cases (47).

In accordance with AD phenotypic features of DS, many papers showed that DYRK1A, which is up-regulated approximately 1.5-fold in DS brains (48), directly phosphorylates tau to cause hyperphosphorylation at multiple sites (37, 40, 49, 50). Hyperphosphorylated tau at multiple Ser/Thr residues (T181, S199, T205, T212, T217, S262, S396, S404, and S422 residues) by DYRK1A impaired microtubule assembly and led to the formation of intracellular aggregates (37, 40, 49-51). These results are further supported by the finding that DYRK1A is present in neurofibrillary tangles of DS and AD brains (51) as well as in phosphorylated tau-enriched sarkosyl-insoluble fraction of AD brains (52). DYRK1A also plays a role in β -amyloid production and senile plaque formation. It was reported to phosphorylate the intracellular domain of APP, which is also

mapped to human chromosome 21 and proteolytically processed by β - or γ -secretase to produce amyloidogenic β -amyloid fragments. In rat hippocampal progenitor cells and mouse brains, the overexpression of DYRK1A caused the increase in the levels of phosphorylated APP at threonine 668 residue as well as β -amyloid fragments (41, 49). Moreover, transcriptional regulation of *DYRK1A* was reported to be mediated by β -amyloid fragments, and the *DYRK1A* transcripts became up-regulated in AD patients and model mice (APP/PS1) (53).

Meanwhile, DYRK1A is reported to phosphorylate α -synuclein directly (54), a key component of LBs observed in PD patients. Phosphorylated α -synuclein showed an enhanced tendency to form the inclusion, eventually leading to neuronal cell death (54). The stable overexpression of DYRK1A also made rat hippocampal progenitor cells more susceptible to serum deprivation-induced cell death (49). These findings are further supported by a recent paper showing that DYRK1A specifically interacts with and phosphorylates a member of guanosine triphosphate hydrolases, septin 4 (SEPT4/Pnutl2/CDCrel-2), which was found to be within the neurofibrillary tangles of AD brains and in α -synuclein-positive cytoplasmic inclusions in PD brains (55).

HD is characterized by involuntary movements, intellectual decline, and dementia (56), and DS has many similar congenital defects. Although more experiments are required to determine whether and how DYRK1A participates in the cellular events of HD pathology, specifically in neuronal defects, our preliminary study suggests the possible involvement of DYRK1A in HD (57). For example, DYRK1A binds to and phosphorylates huntingtin-interacting protein-1, which consequently modulates neuronal differentiation and cell death in hippocampal neuroprogenitor cells (57).

Taken together, these data show that DYRK1A may play a pivotal role in neuronal cell death and the pathogenesis of neurodegenerative diseases, such as AD, PD, and HD (Fig. 1).

Regulators of calcineurin 1 (RCAN1)

Alternative splicing of *RCAN1*

RCAN1, also known as *MCIP1*, *DSCR1*, *adapt78* and *calcipressin*, is located at 21q22.12 and consists of seven exons plus the first alternative one (exon 1 through 4) (58). Among the four possible transcripts, *RCAN1-2* appears not to be synthesized due to the lack of a start codon, and *RCAN1-3* has not been detected through RT-PCR on any tissues so far. *RCAN1-1* encodes a protein of 197 amino acids and is primarily abundant in the fetal and adult brains (58). A recent study revealed an additional start site upstream of exon 1, which leads to the production of *RCAN1-1* with 252 amino acids (59). In order to avoid confusion between these two products, the former short form is referred as *RCAN1-1S* (short form) and the latter as *RCAN1-1L* (long-form). *RCAN1-4* encodes a protein of 197 amino acids, and expressed in fetal kidney, adult heart, skeletal muscle, placenta, and adult brain (58, 60). The intragenic region upstream of *RCAN1-4* exon 4 contains an alternative pro-

motor independent of *RCAN1-1*, and thus, these two isoforms are believed to have different transcriptional regulations.

The initial finding that *RCAN1-4* is inducible by oxidative or Ca^{2+} -mediated stress suggests that *RCAN1-4* has a role in stress response (61). Later, *RCAN1-4* promoter was found to have NFAT- (62) and AP-1-binding sites (63). *RCAN1-4* was also induced by many other factors in diverse cell and tissue types (i.e. VEGF, thrombin, oxidative or Ca^{2+} -mediated stress, β -amyloid fragments, thapsigargin, $TNF\alpha$, and IL-1 β). On the other hand, *RCAN1-1* promoter contains a muscle-specific CAT element (M-CAT) (64). Although *RCAN1-1* is also induced by VEGF, its mechanism differs from the *RCAN1-4*. While the induction of *RCAN1-4* acts through Ca^{2+} -NFAT pathway, *RCAN1-1* becomes induced through the binding of transcription enhancer factor 3 (TEF3) to M-CAT site (64) (Fig. 2). Despite the lack of functional glucocorticoid response elements (GREs) in the promoter region, glucocorticoid also enhanced the expression of *RCAN1-1* (65).

Roles of RCAN1 in various cellular events

RCAN1 physically and functionally interacts with calcineurin A, effectively inhibiting its phosphatase activity induced by PMA/ Ca^{2+} (66, 67). As *RCAN1-4* is induced by the Ca^{2+} -NFAT pathway and *RCAN1-4* inhibits calcineurin, a negative feedback loop is formed (62). In *Saccharomyces cerevisiae*, a decrease of calcineurin activity was observed in the null-mutant of *Rcn1* (the yeast homolog of *RCAN1*) or by *RCAN1-1S* overexpression (68). In addition, calcineurin activity became decreased in *Rcn1*^{-/-} mice, suggesting that RCAN1 is needed for calcineurin to show its maximal activity (69).

Moreover, *RCAN1-4* is induced by thrombin in endothelial cells (ECs), in which NFAT is known to interplay with NF- κ B signaling pathway (70, 71). NF- κ B-inducing kinase (NIK) was shown to phosphorylate *RCAN1-1S*, which leads to the de-

creased proteasomal degradation of *RCAN1-1S* (72). Another study showed that *RCAN1-4* attenuates NF- κ B-mediated transcriptional activation by stabilizing its inhibitory protein, I κ B α (73). Putting these data together, it can be deduced that *RCAN1*-mediated interplay between NFAT and NF- κ B signaling pathways may cause the attenuation of the signal transmission through two signaling pathways.

Functional regulation of RCAN1 by post-translational modification

Mitogen-activated protein kinase kinase kinase-3 (MAP3K3) could phosphorylate *RCAN1*, and phosphorylated *RCAN1* stimulates the activity of calcineurin (74). Big MAPK (BMK1) is also required for angiotensin II-mediated calcineurin-NFAT activation through *RCAN1* phosphorylation (75). Phosphorylated *RCAN1* became dissociated from calcineurin and binds to 14-3-3 ϵ/ξ (75). On the other hand, *RCAN1-1L* was less phosphorylated at the serine-proline (SP) repeat site, and the inhibition effect of calcineurin activity was consequently reduced (59). This inconsistency was explained by Cunningham *et al.*, that a low concentration of phosphorylated *RCAN1* stimulates calcineurin activity, while a high concentration of phosphorylated or non-phosphorylated *RCAN1* inhibits it (76). Furthermore, half-life of *RCAN1-1L* became reduced by phosphorylation (59). We have shown that NIK binds to and directly phosphorylates *RCAN1-1S*, leading to the significant increase of its half-life (72). These results indicated that the remarkable variation of *RCAN1* half-life is likely due to the difference of phosphorylation target site(s) by NIK, compared with other known kinases.

Different RCAN1 isoforms have different function?

With the deletion of the C-terminal region *RCAN1-1* could not bind to or inhibit calcineurin (67, 77), but *RCAN1-4* still could

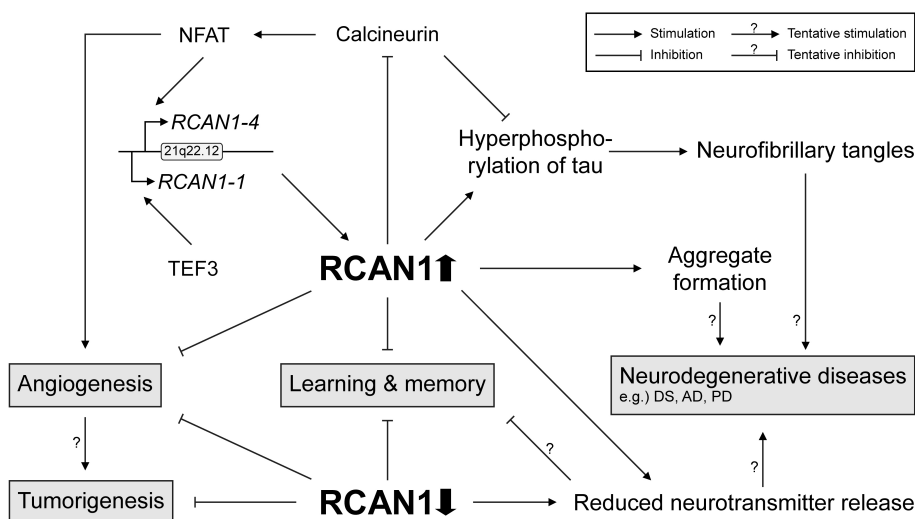


Fig. 2. Contribution of RCAN1 to various cellular events and its implication to the pathogenesis of neural defects in DS.

bind to calcineurin through its N-terminal region, suggesting that the inhibitory effect of RCAN1 on calcineurin acts through these two domains (78). Also, both of RCAN1-1S and RCAN1-4 decreased the cytotoxicity by Ca^{2+} or oxidative stress (60, 79), but overexpression of RCAN1-1S increased the proliferation in PC-12 cells (60). Meanwhile, overexpression of RCAN1-4 in hamster HA-1 cells suppressed cell growth by stopping the cell cycle at G1 (79). Contrary to the previous reports (59, 66-68), overexpression of RCAN1-1L in ECs stimulated calcineurin and proliferation, subsequently promoting angiogenesis (80). On the other hand, overexpression of RCAN1-4 inhibited angiogenesis and its knockdown promoted angiogenesis (80). Thus, it is still questionable whether RCAN1-1 inhibits or stimulates calcineurin, and additional experiments are required to determine the exact role of RCAN1 on calcineurin signaling. However, based on the current findings, the functions of RCAN1-1 and RCAN1-4 appear to be distinct even though they have almost identical amino acid sequence. As these isoforms differ in their promoter region, it is possible that they have unique functions due to the unique control modes during various cellular events. On the other hand, the functions of RCAN1-1S and RCAN1-1L are speculated to be identical, which should be further examined.

RCAN1 and neurodegenerative diseases

Based on the finding that *Rcan1*^{-/-} mice are impaired in spatial learning and memory and show deficit in late-phase long-term potentiation (L-LTP) (81), RCAN1 is thought to play a positive role in L-LTP and memory. The importance of *RCAN1* gene dosage in mental retardation has been reported in *Drosophila melanogaster* (82). The knockout or overexpression of *nebula*, the *RCAN1* homolog of *Drosophila melanogaster*, caused severe learning defects (82). In addition to the mitochondrial localization of *nebula*, its knockout or overexpression caused the increase of ROS levels and mitochondria number as well as the reduction of ATP levels and mitochondrial DNA contents (83). The oxidative damage by H₂O₂ became reduced in the primary neuron from *Rcan1*^{-/-} mouse (84). Moreover, the sensitivity to oxidative stress was increased when *RCAN1-1S* was overexpressed (84). Furthermore, *Rcan1*^{-/-} and *RCAN1-1S* TG mice displayed reduced levels of exocytosis (85). However, calcineurin inhibition did not affect the rate of exocytosis or fusion pore kinetics (85). These results imply that RCAN1 also involved in the regulation of neuronal oxidative stress and the neurotransmission process at synapses.

Overexpression of RCAN1-1S induced microtubule-dependent aggresome-like inclusion body formation in neuronal cells (86). Ubiquitin, huntingtin (Q148), ataxin-3 (Q84) were found to be co-localized with RCAN1 aggregates (86). The overexpression of RCAN1-1S in neuronal cells led to the formation of cytoprotective nuclear aggregates under the condition of zinc stimulation (87). In addition, the levels of intracellular RCAN1-1S aggregates were also increased by NIK, but this aspect did not affect the proliferation of neuronal cells (72). Furthermore, the expression of RCAN1 was induced by β -amyloid fragments (88). A recent study suggested that the decreased calcineurin activity seen in AD pa-

tients might be caused by the significant increase in RCAN1 expression (89). The finding that the overexpression of RCAN1-1S caused the hyperphosphorylation of tau (90) suggests that the occurrence of part of AD phenotypes be contributed by RCAN1 function (Fig. 2). As protein aggregation is deeply connected to neuronal degenerative disease, further studies on the relationships between RCAN1 aggregation and neuronal degenerative disease would be helpful in understanding the neuropathology of these diseases.

RCAN1 and non-neuronal diseases

RCAN1 BAC TG mice showed lethality at E9.5 (91), while cardiac hypertrophy was significantly inhibited in *RCAN1-4* TG mice controlled by cardiac-specific α -myosin heavy chain promoter (α -MHC) (92). Paradoxically, the phosphatase activity of calcineurin was also inhibited in *Rcan1*^{-/-} mice, whereas cardiac hypertrophy was exacerbated when the active form of calcineurin was overexpressed (69). Although *RCAN1-4* is induced by an angiogenic factor VEGF, the overexpression of RCAN1-4 attenuated tube formation and cell cycle progression of ECs, thus inhibiting angiogenesis (70, 93, 94). When *RCAN1* was knocked down, VEGF-stimulated migration of ECs and angiogenesis was also inhibited (95). In the previous model of cardiac hypertrophy and angiogenesis, *RCAN1* deficiency inhibited calcineurin. But *Rcan1*^{-/-} mice showed a hyperactivated calcineurin activity, exhibited premature endothelial apoptosis, inhibited the formation of an effective tumor vasculature, and suppressed tumorigenesis (96). While such deviations may be caused by the different methods to measure calcineurin activity, these results also indicated that RCAN1-4 overexpression or *RCAN1* deficiency inhibits angiogenesis, and RCAN1-4 overexpression inhibits cardiac hypertrophy. Moreover, *RCAN1* deficiency suppresses tumorigenesis. In conclusion, the appropriate level of RCAN1 is a requirement for normal heart development, angiogenesis and tumorigenesis (Fig. 2).

Conclusions and future perspectives

In the current article, we have reviewed recent studies of two critical genes in DSCR, including functional roles, up-regulation effects in mice models, and their putative actions in the pathogenesis of DS and neurodegenerative diseases. Although it is a long way from experimental findings to clinical applications, a few possible therapeutic ideas could be formulated by expanding upon the available information made by these studies. For example, two putative DYRK1A inhibitors were recently developed from *in vitro* high-throughput screening (97, 98). One of them is a polyphenolic constituent of tea, epigallocatechin 3-gallate (EGCG). EGCG is reported to specifically inhibit approximately 90% activity of DYRK1A and p38-regulated/activated kinase among tested 28 protein kinases (97). The other agent, harmine, was shown to inhibit *in vitro* approximately 95% activity of DYRK1A in the nanomolar range, though it also had the effect on other kinases including DYRK2 and DYRK3 (98).

Concerning the suggested roles of RCAN1, its SP repeat peptide could be one of the good targets for this purpose (99). As described previously, hyperactivation of calcineurin signaling led to cardiac hypertrophy, enhanced angiogenesis and inflammation response. RCAN1, as an endogenous calcineurin modulator, could inhibit calcineurin more selectively and efficiently than existing immunosuppressive drug cyclosporine A. Thus, restoration of the normal level or activity of RCAN1 could possibly lessen several neuropathological DS features caused by abnormality of RCAN1.

In conclusion, better understanding of two key genes closely implicated with DS, *DYRK1A* and *RCAN1*, is necessary and expected to provide us some useful ideas how to develop possible therapeutics for neural defects in DS patients.

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